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# Macrophages and Dendritic Cells

## Methods and Protocols

Edited by

Neil E. Reiner

# METHODS IN MOLECULAR BIOLOGY™

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**Neil E. Reiner**

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# Preface

In the face of frequent threats of either exogenous (usually infection) or endogenous (neoplastic transformation) origin, humans and other mammals are endowed with a vigorous and versatile capacity for host defense. This contributes to the maintenance of homeostasis and in the face of millions of years of evolutionary pressure, mammalian species developed two parallel yet highly interdependent systems to protect against infection and malignancy. Thus, the innate and acquired immune systems together perform remarkably well in responding to threats, thereby favoring survival. Not only are these two systems seamlessly integrated, but they also both support and cross-regulate each other at multiple levels. Hence, knowledge of the critical nodes that unite these systems is fundamental to a comprehensive understanding of mammalian host defense. Macrophages and dendritic cells are sentinels and effectors of innate immunity. Far beyond this, however, they are also perhaps more than any other cell type, poised at the interface with the acquired immune system where they critically influence the development of antigen-specific immune responses and long-term memory. The involvement of macrophages and dendritic cells in health and disease is not, however, limited to the realm of classical immunology. Research findings over the past several decades have made it abundantly clear that these cells are also centrally involved in heterogeneous inflammatory conditions and disease processes such as atherosclerosis, neurodegenerative disease, wound healing, and graft-versus-host disease to name only a few. In light of their critical contributions to diverse inflammatory diseases and to immunity and host defense, a contemporaneous understanding of state-of-the-art approaches to investigate the behaviors of macrophages and dendritic cells is useful, and is something this volume seeks to achieve.

An essential starting point to any research concerned with the properties or functions of macrophages and dendritic cells is the availability of adequate numbers of cells representing a highly purified homogeneous population. As such, four chapters are presented that address the isolation and cultures of these cells, including generating growth factor-dependent cell lines, methods for phenotypic characterization, and analysis of ontogeny.

Much of the work of macrophages and dendritic cells is initiated at the level of cell surface receptors that survey the environment for cognate ligands. Recognizing the centrality of receptor ligand interactions to the biology of these cells, two chapters are included that address the identification of novel myeloid cell surface receptors, while two complementary chapters describe approaches aimed at identifying novel ligands.

Phagocytosis is one of the most prominent functional properties of macrophages and to a lesser extent dendritic cells. Consequently, no contemporary volume of research approaches would be complete without addressing this area. Just as whole cell analysis is simplified when adequate quantities of pure starting material are available, this is also one of the challenges facing researchers studying phagosome biology. Furthermore, one of the areas of greatest interest to macrophage biologists is the fate of a phagosome once it is formed or "phagosome maturation." Consequently, chapters are included that address topics including large-scale phagosome isolation, quantitative and real-time analysis of phagosome maturation, and the role of GTPases in regulating phagosome biogenesis.

Macrophages and dendritic cells are inherently resistant to the introduction and expression of foreign genes. This has consistently been a significant obstacle confronting researchers whose aim has been to assign function to individual genes and the proteins they encode. This volume takes advantage of recent advances in the area of genetic manipulation of macrophages and dendritic cells with three chapters addressing this area with a particular emphasis on siRNA approaches.

Macrophages are required to undergo an activation program in order to maximize their effector functions and macrophage activation is an area of intense interest. Controlling cell activation is also critically important to limiting collateral damage resulting from excessive inflammation. In recent years, it has become clear that macrophage activation is not a homogeneous process and that the phenotypic outcome is very much influenced by the local environment, most importantly cytokines, but other factors as well. Currently, two major macrophage activation programs have been described leading to the generation of either M1, proinflammatory, or M2 anti-inflammatory macrophages. Given the importance of this rapidly advancing area of research, three chapters in this volume address the M1 vs. M2 or "classical vs. alternative" paradigm of macrophage activation.

Macrophage activation and for that matter all other stimulus-dependent responses by these cells are brought about through tightly regulated and highly integrated signaling pathways. Cell regulation in these cells has been studied intensively and recently it has become clear that lipid signaling plays an important role in determining phenotype. As such, two chapters address the role of lipid signaling and lipid signaling intermediates in macrophage biology.

Finally, macrophages are endowed with a vast array of degradative enzymes that they use to carry out functional programs and which contribute profoundly to both health and disease. A chapter dealing with the secretion of matrix metalloproteinases by macrophages provides a window into advances in this area and offers a vantage point for future research concerned with how the enzymatic machinery of these cells is linked to tissue inflammation.

It is my hope that readers, both neophytes and seasoned investigators, will find *Macrophages and Dendritic Cells: Methods and Protocols* to be both timely and useful in their research. Most importantly, I thank the authors for valuable time spent in preparing their chapters and for sharing their experience and knowledge. Thanks is also due to John Walker for his editorial advice and Jessica Lin for her critical editorial assistance.

***Vancouver, BC*** ***Neil E. Reiner***

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# Chapter 1

# Generation of Retroviral Macrophage cDNA Expression Libraries and Functional Screening for Surface Receptors

**Georgia Schäfer and Gordon D. Brown**

## Summary

Macrophages, tissue-based phagocytic cells derived from blood monocytes, play important roles in immunity and homeostasis. As professional scavengers, macrophages phagocytose microbes, apoptotic and necrotic cells and take up modified lipoprotein particles. However, many of their complex interactions with other immune cells and/or various ligands are not yet clearly understood. To identify and isolate macrophage cell surface molecules, particularly macrophage receptors, for which ligands are known, a powerful generalized screening method has been established. As discussed in this chapter, this technique based on function has been successfully applied for the identification of dectin-1, the major macrophage receptor involved in the binding and recognition of β-glucans (Nature 413:36–37, 2001).

**Key words:** cDNA expression cloning, Retroviral cDNA expression library, Functional screening, Surface molecule, Ligand binding, Receptor cloning, Stable transfection, Fluorescent microscopy.

## 1. Introduction

Cell surface molecules are important for macrophage cell functions. These molecules may be receptors for microorganisms, co-receptors for signal transduction modulating cell response to various stimuli, complement regulatory proteins, or adhesion molecules crucial in cell–cell and cell–matrix interactions *(1)*. Our laboratory mainly focuses on macrophage surface receptors which mediate innate immune recognition as the first and immediate line of defence against microbial invasion. These so-called pattern recognition receptors (PRR) are germ-lined encoded and

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recognize highly conserved molecular patterns (PAMPS, pathogen-associated molecular patterns) found only in microorganisms. This is in contrast to the slower but more specific adaptive immune system which uses somatically re-arranged antigen receptors clonally distributed on lymphocytes *(2, 3)*. The recognition of PAMPs by their receptors can directly activate effector mechanisms of innate immunity such as phagocytosis and activation of pro-inflammatory pathways. With the subsequent production of effector cytokines and chemokines and presentation of pathogen-derived peptides on the surface of antigen-presenting cells, the innate immune system is directly linked to the adaptive immune system *(4–6)*. Therefore, activated PRRs determine the type of cellular and immunological response mounted by the host, influencing the outcome of infection.

Although a number of phagocyte receptors have been shown to act as PRRs, their initial identification was for other functions. The mannose receptor, for example, was identified by its ability to bind endogenous self-ligands *(7)*, while CD14 was identified by monoclonal antibodies to human peripheral blood monocytes *(8)*. As no concerted effort had been made to identify these molecules directly, and given their importance in the immune response to infection, a generalized method based on cDNA expression cloning and relying on the inherent ability of PRRs to recognize pathogens was established to isolate these receptors from leucocytes based on their function *(9)*.

cDNA expression cloning using retroviral gene transfer is a powerful method for the isolation and identification of expressed genes that are able to confer a readily identifiable phenotype on specific cell types. Essentially, this method involves the directional cloning of cDNA into a retroviral vector and the generation of pools of stable ecotropic virus-producing cells from this DNA. The cells so derived constitute the library, and the virus they yield can be used to efficiently infect a wide range of cell types, with the foreign DNA stably inserted into the genome, for subsequent functional screening for surface receptors using known ligands *(10)*.

Using this technique, a single receptor that bound to the β-glucan-[1] rich particle zymosan has been isolated from a macrophage cDNA expression library and identified as a previously known molecule, Dectin-1 *(9)*. Further studies involved extensive molecular and functional characterization of this molecule *(11–15)*. The rediscovery of Dectin-1, through its ability to bind β-glucans, demonstrates the power of this screening method to identify receptors based on function. This method can be utilized for the identification for any macrophage cell surface molecule for which a ligand is known, for example receptors involved in host-pathogen interactions using live microorganisms.

[1] Glucose polymers found in the cell walls of plants, fungi and bacteria.

## 2. Materials

### 2.1. Cell Culture, Transfection, and Transduction

1. $DMEM_{10}$ (Dulbecco's Modified Eagle's Medium) supplemented with 10% heat-inactivated foetal calf serum (delta bioproducts), 2 mM l-glutamine, 500 U/ml penicillin, and 50 µg/ml streptomycin.
2. $DMEM_0$ (*see* **item 1**, but without serum).
3. Lidocaine hydrochloride dissolved in PBS at 4 mg/ml, supplemented with 10 mM EDTA.
4. G418 at 100 mg/ml; store at 4°C, use at 0.4 mg/ml in $DMEM_{10}$.
5. Hygromycin B at 100 mg/ml; store at 4°C, use at 300 µg/ml in $DMEM_{10}$.
6. Diphtheria toxin at 1 mg/ml; store at 4°C, use at 1 µg/ml in $DMEM_{10}$.
7. FuGENE 6 transfection reagent.
8. 10 mg/ml polybrene.
9. 0.45-µm sterile filters.

### 2.2 Generation, Expression, and Screening of the Library

1. Trizol® reagent (Invitrogen) for RNA isolation.
2. OligoTEX mRNA Kit (Qiagen).
3. *Denaturing formaldehyde/agarose gel for RNA*. Melt 1 g agarose in 85 ml deionized water, add 10 ml 10× MOPS buffer (0.2 M MOPS; 0.05 M sodium acetate; 0.01 M EDTA, pH 5.5–7.0) and 5.4 ml 37% formaldehyde (caution: formaldehyde is a carcinogen and must be handled with care inside a fume hood). Once the gel is solidified cover with 1× MOPS buffer and load RNA samples resuspended in 2× loading buffer: 50% deionized formamide; 13% formaldehyde; 1× MOPS buffer; 1.5% ethidium bromide; 2% sterile glycerol; 0.5% bromophenol blue (the loading buffer is not stable and should not be used more than 12 h after preparation). Electrophorese the gel at 100 V for approximately 1 h and examine under UV illumination.
4. *Agarose/ethidium bromide gels for DNA*. Melt 1 g agarose in 100 ml 0.5× TBE-buffer (50 mM Tris, 50 mM borate, 1 mM EDTA) and add 3% ethidium bromide. Once the gel is solidified cover with 0.5× TBE-buffer and load DNA samples resuspended in 6× loading buffer. Electrophorese the gel at 100 V for approximately 1 h and examine under UV illumination.
5. Creator™ SMART™ cDNA Library Construction Kit (Clontech).
6. Advantage® 2 PCR Kit (Clontech).

7. *Electroporation system*. Bio-Rad Gene Pulser II.
8. *E .cloni*® 10G SUPREME Electrocompetent Cells (Lucigen® Corporation).
9. *Luria Bertani (LB) broth*. 1% NaCl, 1% bactotrypton, 0.5% yeast extract, pH 7.0, 100 µg/ml ampicillin added after autoclaving and stored at 4°C.
10. *Luria Bertani (LB) agar plates (150 mm and 100 mm)*. 1% NaCl, 1% bactotrypton, 0.5% yeast extract, 1.5% agar; pH 7.0, 100 µg/ml ampicillin added after autoclaving when still liquid, poured into plates, and stored at 4°C.
11. PureYield™ Plasmid Midiprep system (Promega).
12. Wizard® Plus SV Miniprep system (Promega).
13. 2× PCR-Mastermix (Fermentas).

### 2.3. Isolation of Positive Cells

1. Dow Corning High Vacuum Grease.
2. Cloning cylinders, sterile, polystyrene, size: 4.7 mm × 8 mm (Sigma).
3. FITC-labelled zymosan (Molecular Probes).
4. QIAamp® DNA Mini Kit (Qiagen).
5. Advantage® 2 PCR Kit (Clontech).
6. TOPO TA Cloning® Kit for Sequencing (Invitrogen).
7. Wizard® SV Gel and PCR Clean-Up System (Promega).
8. pcDNA™ 3.1 Directional TOPO® Expression Kit (Invitrogen).

## 3. Methods

### 3.1. Generation of the Library

The construction of the retroviral cDNA expression library essentially involves the isolation of RNA from macrophages, its reverse transcription into cDNA, and ligation into a suitable vector which allows for subsequent virus production, as discussed in detail in the following.

1. Total RNA from macrophages grown in culture or freshly isolated from the organism/tissue of interest is prepared using the Trizol® reagent (Invitrogen) according to the manufacturer's instruction. This preparation method has been shown to yield high amounts of RNA though it can still contain traces of phenol. To get rid of the phenol and to enrich for mRNA, the total RNA is further purified using the Qiagen OligoTEX mRNA Kit (*see* **Note 1**).
2. For the preparation and cloning of cDNA fragments a commercially available kit is applied with the Creator™ SMART™

cDNA Library Construction Kit (Clontech) preferred in our laboratory. Initially 500 ng mRNA is reverse transcribed into first-strand cDNA using a modified oligo(dT) primer (CDS III/3′ primer) and the SMART IV™ oligonucleotide which serves as a short extended template at the 5 end of the mRNA (*see* **Note 2**). Second-strand DNA synthesis and amplification of the cDNA is then performed by Long-Distance PCR using the Advantage® 2 PCR Kit (Clontech), the CDS III/3′ primer and a 5′ primer complement to the SMART™ anchor. Using 500 ng mRNA as starting material the PCR cycling conditions are as follows: 1 min 95°C; 20× (15 s 95°C; 6 min 68°C). After the amplification the cDNA should be checked on a 1% agarose/ethidium bromide gel and appear as a 0.2–4 kb smear. Following a proteinase K digestion step according to the kit's instruction, the cDNA is digested by the restriction enzyme *Sfi*I for 2 h at 50°C. This enzyme recognizes asymmetrical restriction sites incorporated at the 5′ and 3′ ends of the cDNA fragments by the primers during synthesis and allows for subsequent directional ligation with respect to the transcription initiation site into an *Sfi*I-digested vector containing the same asymmetrical *Sfi*I sites in its MCS (*see* **Note 3**). The *Sfi*I-digested cDNA is size fractionated according to the kit's instruction, and the profile of the fractions is checked on a 1% agarose/ethidium bromide gel. To select for cDNA fragments greater than 500 bp appropriate fractions are collected and pooled accordingly (*see* **Note 4**). The pooled cDNA is then concentrated by precipitation according to the kit's instruction and is then ready for ligation into the *Sfi*I-digested vector.

3. As the cDNA fragments are intended to constitute a retroviral expression library they must be cloned into a suitable vector that allows for their transcription and subsequent packaging of the transcripts into infectious viral particles (*see* **Subheading 3.2**). The vector routinely used in our laboratory is pFBneo (Stratagene) which is derived from the Moloney murine leukaemia virus (MMLV) and can be used to produce high titre viral stocks with MMLV-based packaging cell lines. It contains the bacterial origin of replication and an ampicillin-resistance gene, an extended MMLV-packaging signal (ψ+), a neomycin-resistance gene, and an MCS located between the MMLV 5′ and 3′ long terminal repeat sequences (LTRs) which subsequently provide the retroviral RNA with the requisite *cis*-acting sequences for gene expression. Since pFBneo does not contain the asymmetrical *Sfi*I restriction sites needed for ligation of the cDNA fragments it has to be modified accordingly (*see* **Note 5**).

4. The ligation reaction is then performed under standard conditions. Briefly, approximately 100 ng *Sfi*I-digested pFBneo vector is added to approximately 200 ng *Sfi*I-digested pooled cDNA fragments and incubated at 16°C overnight in the presence of ligation buffer, ATP, and T4 DNA ligase in a total volume of 5 µl as outlined in the kit. The reaction mixture is then purified by precipitation according to the kit's instruction and resuspended in 2 µl sterile $H_2O$. Electrocompetent *E. coli* cells (*E. cloni*® 10G SUPREME Electrocompetent Cells, Lucigen® Corporation) with a transformation efficiency of approximately $5 \times 10^{10}$ cfu/µg DNA are transformed with the ligation mixture according to the manufacturer's instructions and plated out completely onto 20–30 150 mm LB-Amp plates. The resultant colonies should add up to about $1–2 \times 10^6$ independent clones with an insert size range of 0.5–6 kb and an average size of 1.5–2 kb (*see* **Note 6**). The plasmid DNA of a representative number of colonies should be isolated using a commercially available kit such as Promega's Wizard® Plus SV Miniprep Kit and sent off for sequencing to check for complete ORFs and enrichment of macrophage-specific cDNAs. Alternatively, a set of macrophage-specific primers could be tested on the library to confirm the presence of macrophage-specific cDNAs.
5. To proceed with the transfection of the library, colonies are scraped from the plates and pooled individually resulting in 20–30 cDNA pools. Plasmid DNA is isolated using a commercially available kit such as Promega's PureYield™ Plasmid Midiprep Kit to obtain high-quality supercoiled DNA for subsequent transfection of the virus-packaging cell line.

### 3.2. Expression of the Library

Retroviral vectors provide a highly efficient method for gene transfer, particularly for cell types which are often poorly susceptible to transfection methods such as calcium phosphate precipitation or lipid-based transfection agents. Retroviral infection results in the stable integration of one or a few copies of viral DNA into the target genome in a predictable and precise manner which allows subsequent recovery of the insert(s) by PCR. Retroviral vectors are manipulated in DNA form using bacterial plasmids such as pFBneo as described in **Subheading 3.1**, which contain a packaging signal but produce no intact proteins of their own and which are transfected into suitable packaging cells. These cells allow production of replication-defective retrovirus vectors in the absence of helper virus and must encapsidate the plasmid-encoded RNA; provide the viral proteins for infection, reverse transcription, and integration into the genome of target cells; and must produce high titres of virus. A highly transfectable cell line routinely used in our laboratory is the helper-free

ecotropic-packaging cell line Phoenix-Eco (G.P. Nolan, Stanford University) which is capable of producing the three major proteins encoded within a retroviral genome: Gag, Pol, and Env, with different non-Moloney promoters for *gag–pol* and *env* to minimize both their recombination potential with introduced retroviral constructs and their inter-recombination potential (*see* **Note 7**). These retroviral structural gene products (*gag*, *pol*, and *env*) are provided in *trans* by separating them from the elements required for packaging retroviral RNA into virions without the concomitant production of replication competent virus, thus creating helper-free retrovirus-packaging cells. Retroviral particles produced in this way can infect target cells and transmit the gene of interest, but not replicate, because genomic information encoding the packaging proteins is not carried in the packaged retroviral particles.

The following transfection/infection procedure describes the optimized conditions for the cell system routinely used in our laboratory. Depending on the particular cell lines to be transfected/infected the following parameters are suggested to be chosen for optimization: method of transfection of packaging cells, time of infection (stage of growth), polybrene concentration, polybrene exposure time, use of deglycosylation agents such as tunicamycin, temperature of infection, spin/non-spin infection *(16)*. It is recommended to test the efficiency of the transfection/infection procedure on the chosen cell system prior to transfection of the library using a plasmid coding for an easily detectable reporter protein (for example GFP, luciferase, or beta-galactosidase).

1. Phoenix-Eco cells are plated out in 6-well plates in 3 ml $DMEM_{10}$ the day before transfection at a density of $1 \times 10^6$ cells per well (*see* **Note 8**). The following day the Phoenix-Eco cells are transfected with 1 µg of the cDNA plasmid library per well using FuGENE 6 transfection reagent (Roche Applied Science). Growth medium is replaced by 2 ml of fresh $DMEM_{10}$, while the transfection mixture is prepared according to the manufacturer's instructions. Briefly, 94 µl $DMEM_0$ (serum-free!) and 6 µl FuGENE reagent are mixed and incubated at room temperature for 5 min after which 1 µg plasmid DNA is added and incubated for an additional 15 min at room temperature. 100 µl of the reagent mixture is then added carefully in a dropwise fashion to the Phoenix-Eco cells and incubated for 24 h first at 37°C/5%$CO_2$ followed by another 24 h incubation, this time at 32°C/5%$CO_2$ (*see* **Note 9**).
2. The virus-containing supernatant of the Phoenix-Eco cells is harvested after the 48-h culture period and immediately used for infection of the recipient cell line (*see* **Note 10** and **Subheading 3.3** for choice of recipient cell line). For the screening procedure described here, the murine embryonic fibroblast

cell line NIH3T3, which has been plated out on 6-well plates in $DMEM_{10}$ at a cell density of $1 \times 10^5$ the previous day (*see* **Note 11**), is used for infection. NIH3T3 culture medium is removed and the viral supernatant (2 ml per well) containing 5 μg/ml polybrene filtered through 0.45-μm filters is directly added onto the NIH3T3 cells (*see* **Note 12**). The plates are then centrifuged at 1250*g* for 90 min at 25°C to increase infectivity, presumably because of virions attached to cellular debris are spun onto cells. The cells are then incubated overnight at 37°C/5%$CO_2$. The medium is replaced the following day by fresh $DMEM_{10}$ containing 0.4 mg/ml G418 to select for neomycin resistance. The pool of G418-resistant cells now represents the stable library (*see* **Note 13**).

### 3.3. Screening of the Library

To screen cDNA libraries for cell surface molecules various systems have been developed for the isolation of cDNA clones: cDNA probes *(17)*, antibody capture/panning *(18–20)*, FACS *(21)*, magnetic bead capture *(10)*, or the visual isolation by fluorescent microscopy screening *(9)*, the latter being preferred in our laboratory for the reasons discussed later. Although FACS is a standard method to isolate cells based on specific receptor–ligand interactions it is not sensitive enough to rescue rare cDNAs encoding receptors, and the isolation of false-positives being a major disadvantage. The use of magnetic beads coated with antibodies or ligands for the isolation of cells expressing surface molecules proved to be quick, simple, and effective in screening a cDNA library *(10)* but requires the expression of the cDNA library in a non-adherent cell line. One such cell line, FDC-P1, has been successfully used for retroviral expression system construction previously *(22)* as it proliferates rapidly and is considerably infectable under optimized conditions (*see* **Note 14**). However, the adherent cell line NIH3T3 is routinely and very efficiently used for retroviral transduction with an infection efficiency considerably higher than FDC-P1 cells which is essential for the expression of rare inserts of the library. Using NIH3T3 cells as the expressing cell line allows screening for ligand–receptor binding visualized by fluorescent microscopy. Although very laborious and time consuming this method largely overcomes the problems of insensitivity and isolation of false-positives and provides a means of visual estimation of ligand binding (*see* **Fig. 1c**).

1. Using fluorescent microscopy as the method of choice for screening for surface receptors with a known fluorescently labelled ligand, the background binding must be kept as low as possible. This is essential as the eyes become too easily tired with too many non-specific fluorescent particles being present. For any particular application, the library-expressing cells as well as the incubation conditions for ligand binding (time and

temperature of incubation) have to be tested in preliminary experiments very carefully and optimized accordingly to minimize any non-specific background binding. As a control and to test the screening process it is advisable to express a known receptor in the chosen recipient cell line for which a ligand is available. Also, a macrophage cell line (such as RAW 264.7) should be used to optimize the binding conditions for the particular ligand to be screened with. For example, incubation of NIH3T3 cells with FITC-labelled zymosan as done for the isolation of dectin-1 *(9)* does not lead to non-specific background binding of the fluorescent particles as shown in **Fig. 1a**; therefore, NIH3T3 cells are suitable as the expression cell line for the library. In contrast, RAW 264.7 macrophages which were used for the isolation of mRNA and generation of the library clearly bind zymosan as shown in **Fig. 1b**.

2. When choosing a ligand it must be suitable for, and easily detectable by, fluorescent microscopy. Both fluorescence intensity and particle size should be considered with GFP-expressing microorganisms or FITC-labelled particles/beads coupled to a ligand or antibody being optimal. Particle size should not be smaller than approximately 2 μm.
3. In order to screen NIH3T3 cells stably expressing the cDNA library prepared from RAW 264.7 macrophages for expression of the β-glucan receptor, the cells are transferred to G418-free culture medium with approximately ten FITC-labelled zymosan particles per cell added and incubated for 1h at 37°C/5%$CO_2$. Cells are washed three times with culture medium $DMEM_{10}$ to wash off all unbound zymosan particles and then incubated in $DMEM_{10}$ containing 0.4 mg/ml G418. The screening is done by fluorescent microscopy searching for FITC-zymosan binding cells by moving the plate on the microscope stage manually. Once a positive cell is detected the spot is marked with a black marker pen for subsequent isolation under sterile conditions. To isolate the cell(s) at the marked spot(s) growth medium is removed and the spot is ringed by a cloning cylinder using Dow Corning High Vacuum grease. Alternatively, the back of a 100-μl (yellow) or 1,000-μl (blue) standard pipette tip dipped carefully into sterile grease can be used to ring the marked spot. It should be checked by microscope that the grease ring is complete to avoid subsequent leakage. A drop of pre-warmed lidocaine/EDTA is added into the ring, incubated for 1–2 min, and cells are recovered from the ring and transferred into fresh 2 ml $DMEM_{10}$ containing 0.4 mg/ml G418 in a well of 6-well plate (*see* **Note 15**). The cells are then expanded in culture for 4–7 days until they reach approximately 80% confluence. The screening and isolation procedure is repeated until the majority of cells are shown to

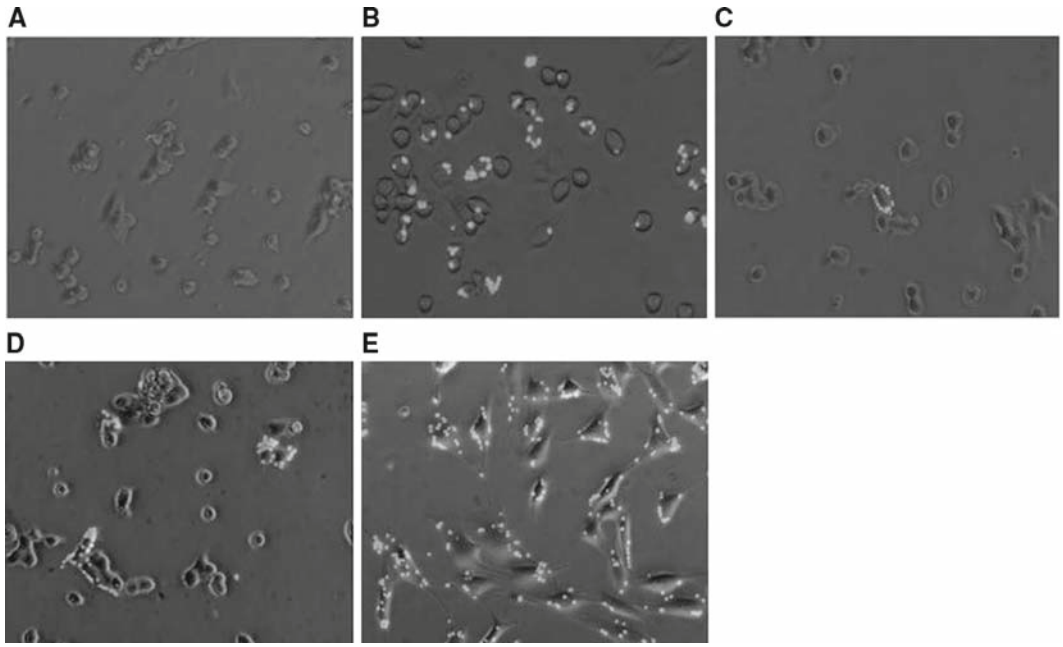


Fig. 1. Binding of FITC-labelled zymosan to negative control cells NIH3T3 (**A**) and positive control cells RAW 264.7 (**B**) using a FITC filter on a Zeiss inverted microscope Axiovert 40 CFL at 20× magnification. **C–E** shows the enrichment of positive FITC-zymosan binding cells during the screening procedure. Starting off with one positive cell (**C**) the following re-screen results in the isolation of a patch of positive cells (**D**) with their subsequent enrichment after a few screening rounds (**E**).

be positive in the functional screen. As shown in **Fig. 1c–e**, a single positive cell which binds FITC-labelled zymosan (**Fig. 1c**) isolated together with many negative cells after the first screening will grow in culture and might give rise to a patch of 5–10 cells seen when re-screened (**Fig. 1d**). Isolation of this cell patch will result in the appearance of many cell patches in the next screen until the cells are finally enriched with the majority binding to the ligand (**Fig. 1e**).

### *3.4. Identification and Functional Testing of Positive Cell(s)*

Once positive ligand-binding cells have been enriched after a couple of screening rounds, genomic DNA is isolated from the cells and conventional PCR is used to recover the proviral cDNA inserts which are stably inserted into the genome.

1. For the genomic DNA isolation approximately $1 \times 10^6$ positive cells are plated out the previous day and harvested using a commercially available kit such as the QIAamp® DNA Mini Kit (Qiagen) according to the manufacturer's instructions (*see* **Note 16**). For the subsequent PCR using the primers 5′ Retro and 3′ pFB-Neo (Stratagene) which correspond to the retroviral vector sequences flanking the multiple cloning site and therefore the individual cDNA fragments, 10 ng of genomic

DNA is used along with the Advantage® 2 PCR Kit (Clontech) (*see* **Note 17**). Due to the complexity of the genomic DNA and the high $T_m$ of the primers the PCR cycling parameters have been optimized as follows: 5 min 95°C; 35× (1 min 95°C; 30 s 80°C; 30 s 75°C; 30 s 70°C; 4 min 72°C). Running an aliquot of the PCR reaction on a 1% agarose/ethidium bromide gel one would expect several fragments of different sizes due to the retroviral MOI of approximately 10 under the described conditions (*see* **Note 13**).

2. The PCR reaction is purified using a commercially available kit such as Promega's Wizard® SV Gel and PCR Clean-Up System and directly cloned into the vector pCR®4-TOPO® using the TOPO TA Cloning® Kit for Sequencing (Invitrogen) according to the manufacturer's instructions (*see* **Note 18**).
3. While selected clones are sent off for sequencing of the inserts they can be further cloned into suitable vectors which allow for their functional testing. To test their expression and ligand binding in transient transfections the inserts of selected clones are amplified in a PCR using the primer 3′ pFB-Neo and a modified primer 5′ Retro containing the sequence CACC at its 5′ end which allows directional cloning into the vector pcDNA™3.1D/V5-His-TOPO® using the pcDNA™ 3.1 Directional TOPO® Expression Kit (Invitrogen) according to the manufacturer's instruction (*see* **Note 19**). Alternatively, the recovered proviral cDNA inserts can be recloned into the retroviral vector pFBneo using the unique *Sfi*I restriction sites present in their 5′ and 3′ flanking regions. As outlined earlier, *E. coli* cells are transformed with this recloned cDNA and plasmid DNA isolated to transfect virus-producing cells for subsequent infection of the recipient cells and generation of stable cell lines expressing the various cloned cDNA fragments individually. For validation of successful recloning of the correct cDNA clone, these cells are then functionally analysed for ligand binding as stated earlier.
4. Sequence data are then analysed by accessing available gene bank databases. This might lead to the re-identification of a known receptor as in the case of dectin-1 *(9)*. In case of the identification of an unknown receptor or surface molecule, sequence analysis will provide information of its nature and helps to group the receptor into its relevant receptor class according to its sequence and structural properties. Further molecular and functional characterization of the identified molecule might then include its expression profile in different macrophage populations and tissues, the determination of its ligand specificity, and the investigation of its immunomodulatory effects.

## 4. Notes

1. The quality and integrity of both the total RNA and the mRNA should be checked on a denaturing formaldehyde/agarose gel before proceeding with the protocol. Total RNA from mammalian sources should appear as a faint smear with two bright bands corresponding to 28S rRNA and 18S rRNA, while intact mammalian mRNA should appear as a smear (usually 0.5–12 kb) with faint 28S and 18S rRNA bands. Also, the concentration of both total RNA and mRNA should be determined by measuring the absorbance at 260 nm ($A_{260}$) in a spectrophotometer (with $A_{260}$ = 1 equivalent to 40 ng/µl RNA) to estimate the mRNA yield – keeping in mind that mRNA accounts for only 1–5% of the total RNA in a typical mammalian cell (although the actual amount depends on the cell type and physiological state) with approximately 12,000 different mRNA species per cell. To ensure highly pure mRNA before proceeding with the library construction the ratio of the readings at 260 nm and 280 nm ($A_{260}/A_{280}$) should be determined with pure RNA having an $A_{260}/A_{280}$ ratio of 1.9–2.1. Both total RNA and mRNA should be stored at –80°C; repeated freezing and thawing should be avoided.
2. For further information on the underlying SMART™ mechanism please refer to the Clontech User Manual for the Creator™ SMART™ cDNA Library Construction Kit.
3. *Sfi*I sites are extremely rare in mammalian DNA; therefore, *Sfi*I-digested cDNA remains intact.
4. It has been observed regularly in our laboratory that pooling 2–3 fractions before the actual peak fractions (i.e. where the cDNA amount is too low to be detected on the gel) usually contains both large cDNA fragments and cDNA fragments in the medium range. Pooling the peak fractions can result in collecting too many small cDNAs (i.e. smaller than 500 bp).
5. The *Sfi*I-digested control insert contained in the Creator™ SMART™ cDNA Library Construction Kit (Clontech) is ligated into the provided *Sfi*I-digested vector pDNR-LIB and afterwards cut out with the restriction enzymes *Eco*RI and *Xho*I flanking the ligated control insert in the vector. The *Eco*RI/*Xho*I-digested insert in turn is ligated into the *Eco*RI/*Xho*I-digested vector pFBneo allowing its subsequent digestion with *Sfi*I and cloning of the *Sfi*I-digested cDNA fragments.
6. Colonies are picked randomly and checked for insert size distribution by colony PCR using a commercially available PCR

kit such as Fermentas' 2× PCR Mastermix and the pFBneo vector primers 5′ Retro and 3′ pFB-Neo with the following cycling parameters: 5 min 95°C; 30× (1 min 95°C; 1 min 65°C; 4 min 72°C).

7. To monitor *gag–pol* expression on a cell-by-cell basis (i.e. with the help of flow cytometry) an IRES-CD8 surface marker has been introduced downstream of the *gag–pol* reading frame.
8. Phoenix-eco cells are kept in selective medium (1 μg/ml diphtheria toxin; 300 μg/ml hygromycin) constantly except for the transfection experiment. For each pool of the library to be transfected prepare two wells of one 6-well plate. Normally three pools of the library can be handled simultaneously in the subsequent screening.
9. The incubation at 32°C overnight has been shown to be crucial for library infection efficiency since prolonged incubation at 37°C leads to reduced virus titre due to instability of the virus particles.
10. The virus-containing supernatant (filtered and polybrene added) can be stored at –80°C although this might reduce infection efficiency by approximately 50%. For library transduction it is recommended to use fresh virus-containing supernatant for infection.
11. It is important to infect replicating cells with those in exponential phase being most efficiently infected since retroviral infection is largely inhibited in non-replicating cells *(16)*.
12. Polybrene facilitates infection, presumably through its polycationicity, neutralizing negative charges on the surface of target cells and reducing electrostatic repulsion of virions. However, it is toxic to cells and should be kept to a minimum concentration. While the infection of NIH3T3 cells requires 5 μg/ml polybrene, infection of the non-adherent cell line FDC-P1 is generally performed in our laboratory in the presence of 20 μg/ml polybrene. Infection of the murine macrophage cell line RAW 264.7 in the presence of 5 μg/ml polybrene is very low but can be improved by addition of 0.2 μg/ml tunicamycin to the growth medium the day before infection.
13. Under the experimental conditions for the transfection and transduction described here one would expect a MOI of the virus titre of approximately 10, i.e. ten infection events per NIH3T3 cell. Therefore no cell death is expected due to G418. The initial number of $1 \times 10^5$ NIH3T3 cells per well might appear low, but the cells will grow for more than 3 days until the start of the screening and should not be confluent then. If the cells are confluent one should consider

splitting them into two or more new wells to avoid them coming off during the screen. Ideally, the cells should be 80–90% confluent on the day of screening.

14. The optimized conditions for infection of the non-adherent cell line FDC-P1 are as follows: $2 \times 10^5$ FDC-P1 cells are added to 1.5 ml viral supernatant in a 6-well plate with 20 μg/ml polybrene. Cells are centrifuged for 90 min at 1250 *g* at 25°C, agitated by rocking the plate gently back and forth to lift adherent cells and incubated at 37°C/5%$CO_2$ overnight. The medium is changed the following day and selected for neomycin-resistant cells 48 h post-infection.
15. The incubation with lidocaine/EDTA for more than 5 min has shown to decrease the viability of the cells. Also, trypsin should be avoided since it might stress the cells too much and decreases viability. Special care must be taken at the first screening and isolation with only one positive cell detectable among lots of negative cells. It should be checked by microscope that all cells in the ring have been transferred. Further re-screening and isolation steps will involve enriched positive cells which do not necessarily need to be isolated completely.
16. It is strongly recommended to use a method for the genomic DNA isolation that is not based on phenol/chloroform, since this has shown to limit the sensitivity of the downstream PCR significantly.
17. This PCR kit has performed best in our laboratory compared to several other polymerases tested in terms of amplified fragment sizes. For any PCR problems such as amplification of fragments greater than 3 kb or very rich in GC one should consider to use a specific PCR kit for long-distance PCR or GC-rich PCR, respectively.
18. The cloning system preferentially ligates small fragments of the PCR mixture into the vector. To clone large DNA fragments the PCR mixture could be run on a preparative gel and the desired DNA sizes cut out, purified, and added to the TOPO-cloning mixture.
19. The direct amplification of the cDNA inserts from the genome using the modified primers allowing directional cloning into the expression vector pcDNA™3.1D/V5-His-TOPO® has been shown to be unsuitable due to decreased primer specificity and decreased efficiency with the proofreading polymerases tested.

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# Chapter 2

# Generation of Murine Growth Factor-Dependent Long-Term Dendritic Cell Lines to Investigate Host–Parasite Interactions

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## Summary

Substantial progress has been made over the last several years in the development of protocols for the isolation of large numbers of dendritic cells (DCs) from different tissues and their short-term culture. Indeed, several stable DC lines and clones have been established from various tissues of mice and humans, providing useful experimental tools for studying the biology of DCs at both molecular and biochemical levels and for the establishment of new DC-based immunotherapies. In this chapter, we will describe the development of long-term DC lines that maintain the growth factor dependence and their immature functional state, thus providing a unique opportunity to study the mechanisms of the initiation of the immune response to infectious agents.

**Key words:** Dendritic cell lines, Host–parasite interaction, GM-CSF, Bone marrow, Bacteria, Immune response.

## 1. Introduction

Dendritic cells (DCs) are distributed especially in tissues that interface with the external environment, such as the skin, the gut, and the lungs *(1–3)*. At these locations, they can perform a sentinel function for incoming pathogens, and have the capacity to recruit and activate cells of the innate immune system upon inflammation *(4–6)*. Uptake of pathogens by DCs induces a state

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of activation, which eventually leads to the migration of the antigen-loaded DCs to the lymphoid organs where the cells of the adaptive immune response can be alerted *(7)*.

Until the last decade, the paucity of DCs in most tissues and the lack of specific markers have hampered the study of DC ontogeny, phenotype, and function. Several groups have succeeded in generating large numbers of functional DCs/Langerhans cells in murine or human systems by treating DC precursors with granulocyte-macrophage colony-stimulating factor (GM-CSF) alone or in combination with other growth factors *(8–13)*. However, such DCs could be propagated only for limited periods of time. Growth factor-dependent long-term DC lines from mouse fetal or newborn skin have been established *(14, 15)*. Nevertheless, although these lines possess some properties of DC precursors and maintain an immature phenotype, they cannot be induced to mature in vitro *(14, 15)*.

In recent years, immortalization of DCs from mouse tissues, using retroviral vectors carrying immortalizing oncogenes, has successfully been achieved *(16–18)*. The immortalized DC lines are homogeneous, easily grown, and do not require growth factors for their propagation, being extensively used for functional and biochemical DC characterization. Indeed, the cells exhibited phenotypic and functional features of immature DCs, including the ability to present exogenous antigens on class I and class II molecules and the capacity to induce primary T-cell response in vitro and in vivo *(19–21)*. However, the inability to induce growth factor arrest in these immortal lines has hampered the complete maturation of DCs upon activation.

In the mouse system, we succeeded in generating long-term growth factor-dependent immature DC lines, derived from adult mouse spleen *(22)* or mouse bone marrow. In this system, proliferation and survival are strictly dependent upon the presence of exogenous murineGM-CSF (mGM-CSF) and fibroblast-derived growth factors *(22)*. Long-term DCs preserve an immature phenotype. Activating signals, such as living bacteria, Toll-like receptors agonists and cytokines, promote full maturation of these DC lines, such as the D1 cells, mimicking and recapitulating the whole natural process of DC differentiation that occurs in response to activation by pathogens in vivo.

This chapter focuses on in vitro techniques leading to the establishment of murine long-term DC lines, similar to the D1 cells that have been widely used *(23–26)*. Using a conditioned medium (R1 medium) containing several cytokines, including mGM-CSF produced by GM-CSF-transfected NIH/3T3, we show that DCs can be stimulated to undergo maturation in response to live bacteria, thus allowing investigation of host–pathogen interactions *(22, 27)*.

## 2. Materials

1. *Iscove's Modified Dulbecco's Medium (complete medium)*. IMDM supplemented with 10% heat-inactivated fetal bovine serum (FBS) of Australian origin by Gibco BRL, 2 mM L-glutamine, 100 IU/mL penicillin, 100 µg/mL streptomycin, and 0.05 mM 2β-mercaptoethanol (*see* **Note 1**).
2. *DC-GM medium (R1 medium)*. IMDM complete medium supplemented with conditioned medium produced by mGM-CSF-transfected NIH/3T3 fibroblasts (available upon request from P. Ricciardi-Castagnoli) corresponding to 10 ng/mL of mGM-CSF as final concentration. DC medium is stable at 4°C for up to 3 weeks. (For conditioned medium preparation *see* **Subheading 3.1** and **Note 2**).
3. *Phosphate-buffered saline (PBS), pH 7.2–7.4*. 154 mM NaCl, 8.1 mM $Na_2HPO_4$ ($7H_2O$), 1.9 mM $NaH_2PO_4$ ($H_2O$).
4. *Red blood cell (RBC) lysis buffer*. 105 mM $NH_4Cl$, 10 mM $KHCO_3$, 0.1 mM $Na_4EDTA$.
5. *PBS-EDTA*. Solution of PBS supplemented with 2 mM EDTA.
6. *Composition of Luria Bertani (LB) medium*. Tryptone 10 g/L, yeast extract 5 g/L, NaCl 10 g/L.

## 3. Methods

### 3.1. Production of Conditioned Medium Containing mGM-CSF

1. NIH/3T3 fibroblasts stably transfected with mGM-CSF ($1 \times 10^6$ in 7 ml) are plated in 100 × 20 mm tissue-cultured-treated petri dish using IMDM medium supplemented with 5% heat-inactivated FBS of Australian origin by Gibco BRL, 2 mM L -glutamine, 100 IU/mL penicillin, 100 µ g/mL streptomycin, and 0.05 mM 2β-mercaptoethanol (*see* **Note 3**).
2. The conditioned medium is collected from culture after 3 days and centrifuged at 270 × *g* for 5 min to pellet floating cells and debris. Then, the supernatant is filtered through a 0.2 µm filter and aliquots stored at –20°C.
3. In order to measure the concentration of mGM-CSF, a specific enzyme-linked immunoabsorbent assay should be performed.

### 3.2. Preparation of Cell Suspensions from Bone Marrow

1. Mice are euthanized using $CO_2$ asphyxiation followed by cervical dislocation (*see* **Note 4**).
2. The skin of mouse hind legs is pulled back and the muscles dissected to expose femur and tibia (*see* **Note 5**).

3. Using a scissors and a pair of tweezers, the knee joint is cut, the muscle along the femur is sliced and the bone is severed from the hip joint. Most of the muscle tissues surrounding the bone should be eliminated with the scissors.
4. After removal of both femurs and tibias, all bones are washed twice in PBS and placed in a 60 × 15 mm petri dish containing 5 mL of IMDM complete medium.
5. Cutting both bone edges using scissors exposes the cavity lumen. Then, the BM is flushed out using a 5-mL syringe and 25-G needle filled with IMDM complete medium. Flush the bone cavity until it appears white.
6. To disaggregate clusters of BM cells, the cell suspension is passed through the syringe and filtered using a cell strainer (pores diameter = 70 μm) into a 50-mL tube.
7. Cells are recovered by centrifugation and the pellet resuspended in 10 mL R1 medium.
8. Finally, cells are counted using a Bürker chamber.

### 3.3. Preparation of Cell Suspensions from Spleen

1. Mice are euthanized using $CO_2$ asphyxiation followed by cervical dislocation (*see* **Note 4**).
2. Spleens are isolated from mice, placed in a 60 × 15 mm petri dish containing 5 mL of IMDM complete medium, and cut into small pieces. A single cell suspension is obtained by smashing the spleens using a 5 mL-siring plunger until mostly fibrous tissue remains.
3. To avoid cell aggregates, the suspension is filtered through 70-μm cell strainers.
4. The suspension is transferred to a 15-mL tube and centrifuged for 5 min at 400 × *g*.
5. At this stage, the removal of RBC from the spleen cell suspension is necessary. For this purpose, the pellet is resuspended in 1–2 mL of RBC lysis buffer and incubated at 4°C for 9 min. 10 mL IMDM complete medium is added and the aggregates removed by filtration through 70-μm cell strainer. The mononuclear cells are recovered by centrifugation at 270 × *g* for 5 min. The supernatant is discarded and the pellet is resuspended in a suitable volume of R1 medium for counting.

### 3.4. Generation of Mouse Long-Term Growth Factor-Dependent DC Lines

1. Unfractionated cell populations from BM or spleen are plated at a density of $4 \times 10^5$ cells/mL in 100 × 20mm suspension culture dishes in R1 medium and incubated at 37°C with 5% $CO_2$ (*see* **Note 6**).
2. The culture is fed with fresh R1 medium without the addition of exogenous IL-4 every 3–4 days (*see* **Note 7**).

3. Usually the first passage of DC-enriched culture is performed around day 7, when the cell density is high, using a solution of PBS-EDTA. Then, the cells are counted and replated at a concentration of 2–3 × $10^5$ cells/mL (*see* **Note 8**).
4. Once a week, both suspended and weakly adherent cells are collected using PBS, centrifugated at 270 × *g* for 5 min, and seeded at a density of 2–3 × $10^5$ cells/mL (*see* **Note 9**).
5. After 3 months of continuous culture, in vitro growing cells can be split every 3–4 days using PBS-EDTA.
6. At this point, long-term growth factor-dependent DC lines are established (*see* **Notes 10** and **11**).
7. The established long-term culture retains a characteristic immature DC phenotype, and the cells can be induced to mature in vitro into terminally differentiated DCs upon activation with a number of stimulatory signals, such as LPS, bacteria, or cytokines. In **Fig. 1,** the phenotypical maturation of BM-derived DC lines induced by LPS is shown.

### 3.5. Growth of Bacterial Culture

1. A single colony of DH5α *E. coli* bacteria is inoculated into 2 mL of LB medium in a loosely capped 15-mL tube. The culture is incubated for ~8 h at 37°C with vigorous shaking. Using a vessel with a volume of at least four times greater than the volume of medium, the starter culture is diluted 1/500 to 1/1,000 into a larger volume of LB medium and grown with vigorous shaking to saturation (12–16 h, overnight). (*see* **Notes 12** and **13**)
2. The bacterial culture is then diluted 1/10 with LB medium and incubated for ~2.5 h at 37°C with shaking. Growth is monitored every 20–30 min by spectrophotometer and stopped in late-log-phase corresponding to $OD_{600}$ ~0.6.
3. The concentration of the bacterial culture can be calculated using the following formula:

   $y = -29 + 1{,}191\ (OD_{600}) \times 10^6$ bact/mL (*see* **Note 14**).
4. The bacterial cells are harvested by centrifugation at 6,000 × *g* for 2 min at 4°C.
5. The LB medium is removed by aspiration, leaving the bacterial pellet as dry as possible. Bacteria are resuspended in 1 mL of R1 medium without antibiotics per mL of bacterial culture (*see* **Note 15**).

### 3.6. Bacterial Infection of DCs

1. The day before the infection, DC lines are harvested using a solution of PBS-EDTA and centrifuged at 250 × *g* for 5 min.
2. After a wash with PBS, DC lines are resuspended in an appropriate amount of R1 medium for counting.

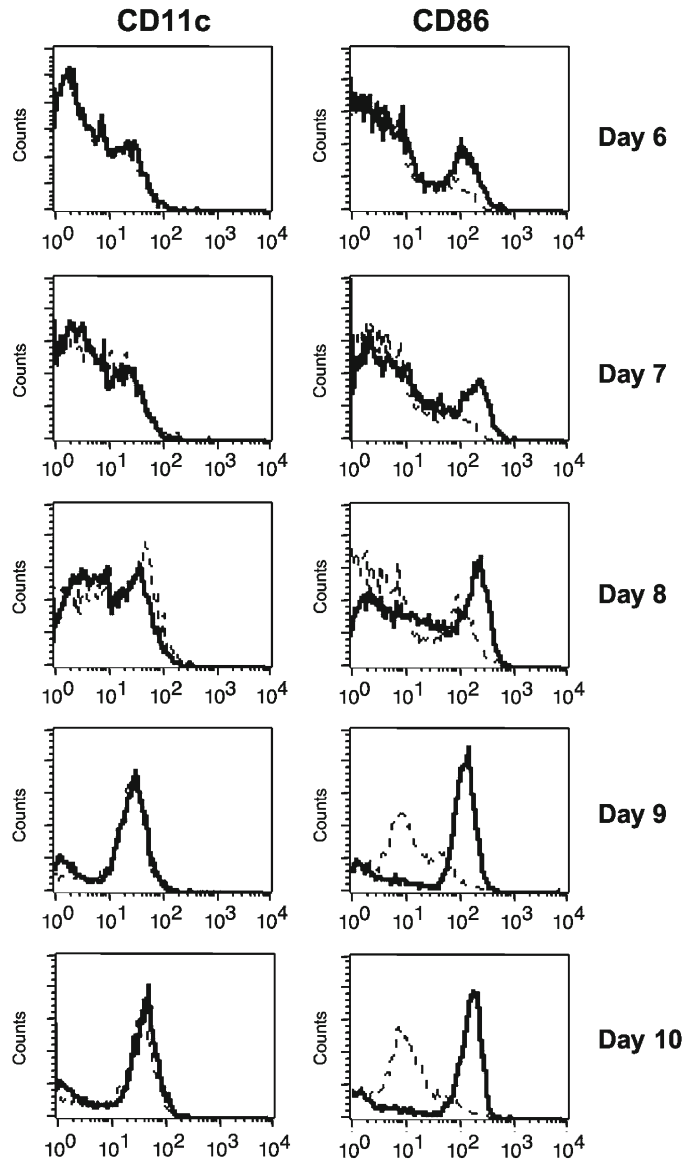


Fig. 1. Analysis of cell surface marker expression in BM-derived DC line cultured in R1 medium. The cells have been harvested at the indicated time and stimulated with LPS (1 μg/mL) for 24 hours. The expression of CD11c and CD86 markers was evaluated by flow cytometry. Dashed histogram: untreated cells; bold histogram: LPS-treated cells.

3. Cells are plated at a concentration of 2–3 × $10^5$/mL in suspension petri dishes and incubated at 37°C 5% $CO_2$ for 16–18 h.
4. The R1 medium is removed and replaced with fresh R1 medium without antibiotics (*see* **Note 16**).
5. DC lines are infected with *E. coli* bacteria at a bacteria:DC ratio of 10:1 and incubated at 37°C 5% $CO_2$ for 1 h (*see* **Note 17**).

6. At this point, the medium is discarded and the petri dishes washed gently with PBS. Finally, fresh R1 medium supplemented with gentamicin (50 µg/mL) and tetracycline (10 µg/mL) is replated, and the cell culture is incubated at 37°C 5% $CO_2$ for the desired time.
7. Bacteria are efficient in upregulating the expression of DC maturation surface markers, such as CD80, CD86, MHC class II, and CD40. In **Fig. 2**, the upregulation of costimulatory molecules induced by bacteria in DC lines derived from BM and spleen is shown, as assessed by FACS analysis.

## 4. Notes

1. To optimize DC growth conditions, different batches of sera should be tested prior to use. In order to avoid phagocytosis of debris by DCs, it is optimal to filter complete medium using 0.2-µm filter.

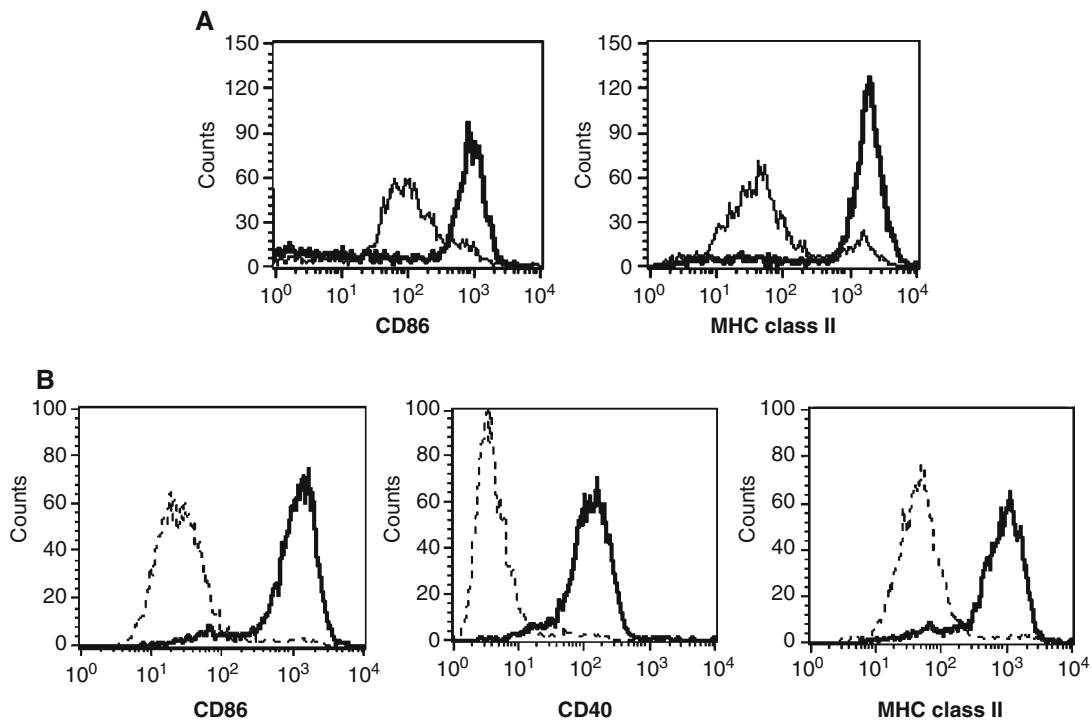


Fig. 2. Phenotypical maturation of BM- and spleen-derived long-term GM-CSF-dependent DCs. BM- (**A**) and spleen-derived (**B**) immature DCs were stimulated with DH5α *E. coli* bacteria (moi: 10) for 1 h, and the expression of CD86, CD40, and I-A MHC class II cell surface markers was measured by flow cytometry. Dashed and bold histograms show untreated cells and cells challenged with DH5α *E. coli* bacteria, respectively.

2. The ideal final concentration of mGM-CSF in the conditioned medium produced by mGM-CSF-transfected NIH/3T3 should be around 30 ng/mL.
3. In order to produce optimal conditioned medium, mGM-SCF-transfected NIH/3T3 fibroblasts should be plated starting from a semiconfluent culture.
4. In general, 8–12 weeks old mice yield sufficient numbers of DCs, precursors, or progenitor cells, and organs of sufficient size to be easily manipulated. Mice younger than 6 weeks may yield fewer cells or to be too small to manipulate easily, and mice older than 12 weeks may be more expensive with no additional gain in cell yield. The use of specific pathogen-free mice is recommended to avoid pathogen-specific effect. To minimize the effect of the anesthetizing or euthanatizing agents that may perturb DC function, $CO_2$ asphyxiation followed by cervical dislocation is used to kill mice.
5. Both femurs and tibias should be removed to isolate maximal numbers of mononuclear cells from a mouse. Femurs provide 80% of total BM cells.
6. DC lines tend to adhere strongly to plastic. Thus, the use of suspension culture dishes is highly recommended to obtain and culture DC lines.
7. Half of the medium is collected and the floating cells harvested by centrifugation. Then, the cell pellet is resuspended in fresh R1 medium and replated.
8. Cellular growth is monitored daily by microscopy. Proliferating cells are visible in suspension as clusters with veiled morphology.
9. Cell types other that DC precursors also respond to mGM-CSF and grow in R1 medium. For example, macrophages differentiate and strongly attach to the petri dish. These are discarded at each passage by harvesting only cells in suspension. Other major contaminants of nonadherent DCs are granulocytes (especially when starting from BM) visible as clusters of round cells from day 2. However, the majority of granulocyte contaminants tend to die in culture and are absent by the time DCs are harvested. It is often necessary to wash the cell pellet with PBS, to discard debris and dead cells.
10. In order to monitor the differentiation process of DCs, cell surface marker expression is analyzed by flow cytometry. Generally, it is found that the approaches described earlier generate CD11c+ DCs at 80–95% purity.
11. The DC lines once established could be continuously maintained for years but should not kept in culture. As these DC lines are not clonally selected, it is necessary to prepare frozen stocks of the cells kept as a backup storage. The cell

growth is strictly dependent on the presence in the culture medium of mGM-CSF. Indeed, growth factor deprivation leads to cell growth arrest and cell death.

12. Bacterial cultures should always be grown from a single colony picked from a freshly streaked plate. Subculturing directly from glycerol stocks, agar stabs, and liquid cultures is a poor microbiological practice. Inoculation from plates that have been stored for a long time may also lead to liquid culture containing different bacteria. The desired clone should be streaked from a glycerol stock onto a freshly prepared agar plate such that single colonies can be isolated. This procedure should then be repeated to ensure that a single colony is picked.
13. To avoid bacterial death, LB medium should be prewarmed at 37°C before bacteria inoculation. It is often convenient to grow the starter culture during the day and the larger culture overnight for harvesting the following morning.
14. Usually, $OD_{600}$ = 0.6 corresponds to $6.85 \times 10^8$ bacteria/mL. Because the relationship between the $OD_{600}$ and the number of viable cells per mL varies substantially from strain to strain, it is essential to calibrate the spectrophotometer. This can be achieved by plating serial dilutions of a bacterial culture onto LB agar plates in the absence of antibiotics. The counted colonies are used to calculate the number of bacteria per mL, which is then set in relation to the measured $OD_{600}$ values.
15. The supernatant can be conveniently withdrawn with a disposable pipette tip attached to a vacuum line. Use gentle suction to avoid drawing the pellet into the pipette tip. Any droplets of liquid adherent to the walls of the tube should be removed by vacuum. It is essential to ensure that the bacterial pellet is completely dispersed.
16. It is critically important to remember to not add antibiotics to the R1 medium at this stage to avoid bacterial death.
17. The amount of bacteria used to challenge DC lines can vary depending on the aim of the experiment. It is possible to use lower bacteria:DC ratios and to extend the incubation period to 16–18 h.

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# Chapter 3

# Macrophage Activation: Classical vs. Alternative


## Andrea Classen, Jorge Lloberas, and Antonio Celada



## Summary

Macrophages are involved in both innate and adaptative immune responses. Depending on the types of cytokines that macrophages are exposed to, these cells are subjected to classical (Th1) or alternative (Th2) activation. In the first case, macrophages, particularly when activated by interferon gamma (IFN-γ) or by lipopolysaccharide (LPS), have the capacity, through the production of NO and other intermediates, to destroy the remaining microorganisms in the inflammatory loci. In the second case, after exposure to cytokines such as IL-4, IL-10, or IL-13, macrophages produce polyamines and proline, which induce proliferation and collagen production, respectively. Interestingly, in both classical and alternative activation, the essential substrate that drives these pathways is the amino acid arginine. NO synthase 2 (NOS2) is induced by IFN-γ or LPS and degrades arginine into OH-arginine and then into NO. Arginase is induced by Th2-type cytokines, which convert arginine into ornithine and subsequently into polyamines and proline. In this chapter, we present simple and direct methods for analyzing the properties of macrophage populations to determine whether they exhibit either a classical or alternatively activated phenotype.



**Key words:** Macrophage, Classical activation, Alternative activation, Nitric oxide synthase 2, NO, NOS2, Arginase 1, Cytokines.


## 1. Introduction

Macrophages play an important role in immune responses, both in innate and in acquired immunity. These cells have multiple functional activities not only in the immune system but also in the regulation of lipid metabolism, iron metabolism, wound healing, etc. When the body is invaded by microorganisms, the first cells that reach the inflammatory loci are neutrophils, which


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generate large amounts of damaging products in order to destroy the invading microorganisms. After 24 h, macrophages are transported in blood to the inflammatory tissues, where they remain until the resolution of the inflammatory process. Initially, macrophages are required to kill the microorganisms that have resisted the action of neutrophils. This is the point at which macrophages interact with several molecules, thereby activating these cells. This activation implies that they undergo a series of functional, morphological, and biochemical modifications produced by the regulation of a large number of genes. When macrophages interact with Th1-type cytokines, such as interferon gamma (IFN-γ), or lipopolysaccharide (LPS), they produce a series of products, such as NO and oxygen-free radicals, that destroy microorganisms. This type of activation is known as classical activation or M1. When the inciting stimulus is removed from the inflammatory loci, a period of reconstruction ensues, with the removal of apoptotic cells, production of collagen, etc. During this period macrophages become activated by Th2-type cytokines, such as IL-4, IL-10, or IL-13. This type of activation is called alternative activation or M2 *(1–3)*. Interestingly, although these phenotypes exhibit quite distinct properties, they both involve metabolism of the amino acid arginine through distinct biochemical pathways to yield their ultimate characteristics. IFN-γ or LPS induce macrophage NO synthase 2 (NOS2), which converts arginine into OH-arginine and then into NO (**Fig. 1**). In contrast, when

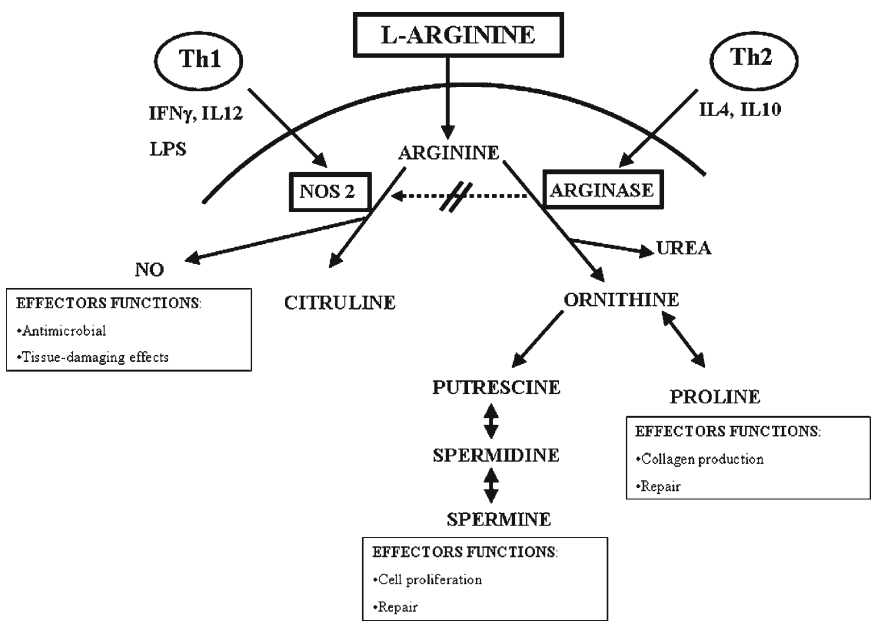


Fig. 1. Catabolism of arginine by macrophages activated by Th1-type cytokines (classical) or by Th2-type cytokines (alternative).

macrophages are activated by IL-4, IL-10 or IL-13, arginase I is induced which degrades arginine into urea and ornithine, which is then subsequently metabolized into proline and polyamines (putrescine, spermidine, and spermine). Proline mediates the production of collagen, while polyamines induce cell proliferation. Thus, alternative activation of macrophages catalyzes the reconstitution of the damaged extracellular matrix, a process that occurs during the final phases of inflammation. Interestingly, the induction of arginase blocks NOS2 at the translational level *(4)*. This observation implies that macrophages are either prepared to destroy by classical activation or to repair by the alternative mode

The entry of arginine to the cell is mediated by specific transporters. We have seen that both classical and alternative activation of macrophages induce the expression of the CAT2 transport system in order to enhance arginine import *(5, 6)*. This transport system limits macrophage activation via either pathway. Arginine is also required for cell proliferation. In this case, it is introduced into the cell by the CAT1 transport system and, once inside it is not degraded but used mainly for new protein synthesis *(7)*.

Investigators are frequently interested to know whether the properties of their particular experimental system favor the generation of M1 vs. M2 macrophages. Here we describe approaches to analyze the properties of macrophage populations including the production of NO and the generation of arginase activity. In addition, we describe a quantitative PCR approach to determine mRNA abundance for both NOS2 and arginase. Finally, we explain how to quantify the amounts of these enzymes by means of Western blots.

## 2. Materials

### 2.1. Cell Culture

1. Dulbecco's Modified Eagle's Medium (DMEM).
2. Heat-inactivate Fetal Bovine Serum at 56°C for 30 min; store aliquots at –20°C.
3. Penicillin/Streptomycin.
4. *Stimulating agents*: IFN-γ, LPS, IL-4, IL-10.
5. Cell scrapers.
6. *1× PBS (500 ml)*. 4 g NaCl; 0.1 g KCl; 0.38 g $Na_2HPO_4$ × $2H_2O$; 0.1 g KH$_2$PO4 filled up with dd$H_2O$ up to 500 ml.
7. *Buffer for cell lysis*. 1% Triton X-100; 10% Glycerol; 50 mM HEPES, pH 7.5; 150 mM NaCl protease inhibitors: add directly before using aproptinin 1 µg/ml; leupeptin 1 µg/ml; iod-acetamidin 8.6 µg/ml; PMSF 1 mM; phosphatase inhibitors: Na – ortho – vanadate ($Na_3VO_4$ mM).

### 2.2. NO Production

1. *100 μM $NaNO_2$*. Dissolve 6.9 mg $NaNO_2$ in 10 ml $H_2O$; use this stock to prepare a standard curve.
2. $H_3PO_4$.
3. Sulfanilamide ($H_2NC_6H_4SO_2NH_2$)
4. Naphthyl-Ethylenediamine-di-Hydrochloride ($C_{10}H_7NHCH_2CH_2NH_2 \cdot 2HCl \cdot MeOH$)

### 2.3. Arginase Activity

1. Eppendorf safe-lock tubes.
2. 0.1% Triton X-100 dissolved in $ddH_2O$.
3. 50 mM Tris–HCl buffer, pH 7.5.
4. 100 mM $MnCl_2$, for measuring total arginase.
5. 0.5 M Arginine, pH 9.7.
6. $H_3PO_4$.
7. $H_2SO_4$.
8. Urea stock solution 600 μg/ml diluted in $ddH_2O$.
9. Prepare an acid mix containing $H_3PO_4$, $H_2SO_4$ and $H_2O$ in a ratio 1/3/7 v/v/v and store it at 4°C.
10. 6% α-isonitrosopropiophenone (α-ISPP) in 100% ethanol; store at 4°C.

### 2.4. Quantitative Real-Time PCR

1. EZ-RNA Total RNA Isolation Kit.
2. Isopropanol.
3. 75% ethanol absolute diluted in RNase-free $H_2O$.
4. DNase and RNase-free $H_2O$.
5. RNase-free 1.5-ml Eppendorf tubes.
6. Sterile filter tips.
7. Quartz glass cuvette, 10 mm.
8. RNase-free 1.5-ml Eppendorf tubes.
9. Sterile pipette tips with filter.
10. M-MLV RT 5× Reaction Buffer, Oligo-(dT) 15 Primer, M-MLV Reverse Transcriptase, dNTP mix. Store these reagents at –20°C.
11. Power SYBR® Green PCR Master Mix.
12. *Primer*. Forward Primer Arginase 1:5′-TTGCGAGACGTAGACCCTGG-3′; $T_m$ = 67.5.
    - Reverse Primer Arginase 1:5′- CAAAGCTCAGGTGAATCGGC-3′; $T_m$ = 66.9.
    - Forward Primer NOS2: 5′-GCCACCAACAATGGCAACA-3′; $T_m$ = 60.
    - Reverse Primer NOS2: 5′-CGTACCGGATGAGCTGTGAATT-3′; $T_m$ = 60.

13. 384-well DNase- and RNase-free PCR plates.
14. PCR ultraclear film.

### 2.5. Western Blotting

1. Bradford, Protein assay Dye Reagent Concentrate.
2. *Bovine Serum Albumin (BSA)*. Prepare a stock solution of 1 mg/ml.
3. Separating gel buffer 1.5 M Tris–HCl, pH 8.8.
4. Stacking gel buffer 0.5 M Tris–HCl, pH 6.8.
5. 10% Ammonium persulfate (APS).
6. *N*,*N*,*N*′,*N*′-Tetramethylethylenediamine (TEMED).
7. *10× running buffer*. 0.25 M Trizma base; 1.92 M glycin; 1% sodium dodecyl sulfate (SDS); dissolve in $H_2O$.
8. *5× loading buffer*. For 10 ml: 0.42 ml 0.5 M Tris–HCl, pH 6.8, 2 g 10% SDS, 2.5 ml 0.7% β-mercaptoethanol. 5 ml 10% glycero, 0.5 mg bromophenol-blue-Na-salt, 0.42 ml $ddH_2O$; store at RT.
9. Prestained SDS-PAGE Standard.
10. *Transfer buffer (for 5 l)*. 15.15 g Trizma base, 72 g glycine, 1 l Methanol; make up to 5 l with water; store at 4°C.
11. Hybond ECL membranes.
12. Whatman paper.
13. *10× TBS (for 1l)*. 60.6 g Trizma base; 87.6 g NaCl; fill with $H_2O$ up to 1 l; adjust pH to 7.5.
14. Tween 20.
15. *Primary antibodies*: Anti-NOS2; anti-Arginase 1; anti-β-Actin.
16. *Secondary antibodies*. Antirabbit-horseradish-peroxidase; antimouse-horseradish-peroxidase.
17. ECL™– Western Blotting Detection reagent.

## 3. Methods

### 3.1. Cell Culture (8)

1. Prepare macrophages from bone marrow of Balb/c mice.
2. Kill mice. Remove the legs and separate the femur and tibiae. Cut open the bones and flush with DMEM using an injection needle.
3. Separate cells by pipetting up and down and culture them in DMEM supplemented with 30% M-CSF, 20% FBS, and 1% Penicillin/Streptomycin. The bone marrow extracted from

one mouse is usually divided into four Petri dishes (diameter: 15 cm).

4. Culture cells for 6 days at 37°C in 5% $CO_2$.
5. After 6 days, detach the adherent cells, an almost homogenous population of macrophages, by scraping. Centrifuge them at 500 g at 4°C for 5 min, and resuspend them in 1 ml DMEM, 10% FBS and 1% Penicillin/Streptomycin.
6. Count cells using a Neubauer cell chamber.
7. Culture cells for 18 h without the macrophage growth factor M-CSF in DMEM 10% FBS and 1% Penicillin/Streptomycin at 37°C in 5% $CO_2$. Under these conditions macrophages stop proliferating and are arrested in the G0 phase of the cell cycle.

### *3.2. NO Production* (9)

1. Harvest cells. Seed from 100,000 up to 200,000 cells per well in a 96-well culture plate. The total volume per well should be 200 µl. Allow cells to adhere well to the plate at 37°C in 5% $CO_2$ overnight.
2. The following day, treat cells with the desired stimulating agents. As a positive control, stimulate cells with LPS (100 ng/ml) in combination with IFN-γ (300 units/ml). After adding the stimulating agent, leave cells at 37°C in 5% $CO_2$ for 24 h.
3. Prepare the working agent before starting the assay because the reagents do not dissolve easily. Add 24.3 ml double-distilled water ($ddH_2O$) and 735 µl $H_3PO_4$ to 0.25 g sulfonamide. Wrap the falcon tube in aluminum foil and leave on a roller for 10 min. Afterward, add 0.025 g naphthyl – ethylenediamine – dihydrochloride and allow the components to dissolve while rolling.
4. Meanwhile prepare a standard curve in a new 96-well plate. Prepare sufficient volume for duplicates of each standard value. Prepare the following dilutions:

   0 mM = 100 µl $ddH_2O$; 5 mM = 5 µl $NaNO_2$ (100 µM) + 95 µl $ddH_2O$; 10 mM = 10 µl $NaNO_2$ (100 µM) + 90 µl $ddH_2O$; 25 mM = 25 µl $NaNO_2$

   (100 µM) + 75 µl $ddH_2O$; 50 mM = 50 µl $NaNO_2$ (100 µM) + 50 µl $ddH_2O$; 75 mM = 75 µl $NaNO_2$

   (100 µM) + 25 µl $ddH_2O$; 100 mM = 100 µl $NaNO_2$. Pipette 100 µl of each dilution per well.
5. Transfer 100 µl of the "cell supernatant" per well to the new 96-well plate. From this point on it is not necessary to work in sterile conditions.
6. Add 100 µl of working reagent per well and incubate the reaction for 10 min at RT and protected from light.
7. Measure NO production using an ELISA reader at 540 nm.

8. Generate a standard curve and use this to determine the amounts of NO produced by experimental samples.

### *3.3. Arginase Activity* (9)

1. Harvest cells and count them using a Neubauer cell chamber. Seed 200,000 cells per well in a 96-well culture plate. Culture cells in DMEM 10% FBS and 1% Penicillin/Streptamycin. The total volume per well should be 200 µl. Allow cells to adhere well to the plate at 37°C in 5% $CO_2$ overnight.
2. The following day treat cells with the stimulating agents of interest. To induce arginase activity in positive control samples, stimulate cells with either IL-4 (10 units/ml) or IL-4/IL-10 (10 units/ml each). Cells are usually incubated for 24 h at 37°C after addition of agonists.
3. The next day, wash cells once with 200 µl 1× PBS and remove PBS by tapping on the plate.
4. Add 100 µl of Triton 0.1% and leave the plate on a rocking platform for 15 min in order to lyse the cells.
5. Add 100 µl of 50 mM Tris–HCl pH 7.5. To measure total arginase, add 10 µl $MnCl_2$. At this point you can stop the reaction and leave the plates wrapped in parafilm at 4°C.
6. Transfer 100 µl per well from the plate in **step 5** to a safe-lock tube (*see* **Note 1**). To measure native arginase, one of the tubes must not contain $MnCl_2$.
7. Incubate the tubes at 56°C for 7 min to activate the enzyme.
8. Add 100 µl of 0.5 M arginine, pH 9.7, and incubate the mixture at 37°C for 2 h. If the stimulus is IL-4, incubate for 1 h; if it is IL-4/IL-10 incubate for only 15 min.
9. Meanwhile prepare the urea standards of the following concentrations:

   0 µg = 100 µl $ddH_2O$; 7.5 µg = 12.5 µl urea (600 µg/ml) + 87.5 µl $ddH_2O$; 15 µg = 25 µl urea

   (600 µg/ml) + 75 µl $ddH_2O$; 30 µg = 50 µl urea

   (600 µg/ml) + 50 µl $ddH_2O$; 45 µg = 75 µl urea

   (600 µg/ml) + 25 µl $ddH_2O$; 60 µg = 100 µl urea (600 µg/ml).
10. Stop the reactions from **step 8** by adding 800 µl of an acid mix containing $H_3PO_4$, $H_2SO4$, and $H_2O$ in a ratio 1:3:7. Add 900 µl of the same solution to the standards. The reaction can be stopped here and the samples can be left at 4°C for days.
11. Add 40 µl of α-ISPP 6% dissolved in EtOH to all tubes including the standards. Vortex for 5 s (*see* **Note 2**).
12. Incubate all tubes at 95°C for 30 min.
13. Incubate all tubes at 4°C for 30 min.

14. Transfer 200 µl from each tube to a new 96-well cell culture plate.
15. Read the optical density at 540 nm and convert this number using the standard curve in µg urea.
16. Analyze data using the following formula: µg urea/60 (molecular weight of urea) × 50 (dilution factor)/*t* (minutes of incubation with arginine) = units of arginase per $1 \times 10^6$ cells; 1 unit = amount of enzyme required to hydrolyze 1 µM arginine per minute.

### *3.4. Determination of mRNA* (10)

All reagents and material must be RNase-free. WORK WITH GLOVES!

1. Harvest day 6 bone marrow-derived macrophages as described in **Subheading 3.1** and seed approximately $2 \times 10^6$ cells per small petri dish (6 cm diameter). Allow cells to adhere and stimulate them the next day.
2. Wash cells once with 2 ml 1× PBS.
3. Add 250 µl of Solution A (EZ-RNA isolation-KIT) per plate and detach cells by scraping (*see* **Note 3**).
4. Transfer the cell suspension to an RNase-free Eppendorf tube and leave the cells for 5 min at RT.
5. Add 250 µl of Solution B (EZ-RNA Total RNA Isolation Kit), invert tubes for 15 s, and incubate the reaction at RT for 10 min.
6. Centrifuge the samples at 12,000 × *g*, 4°C for 15 min.
7. Transfer the upper aqueous phase to a new tube, add 250 µl isopropanol, mix, and incubate for 10 min at RT. You can stop the reaction here and leave the samples at –20°C overnight.
8. Centrifuge the samples at 12,000 × *g* at 4°C for 8 min.
9. Carefully discard the supernatant and add 250 µl 75% EtOH. Mix by tipping the tube.
10. Centrifuge the tubes at 7,500 × *g* at 4°C for 5 min.
11. Discard the supernatant and allow the pellet to dry completely. This might take up to 1 h, but it is important that the pellet is completely dry.
12. Meanwhile prepare a heatblock at 65°C.
13. Dissolve the pellet in approximately 20 µl RNase-free water, depending on the size of the pellet (*see* **Note 4**).
14. Store the samples at –20°C for short-term storage and at –80°C for long-term storage.
15. *Measure RNA*. 1 µl RNA + 400 µl RNase-free $H_2O$.

16. Measure absorbance at 260 nm using a Quartz cuvette; blank = $H_2O$; absorbance × 16.04 = µg/µl RNA.
17. For reverse transcription use Rnase-free Eppendorf tubes.
18. Mix 0.1–2 µg RNA with 1 µl Oligo-dT primer mix and fill with $ddH_2O$ to a final volume of 14 µl.
19. Incubate tubes at 65°C for 5 min.
20. Incubate tubes at 4°C for 5 min to prevent the formation of secondary structures.
21. Meanwhile prepare a mastermix: 2.5 µl M-MLV RT 5× Reaction Buffer; 0.625 µl dNTP mix (10 mM each); 0.5 µl M-MLV Reverse Transcriptase; 8.875 µl $ddH_2O$/per tube.
22. Add 12.5 µl of the mastermix to each tube and spin quickly.
23. Incubate the samples for 2 min at 37°C.
24. Incubate the samples for 50 min at 40°C.
25. Incubate the samples 15 min at 70°C.
26. Spin for a short time and store samples at –20°C.
27. For quantitative Real-Time PCR design primers using the software Primer Express 1.5, Applied Biosystems. The reaction is shown schematically in **Fig. 2**.
28. For normalization, use a gene that is constitutively expressed, like β-Actin or L14.
29. Prepare dilutions of each sample in a ratio 1:4 (sample: $H_2O$).
30. Prepare a curve using the following dilutions: Dilution 1 *(1)* = 5 µl sample + 15 µl $H_2O$; Dilution 2 (0.5) = 10 µl of dilution 1 + 10 µl $H_2O$; Dilution 3 (0.25) = 10 µl of dilution 2 + 10 µl $H_2O$; Dilution 4 (0.125) = 10 µl of dilution 3 + 10 µl $H_2O$; Dilution 5 (0.0625) = 10 µl of dilution 4 + 10 µl $H_2O$. With the values resulting from these dilutions a curve will be made that will allow to evaluate the efficiency of the reaction and will be used to calculate the dilutions of the cycle threshold (CT).
31. Prepare a master mix for each pair of primers:
    - 5.5625 µl SYBR® Green PCR Master Mix
    - 5.5625 µl $ddH_2O$
    - 0.1875 µl Forward Primer 20 µM
    - 0.1875 µl Reverse Primer 20 µM
    - This is the volume required per well. Calculate for triplicates of each sample and include one well without sample. The standards should be done in duplicate for each gene.
32. Add 11.5 µl master mix and 1 µl sample per well, except the negative control.

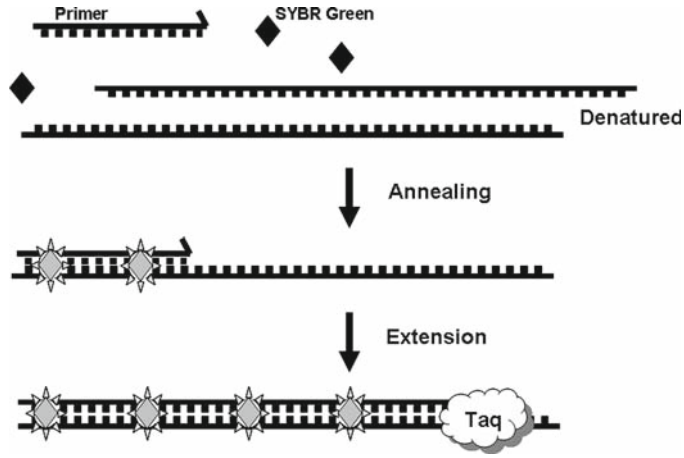


Fig. 2. Schematic representation of real-time PCR with the SYBR® Green I dye. SYBR® Green I dye (DNA nonassociated black diamonds) becomes fluorescent (DNA-bound gray diamonds with spikes) upon binding to double-stranded DNA, thereby providing a direct method for measuring PCR products in real time. (Image provided by Molecular Probes®).

33. Cover the plate with transparent film (*see* **Note 5**).
34. Perform the PCR using the software SDS 2.1. Program the following cycles: 10 min at 95°C; 30 s at 95°C; 30 s at 60°C; 30 s at 72°C. Dissociation stage: 1 min at 95°C during 20 min cooling down to 60°C (1 min); 1 min at 95°C; 35 repeats
35. Analyze data using the software SDS 2.1.

### *3.5. Determination of Protein Abundance for NOS2 and Arginase 1*

All steps should be carried out on ice to avoid protein degradation.

1. Harvest day 6 bone marrow-derived macrophages as described in **Subheading 3.1** and seed approximately $2 \times 10^6$ cells per petri dish (diameter: 60 mm). Allow cells to adhere and stimulate the next day.
2. Wash cells once with cold 1× PBS and incubate the plates on ice.
3. Add 150 µl lysis buffer.
4. Detach cells by scraping.
5. Transfer the cells to Eppendorf tubes and leave them rotating at 4°C for 20 min.
6. Centrifuge at 12,000 × *g* at 4°C for 10 min to remove cell wall components.
7. Store the supernatant at –20°C.

**Table 1**
**Recipes for polyacrylamide separating and stacking gels**

| Reagent | 7.5% separating gel | 12.5% separating gel | 5% stacking gel |
|---|---|---|---|
| 30% Acrylamide/0.8% Bis-Acrylamide | 2.5 ml | 4.2 ml | 1.7 ml |
| 1.5M Tris–HCl pH 8.8 | 2.5 ml | 2.5 ml | |
| 0.5 M Tris–HCl pH6.8 | | | 2.5 ml |
| $ddH_2O$ | 4.9 ml | 3.2 ml | 5.7ml |
| 10% Ammonium persulfate | 100 µl | 100 µl | 100 µl |
| TEMED | 4 µl | 4 µl | 10 µl |

8. Dilute Bradford reagent 1:4.
9. Prepare BSA standards of the following volume: 0, 1, 3, 5, 7, 9 µl BSA (1 mg/ml).
10. Add 1 µl of the sample (vortex before) to 1 ml diluted Bradford reagent.
11. Add 1 ml Bradford reagent to the BSA standards.
12. Mix by inverting the tubes.
13. Incubate for 15–30 min at RT.
14. Measure the absorbances of samples and standards at 595 nm using plastic cuvettes.
15. For SDS-PAGE, prepare gels of a thickness of 1.5 mm. For the detection of Arginase1, prepare a 12.5% resolving gel, and for NOS2 a 7.5% resolving gel (**Table 1**).
16. First prepare the separating gel, leaving space for the stacking gel, and overlay with $H_2O$.
17. As soon as the separating gel is completely polymerized, pour off the water and prepare the stacking gel.
18. Pour in the stacking gel and quickly insert the comb.
19. Prepare the running buffer by diluting 100 ml of the 10× running buffer with 900 ml $H_2O$.
20. Once the stacking gel is polymerized, remove the comb and assemble the gel unit into the apparatus provided.
21. Fill the tank with 1× running buffer.
22. Prepare the samples by adding 5× loading buffer (dilution 1:4) to approximately 50–100 µg protein (the appropriate volume is determined after quantifying the samples using the Bradford assay).

23. Incubate the samples at 95°C for 6 min and transfer them immediately on ice.
24. Load samples on the gel and include one well for a prestained molecular weight marker.
25. Complete the assembly of the gel unit and connect to a power supply.
26. Run the gel at 80–150 V.
27. When the dye fronts are about to run off the gel, stop the run.
28. For Western Blotting, prepare four Whatman papers and one Hybond-C nitrocellulose membrane (9 cm × 6 cm).
29. Incubate the membrane for 5 min in water.
30. Incubate the membrane and the Whatman papers for 10 min in transfer buffer.
31. Set up the transfer unit as shown schematically in **Fig. 3**.
32. Disconnect the gel unit from the power supply and disassemble it. Separate the two glasses plates. Cut off and discard the stacking gel and transfer the separating gel onto the membrane. Add two Whatman papers and close the transfer cassette.
33. Transfer takes place at 0.8–1.4 mA/cm$^2$ for 1 h.
34. When the transfer is finished, disassemble the cassette. Use tweezers to move the membrane.
35. From this point on, perform all wash and incubation steps on a rocking platform/shaker.
36. Incubate the membrane for 5 min in 1× TBS 0.1%Tween (TBS-T).
37. Incubate the membrane for 1 h in 50 ml of 1× TBS-T 5% milk to detect NOS2 and for 2 h in 50 ml of TBS-T 10% milk to detect Arginase1.
38. Wash the membrane 2–3 times in TBS-T for 10 min.
39. Incubate the membrane with the first antibody:

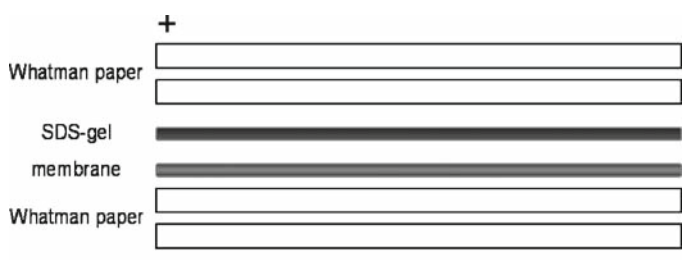


Fig. 3. Schematic representation of the transfer blot.

- *For NOS2.* Anti-NOS2 (1:1000) in TBS-T 0.02% $NaN_3$.
- *For Arginase 1.* Anti-Arginase 1 (1:10 000) in TBS-T 10% milk 0.02% $NaN_3$ (*see* **Note 6**).

40. Incubate the membrane with the primary antibody on a roller at 4°C overnight.
41. The next day wash the membrane 2–3 times in TBS-T for 10 min.
42. Incubate the membrane with the secondary antibody. For Arginase1 and for NOS2, you need horseradish-peroxidase-conjugated antirabbit IgG diluted 1:5,000 in TBS-T. Incubate the membrane with this antibody for 1 h at RT on a roller (*see* **Note 7**).
43. Wash the membrane 2–3 times in TBS-T for 10 min.
44. Prepare the ECL solution by mixing both components in the ratio 1:1.
45. Incubate the membrane in ECL solution for approximately 2 min.
46. From this point on, work in the dark using only red light.
47. Dry the membrane carefully and place it between two plastic films located in an X-ray film cassette.
48. Exposure time depends on the intensity of the signal, but usually 1–5 min is sufficient.
49. Once you have achieved a satisfactory result, wash the membrane intensely (put the shaker on max) in TBS-T for 30 min.
50. Incubate the membrane for 1 h with anti-β-Actin (1:5,000) at RT.
51. Wash the membrane 2–3 times in TBS-T for 10 min.
52. Incubate the membranes for 1 h with horseradish-peroxidase-conjugated antimouse IgG (1:5,000) at RT.
53. Wash the membrane 2–3 times in TBS-T for 10 min and repeat the procedure of developing the film after incubating it again in ECL solution.
54. β-Actin serves as a loading control, so you should see a similar intensity of the bands in all lanes.

## 4. Notes

1. It is important to use safe-lock tubes because later on tubes are incubated at 95°C, which subjects them to high pressure.

2. This step is crucial to avoid a gradient.
3. Ensure that the cell scraper is either new or alternatively incubate it in 0.1M NaOH for 20 min and after that in EtOH for 20 min, before leaving it in 1× PBS.
4. The pellet is normally hardly visible and dissolves very slowly. To shorten the process, incubate the samples at 65°C for 10 min to allow the pellet to dissolve completely.
5. Be careful not to touch the foil except on the edges as this can cause errors in plate reading later on.
6. Primary antibody solutions are prepared in a falcon tube in a total volume of 5 ml. They can be stored at 4°C and be reused for months. Transfer the membrane to the falcon tube using tweezers. The membrane must be rolled carefully to fit in the tube.
7. Secondary antibody solutions should be freshly prepared every time.

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# Chapter 4

# Analysis of Macrophage Phagocytosis: Quantitative Assays of Phagosome Formation and Maturation Using High-Throughput Fluorescence Microscopy

**Benjamin E. Steinberg and Sergio Grinstein**

## Summary

Phagocytosis of invading pathogens by macrophages represents a fundamental component of the innate immune system. In this chapter, we describe protocols designed for high-throughput analysis of phagosome formation and maturation using latex beads as model phagocytic targets. The method takes advantage of an automated fluorescence microscope platform to investigate Fcγ receptor-mediated particle internalization. First, procedures to opsonize and fluorescently label the model particles are outlined. In combination with the robotic fluorescence microscope, these labeling methods provide for the quantitative high-throughput assessment of phagocytosis. Acidification of the phagosomal lumen can be used as an index of maturation. We describe a fluorimetric procedure to assess phagosomal pH based on the partitioning of a membrane-permeant weak base that accumulates in acidic intracellular compartments. Lastly, a description of the hardware and software components of the robotic high-throughput fluorescence microscope platform is provided.

**Key words:** Phagocytosis, Acidification, Vacuolar pH, Fcγ receptor, Macrophage.

## 1. Introduction

A key function of neutrophils and macrophages is to engage and clear invading microorganisms. A variety of receptors present on the plasma membrane of phagocytes mediate interactions with pathogens. These receptors recognize ligands that are either endogenous to the microorganism or are derived from host proteins, such as immunoglobulin G (IgG), components of the complement system or others, that have previously bound (opsonized) the microorganism. IgG molecules that coat the

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bacterial surface are recognized by the Fcγ family of receptors, the most highly characterized of phagocytic receptors. Upon engagement with their cognate ligand, Fcγ receptors are thought to cluster at the cell surface, thereby initiating a signaling cascade that brings about active particle internalization into a membrane-bound organelle termed the phagosome. Following its formation, this nascent organelle undergoes an elaborate maturation process whereby both its membrane and luminal composition are modified through a series of coordinated membrane fusion and fission events *(1)*. During its maturation, the phagosome acquires a large armamentarium of antimicrobial components, including proteases and cationic peptides that allow for pathogen clearance and ultimately for the resolution of infection.

A critical element of the maturation process is the gradual acidification of the phagosome lumen to pH values less than 5.5. This acidification is an active process mediated by the proton-pumping vacuolar ATPase (V-ATPase) *(2,3)*. As well as directly affecting bacterial growth, luminal acidification is required for the optimal activity of several bactericidal components. The activity of the various hydrolytic enzymes is exquisitely pH sensitive, requiring acidic conditions. Generation of hydrogen peroxide is also catalyzed by an acidic pH. Moreover, maintenance of an acidic lumen is seemingly required for proper phagosome maturation since dissipation of transmembrane pH gradients with permeant weak bases impairs fusion between phagosomes and lysosomes *(1,4)*.

Phagocytosis can be conceptually divided into two distinct processes: phagosome formation and phagosome maturation. A variety of assays exist to study these two processes independently, often taking advantage of fluorescence microscopy in view of its high sensitivity. Such fluorescence microscopy-based assays rely on the manual acquisition of images, which are subsequently analyzed to discern internalized particles from those that failed to enter the phagocytic cells. To score maturation, multiple fluorescent markers and acquisition channels are sometimes required. When studying multiple time points or conditions, manual data acquisition and analysis can be extremely labor intensive and time consuming. Screening siRNA or small molecule libraries by this approach would be prohibitive.

Automation of both image acquisition and analysis would circumvent these limitations. In this chapter, we describe protocols that make use of a robotic fluorescence microscope along with accompanying digital imaging software to generate high-throughput measurements and analyses of phagocytosis. Phagosome formation is examined by locating cells that have engaged particles and further discriminates between adherent and internalized particles based on a dual-labeling strategy. A method for high-throughput analysis of phagosome maturation is also presented. The method is based on the quantification of luminal

acidification using a fluorescent acidotropic dye. Important features of the high-throughput microscopy system and of the ancillary analysis software are also discussed.

## 2. Materials

1. The assays described in **Subheading 3** can employ either primary cell preparations or established macrophage cells lines, such as the murine RAW 264.7 (ATCC catalog number: TIB-71) or J774 (ATCC catalog number: TIB-67) lines. It is important to note that regardless of cell type, the described procedures entail plating the macrophages on the surface of standard plastic tissue culture plates (*see* **Note 1**). Protocols are given for 24-well plates, but can be readily adapted to smaller well formats (*see* **Note 2**).
2. *Phosphate-buffered saline (PBS)*. 140 mM NaCl, 5 mM KCl, 8 mM $NaH_2PO_4$, and 2 mM $KH_2PO_4$, adjusted to pH 7.4 with 1 M NaOH.
3. Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5% fetal bovine serum and 4.5 g/L of glucose, L-glutamine and sodium pyruvate, available commercially.
4. Latex beads are available from a variety of commercial sources.
5. Human IgG and fluorescent secondary antibody conjugates can be purchased commercially (e.g., Sigma-Aldrich or Jackson ImmunoResearch Laboratories), as can Hoechst 33342 and acidotropic fluorescent dyes (available from Molecular Probes and elsewhere).
6. Several commercial high-throughput fluorescence microscope systems are available. The assays described in this chapter make use of the KineticScan HCS Reader and accompanying software modules from Cellomics, Inc. The current state of high-throughput fluorescence microscopy has been the subject of a recent review *(5)*, to which the interested reader is referred.

## 3. Methods

### 3.1. Particles

A variety of model particles can be used to carry out phagocytosis assays in macrophages. These include zymosan A, sheep red blood cells, latex beads, and bacteria. We describe protocols employing latex beads as phagocytic targets; nevertheless, other particles can be readily substituted.

### 3.2. Opsonization

The opsonization of latex beads with IgG simply requires that the particle be incubated with the opsonin (*see* **Note 3**). Other opsonins can be used in a similar manner.

1. Add 40 µL of a 10% suspension of latex beads to 500 µL of PBS and spin for 30 s at 6,000 × *g* in a microcentrifuge, aspirate the supernatant, and resuspend the beads in 500 µL of fresh PBS.
2. Wash twice with PBS. Use 500 µL of PBS and the same centrifugation settings outlined in **step 1** for all subsequent washes.
3. Resuspend beads in 190 µL of PBS and add 10 µL of antihuman IgG (30 mg/mL stock).
4. Incubate for 60 min at room temperature with gentle mixing.
5. Wash three times with PBS to remove excess antibody and resuspend in 500 µL of PBS.

### 3.3. Fluorescent Labeling

Analysis of phagocytosis by fluorescence microscopy requires that the particles be identifiable. To this end, the opsonized particles can be fluorescently labeled prior to internalization using fluorophore-conjugated secondary antibodies directed against the opsonin (*see* **Note 4**).

1. Add 5 µL of the appropriate fluorophore-conjugated (e.g., Cy3) secondary antibody (1.5 mg/mL stock) to the 500 µL of PBS suspension of opsonized latex beads.
2. Incubate at room temperature for 60 min. The suspension should be kept rotating to prevent the particles from settling and aggregating.
3. Wash four times with PBS and resuspend in 500 µL of PBS. Prior to initiating phagocytosis, the particles can be extruded through a 25-gauge syringe, to disrupt any particle aggregates.

### 3.4. Particle Presentation and Internalization

#### 3.4.1. Synchronization of Phagocytosis

When assaying multiple conditions for phagocytosis, it is necessary to initiate particle presentation in a temporally defined and homogeneous way. Phagocytosis can be synchronized by rapidly depositing the phagocytic targets onto the cells, but other methods are also available (*see* **Note 5**).

1. Add an aliquot of 15–30 µL of the labeled, opsonized bead suspension (*see* **Subheadings 3.2** and **3.3**) to each well. Ensure that the beads are evenly distributed throughout the bathing medium by briefly shaking the plate.
2. Synchronize phagocytosis by centrifugation at 300 × *g* for 1 min to uniformly settle the beads onto the cells.

3. Incubate the cells at 37°C for 15 min to allow for adequate particle internalization. Different incubation times can be used depending on the experimental objectives and design.

*3.4.2. Internal/External Particle Discrimination by Differential Fluorescence Labeling*

Once phagocytosis is complete, it is necessary to unambiguously distinguish between those particles that have been internalized from those that remain adherent to the cell membrane but failed to be engulfed. This can be achieved by differentially labeling internalized and external particles using an impermeant fluorophore-conjugated secondary antibody that will stain exclusively the external (exposed) beads. By using an antibody labeled with a fluorophore that is distinct from that used for total bead labeling (*see* **Fig. 1** and **Subheading 3.3**), external beads can be visualized as being dual labeled and differentiated from those that were internalized and are only single labeled (**Fig. 1b**, **c**). The secondary antibody used to identify external beads is similarly directed against the opsonizing IgG.

1. Arrest phagocytosis by placing the cells on ice and washing with cold DMEM to remove beads not associated with the cells.
2. Stain the remaining external, mostly adherent particles by adding 5 μL of a 0.3 mg/mL stock of fluorophore-conjugated (e.g., Cy2) antihuman antibody to the 500 μL solution overlaying each well for 5–7 min in the cold.
3. Wash each well 3–5 times using cold PBS to remove excess secondary antibody.

When assaying for phagosome formation or maturation, proceed to **Subheadings 3.4.3** and **3.5**, respectively.

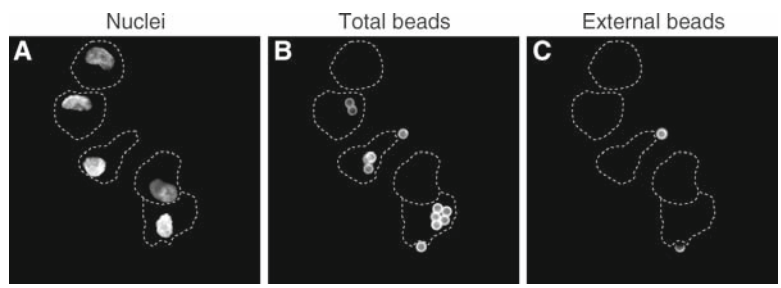


Fig. 1. *Discrimination of internal and external beads by differential fluorescence labeling.* Cy3-labeled, IgG-opsonized latex beads (3.1 μm diameter) were presented to human primary macrophages for 30 min. An image of all the beads associated with the cells after washing is shown in (**B**). External particles were differentially labeled with a Cy2-conjugated secondary antibody and are shown in (**C**). In this way, the external beads can be distinguished by their dual labeling with Cy3 and Cy2, whereas beads that had been successfully engulfed display only the Cy3 label. The cells were fixed and their nuclei stained with Hoechst 33342 prior to imaging. The cell outlines, visualized by differential interference contrast microscopy, are traced by the *dotted lines*, while the fluorescence of the nuclei is shown in (**A**).

#### 3.4.3. Cell Identification by Nuclear Staining

The quantification of phagocytosis requires not only the accurate detection of the internalized particles but also the identification and quantification of the phagocytes themselves. The cells can be seen using either a cytosolic (*see* **Note 6**) or a nuclear stain (**Fig. 1a**) *(6)*.

1. Fix the cells using 4% paraformaldehyde in PBS for 20 min, followed by a wash with 100 mM glycine for at least 10 min, to scavenge excess fixative.
2. Stain the cell nuclei by incubating with the nuclear dye Hoechst 33342 (8 μg/mL) in PBS for 10 min.
3. Wash once and maintain the cells in PBS.

#### 3.4.4. Determination of Phagocytic Index

Subsequent to phagocytosis and external particle labeling, the macrophages are examined using the robotic fluorescence microscope (*see* **Subheading 3.6.1**). Quantification can be performed immediately or digital images can be acquired for subsequent analysis using the image processing software. The efficiency of phagosome formation can be expressed as the phagocytic index (PI), defined as the number of particles internalized per macrophage:

PI = (Total particles – external particles)/total number of macrophages

For each image, the number of internalized beads is simply equal to the difference between the total and external bead numbers. The PI is often normalized per 100 phagocytes.

1. Place the preparation into the microscope chamber, and select the appropriate acquisition protocol.
2. When using dual fluorescent labeling, separately acquire fluorescence images in the channels corresponding to total and external particles, as well as the nuclear stain. Acquire images of multiple fields of view in each well, as needed.
3. Using the object identification software (*see* **Subheading 3.6.2**), identify and count the total number of beads, the total number of external beads, and the total number of macrophages in each field.
4. Compile the data and compute the PI as outlined earlier.

### 3.5. Phagosome Maturation

Markers of acidification, such as acidotropic fluorescent dyes, can be used to assess phagosome maturation. These compounds are permeant weak bases that are selectively retained in cellular compartments of low pH following their protonation. Acidotropic dyes of assorted colors are commercially available through Molecular Probes and other suppliers, and provide a simple, qualitative evaluation of phagosomal pH (*see* **Note 7**). These dyes can be used according to the manufacturer's instructions. In our protocol, acidic compartments are labeled by incubating the cells

with 1 μM LysoTracker® in PBS for 1 min. The cells are washed twice, maintained in cold PBS, and imaged immediately.

### 3.6. Automated High-Throughput Fluorescence Microscope

The robotic high-throughput fluorescence microscopy provides an integrated system for the automated acquisition and analysis of large populations of cells. High-throughput platforms generally consist of the imaging hardware with either parallel or serial image analysis software modules that can be implemented on- or off-line.

#### 3.6.1. Acquisition

Here we described image acquisition in an automated fashion by the KineticScan HCS Reader. Detailed instructions for its use are provided by the manufacturer. The basic platform consists of a temperature- and $CO_2$-controlled imaging chamber, outfitted with a mechanical stage that moves the plate across the light path in a predefined trajectory. The general imaging procedure consists of placing the tissue culture plate into the microscope chamber and selecting the appropriate acquisition protocol. Acquisition protocols are defined by three components (the plate, assay, and kinetic protocols), which can be set prior to imaging. Note that for the live cell acidification assays, the chamber is left at room temperature to minimize any further phagosome maturation during the course of image acquisition. Imaging is performed using the XP93 filter set, which allows for recording of DAPI, green, red, and far-red fluorescence channels, with either the 20× or 40× objectives. Autofocus, a feature of the equipment, can be performed at variable intervals, at the discretion of the operator. It is recommended to start by autofocusing every two fields and decreasing the frequency whenever possible (*see* **Note 2**), to increase the acquisition speed.

For phagocytic index assays using dual fluorescent particle labeling, acquire separately fluorescence images in the channels corresponding to the nuclear stain and total and external particles for multiple fields. It is important to acquire the nuclear stain in the first channel, as this is the channel for which the primary object identification will be carried out (*see* **Note 8**). Images of the three channels in a representative field are shown in **Fig. 2a**, **c**, **e**. Acidification assays will similarly have three fluorescent channels, corresponding to the total and external bead labels and the acidotropic dye. In this case, the total bead fluorescence should be acquired in the first channel.

#### 3.6.2. Analysis

Image analysis centers upon object identification algorithms. Identification is initially implemented based on user-specified intensity thresholds between adjoining pixels in the first fluorescent channel. Objects within the field can be accepted or rejected for subsequent analyses depending on physical parameters including object area, size, and fluorescent intensity. The accepted objects

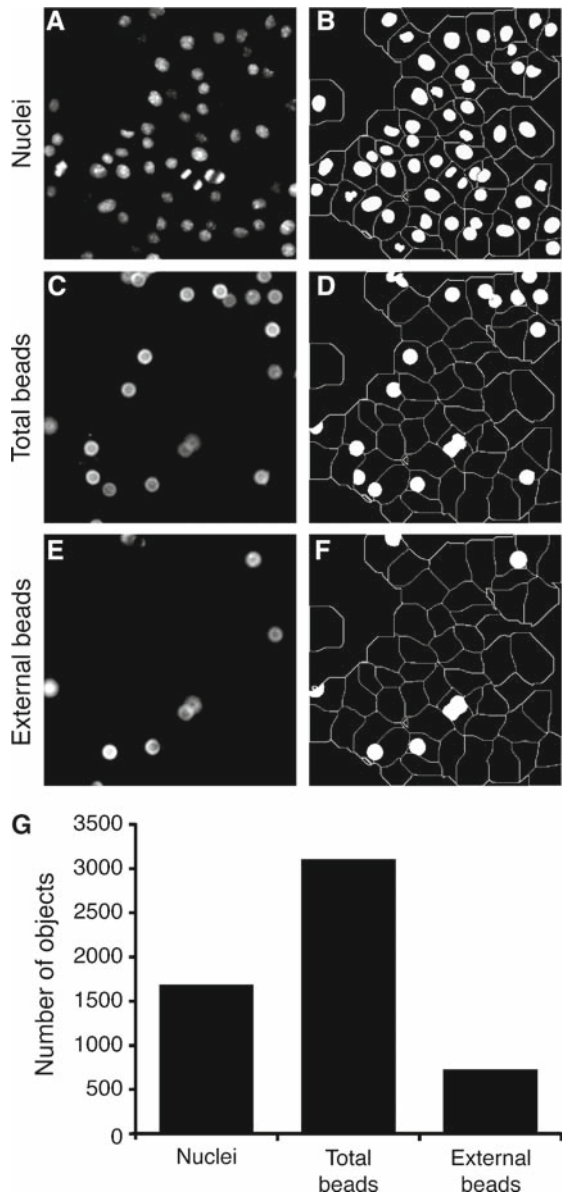


Fig. 2. *Automated quantitation of the phagocytic index.* RAW 264.7 macrophages were allowed to internalize IgG-opsonized, Cy3-labeled 3.1 μm beads as described in the text. Following 15 min of phagocytosis, the remaining external beads were labeled with a Cy2-conjugated antihuman secondary antibody, the cells were fixed, and the nuclei labeled with Hoechst 33342. Imaging was then carried out using the KineticScan HCS Reader. The stained nuclei, used to identify individual cells, are shown in (**A**), all the cell-associated beads (Total beads) are shown in (**C**), while the external beads are displayed in (**E**). The number of objects in each fluorescent channel was quantified using the Spot Detector BioApplication. Objects identified by the software module are shown in for each channel in panels (**B**), (**D**) and (**F**). The cell outlines, defined by the software as an area (a *Circ*) corresponding to 35 pixels beyond the edge of each identified nucleus, are also depicted. These calculated outlines were used to search and quantify objects associated with individual cells. Object counts from a representative experiment are shown in (**G**)

are designated as primary objects, and regions around or within the individual objects are used in the ensuing quantification procedures (*see* **subheading "Regions of Interest"**). Proprietary software modules (termed BioApplications) further refine the analysis of the determined regions of interest. Brief overviews of two such modules are provided as follows, whereas detailed descriptions are available from the manufacturer.

Regions of Interest

Refinement of the image analysis relies heavily on defining appropriate regions of interest. Here we present a summary of the various shapes that can be assigned to such regions. Across the different analysis modules, two different types of regions can be demarcated with reference to the primary object. These are termed the *Circ* and the *Ring*. A *Circ* represents an area similar to, and centered about, that occupied by the primary object. It can be made either smaller or larger than the primary object. In contrast, the *Ring* denotes an annular area defined beyond the edge of the primary object. The user defines the position of the inner and outer perimeters relative to the primary object. In certain applications, individual spots within the regions can be discerned and are termed either *Circ Spots* or *Ring Spots*, depending on the structure of the associated region in which they lie.

Phagocytic Index

The Spot Detector BioApplication is used to calculate the phagocytic index, with the nuclei representing the primary objects (cells). This module offers a generic and adaptable stage for counting well defined cell-associated objects.

1. Following primary object identification, define a region (a *Circ*) delineating approximately 20 μm beyond the edge of each nucleus to fully encompass the cell perimeter. **Figure 2b** illustrates the identified nuclei and the associated regions of interest of a representative field.
2. Determine the number of distinct spots within these regions in both the total and external bead labels. The parameters used for secondary object identification must be appropriately configured. Spot identification is shown for total and external bead channels in **Fig. 2d**, **f**, respectively. Total object numbers in each channel are given in **Fig. 2g**.
3. Compute the PI as defined in **Subheading 3.4.4**.

Phagosome Maturation

The Compartmental Analysis BioApplication is used to quantify individual object fluorescence intensities and thus assess phagosome maturation. In this assay, the total bead label identifies the primary objects. This module offers detailed fluorescence intensity data of individual primary objects and their associated regions in multiple fluorescent channels. Application

examples of this protocol to the study of phagosome maturation are available in *(6)*.

1. Following primary object identification, define a region (a *Circ*) delineating approximately 1 μm beyond the edge of each bead. This region represents the area enclosed by the phagosome.
2. Determine the total fluorescence intensity within the region for the acidotropic dye and establish the presence of the external bead label.
3. Establish which objects represent the subpopulation of external particles based on their external-bead label fluorescence. Uninternalized beads will have both the total and external labels, as described earlier. All remaining objects are considered to be contained within phagosomes (*see* **Figs. 1** and **2**).
4. Compute the fluorescence of the acidotropic dye colocalized with the internalized particles as a measure of acidification and thus maturation.

## 4. Notes

1. Efficient high-throughput acquisition depends on the ability of the microscope to capture accurate (in focus) images with minimal user input. This is accommodated by the autofocusing capabilities (also *see* **Note 2**) of the Cellomics system and of equivalent systems. The ability to autofocus depends on the number of objects on which the algorithm is trying to focus. Should the cells be plated too sparsely, some fields may not contain any identifiable objects. In this case, the system will spend considerable time attempting ineffectively to focus. As this dramatically increases acquisition time, it should be avoided whenever possible. Image analysis requires precise object identification algorithms. When too many objects are captured in the field of view, distinguishing individual particles becomes more difficult. Even with optimal plating conditions, the software will encounter situations where objects will be directly adjacent to each other. To help resolve two adjacent objects, certain software modules include algorithms that discriminate between overlapping particles (termed object segmentation) based on geometry or peak intensities. The geometric method is particularly well suited for resolving adjacent latex beads as they have smooth boundaries and generally uniform size and intensity. While the object segmentation methods provide a valuable tool when imaging at higher object

densities, suitable cell and particle numbers remain crucial for a successful experiment. Ultimately, for efficient imaging, cells should be plated at minimum densities of 40 and 10 cells/mm$^2$ when using the 40× and 20× objectives, respectively. In fact, we have found that plating densities 10–40 times these minima provide significantly better autofocusing without compromising the object identification algorithms.

2. Imaging is performed through the bottom of the plastic tissue culture plates. As a result, autofocusing is necessary due to the nonuniformity in plate thickness. We empirically found that this problem was minimized using smaller well sizes and recommend that at least 24-well plates be used as they are more easily managed by the autofocus feature of the microscope. Smaller well formats further decrease the need for continual refocusing.
3. A variety of different latex beads are available commercially; however, styrene/divinylbenzene (DVB) copolymer latex beads are, in our experience, preferable. In our hands, beads that do not contain DVB are not efficiently opsonized and are therefore ingested poorly by RAW 264.7 macrophages. Once opsonized, the latex beads can be stored at 4°C for several days. Following prolonged storage, it is advisable to ensure that the beads have maintained the opsonin using a fluorophore-conjugated secondary antihuman antibody.
4. For total particle labeling with a fluorophore-conjugated secondary antibody, it is imperative that the fluorophore display little pH sensitivity. As outlined in **Subheading 1**, the phagosome lumen becomes progressively more acidic, which can severely quench the fluorescence of pH-sensitive dyes, such as fluorescein.
5. Placing the cells on ice and allowing the phagocytic targets to slowly sediment at $1 \times g$ can alternatively be used to synchronize phagocytosis. In this way, centrifugation is obviated, yet the particles adhere to the cells without being internalized. The cells are then gently washed and maintained in media prewarmed to 37°C. The protocol is completed as outlined in **Subheading 3.4**.
6. Phagocytosis protocols can similarly be implemented using a cytosolic marker instead of a nuclear stain for cell identification. Cytosol labeling can be accomplished using a variety of dyes, such as the Cell Tracker probes available from Molecular Probes. In this case, the cells are prelabeled with Cell Tracker Green (2 μg/mL) for 30 min at 37°C. The cells are then washed three times with PBS before adding the phagocytic targets. This type of cell labeling is particularly useful when information relating to cell size and shape is required.

7. When using acidotropic dyes, it is important to consider that in some cases dye accumulation is not readily reversible. Even artificial alkalinization using ionophores or a membrane-permeant weak base will not readily disperse the accumulated dye. As a result, treatments that will dissipate the phagosomal pH gradient must be carried out prior to and during application of the acidotropic dye.
8. The automated system does have certain limitations both in terms of its acquisition and analysis that must be considered when designing defined experimental protocols. Some examples are listed here (1) currently, acquisition is limited to five fluorescent channels, with no capacity to acquire a corresponding bright field image; (2) because image acquisition occurs through the tissue culture plate, objectives with suitably long focal distances must be used. The highest possible magnification is currently 40×, which limits the types of structures that can be resolved; (3) primary object identification is carried out on the images acquired in the first channel. The channel number order cannot, at present, be easily modified postacquisition.

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# Chapter 5

# Detection of Activated Rab7 GTPase with an Immobilized RILP Probe

**Jim Sun, Ala-Eddine Deghmane, Cecilia Bucci, and Zakaria Hmama**

## Summary

The dynamic and coordinated exchange of multiple GTPases between the cytosol and the phagosome membrane represents a critical process during phagosome biogenesis. In particular, acquisition of Rab7 is crucial for progression to the stage where formation of phagolysosomes is observed. Optimal Rab7 effector function requires its conversion to the GTP-bound form where it becomes activated. In light of this regulatory node, the GDP/GTP switch on the Rab7 molecule represents a tractable event to dissect the control of phagosome maturation by intracellular pathogen or their products. Direct measurement of Rab7 activation requires $^{32}$P-GTP binding to renatured Rab7 recovered by pull downs and resolved by SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and autoradiography. Here, we describe a novel, alternative, nonradioactive assay to measure Rab7 activity which takes advantage of the specific binding of activated (GTP bound) Rab7 to its effector RILP (Rab7 interacting lysosomal protein). Active Rab7 bound to immobilized recombinant RILP on latex beads can be detected quantitatively by either classical Western blotting or flow cytometry.

**Key words:** Rab7, RILP, Small GTPase, Flow cytometry, Immunostaining, Immunoblotting.

## 1. Introduction

Small GTPases function as molecular switches, alternating between inactive GDP- and active GTP-bound states dependent on signals that influence the remodeling of cell membranes *(1, 2)*. Within the Rab GTPase family, Rab7 has been shown to be a major regulator of phagosome fusion with late endosomes and lysosomes *(3–5)*. Rab7 is translocated from the cytosol to phagosome membranes in a GDP-bound (inactive) form, at which point a guanine nucleotide exchange factor (GEF) mediates exchange

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of GDP for GTP on membrane-bound Rab7 *(2)*. Subsequent inactivation of GTP-bound Rab7 is catalyzed by GTPase-activating protein (GAP) through GTP hydrolysis. Several studies have shown that the GDP/GTP switch allows direct interaction of Rab7-GTP with RILP (Rab7 interacting lysosomal protein), an essential downstream effector involved in phagosomal fusion with lysosomes *(6–8)*. RILP bridges phagosomes with dynein-dynactin, a microtubule-associated motor complex that drives phagosomes toward lysosomes *(7)*. Therefore, the GDP/GTP switch on Rab7 defines a discrete vesicular trafficking checkpoint that determines in part the ability of phagosomes to fuse with the lysosomal compartment.

Activation state of Rab7 may be assessed with the use of a radioactive nucleotide probe ($^{32}$P-GTP) to detect active Rab7 in cell lysates resolved by SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and electrotransferred onto PVDF membranes *(6, 9)*. Alternatively, fluorescent chimeric constructs of Rab7 and RILP, in combination with confocal microscopy may be used to define the kinetics of Rab7-RILP association and thus the state of activation of phagosome-associated Rab7 *(10–12)*. With the goal of delineating a less labor-intensive and nonradioactive method for quantification of intracellular active Rab7, we developed RILP-coated latex beads as a specific probe for accurate detection of Rab7-GTP.

This approach could also be applied to other GTPases involved in intracellular trafficking events. For example, Rab5 is an early endosomal marker required for proper vesicle trafficking, and its activation through the GDP/GTP switch allows for interactions with downstream effectors, one of which is EEA1 (early endosomal autoantigen 1) *(13)*. EEA1 is a known cognate binding protein of Rab5, and it could be used in a similar manner as RILP to pull down active Rab5 from cell lysates. This activation assay could also extend to any other small GTPases for which a specific binding effector has been identified.

We highlight the utility of this assay using macrophages infected with *Mycobacterium bovis* BCG. This illustrates how this powerful tool may be used to dissect mechanisms of microbial pathogenesis at the level of the Rab7 GDP/GTP switch contributing to defects in phagosome trafficking.

## 2. Materials

### 2.1. Cell Culture and Lysis

1. Murine macrophage cell lines J774A.1 and RAW 264.7.
2. Endotoxin-free Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum (FCS)

and 1% of each of the following supplements: penicillin-streptomycin mixture, nonessential amino acids, and HEPES.

3. Protease inhibitor mixture and PMSF.
4. *Macrophage lysis buffer*. 20 mM Tris buffer, pH 8.0 containing 137 mM NaCl, 1% glycerol, 1% TritonX-100, 1 mM EDTA, 1 mM PMSF, and 100 μg/ml mammalian protease inhibitor mixture.

### 2.2. Mycobacterial Culture

1. *Mycobacterium bovis* BCG (Pasteur 1173P2 strain) and *M. smegmatis* ($mc^2155$ strain) were provided by Dr. Richard Stokes (Dept of Pediatrics, University of British Columbia, BC, Canada).
2. *7H9-OADC*. Middlebrook 7H9 supplemented with 10% OADC.

### 2.3. GST Fusion Protein Expression and Purification

1. *E. coli* strain BL21, RILP-GST plasmid (*see* **Note 1**).
2. Luria–Bertani (LB) media, ampicillin, and chloramphenicol.
3. Glutathione-agarose resin and Isopropyl β-D-1-thiogalacto-pyranoside (IPTG).
4. *Bacterial lysis buffer*. PBS plus 1 mM DTT, 0.1 mM PMSF, 1 mg/ml lysozyme, and 100 μg/ml of bacterial protease inhibitors.
5. *Elution buffer*. 10 mM reduced glutathione in 50 mM Tris-HCl, pH 9.5 (*see* **Note 2**).

### 2.4. Cell Transfection

1. OPTI-MEM and HBSS.
2. pEGFP-C1 vector construct expressing wild-type Rab7, Rab7Q67L, or Rab7T22N *(6, 14, 16)*.
3. Lipofectamine 2000.

### 2.5. Latex Bead Coating

1. Aldehyde/sulfate latex beads (4 μm diameter).
2. 25 mM MES buffer, pH 5.86.
3. *PBS*. 8 g NaCl, 0.2 g KCl, 1.44 g $Na_2HPO_4$, 0.24 g $KH_2PO_4$, in 1 L of distilled water, pH 7.4.
4. PBS plus 1% BSA and 0.1% $NaN_3$ (PBS-BSA-Az).

### 2.6. Flow Cytometry Analysis

1. Mouse mAb anti-GST and rabbit polyclonal anti-RILP *(6)*.
2. FITC-conjugated goat antimouse and goat antirabbit IgG.
3. *Binding buffer*. HBSS plus 1% FCS and 0.1% $NaN_3$ (HBSS-FCS-Az).
4. Setting parameters for latex bead analysis (*see* **Fig. 1**).

### 2.7. SDS-Polyacrylamide Gel Electrophoresis

1. *Separating buffer*. 1.5 M Tris-HCl, pH 8.8, 0.4% SDS. Store at room temperature.

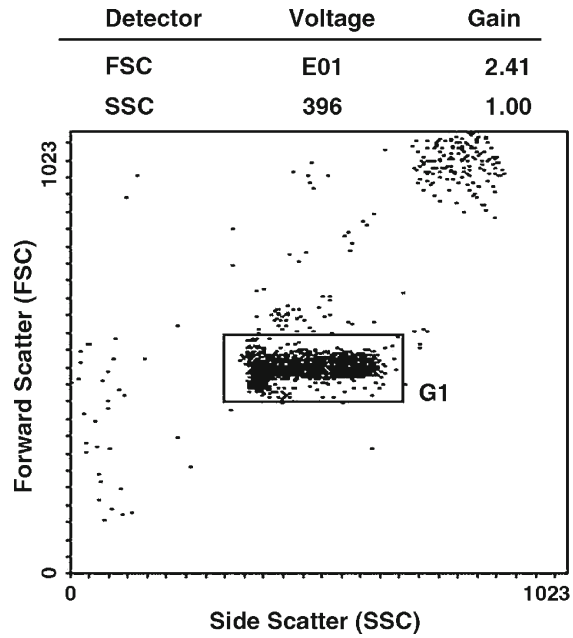


Fig. 1. Setting parameters for FACS analysis of latex beads. RILP-coated 4-μm latex beads (~$10^7$) were incubated with 500 μg of soluble fraction of cell lysate for 3 h at room temperature. Beads were then washed three times with binding buffer, resuspended in the same buffer, and analyzed by flow cytometry. Beads were localized by plotting forward scatter (FSC) vs. side scatter (SSC) channels on a linear scale using the voltage and gain values indicated. Beads (G1 gate) were readily discriminated from remaining contaminating material in the preparation.

2. *Stacking buffer*. 0.5 M Tris-HCl, pH 6.8, 0.4% SDS. Store at room temperature.
3. Acrylamide/bis-Acrylamide (30% solution). Store at 4°C.
4. *N,N,N,N'*-Tetramethyl-ethylenediamine (TEMED).
5. *Sample buffer*. 62.5 mM Tris-HCl, pH 6.8, 20% glycerol, 2% SDS, 5% β-mercaptoethanol.
6. *Ammonium persulfate*. Prepare 10% solution in water and freeze in single-use (100 μL) aliquots at –20°C.
7. Water-saturated isobutanol. Shake equal volumes of water and isobutanol in a glass bottle and allow separation to occur. Store at room temperature. Use the top layer.
8. *Running buffer*. 25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS, pH 8.3. Store at room temperature.
9. Pageruler prestained protein ladder.

### 2.8. Immunoblotting

1. Rabbit polyclonal anti-Rab7, mouse mAb anti-GST, and rabbit polyclonal anti-GFP.
2. Horseradish-peroxidase-conjugated antirabbit and antimouse IgG.
3. Nitrocellulose membranes (0.45 μm).
4. *Transfer buffer*. 25 mM Tris, 0.2 M glycine, 20% methanol, 0.04% (w/v) SDS.
5. *TBS-T (wash buffer)*. 100 mM Tris-HCl, pH 7.5, 0.9% (w/v) NaCl, 0.1%–0.5% Tween 20.
6. *Blocking buffer*. TBS-T plus 3% BSA.
7. Enhanced chemiluminescent (ECL) reagents.
8. *Stripping buffer*. 0.5 N NaOH at 37°C.

## 3. Methods

This assay will allow measurement of the amount of active GTP-bound Rab7 in a prepared cell lysate sample. It utilizes the Rab7 effector, RILP, as a surrogate marker of Rab7 activity. It is important to have highly purified RILP expressed as a fusion protein to GST as well as a GST fusion protein negative control. Purification could be assessed by sample analysis of eluted protein fractions using SDS-PAGE and Coomassie blue staining. The macrophage cell lines, RAW 264.7 and J774, as well as bone marrow-derived mouse macrophages express significant levels of endogenous Rab7. Therefore, there is no need to transfect cells with Rab7 constructs unless the experiment calls for negative mutant (Rab7T22N) or constitutively active (Rab7Q67L) constructs of Rab7. Details for macrophage transfection are provided in materials **Subheading 2.4** and in **Note 3**.

RILP-coated latex beads are used to pull down specifically *active* Rab7 (GTP-bound form) from cell lysate. Bead-associated Rab7 is detected by immunostaining with specific anti-Rab7 antibodies. The extent of Rab7 interaction with its partner molecule RILP is then quantified by flow cytometry. If required, confirmation of the flow cytometry data may be obtained by SDS-PAGE and Western blotting with anti-Rab7 antibodies using equal numbers of beads from various test samples being tested.

To validate this assay, we have transfected cells of the RAW 264.7 macrophage cell line individually with either of three forms of Rab7: wild-type (WT), constitutively active mutant (Q67L), and dominant negative (T22N). Rab7T22N is a GDP-locked mutant and thus unable to interact with RILP. However, the wild-type and constitutively active GTP-bound mutants are competent

to associate with RILP-coated beads in vitro and thus function as a readout of Rab7 activity. As described, validation experiments involving FACS quantification (**Fig. 2a**) as well as immunoblotting analysis (**Fig. 2b**) show that Rab7WT and Rab7Q67L but not Rab7T22N bind to immobilized RILP on latex beads.

Active Rab7 is an important regulator of vesicle trafficking and phagosome maturation. Hence, this assay allows rapid and accurate determination of the activation state of Rab7 in cells undergoing active vesicular trafficking. One example of how this novel assay can be exploited to examine the dynamics of phagosome maturation is provided using macrophages infected with

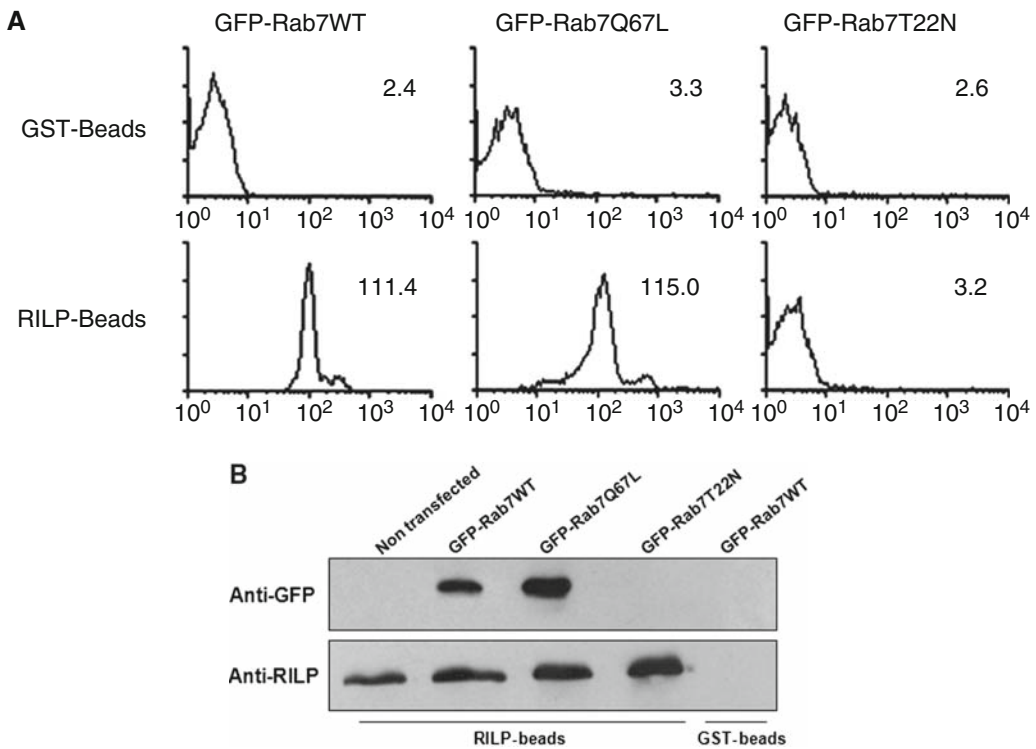


Fig. 2. Validation of Rab7 activation assay. (**A**) adherent RAW cells were transfected with wild-type (GFP-Rab7WT), constitutively active (GFP-Rab7Q67L) or dominant negative (GFP-Rab7T22N) Rab7. Cell lysates were prepared, cleared of insoluble material and then incubated with GST-RILP- or GST (control)-coated latex beads. Beads were then washed and stained with rabbit anti-GFP (1:100 dilution) followed by FITC conjugated to antirabbit (1:50 dilution). Samples were then subjected to analysis by flow cytometry according to parameter sittings shown in **Fig.** 1. Fluorescence signals from 10,000 events were recorded from the G1 gate, and the mean fluorescence intensities were determined (values in top right side of each panel). The shift of fluorescence histogram (binding to GST-RILP-beads relative to binding to GST-beads) shows the extent of Rab7 binding to immobilized RILP. GST-RILP beads were able to pull down GFP-Rab7WT and GFP-Rab7Q67L, but not GFP-Rab7T22N, from cell lysates. (**B**) equal numbers (~$50 \times 10^7$) of latex beads prepared as in (**A**) were subjected to SDS-PAGE and Western blotting with anti-GFP antibodies (*upper panel*). Membranes were stripped and reprobed with rabbit anti-RILP antibodies (lower panel) to control for level of RILP-GST in the reactions. As was observed in the flow cytometry analysis, the dominantly negative form (T22N) of Rab7 was unable to interact with RILP.

pathogenic mycobacteria. The results shown in **Fig.** 3a (FACS analysis) and **Fig.** 3b (Western blotting) indicate that active Rab7 is reduced in cells infected with live *M. bovis* BCG compared to cells exposed to killed BCG or infected with the nonpathogenic species *M. smegmatis*. These findings are consistent with previously published findings showing phagosome maturation arrest in macrophages infected with live BCG but not in cells exposed to either killed BCG or infected with *M. smegmatis (4, 15, 16)*.

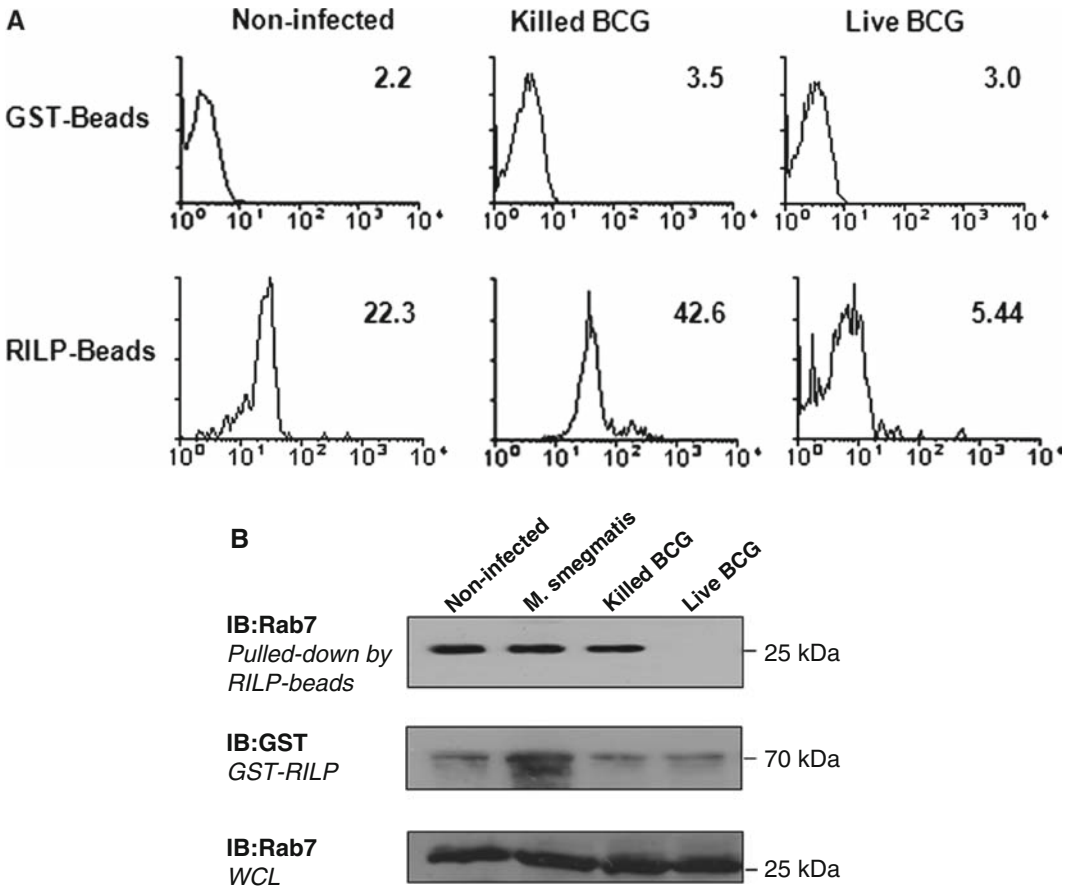


Fig. 3. Live BCG infection disrupts Rab7-RILP interaction in J774 macrophages. (**A**) J774 macrophages were infected with live or killed BCG and cell lysates were prepared at 4 h postphagocytosis. BCG organisms were killed by 2 h treatment with 50 μg/ml gentamycin at 37°C. Lysates were mixed with RILP-GST or GST (control) beads to pull down endogenous active Rab7. Samples were then washed and stained with rabbit anti-Rab7 (1:100 dilution) followed by FITC-conjugated antirabbit (1:50), and subjected to flow-cytometry analysis as described in **Fig. 2a**. The results show that RILP beads failed to pull down Rab7 from lysates of cells infected with live BCG indicating that infection blocked GDP/GTP exchange. (**B**) macrophages were infected with either live or killed BCG or with the nonpathogenic species *M. smegmatis*. Endogenous Rab7 was pulled down as described in (**A**) and equal numbers of beads were subjected to SDS-PAGE and Western blotting with anti-Rab7 antibodies (upper panel). Membranes were stripped and reprobed with rabbit anti-GST antibodies (middle panel) to control for level of RILP-GST in the reactions. Equal amounts of whole-cell lysate (WCL) protein from each treatment sample were subjected to Western blotting with anti-Rab7 antibodies to control for levels of Rab7. Live BCG, unlike either killed BCG or nonpathogenic *M. smegmatis*, blocks the interaction of Rab7 with RILP.

### 3.1 Cell Culture and Infection

1. Macrophage transfection with Rab7 constructs (*see* **Note 3**).
2. Allow RAW cells to adhere overnight in 6-cm diameter culture dishes: ~$10^5/cm^2$.
3. Wash cells twice with HBSS and replenish wells with antibiotic-free media.
4. Infect cells with mycobacteria (multiplicity of infection, 20:1) for 2 h at 37°C and 5% $CO_2$, then wash three times with HBSS and reincubate for an additional 4 h.
5. Wash three times with HBSS and harvest by cell scraper.
6. Lyse cells on ice for 30 min in macrophage lysis buffer.
7. Centrifuge at 13,000 rpm for 10 min at 4°C to remove cell debris. Collect lysate and store at –70°C until ready for incubation with coated latex beads (**Subheading 3.4.1**). This lysate contains the sample of either endogenous Rab7 or transfected GFP-Rab7 for assay of activation state.

### 3.2. Protein Overexpression and Purification

1. Transform RILP-GST plasmid into *E. coli* strain BL21.
   (a) Mix DNA (1–2 μl) and competent BL21 cells (80–100 μl), then keep on ice for 1–1.5 h.
   (b) Heat shock for 3 min at 42°C, then add 900 μl LB media, and incubate 1 h at 37°C with shaking.
   (c) Plate on LB agar plates containing ampicillin (100 μg/ml) and chloramphenicol (15 μg/ml), then incubate overnight at 37°C.
2. Pick one growing colony and transfer into LB medium containing ampicillin (100 μg/ml) and chloramphenicol (15 μg/ml). Allow the culture to grow to an $OD_{600}$ of 0.6 at 37°C.
3. Induce expression of RILP-GST with 0.2 mM IPTG at 22°C overnight.
4. Spin down bacterial cells (15 min, 5,000 rpm, 4°C), resuspend in bacterial lysis buffer for 30 min, and lyse on ice by sonication for 2–3 min at 30 s intervals.
5. Clarify bacterial lysate (30 min, 15,000 rpm, 4°C), then purify GST fusion protein using glutathione-agarose resin by passing through the column several times (*see* **Note 4.**).
6. Elute the fusion protein using 10 mM reduced glutathione in 50 mM Tris-HCl, pH 9.5.
7. Collect three 1-ml fractions and examine on SDS-PAGE for purity and estimate concentration using Bio-Rad protein concentration assay kit.

### 3.3. Latex Bead Coating

1. Adjust MES buffer to pH 5.86 (pI of RILP-GST).
2. Transfer 100 μl of aldehyde/sulfate latex beads (about $10^8$ beads) into an Eppendorf tube and wash once with 1 ml

MES buffer in a microcentrifuge, 5 min at full speed. Resuspend beads in 50 µl MES buffer.

3. Add purified RILP-GST or GST at a final concentration of 250 µg/ml to the beads in a total volume of 500 µl MES buffer and incubate overnight at room temperature on a shaker.
4. Wash the beads three times with PBS in a microcentrifuge, 5 min at full speed and resuspend in 1 ml of PBS-BSA-Az.
5. In order to confirm proper coating of beads (a) incubate 20 µl sample with mouse anti-GST (1:100 dilution in binding buffer) for 20 min at room temperature. (b) Wash once with binding buffer in a microcentrifuge, 5 min at full speed (c) Stain with FITC-conjugated goat antimouse at 1:50 dilution for 20 min. (d) Wash twice with binding buffer and resuspend in 500 µl binding buffer. Evaluate the coating by FACS analysis by reference to uncoated latex beads stained with the same antibodies as the coated samples.

### 3.4. Sample Incubation and Washes

1. Incubate each cell lysate sample with 50 µl of RILP-GST bead preparation; where controls are needed, set aside half of the lysate sample to incubate with GST-coated beads (50 µl).
2. Mix on rocker overnight at 4°C or 3–4 h at room temperature (*see* **Note 5**).
3. Wash three times with PBS in a microcentrifuge, 5 min at full speed and resuspend in 100 µl PBS. Split each sample into two equal fractions; set aside one fraction for FACS and another fraction for immunoblot confirmation.

### 3.5. Flow Cytometry Analysis

1. Stain sample from **Subheading 3.4** with rabbit anti-Rab7 (or rabbit anti-GFP if using transfected Rab7-GFP constructs) diluted 1:100 for 20 min at room temperature.
2. Wash once with 1 ml binding buffer in a microcentrifuge, 5 min at full speed.
3. Stain with FITC-conjugated goat antirabbit IgG at dilution of 1:50 for 20 min.
4. Wash two times with binding buffer and resuspend in 500 µl of the same buffer.
5. Analyze by FACS according to parameter setting shown in **Fig. 1**.

### 3.6. Immunoblot Confirmation

1. Resuspend beads in sample buffer and heat at 90°C for 10 min.
2. Prepare a 1.5-mm thick, 12% gel by mixing 2.5 ml of separating buffer, 4.0 ml of 30% acrylamide/bis solution, 3.4 ml

distilled water, 50 µl ammonium persulfate solution, and 5 µl TEMED. Pour the gel between the glass plates leaving some room for the stacking gel and overlay with water-saturated isobutanol. It will take about 20–30 min to polymerize.

3. Pour off the isobutanol and rinse the top of gel with water.
4. Prepare the stacking gel by mixing 2.5 ml of stacking buffer with 1.3 ml of acrylamide/bis solution, 6.1 ml water, 50 µl ammonium persulfate solution, and 5 µl TEMED.
5. Pour the stacking gel on top of the separating gel until it overflows. Insert 10- or 15-well comb gently without causing bubbles and overflow more gel mixture. The stacking gel should polymerize within 30 min.
6. Add some running buffer to cover the bottom of tank for the electric current. Carefully place prepared gel into the inner chamber and lock tightly into the electrophoresis tank. Take out comb gently and fill inner chamber with running buffer till it overflows.
7. Load wells with samples leaving one for the prestained molecular weight marker (*see* **Note 6**).
8. Place lid on tank and connect to power supply. The gel can be run at 100 V for about 10 min or until the gel front has reached the separating layer. Then it can be run at 150 V for the remainder of the duration. Stop the gel when the gel front is about to or has just run out of the bottom of the glass plates.

### *3.7. Immunoblotting Confirmation (Membrane Transfer and Probing)*

1. The gel will be transferred to a supported nitrocellulose membrane (NC) elecrophoretically. These directions assume the use of an OWL separation system.
2. To set up the transfer cassette, soak three sheets of Whatman paper in transfer buffer and lay on the cassette surface. Remove bubbles within by rolling over with a stirring rod. Then carefully remove your separating gel from the glass plates and place on top of these three sheets of Whatman paper.
3. Cut a piece of NC membrane slightly larger than size of the gel and soak in transfer buffer for 10 s. Then cut one corner to indicate lane order by marker and lay on top of the gel. Finish by placing three more sheets of soaked Whatman paper on top and chase bubbles with a rod again.
4. Cover with lid of apparatus and set power supply to 200 mA for 20–25 min.
5. Carefully disassemble apparatus after completion and check if the marker has transferred appropriately.

6. Place membrane in shallow container for incubation with 10 ml blocking buffer for 1 h at room temperature on rocker.
7. Remove blocking buffer and add anti-Rab7 or anti-GFP (*see* **Note 7**) at a dilution of 1:1,000 in blocking buffer. Incubate for 1.5–2 h at room temperature with shaking.
8. Recycle the primary antibody and perform three washes of 5 min each with TBS-T and shaking.
9. Prepare fresh secondary antibody (HRPO conjugate) at 1:5,000 dilution in TBS-T. Incubate for 30–40 min at room temperature with shaking.
10. Discard secondary antibody and wash thoroughly four times with TBS-T for 10 min each at room temperature with shaking (*see* **Note 8**).
11. Prepare ECL reagent by mixing 1 ml of each solution and then adding it on top of the membrane to cover it fully. Allow 3–5 min of incubation at room temperature without shaking. Using tweezers, stand membrane on Kimwipe to drain off excess solution.
12. Place membrane on top of a cardboard or glass plate and carefully cover with saran wrap. Make sure to avoid bubbles on the surface of membrane in contact with the wrap.
13. Proceed to dark room to develop the membrane by placing a cut piece of film over the membrane, remembering to cut the corner of the film to align with the membrane. Quickly close the developing cassette and develop for 1 min. Open the cassette and carefully take out the film without moving it around in contact with the membrane, and put into developing solution and shake until bands start to appear. Next, transfer the film to water and quickly rinse and transfer to fixative solution. Shake for 10 s then wash with water again.
14. Dry film and examine by aligning the lanes of the membrane according to marker and analyze for presence of Rab7 at about 24 kDa in the samples that detected positive for active Rab7 by FACS.
15. The bands can be quantified as well by densitometry.
16. The membrane may then be stripped by incubation in stripping buffer for 5 min, 37°C. Then wash the membrane well with distilled water to remove traces of NaOH. Proceed to wash once with TBS-T.
17. Reprobe with mouse anti-GST as described in **step 7** and repeat film exposure and development steps. There is no need to reblock the membrane after stripping. The anti-GST probing should demonstrate equal loading of samples in all the lanes.

## 4. Notes

1. RILP-GST was prepared using pGEX-4T3 vector as previously described *(6, 16)*.
2. Prepare also a half dilution of the elution buffer for use as wash buffer. At 5 mM reduced glutathione, RILP-GST should not be eluted, but the contaminants will be. It is important to keep small aliquots of the purified protein and perform normal SDS-PAGE with Commassie blue staining to make sure that RILP-GST is free of contaminant bacterial proteins.
3. The transfections used in the experiments described used Lipofectamine 2000 (Invitrogen). According to the manufacturer's instructions, the optimal parameters for transfections should be defined for the cell line chosen. The purity of the plasmids for transfection is critical for the success of transfection. In our laboratory, J774 and RAW 264.7 macrophage cell lines yield optimal transfection rates when transfected at ~90% confluency in 6-cm diameter culture dishes. For RAW cells, ~10 μg DNA in 250 μl Opti-MEM is mixed with equal volume of Opti-MEM containing 12% Lipofectamine. After 20 min incubation at room temperature, 0.5 ml Opti-MEM is added to the preparation and the mixture is added to adherent cells. A similar procedure was used for J774 cells but with lower amount of DNA (~5 μg) in order to reduce toxicity.
4. It is recommended to passage the lysate through the column at least three times if the flow rate is good. Prior to elution, it is also recommended to wash with one column full of the diluted elution buffer to yield better purity, although this step is optional.
5. This experiment was also successful with a shorter incubation period (3–4 h) of macrophage lysate with RILP-beads. So it is not essential for overnight binding if there are time restrictions. However, it is recommended that the 3–4 h incubation be done at room temperature.
6. Prior to loading, quickly spin down the beads in a microfuge, and be careful to pipette only the liquid and not to load beads into the wells. Each well made with a 1.5-mm comb can hold about 50 μl of sample. Be sure to have equal loading in all wells. Only 6–8 μl of the prestained marker is necessary.
7. Anti-Rab7 can be used for cases of endogenous Rab7 or transfected GFP-Rab7. In the case of transfections, using this antibody will produce two bands of Rab7: one at ~52 kDa (GFP-Rab7) and one at ~25 kDa (Rab7 – endogenous). In both cases, only active GTP-bound Rab7 will appear on the membrane.

8. This is the critical step in removing background noise from immunoblotting. The stated times for this step should be followed strictly, and washes that are longer will not be deleterious.

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# Chapter 6

# Probing In Vivo Origins of Mononuclear Phagocytes by Conditional Ablation and Reconstitution

**Chen Varol, Limor Landsman, and Steffen Jung**

## Summary

Dendritic cells (DCs) and macrophages (MΦs), collectively termed mononuclear phagocytes (MP), are crucial for homeostatic tissue maintenance as well as the innate and adaptive host defense. These pleiotropic functions are likely to be covered by distinct DC and MΦ subsets, defined by anatomic location and molecular make-up. However, task division within the MP system remains poorly defined. A key to understanding of this issue, which might have important implications for the development of future therapeutic strategies, is the elucidation of the in vivo origins of DCs and MΦs, whose study recently gained striking momentum. Here we present methods to investigate the role of MP progenitors, such as monocytes and MΦ/DC precursors (MDPs), in the replenishment of the peripheral MP system.

**Key words:** Mononuclear phagocytes, Macrophages, Dendritic cells, Monocytes.

## 1. Introduction

Mononuclear Phagocytes (MPs) play an important role in homeostasis and the innate and adaptive host defense against pathogens *(1, 2)*. MPs have been divided into two major terminally differentiated subsets: Macrophages (MΦs) and Dendritic cells (DCs). In the organism, both these cell types consist of multiple subpopulations largely defined by distinct anatomic location and phenotypes *(3, 4)*. MΦs include tissue-specific representatives in the serosa, lamina propria (Lp), lung, brain, bone, and liver *(1)*. DCs, on the other hand, have in the mouse been subdivided into epidermal Langerhans cells (LCs), plasmacytoid DCs, and CD11c$^{hi}$ conventional DCs (cDCs) found in the spleen and periphery *(4)*.

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Peripheral MP populations are thought to rely on continuous replenishment from BM-resident hematopoetic stem cells (HSCs). The HSC-to-MP differentiation pathway includes a number of BM intermediates, such as common myeloid precursors (CMPs) *(5)*, granulocyte/MΦ precursors (GMPs) *(5)*, and MΦ/DC precursors (MDPs) *(6)*, characterized by progressively limited developmental plasticity arising from asymmetric cell division. MDPs give rise to BM monocytes, which egress to the blood, where they contribute to the inflammatory response and act as circulating precursors of tissue MΦs and DCs *(7–10)*. Notably, some DC and MΦ populations, such as brain microglia, epidermal LCs, and splenic cDCs, arise in the adult steady state from local precursors without monocytic intermediates *(11–13)*.

As in man, murine monocytes comprise two phenotypically distinct subsets characterized as either $Gr1^{hi}CCR2^{+}$ $CD62L^{+}$ $CX_3CR1^{int}$ or $Gr1^{low}CCR2^{-}CD62L^{-}CX_3CR1^{high}$, *(9)*. Under steady-state conditions, $Gr1^{hi}$ monocytes shuttle between the blood and the BM, where they can convert into $Gr1^{low}$ monocytes *(7, 14, 15)*. Inflammation results in rapid CCR2-mediated recruitment of the $Gr1^{hi}$ monocytes to the site of insult *(7, 9, 16)*, whereas the homing pattern of $Gr1^{lo}$ monocytes remains less understood. With regard to differentiation potential, $Gr1^{hi}$ monocytes were shown to give rise to LCs under inflammation *(17)* and to intestinal and lung DCs under noninflammatory conditions *(18, 19)*. Differentiation of $Gr1^{hi}$ monocytes into lung MΦs, however, requires their prior conversion into $Gr1^{low}$ cells *(18)*.

The most direct way to investigate the origin of MPs is by the adoptive transfer of precursor cells into recipient mice and subsequent tracking of graft descendants. Here we present methods that allow studying the potential of MDPs, BM monocytes, and blood monocytes to differentiate in vivo into splenic, intestinal, and pulmonary MPs. In combination with a novel Diphtheria toxin-based conditional in vivo cell ablation strategy *(20)*, this transfer approach allows for efficient in vivo MP reconstitution and sets the stage for future functional studies.

## 2. Materials

### 2.1. Animals

Our approaches involve the use of the following mouse strains (*see* **Note 1**):

1. *CD11c*:DTR transgenic mice (B6.FVB-Tg(Itgax-DTR/GFP) 57Lan/J, Jackson laboratory, stock #004509) that carry a human diphtheria toxin receptor (DTR) transgene under the murine CD11c promoter *(20)*.

2. $CX_3CR1^{gfp}$ mutant mice (B6.129P-*Cx3cr1tm1Litt*/J, Jackson laboratory, stock #005582) *(21)* harboring a targeted replacement of the *cx3cr1* gene by an enhanced green fluorescent protein (EGFP) reporter gene. $CX_3CR1$ is the receptor of the membrane-tethered chemokine $CX_3CL1$ (fractalkine (Fkn) *(22)*. In the transfers we generally use heterozygote mutant mice ($CX_3CR1^{gfp/+}$) that show a reduced surface receptor expression, but exhibit no overt phenotype *(21)*. $CX_3CR1^{gfp}$ donor mice have been crossed to mice bearing the CD45.1 allotype (B6.SJL-*Ptprc*$^a$ *Pepc*$^b$/BoyJ, Jackson laboratory, stock #002014).

### 2.2. Conditional In Vivo Ablation of MPs

1. Diphtheria toxin (DTx) is dissolved in PBS at a concentration of 1 mg/ml and stored in 10 µl single-use aliquots at –80°C. Aliquots are diluted in PBS prior to use.
2. *FACS buffer contains.* 1% fetal bovine serum (FBS), 2 mM $Na_2EDTA$, and 0.05% sodium azide. Fluorochrome-conjugated antibodies used include: CD11c-APC or CD11c-PE (eBioscience, clone N418), MHCII-PE (eBioscience, clone M5/114.15.2), and CD11b-APC (eBioscience, clone M1/70), stored at 4°C. Dilutions are performed in FACS buffer.
3. *FACS-Blocking reagents.* Antimouse CD16/32-FC (FC) block (eBioscience clone #93) or goat IgG (Cat#I5256, Sigma Aldrich, Israel) stored at 4°C.

### 2.3. Adoptive Transfer of MDPs or Monocytes into MP-Depleted CD11c-DTR transgenic Recipient Mice

1. Ficoll-Paque™ PLUS (Amersham Biosciences AB, Uppsala, Sweden). Ficoll is light sensitive, and therefore should be stored either in the dark or in opaque bottles at RT.
2. *Antibodies.* CD117 (cKit)-PE (BioLegends, clone 2B8), Gr1-APC (eBioscience, clone RB6-8C5), CD11b-PerCP (BD-Pharmingen, clone M1/70), CD115-Biotin (eBioscience, clone AFS98) stored at 4°C.
3. Heparin sodium from bovine intestine is reconstituted at 10,000 units/ml with DDW and stored at 4°C.
4. *Magnetic cell sorting (MACS) buffer contains.* 1% fetal bovine serum and 2 mM $Na_2EDTA$ (*see* **Subheading 2.2.3**) (no Azide!). Five ml of 100 mM EDTA is added to 10 ml of FBS, stored for 10 min at RT, and added to 500 ml of PBS without $Ca^{++}$ and $Mg^{++}$ (PBS–/–), sterile filtered, and degassed.
5. Magnetic Cell Sorting (MACS) instruments and reagents (Miltenyi Biotec, GmbH, Germany): MiniMACS Separator, MS columns, and Streptavidin-Microbeads (cat #130-048-102), which should be stored at 4°C and protected from light are used according to the manufacturer's protocols.

6. Anesthetic: 10% Xylazine (20 mg/ml) and 10% Ketamine (100 mg/ml) in PBS−/−. Mixture volume of 100 µl per 10 g of recipient bodyweight is injected i.p.

### 2.4. Analysis of Precursor Graft-Derived MPs

1. Collagenase D is dissolved in PBS at a concentration of 200 mg/ml and stored in 20 µl (4 mg) single-use aliquots at −20°C. Aliquots are diluted in PBS with $Ca^{+2}$ and $Mg^{+2}$ (PBS+/+) prior to use, to achieve 1 mg/ml solution.
2. *ACK buffer*. 0.15 M $NH_4C$, 0.01 M $KHCO_3$) is made by dissolving of 8 g of $NH_4Cl$ and 1 g of $KHCO_3$ in 1 l of DDW. Solution is then divided into 50 ml aliquots and stored at −20°C.
3. A solution of 1 mM DTT is made by dissolving the DTT powder in PBS−/− to a final concentration of 0.1,543 mg/ml.
4. For intestine digestion, collagenase IV (0.5 mg/ml) and DNAse (0.2 mg/ml) are dissolved in PBS+/+.
5. PFA (2%) solution is made by dissolving 10 g of paraformaldehyde in 200 ml of water at 60°C (solution appears milky). NaOH is then added, drop by drop, until solution is cleared. A volume of 50 ml × 10 concentrated PBS is added, and the solution is cooled down. Volume is then completed to a total of 500 ml, and pH is adjusted to 7.4 using HCl.

## 3. Methods

### 3.1. Conditional In Vivo MP Ablation

#### 3.1.1. Short-Term In Vivo Ablation of Peripheral MPs

1. Conditional ablation of MP is achieved using a Diphtheria Toxin (DTx)-based cell ablation strategy *(23)*. CD11c-DTR transgenic mice carry a transgene encoding a human Diphtheria Toxin receptor (DTR) fused to the green fluorescent protein (GFP) under the control of the murine CD11c promoter *(20)*. CD11c-expressing MP, including all cDCs and specific Mφ subsets *(23–25)*, are sensitive to DTx and are hence depleted upon toxin injection. In this conditional cell ablation system, targeted cells die by apoptosis and are removed without causing major inflammation *(26)*.
2. For systemic short-term MP depletion, mice are injected once intraperitoneally (i.p) with 4 ng/g body weight DTx *(20)*.
3. After i.p injection, toxin-induced MP depletion in spleen, intestine, BM, and LNs persists for 2 days, after which MP numbers are gradually restored *(20)* (**Fig. 1**). Lung DCs and MΦs, however, are not efficiently depleted by this procedure, but can be targeted by intratracheal (i.t.) DTx application (*see* **subheading 3.1.2**) *(18, 24)*.

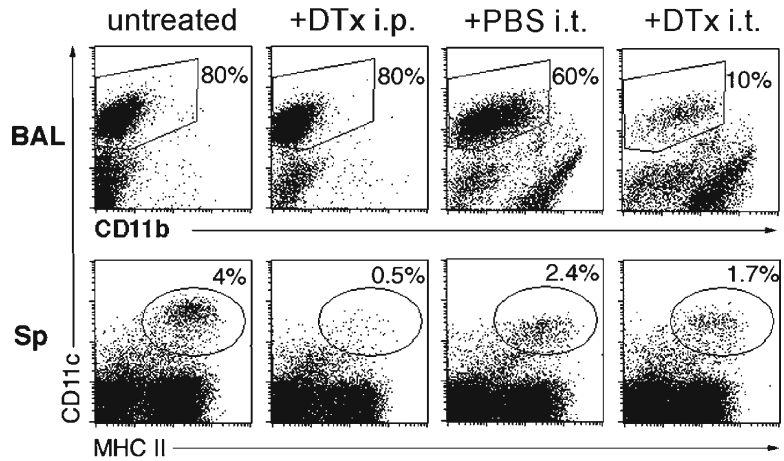


Fig. 1. In vivo depletion of *CD11c*:DTR transgenic CD11c$^+$ lung and spleen cells upon intratracheal or intraperitoneal instillation of Diphtheria toxin (DTx), respectively. *CD11c*:DTR mice were treated with DTx either i.p. (4ng/g, + DTx i.p.) or i.t. (100ng, + DTx i.t.). Control littermates were either treated with PBS i.t. (+PBS i.t.) or left untreated. Lungs and spleens were analyzed one day after treatments. Data show FACS analysis of lung cells for CD11c and CD11b expression (*upper panels*) and of spleen cells for CD11c$^+$ and MHC class II expression (*lower panels*). Numbers indicate percentage of gated cells from total cells.

4. Recipient mice are DTx treated prior to adoptive transfer of donor cells. I.p injection is usually done one day before, whereas i.t instillation of DTx is performed 3 h before transfer.
5. Transient CD11c$^{hi}$ cell depletion is not associated with signs of illness or long-term defects *(20)*. However, repetitive systemic DTx application results in lethality in CD11c-DTR-transgenic mice. For long-term in vivo ablation of peripheral MP see **subheading 3.1.3**.

*3.1.2. Short-Term In Vivo Ablation of Pulmonary MPs*

1. Mice are lightly anesthetized using an isofluorane inhalator (IMPAC$^6$, VetEquip) and placed in a little suspension rack *(27)* with their thorax placed vertically, suspended by their teeth. The tongue is pulled to the side.
2. Using a micropipette with a long nasal tip 80 µl of a 1.25 µg/ml DTx solution (total of 100 ng) is applied into the larynx, while keeping the tongue pulled out. In this way, the mouse is unable to swallow, and the droplet is aspirated. Gasping of treated mice verifies liquid application to the alveolar space.
3. In our hands, intratracheal installation of toxin is the most efficient way to ablate pulmonary MP, while having no or only limited systemic effects (**Fig. 1**).

*3.1.3. Long-Term In Vivo Ablation of Peripheral MPs*

1. In contrast to CD11c-DTR transgenic mice, syngeneic [DTR > wt] BM chimeras, generated by reconstitution of lethally irradiated wt recipient mice with CD11c-DTR BM, can be treated with DTx for prolonged periods of time without adverse side effects *(28)*.
2. *BM chimera generation*. C57BL/6 wt mice are exposed to a single lethal total body irradiation of 950 rad, followed by intravenous (i.v.) transfer of $5 \times 10^6$ CD11c-DTR transgenic BM cells one day after (*see* **Note 4**). BM cells are isolated according to the procedure described later (*see* **Subheading 3.2.2**). Mice are allowed to rest for 8 weeks before use.
3. For systemic long-term MP depletion [DTR > wt] BM chimeras are injected every other day i.p. with DTx (8 ng/g body weight).

*3.1.4. Analysis of Depletion Efficiency by FACS*

1. Cells are isolated from the examined tissues (*see* **Subheading 3.3**) and stained with fluorochrome-conjugated monoclonal antibodies (*see* **Notes 2** and **3**).
2. Prior to the staining, cells are incubated with a 1:100 dilution of goat IgG or FC block, for 15 min on ice to block Fc receptors (staining volume ~10 µl/$10^6$ cells).
3. Cells are then stained for cell-specific markers. We routinely add a 2× concentrated mix of fluorochrome-conjugated CD11c and MHCII or CD11b antibodies and keep the cells 15 min on ice (final staining volume ~20 µl/$10^6$ cells). All dilutions are done in FACS buffer.
4. Following staining, cells are washed with FACS buffer (~50× staining volume) and centrifuged at 1,200 rpm (290 ×*g*) for 7 min at 4°C.
5. Cells are resuspended in FACS buffer and analyzed by multicolor flow cytometry on a FACSCalibur cytometer (BD Biosciences) using CellQuest Pro software (BD Biosciences) (**Fig. 1**).

### *3.2. Adoptive Transfer into MP-Depleted CD11c-DTR Recipients*

*3.2.1. The $CX_3CR1^{gfp/+}$ Transgenic Donor Mice*

1. To study in vivo origins of MP, recipient mice are engrafted with $CX_3CR1^{gfp/+}$ MP precursor cells, e.g., MDPs or monocytes. $CX_3CR1$ is expressed on MDPs *(6)*, as well as BM and blood monocytes *(9, 21)*, which are therefore green fluorescent in $CX_3CR1^{gfp}$ mice.
2. The long half-life of the GFP label allows the detection of adoptively transferred GFP-expressing precursors and their descendants in recipient mice, even if the progeny cease to express $CX_3CR1$/GFP *(7, 18)*. However, the label can be lost in the $CX_3CR1^-$ cell populations by cell division.

3. In addition to the GFP label, it is advisable to include allotypic markers that identify the grafted cells, such as CD45. We therefore use CD45.1; $CX_3CR1^{gfp}$ mice as donors.

*3.2.2. Isolation of Precursor Cells from the Bone Marrow*

1. Femura and tibiae of $CX_3CR1^{GFP/+}$ CD45.1 donor mice are isolated, and bone surface is exposed by removing the surrounding soft tissue.
2. Bone is opened at both ends, and using a 27 gauge fitted to a 1-ml syringe filled with 1 ml of cold PBS–/–, the marrow is flushed out of the bone cavity into a 15-ml tube. This step is repeated several times until the bone is empty and becomes white.
3. BM cells are then gently resuspended using 21G1 3-ml syringe until the suspension is homogenous. Cells are washed with 10 ml of PBS–/– by centrifugation at 1,200 rpm (290 × *g*) at 4°C for 7 min.
4. Mononuclear cells are subsequently enriched by granulocyte- and erythrocyte depletion on a Ficoll density gradient: the cell pellet is resuspended in 4 ml of PBS–/– in a 15-ml tube and the cell suspension is gently underlaid with 4 ml of Ficoll. Two layers with sharp interface should be preserved. The cell-containing tube is then centrifuged at 2,200 rpm (974 × *g*) without acceleration and break (!), 22°C (RT), for 15 min. The tube is gently removed from the centrifuge and the cloudy ring at the border between the two layers, which contains the enriched BM mononuclear cells, is isolated using a long Pasteur pipette. Cells are washed from residual Ficoll in 10 ml of PBS–/–, followed by centrifugation at 1,200 rpm (290 × *g*), 4°C for 7 min.
5. Enriched BM mononuclear cells are stained with the following fluorochrome-labeled monoclonal antibodies: CD-17-PE, Gr1-APC, and CD11b-Percp (*see* **Notes 2** and **3**). This staining allows the identification of BM MDP ($CX_3CR1$/$GFP^+$ CD117 (cKit)$^+$ $CD11b^-$ $Gr1^-$ *(6)* and BM monocytes ($CX_3CR1$/$GFP^+$ $CD11b^+$ $Gr1^{+/-}$) *(7)* (*see* **Note 5**). The staining is performed as described earlier (*see* **Subheading 3.1.4** and **Note 6**).
6. Following immunostaining, cells are purified by high-speed sorting using a FACS Aria (Beckton–Dickinson). Purity is evaluated by flow cytometry prior to the transfer (**Fig. 2a**).
7. Alternatively, a mixed population of BM MDP and monocytes can be isolated on the basis of CD115 expression using magnetic separation (*see* **Subheading 3.2.3**).

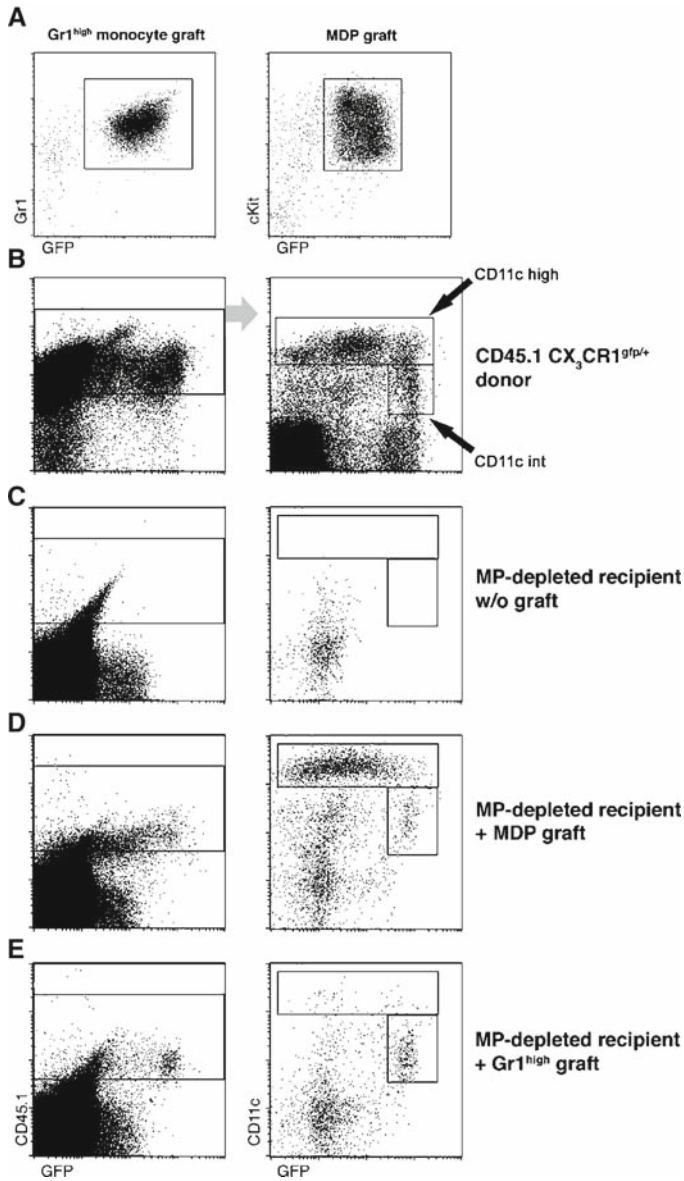


Fig. 2. MΦ/DC precursors (MDP), but not monocytes, replenish splenic conventional CD11c$^{high}$ DCs. MDP and Gr1$^{hi}$ monocytes were isolated from $CX_3CR1^{gfp}$ donor mice and adoptively transferred into [DTR > wt] chimeras. Recipients were i.p injected with DTx every 2 days to maintain the state of DC depletion. At day 7 post transfer, recipient mice were killed and spleens were isolated and analyzed by flow cytometry. (**A**) Flow cytometry analysis of Gr1$^{high}$ BM monocyte and cKit$^{+}$ MDP grafts isolated by high-speed cell sorting. (**B**) Flow cytometry analysis of $CX_3CR1^{gfp}$ donor spleen (CD45.1$^{+}$) showing presence of CD45.1$^{+}$ CD11c $^{high}$ DC (positive control), (**C**) spleen cells from DTx-treated, MP-depleted mouse without engraftment showing lack of CD11c$^{high}$ DC (negative control), (**D**) spleen cells from MP-depleted mouse that received $CX_3CR1^{gfp}$ MDP (CD45.1$^{+}$) graft ($7 \times 10^4$ cKit$^{+}$ CD115$^{+}$ Gr1$^{-}$ CD11b$^{-}$ cells; purity: 96%) showing presence of donor cell-derived CD11c $^{high}$ DC, and (**E**) spleen cells from MP-depleted mouse that received a $CX_3CR1^{gfp}$ Gr1$^{high}$ monocyte graft (CD45.1$^{+}$) ($9 \times 10^5$ CD115$^{+}$ Gr1$^{+}$ CD11b$^{+}$ cells; purity: 85%). Note lack of graft-derived CD11c$^{high}$ dendritic cells, but presence of CD11c$^{low}$ cells. The *right panels* of (**B**–**E**) show splenocytes of the respective mice gated for live cells according to scatter. The *left panels* of (**B**–**E**) show the same splenocytes gated in addition according to CD45.1 expression (as indicated in the *right panels*). Boxes highlight CD11c$^{high}$ DC and CD11c$^{low}$ cells.

*3.2.3. Isolation of Monocytes from the Blood*

1. For blood monocyte transfers approximately 75 mice are bled from their tail veins to collect 12–15 ml of blood (~200 µl blood/mouse). Alternatively, 20 mice are killed after overdosing with a Xylazine/Ketamine mix (120 µl/10 g bodyweight) and bled to obtain 15–18 ml blood. Heparin is added (50 µl/ml blood) to avoid coagulation.
2. Mononuclear cells are enriched by a Ficoll density gradient, which depletes granulocytes and erythrocytes: in a 15-ml tube, 1 ml PBS−/− is added to 2 ml blood, followed by underlying with 4 ml of Ficoll without interfering with the 3-ml blood layer. Two layers with a sharp interface should be preserved. Continue as described under **Subheading 3.2.2**, **step 4**.
3. Blood monocytes, defined as CD115 (MCSF-R)-positive cells, are isolated to high purity by magnetic cell sorting.
4. Blood cells are first blocked by incubation with goat IgG, diluted in MACS buffer 1:100, for 15 min.
5. Following Fc receptor blockade, cells are stained for 15 min with biotinylated antimouse CD115 antibody (final dilution of 1:100) (*see* **Notes 2** and **3**).
6. Cells are then washed with MACS buffer and centrifuged at 1,200 rpm (290 × *g*), 4°C for 7 min to remove unbound primary antibody.
7. The supernatant is removed with a pipette and cells are resuspended in 90 µl of MACS buffer (per $10^7$ total cells) (*see* **Note 7**).
8. Ten microliter of Streptavidin-Microbeads per $10^7$ total cells is added; the tube is mixed and incubated for 15 min at 4–8°C.
9. Cells are then washed with 10 ml of MACS buffer (1,200 rpm (290 × *g*), 4°C for 7 min) and the supernatant is removed with a pipette.
10. Cells (up to $10^8$) are resuspended in 500 µl of MACS buffer.
11. For magnetic separation, miniscale (MS) columns are used for up to $10^7$ labeled cells ($10^8$ total cells), and large-scale (LS) are used for up to $10^8$ labeled cells ($10^9$ total cells). Magnetic separation is performed according to the manufacturer's protocols (*see* **Note 8**).
12. After isolation of the column-bound cell fraction, cells are counted and purity is evaluated by staining a small fraction of the positive cells with Streptavidin-fluorochrome, or fluorochrome-conjugated anti-CD115 antibody and analysis by FACS **(Fig. 3a**).

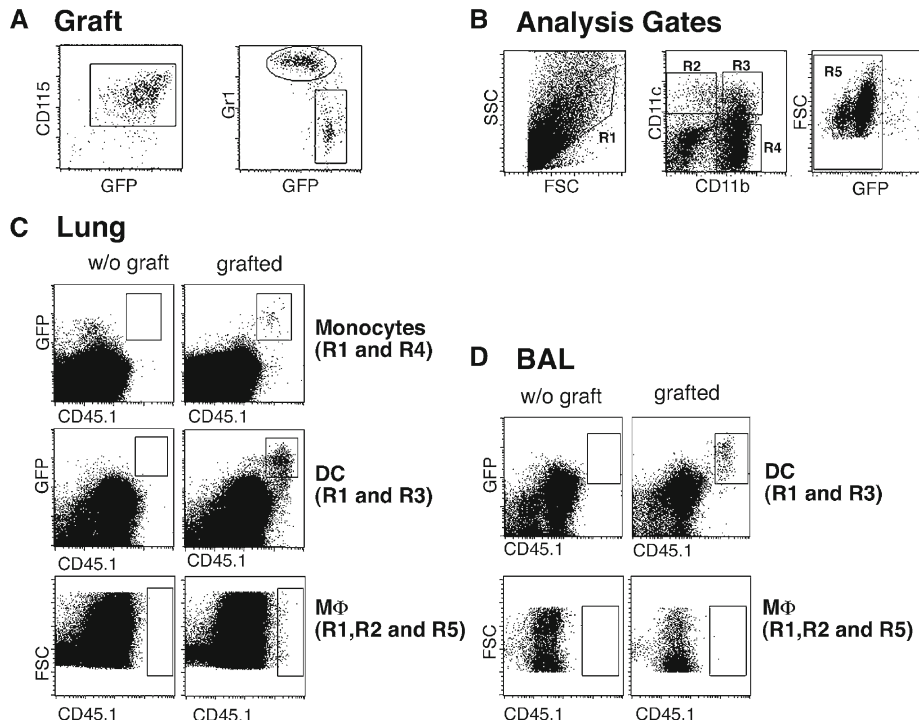


Fig. 3. The fate of adoptively transferred blood monocytes in the lung. Monocytes were isolated from the blood of $CX_3CR1^{gfp}$ donor mice and adoptively transferred into DTx-instilled, pulmonary mononuclear phagocyte (MP)-depleted *CD11c*:DTR transgenic mice. At day 7 post transfer, recipient mice were killed and analyzed by flow cytometry. (**A**) Flow cytometric analysis of representative magnetic cell sorting-enriched $CD115^+$ blood monocyte graft isolated from *cx*$_3$*cr1*$^{gfp/+}$;CD45.1 donor mice. Note presence of both $Gr1^{hi}CX_3CR1/GFP^{int}$ and $Gr1^{low}CX_3CR1/GFP^{hi}$ monocyte subsets. (**B**) Flow cytometric analysis of lung cells of an untreated *CD11c*:DTR mouse to indicate gates used in (**C**). *Left panel* shows FSC/SSC gates for living cells (R1). *Middle dot* plot shows CD11c/CD11b expression pattern of cells from the R1 gate that were distributed into three secondary mononuclear phagocyte gates (R2–R4) used in the analysis shown in (**C**): (R2, MΦ $CD11c^+$ $CD11b^-$, (R3, DC) $CD11c^+$ $CD11b^+$ and (R4, monocytes), $CD11c^-$ $CD11b^+$. *Right dot* plot shows GFP expression of cells gated in the R1 region and position of R5 gate. This gate is used to further define MΦ which are $CX_3CR1$/GFP-negative (*see* ref. 18). (**c**, **d**) DTx-treated *CD11c*:DTR monocyte-recipient lung, day 4 after transfer. *CD11c*:DTR;CD45.2 mice pretreated i.t. with DTx (100 ng, d.0) either received *cx*$_3$*cr1*$^{gfp/+}$;CD45.1 $CD115^+$ blood monocyte graft ($10^6$ cells, grafted) or no graft (w/o graft) 2 h after DTx treatment and were analyzed on day 4. Panel (**C**) shows enclosed in boxes: donor-derived monocytes (R1 and R4), DC (R1 and R3) and MΦ (R1, R2, and R5) found in lung parenchyma. Figure (**D**) shows analysis of bronchoalveolar lavage (BAL): DC (R1 and R3) and MΦ (R1, R2, and R5). Cells were gated as indicated in (**B**). The data show that grafted monocytes give rise to MΦ and DC in the lung parenchyma (**C**) and to DC, but not MΦ in the alveolar space (BAL, **D**).

*3.2.4. Adoptive Transfer into the Tail Vein (i.v.)*

1. Following their isolation, cells are resuspended in 200 µl of PBS−/−.
2. Recipient mice are moderately heated with an infrared lamp in order to promote vasodilatation, to allow easier injection to the tail vein
3. Cells are injected into recipient mice via the tail vein (*see* **Note 9**).

4. In order to reconstitute the splenic CD11c$^{high}$ conventional DC compartment, BM MDP should be transferred. Monocytes do not give rise to these cells (**Fig. 2**) *(7)*.
5. In order to reconstitute mucosal compartments, such as the intestinal lamina propria (**Fig. 4**) and the lung (**Fig. 3**), MDPs and monocytes can be transferred *(7, 18)*. MDPs differentiate in vivo into monocytes *(7)*.
6. We generally prefer to transfer near physiological numbers, e.g., 2–5 × $10^4$ BM MDP and 0.5–1 × $10^6$ BM and blood monocytes, respectively *(7, 18)*.

#### *3.2.5. Intrabone Cavity (IBC) Transfer*

1. To study precursor differentiation within the marrow, MDPs and monocytes can be transferred directly into the femural bone cavity *(7)*.
2. Mice are anesthetized by i.p injection of Xylazine and Ketamine mixture.
3. The skin covering the knee is incised and the knee is exposed.
4. The knee side of the femur is pierced with a 27G1/2 needle.
5. The cells to be transferred are concentrated in a volume of 5–10 µl sterile PBS−/− and injected into the femural BM cavity using a U-100 insulin syringe (with a 30G needle). The incision is closed (with MikRon Autoclips (Clay Adams Brand, BD, USA) or suture).

#### *3.2.6. Intrasplenic Transfer*

1. To directly investigate intrasplenic differentiation pathways (and avoid monocyte differentiation), MDPs can be injected directly into the spleen where they differentiate into splenic conventional CD11c$^{high}$ DCs in situ *(7)*.
2. Mice are anesthetized by i.p injection of Xylazine and Ketamine mixture. A small incision is made in the skin covering the spleen area, to expose the spleen, which is then pulled out gently without damaging blood vessels connected to it.
3. The cells to be transferred are concentrated in a volume of 20 µl and injected slowly into the spleen capsule by using a U-100 insulin syringe (with a 30G needle).
4. A Gel-foam melting sponge (Pfizer Inc, U.S.A) is inserted to prevent bleeding, and the incision is closed (with MikRon Autoclips (Clay Adams Brand, BD, USA) or suture).

### *3.3. Analysis of Precursors Graft-Derived MPs*

The precursor grafts can be expected to seed all organs of the recipient. Here we will focus on the analysis of three tissue compartments of the recipients: the spleen (as representative of a lymphoid tissue), and the lung, and the intestinal terminal ileum (as representatives of mucosal, nonlymphoid tissues).

*3.3.1. Analysis of Splenocytes for Graft-Derived MPs*

1. Mice are killed and the spleen is removed and placed in PBS in a 5-cm petri dish.
2. The efficient isolation of MPs requires, as opposed to lymphocytes, prior digestion of the tissues with matrix-degrading proteases. To this end the spleen is injected with 1 ml of collagenase D (1 mg/ml in $PBS^{+/+}$) using a 28 G 1/2 needle and incubated for 45 min at 37°C.
3. Following the collagenase D digest, spleen is minced and transferred via passage of an 80-μm mesh strainer into a 15-ml tube.
4. The cell suspension is then washed with 10 ml of PBS−/− and centrifuged at 1,200 rpm (290 × *g*), 4°C for 7 min.
5. Erythrocytes in the suspension are lysed by incubation with 1 ml of hypotonic ACK buffer for 2 min at room temperature (RT). Lysis is stopped by dilution of the ACK buffer with PBS−/− (tenfold volume at least). The suspension is then centrifuged and the pellet is washed.

*3.3.2. Analysis of Small Intestinal Lamina Propria MPs*

1. Mice are killed and the small intestine is isolated. By choosing proximal, central, or distal portions of the intestine the analysis can be focused on the duodenum, jejunum, and ileum, respectively.
2. Mesenteric tissue (lymph nodes) and fat are removed.
3. The intestine is flushed with PBS−/− to remove all its fecal content.
4. The intestinal tube is flipped inside out and incubated with RPMI for 20 min at 37°C (*see* **Note 10**).
5. The tissue is washed three times with PBS−/− at RT and then incubated for 10 min in 10 ml of 1 mM DTT (in PBS−/−). Following that, the intestine is washed once in PBS−/− (*see* **Note 11**).
6. The intestine is incubated for 10 min in 10 ml of EDTA 30 mM (diluted in PBS−/−) in a 15-ml tube with gently agitation by constant rotation. Incubation is once more repeated, using a fresh EDTA solution. The intestine is then transferred into a fresh 15-ml tube and washed four times with PBS−/− at RT.
7. Collagenase IV digestion is performed with the addition of DNAse, for 1 h at 37°C. (This stage is performed in a dish, with the intestine spread.)
8. The tissue is gently pipetted up and down using 1-ml tips (with their end cut-off) in order to physically release cells from the mucus layer.

9. The cell suspension is then passed through an 80-μm mesh strainer into a 15-ml tube. The dish is washed with PBS−/− in order to collect all released cells.
10. The cells are centrifuged at 1,200 rpm (290 × *g*), 10 min, 4°C (*see* **Note 12**).

*3.3.3. Analysis of MPs in the Bronchoalveolar Space and the Lung Parenchyma*

1. Mice are killed, their abdomen is opened, and the main artery is cut to allow a maximum blood volume to exit the circulation (*see* **Note 13**).
2. The mouse head is fixed on a Styrofoam board and the neck skin, and salivary glands are removed to expose the trachea.
3. The thin tissue layer covering the trachea is carefully removed using watch-maker forceps and scissors. The trachea rings are now exposed.
4. A 20-cm-long sewing thread is placed beneath the trachea and tied around it in proximity to the mouse head. Holding the string will allow to stretch the trachea for the next stages.
5. Using fine scissors a small hole is cut in the upper side of the trachea, next to, but below, the string.
6. A blunted (cut) 21G butterfly needle is inserted into the hole in trachea, and a 3-ml syringe, filled with 1.5 ml of PBS−/−, is attached to the needle.
7. A volume of 1 ml PBS−/− is slowly inserted into the mouse trachea through the infusion needle, resulting in the filling of the lungs with liquid.
8. The bronchoalveolar lavage (BAL) containing the cells is slowly withdrawn by pulling the syringe plunger.
9. The syringe is then removed from the infusion needle tube, and the BAL is transferred into a 15-ml tube.
10. **Steps 7–9** should be repeated three times.
11. The isolation procedure for MPs from the lung parenchyma is similar to the procedure for the splenic MPs, except that the lung tissue is injected with 1 ml of collagenase D solution at a concentration of 4 mg/ml (in PBS+/+).

*3.3.4. Analysis of Graft-Derived MPs by FACS*

1. The detection of $CX_3CR1^{gfp}$ graft-derived cells in recipient mice is based on two markers: the expression of EGFP in the grafted cells and their descendants, and the congenic CD45.1 marker. The analysis can rely on either or both the markers (*see* also **Subheading 3.2.1**, **step2**) (**Figs. 2** and **3b**)
2. FACS staining is performed as described earlier (*see* **Subheading 3.1.4**).

3. Additional markers such as CD11c, CD11b, and CD8 can be used to differentiate between DCs and MΦs, as well as different DC subsets derived from the grafted-precursors *(6, 7, 18, 25)*. (*see* **Note 14**).

*3.3.5. Analysis of Graft-Derived Lamina Propria DC by Fluorescent Microscopy*

1. In CD11c-DTR transgenic mice, the GFP protein is fused to the DTR *(20)* resulting in relatively poor fluorescence intensity. Using conventional fluorescence microscopy, CD11c-DTR transgenic cDCs can therefore not be reliably detected in histology without using an anti-GFP amplification *(29)*. $CX_3CR1^{gfp}$ precursor descendants, on the other hand, harbor an intense GFP label, in particular if they express $CX_3CR1$ (*see* **Subheading 3.2.1**, **step 2**). Together, this allows the GFP-based detection of $CX_3CR1^{gfp}$ graft-derived cells in CD11c-DTR transgenic mice *(7, 18)*.
2. In a typical experiment, $CX_3CR1^{gfp}$ graft recipients are killed, and the small intestinal ileum is isolated.
3. Mesenteric material and fat are removed.
4. The ileum is flushed with PBS−/− to remove all content.
5. A longitudinal incision is made along the ileum.
6. The tissue is placed on a microscope slide with its luminal side up.
7. The tissue is examined and imaged with a Zeiss Axioskop II fluorescent microscope using appropriate imaging software, such as Simple PCI.
8. In another approach, a systemic scan and visualization of large areas of the intestine is enabled by using the "Swiss-role" technique.

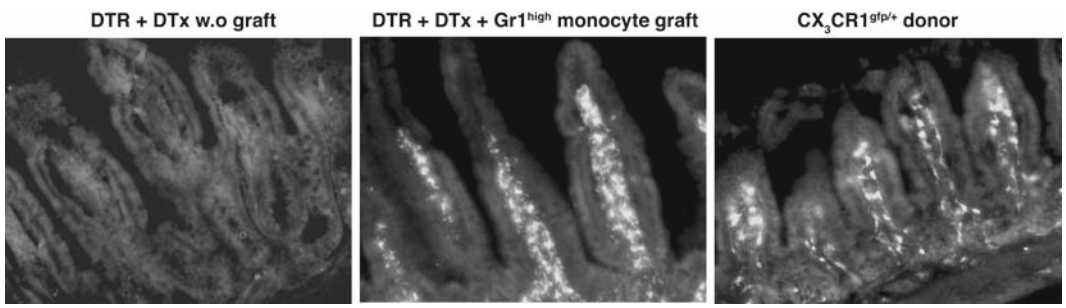


Fig. 4. Monocytes are precursors of intestinal lamina propria (Lp) DC. Fluorescence microscopy analysis of small intestinal villi of $CX_3CR1^{gfp}$ donor mouse (*right-hand panel*) and of mice depleted for LpDC and LpMΦ with (*middle panel*) or without (*left-hand panel*) adoptive transfer of $CX_3CR1^{gfp}$ BM $Gr1^{high}$ monocytes ($1 \times 10^6$ $CD115^+$ $Gr1^{high}$ $CD11b^+$ cells; purity: 85%). Note presence of graft-derived $CX_3CR1/GFP^+$ LpDC in the fixed cryostat sectioned tissue of BM monocyte recipient (Original magnification, 20×). Graft-derived cells can be identified as DC because of their location, morphology, and functional features (such as the formation of trans-epithelial dendrites *(25)* and migration to the mesenteric LNs (data not shown).

9. Ileum is longitudinally opened along the mesenteric wall, and rolled up, keeping its luminal side out.
10. Rolled tissue is fixed overnight (o.n.) with 2% PFA at RT.
11. Fixed tissue is then incubated o.n. with 15% sucrose at RT.
12. Rolled intestine is embedded in Tissue-Tek O.C.T compound at –20°C.
13. Tissue segments are sectioned into 10-μm segments, using a cryostat, and segments are then imaged with a Zeiss Axioskop II fluorescent microscope (**Fig. 4**).

## 4. Notes

1. All mice are maintained under Specific Pathogen-Free (SPF) conditions and handled under institutional protocols according to international guidelines.
2. Antibodies are used according to the manufacturer's protocols. All antibodies should be tested and titrated in advance to determine the best staining conditions, before analysis of samples from recipient mice.
3. The suggested total staining volume is 20 μl for up to $1 \times 10^6$ cells, 100 μl for $5 \times 10^6$ cells. A staining time of 10–15 min on ice is usually sufficient.
4. Irradiated mice receive transiently (for 1 week) an antibiotic (Ciproxin 0.2%) in their drinking water (20 μg/ml) to prevent bacterial infections during the period of immunodeficiency (the water bottle should be either dark or covered with aluminum foil to protect the light-sensitive drug).
5. Both BM MDPs and monocytes are $CD115^+$, which can be used as a marker for identification and isolation of these cells *(7, 18)*.
6. Presorting staining is performed in MACS buffer (instead of FACS buffer) to avoid azide exposure of the cells.
7. When working with less than $10^7$ cells, use the indicated volumes. When working with higher cell numbers, scale up all the reagents and total volumes, accordingly.
8. To increase the purity of the magnetically labeled fraction, it can be repassed over a freshly prepared column.
9. Volumes lower than 200 μl can also be injected i.v.
10. The intestine is mounted on a polyethylene tube with a diameter of 2.42 mm. One end of the intestine is tied with a sewing thread, and the rest is flipped on top of this end in order to

invert it. The tissue should be kept wet through the inversion process to avoid ripping. The inversion is a crucial step, as the digestion should be performed on the luminal side.

11. Washing is done by addition of PBS−/− to the tissue in the tube, shaking, and pouring off the fluid.
12. For an updated protocol of small intestinal lamina propria MPs isolation see the reference: Denning et al, Nature immunology, October 2007, vol 8, PP 1086–1094.
13. Blood is absorbed using Kim wipes, in order to prevent blood contamination of BAL fluid at later stages.
14. We suggest that each experiment will include at least two controls: a CD45.1 $CX_3CR1^{gfp/+}$ positive control mouse, and a DTx-treated CD11c-DTR transgenic mouse, that did not receive a graft to evaluate the background.

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# Chapter 7

# Multivalent Protein Probes for the Identification and Characterization of Cognate Cellular Ligands for Myeloid Cell Surface Receptors

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## Summary

Cell–cell interactions mediated by cell surface receptor–ligand pairs in the immune system are often of low affinity and transient in nature. To begin to study these weak interactions, it is desirable to devise a generally applicable method for screening for and enriching cells expressing low-affinity ligands for specific cell surface receptors. We describe here an experimental strategy that uses a multivalent form of protein as a probe to identify and characterize cognate ligand(s) of myeloid cell surface receptors. Recombinant fusion proteins containing the receptor protein fragment of interest fused to a truncated mouse Fc domain and a unique biotinylation signal are produced, biotinylated, and coupled to (strept) avidin-coated fluorescent or paramagnetic microspheres. These multivalent microparticle probes are then used to screen or capture cells expressing the cognate cellular ligand(s).

**Key words:** Biotinylation, Cell surface receptor, Fc, Ligand, Low Affinity, Multivalent Protein Probe, Protein–protein Interaction.

## 1. Introduction

Cell surface proteins play an important role in the functions of the immune system and are also critically involved in regulating maturation and differentiation of various immune cells. In addition, cell surface proteins also are one of the major determinants in directing and regulating the effector functions of immune cells *(1–5)*. For example, the interaction of the cell surface proteins

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and their ligands can often determine the fate of $T_H$ cells as either $T_H1$ or $T_H2$ cells *(6, 7)*.

While certain surface proteins such as cytokine receptors bind their cytokine ligands with relatively high affinity, the majority of the cell surface adhesion molecules show specific but weak and transient interactions with their cellular ligands, which themselves are usually cell surface molecules. A typical example is the E-selectin and E-selectin ligand-1, which have been shown to have a weak association constant of $K_a = \sim 7.4 \times 10^4\ M^{-1}\ S^{-1}$ *(8)*. It is thus essential in order to study the functional properties of the cell surface receptors to be able to characterize and identify their weak interacting cellular ligands.

We have previously demonstrated that the binding of the predominantly leukocyte-restricted CD97 receptor to its cellular ligand, CD55 is of both low affinity (86 μM) and transient (off-rate, 0.6 $s^{-1}$*9) (9)*. During this investigation, we devised an efficient method for characterizing the weak CD97–CD55 interaction. Our method is based on (a) the construction of an expression vector encoding the receptor protein of interest fused to a mouse Fc (mFc)-fragment and a specific C-terminal biotinylation signal sequence (b) the efficient production and purification of mFc-fusion proteins (c) the in vitro biotinylation of the mFc-fusion proteins (d) the generation of multivalent protein probes by coupling the biotinylated mFc-fusion proteins to (strept) avidin-coated fluorescent or paramagnetic microspheres, and (e) detection and selection of ligand-expressing cells by the multivalent protein probes *(10, 11)*. A general methodology that should be applicable to most cell surface proteins is described later using the CD97–CD55 interaction as an example.

## 2. Materials

### *2.1. Cell Lines and Cell Culture*

1. HEK-293T cells were derived from the human embryonic kidney (HEK) 293 cell line transformed with Ad5. CHO-K1 was derived from Chinese hamster ovary cells. K562 cells were derived from chronic myelogenous leukemia cells. The full details of cell lines used can be obtained from the American Type Culture Collection (http://www.atcc.org/).
2. *Cell culture media*. Ham's F12, RPMI-1640, OPTI-MEM I, and Dulbecco's Modified Eagle's Medium (DMEM).
3. *Media supplements.* 50 IU/ml penicillin, 50 μg/ml streptomycin, 2 mM L-glutamine, and 10% (v/v) heat-inactivated fetal calf serum (FCS).
4. Trypsin–EDTA (10×), liquid.
5. Tissue culture plasticware.

### 2.2. Construction of Expression Vectors

1. General chemicals are of analytical grade and obtained from Sigma or BDH-Merck, unless otherwise stated.
2. The expression vectors are constructed on the pcDNA3.1 (+) plasmid unless otherwise specified (*see* **Note 1**).
3. Advantage™-HF 2 PCR Kit for the high-fidelity PCR amplification of cDNA fragments (Clontech, Mountain View, CA).
4. UltraPure™ Agarose.
5. QIAquick Gel Extraction Kit (Qiagen, Valencia, CA).
6. 1 Kb Plus DNA Ladder and the PCR®4-Topo TA cloning kit (Invitrogen).
7. Enzymes for the restriction digestion, dephosphorylation, and ligation of DNA (New England Biolabs, Beverly, MA).
8. QIAprep® Spin Miniprep kit and HiSpeed® Plasmid Midi and Maxi kits (Qiagen, Valencia, CA).
9. The murine IgG2b genome DNA for the amplification of the truncated mouse Fc region was kindly provided by Dr. Lisa Gilliland (University of Oxford) *(11, 12)*.
10. *Primers.* XbaBio (5′-AATCTAGAGATCCAAATTCCGGA-3′) and Bio3 (5′-TAGTAGGGGCCCTTAACGATGATT CCACACC-3′) are for the amplification of the biotinylation signal sequence. NotmFc (5′-AGGATCCTGAATTCGCG-GCCGCAGAGCCCAGCGGGCCT-3′) and mFcXba (5′-TTCTCTAGATTTACCCGGAGACCGGGA-3′) are for the mFc fragment. The oligonucleotide primers were produced and purified by Sigma.
11. *6× DNA-loading dye.* 0.05% (w/v) Orange G and 30% (v/v) glycerol
12. *1× TBE buffer (pH8.0).* 0.09 M Tris–base, 0.09 M boric acid, 2 mM EDTA.
13. Ethidium bromide solution.
14. The CD97 full-length cDNA containing the EGF-like motifs 1, 2, and 5 was kindly provided by Dr. Jörg Hamann (University of Amsterdam).

### 2.3. Large-Scale Calcium Phosphate Transfection of HEK-293T Cells

1. Highly purified expression vector plasmid DNA is prepared using the HiSpeed® Plasmid Maxi kit (Qiagen, Valencia, CA).
2. 14-ml polypropylene round-bottom tubes.
3. *2× HBS.* 1.64% (w/v) NaCl, 1.19% (w/v) HEPES, 0.04% (w/v) $Na_2HPO_4$, adjusted to pH 7.1 with HCl, filtered through a 0.22-μm filter.
4. Cell-culture-grade $dH_2O$.
5. 1 M $CaCl_2$, sterile.
6. 1 N HCl, sterile.

### 2.4. Purification of mFc-Fusion Protein

1. Protein G sepharose™ 4 Fast Flow (GE Healthcare).
2. Glass Econo-Column® Chromatography columns (Bio-Rad, Hercules, CA).
3. *Washing buffer*. 50 mM Tris–HCl, pH 7.5, 10 mM $CaCl_2$, and 150 mM NaCl.
4. *Elution buffer*. 100 mM Glycine, pH 2.9.
5. *Neutralization buffer*. 1 M Tris–base solution, pH 9.0.
6. The BCA™ Protein Assay kit (PIERCE, Rockford, USA).
7. DU 800 spectrophotometer (Beckman Coulter, Fullerton, CA).

### 2.5. Protein Biotinylation

1. Slide-A-Lyzer® Dialysis Cassettes (PIERCE, Rockford, USA).
2. 10 mM Tris–HCl buffer, pH 8.0.
3. The BirA enzyme and BioMix A and BioMix B buffers (Avidity, Denver, CO).
4. 10 mM Tris–HCl buffer, pH 7.5 containing 100 mM NaCl and 10 mM $CaCl_2$.

### 2.6. Production of Multivalent Fluorescent and Paramagnetic Protein Probes

1. *Avidin-coated green fluorescent beads*. 0.1% w/v, 5 ml, 0.4–0.6 μm. (Spherotech Inc., Libertyville, IL).
2. Dynabeads® M-280 streptavidin-conjugated paramagnetic polystyrene beads, 2.8 μm in diameter (Dynal A.S., Oslo, Norway).
3. The 6-tube magnet stand, Dynal MPS®-S (Dynal A.S., Oslo, Norway).
4. *Phosphate-buffered saline (PBS)*. Prepare 10× stock with 1.37 M NaCl, 27 mM KCl, 100 mM $Na_2HPO_4$, 18 mM $KH_2PO_4$, (adjust to pH 7.4 with HCl). The solution is autoclaved before storage at room temperature. Prepare 1× working solution by adding nine parts $dH_2O$ with one part 10× stock solution.
5. Hanks' balanced salt solution with calcium and magnesium, (HBSS) (1×), liquid.
6. *PBS/BSA*. 10 mg BSA dissolved in 1 ml PBS.
7. *HBSS/BSA*. 5 mg BSA dissolved in 1 ml HBSS.

### 2.7. Identification and Characterization of Ligand-Expressing Cells

1. 96-well U-bottom tissue culture plates.
2. 10 mM EDTA in PBS, pH 8.0.
3. The sonicator (Heat Systems, Farmingdale, NY).
4. FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ).

### 2.8. Enrichment of Ligand-Expressing Cells

1. The 6-tube magnet stand, Dynal MPS®-S (Dynal A.S., Oslo, Norway).
2. Trypan Blue Stain (GIBCO™, Invitrogen).

3. Hemocytometer (Hausser Scientific, Horsham, PA).
4. End-over-end rotator (Digisystem Laboratory Instrument Inc., Taiwan).

## 3. Methods

It is critical for this application that a large quantity of purified fusion protein is obtained. Care should be taken to optimize the large-scale cell transfection procedure in house. Both HEK-293T and CHO-K1 cells are good protein-producing cells in our experience. We have noticed that an optimal pH is very important for the calcium phosphate precipitation of DNA and hence the transfection efficiency. A good negative control using the fluorescent microspheres or paramagnetic beads alone is also essential and is always included. In addition, we also routinely include a recombinant mFc fragment as a negative protein control *(11)*. Choice of cell types is virtually unlimited, but care must be taken to ensure cell surface or extracellular proteins are not damaged in the cell preparation step *(10, 11)*. An outline of the following experimental procedures described is depicted in **Fig. 1**.

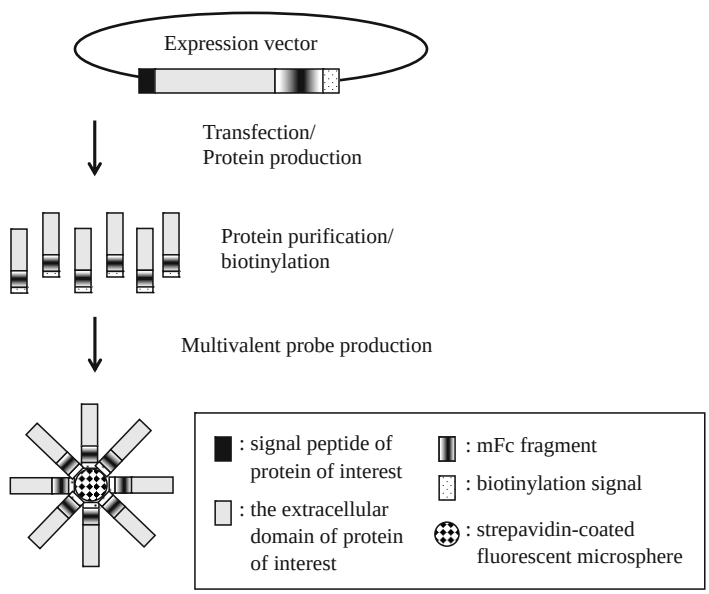


Fig. 1. Schematic diagram showing the experimental procedures for the production of multivalent protein probes. The cDNA of the protein of interest containing its own signal peptide (to ensure secretion) and the extracellular domain is cloned in frame into the mFc-Bio expression vector. The recombinant protein is produced and affinity purified from the conditioned media of transiently transfected cells. Purified proteins are then biotinylated in vitro and coupled to the strepavidin-conjugated fluorescent microspheres to produce the multivalent probes.

### 3.1. Construction of Expression Vectors

1. The cDNA fragment encoding the peptide sequence DPNSGSLHHILDAQKMVWNHR* (*see* **Note 2**), recognized by the *Escherichia coli* biotin holoenzyme synthetase BirA, is generated using the Advantage™-HF 2 PCR Kit according to the manufacturer's protocol. The XbaB10 and Bio3 primers are used to amplify an HLA-A2 plasmid template, which contains the biotinylation signal peptide sequence *(13)*. The hinge-CH2 and CH3 region of a non-Fc receptor-binding form of murine IgG2b is generated by High-Fidelity PCR using mFcXba and NotmFc primers and a murine IgG2b genomic DNA as a template *(11, 12)*. The cycling parameters used are as follows: the denaturation step (94°C, 30 s), five cycles of 94°C for 30 s and extension at 72°C, five cycles of 94°C for 30 s and extension at 70°C followed by 20–30 cycles of 94°C for 30 s of annealing/extension at 68°C.
2. The amplified DNA fragment is separated by gel electrophoresis in a 1% agarose gel. Agarose gels are prepared in 1× TBE containing 0.2 μg/ml ethidium bromide. DNA samples are mixed in 6× DNA loading dye and run at 50 mA. 1 kb DNA Plus ladder is used as a reference.
3. The amplified DNA band is examined with UV light, excised, and purified from agarose gels using the QIAquick gel extraction kit (*see* **Note 3**).
4. The purified PCR products are individually subcloned into the PCR4-Topo TA cloning vector for subsequent plasmid isolation and DNA sequencing to confirm the integrity of the amplified DNA fragments.
5. The biotinylation peptide signal sequence is cloned into the pcDNA3.1 (+) vector via *Xba* I and *Apa* I sites, while the mFc fragment is cloned subsequently into the resulting construct via the *BamH* I and *Xba* I sites. In general, the vectors are dephosphorylated to prevent religation. The ligation reaction mixture is incubated at 16°C overnight before transformation of competent bacteria.
6. The completed vector, called mFc-Bio/pcDNA3.1, is a pcDNA3.1(+) plasmid containing an mFc fragment followed by a biotinylation signal sequence. To make the final expression vector, the cDNA sequence coding for the protein of interest is inserted in frame immediately before the mFc fragment via appropriate restriction sites. In the case illustrated here, the CD97(EGF 1, 2, 5) fragment coding for the N-terminal EGF motifs 1, 2, and 5 of CD97 is placed in front of the mFc fragment via *Hind* III and *EcoR* I sites to generate the CD97-mFc-Bio expression vector.
7. All expression vectors are purified and subjected to DNA sequencing in both directions to confirm the fidelity of the coding sequence (*see* **Note 4**).

### 3.2. Large-Scale Cell Transfection by Calcium Phosphate Precipitation of DNA

1. Mock precipitations are first performed to adjust the optimal pH used for the transfection. This is achieved by making *solution I:* mixing 1 ml 2× HBS and different volumes of 1N HCl ranging from 0 to 10 µl in a series of 14-ml polypropylene round-bottom tubes.
2. 1 ml of *solution II*. A mixture of 248 µl of 1 M $CaCl_2$ and 752 µl $dH_2O$ is then slowly added to each tube containing solution I by gradually air bubbling the mixture continuously with a pipette.
3. The tubes are left for 30 min at room temperature for the precipitates to form. The sizes of the precipitates are monitored under a microscope to find the optimal pH condition, i.e., the optimal amounts of 1N HCl for solution I (*see* **Note 5**).
4. When the optimal condition is found, all precipitation is done using the same amount of 1N HCl for solution I. For solution II, 20 µg plasmid DNA and appropriate amount of sterile $dH_2O$ are added to 248 µl of 1 M $CaCl_2$ to a final 1 ml.
5. HEK-293T cells are cultured in DMEM supplemented with 10% (v/v) FCS until they reach about 60–70% confluence. To this was added 4 ml of transfection mixture containing 40 µg DNA which was found to be adequate for one T-175 flask of HEK-293T cells (*see* **Note 6**).
6. Typically, 5 T-175 flasks are used for one expression construct.
7. Cells are fed with fresh medium 1 h before transfection (20 ml/T-175 flask) and the transfection mix was slowly pipetted into the culture medium ensuring an even distribution (*see* **Note 7**).
8. Cells are then left in standard culture conditions for up to 16 h. Afterward, the transfection medium is removed and cells are rinsed carefully with PBS, followed by the addition of 40 ml fresh serum-free OPTI-MEM I medium. Cells can be cultured for up to 7 days (*see* **Note 8**). The conditioned medium is harvested and spun at 2000 *g* for 15 min at 4°C to remove cell debris. The supernatant is then filtered through a 0.45-µm filter and stored at –80°C until protein purification.

### 3.3. Purification of mFc-Fusion Protein by Protein G Affinity Chromatography

1. Mouse Fc-fusion proteins are purified from conditioned media using protein G sepharose chromatography (*see* **Note 9**). Protein G sepharose (1 ml) was first packed into the chromatography column and equilibrated with 50 ml washing buffer. Conditioned medium is then passed through the column twice under gravity (*see* **Note 10**).
2. The column is washed once with at least 50 ml washing buffer (*see* **Note 11**).
3. The bound mFc-fusion protein is eluted with 100 mM glycine, pH 2.9 elution buffer.

4. ~0.9 ml of eluate/fraction is collected into a 1.5-ml Eppendorf tube containing 100 µl of 1 M Tris–HCl, pH9.0 solution for neutralization. A total of ten fractions are collected (*see* **Note 12**).
5. Eluates are assessed by absorbance at O.D. 280.
6. Fractions containing proteins are combined and dialyzed in a Slide-A-Lyzer® Dialysis Cassette against 10 mM Tris–HCl, pH 8.0 buffer at 4°C overnight.
7. The final protein concentration is measured by the BCA™ protein assay kit according to the manufacturer's protocol, and the purified protein is aliquoted and stored at –80°C.

### 3.4. Protein Biotinylation

1. The purified mFc-fusion proteins are biotinylated based on the following formula: Eight parts of dialyzed protein are incubated with one part BioMix A buffer, one part BioMix B buffer, and 1 µl BirA enzyme overnight at room temperature *(9, 11)* (*see* **Notes 13** and **14**).
2. Excess biotin is subsequently removed by dialysis against 10 mM Tris–HCl, pH 7.3 buffer containing 10 mM $CaCl_2$ and 100 mM NaCl at 4°C overnight (*see* **Note 15**).
3. The biotinylated mFc-fusion proteins are quantified by dot-blot analysis using myelin basic protein biotin (Avidity, Denver, CO) as standard (*see* **Note 16**).
4. The biotinylated mFc-fusion proteins are then aliquoted and stored at –80°C.

### 3.5. Production of Multivalent Fluorescent Protein Probes

1. 10 µl of avidin-coated fluorescent microspheres are placed in a 1.5-ml eppendorf tube, spun at 100 *g* at 4°C for 5 min, and washed twice with 1 ml PBS.
2. The fluorescent beads are then blocked with 1 mL PBS/BSA at 4°C for 1 h by end-over-end rotation, followed by centrifugation to discard the blocking buffer.
3. The fluorescent beads are then mixed with 1 µg biotinylated fusion proteins in HBSS/BSA in a total volume of 100 µl and incubated at 4°C for 1 h by end-over-end rotation.
4. The fluorescent bead–protein complexes are spun at 100 g at 4°C for 5 min and washed twice with 1 ml HBSS/BSA to remove unbound biotinylated proteins.
5. The fluorescent bead–protein complexes are resuspended in 50 µl HBSS/BSA, placed on ice, and sonicated immediately before use at 20% power for 20 s at 4°C three times with 10-s intervals in between *(9, 11)* (*see* **Note 17**).

### 3.6. Production of Multivalent Paramagnetic Protein Probes

1. For the magnetic selection of ligand-expressing cells, biotinylated proteins are coated onto streptavidin-conjugated paramagnetic beads *(10)*.

2. 10 μL of paramagnetic beads are placed in a 1.5-ml eppendorf tube next to a magnet for 1 min. The supernatant is discarded carefully not to disturb the beads adhered to the side of the tube.
3. After removal of the tube from the magnet, the beads are blocked with 1 mL HBSS/BSA at 4°C for 1 h.
4. The paramagnetic beads are washed three times with HBSS/BSA and then resuspended in 50 μl HBSS/BSA.
5. The paramagnetic beads are incubated with 2 μg biotinylated proteins at 4°C for 30 min by end-over-end rotation.
6. The tube is then placed next to the magnet for 1 min and the supernatant is carefully discarded to remove unbound biotinylated proteins by magnetic separation.
7. The tube is then removed from the magnet, and the bead–protein complexes are washed three times with 1 ml HBSS/BSA.
8. The paramagnetic bead–protein complexes are then resuspended in 10 μl HBSS/BSA and are ready for use.

### *3.7. Identification and Characterization of Ligand-Expressing Cells*

1. Various cell types can be used to screen for candidate ligands. If adherent cell types such as macrophages or dendritic cells are to be screened, they must be lifted by nonenzymatic dissociation buffer to avoid digestion of the potential cell surface or extracellular matrix ligand(s) *(9–11, 14, 15)* (*see* **Note 18**).
2. To screen for the candidate ligand-expressing cells, the fluorescent bead–protein probes are incubated with the cells to be screened in U-bottom 96-well plates at $1 \times 10^6$ cells/50 μl bead–protein probes/well (*see* **Note 19**).
3. The cell–bead mixture is spun at 100 *g* at 4°C for 20 min and incubated for a further 40 min at 4°C in the dark.
4. The cell–bead mixture is resuspended in 200 μl of PBS and placed in a tube for analysis by flow cytometry. Putative ligand-expressing cells will bind the fluorescent probes and give an increased FL-1 signal. In the example illustrated here **(Fig. 2)**, $CD55^+$ K562 cells bind the CD97 probe readily and show strong fluorescent signals compared to the negative controls consisting of cells incubated with either fluorescent beads alone or fluorescent beads coupled to only mFc fragment. On the other hand, CHO-K1 cells, which do not express human CD55, show no binding at all and thus no enhanced fluorescent signal (data not shown).
5. Negative controls consisting of either fluorescent beads alone or beads coated with only mFc fragment are always included in each experiment.
6. After the identification of the putative positive ligand-expressing cells, various treatments such as protease digestion and Ab

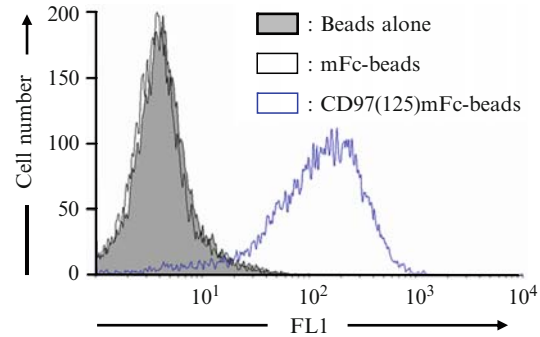


*Fig. 2* A typical flow cytometry profile demonstrating the interaction of CD97 fluorescent multivalent probes with CD55 on K562 cells. Negative controls consist of incubating K562 cells with either fluorescent beads alone or fluorescent beads coupled to only mFc fragment.

blocking can be used to confirm the specific receptor–ligand interaction and further characterize the potential cellular ligand(s) *(11)* (*see* **Note 20**).

### *3.8. Enrichment of the Ligand-Expressing Cells*

1. The ligand-expressing cells can be enriched from mixed cell populations such as blood for potential downstream application using the paramagnetic separation *(10)*.
2. Mixed cell populations containing potential ligand-expressing cells are washed with 5 ml PBS/BSA, adjusted to a cell density of $3 \times 10^6$ cells/ml, and incubated at 4°C with the paramagnetic bead–protein complexes (20 µl bead–protein complexes/ml cell solution/reaction) for 1 h on an end-over-end rotator at 4°C.
3. The cell–bead mixture in 1.5-ml eppendorf tube is then placed next to a magnet for 1 min and the supernatant carefully discarded. The unbound cells are removed by five gentle but thorough washes in 1 ml PBS/BSA coupled with magnetic separation.
4. The remaining cells are separated and isolated from the beads by incubation in HBSS/BSA containing 5 mM EDTA in PBS for 10 min at room temperature followed by magnetic separation (*see* **Note 21**).
5. The total number of recovered cells is determined by counting in a hemocytometer.
6. The isolated ligand-expressing cells are now ready for further analysis such as RNA isolation, protein purification, and immunocytochemistry.
7. Based upon the characteristics of the receptor–ligand interaction, it is possible to molecularly identify the putative cellular ligand(s)

expressed on the isolated ligand-expressing cells. Methods of choice include affinity purification using the multivalent protein probes described here, or expression cloning strategies described elsewhere. We have successfully identified a cellular ligand for myeloid-restricted EMR2 receptor using a panel of mutant cell lines defective in a specific biosynthetic pathway *(11)*.

## 4. Notes

1. If the mFc fragment is desired as a negative control, an mFc expression vector constructed on the pSecTag2A plasmid (Invitrogen), called pSecTag-mFc, may be used. The pSecTag-mFc construct was made by subcloning the mFc fragment and the biotinylation signal into the pSecTag2 A vector via the same restriction sites described in the text.
2. The biotinylation signal sequence described here is amplified from an HLA-A2 plasmid template. This sequence is recognized and efficiently biotinylated by BirA enzyme purchased from Avidity. However, the BirA enzyme has since been engineered and modified by the company and no longer efficiently works on the sequence listed earlier. The optimal biotinylation signal sequence for the new BirA enzyme is GGGLNDIFEAQKIEWHE*, which can be obtained from a template plasmid from Avidity.
3. Observe the amplified DNA fragments under long-wave UV light if the DNA is to be used for subsequent cloning.
4. HiSpeed® Plasmid Maxi kits are used to obtain large quantities of plasmid DNA for the subsequent large-scale cell transfections. If desired, CsCl banding of plasmid DNA isolation can also be considered.
5. The optimal size of the calcium-phosphate DNA precipitate is approximately the tip of a needle under the microscope at 200× magnification. pH influences the precipitate formation greatly. If no precipitate is seen, it is likely that the pH is too low, whereas if the precipitate is too large, it is likely that the pH is too high. The formation of large precipitates or aggregates affects the ability of cell to take up DNA. This should be avoided and the mock precipitation step should be repeated until an optimal pH condition is found.
6. The amount of solutions I and II is determined by how many flasks of cells are to be transfected. Usually, we prepare the solutions in bulk keeping the amounts of individual components in proportion.

7. HEK-293T cells are only loosely adherent especially when reaching confluence, so care should be taken to minimize disturbing the monolayer when adding reagents or changing medium.
8. Observe the transfected cells daily to check their health in order to decide when to collect the conditioned medium.
9. Can also use protein A sepharose as it binds to the mFc fragment equally well.
10. Care should be taken to make sure the protein G sepharose resin remains in solution and does not dry out.
11. The column should be washed with at least 50× void volume of the sepharose resin.
12. The mFc-fusion proteins usually are eluted in the first 2–4 tubes depending upon the starting amount.
13. Alternatively, the biotinylation reaction can be incubated at 4°C for longer periods or 3–4 h at 30°C.
14. If the protein concentration of starting material is high (>2 mg/ml), use eight parts of protein, one part BioMix A buffer, one part BioMix A buffer, and one part supplemental Biotin.
15. We usually change the dialysis buffer every few hours to facilitate dialysis.
16. A serial dilution of the biotinylated protein is prepared and dot-blotted onto the nitrocellulose filter paper next to the standard biotin-myelin basic protein. After extensive blocking in PBS/BSA, Strepavidin-HRP is applied to react with the biotinylated protein, followed by thorough washes with PBS/BSA and signal detection by ECL reagent (Amersham).
17. Be sure not to overheat the protein–bead complexes.
18. We routinely used 5 mM EDTA/PBS to lift adherent cells from tissue culture plates.
19. The U-bottom plates ensure that the cells interact optimally with the microspheres.
20. Cells can be pretreated with various proteolytic enzymes such as trypsin, α-chymotrypsin, dispase, etc. to compare with untreated cells to determine whether the putative cellular ligand is protease sensitive. Similarly, exogenous chemical reagents such as EGTA can also be included to test whether the ligand-receptor interaction is $Ca^{2+}$ dependent. Finally, blocking Abs can be used to test candidate ligand molecules.
21. In our case, $CD55^{+}$-red blood cells can be efficiently enriched from whole blood cell sample using the CD97-mFc-fusion protein probe on paramagnetic beads.

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# Chapter 8

# Exploiting Fc Chimaeric Proteins for the Identification of Ligands Specific for the Mannose Receptor

Luisa Martinez-Pomares

## Summary

The use of chimaeric molecules bearing tags easily recognised by secondary reagents has facilitated the discovery of protein–protein and protein–carbohydrate interactions using binding assays in situ, in solution and in solid phase. In this chapter we describe our experience in the use of proteins containing selected regions of the mannose receptor fused to the Fc region of human IgG1 or murine IgG2b. Using these reagents we have discovered new and unexpected ligands for the mannose receptor. These ligands were first detected in tissue section using standard histological techniques or ligand blots of whole tissue lysates, identified using affinity chromatography and N-terminal protein sequencing and confirmed using ligand blots or solid-phase-binding assays using purified proteins. These findings have dramatically changed the way we think about this molecule.

**Key words:** Mannose receptor, Ligands, Carbohydrates, Glycoproteins, Collagens.

## 1. Introduction

The mannose receptor (MR) is a 175-kDa type I integral membrane receptor. It recognises sulphated carbohydrates terminated in $SO_4$-4-GalNAc-, $SO_4$-3-GalNAc-, or $SO_4$-3-Gal through its cysteine-rich domain (CR) *(1, 2)*, native and denatured collagens through its fibronectin type II domain (FNII) *(3)* and mannose, fucose, and *N*-acetylglucosamine in a $Ca^{2+}$- and pH-dependent manner, via C-type lectin-like domain (CTLD)-4, with CTLD4-8 providing similar high-affinity binding as the full-length receptor *(4, 5)* (*see* **Fig. 1**). MR is an endocytic receptor that resides in the recycling endocytic compartment; it is expressed by most murine

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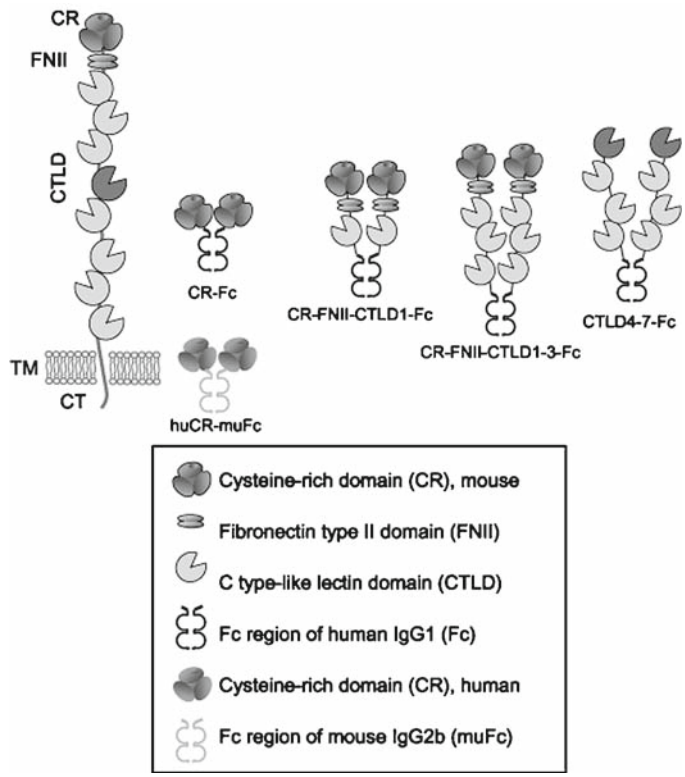



Fig. 1. Schematic representation of the domain structure of MR and Fc proteins used for studying MR-binding properties. *CR* cysteine-rich domain of mouse MR; *FNII* fibronectin-type II domain of mouse MR; *CTLD* C-type lectin-like domain of mouse MR; *TM* transmembrane domain of mouse MR; *CT* cytoplasmic tail of mouse MR; *huCR* cysteine-rich domain of the human MR; *Fc* Fc region of human $IgG_1$; *muFc* Fc region of murine IgG2b.

macrophage populations, hepatic and lymphatic endothelia, glomerular mesangial cells in the kidneys, tracheal smooth muscle cells, and retinal pigment epithelium. In humans MR has been detected in skin dermis, mucosal lamina propria, and the T-cell areas of tonsil. MR was initially discovered through its involvement in the clearance of endogenous glycoproteins; indeed, MR deficiency does not have a major impact in immunity against *Candida albicans* or *Pneumocystis carinii* but leads to a drastic increase in the circulating levels of multiple lysosomal hydrolases (*see* **ref.** *(6)* for review). MR-derived Fc chimaeric proteins have provided useful information regarding the binding properties of MR. They led to the discovery of cell-associated ligands for the MR domain CR (CR ligands) in lymphoid organs *(7, 8)*, ligands for the CTLD4-7 region in secretory organs *(9)*, one of them being thyroglobulin, and for the FNII region *(3)*. In this chapter we have outlined the methodology used for these studies.

## 2. Materials

### *2.1. Detection of Ligands In Situ*

#### *2.1.1. Tissue Collection*

1. Dissecting tools.
2. Dissecting board.
3. OCT Tissue-Tek compound.
4. Plastic moulds.
5. Iso-pentane.
6. Dry ice.
7. Ice container.
8. Gelatine-coated slides.
9. Charged slides.
10. Cryostat.

#### *2.1.2. Tissue Labelling*

1. Fixative: Methanol-free paraformaldehyde (16% solution). To prepare 200 ml of fixative: 25 ml paraformaldehyde, 175 ml $dH_2O$, 1.8 g NaCl, 0.08 g $CaCl_2$, 1.5 ml 1 M Hepes buffer, pH 7.4.
2. Phosphate-buffered saline (PBS).
3. Lectin-binding buffer: 10 mM Tris–HCl, pH 7.4, 154 mM NaCl, 10 mM $CaCl_2$.
4. Permeabilising solution: 0.1% Triton X-100 in PBS.
5. Quenching solution: add 50 µl of 1 M $NaN_3$ and 0.09 g glucose to 50 ml of 0.1 M phosphate buffer. Warm to 37°C and add 20 µl glucose oxidase just before use.
6. Avidin/Biotin Blocking Kit.
7. Blocking solution: 5% normal serum in PBS sources of normal sera: donkey, goat, mouse, rabbit or rat. Purified rat IgG.
8. Shandon Sequenza® Slide Rack (Thermo Scientific, 7331017).
9. Shandon Coverplate™ (Thermo Scientific, 72110013).
10. Biotinylated goat anti-human IgG.
11. Peroxidase-conjugated goat anti-Human IgG and peroxidase-conjugated horse anti-mouse IgG.
12. Alkaline phosphatase-conjugated goat anti-Human IgG.
13. Alexa 488-conjugated Goat anti-human IgG.
14. ABC Peroxidase Vector Vectastain Elite Kit.
15. ABC Alkaline Phosphatase Vector Vectastain Elite ABC Kit. For both ABC kits add two drops of solution A to 10 ml PBS and mix well then add two drops of solution B mix well and incubate 30 min at room temperature before using.

16. Cy5-conjugated Streptavidin.
17. Alexa 488-conjugated Streptavidin.
18. 3,3′-Diaminobenzidine tetrahydrochloride (DAB) (5 × 10 mg). To be used with peroxidase-conjugated secondary reagents. DAB is a potential carcinogen and it should be used in a ventilated hood. It is most dangerous in powder form and is not to be removed from the container in this state. Equipment: 20-ml syringe, 22-μm syringe filter, two needles, 0.5- to 10-μl pipette and tip, two beakers, 10 mM imidazole solution (kept at 4°C), hydrogen peroxide. Preparation of developing solution: pour imidazole into beaker and draw 20 ml into syringe. Pour any excess back into the bottle. Push two needles into DAB vial and inject contents of syringe through. Mix. Pipette 6 μl $H_2O_2$ into beaker. Withdraw DAB solution with syringe and filter into beaker containing $H_2O_2$. Mix. The solution is now ready for use.
19. Substrate Kit IV BCIP/NBT. To be used with alkaline phosphatase-conjugated secondary reagents. To 5 ml of 100 mM Tris–HCl pH 9.5 buffer add one drop of levamisole (a reagent to quench endogenous alkaline phosphatase activity within tissues) and mix. Add two drops of reagent 1 and mix well. Add two drops of reagent 2 and mix well. Add three drops of reagent 3 and mix well.
20. Methyl green.
21. Histoclear.
22. 4 ,6-Diamidino-2-phenylindole,dilactate. Prepare concentrate stock in PBS (1 mg/ml), aliquot, and store at –20°C. Just before use, dilute in PBS to 400 ng/ml.
23. DPX (VWR, 36125 4D).
24. AquaPerm.
25. Fluorescence-mounting medium.

### *2.2. Detection of Ligands in Tissue Lysates*

#### *2.2.1. Preparation of Tissue Lysates*

1. Lysis buffer for preparations of total protein lysates: 10 mM Tris–HCl, pH 8, 2% (v/v) Triton X-100, 10 mM $NaN_3$, 150 mM NaCl, 2 mM EDTA.
2. Buffer A: 10 mM Tris–HCl, pH 8, 2.5% (v/v) Tween 40, 150 mM NaCl, 10 mM $NaN_3$, 2 mM EDTA.
3. Buffer B: 10 mM Tris–HCl, pH 8, 10 mM $NaN_3$, 150 mM NaCl, 2 mM EDTA.
4. Complete Protease Inhibitor Cocktail Tablets.
5. Polytron Homogeniser.
6. Dounce Homogeniser.
7. TC100 centrifuge.

8. Preparative ultracentrifuge.
9. BCA Protein assay kit.

*2.2.2. Ligand Blots*

1. Non-reducing SDS–PAGE sample buffer: 10 mM Tris–HCl, pH 6.8, 2% (w/v) SDS, 20% (v/v) glycerol, 0.001% (w/v) bromophenol blue.
2. Separating gel (20 ml): 5 ml of 1.5 M Tris–HCl pH 8.8, 0.2 ml of 10% SDS, acrylamide:bis-acrylamide (30%:0.8%) solution (amount depends on % of acrylamide required), MilliQ $H_2O$ to 20 ml, 0.2 ml of 10% (w/v) ammonium persulphate, 30 µl of temed.
3. Stacking gel (10 ml): 2.5 ml of 0.5 M Tris–HCl, pH 6.8, 0.1 ml of 10% SDS, 1.34 ml acrylamide:bis-acrylamide (30%:0.8%) solution, 5.94 ml MilliQ $H_2O$, 0.1 ml of 10% ammonium persulphate, 20 µl of temed.
4. Running buffer: 25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS, pH 8.3.
5. Rainbow Markers Full Range.
6. Heating block.
7. Western blot transfer buffer: 25 mM Tris, 192 mM glycine, 20% methanol.
8. Western blot apparatus.
9. Hybond-C extra membranes.
10. Blocking buffers: 5% (w/v) non-fat dry milk, 0.1% (v/v) Tween 20 in phosphate-buffered saline or 5% (w/v) non-fat milk, 0.1% (v/v) Tween 20 in 10 mM Tris–HCl, pH 7.4, 154 mM NaCl, 10 mM $CaCl_2$.
11. Washing buffers: 0.1% (v/v) Tween 20 in PBS or 0.1% (v/v) Tween 20 in 10 mM Tris–HCl, pH 7.4, 154 mM NaCl, 10 mM $CaCl_2$.
12. Peroxidase-conjugated secondary Ab against human or mouse IgG.
13. Rotating shaker.
14. ECL Western Blotting Reagents.
15. Hyperfilm ECL.

*2.2.3. Detection of Ligands by Enrichment in Solution Using Protein A- or G-Sepharose and Ligand Blot Analysis*

1. Lysis buffer for preparations of total protein lysates (*see* **Subheading 2.2.1**).
2. Protease inhibitors.
3. Protein A-sepharose or protein G-sepharose.
4. Rotating wheel.
5. Microcentrifuge.

6. Digoxigenin protein labelling kit.
7. Peroxidase-conjugated anti-Digoxigenin Ab.

*2.2.4. Purication of Ligands Using Fc Chimaeric Proteins Directly Conjugated to Protein A- or G-Sepharose*

1. PBS.
2. 0.1 M Sodium borate, pH 9.
3. Coupling reagent: dissolve 0.5 g of dimethylpimelimidate in enough 0.1 sodium borate (pH not adjusted) to give a pH of 9. Add 0.1 M sodium borate, pH 9, to give a final volume of 50 ml.
4. Econo Columns.
5. Protein A-sepharose or protein G-sepharose.
6. Lysis buffer for preparations of total protein lysates (*see* **Subheading 2.2.1**).
7. High pH elution buffer (>11): 0.5% (v/v) diethylamine in 0.1% (v/v) Triton X-100.
8. Low pH elution buffer (<3): 0.1 M glycine, pH 2.9 in 0.1% (v/v) Triton X-100.
9. Calcium quelator: EDTA in 0.1% (v/v) Triton X-100.
10. Neutralisation buffer: 1 M Tris–HCl, pH 7.

*2.2.5. Assessment of Binding to Candidate Molecule Immunoprecipitated from a Protein Lysate Using a Monoclonal Ab*

1. CNBr-activated sepharose or protein A/G-sepharose.
2. Immunoprecipitation washing buffer: A: 10 mM Tris–HCl, pH 8, 500 mM NaCl, 0.5% (w/v) deoxycholate, 0.5% (v/v) Triton X-100, 0.05% (w/v) SDS.
3. Immunoprecipitation washing buffer: B: 10 mM Tris–HCl, pH 8, 150 mM NaCl, 0.5% (w/v) deoxycholate, 0.5% (v/v) Triton X-100, 0.05% (w/v) SDS.
4. Immunoprecipitation washing buffer: C: 10 mM Tris–HCl, pH 8, 0.05% (w/v) SDS.

### *2.3. Assessment of Binding to Candidate Ligands in Solid Phase Assays*

1. MaxiSorp 96-microwell plates.
2. 96-Microwell plates.
3. PBS.
4. Saline (154 mM NaCl).
5. Lectin buffer: 10 mM Tris–HCl, pH 7.5, 10 mM $Ca^{2+}$, 154 mM NaCl, and 0.05%(w/v) Tween 20.
6. PBS–0.3% bovine serum albumin (BSA).
7. Alkaline phosphatase-conjugated anti-human Fc.
8. Alkaline phosphatase substrate buffer: 100 mM Tris–HCl, 100 mM NaCl, 1 mM $MgCl_2 \cdot 6H_2O$, pH 9.5.
9. Substrate: 4-nitrophenyl phosphate disodium salt hexahydrate 1 mg/ml in substrate buffer.
10. Plate reader with 405-nm filter.

## 3. Methods

### 3.1. Detection of Ligands In Situ

1. After collection, tissues are carefully placed in a mould containing OCT-LabTek compound (*see* **Note 1**).
2. Put moulds within a glass beaker containing iso-pentane previously placed on dry ice within an ice container.
3. Once the contents of the mould are frozen, they can be safely placed at –20°C until cutting.
4. Perform cutting in a cryostat at –20°C at 5–6 μm.
5. Sections are collected onto gelatine-coated slides or charged slides (*see* **Note 2**), left for 1 h at room temperature and placed at –20°C in sealed boxes containing silica gel in porous bags.
6. Thaw tissues at room temperature for 30–60 min and place in ice-cold 2% paraformaldehyde solution for 10 min on ice in a Coplin jar (*see* **Note 3**).
7. Rinse three times in PBS, cover slides with a Shandon Coverplate™ (*see* **Note 4**), and place both in the Shandon Sequenza® Slide Rack.
8. Permeabilise the tissues in PBS–0.1% Triton for 10 min (*see* **Note 5**) at room temperature. If using peroxidase-based detection reagents place the slides in quenching solution for 15 min at 37°C.
9. After washing (*see* **Note 6**) block slides with appropriate blocking buffer (*see* **Note 7**) for 30 min. If using a biotin/streptavidin-based detection system, block endogenous biotin with the avidin-biotin blocking kit to (*see* **Note 8**).
10. Dilute MR-Fc chimaeric protein of interest in blocking buffer (*see* **Notes 9** and **10**, and **Figs. 1** and **2**) and add to slide.
11. Incubate at room temperature for 90 min (*see* **Note 11**).
12. After washing detection of binding can be performed using techniques outlined in **Table 1**. Washes should be performed in between all steps. *See* **Note 15** for additional information.

### 3.2. Detection of Ligands in Tissue Lysates

A general overview of the strategies that can be used for the identification of ligands for proteins of interest is described in **Table 2**.

#### 3.2.1. Preparation of Total Tissue Lysates

1. Weigh frozen tissues (*see* **Note 16**) and add ice-cold freshly prepared lysis buffer containing protease inhibitors (approx. 1 ml of buffer/100 mg of sample).
2. Depending of the size of the sample use a polytron homogeniser or a dounce homogeniser to break up the tissue (*see* **Note 17**).

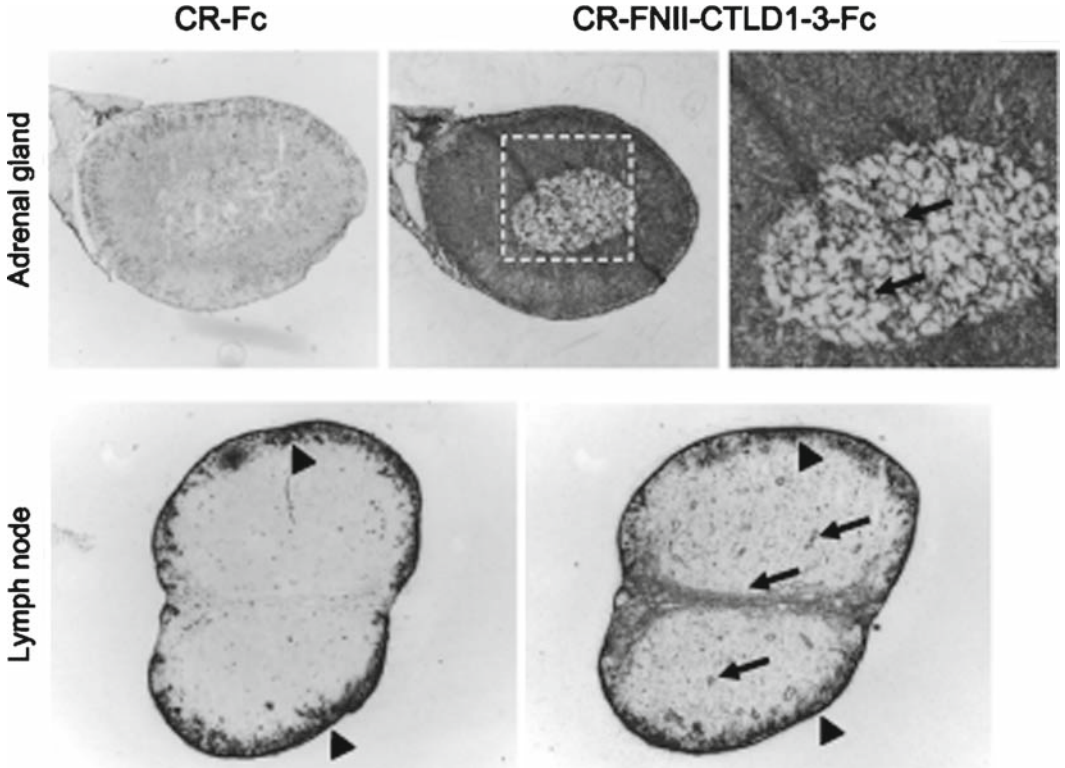



Fig. 2. Detection of MR CR and FNII ligands in situ. Tissues from adult mice were collected, processed, and incubated with cross-linked MR-derived Fc constructs CR-Fc and CR-FNII-CTLD1-3-Fc. Proteins containing FNII bound to structures resembling basement membrane (*arrows*) in the adrenal gland and lymph node. In the lymph node CR-FNII-CTLD1-3-Fc displayed dual binding activity with recognition of ligands (probably sulphated sugars) in the subcapsular sinus (also recognised by CR, *arrow heads*) in addition to basal membrane components in the T-cell region and medulla (*arrows*). Magnification ×10. Area in *inset* has been electronically magnified. Reproduced from *(3)* with permission.

3. After homogenisation, incubate at 4°C for 30–60 min, centrifuge lysate at 1,000 *g* at 4°C for 5 min, transfer the supernatant to another centrifuge tube, and centrifuge at 100,000 × *g* at 4°C for 30 min (*see* **Note 18**).
4. Collect the supernatant, quantify protein using the BCA protein assay kit, aliquot if necessary and store at –20 or –70°C (*see* **Note 19**).

*3.2.2. Preparation of Membrane Lysates*

This procedure is based on the method described by Williams and Barclay *(10)*.

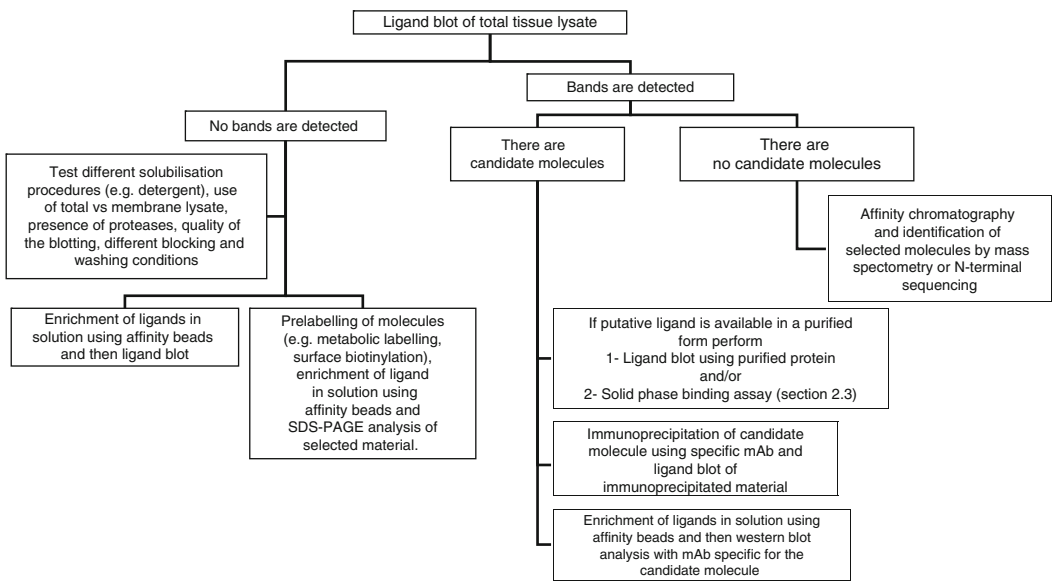
1. Tissues are homogenised as before using buffer A containing protease inhibitors.
2. Nuclei are removed by centrifugation at 500 × *g* for 15 min at 4°C (*see* **Note 20**), and the supernatant is centrifuged at 35,000 rpm for 60 min at 4°C using a 60.Ti rotor (100,000 × *g*) to pellet the membranes (*see* **Note 21**).

## Table 1
## Strategies to detect binding of MR-Fc fusion proteins to tissues

| | Brightfield | | |
|---|---|---|---|
| | **Peroxidase[a]** | **Alkaline phosphatase[b]** | **Fluorescence[c]** |
| Two-step procedure | Peroxidase-conjugated anti-Fc (30–45 min) | Alkaline-phosphatase-conjugated anti-Fc (30–45 min) | Fluorochrome-conjugated anti-Fc (30–45 min) |
| | DAB | BCIP/NBT | |
| Three-step procedure | Biotin-conjugated anti-Fc (30–45 min) | Biotin-conjugated anti-Fc (30–45 min) | Biotin-conjugated anti-Fc (30–45 min) |
| | ABC Peroxidase Vector Vectastain Elite Kit (30–45 min) | ABC Alkaline Phosphatase Vector Vectastain Elite Kit (30–45 min) | Fluorochrome-conjugated-streptavidin (30–45 min) |
| | DAB | BCIP/NBT | |
| Counterstaining | Methyl green | Not recommended | DAPI |
| Mounting media | DPX (after dehydration) | AquaPerm | Fluorescence-mounting medium |

[a] *See* **Note 12**
[b] *See* **Note 13**
[c] *See* **Note 14**

## Table 2
## Strategies to characterise ligands in tissue lysates using MR-Fc chimaeric proteins



3. The membrane pellet is washed twice in buffer B containing protease inhibitors and proteins are solubilised by incubation in lysis buffer with protease inhibitors. Centrifuge lysate at 100,000 × *g* at 4°C.
4. Collect the supernatant, quantify protein using the BCA protein assay kit, aliquot if necessary and store at –20 or –70°C (*see* **Note 19**).

*3.2.3. Ligand Blots*

1. Separate proteins prepared in **Subheading 3.2.1** or **3.2.2**, using a non-continuous SDS–PAGE following standard procedures and solutions provided in **Subheading 2.2.2** (*see* **Notes 22** and **23**).
2. Transfer proteins to nitrocellulose filter (*see* **Notes 24** and **25**).
3. Place the filter in blocking buffer on a rotating shaker for 60 min at room temperature.
4. Pour out the blocking solution and add MR-Fc chimaeric proteins in blocking buffer (*see* **Note 26**).
5. Incubate for 60 min in the shaker at room temperature.
6. Wash in appropriate blocking buffer (*see* **Notes 26** and **27**).
7. Add peroxidase-conjugated anti-Fc or muFc secondary Ab diluted in blocking buffer (*see* **Note 28**).
8. Incubate for 60 min at room temperature, wash filter in appropriate washing buffer (*see* **Note 29**).
9. Develop using ECL Western Blotting Reagents (*see* **Note 30**).
10. Expose filter to film (*see* **Note 31**) in the dark and develop.

*3.2.4. Detection of Ligands by Enrichment in Solution Using Protein A- or G-Sepharose and Ligand Blot Analysis*

Immobilisation of purified recombinant proteins onto beads enables the selection of ligands for these proteins from complex protein solutions such as total or membrane lysates (*see* **Subheadings 3.2.1** and **3.2.2**). In the case of chimaeric proteins bearing the Fc region of human IgG1 (Fc) or mouse IgG2b (muFc), protein A- and/or protein G-sepharose are highly suitable for this purpose as they will readily interact with the Fc region of the recombinant proteins and provide a solid support for the selection for ligands in solution (*see* **Notes 32** and **33**).

1. Thaw protein lysate(s) on ice and examine for the presence of protein aggregates that might have formed during the freezing and thawing process. If present, remove these by centrifugation.
2. To pre-clear lysates of ligands that might bind non-specifically, wash protein A/G-sepharose -that has not been incubated with Fc-fusion proteins- in lysis buffer with protease inhibitors three times.

3. Pellet the sepharose after each wash by centrifugation at 12,000 *g* for 2 min in a microfuge and resuspend in 1 ml of buffer.
4. After the final wash, resuspend the sepharose as a 50% slurry in lysis buffer and add to protein lysate (typically 20–50 µl of slurry/250–1,000 ml lysate) (*see* **Note 34**).
5. Incubate in a rotating wheel at 4°C for 2 h.
6. Centrifuge the lysate to remove sepharose at 12,000 *g* for 5 min in a microfuge and collect the supernatant. This step ensures that compounds that bind to protein A/G-sepharose non-specifically are removed.
7. Add chimaeric protein to pre-cleared lysate (10–20 µg/ml of lysate) together with protein A/G-sepharose washed as before in lysis buffer.
8. Leave in a rotating wheel overnight at 4°C.
9. On the following day, collect the beads by centrifugation and wash in lysis buffer three times.
10. Elute bound proteins by resuspending beads in 1× SDS–PAGE loading buffer and boiling for 5 min.
11. Run sample in a SDS–PAGE, transfer as before, and probe with Fc chimaeric protein labelled with digoxigenin and peroxidase-conjugated anti-digoxigenin Ab.
12. Develop using ECL reagent (*see* **Note 35**).

*3.2.5. Purification of Ligands Using Fc Chimaeric Proteins Directly Conjugated to Protein A- or G-Sepharose*

Conjugate recombinant Fc fusion protein to protein A/G-sepharose as follows *(11)*:

1. Wash sepharose in PBS by centrifugation and incubate with recombinant protein (2 mg protein/ml of sepharose) in a rotating wheel for 2 h at room temperature.
2. Wash the sepharose with 10–20 volumes of PBS followed by two washes with 10 volumes of 0.1 M sodium borate, pH 9.
3. Prepare the coupling reagent, remove the buffer from the sepharose-Fc protein complex, and add 10 volumes of the coupling reagent.
4. Incubate in a rotating wheel for 30 min at room temperature.
5. Wash the beads with 0.02 methanolamine pH 9 for 10–20 min and then in buffer to be used for the affinity chromatography (*see* **Note 36**).
6. Prepare two columns, one containing the protein A/G-sepharose (pre-clearing column, *see* **Note 37**) and another containing the Fc chimaeric protein coupled to protein A/G-sepharose.

7. Wash both columns with 10 volumes of lysis buffer and then apply the tissue lysate (total or membrane lysate) to the pre-clearing column.
8. Collect the flow through and apply it to the column containing the Fc chimaeric protein (*see* **Note 38**).
9. Wash the column with 50–100 volumes of lysis buffer, PBS, or lectin buffer and elute bound proteins with suitable elution buffer (*see* **Note 39**).
10. Examine eluted proteins by SDS–PAGE (see examples of ligand blot and of ligand enrichment and probing with Ab against candidate molecules in **Fig. 3**).

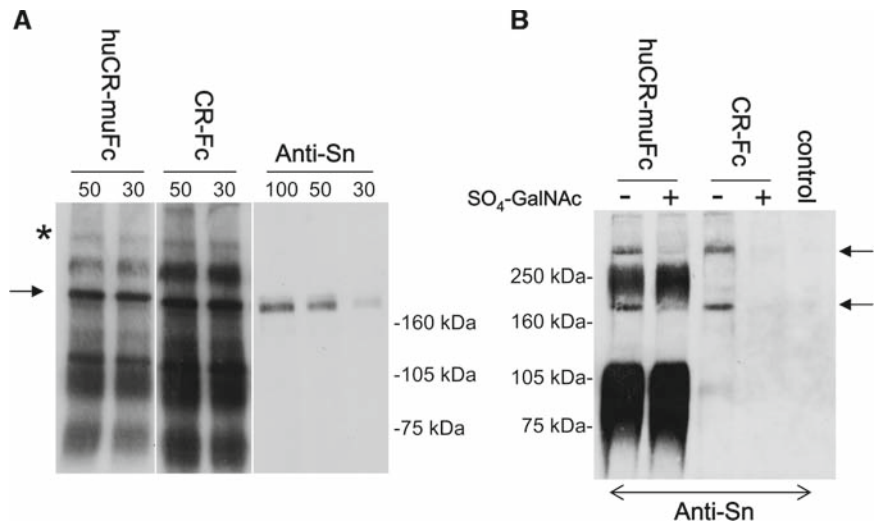



Fig. 3. Analysis of CR ligands in rat spleen lysates. (**A**) Detection of ligands for huCR and CR in rat spleen lysates by ligand blot. Different amounts of protein lysates from rat spleen were separated by SDS–PAGE followed by transfer to nitrocellulose and incubated with huCR-muFc or CR-Fc (50 and 30 μg) or with a monoclonal Ab against the candidate ligand sialoadhesin (Sn) (100, 50, and 30 μg). huCR-muFc and CR-Fc recognised a major band situated between the 160 and 250 kDa MW markers (see *arrow*) that based on size, co-migrated with the Sn band that was recognised by the anti-Sn mAb. Other minor species with rMW above 50 kDa were also detected. The band marked with (asterisk) could correspond to the higher rMW band of Sn that appeared enriched in the preparation of huCR and CR ligands (see band marked with *arrowhead* in **B**). Similar to what was observed previously in murine spleen cells *(7,8)*, a major 250-kDa ligand was detected in rat spleen using this technique. No information regarding the identity of this ligand is available. No binding of the secondary reagent used for detection was observed (data not shown). (**B**) Sn is present in preparations of huCR and CR ligands. HuCR and CR ligands were enriched in the presence (+) or absence (–) of $SO_4$-4-GalNAc using huCR-muFc or CR-Fc conjugated to protein A-sepharose. Selected proteins were analysed by Western blot using anti-Sn mAb. Two bands immunoreactive with this mAb (marked with *arrow* and *arrowhead*) were detected in the absence of inhibitor. Additional bands present in the preparation of huCR ligands in the presence and absence of inhibitor (first two lanes) may correspond to huCR-muFc eluted from the sepharose as they were recognised by the anti-mouse IgG reagent used for detection (data not shown). The control sample was included to detect proteins that bound non-specifically to protein A-sepharose alone.

*3.2.6. Assessment of Binding to Candidate Molecule Immunoprecipitated from a Protein Lysate Using a Monoclonal Ab*

This procedure can be used if prior knowledge is available regarding the nature of the ligand; for instance, if the labelling pattern obtained with the Fc chimaeric protein in situ is similar to the distribution of a particular molecule or if the rMW of a ligand detected using ligand blot is characteristic of a putative candidate.

1. Prior to immunoprecipitation, lysates are pre-cleared using protein A-Sepharose for 2 h at 4°C as before.
2. After pre-clearance, lysates are incubated with mAb coupled to sepharose (*see* **Note 40**) overnight at 4°C in a rotating wheel.
3. Beads are collected on the following day and are washed in lysis buffer or in immunoprecipitation washing buffers A, B, and C (3× in A, 2× in B and 1× in C).
4. Proteins are eluted by boiling in 1× SDS-loading buffer and subjected to electrophoresis and transfer as before.
5. Probe membrane with Fc chimaeric protein as described previously (*see* **Note 41**).

### *3.3. Assessment of Binding to Candidate Ligands in Solid Phase Assays*

*3.3.1. For the Detection of Binding to Potential Carbohydrate Containing Ligands*

1. Coat MaxiSort plates with 50 µl of candidate ligands diluted at 5 µg/ml as follows:
   (a) In the case of purified sugars make dilutions in 0.154 mM NaCl or PBS and incubate at 37°C.
   (b) In the case of PBS-soluble glycoproteins, make dilutions in PBS and incubate at 4°C.
2. Seal plates in parafilm and incubate overnight in a sealed damp box.
3. Wash with lectin buffer (*see* **Note 42**).
4. Add Fc constructs (2 µg/ml) diluted in lectin buffer (*see* **Note 43**).
5. Incubate for 60–90 min at room temperature.
6. After washes detect binding with alkaline phosphatase-conjugated anti-human IgG Fc-specific (typically diluted 1:1,000 in lectin buffer) (*see* **Note 44**).
7. Wash plates with lectin buffer and then twice with alkaline phosphatase substrate buffer and develop with freshly prepared *p*-nitrophenyl phosphate substrate dissolved in alkaline phosphatase substrate buffer (100 µl/well).
8. Measure absorbance at 405 nm (*see* **Note 45**).

*3.3.2. For the Detection of Binding to Ligands in a Carbohydrate-Independent Fashion*

1. E.I.A./R.I.A high-binding plates are coated with appropriate candidate proteins diluted at 10 µg/ml (50 µl/well) in PBS (*see* **Note 46**).
2. Incubated overnight at 37°C.

3. Plates are washed three times with PBS–0.3% BSA and blocked with 0.3% BSA in PBS for 60 min at room temperature.
4. Fc chimeric proteins (diluted at 2–10 μg/ml in PBS–0.3% BSA) (*see* **Note 47**) are then added and incubated for 1 h at RT.
5. After three washes in PBS–0.3% BSA, plates are incubated with alkaline phosphatase-conjugated anti-human Fc for 1 h at RT in PBS–0.3% BSA.
6. After washing three times with PBS–0.3% BSA, wells are washed twice with alkaline phosphatase substrate buffer and 100 μl of substrate solution are added to each well.
7. Measure absorbance at 405 nm (*see* **Note 45**).

## 4. Notes

1. When working with mouse or rat tissues we routinely place several lymphoid organs per block. This does not affect cutting if organs with similar consistency are grouped together.
2. If detecting ligands for proteins able to recognise collagen, such as CR-FNII-CTLD1-Fc and CR-FNII-CTLD1-3-Fc 3 *(12)*, use of charged slides is recommended to avoid binding of the FNII-containing proteins to the gelatine used for coating (*see* **Figs. 1** and **2**).
3. Detection of ligands in specific organs may be fixation sensitive. We have found that fixation with paraformaldehyde is suitable for most of our studies. Alternatives include fixation of fresh frozen tissues with acetone or methanol or the use of perfused tissues *(9)*.
4. The Shandon Sequenza® system enables the labelling of up to 50 slides simultaneously. Assembly needs to be performed with slide and coverplate immersed in buffer and avoiding formation of bubbles. The shape of the coverplate (similar to a funnel) allows the easy application of all the following solutions. For washes, permeabilisation and quenching, the upper reservoir should be filled. In the case of washes, the slide is considered washed once the reservoir has drained. For permeabilisation, quenching and DAPI staining, slides are maintained in the appropriate buffers for specific incubation times.
5. Permeabilisation for 10 min is optimal for most applications. If there is concern about accessibility of the ligands, longer permeabilisation times (30 min) are recommended *(3)*.

6. The buffer to use for washes will depend on the properties of the protein of interest. If binding is expected to take place in the absence of calcium (such as in the case of CR-Fc, huCR-muFc, CR-FNII-CTLD1-Fc and CR-FNII-CTLD1-3-Fc) PBS would be appropriate, but if binding is expected to require calcium (such as in the case of CTLD4-7-Fc), use of the Lectin-binding buffer (10 mM Tris–HCl, pH 7.4, 154 mM NaCl, 10 mM $CaCl_2$) is recommended.
7. The species of origin of the normal serum used for blocking should correspond to that used to raise the secondary Ab used for detection of binding. Absence of ligands in serum should be ensured before using this procedure.
8. New streptavidin-derived proteins with reduced unspecific binding are also available (e.g. NeutrAvidin). The use of these reagents will help to reduce background labelling.
9. We have been successful in detecting ligands for the CR, FNII, and CTLD4-7 regions of the mannose receptor using human IgG1 Fc and mouse IgG2b Fc chimaeric proteins *(3, 8, 9, 13)*. Other examples of the successful use of Fc-derived proteins for detection of ligands in situ are: the lectin macrophage galactose-type C-type lectin 1 and Endo 180 *(12, 14)*.
10. In some instances where recognition of ligands is highly dependent upon protein multimerisation, it is desirable to pre-incubate the chimaeric Fc fusion protein with the anti-Fc reagent use for detection to promote cross-linking of individual molecules. This approach made it possible to observe binding by CR-FNII-CTLD1-Fc and CR-FNII-CTLD1-3-Fc and improve recognition by CR-Fc *(3)* (**Fig. 2**). If using chimaeric proteins with a mutated Fc portion, it is likely that cross-linking would be essential to detect binding *(15)*. The presence of suitable competitors during this incubation will determine the specificity of the interaction. For instance, for CR-Fc we and others have used 10 mM $SO_4$-4-GalNAc *(2, 13)* and for CTLD4-7-Fc we have used 100 mM mannose *(9)*.
11. In the case of CR-FNII-CTLD1-Fc and CR-FNII-CTLD1-3-Fc in mouse tissues and of huCR-muFc in human spleen we observed binding by incubating overnight incubation at 4°C (*(3, 13)*, and **Fig. 2**).
12. Peroxidase-based detection systems are suitable for strong signals and facilitate location of signal by the use of counter-staining. The use of the ABC kit enhances detection, though it is difficult to quantify. The development step has to be performed using a traditional staining tray.

13. Alkaline phosphatase-based detection systems are suitable for weak signals as the reaction can be developed for longer periods. It provides a less-defined signal and is incompatible with multiple counterstains and DPX. The use of the ABC kit enhances detection. It is difficult to quantify but is very robust. The development step has to be performed using a traditional staining tray.
14. Fluorescence labelling is highly versatile and quantitative. The fluorochrome of choice will depend on the strength of the interaction between the protein and its ligand(s), abundance of the ligand, and the microscope available. For weak signals and fluorescence microscopy, Alexa-488 or FITC and the use of three steps labelling procedures is recommended. The use of Texas red or Cy3 is not suitable for tissues with high levels of autofluorescence if only a fluorescence microscope is available.
15. Combining detection of ligands with Fc chimaeric proteins and labelling with cell type-specific markers will provide information regarding the nature of the cells bearing the ligands and their anatomical location *(15)*.
16. To avoid protein degradation it is recommended that tissues are snap frozen in liquid nitrogen or collected in pre-cooled microfuge tubes maintained on dry ice.
17. Homogenisers have to be washed and rinsed thoroughly and then soaked in lysis buffer with protease inhibitors prior use. Samples must be maintained on ice during the lysis and formation of foam must be avoided. Cutting tissue into smaller fragments with a sterile scalpel in a sterile petri dish and in the presence of lysis buffer with protease inhibitors will facilitate the preparation of lysates using a dounce homogeniser.
18. Centrifugation at 100,000 × *g* will provide a lysate suitable for affinity chromatography, immunoprecipitation, and enrichment of ligand in solution using affinity beads. If lysate is going to be used for ligand blot or Western blot, centrifugation in a refrigerated microfuge at 12,000 *g* for 15 min is also suitable.
19. Avoid repeated freezing and thawing.
20. In some instances, several rounds of centrifugation are required to eliminate all the nuclei.
21. This would depend on the scale of the preparation; for the preparation of small samples (1–5 ml) use a TC100 centrifuge.
22. It is recommended to use non-reducing conditions initially so as not to disturb di-sulphide bonds. The use of 4× concentrated loading buffer will facilitate loading of larger amounts

of protein lysates. Samples should be boiled immediately after preparation. In some instances, boiling could induce aggregation of some proteins; in this case keeping the samples at 65°C for 10–30 min may solve the problem but the risk of protein degradation might increase.

23. The percentage of acrylamide in the gel will depend on the size range that needs to be tested. For initial studies running of gradient gels or a low and a high percentage gel would increase the range of proteins tested.
24. The choice of filter might have major implications for the final results. Hybond C is suitable for most of our studies, but PVDF membranes can also be used as an alternative. These membranes need to be activated prior use.
25. We routinely perform protein transfers overnight at 200 mA using the transfer buffer described in **Subheading 2.2.2**.
26. The blocking buffer to use for incubations and washes will depend on the properties of the protein of interest. If binding is expected to take place in the absence of calcium (such as in the case of CR-Fc and huCR-muFc, *see* **Fig. 1**) 5% (w/v) non-fat dry milk, 0.1% (v/v) Tween 20 in PBS would be appropriate, but if binding is expected to require calcium (such as in the case of CTLD4-7-Fc, *see* **Fig. 1**) use 5% (w/v) non-fat dry milk, 0.1% (v/v) Tween 20 in 10 mM Tris–HCl, pH 7.4, 154 mM NaCl, 10 mM $CaCl_2$. We have never encountered problems using non-fat dry milk as blocking reagent, but if there are concerns, 2–3% bovine serum albumin or casein can be used instead.
27. Washes consist in one rinse in buffer and two 10-min incubations.
28. We have obtained good results using our secondary reagents diluted 1:1,000. A control testing of the binding of the secondary Ab to the lysate should be included, in particular when immunoglobulins might be present in the lysate.
29. The washing buffer to use for the last washes will depend on the properties of the protein of interest. If binding is expected to take place in the absence of calcium (such as in the case of CR-Fc and huCR-muFc) 0.1% (v/v), Tween 20 in PBS would be appropriate, but if binding is expected to require calcium (such as in the case of CTLD4-7-Fc) use 0.1% (v/v) Tween 20 in 10 mM Tris–HCl, pH 7.4, 154 mM NaCl, 10 mM $CaCl_2$.
30. If more sensitivity is required, ECLplus offers a suitable alternative.
31. Avoid direct contact between the film and the filter using cling foil or an acetate sheet.

32. The differences in specificity between protein A and protein G should be taken into consideration when designing the experimental approach.
33. During the preparation of the lysate it should be taken into consideration that the incubation of the protein–bead complex with the lysate has to be done under conditions optimal for binding. For instance, presence of $Ca^{2+}$ is essential for CRD4-7-Fc recognition; therefore, use of EDTA in the lysis buffer would not be recommended unless it is removed at a later stage.
34. To ensure accurate distribution of the sepharose between samples use cut tips for pipetting and vortex between pipettings to maintain the sepharose in suspension.
35. Elution with SDS–PAGE sample buffer will also release the Fc chimaeric protein bound to the protein A/G-sepharose, which will then be loaded into the gel. The use of a digoxigenin-based detection method will ensure that when performing the ligand blot, the Fc protein present in the gel will not be recognised by the reagent used to detect binding of the Fc protein used to probe the filter. This problem can also be avoided by using Fc proteins directly conjugated to protein A/G-sepharose (*see* **Subheading 3.2.5**).
36. Washing the beads with glycine pH 2.9 will remove proteins not covalently bound to the protein A-sepharose.
37. Control Fc protein or purified immunoglobulin coupled to protein A/G-sepharose, prepared as described earlier, can be used as pre-clearing columns.
38. This step can be repeated twice. Pre-cleared lysate can be passed through the column twice to increase recovery of ligands.
39. Choice of elution buffer will depend on the characteristics of the interaction. They can be determined by enriching ligands using Fc protein–protein A/G beads and performing ligand blot analysis on samples eluted using low or high pH, chelating $Ca^{2+}$ or by incubation in 1× SDS-loading buffer. If using high or low pH elution buffers avoid damage of the sample by collecting fractions (500–1,000 μl) in tubes containing 50–100 ml of 1 M Tris–HCl, pH 7.
40. mAb can be coupled to CNBr-activated sepharose (5 mg Ab/ml sepharose) or to protein A/G-sepharose (*see* **Subheading 3.2.5** for suitable protocol).
41. Part of the sample should be probed with the mAb used for the immunoprecipitation using standard western blot procedures to ensure that the procedure worked and as size control.
42. All washes involve three rinses with 250 μl of lectin buffer.
43. To test for specificity, proteins can be pre-incubated with D-mannose, D-galactose, D-fucose at 100 mM for 30 min on

ice with prior to plate application. These steps should be performed in lectin buffer containing 1 M NaCl *(16)*.

44. Soluble MR *(17)* can be used for these assays. Bound soluble MR can be detected by incubation with the anti-MR mAb MR5D3 *(18)* (2 μg/ml) for 120 min at room temperature followed by alkaline phosphatase-conjugated anti-rat IgG (Chemicon).
45. Keep plates in the dark. It is useful to take several readings during the development reaction. The reaction can be stopped with EDTA.
46. Collagen and gelatin (denatured collagen) are diluted at 10 μg/ml in 10 mM acetic acid.
47. Proteins bearing a human Fc region can be pre-incubated or not with alkaline-phosphatase-conjugated anti-human Fc, 1:100–1:200, for 60 min at room temperature in PBS–0.3% BSA. This step is essential to detect binding of CR-FNII-CTLD1-Fc and CR-FNII-CTLD1-3-Fc to collagens *(3)*.

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# Chapter 9

# The Expression of Exogenous Genes in Macrophages: Obstacles and Opportunities

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## Summary

Over the past three decades many techniques for expressing exogenous genes in a variety of cells and cell lines have been developed. Exogenous gene expression in macrophages has lagged behind that of other nonhematopioetic cells. There are many reasons for this, but most are due to technical difficulties associated with transfecting macrophages. As professional phagocytes, macrophages are endowed with many potent degradative enzymes that can disrupt nucleic acid integrity and make gene transfer into these cells an inefficient process. This is especially true of activated macrophages which undergo a dramatic change in their physiology following exposure to immune or inflammatory stimuli. Viral transduction of these cells has been hampered because macrophages are end-stage cells that generally do not divide; therefore, some of the vectors that depend on integration into a replicative genome have met with limited success. Furthermore, macrophages are quite responsive to "danger signals," and therefore several of the original viral vectors that were used for gene transfer induced potent anti-viral responses in these cells making these vectors inappropriate for gene delivery. Many of these difficulties have been largely overcome, and relatively high efficiency gene expression in primary human or murine macrophages is becoming more routine. In the present chapter we discuss some of the gene expression techniques that have met with success and review the advantages and disadvantages of each.

**Key words:** Adenovirus, DEAE-dextran, Electroporation, Lentivirus, Nucleoporation, Transduction, Transfection, Retrovirus, Vector.

## 1. Introduction

Macrophages are cells that participate in a number of important immunological processes, including phagocytosis, antigen processing and presentation, and the secretion of a wide variety of mediators that can influence host defense. A fundamental property of macrophages is their ability to accumulate in affected tissue

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and form an inflammatory focus there. The accumulation of macrophages at inflammatory sites has been associated with many pathogenetic settings, including infectious granulomas, atherosclerosis, rheumatoid arthritis, tumors, etc. Macrophages have great potential as drug delivery vehicles because of their ability to home to inflamed tissue. This potential has led to attempts to design macrophage-specific gene delivery systems that allow the efficient expression of therapeutic genes in macrophages. Thus, the delivery of exogenous gene products using genetically modified macrophages is an area with great therapeutic and/or immunomodulatory potential.

The process of introducing nucleic acid across the host cell membrane by mechanical or physical disruption is termed "transfection." This is distinct from "transduction," a process for delivery of nucleic acid into cells by viral vectors, which we will discuss subsequently. Most of the original transfection techniques enjoyed only limited success with macrophages. This is due to the requirement of fairly high gene copy numbers required to efficiently transfect cells and the relatively high degree of toxicity associated with the process whereby the host cell membrane is made permeable to DNA. However, because nonviral-based gene transfer methods continue to have some conspicuous advantages over viral-based transduction, efforts continue to be devoted to improving these methods. The various transfection methods used to introduce foreign DNA into mammalian cells include: DEAE-dextran, calcium phosphate coprecipitation, cationic lipid vehicles, and physical disruption of the host cell membranes by electroporation or nucleofection. All these approaches have been used with varying degrees of success on macrophages.

In the 1960s, Pagano and his colleagues used DEAE-dextran for nucleic acid transfer into mammalian cells *(1, 2)*. DEAE-dextran is a cationic polymer that can interact with negatively charged nucleic acids. The overall positive charge of the polymer in the nucleic acid/DEAE-dextran complex promotes its interaction with the negatively charged cell membrane, facilitating endocytosis. The calcium phosphate coprecipitation-mediated transfection method was introduced by Graham and van der Eb *(3)*. This method involves mixing the nucleic acid with calcium chloride, dropping this mixture in a controlled fashion into a buffered saline/phosphate solution, and then incubating the final mixture to generate a precipitate. The precipitate is then dispersed onto cells and taken up via endocytosis. This transfection technique gained popularity due to its simplicity and low cost. Another advantage of this technique was the discovery that the concentrations of calcium phosphate used for this technique could inhibit host cell nucleases, protecting exogenous DNA from extensive degradation *(4)*. Although these two methods have been fairly widely used for the transfection of various cultured cell lines, their utility for monocytes and macrophages is limited *(5)*.

Using the macrophage-like cell line RAW264.7, Thompson and colleagues *(6)* made a systematic comparison of different transfection methods and concluded that DEAE-dextran and calcium phosphate coprecipitation had the lowest efficiency and the highest rate of interassay variability.

In 1980, a group led by Papahadjopoulos first reported the successful delivery of DNA into a monkey cell line by using unilamellar phospholipid vesicles *(7)*. Later this technique was refined due to the application of a novel synthetic cationic lipid: *N*-[1-(2,3-dioleyloxy) propyl]-*N*,*N*,*N*-trimethylammonium chloride (DOTMA). In comparison with DEAE-dextran and calcium phosphate coprecipitation, liposome-mediated transfection or "lipofection" had 5-fold to as much as 100-fold greater efficiency of delivery, depending on the cell tested *(8)*. The advantages of lipofection included the simplicity of the methodology and the fact that both transient and stable transfections could be similarly accomplished using this technique. Another conspicuous advantage was that liposome/nucleic acid complexes could be administered in vivo to animals and humans for therapeutic purposes *(9)*.

Liposomes are typically formed by mixing a cationic lipid with a neutral lipid. The positively charged cationic lipid interacts with the negatively charged nucleic acid to form a liposome/nucleic acid complex, which can be taken up by cells via endocytosis. After entering the cells, liposome/nucleic acid complexes appear within endosomes. The endosomes release their contents into cytoplasmic compartments and the nucleus *(10)*. The release of nucleic acids from the endosome is believed to be facilitated by the neutral lipid in the liposome/nucleic acid complex. Some neutral lipids may also assist fusion of the complex with the outer cell membrane to enhance the lipofection efficiency *(11)*. Recent refinements of the liposome technology have led to the development of uniform-sized micelles to deliver DNA with greater efficiency and reproducibility. Many lipid-based reagents are commercially available, including LIPOFECTAMINE2000® from Invitrogen Corporation and the FUGENE® Transfection Reagents from Roche Applied Sciences. Additional components can be added to these basic formulas to more efficiently condense the nucleic acid, or to facilitate their release from endosomes (ExGen® 500 reagent from Fermanta). The FUGENE® reagents have been used to successfully transfect the mouse macrophage-like cell line, RAW264.7 (http://www.roche-applied-science.com), and the human macrophage-line cell line, U937 *(12)*. These methods are quite straightforward and commercially available. Detailed instructions are provided by the manufacturer.

The use of recombinant viruses to infect mammalian cells has emerged as one of the preferred means to deliver exogenous genes to mammalian cells. Adenoviruses, adeno-associated viruses, and retroviruses/lentiviruses have all proven to have utility for gene transfer. All these methods carry the conspicuous

advantages of high efficiency gene transfer into mammalian cells, which can result in high expression of the gene product. However, all these systems have some potential disadvantages that must be addressed before they enjoy routine use. The immunogenicity of adenoviruses is a limitation that is being overcome in many of the newer generation viruses. The genotoxicity associated with retroviral integration continues to be an area of concern *(13–15)*. Clinical gene therapy trials with retroviral vectors were dealt a serious blow in 2003 when serious side effects in gene therapy for X-SCID patients were revealed *(14, 16)*. Thus, significant hurdles in vector selection have been cleared but several still remain before large clinical trials can be initiated. Optimization of these viral infection methods to deliver and express genes in hard-to-transfect cells is still in progress, and this is particularly exciting for gene delivery into macrophages.

## 2. Materials

### 2.1. Cell Culture

1. RPMI-1640-10: Roswell Park Memorial Institute-1640 (RPMI-1640) (Mediatech, Inc., cellgro.bizatomic.net) supplemented with 10% fetal bovine serum (FBS, HyClone, http://www.HyClone.com), 10 mmol/L glutamine, 100 IU/mL penicillin, and 100 μg/mL streptomycin (penicillin and streptomycin can be replaced by 50 μg/mL gentamicin sulfate). Storage at 4°C.
2. DMEM/F12-10: Dulbecco's Modified Eagle's Medium Nutrient Mixture F-12 (Ham) (1:1) (DMEM/F12) (GIBCO 10565, Invitrogen, Carlsbad, California 92008, http://www.invitrogen.com) supplemented with 10% fetal bovine serum (FBS, HyClone, http://www.HyClone.com), 10 mmol/L glutamine, 100 IU/mL penicillin, and 100 μg/mL streptomycin (penicillin and streptomycin can be replaced by 50 μg/mL gentamicin sulfate). Storage at 4°C.
3. L-929-conditioned medium: L-929 is a murine fibroblast cell line used as a source of M-CSF for culture of bone marrow-derived macrophages. L-929 cells (L-929 cells, CCL-1™, ATCC, http://www.atcc.org) are cultured in DMEM/F12-10 until confluent. L-929-conditioned medium is then harvested at confluence. Cells are removed by centrifugation and supernatants are stored at –80°C until use.
4. Cellstripper™ Nonenzymatic cell dissociation solution (Mediatech, Inc., cellgro.bizatomic.net). Storage at room temperature.
5. Falcon® Petri dishes (100 × 15 mm Style) (Becton Dickenson Labware).

6. All mammalian cell lines and primary cells are cultured in their corresponding media in an atmosphere of 5% $CO_2$ at 37°C.

### 2.2. Macrophage Cell Lines and Bone Marrow-Derived Macrophages

1. THP-1 (TIB-202™, ATCC, http://www.atcc.org) and U937 (CRL-1593.2™, ATCC, http://www.atcc.org) are cultured in RPMI-1640-10.
2. RAW 264.7 (TIB-71™, ATCC, http://www.atcc.org) and J774A.1 (TIB-67™, ATCC, http://www.atcc.org) are cultured in DMEM/F12-10.
3. Bone marrow-derived macrophages: mouse bone marrow is flushed from the femurs and tibias of mice at 6–10 weeks of age. The cells are plated in petri dishes in DMEM/F12-10 supplemented 20% L-929-conditioned medium. Cells are fed on days 2 and 5. On day 7, cells are removed from petri dishes and cultured on tissue culture dishes in DMEM/F12-10. On the next day, cells are subjected to experiments.

### 2.3. Electroporation Apparatus

1. BioRad Gene Pulser® II apparatus are obtained from BioRad Laboratory (http://www.biorad.com).
2. Electroporation cuvettes (gap width of 4 mm) can be obtained from numerous vendors including BioRad Laboratory.

### 2.4. Nucleofection

1. A Nucleofector device is purchased from Amaxa Biosystem (Amaxa GmbH, Cologne, Germany. http://www.amaxa.com).
2. Cell Line Nucleofector™ Kit T (VCA-1002, Amaxa GmbH, Cologne, Germany. http://www.amaxa.com) is used for the transfection of bone marrow-derived murine macrophages as described in **Subheading 3**. Storage conditions are listed in the accompanying manual.

### 2.5. Adenoviral Transduction

1. A method described by He et al. *(17)* has been widely accepted as a fast and easy alternative to the traditional methods of generating recombinant adenovirus. This system has been improved and commercialized as the AdEasy™ System by many companies including Stratagene® (http://www.stratagene.com), QBiogene® (http://www.qbiogene.com), and the ATCC® (http://www.atcc.org). Storage conditions are listed in the accompanied manual.
2. AdEasy™ vectors are pShuttle, pShuttle-CMV, and pAdEasy-1. The pAdEasy-1 vector is devoid of E1 and E3 regions so that the recombinant virus will not replicate in cells other than complementing cells, such as human embryonic kidney 293 (HEK293).
3. BJ5183 (*rec*A+) (BJ5183-AD1 Electrocompetent Cells, http://www.stratagene.com) is used for in vivo homologous recombination, and DH5a (MAX Efficiency® DH5a™ Chemically Competent Cells, Invitrogen, http://www.invitrogen.com) is used for scale-up production of viral vectors. Storage at –80°C.

4. EndoFree® Plasmid Maxi kit is obtained from QIAGEN (Cat. No.: 12362, QIANGEN, http://www1.qiangen.com). Storage conditions are listed in the accompanied manual.
5. HEK293 cells (CRL-1573™, ATCC, http://www.atcc.org) are used as a host cell to generate recombinant adenovirus and are cultured DMEM/F12-10.
6. Adeno-X™ Rapid Titer Kit is supplied from Takara Bio Company (Cat. No.: 631028, Clontech, http://www.clontech.com). Storage conditions are listed in the accompanied manual.

### 2.6. Viral Transduction by Retroviruses and Lentiviruses

1. The ViraPower™ Lentiviral Expression System (Invitrogen, Carlsbad, California 92008, http://www.invitrogen.com) is loosely based on the HIV-1 strain NL4-3. This system contains Invitrogen's Directional TOPO and Gateway Lentiviral vectors that can accommodate up to 6 kb of inserted exogenous gene. The packaging mix contains three supercoiled packaging plasmids (gag/pol, rev, and VSV-G envelop) that are needed for helper functions and supplying viral proteins in trans. Also contained in this system are Stbl3 *E. coli* cells for transformation and the 293FT packaging cell line that can facilitate production of high-titer virus. The system also contains EmGFP lentiviral expression vectors for assessment of transfection and transduction efficiency and HT1080 cells for titering viral stock.
2. HT1080 is a human fibrosarcoma cell line (CCL-121™, ATCC, http://www.atcc.org) and culture in DMEM/F12-10.
3. Polybrene® can be obtained from Sigma-Aldrich (Catalog No. H9268, http://www.sigma-aldrich.com).
4. G418 is used to maintain regular culture of 293FT cells and can be obtained from Invitrogen (Geneticin™, Invitrogen, http://www.invitrogen.com).
5. Blasticidin and Zeocin™ can be obtained from InvivoGen (http://www.invivogen.com).
6. Lipofectamine™ 2000 is the product from Invitrogen (http://www.invitrogen.com). Fugene™ 6 transfection reagent is supplied from Roche Applied Science (http://www.roche.com).

## 3. Methods

### 3.1. Electroporation

Electroporation is an established technique that has been used in a variety of applications, including but certainly not limited to introducing exogenous nucleic acids into cells. In 1982,

Neumann et al. applied the electroporation technique to introduce DNA into mouse fibroblasts *(18, 19)*. Potter and his colleagues later improved the technique to introduce DNA into a broad range of established cell lines *(20, 21)*. Electroporation has the unique advantage over methods that involve endocytosis and/or phagocytosis since direct uptake of DNA bypasses the lysosomal compartment, limiting the possibility of enzymatic degradation. During the electroporation process, cells are exposed to several electric pulses of varying intensity and duration, creating a trans-membrane potential across the poorly conducting cell membrane. Once this trans-membrane potential reaches a certain threshold, which is proportional to the cell radius and the applied electric field strength, the molecular structure of the membrane will be rearranged to create hydrophilic pores that are permeable to DNA. These pores can be transient in nature and resealed to preserve the integrity of the cell. This process is known as reversible electroporation. An excessive electric field can cause a trans-membrane potential exceeding a tolerable threshold, resulting in pores that cannot be resealed, leading to the destruction of the cell.

Electroporation-based gene transfection into macrophages has received mixed reviews. Since electroporation bypasses lysosomes and causes cell membrane permeabilization that can inhibit phagosomal acidification *(22)*, the potential problems associated with the degradation of DNA by macrophage lysosomal nucleases are minimized. Several groups have successfully used electroporation to introduce exogenous DNA into macrophage-like cell lines. However, the use of electroporation on primary macrophages appears to have been met with more limited success. For example, Stacey et al. found that despite the successful transfection of the macrophage-like RAW264.7 cell line via electroporation, a similar application to primary bone marrow-derived macrophages was much less efficient *(23)*. In many instances, electroporation of primary macrophages results in excessive cell death, regardless of source (synthetic, genomic, or plasmid) or sequence of the DNA.

The following procedures are successfully applied for transfection of macrophage cell lines such as RAW 264.7 cells.

1. Log-phased cells are split 24 h before electroporation to ensure that cells are growing and healthy. Before electroporation, the cells are centrifuged and then resuspended in fresh medium (RPMI-1640) without fetal calf serum or other additives, such as penicillin and streptomycin.
2. Approximately $4 \times 10^6$ cells in a 0.25-mL volume are added to the cuvette (4 mm) and then mixed with DNA. The mixture is allowed to stand at room temperature for 10 min (*see* **Note 1**).
3. Gene Pulser® II apparatus is set for 950 μF and the Pulse Controller for 200 Ω. Efficient transfection can be achieved at a voltage of 250 V. Normally by these parameters, a clump of dead cells will be noticeable (*see* **Note 2**).

4. The electroporated cells are removed from the dead cells and transferred into prewarmed complete medium (*see* **Note 3**). The cells are plated in 24 wells of a 48-well plate and allowed to recover overnight.
5. After overnight recovery, the media is removed and replenished with a fresh media, at which time the cells are ready for experimental studies.

### 3.2. Nucleofection

Nucleofection is a technology developed by Amaxa GmbH (Cologne, Germany). This proprietary technology includes two components: (1) a Nucleofector device that can deliver unique preprogrammed electrical parameters for electroporation, and (2) solutions that are specific for the individual cell types that are targeted for transfection. By varying these combinations, this technology can efficiently deliver DNA into a variety of cell types. The DNA rapidly travels to the nucleus, after which expression of transfected genes can be detected within a short period of time. In 2003, Martinet et al. reported that transfection efficiencies up to 80% without significant cell toxicity could be achieved on human U937 and THP-1 cells *(24)*. Lenz et al. reported similar transfection efficiencies of human monocyte-derived dendritic cells *(25)*. Others have not experienced a similar level of success with nucleofection. For example, nucleofection of DNA into the murine macrophage-like J774A.1 cells resulted in a substantial amount of apoptosis with little exogenous gene expression *(26)*. This group did, however, see up to 75% transfection efficiency when using mRNA rather than DNA.

We have used the following nucleofection procedures to efficiently transfer DNA into primary mouse bone marrow-derived macrophages and RAW264.7 cells *(27, 28)*. Using GFP expression as a readout, we obtained transfection efficiencies of approximately 90% in primary murine macrophages (**Fig. 1**) *(27)*. We have also found this technique to be particularly effective for the delivery of siRNA into primary macrophages. Using siRNA to the MAPKs we were able to achieve a near complete knockdown of ERK mRNA levels (**Fig. 2**) and ERK kinase activity in primary murine macrophages *(28)*.

1. All growth media is warmed to 37°C. Prepare cell culture plates containing growth media and preincubate in a 37°C/5% $CO_2$ incubator. Bone marrow-derived macrophages are removed from the femurs of mice and grown for 6–7 days in CSF until ready for use. A total of approximately $5 \times 10^6$ cells are used per Nucleofection. Cells are centrifuged at $300 \times g$ for 10 min at room temperature and resuspended at a concentration of $5 \times 10^6$ cells/mL in PBS. One milliliter of cells is added to 1.5-mL Eppendorf® tubes and centrifuged for 10s at $7{,}000 \times g$ by Eppendorf microcentrifuge.

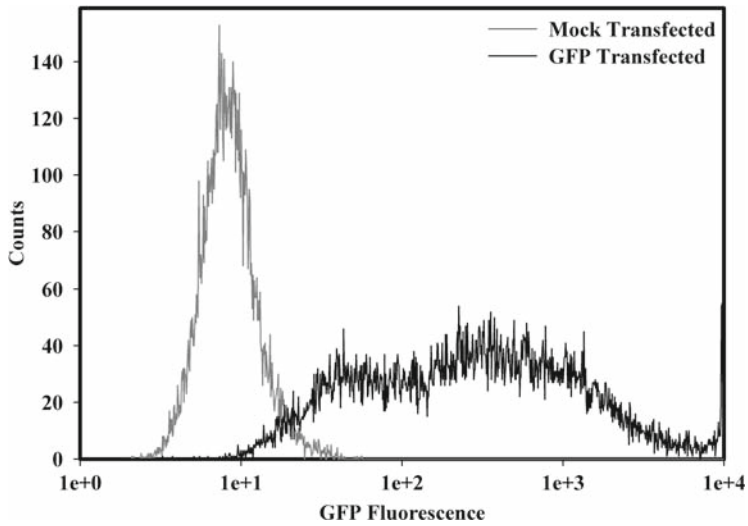


Fig. 1. GFP transfection of bone marrow-derived macrophages (BMMΦ). $5 \times 10^6$ cells at day 6 (BMMΦ) were subject to nucleofection using program T-20, cell line kit-T, and 3.5 μg of pmaxGFP plasmid (GFP) or no DNA (Mock). GFP expression was assessed by flow cytometry.

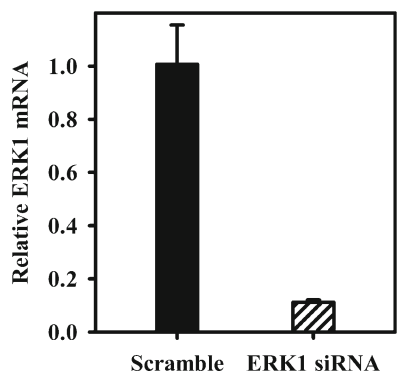


Fig. 2. ERK1 mRNA after nucleofection with ERK1-specfic siRNA. $5 \times 10^6$ cells at day 6 (BMMΦ) were subject to nucleofection using an ERK1-specific siRNA (*black bar*) or nonspecific scramble dsRNA (*hatched bar*) at a final concentration of 100 nM. ERK1 mRNA levels were assessed 48 h after nucleofection by real-time PCR.

2. PBS is gently aspirated from each tube, and the pellets are resuspended in 100 μL of room temperature Nucleofection media (avoiding bubbles) along with 3–4 μg of plasmid or siRNA at a final concentration of 10–1,000 nM (in 1–2 mL) and gently mixed. The mixture is incubated at room temperature for approximately 5 min.
3. Cells are gently resuspended using plastic pipettes and transferred to a Nucleofection cuvette. It is critical to avoid the formation of bubbles during resuspension and transfer.

4. The cuvette is placed in the Nucleofector and the T-20 program is run. After the program ends, the cuvette is removed and 1 mL of prewarmed growth media is added. The cuvette is recapped and gently tapped to allow cell debris to rise to the top. Cells are gently resuspended using a plastic pipette, being careful to avoid cell debris that has risen to the top.
5. Depending on the application, cells may be counted and directly transferred to preincubated growth media, or if multiple transfections are performed using the same plasmid, cells may be combined, then counted, and then transferred to preincubated growth media.
6. Growth medium is changed the following morning at which time gene expression and/or transfection efficiency may be assessed. After the medium is changed, the transfected cells are cultured for an additional 24 h before gene expression is assessed for either knockdown or gain of function transfections (*see* **Note 4**).

### 3.3. Adenovirus Transduction

Adenoviruses are virions that have a double-stranded DNA genome with sizes ranging from 26 to 45 kb. Adenoviruses can replicate in either quiescent or dividing cells after infection. A relatively high level of protein expression can be achieved following transduction, and estimates of as high as 35% of total cellular protein have been reported *(29, 30)*. Since transduced genes remain epichromosomal, activation or inactivation of host genes following integration is avoided. Postinfection viability of the host cells remains at nearly 100% for most mammalian cell types; thus, it is well tolerated. For these reasons recombinant adenovirus is the vector of choice for many protein overexpression studies.

Most adenoviruses utilize the ubiquitously expressed coxackie and adenovirus receptor (CAR) for initial attachment and integrin-alpha-V glycoproteins for internalization. The heparan sulfate glycosaminoglycan receptor has also been shown to be involved in the binding of adenoviruses. These features lead to gene delivery in nontarget tissues and make the prospect of systemic gene delivery less plausible. Modifications to refine the specificity in attachment of adenovirus are under development, and these are likely to be especially important for macrophage transduction. Since macrophages do not express the high-affinity adenovirus receptor and only low levels of alpha-V-beta 5 integrin *(31, 32)*; transduction efficiency in these cells is sometimes low. The development of vectors that are targeted to specific receptors on macrophages has begun to address this limitation for in vitro transduction *(33)*. It should be noted, however, that the infection of alveolar macrophages by adenoviruses in vivo appears to proceed reasonably well. This may be due to the presence of surfactant proteins in the lung that may enhance viral uptake *(34, 35)*.

Adenoviruses can be highly immunogenic and induce proinflammatory cytokine production *(36)*. Additionally they induce dendritic cells to become mature antigen-presenting cells *(37)*. In early clinical trials, an Ad5-based gene delivery vector was associated with a fatal systemic inflammatory response *(38)*. Such problems have largely been addressed by engineering nonimmunogenic vectors that are devoid of viral-coding sequences (gutless) and/or engineered viruses that coexpress immunomodulatory molecules *(39)*.

The adeno-associated viruses (AAV) are single-stranded nonpathogenic DNA parvoviruses with a genome of roughly 4.7 kb. AAV needs the presence of an adenovirus or a herpes simplex virus coinfection in order to fulfill its life cycle. In the absence of these helper viruses, AAV integrates into human chromosome 19 via specific AAVS1 site to establish latency *(40)*. This property makes them superior to other viral methods, especially retroviruses, which randomly insert into host chromosomes. There are two open reading frames (ORFs) that encode viral proteins: the left one encodes the proteins essential for replication and the right one encodes the structural proteins for the capsid. Palindromic inverted elements that flank the two ends of the genome are the minimal cis-acting elements necessary for the integration and rescue of the viral genome during the latent stage. These elements are also essential for the replication of the viral genome and packaging into the capsid *(41, 42)*. AAV infection begins with virus binding to the cell surface via its primary receptor, heparan sulfate proteoglycans (HSPG), followed by viral internalization, intracellular trafficking, nuclear entry, and virion disassembly and release of a single-stranded DNA template for second-strand DNA synthesis. Fibroblast growth factor receptor 1 and alpha-V-beta 5 integrin have been identified as coreceptors for AAV infection *(43)*. In addition, adeno-associated viruses can use O-linked or N-linked sialic acids for cell binding. Different AAV serotypes exhibit different tissue or cell tropisms, which may be exploited for the development of AAV tissue or cell-specific-targeting vectors. The tropism of AAV can also be engineered through modification of the capsid. A chimeric capsid protein containing the variable region of an antibody against CD34 was used to specifically target hematopoietic progenitor cells *(44)*.

McEacher et al. demonstrated a correction of a murine model of Gaucher disease by the intravenous administration of adeno-associated virus vector that contained the human glucocerebrosidase gene *(45)*. Efficient gene knockdown of HIV-1-transactivating region (TAR) in human hematopoietic stem cells by transduction with AAV was demonstrated *(46)*. In this work, antisense RNA resulted in a continuous inhibition of viral replication without selective pressure. Thus, although not

covered here in detail, adeno-associated viruses remain a viable alternative for the delivery of exogenous genes into macrophages.

The following procedures are described for adenovirus-mediated transduction of genes into macrophages:

1. The gene of interest is cloned into a shuttle vector. Shuttle vectors that are devoid of a promoter allow the insertion of the gene of interest under control of its own promoter. Alternatively, vectors containing strong constitutive promoters, such as CMV or EF-1 (elongation factor-1) promoter, are available and allow for high level of gene and protein expression (*see* **Note 5**).
2. To achieve homologous recombination of recombinant adenovirus, the shuttle vector containing the gene of interest has to be linearized by *Pme* I and dephosphorylated. One microgram of linearized recombinant transfer vector and 0.1 μg of the pAdEasy-1 vector are then cotransfected into BJ5183 bacterial cells by electroporation. A control transformation lacking the pAdEasy-1 vector should be included.
3. Typical parameters for electroporation of BJ5183 bacterial cells are: 25 μF of capacity, 200 Ω of resistance and 2.5 kV for a Bio-Rad instrument, 5 Ω of resistance, and 2.5 kV with 50 μF capacity for a BTX instrument.
4. After recovery at 37°C for 1 h with 1 mL SOC medium, the electroporated mixture is plated onto three LB plates in the presence of antibiotic for 24 h at 37°C, with 100, 300, and 600 μL on each. Approximately twenty of the smallest colonies are picked up and individually grown in LB medium with antibiotic. Restriction enzyme analysis is performed on miniprepared plasmid DNA to identify appropriate recombinants.
5. Selected recombinants can be amplified in an electrocompetent *rec*A-strain of *E. coli*, such as DH5α. The procedure is similar to that described earlier for BJ5183. Recombinant plasmid DNA is purified by EndoFree® Plasmid Maxi kit (QIAGEN). Purified plasmid is then linearized by *Pac* I and purified through phenol/chloroform extraction and EtOH precipitation under sterile conditions. Five micrograms of purified linearized recombinant plasmid is used for transfection into HEK293 cells.
6. Approximately $1 \times 10^6$ HEK293 cells are plated on a 60-mm-diameter culture dish in DMEM with 5% FBS for 24 h before transfection, by which time they will reach ~70% confluency (*see* **Note 6**). Cells are changed with fresh medium at least 1 h before transfection. Transfection can be accomplished by lipofection or nucleoporation (*see* **Note 7**). The transfected cells are then collected 7–10 days later by scraping cells off the dish and subsequent centrifugation. The pelleted cells are

resuspended in a small volume of media and subject to three freeze/thaw cycles. After centrifugation at maximum speed for 10 min, the supernatants are collected as the original viral stock (*see* **Note 8**). One milliliter of the collected supernatant is generally ready to infect $3 \times 10^6$ cells to amplify virus (*see* **Note 9**). The remaining original stock solution is stored at −80°C (*see* **Note 10**).

7. To obtain high-titer viral stocks, HEK293 cells are infected at a multiplicity of infection (MOI) of 0.1–1 as determined in 8 and grown for 4 days, at which time virus is collected as described earlier. The process is repeated 1–3 times with a final repeat using a total of $5 \times 10^8$ HEK293 cells and an MOI of 1–5. Lysis of 50% HEK293 will be found between the third and fifth day following infection. At the fifth day postinfection, the viruses are harvested and purified using a double cesium chloride gradient (*see* **Note 11**), as previously described *(47)*. This is the virus preparation used to transduce macrophages.
8. A viral particle titration should be performed before transduction of the final target macrophages. Protocols used for titration can be divided into two types: physical methods (optical viral unit or viral particle) and biological ones. A fast, easy, and reliable biological assay developed by Bewig and Schmidt *(48)* and commercialized by Clontech Laboratories, Inc. (http://www.clontech.com) as the Adeno-X™ Rapid Titer Kit can be used. Alternatively, other methods can be used to fit the needs of each laboratory.
9. Human macrophages, particularly alveolar macrophages, are known to have low expression levels of the high-affinity coxsackie-adenovirus receptor (CAR). Thus, in addition to keeping cells in optimal condition and using viral titers as high as possible, special steps are needed to facilitate adenovirus infection. Viral particles in prewarmed medium without serum are mixed with cholesterol at 3 μg/mL (Sigma-Aldrich, St. Louis, MO) and incubated at 37°C for 30 min *(49)*. Macrophages are washed and resuspended at $5 \times 10^6$ cells/mL in complete medium typically with M-CSF (20 ng/mL) and 10% FCS. $5 \times 10^5$ cells are transferred into a nonadherent tube (Cat. No. 466982, Nunc, Roskilde, Denmark) and infected with the cholesterol/adenovirus mixture at an MOI of 100:1. The mixture is then centrifuged at 2,000 × *g* at 37°C for 2 h. After infection, the cells are washed three times with serum-free medium, and then complete medium is added to the culture *(50)*. After culture for 24–48 h, successful delivery of genes of interest can be examined by methods such as Western blot, flow cytometry, quantitative PCR, etc. as suitable for individual purposes.

### 3.4. Transduction by Retroviruses and Lentiviruses

Over the past decade, retroviruses have been used as a means to deliver genes into mammalian cells. Retroviruses are named for their ability to reverse-transcribe their RNA genome into DNA after infection. The general limitation associated with retroviral transduction is that they typically integrate only into a dividing genome, and therefore they cannot be used on terminally differentiated, nondividing cells, such as macrophages. The lentiviruses, however, are a subgroup of retroviruses that integrate into the genomes of nondividing cells. Consequently, these viruses have the most potential for macrophage gene delivery. All retroviruses have three coding domains: the *gag* domain that generates the matrix-, capsid-, and nucleoproteins; the *pol* domain that yields the viral protease, reverse transcriptase, and integrase; and the *env* domain that encodes envelope glycoprotein. The life cycle of retroviruses can be simply defined as follows: binding of viruses to their receptors, entry and internalization, reverse transcription, assembly of preintegration complex, nuclear transportation, transcription of integrated virus, translation from spliced mRNA, virion assembly, and exit from the cell.

Lentiviruses (e.g., HIV) are naturally attractive candidates as vectors for gene expression in primary nondividing macrophages. In 1995, a team led by Verma and Trono first detailed the construction of a harmless HIV-derived lentiviral vector that was able to deliver genes into a variety of cells, including human primary macrophages *(51)*. These authors used an HIV-derived lentivirus-based luciferase vector pseudotyped with the VSV G protein to transduce human monocyte-derived primary macrophages. A significant increase in luciferase activity could be detected in an envelope-dependent fashion. The possibility that the vector transduced a small portion of proliferating macrophages was ruled out by mutating Vpr and MA, two virion proteins that are central for HIV infection of primary macrophages. Since then, numerous studies using this or a modified delivery system have been reported. Rossi et al. demonstrated that similar HIV-1-based lentiviral vectors could mediate efficient gene transfer into primary murine macrophages and mature B lymphocytes without the participation of HIV-1 accessory proteins Nef, Vpr, Vif, and Vpu *(52)*. The lentiviral vector-mediated method was used to genetically modify monocytes so that the autocrine and paracrine production of GM-CSF and IL-4 could be used to differentiate human CD14+ monocytes into immature dendritic cells *(53)*.

The subgroup lentivirus includes human immunodeficiency viruses (HIV-1 and HIV-2), simian immunodeficiency viruses, feline immunodeficiency virus (FIV), equine anemia infectious virus, and maedi-visna virus. All these viruses can integrate into the genome of nondividing cells, which makes them attractive delivery vehicles for the expression of exogenous genes or small interfering RNAs. Many optimized lentiviral systems such as the

FIV-based vector have been developed to circumvent safety hurdles for potential application in human subjects. All of them are based on a genetically split gene expression design. The essential viral elements include the minimal LV packaging helper proteins (gag-pol genes), the LV transfer vector RNA containing the transgene expression cassette, and heterologous glycoproteins. The following procedure is based on an HIV-1-based vector system developed by Invitrogen (http://www.invitrogen.com) for academic applications (*see* **Note 12**). The HIV-1-based system is well developed and characterized and been proven to be successful for use on primary macrophages *(51)*.

The procedure can be divided into five basic steps: (a) cloning the gene of interest into the lentiviral vector; (b) transforming *E. coli* and isolating plasmid DNA; (c) testing the construct by transient transfection; (d) harvesting and titering the viral stock; and (e) transducing macrophages with virus.

1. TOPO-technology-based vectors are designed to facilitate PCR-based cloning using a proofreading polymerase. Taq polymerase should not be used due to the inhibitory effect of 3′-A on the D-TOPO reaction. An excess of PCR product inhibits the D-TOPO reaction, thus 1:1 ratio of insert:vector should generally be maintained.
2. All retroviral vectors have two LTRs with 180 bp of repeats; thus, they can recombine in bacteria, easily resulting in smaller products. To reduce the possible occurrence of inappropriate recombination between the LTRs, Stbl3 *E. coli* cells are used for transformation. The choice of LB plates with 100 μg/mL ampicillin also reduces recombination. Only small colonies are picked for subsequent minipreparation, as recombination leads to loss of most of the plasmid, conferring a growth advantage and favoring the formation of the large colonies. EDTA should be included in the miniprep solution to kill Stbl3 *E. coli* cells containing a thermostable periplasmic endonuclease. Phenol/chloroform extraction is added to denature the enzyme. Restriction endonuclease analysis is performed to determine whether unwanted recombinants exist in the preparation.
3. The 293FT cell line is derived from HEK293 cells. Both these cells contain a stably integrated T antigen, but the 293FT cells have the added convenience of rapid growth in culture. Plasmids with an SV40 ori are not appropriate since it can be recognized by T antigen and lead to cell death. Transient transfection of the newly constructed viral vector and packaging vectors (packaging mix) in 293FT cells can be performed by using Lipofectamine 2000™ (Invitrogen) or Fugene™ 6 (Roche). Multinucleated syncytia should be found after transfection since VSV-G glycoproteins (packaging mix) can cause cells to fuse. Cells should be at approximately 90% confluence

by plating $5 \times 10^6$ cells per 10 $cm^2$ 1 day before transfection in complete media.

4. Virus-containing supernatants are collected 48–72 h after transfection. Due to the infectious nature of the viruses, BSL-2 guidelines should be followed. The supernatants are centrifuged at 1,800*g* at 4°C for 15 min and then filtered through a 0.45-μm filter. The virus can be kept in the medium used to culture 293 cells (in the absence of G418) in small aliquots at −80°C. The virus can also be concentrated by ultracentrifugation as previously described *(54)*.
5. Viral titering is done on HT1080 cells (human fibrosarcoma cell line from ATCC: http://www.atcc.org). Tenfold serial dilutions of lentiviral stock are prepared and transduced into HT1080 cells in the presence of Polybrene (Sigma, Catalog No. H9268). Twenty-four hours after viral transduction, cells are trypsinized from 6-well culture plates and expanded to 100-mm plates. Twenty-four hours later, 10 μg/mL blasticidin or 100 μg/mL zeocin are added for selection. One week after blasticidin selection or 3 weeks after zeocin, crystal violet staining is carried out to visualize plaques. Cells are washed with PBS, then treated with 1 mL of crystal violet solution (1% crystal violet in 10% ethanol) for 10 min at room temperature, and then washed with PBS twice. The infected plaques are visible to the naked eye for counting to determine the viral titer of the original collected viral supernatant.
6. Cell lines or primary macrophages and monocytes are obtained and maintained as described earlier. One day before transduction, macrophages are plated so that they will be approximately 30% confluent the next day. Virus solutions with a range of MOI from 1 to 100 are prepared in cell culture media with or without Polybrene or DEAE-dextran (*see* **Note 13**). Virus should be kept at a ratio no greater than 1:2 in the final volume of cell culture medium. The final volume in a well of 24-well plates should not exceed 250 μL. The lower the volume, the better. Twenty-four hours after incubation with virus, culture supernantants are removed and fresh culture medium is added. For nonadherent monocytes, on the day of transduction cells are plated at a density so that they will not need to be subcultured for 3 days. The cells are plated in a small volume (100 μL for 24-well plates). Virus solutions with different MOI are added and mixed well with the cells. The final volume should be kept under 200 μL in total. Cells and viruses are mixed every couple of hours at least twice during the daytime and incubated at 37°C for an additional 24 h. Fresh medium (100 μL) is then added, or virus-containing medium is removed by centrifugation and replaced with fresh culture medium. Expression of inserted genes can be monitored beginning at day 2 and continuing until 7 days post-transduction (*see* **Note 14**).

## 4. Notes

1. Precooling the cuvette and cells before electroporation is not necessary.
2. The BioRad Gene Pulser II apparatus generates exponential pulses by the Pulse Trac™ waveform delivery system for optimal transfection in an electroporation cuvette. Other electroporation devices that generate square wave pulses such as BTX® molecular delivery system (http://www.btxonline.com) have also been well established for the transfection of mammalian cells including human and mouse macrophage cell lines. The parameters are: 260 V and three pulses of 10 ms with a pulse length set to BCM 820 when 4-mm gap cuvette is used. Ten micrograms of DNA in 0.4 mL volume with $5 \times 10^6$ cells in total is used. These parameters are also applicable to human THP-1 and U937 cell lines.
3. It is common to lose up to 50% of the cells during the electroporation procedure.
4. We have achieved a transfection efficiency of approximately 90% using this technique on primary bone marrow macrophages (**Fig. 1**).
5. The CMV promoter may not be suitable for the expression of inserted genes in rodent cells due to methylation-dependent downregulation *(55)*. EF-1 (elongation factor-1) promoter or other macrophage-specific promoters are alternative choices.
6. To avoid the occurrence of replication competent adenoviruses (RCAs), passage numbers of HEK293 cells should not be higher than four.
7. Addition of GeneJammer (Stratagene: http://www.stratagene.com) during the adenovirus infection can lead to increases in both the total numbers of infected cells and the level of transgene expression per cell in the absence of CAR *(56)*.
8. In order to avoid the generation of the unwanted recombinants, extraction of recombinant adenovirus plasmid from HEK293 cells should be performed as quickly as possible using the minipreparation procedure for low copy plasmid. Furthermore, a recombinant adenovirus plasmid with a large size insertion is genetically not stable in a *rec*A+ strain such as BJ5183.
9. Good management of the first amplification of viral stock or use of low passage viral stock is critical because they contain the lowest possible ratio of RCA/recombinants. The frequency of RCA occurrence is about one revertant per $10^7$ viruses.
10. Nyberg-Hoffman virus storage buffer (Tris 10 mM, pH 8.0, with 2 mM $MgCl_2$ and 4% sucrose) allows the viral particles

to be stored at concentrations up to $1 \times 10^{13}$ viral particles/mL without precipitation.

11. Cesium chloride purification is essential for in vivo gene delivery because it can remove contaminants that can elicit an immune response in vivo.
12. Other Lentiviral vector-based systems are also available through commercial vendors, such as a series of feline lentiviral vectors supplied by SBI (http://www.sbi.com) and a lenti-siRNA vector from GenScript (http://www.genscript.com). Feline or other nonhuman lentiviral vectors-based gene delivery systems are particularly suitable for gene therapy in human subjects *(57)*.
13. Polybrene (hexadimethrine bromide) is a small positively charged molecule that can enhance transduction by retroviruses because it can reduce repulsion between sialic acid-containing molecules and neutralize surface charge to allow the viral glycoproteins to bind more efficiently to their receptors. However, polybrene must be used with caution, particularly for cells derived from hematopoietic precursors because of its toxicity to T and B cells. DEAE-dextran is an alternative, but both reagents should be titrated before being added to macrophages or monocytes. Titration of polybrene generally begins at 1 μg/mL, and DEAE-dextran at 3 μg/mL. Inclusion of polybrene or DEAE-dextran generally increases transduction efficiency by twofold *(58)*.
14. Because macrophages are particularly adept at sensing 'danger' and migrating to areas of infection or tissue damage, these cells become potential drug delivery vehicles *(63)*. The development of innovative new methods for introducing exogenous macromolecules into macrophages is not only useful for basic bench experimentation, but has also led to the design of new protocols to be applied to clinical gene therapy or drug delivery. Chemical and lipofection methods do not have the concern of the host immune response that is often associated with viral infection. Adenovirus-based gene delivery may have the disadvantage of transient expression of the transduced gene, and it can lead to some side effects in vivo, such as hepatotoxicity *(59)*. Furthermore, the repeated administration of adenovirus will often result in unwanted host immune responses. Lentivirus-based constructs have the advantages of macrophage-specific targeting and continuous expression of the transduced gene due to integration into chromosomal DNA. Thus, lentivirus-based systems are quite promising for both research and clinical application. However, dangers associated with HIV genotoxicity continue to be major limitations for the use of these vectors. Importantly, many new and refined vectors are under development to obviate many of these concerns. New methods, including the use of nanoparticles with cell-specific-targeting moieties,

have been reported *(60)*. Osmotic delivery of small interfering RNA (siRNA) *(61)* and even the use of bacterial ghosts loaded with DNA have been used as methods to efficiently transfer nucleic acids into macrophages *(62)*. The goal is to obtain high transfection efficiency and cell specificity so that genetic manipulation of macrophages will become a routine task.

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# Chapter 10

# Genetic Modification of Murine Dendritic Cells by RNA Transfection

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## Summary

The ability to manipulate in vitro cultured dendritic cells (DCs) by transfection represents an attractive strategy to load these antigen-presenting cells with genetic material encoding various immunogenic epitopes. The gene transfer approach can also be applied to DCs with the aim of expressing immunologically active molecules such as cytokines, costimulatory molecules, or simply to transiently express proteins to perform cell biology studies. Available gene transfer technologies for DCs include both viral and non-viral vector-based approaches. In this chapter, we describe non-viral strategies of RNA transfection. Special emphasis is given to murine bone-marrow-derived DCs, since gene transfer to human DCs has been extensively described in the literature, especially in the context of cancer immunotherapy and other clinical applications. Methods to deliver small interfering RNA (siRNA) to DCs are described as well. Finally, the potential of exogenously delivered RNA to activate DCs is discussed and some practical advice to avoid DC activation is described.

**Key words:** Dendritic cells, Mice, Non-viral gene transfer, RNA transfection, RNA electroporation, RNA lipofection, DC activation, Gene expression, Translation.

## 1. Introduction

Different approaches have been developed to load DCs with antigens *(1)*. Direct use of antigenic peptides is efficient, but is limited to a finite number of MHC-restricted epitopes. DCs can be loaded with recombinant proteins encoding various immunogenic epitopes, but this requires the laborious and expensive production of proteins. These limitations can be overcome through either non-viral or viral gene transfer technologies developed for DC loading *(2)*. Although viral vectors will efficiently transfect

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DCs *(3, 4)*, their use is complicated by important safety concerns. Infection of bone-marrow progenitors has also been developed, but the time required for DC differentiation (2–3 days) affects the outcome of the experiments and obviously the conclusions drawn from heterologous gene expression over 48–72 h. Plasmid DNA transfer into DCs does not represent a valid alternative, because of its low transfection efficiency *(5)*. This contrasts profoundly with RNA delivery, with which the efficient strategy of RNA electroporation has recently been described *(6, 7)*. RNA electroporation is by far the most efficient and safest transfection technique for DCs (*see* **Table1**), and is described in detail in this chapter.

**Table 1**
**RNA-transfection procedures, transfection efficiencies, and immunological outcomes measured in dendritic cells**

| RNA-transfection procedure | DC type | Transfection efficiency | T-cell stimulation/ cytokine secretion | Reference |
|---|---|---|---|---|
| **Passive pulsing** | | | | |
| | Human MoDCs | ND | CTL induction | Nair et al. *(12)* |
| | Human MoDCs | ND | CTL induction | Heiser et al. *(22)* |
| | Human MoDCs | ND | CTL induction/IFNγ | Heiser et al. *(21)* |
| | Porcine MoDCs | 1%[a] | ND | Ceppi et al. *(18)* |
| **Lipofection (employed reagent)** | | | | |
| Lipofectin | Human MoDCs | 508 pg LUC/$10^5$ DCs | $CD4^+$ and $CD8^+$ proliferation<br>CTL induction/IFNγ | Weissman et al. *(9)* |
| DOTAP | Murine BMDCs | ND | CTL induction | Boczkowski et al. *(15)* |
| DMRIE-C | Human MoDCs | 4–8% | CTL induction/IFNγ | Van Tendeloo et al. *(13)* |
| | Human MoDCs, Murine BMDCs | ND | CTL induction | Boczkowski *(27)* |
| | Human MoDCs | ND | CTL induction | Thornburg *(28)* |
| TransFast | Human MoDCs | 21% | CTL induction/IFNγ | Strobel et al. *(7)* |
| | Porcine MoDCs | 15–20% | IFN type I induced by RNA lipofection | Ceppi et al. *(10, 18)* |
| DAC-30 | Human MoDCs | 14% | ND | Strobel et al. *(7)* |

(continued)

**Table 1 (continued)**

| RNA-transfection procedure | DC type | Transfection efficiency | T-cell stimulation/ cytokine secretion | Reference |
|---|---|---|---|---|
| **Electroporation** | | | | |
| | Human MoDCs, $CD34^+$ DCs, and LCs | 63, 73, and 50% | CTL induction/IFNγ | Van Tendeloo et al. *(6, 13)* |
| | Human MoDCs | 11% | CTL induction/IFNγ | Strobel et al. *(7)* |
| | Murine BMDCs | 70–80% | $CD4^+$ and $CD8^+$ proliferation | Van Meirvenne et al. *(17)* |
| | Porcine MoDCs | 70–80% | CTL induction/IFNγ | Ceppi et al. *(10, 18)* |

*MoDCs* monocyte-derived DCs; *ND* not determined; *CTL* cytotoxic T-lymphocytes; *CD34+* $CD34^+$ progenitor-derived DCs; *LCs* Langerhans cells
[a]% of cells expressing a GFP reporter gene analysed by flow cytometry

RNA transfection of DCs may be advantageous for several reasons. For protein production, the transfected mRNA molecules need to be delivered only into the cytosol of the target cells, while in comparison plasmid DNA also requires entry into the nucleus and subsequent transcription to generate the encoded message *(8)*. This is especially true in non-dividing cells and may partially explain why DNA transfection is so inefficient in DCs *(5)*. In contrast, RNA transfection results in high transfection efficiency (80–90% after electroporation) *(6)* and rapid protein expression (detectable within 2 h of electroporation) *(9)*. Another major advantage of RNA transfection is that several mRNA species can be simultaneously delivered into the target cells leading to the translation of different gene products. In the context of antigen presentation, RNA transfection permits loading of DCs with mRNA encoding multiple antigens, which may then be processed and displayed on the surface of the DCs as defined antigenic peptides, potentially leading to the activation of multiple T cells *(10)*.

Independent of transfection and gene expression, maturing DCs tightly regulate levels of mRNA translation. Cap-mediated translation is considerably inhibited at late time of activation (from 16 to 24 h), thus rendering heterologous protein expression relatively inefficient at these late stages of DC maturation. This inefficiency is illustrated in **Fig. 1**. Here it is evident that the level of luciferase enzymatic activity in mRNA-transfected maturing DCs

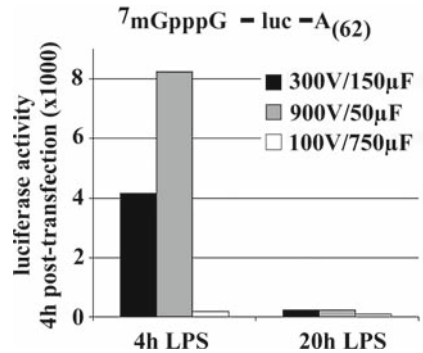


Fig. 1. Immature and LPS-stimulated (16 h) DCs were electroporated with firefly luciferase reporter mRNA applying different voltage (V) and capacitance (μF) as indicated in the figure. DCs were then incubated for further 4 h with fresh complete medium in presence of LPS. Cells were harvested and luciferase activity was measured using a reporter assay system that measures luminescence. The best transfection efficiency is obtained using an electroporation setup of 900 V/50 μF. A different setup may be applied, if the EGFP reporter mRNA is electroporated

is considerably reduced at 20 h of maturation when compared to 4 h, despite the fact that transfected mRNAs were found to be equivalent in the two cell populations *(29)*.

## 2. Materials

### *2.1. Cell Culture*

1. 7- to 8-Week old male C57-BL6 or Balb/c mice.
2. To isolate bones from mice: gauze, sharp curved tip scissors, and forceps.
3. To isolate bone marrow: petri dishes, 10-ml syringes, 25-gauge needles, and cell strainers.
4. 70% EtOH.
5. RPMI 1640 medium.
6. Red Blood Cells (RBC) lysis buffer: 1.66% $NH_4Cl$ solution.
7. DC Wash Medium (DC-WM): RPMI supplemented with 5% de-complemented foetal bovine serum, 50 μM β-mercaptoethanol, 20 μg/ml gentamicin.
8. DC Growth Medium (DC-GM): DC-WM supplemented with recombinant murine cytokine GM-CSF, produced as a culture supernatant from J558L cells transfected with murine GM-CSF cDNA, as described previously *(11)*.
9. Lipopolysaccharide (LPS).

### 2.2. Plasmids and In Vitro Transcription of mRNA

1. Several plasmids have been described in the literature for the in vitro transcription of mRNA molecules coding for reporter genes (*see* **Note 1**). In our laboratory, optimal transfection efficiencies of murine and human DCs have been achieved after RNA electroporation of mRNA molecules that had been transcribed in vitro using the following linearized DNA plasmids:
   - *pGEM4Z/GFP/A64* encoding the enhanced green fluorescent protein (EGFP) (*see* **Note 2**). The plasmid can be linearized with the restriction enzyme SpeI, and for in vitro transcription a T7 RNA polymerase should be employed *(7, 12)*.
   - *pSP64/GFP* encoding for EGFP. The plasmid can be linearized with the restriction enzyme EcoRI and for in vitro transcription a SP6 RNA polymerase is used *(13)*.
   - *pT3LUC(pA)* encoding firefly luciferase (LUC). The plasmid can be linearized with the restriction enzyme NotI, and for in vitro transcription a T3 RNA polymerase is used *(14)* (Lelouard et al., in press; Schmidt et al., in press).
2. QIAquick PCR purification system (QIAGEN).
3. mMESSAGE mMACHINE in vitro transcription system (AMBION). The monomethyl guanosine ($^{7}$mGpppG) cap analogue is provided in the kit.
4. RNeasy Mini RNA purification system (QIAGEN).
5. 1% Denaturing agarose gel.
6. Spectrophotometer.

### 2.3. RNA Transfection

1. PBS.
2. OptiMEM (Gibco).
3. Gene Pulser II apparatus (Biorad, *see* **Note 3**).
4. 4-mm Gap electroporation cuvette (Biorad, *see* **Note 3**).
5. Lipofection reagent TransFast (Promega, *see* **Note 4**).

### 2.4. Reporter Gene Expression Analysis

1. Reporter assay system Dual-Luciferase kit (Promega).
2. FACS-staining buffer (FSB): 1% foetal bovine serum in PBS.
3. FACS-fixing buffer (FSB-fix): 2% paraformaldehyde in FSB.
4. Propidium iodide (10 μg/ml).
5. FACScan analytical flow cytometer (Becton Dickinson).

### 2.5. Antibodies for Immunophenotyping

For the analysis of the surface markers, bone-marrow-derived DCs (BMDCs) should be stained with following fluorochrome-conjugated antibodies:

1. CD11c-FITC or -PE (clone N418, e-Bioscience).
2. CD80-FITC (clone 16-10A1, Miltenyi).
3. CD86-APC (clone GL1, e-Bioscience).

4. CD40-FITC (clone HM40-3, Pharmingen).
5. MHC class I (tested for C57-BL6 mice): H-2K$^b$ – PE or – FITC (clone AF6-88.5, Pharmingen).
6. MHC class II (tested for C57-BL6 mice): IA/IE – PE (clone M5/114.15.2, Pharmingen).

## 3. Methods

### *3.1. Generation and Culture of Murine Bone-Marrow Derived DCs*

1. Kill the desired number of mice. Typically, 40 × 10$^6$ bone-marrow cells can be isolated from one C57-BL6 mouse.
2. Remove femurs and tibias from the mice, and clean off bones from tissue as much as possible using gauze.
3. Transfer the bones in a tube containing RPMI 1640 medium.
4. Remove medium by aspirating and wash bones twice with 70% EtOH.
5. In sterile hood aspirate EtOH and wash bones twice with RPMI 1640 medium. Also sterilize scissors and forceps with EtOH.
6. Place cleaned bones into a Petri dish containing a drop of fresh DC-WM, large enough to wet all bones.
7. Hold the bones with curved forceps and use sharp scissors to snip off both large tips of bones. Put bone tips in a 6-mm dish containing 1–2 ml of medium.
8. Flush the bone marrow out of bone shafts using a 10-ml syringe and a 25-gauge needle.
9. Pipet bone marrow up and down until you have a single-cell suspension.
10. Place a cell strainer (BD Falcon #352350) in a 50-ml Falcon tube. Filter the cell suspension through the strainer to remove extra pieces of bone and connective tissue.
11. Using curved tip scissors, cut bone tips from **step 7** so that marrow is released. Then pipet into a single-cell suspension and pass through the same strainer.
12. Spin cells at 300*g* for 5 min.
13. Resuspend cells in 10 ml of DC-WM (if using ten mice). Add 10 ml of ice-cold RBC lysis buffer.
14. Incubate on ice for 5 min.
15. Fill up the tube with DC-WM. Centrifuge at 1,200 rpm for 5 min. Wash once with medium.

16. Resuspend cell pellet in 10 ml of DC-GM. Pass cell onto a new cell strainer to remove dead cell debris. Wash strainer with extra medium.
17. Count cells.
18. Dilute cell suspension in DC-GM to a cell density of $0.8 \times 10^6$ cells/ml. Transfer 5 ml of the cell suspension ($4 \times 10^6$ cells) into each well of a 6-well plate and incubate at 37°C in a humidified atmosphere supplemented with 5% $CO_2$.
19. Replace medium every 2 days: remove most of the medium in each well by lifting the plate at 45° and gently aspirating; then gently add 5 ml of fresh pre-warmed DC-GM. Avoid swirling or agitating cells at each manipulation in order to prevent spontaneous maturation.
20. Maturation of BMDCs can be induced by adding 100 ng/ml LPS at day 5.

### 3.2. mRNA In Vitro Transcription

1. Linearize at least 20 μg of the plasmid DNA template with the recommended restriction enzyme.
2. Purify the linearized plasmid using the QIAquick PCR purification system, following the manufacturer's instruction. Alternatively, an EtOH precipitation step can be performed to purify DNA (*see* **Note 5**).
3. Perform the in vitro transcription reaction on 1 μg purified DNA template choosing the mMESSAGE mMACHINE system having the appropriate RNA polymerase. Follow the manufacturer's instruction. Typical RNA yields from one reaction are 20–30 μg for the T7 and the T3 kits and 15–25 μg for the SP6 kit. If more RNA is needed, the in vitro transcription reaction can be scaled up.
4. Purify the in vitro transcribed RNA with the RNeasy Mini system, following the manufacturer's instructions.
5. Check the integrity of the RNA fragment on a 1% denaturing agarose gel, and measure the $OD_{260}$ with a spectrophotometer to determine RNA concentration.

### 3.3. mRNA Transfection of DCs

During the whole procedure it is very important to work under sterile and RNase-free conditions. Typically, BMDCs are transfected on day 5 in their immature stage. After careful washing, cells are RNA transfected, either by electroporation or by lipofection. Alternatively, RNA can be 'passively pulsed' to DCs in absence of any transfection reagent *(15)*.

#### 3.3.1. Electroporation

1. Collect cells and wash them twice with ice-cold PBS. Generally $10–15 \times 10^6$ cells can be isolated out of one 6-well plate.
2. Resuspend cells in OptiMEM at a final cell density of $20 \times 10^6$ cells/ml.

3. Pre-incubate 200 µl of cell suspension ($4 \times 10^6$ cells) in a 4-mm gap electroporation cuvette (Biorad) for 15 min on ice.
4. If an EGFP mRNA molecule is transfected, add 10–20 µg of in vitro transcribed mRNA (or siRNA, *see* **Note 6**) to the cell suspension and perform the electroporation with a Gene Pulser II apparatus (Biorad), applying a voltage of 300 V and a capacitance of 150 µF (measured pulse times: 5–10 ms). If a LUC mRNA molecule is transfected, add 2.5 µg mRNA to the cells, applying a voltage of 900 V and a capacitance of 50 µF (measured pulse times: 3–4 ms).
5. After electroporation, immediately resuspend cells in 4 ml fresh pre-warmed DC-GM at a final cell density of $10^6$ cells/ml. Transfer 2 ml of the transfected cell suspension ($2 \times 10^6$ cells) into 12-well plates and incubate at 37°C in a humidified atmosphere supplemented with 5% $CO_2$.
6. A maturation signal such as LPS can be added either just after electroporation or between 4 and 24 h after RNA transfection, depending on the experimental goals.

#### *3.3.2. Lipofection*

1. Collect cells and wash them twice with ice-cold PBS.
2. Resuspend $2 \times 10^6$ cells in 500 µl pre-warmed OptiMEM in a 2-ml test tube.
3. Mix 4 µg mRNA (or the synthetic double-stranded RNA poly I:C, *see* **Note 7**) diluted in 500 µl pre-warmed OptiMEM with 12 µl of the lipofection reagent TransFast at a lipid/RNA ratio of 3:1. Incubate the suspension 15 min at RT to allow mRNA/lipid complexes to form.
4. Add the 500 µl lipid/RNA mix to the 500 µl cell suspension and incubate 1 h at 37°C. Gently mix the suspension by inverting the tube 3–4 times during the incubation time.
5. After lipofection, wash cells twice with pre-warmed OptiMEM and resuspend cells in 2 ml fresh pre-warmed DC-GM at a final cell density of $10^6$ cells/ml. Transfer cell suspension ($2 \times 10^6$ cells) into 12-well plates and incubate at 37°C in a humidified atmosphere supplemented with 5% $CO_2$.
6. If needed, add a maturation signal.

### *3.4. Determination of Transfection Efficiency and Cell Viability of DCs*

Transfection efficiency is generally determined following the expression of the reporter gene EGFP qualitatively by microscopy and quantitatively by flow cytometric (FCM) analysis. If the reporter gene LUC is transfected, expression can be measured quantitatively using a reporter assay system which measures luminescence, such as the Dual-Luciferase kit (Promega). Gene expression can be detected as early as 4-h post RNA transfection and it can last for 7 days *(16)*.

The procedure for FCM analysis of EGFP is as follows:

1. Wash cells with FSB.
2. Resuspend cell pellet in 200 µl FSB-fix.
3. Just before FCM analysis, add 10 µl of propidium iodide (10 µg/ml) to a final concentration of 0.5 µg/ml, to access cell viability. Alternatively, ethidium bromide can be used (*see* **Note 8**).
4. Perform FCM analysis by gating on cells (the DCs) exhibiting a large forward scatter (FSC) and side scatter (SSC) profile, in order to allow exclusion of dead cells. Positively transfected $EGFP^+$ cells are detected on FL-1 by 'green signals', whereas dead cells are detected on FL-2 by 'red signals'. Typically, about 80% of viable DCs show high-level EGFP expression after RNA electroporation, while electroporation-related cell mortality on total DC cultures should be around 10% *(13, 17, 18)*. After RNA lipofection 10–20% of DCs should show high-level EGFP expression, while lipofection-related cell mortality should be from 10 to 20% *(6, 7)*.

### 3.5. Immunophenotyping of DCs

The maturation status of BMDCs should be followed by FCM analysis, before and after RNA transfection, in the absence or presence of the maturation signal LPS. BMDCs should be analysed for the surface markers MHC class I, MHC class II, CD80, CD86, and CD40. FCM analysis should be performed by gating on the CD11c cells (mostly DCs), which under optimal conditions represent 80–90% of the total cell population. mRNA electroporation by itself should not induce DC maturation, while after LPS stimulation mRNA-electroporated DCs should mature like non-transfected DCs *(13, 17, 18)*. In contrast, mRNA lipofection has been reported to induce DC maturation both in murine *(19, 20)* and human *(9, 21, 22)* DCs.

## 4. Notes

1. Incorporation of the 5′ and 3′ untranslated regions (UTRs) of β-globin mRNA greatly stabilizes RNA molecules transfected into cells and leads to over a 1,000-fold increase in reporter gene expression in transfected cells *(23)*. Lengthening the polyA-tail (e.g. 64 × A) in the mRNA transcript also increases expression of GFP in target cells *(6, 24)*. The impact of different structural modifications of RNA on protein expression in RNA-transfected DCs has been recently reported *(25)*. Holtkamp and colleagues demonstrated that the elements most important for enhancing RNA stability and translational

efficiency are located 3′ of the coding region as was observed for two sequential β-globin 3′ UTR followed by an unmasked poly(A) tail of 120 nucleotides.

2. The 741-bp BamH1–NotI fragment containing the GFP coding region was isolated from pEGFP-N1 (Clontech). This fragment was cloned into the BamH1 and Nar1 sites of pGEM4Z (Promega) along with oligonucleotides containing a Not1 half-site, the pGEM4Z polylinker sequence from BamH1 to EcoRI, 64 A-T bp, a SpeI site, and a Nar1 half-site to create pGEM4Z/GFP/A64. To generate a GFP chimeric protein, the gene of interest can be sub-cloned N-terminal of GFP using the restriction site HindIII, which is located downstream of the T7 RNA polymerase promoter.
3. In our laboratory the Biorad electroporation system has been also successfully used for RNA transfection of human monocyte-derived DCs. However, the best transfection efficiencies for human DCs have been obtained using the Amaxa Nucleofector system in combination with RNA (electroporation program U-002).
4. Few comparative studies have been published for RNA lipofection of DCs, but Strobel et al. *(7)* reported that the strongest expression of a reporter gene mRNA was achieved with the liposomal reagent TransFast (20% $GFP^+$ cells). Similar results have been reported for the liposomal reagent DMRIE-C *(6)*. Experience with DNA lipofection has taught us that the efficiency of gene transfer is greatly influenced by the choice of the lipid, the concentration versus toxicity of the complexes, and the cell type one desires to transfect *(24)*.
5. EtOH precipitation procedure to purify DNA:
   - Add 0.1 × vol 3 M NaCl and 3 × vol 100% EtOH to the 1 × vol DNA solution
   - Incubate the resulting mix at –20°C for 20 min
   - Centrifuge the mix (30 min, 4°C, 16,000*g*)
   - Wash the DNA pellet with ice-cold 70% EtOH
   - Centrifuge the washed DNA pellet (5 min, 4°C, 14 krpm)
   - Dry the pellet for 20 min
   - Resuspend the DNA pellet in 10 μl TE
6. The Gene Pulser II apparatus (Biorad) can also be used to transfect siRNA molecules into murine BMDCs, to achieve RNAi-mediated gene silencing. The siRNA electroporation should be performed using 4–5 μg of siRNA and a voltage of 900 V and a capacitance of 50 μF.
7. Lipofection of synthetic double-stranded RNA (poly I:C) has been successfully applied to switch non-plasmacytoid dendritic cells into high interferon producers *(26)*. This response is

partially dependent on the cytosolic dsRNA-binding enzyme protein kinase R (PKR).

8. Just before FCM analysis, add ethidium bromide to the fixed cells to a final concentration of 10 μg/ml, to access cell viability.

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# Chapter 11

# Recording Phagosome Maturation Through the Real-Time, Spectrofluorometric Measurement of Hydrolytic Activities

**Robin M. Yates, Albin Hermetter, and David G. Russell**

## Summary

The efficient degradation of internalized particulate matter is a principal objective of the macrophage's phagosome. Assessment of the true hydrolytic capacity within the phagosomal lumen is often difficult as it is subject to many factors beyond recruitment of lysosomal hydrolases. Here we outline three assays that allow quantitative measurements of serine-cysteine protease, triglyceride lipase, and β-galactosidase activities within the phagosomes of macrophages, in real time. The assays utilize ratio fluorometry between particle-associated fluorogenic substrates and calibration fluorochromes to yield internally controlled values that record rates of substrate hydrolysis. The methods described utilize a spectrofluorometer for fluorometric measurements from a population of macrophages. These assays, however, can be expanded to high-throughput or single cell formats. In addition, this approach can be applied to measure a wide variety of phagosomal hydrolytic properties with the design of suitable fluorogenic substrates.

**Key words:** Phagosome, Lysosome, Phagocytosis, Macrophage, Hydrolase, Lipase, Proteolysis, Lumen.

## 1. Introduction

The ability of macrophages to efficiently degrade phagocytosed particles is essential to their function as mediators of homeostasis and immunity. While recruitment of individual hydrolases to the phagosome has been documented extensively both biochemically and by immunofluorescence *(1–3)*, the true hydrolytic status of the intralumenal milieu of the phagosome is only now being assessed *(4)*. Delivery of degradative enzymes to the phagosome is a necessary first step for the development of hydrolytic activities within the organelle, but it is the complex interplay with the

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physiology of the phagosome that determines the actual hydrolytic activity within. Factors such as pH, ion concentrations, redox potential, activation of proenzymes, and the regulation by cofactors all impact on intraphagosomal hydrolytic activities *(4)*. It is this complexity that precludes an accurate assessment of true phagosomal enzymatic activities based solely on the localization, or even concentration, of recruited hydrolases. In this chapter, approaches that quantify specific hydrolytic activities within the phagosome in real time, in live macrophages, are detailed.

The assays described measure rates of hydrolase activities in real time, exploiting ratio fluorometry of phagocytosed, experimental particles complexed with a fluorogenic substrate and a calibration fluor. Three hydrolytic assays are described that record the degradation of fluorogenic substrates specific for phagosomal cysteine-serine proteases, triglyceride lipases, and β-galactosidases. This approach should not be regarded as limited to the substrates described. Fluorogenic substrates that are pH insensitive and which can be coupled to an experimental particle could readily be used to measure a specific hydrolytic activity of interest. Thus, with the design of suitable substrates, this approach could be used to measure the activities of nucleases, phosphatases, disulfide reductases, and specific cathepsins, to name only a few.

The assays described are population based in design that follow the synchronous uptake of experimental particles by a monolayer of macrophages. This is a convenient and efficient approach as it returns statistically robust data with excellent temporal resolution. In addition, the influence of operator bias is virtually eliminated as minimal qualitative assessment is required. Furthermore, although not documented here, these methodologies could be applied to confocal-based technologies to permit recording of single cell or single phagosome measurements, and thus could assess the variation between individual phagosomes *(5)*. This chapter is intended as a companion to a chapter entitled "Real-Time Spectrofluorometric Assays for the Lumenal Environment of the Maturing Phagosome" published in Humana Press Methods in Molecular Biology series volume 445.

## 2. Materials

### *2.1. Cells, Reagents, and Buffers*

1. Macrophages: Both bone-marrow-derived murine macrophages (BMMØ) and macrophage-like cell lines have been used (*see* **Note 1**). BMMØs are derived from the bone marrow extracted from the femurs, tibias, and iliums of euthanized mice and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS),

5% horse serum, 2 mM L-glutamine, 1 mM sodium pyruvate, and 20% L-cell conditioned media (BMMØ media). RAW 264.7 cells (available from the American Type Culture Collection, Rockville, MD, USA) are maintained in DMEM supplemented with 10% FBS and 1.5 g/ml sodium bicarbonate.

2. Cover slips: Clean 0.13 × 12.5 × 25 mm cover glass (*see* **Note 2**). Sterilize by autoclave.
3. Binding buffer: Tissue-culture-tested PBS pH 7.0 adjusted to contain 1 mM $CaCl_2$, 2.7 mM KCl, 0.5 mM $MgCl_2$, 5 mM dextrose, 10 mM HEPES, and 5% FBS.
4. Cuvette buffer: Tissue-culture-tested PBS pH 7.2 adjusted to contain 1 mM $CaCl_2$, 2.7 mM KCl, 0.5 mM $MgCl_2$, 5 mM dextrose, 10 mM HEPES, and 0.1% calf skin gelatin (*see* **Note 3**).
5. Binding dish: A microbiological Petri dish containing a square piece of parafilm adhered to the lower plate surrounded by a damp Kimwipe® tissue.
6. 0.4% Trypan blue.
7. 3.0 μm Carboxylate-modified silica particles (Si–COOH) 5% suspension (*see* **Note 4**).
8. Streptavidin. Store as desiccate at –20°C.
9. Human IgG. Store as desiccate at 4°C.
10. Cyanamide. Store as desiccate at 4°C.
11. Coupling buffer: 0.1 M sodium borate in $ddH_2O$. Adjust pH to 8.0 with 10 M NaOH. Filter sterilize through 0.22-μm filter.
12. Quenching buffer: 250 mM glycine in PBS pH 7.2. Filter sterilize through 0.22-μm filter.
13. Sodium azide 2% aqueous solution. Very toxic.
14. Alexa Fluor 594® carboxylic acid, succinimidyl ester (mixed isomers) (Alexa594-SE) (Molecular Probes, Eugene, OR, USA). Dissolve in high-quality anhydrous dimethylsulfoxide (DMSO) at 5 mg/ml before use. Stock solutions can be aliquoted and stored at –20°C. Protect from light and moisture.
15. Biotinylated cysteine-serine protease substrate: (Biotin-LC-Phe-Arg)$_2$-Rhodamine 110. Dissolve in high-quality anhydrous dimethylsulfoxide (DMSO) at 1 mg/ml before use. Stock solutions can be aliquoted and stored at –20°C. Protect from light and moisture.
16. Nucleosil 120-3 $C_{18}$ 3 μm silica core reverse-phase HPLC matrix. 10 mg/ml suspension in chloroform.
17. Triglyceride lipase substrate: 1-trinitrophenyl-amino-dodecanoyl-2-pyrenedecanoyl-3-*O*-hexadecyl-sn-glycerol (donated by Albin Hermetter, Graz University of Technology, Graz, Austria).

Dissolve in 2:1 choloroform:methanol at 1 mg/ml and store at –20°C under nitrogen. Protect from light and moisture.

18. 1-Palmitoyl-2oleoyl-sn-glycero-3-phospho-rac-(1-glycerol). Dissolve in 2:1 choloroform:methanol at 10 mg/ml and store at –20°C under nitrogen.
19. Octadecyl rhodamine B chloride. Dissolve in methanol at 1 mg/ml and store at –20°C under nitrogen.
20. 1,2-Dipalmitoyl-sn-glycero-3-phosphoethanolamine-*N*-(Cap Biotinyl). Dissolve in 2:1 choloroform:methanol at 1 mg/ml and store at –20°C under nitrogen.
21. Cholesterol. Dissolve in 2:1 choloroform:methanol at 1 mg/ml and store at –20°C under nitrogen.
22. Mouse monoclonal (clone BN-34) antibiotin IgG as ascite fluid.
23. β-Galactosidase substrate: 5-dodecanoylaminofluorescein di-β-D-galactopyranoside ($C_{12}$FDG). Dissolve in 2:1 choloroform:methanol at 10 mg/ml and store at –20°C under nitrogen. Protect from light and moisture.

### 2.2. Instruments

1. Temperature-controlled spectrofluorometer with variable excitation and emission monochromators. The authors use the QMSE4 model spectrofluorometer from Photon Technologies International (Lawrenceville, NJ, USA) equipped with a thermostat-controlled four chambered turret for simultaneous measurement of four experimental variables. The QMSE4 is interfaced with a PC compatible computer and is managed by Felix32 software (Photon Technologies International, Lawrenceville, NJ, USA).
2. Quartz 10 × 10 × 45 mm cuvettes.
3. Fluorescent microscope with standard FITC and Texas Red filter sets. The authors use the Zeiss Axioskop 2 plus (Carl Zeiss MicroImaging Inc., Thornwood, NY, USA).

## 3. Methods

### 3.1. Macrophage Monolayer Preparation and Handling

1. Fully differentiated BMMØ monolayers are grown to confluency in untreated Petri dishes. Growth media is removed and replaced with cold PBS pH 7.2 (without $Ca^{2+}$ and $Mg^{2+}$), and incubated at 4°C for 10 min to facilitate BMMØ detachment from the plastic. BMMØs are then gently dislodged with a rubber policeman and centrifuged at 230 × *g* at 4°C for 10 min.
2. Sterile, clean 12.5 × 25 mm cover slips are placed in a sterile Petri dish using fine-point forceps that have been dipped in 70% ethanol and flamed (*see* **Note 5**).

3. BMMØs are gently resuspended in 1 ml BMMØ media, counted using a hemocytometer, and diluted appropriately in BMMØ media to achieve ~1.25 × $10^6$ macrophages/ml.
4. Ten milliliters of BMMØ suspension is added to the Petri dish and incubated at 37°C for 24 h to allow a monolayer to establish on the cover slips. Care should be exercised to prevent excessive movement of the cover slips in the Petri dish. The BMMØ monolayers covered on cover slips (subsequently referred to as monolayers) are then ready for fluorometric analysis.

### 3.2. Spectrofluorometer Setup and Operation

1. The spectrofluorometer should be set up according to manufacturer's directions such that optimal measurements can be taken using the desired wavelengths (*see* **Note 6**).
2. Clean quartz cuvettes containing cuvette buffer are inserted into a thermostat-controlled sample holder and warmed to 37°C prior to the loading of the monolayers (*see* **Note 7**).
3. Using fine-point forceps, a monolayer-covered cover slip is grasped at one end (*see* **Note 8**) and dipped 10 times into a sterile 50-ml tube containing binding buffer to remove BMMØ growth media and loosely adhered cells. The cover slip is then placed in the cuvette with a vertical orientation (length of the cover slip parallel to the long axis of the cuvette), on the diagonal (width of the cover slip at 45° to the short axes of the cuvette) and with the cellular side facing the emission slit, as shown in **Fig. 1**.

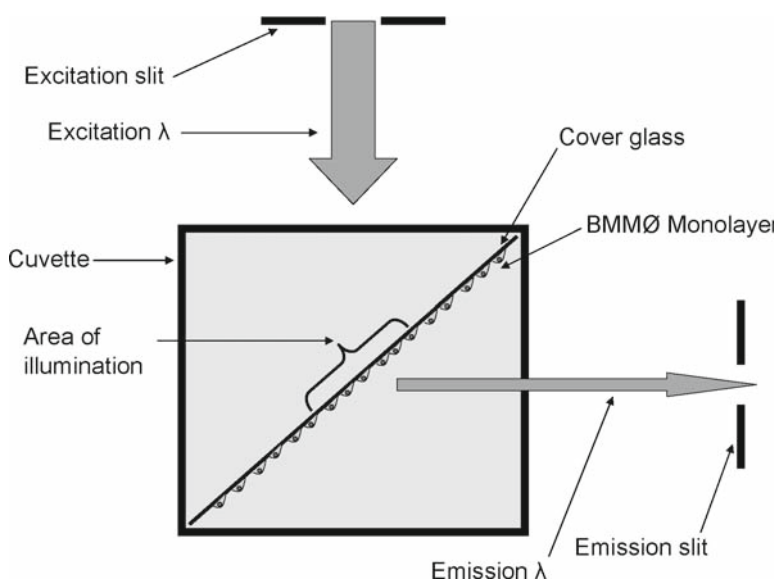


Fig. 1. Orientation of the BMMØ monolayer in the spectrofluorometer. Cover glass is oriented to achieve a 45° incidence with the excitation beam (excitation λ) and with the cells facing the emission slit.

4. At this point, sufficient background measurements of each monolayer are recorded for the wavelengths required.
5. At the conclusion of the background determination, the cover slips are carefully removed from the cuvettes using forceps and placed with the cellular side up, on the parafilm within the binding dish.
6. Eighty-five microliters of a suspension of the appropriate experimental particles is carefully laid over each monolayer. The meniscus should be maintained over the cover slip at room temperature for 3 min (*see* **Note 9**).
7. The cover slips are once again dipped 10 times in binding buffer to remove unbound particles and are placed in the cuvettes with the same orientation.
8. The appropriate successive fluorescent measurements are recorded, alternating between each sample.
9. At the conclusion of the assay, the cover slips are removed by forceps and placed cellular side down onto 30 µl of 0.4% trypan blue on a glass slide and examined with bright-field and fluorescence microscopy. Careful attention should be paid to macrophage viability, optimal bead to BMMØ ratio, and the presence of extracellular experimental particles. This is an extremely important control and must be completed with every sample. Data should be disregarded should there be a decrease in macrophage viability, overloaded macrophages, or the presence of any extracellular particles.

### *3.3. Kinetic Analysis of Intraphagosomal Cysteine-Serine Protease Activity*

#### *3.3.1. Preparation of IgG/Streptavidin-Coupled Particles*

1. Fifty micrograms of carboxylate-modified silica particles is washed three times in 1 ml of PBS by brief vortexing and centrifugation in a tabletop microfuge at 2,000 × *g* for 60 s.
2. Particles are resuspended in PBS (pH 7.2) with 25 mg/ml of the heterobifunctional crosslinker cyanamide (freshly made) and incubated at room temperature with agitation for 15 min. Excess cyanamide is removed by washing the particles twice with coupling buffer.
3. Particles are resuspended in 500 µl of coupling buffer with 1 mg of streptavidin and 250 µg of human IgG, and incubated with agitation for 12 h. This step covalently attaches the streptavidin and IgG to the particles through the cyanamide crosslinker. The IgG serves as an opsonin, while the streptavidin allows coupling of the biotinylated substrate onto the particle (*see* **Note 10**).
4. Particles are washed twice with quenching buffer to quench unreacted cyanamide and twice with coupling buffer to remove soluble amine groups.
5. The particles are resuspended in 1 ml of coupling buffer, and 10 µl of the 5 mg/ml stock of the calibration fluor (Alexa Fluor 594®-SE) in DMSO is added (*see* **Note 11**). Incubation at room

temperature with agitation for 1 h allows the particle-bound albumin to be sufficiently labeled with the amine-reactive fluor.

6. The now-fluorescent particles are washed three times with quenching buffer and finally resuspended in 1 ml PBS (*see* **Note 12**). Ten microliters of a 2% solution of the preservative sodium azide can be added for storage at 4°C.

*3.3.2. Fluorometric Measurement of Phagosomal Cysteine/Serine Protease Activity*

1. 50 µl of IgG/streptavidin particles is washed twice with 500 µl sterile PBS to remove traces of sodium azide and resuspended in 80 µl of PBS.
2. 20 µl of the 1 mg/ml solution of the substrate (Biotin-LC-Phe-Arg)$_2$-Rhodamine 110 in DMSO is added to the washed beads and incubated in the dark, on ice, for 1 h with occasional gentle vortexing.
3. The now-substrate-loaded particles are washed twice with 500 µl sterile PBS to remove unbound substrate and resuspended in an appropriate volume of binding buffer to achieve ~1 × $10^7$ beads/ml (*see* **Note 13**).
4. After 594|620 nm and 490|515 nm (excitation λ|emission λ) background measurements are recorded for BMMØ monolayers alone, 85 µl of the diluted particle suspension is laid onto the monolayers in the binding dish and incubated at room temperature for 3 min (as per **Subheading 3.2**).
5. Unbound particles are washed from the monolayers by dipping in binding buffer and the coverslips are returned to the spectrofluorometer.
6. Both calibration and substrate fluorescent intensities are measured. The fluorescence of the calibration fluor Alexa Fluor 594® (emission at 620 nm with excitation at 594 nm) should remain constant throughout the assay (*see* **Note 14**). The rhodamine 110 fluorescence (emission at 515 nm with excitation at 490 nm) will increase as it is dequenched with sequential cleavage of the amine-linked peptides by cysteine or serine proteases, particularly cathepsin L *(6)*. Typically, an integration time of 1 s per data point is optimal (*see* **Note 15**). Data are collected for at least 60 min or until exhaustion of the substrate (*see* **Note 16**). At the conclusion of the assay, monolayers are examined via microscopy with trypan blue (as per **Subheading 3.2**).
7. Data are exported into a standard spreadsheet application such as Microsoft Excel®.
8. The appropriate background values are deducted (if not already deducted by the acquisition software) and the ratio $S_{RT}/C$ (where $S_{RT}$ is the substrate fluorescence in real time and $C$ is the calibration fluorescence) is plotted against time (**Fig. 2**).

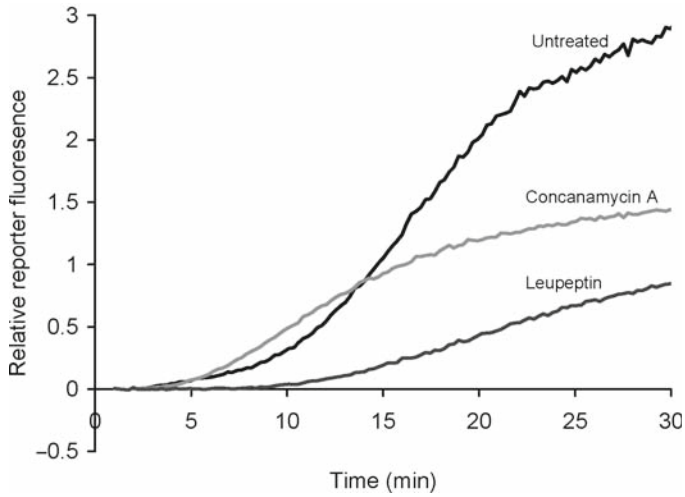


Fig. 2. Phagosomal serine-cysteine protease activity in BMMØs. Serine-cysteine protease activity can be diminished with the serine-cysteine protease inhibitor leupeptin (100 μg/ml) and the V-ATPase inhibitor concanamycin A (100 nm).

9. Gradients of portions of the profiles will give relative values of the cysteine and serine protease activities of the phagosomal population over that period (*see* **Note 17**).

### *3.4. Kinetic Analysis of Intraphagosomal Triglyceride Lipase Activity*

#### *3.4.1. Preparation of Lipase Lipid Monolayer Particles*

1. Two hundred microliters of the 10 mg/ml suspension of the Nucleosil 120-3 $C_{18}$ reverse-phase HPLC matrix in chloroform is added to a clean glass test tube along with the following lipids dissolved in 2:1 chloroform:methanol (*see* **Note 18**): 25 μl of the substrate 1-trinitrophenyl-amino-dodecanoyl-2-pyrenedecanoyl-3-*O*-hexadecyl-sn-glycerol (1 mg/ml); 5 μl of the calibration fluor octadecyl rhodamine B chloride (1 mg/ml); 30 μl of the phospholipid 1-palmitoyl-2oleoyl-sn-glycero-3-phospho-rac-(1-glycerol); 5 μl of the biotinylated phospholipid 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-*N*-(CapBiotinyl)(1mg/ml); 5 μl of cholesterol (10 mg/ml) (*see* **Note 19**).
2. The mixture is briefly sonicated in a bath sonicator at 40°C and then dried under nitrogen (*see* **Note 20**).
3. The test tube is transferred to the bath sonicator at 40°C and 500 μl of sterile PBS is added to the tube during sonication. This facilitates the arrangement of the amphipathic lipids to surround the hydrophobic $C_{18}$-coupled particles, allowing the particles to be miscible in the PBS. The mixture is sonicated until a homogenous suspension is achieved (*see* **Note 21**).
4. The suspension is cooled immediately by placing the test tube on ice. To avoid disruption of the lipid monolayer on the surface of the particles, which results in clumping of the beads,

the suspension must be kept ice cold during subsequent manipulation and not be subjected to excessive shearing or centrifugal forces.

5. Once cooled, the suspension is transferred to 1.5-ml polypropylene tubes and the lipid particles are washed twice with 500 µl ice-cold PBS using gentle centrifugation at 100 × *g* for no more than 30 s.
6. Lipid particles are resuspended in 100 µl PBS to which 5 µl of antibiotin IgG-containing mouse ascite fluid is added. Incubation on ice for 30 min with occasional gentle agitation is sufficient to permit IgG-opsonization of the particles.
7. The opsonized lipid particles are gently washed twice with 500 µl ice-cold PBS to remove excess IgG and finally suspended in 500 µl of PBS (*see* **Note 12**). The particles are stable for 24 h if kept on ice in the dark.

*3.4.2. Fluorometric Measurement of Phagosomal Triglyceride Lipase Activity*

1. The lipid-coated particles are diluted in an appropriate volume of binding buffer to achieve ~ 1 × $10^7$ beads/ml (*see* **Note 13**).
2. After 555|610 nm and 342|400 nm (excitation λ|emission λ) background measurements are recorded for BMMØ monolayers alone, 85 µl of the diluted particle suspension is laid onto the monolayers in the binding dish and incubated at room temperature for 3 min (as per **Subheading 3.2**).
3. Unbound particles are washed from the monolayers by dipping in binding buffer and returned to the spectrofluorometer.
4. Both calibration and substrate fluorescent intensities are measured. The fluorescence of the calibration fluor octadecyl rhodamine B chloride (emission at 610 nm with excitation at 555 nm) should remain constant throughout the assay (*see* **Note 14**). The substrate fluorescence (emission at 400 nm with excitation at 342 nm) will increase with progressive esterolysis of the triglyceride analog as it liberates the fluorescent pyrenedecanoic acid and the quenching trinitrophenylamino acyl residues from the glycerol backbone *(7)*. Typically, an integration time of 1 s per data point is optimal (*see* **Note 15**). Data are collected for at least 60 min or until exhaustion of the substrate. At the conclusion of the assay, monolayers are examined via microscopy with trypan blue (as per **Subheading 3.2**).
5. Data are exported into a standard spreadsheet application such as Microsoft Excel®.
6. The appropriate background values are deducted (if not already deducted by the acquisition software) and the ratio $S_{RT}/C$ (where $S_{RT}$ is the substrate fluorescence in real time and $C$ is the calibration fluorescence) is plotted against time (**Fig. 3**).

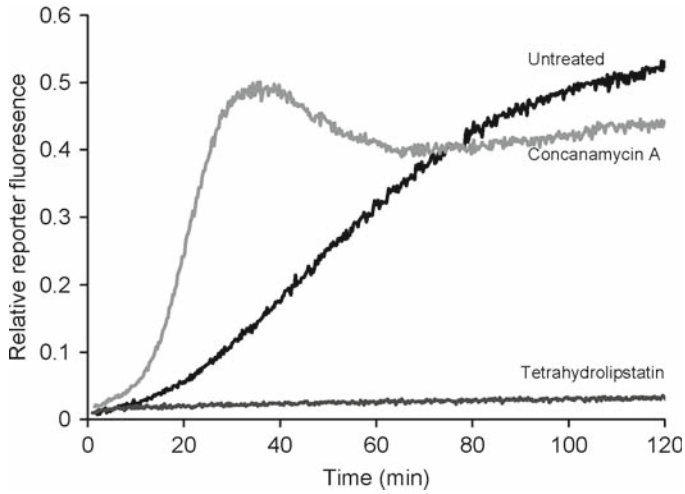


Fig. 3. Phagosomal triglyceride lipase activity in BMMØs. Lipase activity can be diminished with the specific lipase inhibitor tetrahydrolipstatin (50 μg/ml) and is increased in the early phagosome by the V-ATPase inhibitor concanamycin A (100 nm).

7. Gradients of portions of the profiles will give relative values of the triglyceride lipase activity of the phagosomal population over that period (*see* **Note 17**).

### *3.5. Kinetic Analysis of Intraphagosomal β-Galactosidase Activity*

#### *3.5.1. Preparation of β-Galactosidase Lipid Monolayer Particles*

1. The lipid monolayer particle is used as the carrier for the β-galactosidase substrate and is essentially prepared as described for the lipase lipid monolayer particles in **Subheading 3.4.1**. However, the lipid composition of the particles is modified to allow for a greater proportion of the β-galactosidase substrate. To 200 μl of the 10 mg/ml suspension of the Nucleosil 120-3 $C_{18}$ reverse-phase HPLC matrix in chloroform the following lipids are added: 10 μl of the substrate 5-dodecanoylaminofluorescein-di-β-D-galactopyranoside ($C_{12}$FDG) (10 mg/ml); 5 μl of the calibration fluor octadecyl rhodamine B chloride (1 mg/ml); 20 μl of the phospholipid 1-palmitoyl-2oleoyl-sn-glycero-3-phospho-rac-(1-glycerol); 5 μl of the biotinylated phospholipid 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-*N*-(Cap Biotinyl) (1 mg/ml); 5 μl of cholesterol (10 mg/ml) (*see* **Note 19**).
2. The mixture is dried and resuspended in PBS, and particles washed, and IgG opsonized as described in **Subheading 3.4.1**.

#### *3.5.2. Fluorometric Measurement of Phagosomal β-Galactosidase Activity*

1. The lipid particles are diluted in an appropriate volume of binding buffer to achieve ~1 × $10^7$ beads/ml (*see* **Note 13**).
2. After 555|610 nm and 488|520 nm (excitation λ|emission λ) background measurements are recorded for BMMØ monolayers alone, 85 μl of the diluted particle suspension is laid

onto the monolayers in the binding dish and incubated at room temperature for 3 min (as per **Subheading 3.2**).

3. Unbound particles are washed from the monolayers by dipping in binding buffer and returned to the spectrofluorometer.
4. Both calibration and substrate fluorescent intensities are measured. The fluorescence of the calibration of fluor octadecyl rhodamine B chloride (emission at 610 nm with excitation at 555 nm) should remain constant throughout the assay (*see* **Note 14**). The fluorescent intensity of the fluorescein-based substrate (emission at 515 nm with excitation at 490 nm) will increase as it is dequenched with sequential cleavage of the two galactopyranosides (*see* **Note 22**). Typically, an integration time of 1 s per data point is optimal (*see* **Note 15**). Data are collected for at least 90 min or until exhaustion of the substrate. At the conclusion of the assay, monolayers are examined via microscopy with trypan blue (as per **Subheading 3.2**).
5. Data are exported into a standard spreadsheet application such as Microsoft Excel®.
6. The appropriate background values are deducted (if not already deducted by the acquisition software) and the ratio $S_{RT}/C$ (where $S_{RT}$ is the substrate fluorescence in real time and $C$ is the calibration fluorescence) is plotted against time (**Fig. 4**).
7. Gradients of portions of the profiles will give relative values of the β-galactosidase activity of the phagosomal population over that period (*see* **Note 17**).

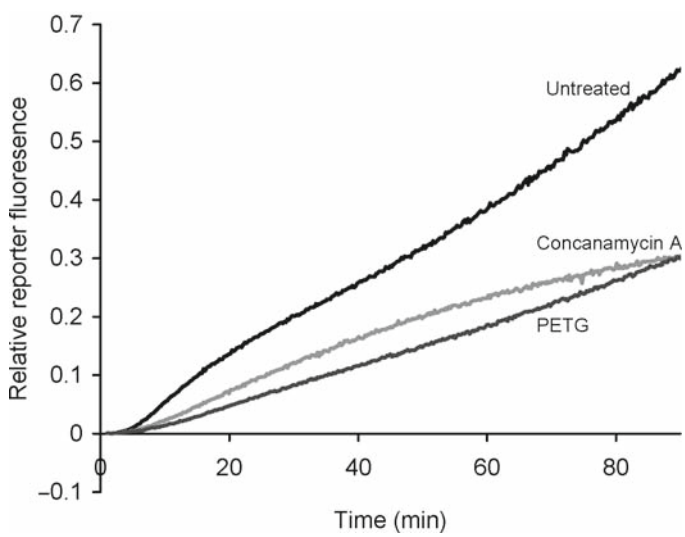


Fig. 4. Phagosomal β-galactosidase activity in BMMØs. β-Galactosidase activity can be diminished with the competitive inhibitor 2-phenylethyl-β-D-thiogalactoside (PETG) (200 μg/ml) and the V-ATPase inhibitor concanamycin A (100 nm).

## 4. Notes

1. Primary BMMØs are generally preferred for their enhanced phagocytic proficiency and adhesion.
2. 0.13 × 12.5 × 25 mm cover glass is not commercially available. Cover glass can be custom ordered from ProSciTech (Thuringowa, Qld, Australia). Alternatively, 25 × 25 mm cover glass is available from Fisher Scientific (Pittsburgh, PA, USA) and can be cut into half by diamond pencil in house.
3. FBS is substituted for gelatin for spectrofluorometric assays as it has low autofluorescence with excitation wavelengths above 450 nm. If assays are expected to take greater than 6 h, FBS is preferred for sustained macrophage viability.
4. The authors prefer the 3.0-μm –COOH-modified silica particles due to their ease of handling and low autofluorescence; however, polystyrene particles and particles of smaller size can be used successfully. Generally, macrophage-like cell lines do not efficiently phagocytose particles with diameters over 2.0 μm. Therefore particles >2.0 μm are not advised for those cell types.
5. Arrange cover slips so as not to overlap, taking care not to overcrowd them in the Petri dish as cover slips can move after monolayers have been established and damage to BMMØs can occur. Alternatively, cover slips can be separated from each other using partitioned Petri dishes or 6-well plates.
6. Some general considerations are: the focusing of illumination on sample, the addition of long-pass and short-pass filters, and the adjustment of excitation and emission slit width to maximize signal-to-noise ratio and to minimize photobleaching.
7. Cuvette buffer should be of a similar temperature to the cuvettes at addition. This prevents bubble formation that can create unwanted scatter of light.
8. Cover slips should only be grasped by forceps at the uppermost edge to avoid damaging the area of the monolayer that is illuminated in the spectrofluorometer.
9. Binding time may need to be increased if smaller particles or polystyrene beads are used.
10. Particles can be directed through the mannose receptor by substitution of the human IgG with the equivalent amount of α-D-mannosylated-PITC-albumin (Sigma St Louis, MO, USA).
11. Ensure that the fluor/DMSO aliquot is fully thawed and at room temperature before opening to avoid wetting the DMSO.

12. Labeled particles should be microscopically examined initially to ensure adequate fluorescence and absence of clumping.
13. A suitable dilution of beads should be determined for each batch. This should be done by applying the bead suspension to a BMMØ monolayer on cover glass for the desired binding time, washing off unbound beads, and assessing degree of bead adhesion under magnification. For most applications, a target of 1–2 beads/macrophage is desired. Due to their low density, significantly higher concentrations of polystyrene beads are needed to achieve optimal binding to monolayers.
14. It is preferred, but not usually necessary, to record the calibration fluorescence throughout the entire assay. Recording a calibration fluorescence at the beginning and/or conclusion of the assay will increase time resolution of reporter fluor profiles and may allow the use of filter sets that improve signal-to-noise ratios.
15. The integration time is the period over which each fluorescent measurement is taken. The emission value for each measurement is calculated by signal averaging over the integration period performed by the acquisition software. A lower signal noise can be achieved by increasing the integration time. However, as fewer measurements would be taken per minute, the time resolution of the resulting readout will be diminished.
16. The free rhodamine 110 is moderately membrane permeable and thus will leach from the cell over time. This may result in a decrease in fluorescent intensity.
17. The data can be expressed as hydrolase activity which is proportional to the rate of decay of the substrate. This is can be calculated over any period of time by determining the linear gradient of the fluorescent ratio plotted against time using line fitting software in Microsoft Excel® or other mathematical software.
18. Warm the stock aliquots to room temperature before opening to ensure that the sample remains anhydrous.
19. The goal of this particle preparation is to create a monolayer of amphipathic lipids that surrounds the particle core. This presents the lipids to the macrophage in an orientation akin to a phospholipid bilayer. As the $C_{18}$-coupled particles are extremely hydrophobic, a minimum of amphipathic lipid must be included to completely surround the particle allowing it to be miscible in an aqueous environment without clumping. As a general guideline, for complete suspension of the particles, the combined weight of the coating lipids should be greater than 20% of the mass of the particles. The specific lipid composition can be modified to best suit

the needs of the investigator; however; the following points should be considered. The lipid composition itself will affect the nature of the particle, especially when charged or long chain lipid complexes are included. It is usually not feasible to coat the particle entirely with the desired substrate and calibration fluor. Thus, an inert "base" lipid is included to provide the majority of the monolayer structure. We have found that a monounsaturated phosphatidylglycerol such as 1-trinitrophenyl-amino-dodecanoyl-2-pyrenedecanoyl-3-*O*-hexadecyl-sn-glycerol performs well in this role. We have also found that the inclusion of cholesterol to the lipid mixture increases the monolayer's stability on the particles and reduces clumping. Since the monolayer creates a "natural" surface that does not inherently engage scavenger receptors, the incorporation of a phagocytic ligand is usually required to mediate its phagocytosis by the macrophage. We have included the biotinylated phospholipid 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-*N*-(Cap Biotinyl) to provide an incorporated antigen for subsequent IgG opsonization. Other phagocytic ligands such as phosphatidylserine or mannosylated lipids can be incorporated to target specific phagocytic receptors.

20. Gently swirl the test tube while under a gentle stream of nitrogen to dry the material in a film surrounding the bottom of the test tube. This will help in the proper resuspension of the particles in the sonication step.
21. It may be necessary to pipette the suspension up and down to help break the surface tension.
22. The fluorescent properties of fluorescein-based compounds are commonly sensitive to changes in pH and hence unsuitable for real-time measurements within acidifying compartments. However, once incorporated in the lipid monolayer of the experimental particle, the fluorescent intensity of the hydrolyzed $C_{12}$FDG is less sensitive to changes in pH (data not shown) making it more suitable for phagosomal assays.

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# Chapter 12

# Derivation and Characterization of Murine Alternatively Activated (M2) Macrophages

**Victor W.H. Ho and Laura M. Sly**

## Summary

Diversity in macrophage responsiveness to inflammatory stimuli has resulted in the description of a new paradigm wherein macrophages are referred to as polarized into one of two distinct phenotypes, classically activated (M1) macrophages and alternatively activated (M2) macrophages. Classically activated, M1 or "killer" macrophages are thought to play a critical role in destroying foreign organisms and tumor cells, while alternatively activated M2 or "healer" macrophages are thought to be important in debris scavenging, wound healing, and angiogenesis. M2 macrophages may also play key roles in chronic infections, tumorigenesis, and tumor metastasis. It is therefore important to establish models of M1 and M2 polarized macrophages to study their characteristics and amenability to manipulation. M1 macrophages are typically derived from myeloid progenitors with murine macrophage-colony-stimulating factor (M-CSF, also known as CSF-1), while M2 macrophages are thought to be derived from mature M1 macrophages by treatment with interleukin-4 (IL-4) or IL-13. M2 macrophages can also be isolated from SH2-containing inositol 5 -phosphatase (SHIP)–/– mice by harvesting macrophages from peritoneal lavage fluids or they can be derived from SHIP–/– bone marrow aspirate cells with addition of 5% human serum. Upon stimulation with lipopolysaccharide (LPS), M1 macrophages produce high levels of proinflammatory cytokines, low levels of anti-inflammatory cytokines, and high levels of inducible nitric oxide synthase (iNOS), which leads to nitric oxide (NO) production. M2 macrophages, on the other hand, express high levels of M2 markers Ym1 and arginase I (ArgI) and, upon stimulation with LPS, produce relatively lower levels of proinflammatory cytokines and NO and higher levels of anti-inflammatory cytokines. In this chapter, we describe methods used in our laboratory to generate and characterize alternatively activated (M2) macrophages.

**Key words:** Macrophage phenotype, Macrophage polarization, Classically activated macrophage, Alternatively activated macrophage, Arginase, Ym1, SHIP, IL-4.

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## 1. Introduction

Macrophages have been described and characterized that are capable of displaying distinct phenotypes because of exposure to different cytokines and, more recently, because of different genetic backgrounds *(1–3)*. Classically activated (M1) macrophages are critical in fighting infectious agents by both initiating the innate immune response and by directing a subsequent acquired immune response *(4)*. These cells produce large amounts of proinflammatory cytokines such as TNFα, IL-12, and IL-23 *(5, 6)*, low amounts of the anti-inflammatory cytokine IL-10 *(5, 6)*, and large amounts of NO upon activation *(3, 5)*. Alternatively activated (M2) macrophages are important in the resolution of the inflammatory response and serve roles in debris scavenging, tissue remodeling, and angiogenesis *(1, 7, 8)*. In response to inflammatory stimuli, M2 macrophages produce relatively lower levels of proinflammatory cytokines, higher levels of IL-10, and produce little or no NO *(3, 7)*.

Alternatively activated macrophages can be derived from mature M1 macrophages by the addition of IL-4 or IL-13, which acts through STAT6 *(8–10)*. We have recently described the first genetic model of alternatively activated macrophages wherein in vivo differentiated macrophages from the SHIP knockout mouse display an M2 phenotype *(3)*. Furthermore, bone marrow progenitors from the SHIP knockout mouse can be skewed to an M2 phenotype by the addition of human or mouse serum, TGFβ, or IL-10 early during differentiation *(3)*. Additional markers of alternatively activated macrophages have been described and include FIZZ1 and the mammalian chitinase Ym1 *(3, 10–13)*. Arginase I expression and activity are also upregulated in M2 macrophages, and this is part of the critical metabolic switch that occurs in murine macrophages during conversion from an M1 to an M2 phenotype *(3)*. ArgI competes with inducible nitric oxide synthase (iNOS) for their common substrate, L-arginine, thereby dramatically reducing or inhibiting NO production by alternatively activated macrophages despite induction of iNOS *(3)*.

Characteristics of alternative macrophage activation have been described in tumors *(1, 14–16)* and during infection *(11, 15)*. Alternatively activated macrophages have also been found in patients with severe chronic inflammatory disorders, where they have been hypothesized to protect the host from damage by dampening down the innate immune response *(7)* and hence are thought to be beneficial during systemic inflammatory response syndrome (SIRS) and sepsis *(17)*. Further study of these models of alternatively activated macrophages and their amenability to manipulation is critical to understanding their role in human diseases and to harnessing them for therapeutic purposes.

## 2. Materials

### 2.1. Cell Culture and Stimulations

1. Iscove's Modified Dulbecco's Medium (IMDM).
2. Fetal bovine serum.
3. Recombinant murine macrophage-colony-stimulating factor (M-CSF also known as CSF-1).
4. Monothioglycerol (MTG).
5. Cell dissociation buffer.
6. Lipopolysaccharide from *Escherichia coli* serotype 0127:B8 (LPS).
7. Recombinant murine interleukin-4 (IL-4).

### 2.2. SDS–Polyacrylamide Gel Electrophoresis, Adapted from (18)

1. Laemmli's solubilization mix: 75 mM Tris–HCl pH 6.8, 7.5% (w/v) glycerol, 200 mM β-mercaptoethanol, 1.5% (w/v) bromophenol blue.
2. Separating gel mix (4×): 1.5 M Tris–HCl pH 8.8, 0.4% SDS.
3. Stacking gel mix (4×): 0.3 M Tris–HCl pH 6.8, 0.4% SDS.
4. 40% Acrylamide/bisacrylamide solution (37.5:1 with 2.6% C).
5. *N*,*N*,*N*′,*N*′-Tetramethyl-ethylenediamine (TEMED).
6. 10% (w/v) Ammonium persulfate (APS).
7. Pageruler prestained protein ladder.
8. Running buffer (10×): 0.25 M Trizma base, 1.9 M glycine, 1% (w/v) SDS.

### 2.3. Western Blotting Analyses, Adapted from (19)

1. Transfer buffer (10×): 0.5 M Trizma base, 3.84 M glycine, 1% (w/v) SDS.
2. Immobilon-P membrane 0.45-μm pore polyvinyldifluoride (PVDF).
3. 3-MM chromatography paper.
4. Tris-buffered saline with Tween-20 (TBST): 137 mM NaCl, 2.7 mM KCl, 25 mM Tris–Cl pH 7.4, 0.1% Tween-20.
5. Blocking buffer: 5% (w/v) bovine serum albumin (BSA) fraction V, 0.02% $NaN_3$ in TBST.
6. Primary antibodies: anti-SHIP, P1C1; anti-GAPDH; anti-ArgI; anti-Ym1. Anti-Ym1 is a rabbit polyclonal antibody and all others are mouse monoclonal antibodies. All antibodies are used at a dilution of 1 in 1,000 in primary antibody buffer.
7. Primary antibody buffer: 2% (w/v) BSA fraction V, 0.008% $NaN_3$ in TBST.

8. Secondary antibody: antimouse conjugated to horse radish peroxidase (HRP) or antirabbit-HRP diluted 1 in 10,000 in TBST.
9. Western Lightning chemiluminescence reagent plus, enhanced luminal reagent and oxidizing reagent.
10. X-OMAT Blue film.

***2.4. ELISAs for Murine TNFα, IL-12p40, IL-10***

1. ELISA kits for TNFα, IL-6, IL-12p40, and IL-10.
2. Coating buffer: 0.2 M sodium phosphate pH 6.5 for IL-12p40 and IL-10.
3. Coating buffer: 0.1 M sodium carbonate pH 9.5 for TNFα and IL-6.
4. Assay diluent: 10% heat-inactivated (55°C for 30 min) FBS in Dulbecco's PBS pH 7.4.
5. ELISA substrate: Substrate Reagent A and Substrate Reagent B.
6. Stop solution: 2N $H_2SO_4$.

***2.5. Arginase Assay, Adapted from (20)***

1. Cell lysis buffer: 0.1% Triton X-100, 25 mM Tris–Cl pH 8.0 + protease inhibitors aprotinin (40 μg/mL), leupeptin (8 μg/mL), PMSF (100 μM).
2. Acid solution: (7:3:1) $H_2O$: $H_2PO_4$ 44.6N, 85%: $H_2SO_4$ 36N.
3. Colorimetric substrate: 9% (w/v) α-isonitrosopropiophenone (ISPF) in absolute ethanol. Store in the dark.
4. 0.5 M l-Arginine in water pH 9.7.
5. 10 mM $MnCl_2$ in $H_2O$.
6. Standard stock solution: 50 mM urea in $H_2O$.

***2.6. Griess Assay, Adapted from (21)***

1. Solution A: 1% (w/v) sulfanilamide in 2.5% $H_3PO_4$.
2. Solution B: 0.1% (w/v) naphthylethylene diamine dihydrochloride in 2.5% $H_3PO_4$.
3. 2 mM $NaNO_2$ in IMDM, 10% FBS.

## 3. Methods

The steadfast hallmark of alternative macrophage polarization in humans and in mice is low expression of proinflammatory cytokines (IL-12) and high expression of anti-inflammatory cytokines (IL-10) in response to toll-like receptor (TLR) activation *(5, 6)*. In addition, L-arginine metabolism via iNOS to NO

(M1) versus ArgI to ornithine and polyamines (M2) provides a key switch in murine macrophage polarization *(3)*. The M1/M2 macrophage polarization paradigm is likely an oversimplification of activated macrophage states that likely reflects a continuum of activation potential from M1 to M2. Alternatively, there may be many subtypes of alternatively activated macrophages. Western blot analyses allow us to examine directly phenotypic markers of M2 macrophages, while the arginase activity assay and Griess assay are quantitative assays of M1/M2 macrophage metabolic function and therefore phenotype severity. Techniques described herein will be valuable in establishing similarities or differences among unique populations of alternatively activated macrophages derived in vitro or in vivo.

### 3.1. Cell Culture and Stimulations

1. Harvest bone marrow aspirates from femura of 8- to 12-week old SHIP+/+ (wild type) and SHIP−/− mice using a 5-mL syringe and a 26-gauge needle to flush the marrow out with IMDM, 10% FCS (*see* **Note 1**).
2. Suspend bone marrow aspirates in 40 mL IMDM, 10% FCS and allow cells to adhere overnight at 37°C, 5% $CO_2$ in a 75-$cm^2$ tissue culture flask.
3. Remove culture supernatant, spin down nonadherent cells, and resuspend at $0.5 \times 10^6$ cells/mL (i.e., about 50 mL) in complete bone marrow macrophage medium (IMDM, 10% FCS, 150 μM MTG, 5 ng/mL M-CSF) in a 175-$cm^2$ filter top tissue culture flask. Replace medium at day 4, spinning down nonadherent cells and returning them to the flask and at day 7, discarding nonadherent cells (*see* **Notes 2** and **3**).
4. Macrophage cultures can be supplemented with 5% human serum on day 0 or 10 ng/mL IL-4 can be added with fresh complete medium at day 10 for three additional days (*see* **Note 4**).
5. Peritoneal macrophages from SHIP+/+ and SHIP−/− mice are isolated by flushing the peritoneal cavity 3 × 5 mL with IMDM, 10% FCS, 150 μM MTG (no M-CSF). An aliquot is counted using a hemocytometer. Macrophages are large and gold in appearance. They can be plated at an appropriate density ($0.5 \times 10^6$ cells/mL) and become adherent overnight. Adherent cells are washed the next day two times with PBS to remove nonadherent or only loosely adherent cells and can be used in stimulations or can be harvested for SDS–PAGE analysis.
6. At day 10, adherent cells derived from bone marrow aspirate cultures are lifted and replated for stimulations and analyses. Cells are lifted off the tissue culture flask using 5 mL per 75 $cm^2$ tissue culture flask of Cell Dissociation Buffer. Place buffer on cells for 3 min and then bang the side of the

flask with the heel of your palm firmly several times. Ensure that cells have lifted off of the flask under the microscope, remove resuspended cells, and spin down at 1,200 × g for 5 min. Resuspend cells in a small volume and count viable cells using a hemocytometer. Replate at a concentration of $0.5 \times 10^6$ cells/mL in IMDM, 10% FCS, 150 µM MTG (no M-CSF).

7. Stimulate peritoneal macrophages the day after plating or the day after replating for bone marrow-derived macrophages by changing the medium to 0.5 volume IMDM, 10% FCS, 150 µM MTG (no M-CSF), and 100 ng/mL LPS.

### 3.2. SDS–PAGE

1. To harvest cells for SDS–PAGE and Western analyses, remove cell supernatants, wash cells twice with Hank's Balanced Salt Solution, and add 80 µL of LDM to $1 \times 10^6$ cells in a well. Pipet the cells up and down and transfer to a new Eppendorf tube. The lysed cell suspensions are very viscous. Shear DNA in samples by passing five times through a 26-gauge needle. Boil 1 min. Store samples in the freezer at –20°C until ready to load on SDS–PAGE.
2. SDS–PAGE instructions provided herein are for preparation of a 40-mL separating gel (1.5 mm thick × 14.5 cm wide × 16 cm long) to be used with the Bio-Rad Protean II xi Cell (Bio-Rad Laboratories Inc, Hercules, CA). One long and one short glass plate are cleaned thoroughly with Sparkleen detergent (Fischer Scientific Co., Pittsburgh, PA), rinsed with water and then rinsed with 95% ethanol, and allowed to air dry immediately before use. Spacers and combs are cleaned with 95% ethanol and the pieces are assembled as per manufacturers' instructions (*see* **Note 5**).
3. A 10% separating gel solution is prepared by combining 20 mL distilled water, 10 mL 4× separating buffer, and 10 mL acrylamide solution in a 50-mL Falcon tube and mixing by inversion several times. This mixture is degassed under house vacuum for 10 min. APS (80 µL) and 20 µL TEMED are added and the solution is mixed gently but thoroughly by rocking the Falcon tube back and forth so as to not introduce any air bubbles. Pour the entire 40 mL solution between the glass plates. Using a Pasteur pipet, gently add 5 mL of isopropanol to overlay the top of the gel. Be careful not to cause mixing with the denser gel solution. Allow gel to polymerize about 30 min (*see* **Notes 6** and **7**).
4. Pour off the isopropanol overlay. Rinse the gel top two times with distilled water and drain water well (*see* **Note 8**).
5. Prepare the stacking gel mix by combining 9.75 mL water, 1.5 mL acrylamide solution and 3.75 mL 4× stacking gel

buffer, 75 μL APS, and 15 μL TEMED in a 50-mL Falcon tube. Mix by inversion and pipet onto the top of the separating gel. Place the well comb into the top of the gel. Avoid trapping air bubbles below or on the side of the wells. Allow to polymerize for 30 min before removing comb.

6. To load samples into wells, use gel loading tips and apply the sample to the bottom of wells. Prepare 1,400 mL of running buffer by diluting 140 mL of 10× buffer stock solution to 1,400 mL with distilled water. Gently add running buffer to top up the wells with a Pasteur pipet and then fill the upper buffer chamber with running buffer. Pour the remaining running buffer into the bottom buffer reservoir of the gel apparatus.
7. Fill the inner chamber of the gel apparatus with cold water and run gel overnight at 65 V.

### 3.3. Western Blotting Analyses

1. Instructions provided are for use with the Bio-Rad Trans-Blot Cell (Bio-Rad Laboratories Inc, Hercules, CA). Cut the PVDF membrane and four pieces of 3-MM filter paper to 14.5 cm × 16 cm.
2. In a plastic container, wet PVDF membrane in methanol, pour off the methanol, add 50 mL distilled water, and shake at 23°C on an orbital shaker while preparing for transfer. The membrane is hydrated when water no longer beads or streaks off the membrane surface.
3. Prepare transfer buffer using 400 mL of 10× transfer buffer, 3,200 mL distilled water, and 400 mL methanol (*see* **Note 9**).
4. Disassemble gel apparatus, cut the stacking gel off and discard, and soak the separating gel in transfer buffer along with the PVDF membrane.
5. Assemble the gel sandwich on the clear side of the transfer tank holder wetting each piece generously in transfer buffer as you assemble. The gel sandwich is assembled in the following order: one scotch brite pad, two pieces of 3MM Whatman filter paper, PVDF membrane, gel (from left to right, noting the orientation), two pieces of filter paper, scotch brite pad. Firmly roll out the gel assembly with a disposable 2-mL pipet to remove air bubbles trapped between the transfer layers.
6. Secure the gel sandwich in its holding apparatus and move it into the transfer tank. Fill the transfer tank with transfer buffer. Run cold water through the transfer tank constantly during transfer. Transfer gels for 4 h at 0.6 A. Check occasionally that buffer tank is not getting too warm. If the transfer apparatus feels warm to the touch, place the entire transfer assembly into a secondary plastic container and pack ice around it.

7. Remove and disassemble the gel sandwich. Remove the membranes and mark the molecular weight markers on the membrane with an indelible pen. Place the membrane in 50 mL of blocking solution in a plastic container. Incubate for 1 h at 23°C on an orbital shaker.
8. Incubate the blocked membrane in primary antibody overnight at 4°C on an orbital shaker.
9. Wash the membrane 3 × 10 min in TBST at 23°C with shaking on an orbital shaker.
10. Incubate with secondary antibody for 45 min at 23°C with shaking on an orbital shaker.
11. Wash the membrane 3 × 10 min in TBST at 23°C with shaking.
12. Mix 7.5 mL of ECL reagents A and B together and cover membrane. Gently rock back and forth by hand for 1 min.
13. Place the membrane between two layers of plastic wrap and expose in a dark room to film. Exposure times for these antibodies are very short, typically in the range of 1–5 s for SHIP, Ym1, and GAPDH and 15 s for ArgI. Develop film in a film processor. An example of the results produced is shown in **Fig.** 1.

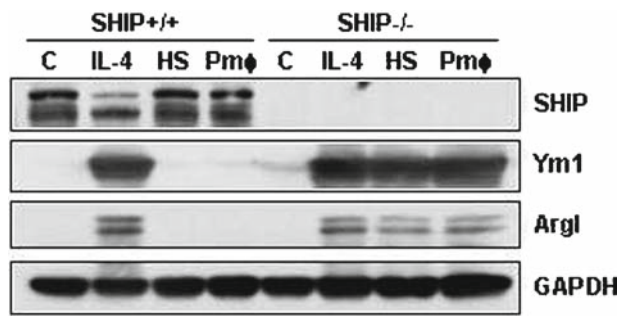


Fig. 1. M2 macrophage marker expression in SHIP+/+ (wild type) and SHIP–/– macrophages. Macrophages ($1 \times 10^6$) were derived in complete medium with 5 ng/mL recombinant murine M-CSF for 10 days (C), were treated with 10 ng/mL IL-4 for an additional 3 days (IL-4), or were derived in complete medium plus 5% human serum (HS) from day "0." Also shown, in vivo differentiated macrophages were harvested from the peritoneal cavity by lavage (Pmϕ). Cells were harvested for SDS–PAGE and Western blotting with antibodies to SHIP (145 and 135 kDa), M2 macrophage markers Ym1 and Arg1 (38 and 35 kDa) and GAPDH, as a loading control. IL-4 treatment post differentiation skewed both SHIP+/+ and SHIP–/– macrophages to an M2 phenotype expressing phenotypic markers Ym1 and Arg1. Human serum provided during differentiation skewed only SHIP–/– cells to an M2 phenotype. Peritoneal macrophages develop an M2 phenotype only in the SHIP–/– mice.

### 3.4. ELISAs

1. To harvest cell supernatants 24 h after stimulation, pipet the supernatant into a 1.5-mL Eppendorf tube. Spin the contents at 1,200 × g to remove any cell debris in the samples. Transfer clarified supernatants to new tubes storing each sample in 0.3- to 0.5-mL aliquots to be thawed individually for assay as needed. Store samples at –80°C until ready to assay.
2. ELISA kits for TNFα, IL-6, IL-12p40, and IL-10 are available from BD Biosciences (Mississauga, ON, Canada), and assays are performed as per manufacturer's instructions (*see* **Note 10**). Cytokine production ± SD from three independent experiments assayed in duplicate is shown in **Fig. 2**.

### 3.5. Arginase Assay

1. Lyse macrophages ($10^6$) in 200–400 µL cell lysis buffer.
2. Quantitate protein in cell lysates using Bio-Rad protein quantification assay (Bio-Rad, Hercules, CA).

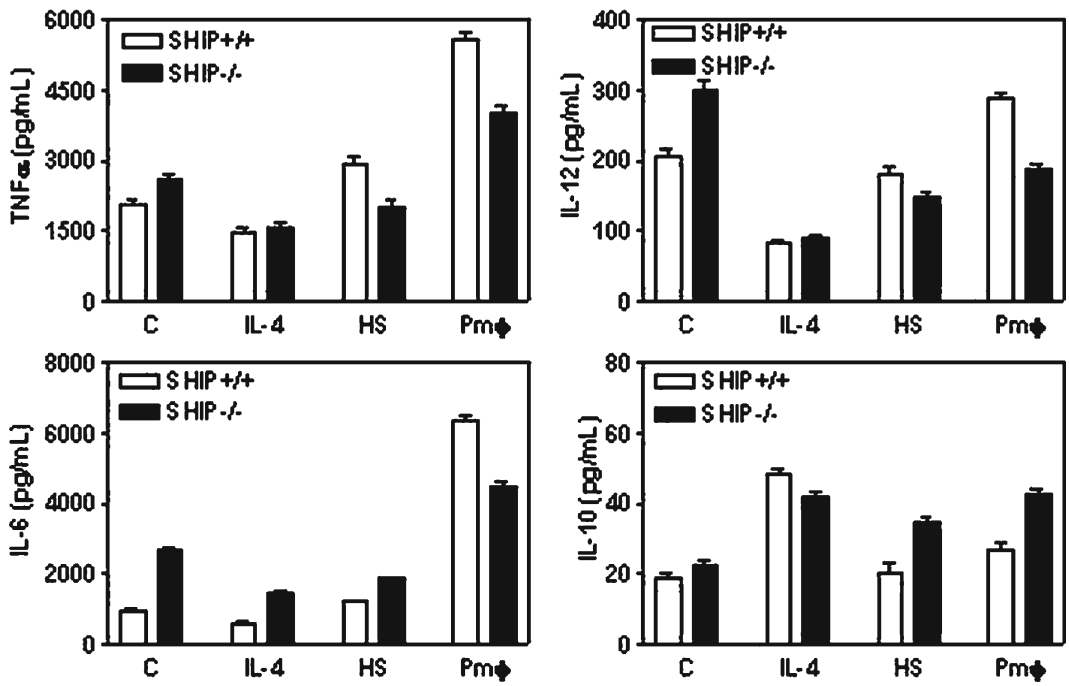


Fig. 2. Proinflammatory (TNFα, IL-12, and IL-6) and anti-inflammatory (IL-10) cytokine production by SHIP+/+ (wild type) and SHIP–/– macrophages in response to LPS stimulation (100 ng/mL for 24 h). Macrophages ($1 \times 10^6$) were derived in complete medium with 5 ng/mL recombinant murine M-CSF for 10 days (C), were treated with 10 ng/mL IL-4 for an additional 3 days (IL-4), or were derived in complete medium plus 5% human serum (HS) from day "0." As well, in vivo differentiated macrophages were harvested from the peritoneal cavity by lavage (Pmϕ). Compared with cells differentiated under control conditions, cells treated with IL-4 showed reduced production of proinflammatory and increased production of anti-inflammatory cytokines in both SHIP+/+ (wild type) and SHIP–/– macrophages. SHIP–/– macrophages differentiated in the presence of human serum, but not their wild-type counterparts, also reduced production of proinflammatory and increased production of anti-inflammatory cytokines in response to LPS. In vivo differentiated SHIP–/– macrophages (Pmϕ) produced less TNFα, IL-6, and IL-12 and more IL-10 than SHIP+/+ (wild type) macrophages (C).

3. Use 5–15 μg protein for each arginase assay, pipetting the desired amount of protein into an Eppendorf tube and topping up the volume to 100 μL with cell lysis buffer.
4. Add 10 μL of 10 mM $MnCl_2$ and heat the samples at 55°C in a water bath for 10 min.
5. Add 100 μL 0.5 M L-arginine into each sample (total volume of 210 μL in each sample), and incubate at 37°C for 1 h (*see* **Note 11**).
6. Add 800 μL acid solution to each sample. Add 40 μL of ISPF solution into each reaction and pipet up and down 3 times (*see* **Note 12**).
7. Prepare serial twofold dilutions of urea stock solution in distilled water to make a standard curve and use distilled water as a blank. Add 100 μL of diluted stocks or distilled water to an Eppendorf tube. Add 400 μL acid solution and then add 25 μL ISPF to each tube.

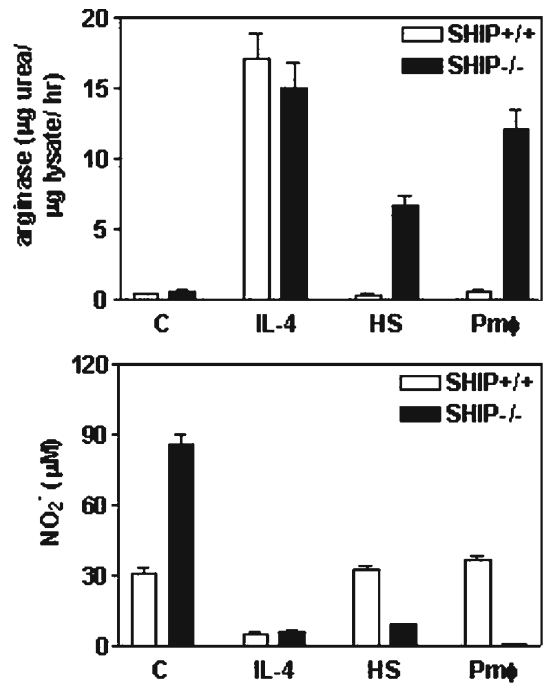


Fig. 3. Arginase activity in cells and $NO_2^-$ in cell culture supernatants of SHIP+/+ (wild type) and SHIP–/– macrophages in response to LPS stimulation (100 ng/mL for 24 h). Macrophages ($1 \times 10^6$) were derived in complete medium with 5 ng/mL recombinant murine M-CSF for 10 days (C), were treated with 10 ng/mL IL-4 for an additional 3 days (IL-4), or were derived in complete medium plus 5% human serum (HS) from day "0." In addition, in vivo differentiated macrophages were harvested from the peritoneal cavity by lavage (Pmϕ). IL-4 treatment resulted in increased arginase activity and decreased nitrite production in both SHIP+/+ (wild type) and SHIP–/– macrophages. Derivation in the presence of human serum and in vivo differentiation resulted in increased arginase activity and decreased nitrite production in SHIP–/– macrophages only.

8. Heat all samples and standards at 100°C for 30 min. Snap-top Eppendorf tube caps must be secured during this step because tubes will pop open. Let samples cool to 23°C in the dark, approximately 10 min (*see* **Note 12**).
9. Read absorbance at 550 nm within 30 min. Arginase-activity-detected ± SD for three independent assays performed in duplicate is shown in **Fig. 3**, upper graph.

### 3.6. Griess Assay

1. Dilute 2 μM $NaNO_2$ stock to 125 μM in cell culture medium. Make serial twofold dilutions from the 125 μM dilution to prepare a standard curve using cell culture medium as a blank.
2. Aliquot 50 μL of standard from each dilution, blank or 50 μL of culture supernatant into a 96-well flat bottom polystyrene nontissue culture treated plate.
3. Add 50 μL of solution A into each sample or standard.
4. Add 50 μL of solution B into each sample or standard.
5. Incubate the plates for 10 min in the dark. Read the absorbance at 550 nm within 30 min (*see* **Note 13**). Nitrite-detected ± SD for three independent assays performed in duplicate is shown in **Fig. 3**, lower graph.

## 4. Notes

1. SHIP−/− mice that are 5–8 weeks old show a considerably less profound M2 skewing of their resident peritoneal macrophages, so SHIP−/− mice used to isolate resident M2 macrophages should be 8 weeks of age or older.
2. We have assayed several different sources of L-cell-conditioned media for use as a source of M-CSF and currently use recombinant M-CSF because we have not found a reliable source for M-CSF production. If using L-cell-conditioned media for derivation of bone marrow macrophages, assay M-CSF to ensure that you are providing sufficient growth factor ( 5 ng/mL) to your cells for differentiation.
3. Macrophages derived as described here are consistently more than 95% positive for macrophage markers, F4/80 and Mac-1.
4. M2 macrophages can also be derived by addition of recombinant murine IL-13 (in place of IL-4) to mature cultures.
5. Check your SDS–PA gel set up to ensure that it will not leak before adding your gel solution by adding distilled water into the assembly while you prepare and degas your gel solution. Ensure that you drain the water thoroughly before adding your gel mix.

6. Unpolymerized acrylamide is a neurotoxin, so care should be taken when handling solutions or gels to avoid exposure.
7. Ammonium persulfate (APS) can be stored in aliquots at –20°C for up to 3 months and once thawed, should be stored at 4°C, used for 1 week and then discarded. Alternatively, APS can be prepared for each use.
8. Do not leave the isopropanol on the top of the running gel for more than the 30 min required to polymerize the gel or the gel will dehydrate, separating from the side spacers.
9. To detect SHIP by Western blotting, wet-gel transfers are required and transfer buffer should contain 10% methanol. Semi-dry transfers and wet transfers performed without methanol are not sufficient to transfer high molecular weight proteins.
10. IL-10 standards can be diluted serially twofold from 250 pg/mL to 3.9 pg/mL. Concentrations above 250 pg/mL are on the plateau of the standard curve and so are of no value. However, because IL-10 levels are typically very low in medium conditioned by stimulated macrophages, the linear range of the standard curve can be extended at the lower range reliably by further diluting the IL-10 standard.
11. The 37°C incubation time for the arginase assay can be lengthened from 1 to 2 h to increase the sensitivity of the assay.
12. ISPF will form a precipitate in the reaction mixture.
13. The Griess assay product is light sensitive, so after performing the assay, samples should be protected from light until the absorbance is read.

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# Chapter 13

# Isolation and Culture of Human Hematopoietic Progenitors for Studies of Dendritic Cell Biology

**Mattias Svensson**

## Summary

Understanding the regulation of distinct dendritic cell (DC) function and differentiation pathways is important in many physiological and pathophysiological processes. This includes infectious and neoplastic diseases, vaccination and immunotherapy, allograft rejection, and the pathogenesis of autoimmune diseases. Isolation and culture of human hematopoietic progenitor cells provide a valuable model for studies on DC biology and may help uncover new means to manipulate DC differentiation and function in therapeutic settings. Here, a detailed protocol for the isolation of $CD34^+$ hematopoietic progenitor cells from human cord blood is described. The isolated cell population consists of approximately 85% $CD34^+$ $CD45^+$ hematopoietic progenitor cells that in response to granulocyte-macrophage colony-stimulating factor (GM-CSF) plus tumor necrosis factor (TNF) expand and differentiate into $CD11c^+$ $HLA\text{-}DR^+$ DC-expressing CD1a.

**Key words:** Dendritic cells, Hematopoietic progenitor cells, Cell separation, Flow cytometry, Growth factors, T cell activation.

## 1. Introduction

DC are a heterogeneous family of cells produced mainly from hematopoietic progenitors in the bone marrow *(1, 2)*. They circulate in the bloodstream and then move into tissues throughout the body. However, several developmental intermediates of DC have been isolated from blood and various tissues, some being more restricted than others in their potential to proliferate and differentiate into distinct subsets of DC with specialized functions *(3–7)*. Research focusing on understanding tissue-specific component interactions with progenitors and DC, that lead to

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programming of distinct DC differentiation pathways and functions *(8)*, can help understanding local immunoregulation and the development of new strategies for immune intervention. In vitro studies on human hematopoietic progenitors and DC under specific culture conditions serve as an accessible model aimed at revealing discrete DC developmental pathways and functions.

The existence of human progenitor cells that can generate DC in vitro was first described by Reid et al. *(9)*, and subsequently it was discovered that DC can develop from human $CD34^+$ hematopoietic progenitors cells *(10)*. In response to granulocyte-macrophage colony-stimulating factor (GM-CSF) plus tumor necrosis factor (TNF), $CD34^+$ progenitors expand and differentiate along at least two unrelated developmental pathways generating $CD11c^+$ $HLA\text{-}DR^+$ DC resembling Langerhans cells (expressing CD1a and Langerin) and a subpopulation of DC found in interstitial tissues (expressing CD1a and coagulation factor XIIIa) *(11)*. Human $CD34^+$ hematopoietic progenitors are found at low frequencies in bone marrow and cord blood. These progenitors can be isolated using commercially available magnetic bead cell separation technologies. In this protocol, cord blood progenitor cells are isolated by positive selection using beads coated with antibodies recognizing the CD34 molecule, and this is followed by detachment of the beads from isolated progenitors. Flow cytometry analysis allows determination of purity and phenotypic characteristics of CD34-enriched cells. This also allows verification that enriched cells are devoid of contaminating DC (CD1a, CD1c, CD11c, and CD303), monocytes (CD14), T cells (CD3), B cells (CD19), and NK cells (CD56). DC differentiation potential is confirmed by the capacity of isolated progenitors to develop into immunostimulatory DC in response to GM-CSF plus TNF.

## 2. Materials

### 2.1. Isolation of Hematopoietic Progenitor Cells

1. Blood collection tubes containing anticoagulant ethylenediamine tetraacetic acid (EDTA).
2. Cord blood (*see* **Note 1**).
3. Lymphoprep to isolate nucleated cells by gradient centrifugation.
4. Dynabeads M-450 CD34 kit for positive selection of $CD34^+$ progenitor cells and detachment of beads.
5. Dynal Magnetic Particle Concentrator (MPC-L or MPC-15).

6. Phosphate-buffered saline (PBS) pH 7.4: (0.2 g $KH_2PO_4$, 0.2 g KCl, 1.44 g $Na_2HPO_4 \cdot 2H_2O$, and 8.0 g NaCl, all from Sigma to 1 L).
7. Isolation buffer: PBS with 2 (w/v) % bovine serum albumin (BSA), 0.6 (w/v) % tri-Na-citrate dehydrate, and 100 IU/ml Penicillin and Streptomycin.
8. Washing buffer: PBS with 0.1 (w/v) % BSA.

### 2.2. Flow Cytometry

1. Flow cytometry buffer: PBS supplemented with 2% fetal bovine serum (FBS), 2.5 mM EDTA, and 0.01% sodium azide.
2. Blocking solution, flow cytometry buffer containing 2 μg/ml of purified immunoglobulins from human serum.
3. Antibodies:
   (a) Alexa fluor 488-conjugated anti-CD11c antibody (clone BU15) (Serotec, Oxford, UK).
   (b) Fluorescein isothiocyanate (FITC)-conjugated anti-CD83 (clone HB15e) (eBioscience, San Diego, CA), anti-CD116 (clone 4H1) (eBioscience), anti-CD1c (clone AD5-8E7) (Miltenyi Biotech, Bergisch Gladbach, Germany), anti-CD14 (clone MEM-18) (ImmunoTools, Friesoythe, Germany), anti-CD3 (clone MEM-57) (ImmunoTools), anti-CD4 (clone MEM-241) (ImmunoTools).
   (c) Phycoerythrin (PE)-conjugated anti-CD1a (clone H148) (eBioscience), anti-CD115 (clone 12-3A3-1B10) (eBioscience), anti-CD56 (clone MEM-188) (ImmunoTools), anti-CD19 (clone LT19) (ImmunoTools), anti-CD14 (clone 61D3) (eBioscience), anti-CD8 (clone MEM-31) (ImmunoTools), anti-CD303 (clone AC144) (Miltenyi Biotech).
   (d) Allophycocyanin (APC)-conjugated anti-CD14 (clone 61D3) (eBioscience), anti-CD11c (clone B-ly6) (BD Biosciences, Mountain View, CA), anti-CD34 (clone 581) (BD Bioscience) San Diego, CA), anti-CD40 (clone 5C3) (BD Bioscience).
   (e) Peridinin Chlorophyll-a protein (PerCp)-conjugated anti-HLA-DR (clone L243) (BD Bioscience).
   (f) Biotinylated anti-CD1c (clone AD5-8E7) (Miltenyi Biotech), anti-CD303 (clone AC144) (Miltenyi Biotech), anti-CD45 (clone H130) (BD Bioscience).
4. Isotype controls and secondary reagents: Labeling with biotinylated antibodies is detected with PerCP-streptavidin and APC-streptavidin, both from BD Biosciences. FITC-conjugated,

PE-conjugated, APC-conjugated, and biotin-conjugated isotype-matched control antibodies all from BD Biosciences.

5. Fix solution: PBS with 4% formaldehyde (freshly made).
6. Flow cytometry analysis: FACSCalibur (BD Biosciences) and the CellQuest software (BD Biosciences).

### 2.3. Culture of Progenitor Cells to Generate DC

1. Dulbecco's Modified Eagle's Medium (DMEM) is supplemented with 10% FBS, 10 mM Hepes, nonessential amino acids from a 100× stock solution, 100 IU/ml Penicillin and Streptomycin, and 50 μM β-mercaptoethanol (*see* **Note 2**).
2. Growth factors GM-CSF and TNF are dissolved in PBS at 10 μg/ml and stored in single-use aliquots at –20 or –80°C.
3. Lipopolysaccharide is dissolved in PBS at 1 mg/ml and stored at 2 to 8°C.
4. Inverted microscope Nikon Eclipse TS100 and digital camera Nikon CoolPix 4500, both from Nikon, Tokyo, Japan.

### 2.4. T Cell Isolation and Mixed Leukocyte Reaction

1. Buffy coat (*see* **Note 3**).
2. Pan T cell isolation kit II, CD8 MicroBeads, CD4 MicroBeads, LS columns, and a MidiMACS separator all from Miltenyi Biotech.
3. Buffer for T cell isolation: PBS with 0.5 (w/v) % BSA and 5 mM EDTA.
4. RPMI 1640 with sodium pyruvate is supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 10 mM Hepes, 100 IU/ml Penicillin and Streptomycin, and 50 μM β-mercaptoethanol. This is referred to as complete RPMI.
5. For $^{3}$H-thymidine incorporation and harvesting of cells: $^{3}$H-thymidine, Tomtec harvesting machine, Glass fiber filter, Melt-on scintillation sheet, Sample bags, and 1450 Microbeta counter (Perkin Elmer, Wellesley, MA).

## 3. Methods

The frequency of CD34$^{+}$ hematopoietic progenitors in human bone marrow and cord blood is in the range of 0.5–1% and similar techniques can be applied for isolation of progenitors from both specimens. Although bone marrow will be the most suitable source of progenitors for generation of autologous DC in vitro for future use in clinical DC-based therapies, cord blood, when accessible, provides a good and reliable source of progenitor cells for studies on DC differentiation and function under

specific culturing conditions. Using this protocol for positive selection of cord blood $CD34^+$ hematopoietic progenitor cells, purity of $CD34^+$ $CD45^+$ cells is approximately 85%. If desired, further purification and separation into subpopulations of enriched progenitors can be accomplished by fluorescence-activated cell sorting (FACS).

It is important to confirm that isolated $CD34^+$ progenitors respond to hematopoietic growth factors known to support DC development in vitro, and therefore progenitors are cultured in GM-CSF plus TNF *(11)*. On day 14 of culture a majority of cells are floating and lightly adherent and show a stellar morphology with elongated cytoplasmic projections, typical for DC. Floating and lightly adherent cells are collected and characterized phenotypically and, as determined by flow cytometry analysis, approximately 70% of cells are $CD11c^+$ $HLA\text{-}DR^+$. A majority of $CD11c^+$ $HLA\text{-}DR^+$ cells express the CD1a molecule. Upon generating DC from progenitors using specific culture conditions to explore new function and differentiation pathways it is also important to perform functional assays with these cells, and the DC generated with GM-CSF and TNF can provide a positive control in T cell proliferation assays. In particular, DC stimulated with LPS upregulate their surface expression of HLA-DR and costimulatory molecules including CD40, CD80, and CD83, and thereby become efficient inducers of T cell proliferation in the mixed leukocyte reaction (MLR).

### 3.1. Isolation of Hematopoietic Progenitor Cells

1. Collect the cord blood in sterile collection tubes containing EDTA. Process the cells within 24 h of collection (*see* **Note 4**).
2. Dilute the cord blood at 1:4 in isolation buffer and then isolate mononuclear leukocytes from the cord blood using Lymphoprep density centrifugation. Transfer up to 35 ml of cell suspension onto 15 ml of Lymphoprep in 50-ml tubes (*see* **Note 5**). Spin down samples for 20 min at $800 \times g$. After centrifugation, transfer cells at the interface to a new 50-ml tube and fill the tube with isolation buffer. Count the number of cells and spin down the cells for 10 min at $300 \times g$. Resuspend the cell pellet in 50 ml of isolation buffer and spin down again. Resuspend the cell pellet at $4 \times 10^7$ cells per 900 µl of isolation buffer and then place the cell suspension on ice.
3. Resuspend the Dynabeads thoroughly in the vial and transfer the desired amount (100 µl per $4 \times 10^7$ cells) into a 15-ml washing tube. Place the washing tube in the Dynal MPC for 1 min and then aspirate fluid while the tube is still exposed to the magnet. Remove the tube from the magnet and resuspend beads in 2 ml of washing buffer. Repeat the washing step once and then resuspend the Dynabeads in 100 µl of washing buffer and place the suspension on ice.

4. Resuspend the cells and Dynabeads thoroughly, and then mix them together (*see* **Note 6**). Incubate the cell-bead suspension on a rotating wheel at 15 rpm for 30 min at 2–8°C. After incubation, add isolation buffer to the cell-bead suspension to the height of the magnet. Place the tube in the Dynal MPC for 2 min and aspirate the supernatant. Repeat this washing step three times. After the final wash resuspend beads with positively selected cells in 100 µl of isolation buffer per $4 \times 10^7$ cells.
5. To detach beads add 100 µl DetachaBead CD34 per $4 \times 10^7$ beads. Vortex the tube for 2–3 s and incubate the tube on a rotating wheel at 15 rpm for 15 min at 37°C. After the incubation, add 2 ml of isolation buffer to the bead-cell suspension and vortex for 2–3 s. Place the sample tube in the Dynal MPC for 2 min. Collect the fluid, which contains released cells, to a new tube. Repeat this step three times and add 2 ml of isolation buffer at each time and pool released cells. To remove any residual beads place the tube with released cells in the Dynal MPC for 2 min and transfer the cell suspension into a new tube. Wash the isolated cells twice in 10 ml of isolation buffer and determine the number of cells (*see* **Note 7**). After isolation, resuspend the hematopoietic progenitor cells in complete DMEM and use them in cultures to generate DC or use the isolated cells for flow cytometry analysis and stain cells with anti-CD34, anti-CD45, anti-CD117, anti-CD116, anti-CD115, anti-CD11c, anti-CD1a, anti-CD1c, anti-CD303, anti-CD14, anti-CD56, anti-CD3, and anti-CD19 antibodies (**Figs. 1** and **2**). Most of $CD34^+$ $CD45^+$ progenitor cells stain positive for CD117 (c-kit), whereas cells positive for the growth factor receptors CD115 (M-CSFR) and CD116 (GM-CSFR) are less than 8% (**Fig. 2a**). Together, this demonstrates that a majority of the isolated $CD34^+$ $CD45^+$ hematopoietic progenitor cells probably still have multilineage potential and are not yet committed to either a monocyte or DC differentiation pathway. To confirm that the presence of contaminating, differentiated leukocytes is at a minimum level in the total population of isolated cells CD34 + cells (R1 gate) the cells are stained for several markers indicative of both myeloid and lymphoid cells. This includes staining for various subsets of DC (CD11c, CD1a, CD1c and CD303), monocytes (CD14), NK cells (CD56), T cells (CD3), and B cells (CD19) (**Fig. 2b, c**).

### 3.2. Flow Cytometry

1. Transfer cells into 5-ml tubes (BD Biosciences) and wash once with flow cytometry buffer (2 ml) (*see* **Note 8**).
2. Resuspend cells in 50–100 µl of blocking solution and incubate for 30 min on ice. If no biotinylated antibodies are used proceed to **step 4**.

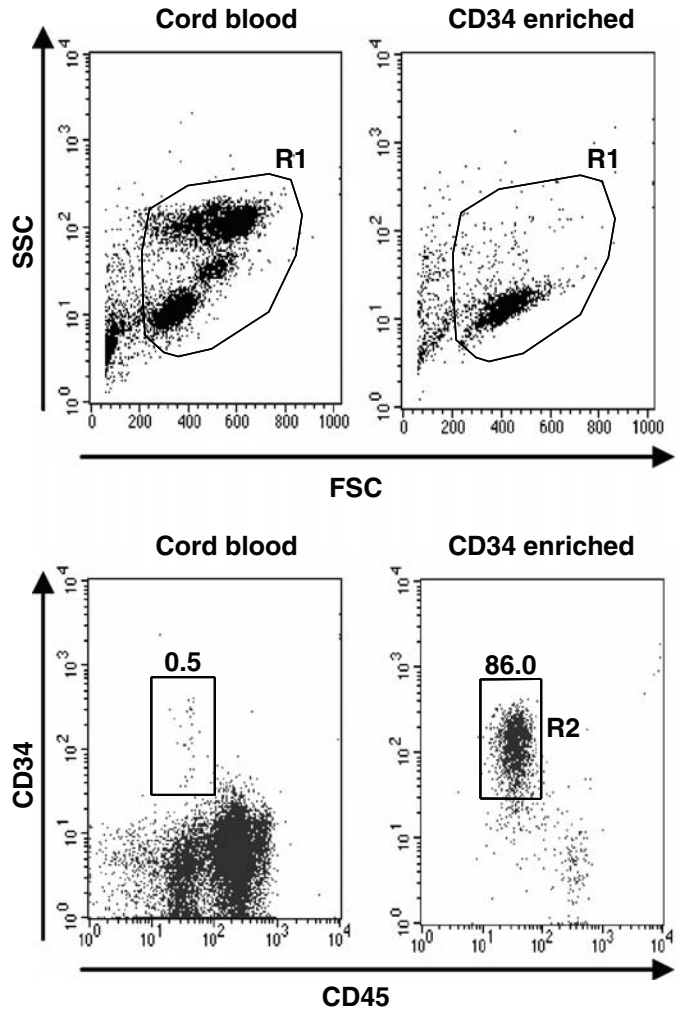


Fig. 1. Frequency of CD34+ CD45+ cells among cells isolated by CD34 enrichment. Whole cord blood or CD34-enriched cells were analyzed by flow cytometry after staining with anti-CD34 and anti-CD45 antibodies. Dot plots show the forward/side scatter size profile and the percentage of cells that are positive for CD34 and CD45. Total cord blood cells and isolated CD34+ progenitors were gated (R1) to include viable cells based on the forward/side scatter profile and hematopoietic progenitor cells were defined as cells expressing CD34 and CD45 (R2).

3. Spin down cells and resuspend in 50–100 µl of flow cytometry buffer containing biotin-conjugated antibodies. Incubate for 45 min on ice and wash cells 2–3 times in flow cytometry buffer.
4. Spin down cells and resuspend cells in 50–100 µl flow cytometry buffer containing fluorochrome-conjugated antibodies and if biotin-conjugated antibodies were added in **step 2** also use fluorochrome-conjugated streptavidin reagents. Incubate

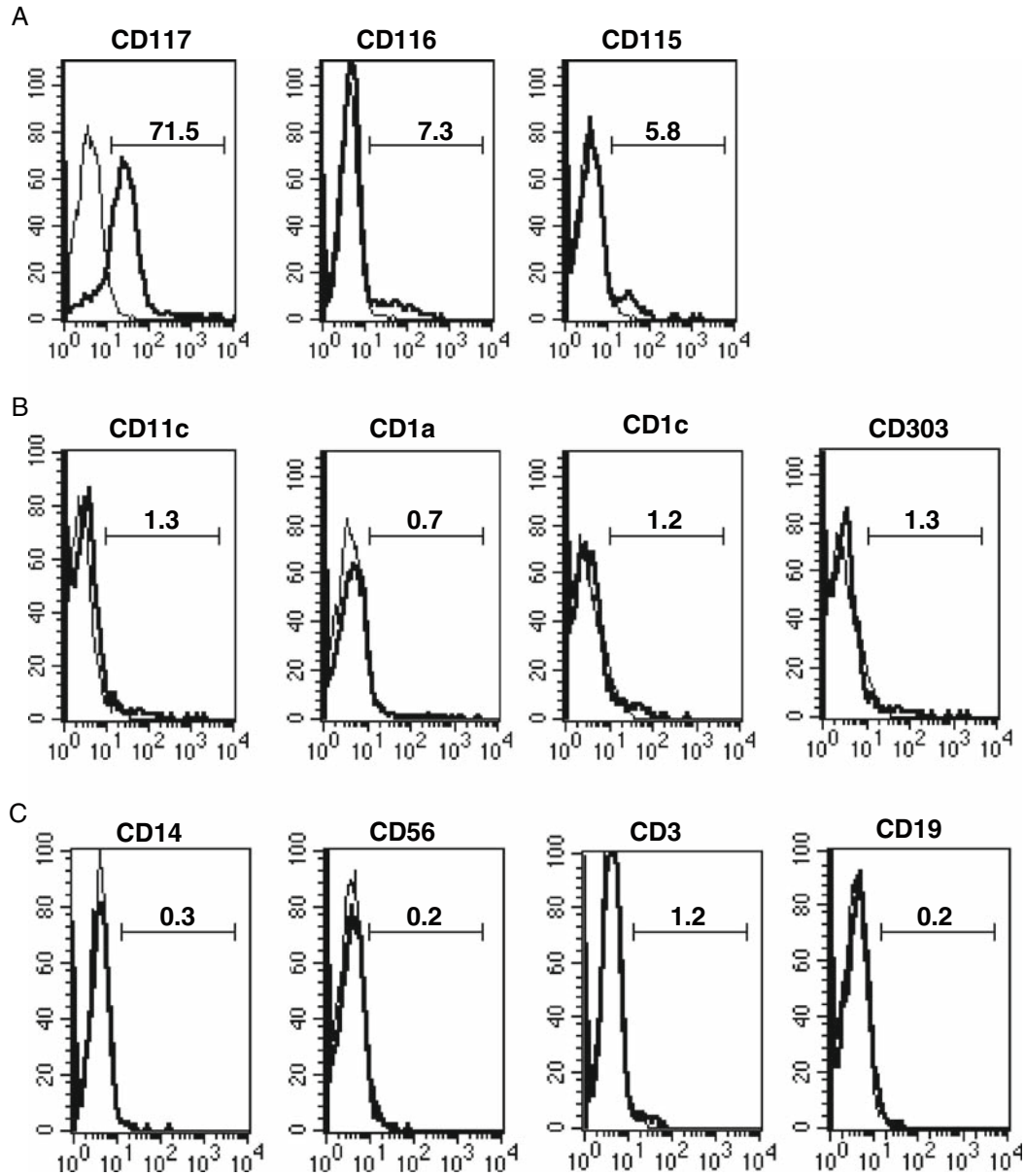


Fig. 2. Surface molecule expression on cells isolated by CD34 enrichment. Isolated CD34+ cells were stained with anti-CD34, anti-CD45, anti-CD117, anti-CD116, anti-CD115, anti-CD11c, anti-CD1a, anti-CD1c, anti-CD303, anti-CD14, anti-CD56, anti-CD3, and anti-CD19. (**A**) Histograms show the surface molecule expression of CD117, CD116, and CD115 (*thick line*) on $CD34^+$ $CD45^+$ cells (gated on R1 and R2, *see* **Fig. 1**). (**B**) Histograms show the surface molecule expression of CD11c, CD1a, CD1c, and CD303 on cells within the R1 gate (total population of isolated CD34+ cells, *see* **Fig. 1**). (**C**) Histograms show the surface expression of CD14, CD56, CD3, and CD19 on cells within the R1 gate (total population of isolated cells, *see* **Fig. 1**). Thin lines represent unstained cells. In (**a**) bars indicate the percentage of positive cells among total $CD34^+$ $CD45^+$ cells and in (**B**, **C**) bars indicate the percentage of positive cells among total cells (*see* **Fig. 1**).

for 45 min on ice and wash cells 2–3 times in flow cytometry buffer.

5. Resuspend cells in fix solution. Incubate for 30 min at room temperature (*see* **Note 9**).
6. Wash cells once in flow cytometry buffer and resuspend in the same buffer prior to acquisition and analysis of the samples using a FACSCalibur (BD Biosciences) and the CellQuest software (BD Biosciences), respectively.

### *3.3. Culture of Progenitor Cells to Generate DC*

1. Wash isolated progenitors in complete DMEM once and resuspend cells at $5 \times 10^4$ per ml in complete DMEM containing 50 ng/ml of GM-CSF and 2.5 ng/ml of TNF. Seed 500 µl of cell suspension per well in a 24-well plate (*see* **Note 10**).
2. On day 6 of culture carefully aspirate 400 µl of medium from the wells followed by adding 400 µl of fresh complete DMEM with growth factors. An example of the typical appearance of cultures at day 6 is shown in **Fig. 3a**.
3. On day 12 collect loosely and nonadherent cells. Pool cells and wash once in complete DMEM. Resuspe 3.3 Culture of Progenitor Cells to Generate DC nd cells at $5 \times 10^4$ per ml in complete DMEM with GM-CSF and TNF. Seed 2 ml of cell suspension per well in a 12-well plate (*see* **Note 11**). To activate DC add LPS to a final concentration of 1 µg/ml and incubate for 48 h.
4. On day 14 of culture, floating and lightly adherent cells are collected and washed twice in DMEM. An example of the typical appearance of cultures at day 14 is shown in **Fig. 3a**. Use the collected cells for flow cytometry analysis and stain with anti-CD11c, anti-HLA-DR, anti-CD1a, anti-CD1c, anti-CD14, anti-CD83, anti-CD80, and anti-CD40 antibodies (**Fig. 3b, c**). The detection of CD1a and CD1c on $CD11c^+$ $HLA\text{-}DR^+$ cells demonstrates that the isolated progenitor cells differentiated into DC (**Fig. 3b**). In addition, the lack of CD14 expression on the $CD11c^+$ $HLA\text{-}DR^+$ population confirms that these cells are DC rather than monocytes (**Fig. 3b**). The relatively low surface molecule expression of HLA-DR and costimulatory molecules (CD40, CD80, and CD83), as shown in **Fig. 3c**, is typical for DC that have not yet been activated. However, upon encounter with microbial products, such as LPS, DC are known to respond and undergo a process of cellular activation termed maturation. Upon maturation DC enhance their antigen-presenting capacity by changes to the surface phenotype involving increased expression of HLA-DR and costimulatory molecules. DC generated from progenitor cells also respond to LPS where increased surface expression of HLA-DR and CD40, CD80, and CD83 is evident (**Fig. 3c**) (*see* **Note 12**). The LPS-matured DC can act as efficient inducers of T cell

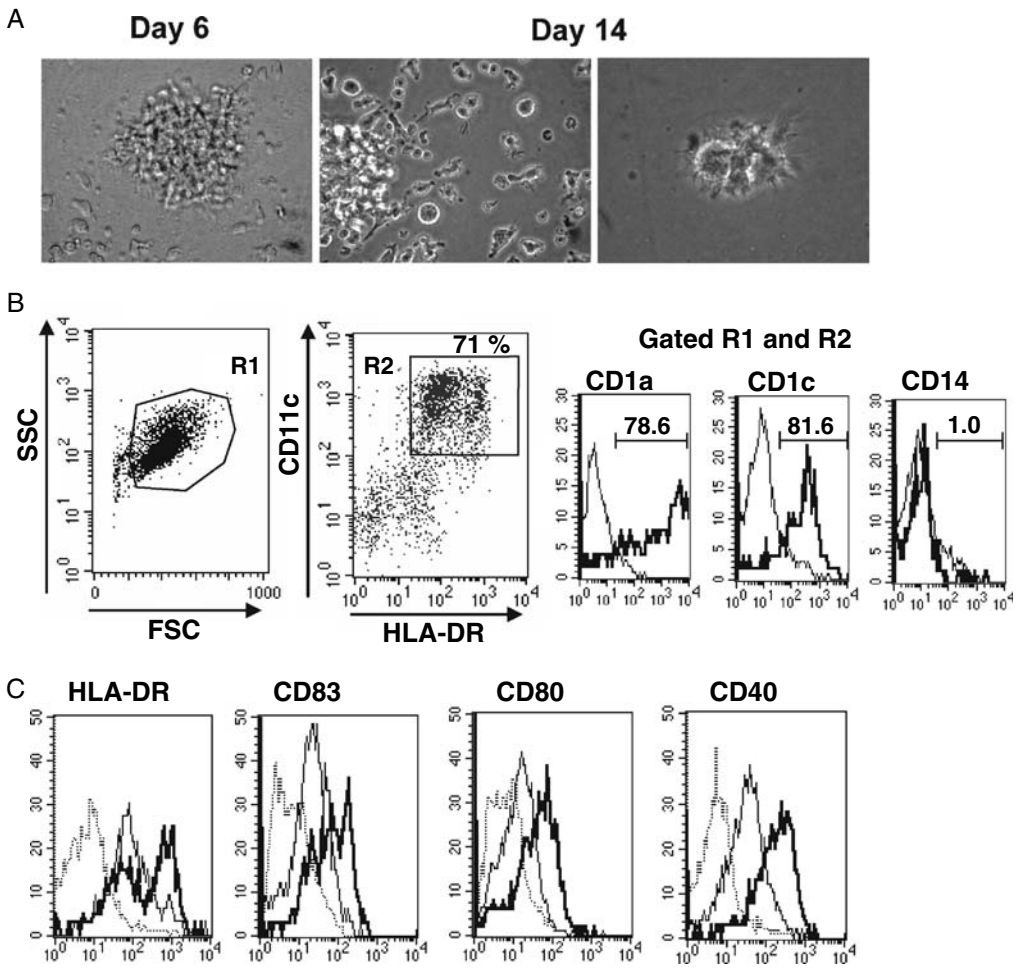


Fig. 3. Morphology and surface molecule expression of DC developing from progenitor cells in response to GM-CSF and TNF. (**A**) The images of day 6 and 14 cell cultures were obtained using an inverted microscope and a digital camera. On day 6 most cells appear in aggregates typical of proliferating hematopoietic cells, and on day 14 cultures contain numerous floating and lightly adherent cells with a DC morphology. (**B**, **C**) Floating and lightly adherent cells were collected on day 14 and analyzed by flow cytometry after staining with antibodies. Cells were stained with anti-CD11c, anti-HLA-DR, anti-CD1a, anti CD1c, anti-CD14, anti-CD83, anti-CD80, anti-CD40, or isotype control antibodies. (**B**) Dot plots show the forward/side scatter size profile and the percentage of cells that are positive for CD11c and HLA-DR. Histograms show the staining of anti-CD1a, anti-CD1c, anti-CD14 (*thick line*), and control antibodies (*thin lines*) on $CD11c^+$ $HLA\text{-}DR^+$ cells (gated on R1 and R2). The bars indicate the percentage of positive cells among total $CD11c^+$ $HLA\text{-}DR^+$ cells. Together, these results confirm that the isolated progenitor cells differentiated into $CD11c^+$ $HLADR^+$ DC-expressing CD1a and CD1c which is consistent with previous reports *(13)*. (**C**) Histograms show the staining of anti-HLA-DR, anti-CD83, anti-CD80, and anti-CD40 on either nonstimulated (*thin line*) or LPS-stimulated (*thick line*) $CD11c^+$ $CD1a^+$ cells. Dotted lines represent staining with isotype-matched control antibodies on LPS-stimulated cells.

activation and can be used as allogeneic stimulators in the mixed leukocyte reaction (MLR).

### *3.4. T Cell Isolation and Mixed Leukocyte Reaction*

1. Isolate mononuclear leukocytes from samples of normal peripheral blood using Lymphoprep density centrifugation (*see* **Note 13**). Transfer 25 ml of blood cell suspension onto

15 ml of Lymphoprep in 50-ml tubes. Spin samples for 20 min at 800 × *g*. After centrifugation transfer cells at the interface to a new 50-ml tube and fill the tube with PBS. Determine the number of cells and spin down the cells for 10 min at 300 × *g*. Resuspend the cell pellet in 50 ml of PBS and spin down cells again. Resuspend the cell pellet in T cell isolation buffer at $1 \times 10^7$ cells per 40 µl T cell isolation buffer and place the cell suspension on ice (*see* **Note 14**).

2. Transfer 80 µl of cell suspension into a 15-ml tube and add 20 µl of the Pan T cell biotin-antibody cocktail (*see* **Note 15**). Incubate at 2–8°C for 10 min.
3. Add 60 µl of T cell isolation buffer and 40 µl of beads conjugated with antibiotin MicroBeads. Incubate at 2–8°C for 15 min.
4. Wash the bead-cell suspension by adding 4 ml of T cell isolation buffer and spin down 10 min at 300 × *g*. Repeat this step once.
5. Resuspend cells in 500 µl of T cell isolation buffer. Transfer the cell suspension to an LS column placed in a MidiMACS separator.
6. Apply 3 × 3 ml of T cell isolation buffer to the column and collect the flow through containing the CD3-enriched fraction. Count the cells and take an aliquot to check purity using an anti-CD3 antibody following the protocol for flow cytometry analysis of isolated progenitor cells (**Subheading 3.2**). The purity of isolated cells as determined by their CD3 expression is shown in **Fig. 4a**.
7. Wash CD3-enriched cells once in T cell isolation buffer.
8. Resuspend the cells at $1 \times 10^7$ cells per 80 µl of T cell isolation buffer and add 20 µl of CD4 or CD8 MicroBeads per $1 \times 10^7$ cells. Incubate at 2–8°C for 15 min.
9. Add 10 ml of T cell isolation buffer and spin down.
10. Resuspend cells in 500 µl T cell isolation buffer. Transfer the cell suspension to an LS column placed in a MidiMACS separator.
11. Apply 3 × 3 ml of T cell isolation buffer to the column. Remove the column from the separator and place in a new 15-ml tube to collect positively selected cells. Add 3 ml of T cell isolation buffer to the column and immediately flush out the positively selected cells using the plunger supplied with the column.
12. Wash once and count the number of cells. Take an aliquot to check purity using anti-CD3, anti-CD4, anti-CD8 antibodies following the protocol for flow cytometry analysis (**Subheading 3.2**). The purity of isolated CD4 T cells and CD8 T cells

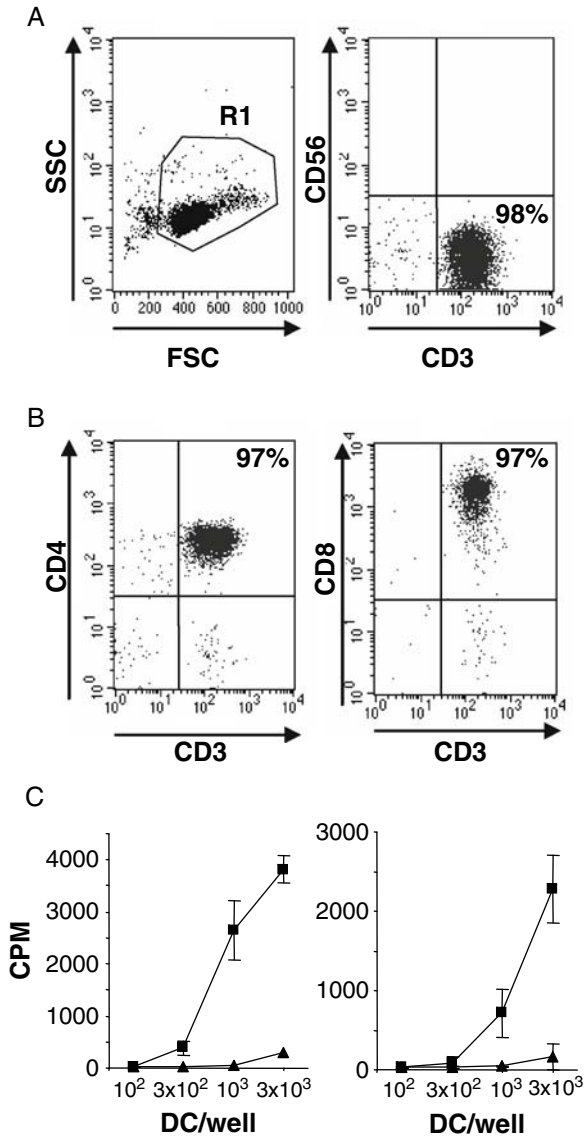


Fig. 4. Isolation of allogeneic T cells and stimulation of T cells in mixed leukocyte reactions using DC derived from progenitor cells. (**A**) CD3-enriched cells were analyzed by flow cytometry after staining with anti-CD3 and anti-CD56 antibodies. Dot plots show the forward/side scatter size profile and the percentage of CD3 single positive cells (gated on R1). (**B**) Positively selected CD4 and CD8 T cells were analyzed by flow cytometry after staining with anti-CD3 and anti-CD4, and anti-CD3 and anti-CD8, respectively. Dot plots show the percentage of cells that are positive for CD3 and CD4 (*left*) and for CD3 and CD8 (*right*) (gated on R1 as in (**A**)). (**C**) Isolated CD4 (*left*) and CD8 (*right*) T cells were used as responder cells in an MLR with DC derived from progenitor cells as stimulators. $1 \times 10^5$ responder $CD4^+$ or $CD8^+$ T cells were mixed together with $10^2$, $3 \times 10^2$, $10^3$, or $3 \times 10^3$ stimulator DC in triplicate wells. The stimulating activity of DC (*triangles*) or DC stimulated with LPS (1 μg/ml) for 48 h (*squares*) was determined by the degree of [$^3$H]thymidine incorporation in proliferating T cells and is shown as counts per minute (CPM). Wells containing only T cells or irradiated APC incorporated <100 cpm.

is determined by the CD3 and CD4, and CD3 and CD8 surface expression, respectively, and is shown in **Fig. 4b**.

13. Wash cells twice in complete RPMI and then resuspend cells in complete RPMI at $1 \times 10^6$ cells per ml.
14. Set up primary mixed leukocyte reactions in flat-bottom 96-well plates with $1 \times 10^5$ (100 µl) responder $CD4^+$ or $CD8^+$ T cells per well. In addition, add $10^2$, $3 \times 10^2$, $10^3$, or $3 \times 10^3$ day 14 DC derived from progenitor cells as allogeneic stimulator cells in triplicates. Stimulator cells should be irradiated with 2,000 rad prior to mixing with T cells. Incubate the MLR for a total of 96 and 8 h before termination; add 0.5 µCi [$^3$H]thymidine per well (*see* **Note 16**).
15. After pulsing with [$^3$H]thymidine harvest cells onto glass fiber filters and count. Then responses can be reported as mean cpm ± standard deviation (**Fig. 4c**).

## 4. Notes

1. Cord blood specimens are obtained according to institutional guidelines and approved by the regional ethical review board. Cord blood is collected after cesarean deliveries after clamping and cutting of the cord, and usually 15–20 ml of cord blood can be collected. From this amount of blood the $CD34^+$ selection procedure generates between $0.5 \times 10^6$ and $1 \times 10^6$ cells.
2. As basal medium DMEM with L-glucose, L-glutamine and pyruvate (Invitrogen) is used. The FBS is heat inactivated at 56°C for 30 min before use. Unless stated otherwise, DMEM with all the supplements is referred to as complete DMEM. All cell cultures are kept at 37°C in humidified, 5% $CO_2$ incubators.
3. Buffy coats are obtained according to institutional guidelines and approved by the regional ethical review board. From one buffy coat at least $5 \times 10^7$ T cells can be isolated.
4. If the cord blood is not processed immediately it can be stored for at least 24 h tilting at room temperature.
5. At the time of doing gradient centrifugations it is critical that all blood, isolation buffer, and the Lymphoprep are adjusted to room temperature. Do the centrifugation at room temperature with low acceleration and no breaks.
6. At the time of adding the 100 µl of Dynabeads M-450 CD34 to the cell suspension it is critical that both solutions have been stored on ice for at least 15 min to prevent nonspecific

attachment of phagocytic cells to the Dynabeads. The final concentration of cells after adding beads is $4 \times 10^7$ per ml. After mixing beads and cells, immediately place the tubes on a rotating wheel at 2–8°C. During the washing procedures ensure that the isolation buffer is ice cold. At the steps following addition of DetachaBead CD34 isolation buffer at room temperature is used.

7. Isolated CD34 cells can be cryopreserved in FBS with 10% dimethyl sulfoxide. Also, if necessary isolated cells can be stored in complete DMEM at 2–8°C and used for flow cytomtery analysis the following day.
8. Following this protocol perform all incubations, except fixation, on ice and use ice-cold flow cytometry buffer to wash cells and for dilution of reagents.
9. After fixation cells can be stored in fix solution at 2–8°C for up to 3 days prior to analysis.
10. Starting with $2.5 \times 10^4$ per well in a 48-well plate avoids overgrowth of cells during the culture period. The number of cells collected at day 12 is in the range of 5- to 10-fold that of the starting population. Interestingly, addition of stem cell factor (SCF) together with the GM-CSF and TNF in cultures results in an additional 5- to 10-fold increase of the cell yield on day 12 compared to the cultures supplemented with GM-CSF and TNF only. This may be explained by the fact that at least 70% of isolated $CD34^+$ $CD45^+$ cells express CD117 which is the receptor for SCF. In contrast, less than 10% of isolated $CD34^+$ $CD45^+$ cells express the GM-CSF receptor CD116. Importantly, the addition of SCF to progenitor cultures generates more cells in total, but the frequency of $CD11c^+$ HLA-$DR^+$ $CD1a^+$ cells is decreased.
11. A majority of cells that are collected on day 12 remain $CD11c^+$ HLA-$DR^+$ $CD1a^+$ and floating also when cultured for 2 days in the presence of complete DMEM with M-CSF (50 ng/ml). This demonstrates that the majority of collected cells are DC rather than monocytes which would develop into adherent macrophages in response to M-CSF.
12. Recently it was demonstrated that Langerhans cells isolated from human skin lack the expression of mRNA for TLR4 and do not respond to LPS ex vivo. Whether the DC that are generated from $CD34^+$ progenitors and resemble Langerhans cells can directly respond to LPS is not yet known. However, the DC population generated from $CD34^+$ progenitors is heterogeneous, and it is possible that the observed response to LPS by DC in these cultures involves both direct and indirect mechanisms *(12)*.

13. Adjust blood and Lymphoprep to room temperature and do the centrifugation at room temperature with low acceleration and no breaks.
14. Always keep the MACS buffer on ice and try to keep cell suspensions on ice as much as possible doing MACS purifications. Also, perform all centrifugation steps at 4°C.
15. Exceeding this number of cells and the corresponding amount of Pan T cell biotin-antibody cocktail per tube may result in poor purity of isolated T cells. It is preferable to isolate $CD4^+$ and $CD8^+$ T cells from the CD3-enriched fraction rather than directly from the mononuclear fraction as this will prevent the copurification of $CD8^+$ NK cells and $CD4^+$ DC present in blood.
16. The stimulating activity of DC is determined by the degree of proliferation in responding T cells according to the amount of [$^3$H]thymidine incorporated into DNA of replicating cells.

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# Chapter 14

# Generation, Culture and Flow-Cytometric Characterization of Primary Mouse Macrophages

**Ulrike Schleicher and Christian Bogdan**

## Summary

Macrophages are not only host cells for many pathogens, but also fulfill several key functions in the innate and adaptive immune response, including the release of pro- and anti-inflammatory cytokines, the generation of organic and inorganic autacoids, the phagocytosis and killing of intracellular microorganisms or tumor cells, and the degradation and presentation of antigens. Several of these functions are shared by other immune cells, including dendritic cells, granulocytes, NK cells, and/or T lymphocytes. Thus, the analysis of macrophage functions in vitro using primary mouse cell populations requires standardized methods for the generation and culture of macrophages that guarantee high cell purity as well as the absence of stimulatory microbial contaminants. This chapter presents methodology to achieve these aims.

**Key words**: Macrophages, Bone marrow-derived macrophages, Peritoneal exudate macrophages, Resident peritoneal macrophages, Teflon bags, Flow cytometry, Cell sorting, Cytokines, Interferon-gamma.

## 1. Introduction

Macrophages (MΦ) are at the center stage of the immune response of mammalian organisms to microbial pathogens and tumor cells and also participate in both the initiation and the effector phases of autoimmune processes. They are potent producers of cytokines, chemokines, and numerous other pro- or anti-inflammatory organic and inorganic mediators such as prostaglandins, enzymes, or nitric oxide (NO); they phagocytose, kill, and degrade viral, bacterial, and protozoan pathogens

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as well as apoptotic or necrotic cells in the tissues of the mammalian hosts; and they are capable of presenting exogenous and endogenous antigens in the context of MHC class I or II antigens *(1–4)*. The multiple functions of MΦ are reflected by their reciprocal interactions with other cell types, notably with T lymphocytes ($CD3^+CD4^+NK1.1^-DX5^-$ or $CD3^+CD8^+NK1.1^-DX5^-$), natural killer (NK) cells ($CD3^-NK1.1^+DX5^+$), and NKT cells ($CD3^+NK1.1^+DX5^+$), all of which can confer soluble and/or cell contact-dependent stimulatory signals to MΦ *(5–14)*.

Immunologists frequently use in vitro cultures of primary mouse MΦ to study (a) the activation of signaling cascades of antimicrobial effector mechanisms and of the release of cytokines and other mediators in response to novel microbial or lymphocyte-derived stimuli, and (b) the regulation (activation or suppression) of T lymphocyte functions. In order to avoid false conclusions, these experiments require the use of highly purified MΦ populations, the absence of MΦ-stimulatory contaminants in all reagents during all steps of MΦ preparation, and the application of cell culture conditions that do not lead to a priori activation or suppression of MΦ functions. In this chapter we present methods of MΦ generation, culture, and phenotypic characterization that are specifically designed to fulfill these criteria.

## 2. Materials

The MΦ discussed here are primary MΦ derived from inbred or outbred mouse strains (e.g., BALB/c, C57BL/6, 129Sv, CD1). It is crucial to use only mice from specific pathogen-free (SPF) facilities, because various mouse pathogens (e.g., mouse hepatitis virus, Sendai virus) have been demonstrated to strongly influence macrophage functions *(15–20)*.

### *2.1. Peritoneal MΦ*

1. $Ca^{2+}$- and $Mg^{2+}$-free Dulbecco's phosphate-buffered saline (PBS), prepared from PBS powder and deionized, endotoxin-free water (*see* **Note 1**) or commercially purchased (e.g., from Invitrogen, Karlsruhe, Germany).
2. 4% (w/v) Brewer's thioglycolate broth. The broth is prepared by suspending 40.5 g of the dehydrated Bacto Brewer thioglycolate medium (*see* **Note 2**) in 1 L of endotoxin-free deionized water (*see* **Note 1**), boiling in a microwave oven to dissolve completely, and immediate sterilization in an autoclave (121°C, 15–20 min). Prepare 50- to 100-mL aliquots, which can be stored at room temperature for many months.
3. 0.9% (w/v) NaCl solution containing 5 mM sodium periodate. Prepare fresh immediately before use (*see* **Note 1**).

4. 6% (w/v) Sodium caseinate. The 6% suspension is prepared by suspending 6 g of sodium caseinate in 100 mL of endotoxin-free deionized water or 0.9% (w/v) saline (*see* **Note 1**), stirring with medium heat (ca. 30 min), and immediate sterilization in an autoclave (121°C, 15–20 min). The suspension will look pink. Continue stirring it while still warm until the suspension is uniform. Prepare aliquots (e.g., 5 mL), store at 4°C, and warm to room temperature before use.
5. 1 or 3% (w/v) Proteose peptone. Add 1 or 3% (w/v) proteose peptone to endotoxin-free deionized water (*see* **Note 1**), boil, and autoclave the solution. Prepare aliquots, store up to 3 months at 4°C, and warm to room temperature before use.
6. 1% (w/v) Polyacrylamide beads in sterile saline or PBS (*see* **Note 1**). Prepare freshly before use.
7. 10-mL Sterile, pyrogen-free plastic syringes and needles (20G and 27G), small sterile scissors and forcipes.
8. 70% (v/v) Ethanol.
9. 50-mL Sterile polypropylene tubes.
10. Cell culture medium RPMI1640, supplemented with 2 mM L-glutamine, 23.8 mM $NaHCO_3$, 10 mM HEPES, 50 µM 2-mercaptoethanol, 100 U/mL penicillin/100 µg/mL streptomycin, and varying amounts of fetal calf serum (FCS; 1–5%) (*see* **Note 3**).
11. Styrofoam box with ice.
12. Multicentrifuge 3-SR Sorvall-Heraeus or equivalent.
13. Shandon Cytospin® 4 Cytocentrifuge and accessories (glass slides, filtercards, funnels).
14. Diff-Quick® fixative and staining solutions.
15. 0.04% (w/v) Trypan blue solution.

### *2.2. Bone Marrow-Derived MΦ (BMMΦ)*

#### *2.2.1. Preparation of Teflon bags*

1. Biologically inert, transparent, gas-permeable membrane of thermoplastic fluoro-ethylene-propylene copolymer (*see* **Note 4**).
2. Continuous band sealer machine to seal FEP-Teflon® membrane, e.g., Polystar 350/5 DSM.
3. Anprolene® ampoules (containing ethylene oxide) for gas sterilization (*see* **Note 5**).
4. Ethylene oxide indicator tape.
5. Sterilization and aeration cabinet. Alternatively, a commercially available cooling (ice) box (as used in households) will also do, provided it is placed in a fume hood during the entire sterilization and evaporation phase.
6. 70% (v/v) Ethanol.
7. Sterile scalpels.

*2.2.2. Generation of BMMΦ*

1. Dulbecco's modified Eagle medium (DMEM) containing 3.7 g $NaHCO_3$/L (23 mM), 4.5 g glucose/L (40 mM), 4 mM L-glutamine, and 110 mg/L sodium pyruvate (*see* **Note 1**).
2. FCS (*see* **Note 3**).
3. Horse serum (*see* **Note 3**).
4. 10 mM 2-mercaptoethanol in PBS, sterile filtrated, stored in 5-mL aliquots at –20°C (*see* **Note 1**).
5. Nonessential amino acid solution (100×), store at +4°C (*see* **Note 1**).
6. Penicillin/streptomycin solution (100×; 10,000 U/mL penicillin, 10,000 µg/mL streptomycin; Invitrogen), 5-mL aliquots stored at –20°C (*see* **Note 1**).
7. L929 cell culture supernatant (L929 SN, *see* **Subheadings 2.2.3** and **3.2.3**, stored in aliquots at –20°C).
8. Recombinant murine macrophage-colony-stimulating factor, 10 µg/mL stock solution stored in single-use aliquots at –80°C as recommended by the manufacturer (*see* **Note 1**).
9. Sterile PBS (*see* **Note 1**).
10. 70% (v/v) Ethanol.
11. 0.04% Trypan blue solution.
12. Sterile Teflon® bags, 5 × 30 cm (inner side of the Teflon® bag is hydrophobic).
13. Sterilized pairs of forcipes and scissors for the preparation of mouse femurs (kept in sterile stainless steel box).
14. Sterile, pyrogen-free syringes (10 or 20 mL), i.v. indwelling cannulas (14G), and disposable hypodermic needles (27G).
15. Anatomical clamp (the grooved ends need to be covered with adhesive tape, with the sticky surface facing outward).
16. Plastic closures (normally used for closing dialysis tubings) to lock the Teflon bags after filling.
17. Perforated trays for the incubator.

*2.2.3. Preparation of L929 Cell Culture Supernatant (L929 SN)*

1. Cell culture medium as described in **Subheading 2.2.2.**
2. Sterile Teflon® bags, 20 × 30 cm (inner side of the Teflon® bag is hydrophilic).
3. L929 fibroblasts, obtained from ATCC.
4. Cell scrapers with thin, flexible, 2-position blades.

***2.3. Culture of MΦ for Induction of Cytokine Production and/or Effector Molecules***

1. Polystyrene tissue culture plates (e.g., NUNC, Wiesbaden, Germany: 96-well flat-bottomed plates (cat. no. 167008, 0.33 $cm^2$/well), 24-well plates (cat. no. 142475, 1.9 $cm^2$/well), small Petri dishes (cat. no. 150288, Ø 5.3 cm, 21.5 $cm^2$/dish), large Petri dishes (cat. no. 150350, Ø 8.7 cm,

56.7 $cm^2$/dish), 8-well Permanox™ Lab-Tek™ Chamber Slides™ (cat. no. 177445, 0.8 $cm^2$/well).

2. Macrophage cell culture medium (*see* **Subheading 2.1**, **item 10**; **Subheading 2.2.2**, **item 1**; **Subheading 3.2.2**, **step 1**) (*see* **Note 1**).
3. PBS (*see* **Subheading 2.1, item 1**; **Note 1**).
4. rmIFN-γ: 1 mg/mL stock solution in 20 mM Tris–HCl, 0.15 M NaCl, pH 7.5 from Genentech, South San Francisco; stored without loss of activity at 4°C for more than 14 years. Prepare 1-mL aliquots with 10 µg IFN-γ/mL in RPMI1640 plus 5% FCS (long-term storage at –80°C, aliquots for ongoing experiments are stored at 4°C and are stable for at least 4 years if kept sterile; do not refreeze (*see* **Note 1**)).
5. Lipopolysaccharide (LPS), e.g., from *E. coli* O111:B4 (Sigma-Aldrich, Taufkirchen, Germany). Dissolve at 10 mg/mL in deionized water using a sonicating water bath and prepare 50-µL aliquots (stored at –80°C). Working solutions (1 mL) of 10 or 100 µg LPS/mL are prepared in RPMI1640 plus 5% FCS (kept at –80°C; vortex after thawing).
6. rmTNF (R&D Systems, Wiesbaden, Germany: 10 µg/mL stock solution prepared in sterile PBS plus 2.5 mg/mL human serum albumin; store in 20-µL aliquots at –80°C) (*see* **Note 1**).
7. PBS (*see* **Subheading 2.1**, **item 1**) plus 10 mM EDTA (*see* **Note 1**).
8. Accutase™ (PAA Laboratories, Coelbe, Germany), ready-to-use solution, stored in single-use aliquots at –20°C.
9. Cell scrapers (*see* **Subheading 2.2**, **item 4**).

### *2.4. Phenotypic Analysis of MΦ by Flow Cytometry*

1. PBS (*see* **Subheading 2.1**, **item 1**; **Note 1**).
2. FACS (fluorescence-activated cell sorting) staining buffer: PBS, 2 mM EDTA plus 1% FCS (PAA Laboratories, Coelbe, Germany) (*see* **Note 6**).
3. Propidium iodide (PI) solution. PI (Sigma-Aldrich, cat. no. P4170) is dissolved in PBS. Stock solution (1 mg/mL) is stored in aliquots at –80°C, working aliquot (100 µg/mL) is stored at +4°C in the dark (light sensitive!). PI is potentially mutagenic (DNA-intercalating agent).
4. Purified rat antimouse CD16/CD32 mAb (mouse Fc block) (BD Biosciences, Heidelberg, Germany), stored at +4°C.
5. Fluorochrome-labeled or biotinylated monoclonal antibodies (mAb), fluorochrome-labeled streptavidin, and the respective fluorochrome-labeled isotype control mAbs. Storage as recommended by the manufacturer (mostly at +4°C in the

dark (light-sensitive dyes)). The concentration for optimal staining of the respective macrophage population has to be determined by titration of the mAb.

6. 12 × 75 mm FACS test tubes (polypropylene, not polystyrene!) (e.g., Sarstedt, Nümbrecht, Germany).
7. Accutase (*see* **Subheading 2.3**, **item 8**).
8. PBS (*see* **Subheading 2.1**, **item 1**) with 10 mM EDTA (*see* **Note 1**).
9. Cell scrapers (*see* **Subheading 2.2.3, item 4**).
10. 100-µm Nylon mesh (e.g., Fa. Eckert, Waldkirch,Germany).

## 3. Methods

### *3.1. Generation of Peritoneal MΦ*

#### *3.1.1. General Remarks*

1. Resident peritoneal MΦ (RPMΦ) are derived from the peritoneal cavity of naive mice, i.e., they are generated from resident peritoneal cells (PCs). Peritoneal exudate MΦ (PE-MΦ), in contrast, are generated from peritoneal exudate cells (PECs) that are recruited to the peritoneal cavity after intraperitoneal injection of sterile inflammatory agents.
2. The percentage of MΦ within resident PCs is in the range of 40–60%, whereas PECs contain 75–95% MΦ depending on the eliciting agent and the time point of harvesting.
3. Harvesting of peritoneal MΦ does not require a laminar-flow hood as long as the investigator carefully disinfects the mice and wears a gown, facial mask, and disposable gloves.
4. Peritoneal cells should always been evaluated for their cellular composition (% MΦ, lymphocytes, neutrophils, eosinophils, basophils, and mast cells) using cytocentrifugation and Diff-Quick®-staining. An altered cellular composition from what is normally expected may be indicative of an infection in the mouse colony.

#### *3.1.2. Resident Peritoneal Cells*

1. Naive mice are euthanized by $CO_2$ asphyxiation and subsequent cervical dislocation with a large pair of tweezers (*see* **Note 7**). Do not prepare more than 4–5 mice at a time.
2. Fix the dead mice with four pins at the extremities using a Styrofoam™ board.
3. Carefully wet the fur of each of the mice with 70% ethanol, thereby disinfecting the skin and avoiding loose hair which might later contaminate the peritoneal lavage fluid.

4. Make a small incision in the center of the abdominal skin (without damaging the peritoneum!) using sterile scissors. Carefully pull apart the abdominal skin (cranially and caudally) so that the abdominal wall is completely exposed. Do *not* disinfect the abdominal wall, because 70% ethanol will make the peritoneum rigid due to protein denaturation.
5. Using a 10-mL syringe and a 20-G needle inject 10–11 mL sterile PBS (without FCS) into the peritoneal cavity. Insert the needle in the midline of the lower abdomen (between the umbilical area and the bladder) and, while forcefully injecting the PBS (which keeps away the intestine), push the needle slowly toward the right upper quadrant of the peritoneal cavity (where no omental fat will later block the needle during reaspiration). Throughout the procedure the beveled edge of the needle is facing upward.
6. Turn the syringe so that the beveled edge of the needle is facing downward and raise the needle, which will cause tenting of the peritoneal wall (keep the needle strictly parallel to the longitudinal axis of the mouse to avoid perforation of the peritoneum). Withdraw the peritoneal lavage fluid (expected volume ca. 9–10 mL) *slowly* to avoid aspiration of mesenterial tissue. After removal of the needle dispense the lavage fluid into 50-mL polypropylene centrifuge tubes on ice.
7. Repeat **steps 5** and **6** (insert the needle into the previous peritoneal perforation). Pool the lavage fluid from several mice, but discard lavages that contain blood.
8. Centrifuge the cells at 300 × g (8–10 min, 4°C). Resuspend the cell pellet in RPMI1640 cell culture medium **(Subheading 2.1, item 10)** so that the cell density is at least $5 \times 10^6$/mL. For the expected cell yields, *see* **Note 8** and **Table 1**.
9. Remove a 10 (or 20) µL sample, mix it with 90 (or 80) µL trypan blue solution and count the cells (MΦ, lymphocytes, granulocytes) using a hemocytometer (*see* **Note 8** and **Table 1**).
10. Prepare a cytospin slide (using ca. $1 \times 10^5$ peritoneal cells in 250 µL PBS) for Diff-Quick® staining, perform a differential cell count, and adjust the peritoneal cell suspension according to the determined macrophage content.

*3.1.3. Peritoneal Exudate MΦ*

1. Inject the mice intraperitoneally with a defined volume of the elicitating agent (*see* **Note 8** and **Table 1**).
2. At the time point indicated (**Table 1**) harvest the cells from the peritoneal cavity by two sequential peritoneal lavages with serum-free PBS as described earlier **(Subheading 3.1.2 Steps 1–10)** (*see* **Note 8** and **Table 1**).

**Table 1**
**Eliciting agents, expected yields, and cellular composition of peritoneal cells**

| Type of peritoneal cell population | Volume of i.p. injected inflammatory agent | Hours after i.p. injection when the peritoneal cells are harvested | Expected yield of total peritoneal cells per mouse[a] | % of Macrophages expected prior to adherence (reference) | % of Macrophages expected after adherence (reference) |
|---|---|---|---|---|---|
| Resident peritoneal cells | None | n.a. | $1–2 \times 10^6$ | ca. 40–60% | 83–88% *(30, 42)* |
| Thioglycolate-elicited PEC | 2–3 mL 4% Brewer?s thioglycolate medium | 96 h (–120 h) | $15–25 \times 10^6$ | >86–95%[b] *(29, 32, 35)* | 99% *(29, 30)* |
| Proteose peptone-elicited PECs | 2–3 mL 3% proteose peptone solution | 72 h | $5–10 \times 10^6$ | 75–80% | 90–94% *(42)* |
| Caseinate-elicited PECs | 2 mL 6% sodium caseinate solution | 72 h | $5–10 \times 10^6$ | 75–80% | 92–96% *(30, 42)* |
| Sodium periodate-elicited PECs | 2 mL 5 mM $NaIO_4$ solution | 72 h | $5–15 \times 10^6$ | 75–80% | 78–93% *(30, 42)* |
| Biogel-elicited PECs | 2 mL 1% Biogel P100 bead suspension | 120 h | $5–15 \times 10^6$ | 60% | 97% *(24)* |

n.a. not applicable
[a]Numbers refer to female CD1 outbred mice (10–12 weeks of age, 20–24 g of weight) (unpublished data of the authors as well as published data)
[b]Thioglycolate-elicited PECs contain numerous vacuoles that are filled with ingested thioglycolate medium *(43)*
[c]Biogel-elicited macrophages do not contain the bead particles, because these are too big to be ingested by the macrophages *(24)*

### *3.2. Generation of BMMΦ*

#### *3.2.1. Preparation of Teflon® Bags*

1. The FEB-Teflon® foil is unrolled on a clean surface. The inner side of the Teflon® bag (*see* **Note 9**) is wiped off with 70% ethanol and cut into 30 × 10 cm (BMMΦ) or 30 × 40 cm pieces (L929 cells) using a sterile scalpel. It is recommended to wear unpowdered gloves during the preparation of Teflon® bags.
2. The foil is then folded and sealed so that bags of 30 × 5 cm (BMMΦ) or of 30 × 20 cm (L929) are obtained. One of the

short sides of the bags remains unsealed, but otherwise take care that the sealing seam is tight (indicated by an opaque color).

3. Fifteen to twenty of the BMMΦ Teflon® bags or 10 of the L929 Teflon® bags, all lined up in the same orientation, are wrapped with aluminum foil. The aluminum bags are closed by folding except for the side where the open ends of the Teflon® bags are. A piece of ethylene oxide indicator tape is attached to the outside of the aluminum foil.
4. For sterilization, the aluminum bags, an Anprolene® ampoule (which is enclosed in a gas-permeable plastic bag), and wet paper towels are put together into the so-called liner bag (a gas-impermeable plastic bag) and transferred into the sterilizer container. The wet towels serve to achieve an ambient relative humidity of at least 30% which is required for efficient sterilization. After opening of the Anprolene® ampoule, the sterilizer will be locked and placed in a running fume hood for 24 h. To allow evaporation of residual ethylene oxide at the end of the 24-h sterilization period, the sterilizer container and the liner bag are opened and left in the fume hood for another 24 h. Thereafter, the aluminum bags are placed in a running laminar-flow hood overnight to further aerate the Teflon® bags. Finally, the open side of the aluminum bags is tightly closed by folding over, which allows storage at room temperature until use for cell culture.

*3.2.2. Culture of Bone Marrow Cells for the Generation of BMMΦ*

1. The following media have to be prepared:
   (a) *Conditioned DMEM medium*: DMEM supplemented with 50 μM 2-ME, 1% nonessential amino acids, 10% FCS, 5% horse serum to inhibit growth of granulocytes, 15% L929 SN as a source of M-CSF (*see* **Subheadings 2.2.3** and **3.2.3**) or 5 ng/mL rmM-CSF (*see* **Note 10**).
   (b) *DMEM medium*: DMEM supplemented with 50 μM 2-ME, 1% nonessential amino acids, 5% FCS, 1% penicillin/streptomycin solution
2. Mice are euthanized and thoroughly disinfected with 70% ethanol. Wear gloves. The skin is peeled from both hind legs by pulling it down over the feet. Without damaging the femurs and tibiae, the muscles are completely removed with forceps and scissors. The bones are stored in cold sterile DMEM medium on ice until the bone marrow is prepared under laminar-flow conditions.
3. The leg bones are severed between the joints, the epiphyses of femur and tibia are opened with sterile scissors, and a 27-G needle (connected to a 10-mL syringe filled with

sterile PBS) is inserted into the bone marrow cavity, which is then flushed until the PBS remains clear and the bones appear white. The washed-out bone marrow is collected in 50-mL polypropylene centrifuge tubes.

4. Bone marrow (BM) cells are centrifuged at 300 × *g*, 5–10 min, 4°C. The total number of nucleated cells (i.e., excluding erythrocytes) is determined using a hemocytometer (*see* **Subheading 3.1.2**, **item 9**). Depending on the mouse strain expect to recover 30–50 × $10^6$ nucleated cells per mouse (two hind femurs and tibiae).
5. 6 × $10^6$ Nucleated BM cells are resuspended in 50 mL conditioned DMEM medium.
6. Sterile Teflon® bags are taken from the aluminum foil bag. The open side of the Teflon® bag is stretched between the ends (covered with adhesive tape) of an anatomical clamp. Each bag is filled with 6 × $10^6$ BM cells (in 50 mL conditioned DMEM medium) and closed with a clip after removal of air bubbles. Be careful to avoid contaminations especially of the inner side of the Teflon® bag. To allow for sufficient gas exchange the Teflon® bags are incubated at 37°C and 10% (!) $CO_2$/95% humidified air on perforated trays *(21)* (*see* **Note 11**).
7. After 7–10 days a monolayer of MΦ is present at the bottom of the Teflon® bag. To detach the loosely adherent MΦ from the hydrophobic membrane, the bag is turned over and the medium in the bag is carefully moved back and forth. The Teflon® bag is sprayed with 70% ethanol, which is allowed to evaporate, and the resuspended MΦ are harvested with a 14-G indwelling cannula. Expect to recover ca. 10–20 × $10^6$ BMMΦ per bag (*see* **Note 10**).

*3.2.3. Culture of L929 Cells for the Generation of L929 Culture Supernatant*

1. L929 fibroblasts are grown in DMEM medium (*see* **Subheading 3.2.2**, **item 1**) in dishes or cell culture flasks and harvested by use of a cell scraper (*see* **Subheading 2.2.3**, **item 4**).
2. For production of L929 SN, fibroblasts are adjusted to 1 × $10^5$/mL in DMEM medium and incubated in Teflon® bags (*see* **Subheading 3.2.1** and **Note 9**) for 7 days at 10% $CO_2$/95% humidified air, with 250–300 mL per bag. Alternatively, the L929 cell suspension can also be seeded in 175-$cm^2$ tissue culture flasks, if Teflon® bags are not available.
3. At day 7 (the medium has already turned yellow) bags are sliced at one side, the cell culture supernatant is transferred into 50-mL polypropylene tubes and centrifuged at 300 × *g*, 5–10 min, 4°C.

4. The SN is subjected to sterile filtration (0.2-µm pores) and stored frozen in aliquots at –20°C.

### 3.3. Culture of MΦ for Induction of Cytokine Production and/or Effector Molecules

This section covers general aspects of macrophage culture and stimulation, but does not provide detailed information on specific questions of macrophage activation.

#### 3.3.1. Culture Medium and Plating of the MΦ

Cell Density

Mouse MΦ are routinely seeded at $2 \times 10^6$/mL. As the cell density and the ratio of cell number and culture volume can influence the outcome of certain macrophage functions (e.g., arginine metabolism, uptake of microbes) *(22, 23)*, it is important to apply comparable conditions when using different culture devices (**Table 2**).

Culture Medium

The most commonly used media are RPMI1640 (*see* **Subheading 2.1**, **item 10**) and DMEM (*see* **Subheading 2.2.2**, **item 1** and **Subheading 3.2.2**). The media differ in their composition (notably in their content of arginine and glucose). Although routinely added by many authors including us, neither 2-mercaptoethanol (an antioxidant and SH donor) nor HEPES (a $CO_2$-independent buffer) are essential for culturing MΦ. Prolonged periods of alkaline pH diminish the responsiveness of MΦ to activating stimuli. It is therefore highly advisable to maintain well-acidified conditions. If prolonged pipetting under laminar-flow conditions (i.e., outside of $CO_2$ incubator) is necessary, HEPES helps to achieve this goal.

FCS, which is an ill-defined but routinely used source of growth factors, cytokines, hormones, inorganic mediators, and

**Table 2**
**Numbers of MΦ seeded in different tissue culture devices**

| Cell culture device | Culture area ($cm^2$) (per well)[a] | No. of seeded MΦ | Culture volume (mL) | No. of cells per $cm^2$ | No. of cells per mL medium |
|---|---|---|---|---|---|
| 96-Well flat-bottomed plate | 0.33 | $1 \times 10^5$ | 0.05 | $3 \times 10^5$ | $2 \times 10^6$ |
| 24-Well plate | 1.9 | $7 \times 10^5$ | 0.35 | $3.6 \times 10^5$ | $2 \times 10^6$ |
| 8-Well LabTek® Chamber Slides® | 0.8 | $4 \times 10^5$ | 0.2 | $5 \times 10^5$ | $2 \times 10^6$ |
| Small Petri dishes (Ø 5.3 cm) | 21.5 | $6 \times 10^6$ | 3 | $2.8 \times 10^5$ | $2 \times 10^6$ |
| Large Petri dishes (Ø 8.7 cm) | 56.7 | $16 \times 10^6$ | 8 | $2.8 \times 10^5$ | $2 \times 10^6$ |

[a]The numbers given refer to the products from NUNC International. Comparable products with slightly different sizes are available from other suppliers

various other stimulatory or inhibitory compounds, can be readily omitted in macrophage cultures, if necessary. Both inflammatory MΦ and BMMΦ become surface adherent, survive, and respond to various stimuli (e.g., with IFN-γ, TNF, IFN-γ plus LPS) in the absence of FCS *(23, 24)*, although an optimal response to other stimuli (e.g., LPS alone) might require the presence of FCS *(25)*. We did not observe different responses, when we stimulated the MΦ in the presence of 2.5, 5, or 10% FCS.

*3.3.2. Adherence*

When primary mouse MΦ are seeded into tissue culture plastic plates or dishes, they adhere to the surface within 60 min. After 2 h they are nicely spread out and form a monolayer. MΦ also adhere to glass surfaces and, much less well, to bacteriological (i.e., not surface-treated) Petri dishes. Adherence does not require FCS. Surfaces that impede MΦ adherence are polypropylene *(26)* or hydrophobic Teflon® membranes *(21)*. Adherence or prolonged incubation under adherent conditions prior to stimulation affects various MΦ functions *(23, 27, 28)*.

*3.3.3. Washing of the MΦ Monolayer*

In order to remove nonadherent cells, MΦ monolayers are washed at least three times with PBS (37°C). The most efficient way to remove the washing fluid is to use a vacuum pump with a sterile 100-µL pipet tip attached to the suction tubing. In the case of PE-MΦ the percentage of contaminating lymphoid cells clearly decreases with an increased number of washing steps. However, lymphoid contaminants remain detectable even after 10 washings (U. Schleicher and C. Bogdan, unpublished observations) *(29)*.

*3.3.4. Stimulation of the MΦ Monolayer*

1. Standard stimuli for the priming and/or activation of MΦ are IFN-γ (2–20 ng/mL), LPS (1–500 ng/mL), TNF (2–20 ng/mL), and combinations thereof. Production of reactive oxygen intermediates (ROI) by IFN-γ- or TNF-primed MΦ can be triggered by phorbol myristate acetate (PMA). The necessary duration of stimulation depends on the MΦ function under examination (e.g., ROI release: 10–60 min; nitric oxide (NO) release: 24–48 h; TNF release: 8–24 h) *(30–32)*.
2. The various MΦ populations differ in their secretory functions. For example, PE-MΦ are already potent producers of NO in response to IFN-γ alone, whereas resident peritoneal MΦ or BMMΦ usually require combined stimulation with IFN-γ plus LPS or TNF *(30)*. Resident peritoneal MΦ and periodate- or caseinate-elicited PE-MΦ efficiently release ROI after priming with IFN-γ and triggering with PMA, whereas thioglycolate-elicited PE-MΦ fail to do so *(30)*.

*3.3.5. Detachment of Adherent MΦ*

The detachment of tightly adherent MΦ for subsequent analyses is a challenge, because all procedures can damage the cells

(*see* **Note 11**). In our hands, treatment of the MΦ monolayers with Accutase proved to be most effective.

1. The medium is removed and the adherent MΦ are rinsed with sterile PBS (room temperature).
2. Accutase, a mixture of proteolytic and collagenolytic enzymes, is added (10 mL per 75 $cm^2$ surface).
3. The cultures are returned to the 37°C incubator, incubated for 15–45 min.
4. The detached MΦ are harvested by vigorous washing with PBS. MΦ, which do not lose their adherence, have to be harvested using a cell scraper.

#### *3.3.6. Lysis of Adherent MΦ*

For several experimental purposes (e.g., Western blot analysis, immunoprecipitation, electromobility shift assays, preparation of RNA) MΦ have to be lysed. In the case of adherent MΦ the most convenient procedure is to rapidly wash the monolayers with ice-cold PBS (3×) followed by the addition of the respective lysis buffer *(23, 33–35)*. For protein analyses the MΦ are directly scraped in the added volume of lysis puffer (5.3 cm Ø Petri dish: 0.5 mL; 8.7 cm Ø Petri dish: 1 mL). For the preparation of RNA, the added lysis puffer (5.3 cm Ø Petri dish: 2 mL; 8.7 cm Ø Petri dish: 4 mL) is rapidly pipetted up and down until all cells are lysed.

### *3.4. Phenotypic Analysis of MΦ by Flow Cytometry*

In the protocol described later MΦ are stained simultaneously with sets of three differently labeled mAbs (three-color immunofluorescence). Propidium iodide (PI) is added to discriminate viable and dead cells (*see* **Fig. 1**). If the analysis is performed on a flow cytometer with two lasers (blue laser: 488 nm, red laser: 633 nm) (e.g., BD FACS Calibur®) as in this protocol, PI should be detected via the FL3 channel. It is therefore recommended to use fluorescein isothiocyanate (FITC) (FL1 channel)-, phycoerythrin (PE) (FL2 channel)-, and allophycocyanin (APC) (FL4 channel)-labeled mAbs or other suitable fluorochrome conjugates for staining of cell surface markers. Fluorochrome conjugates that are detected via the FL3 channel (e.g., peridinin chlorophyll protein (PerCP)) cannot be used for surface staining in this case. The analysis on a flow cytometer with three lasers (488, 633 nm, and violet laser 405 nm) (e.g., BD FACS Canto II, BD LSR II) allows simultaneous detection of more than three surface markers. In this case the staining protocol and the choice of fluorochrome-conjugates have to be altered accordingly, and it is recommended to use 4 ,6-diamidino-2-phenylindole (DAPI; excited by ultraviolet light) instead of PI for exclusion of dead cells. If biotin-conjugated mAbs are used for staining, a two-step staining procedure is required. As biotin has an extremely strong affinity to avidin, biotin-conjugated mAbs can be detected with fluorochromes (FITC, PE, or APC) coupled to streptavidin.

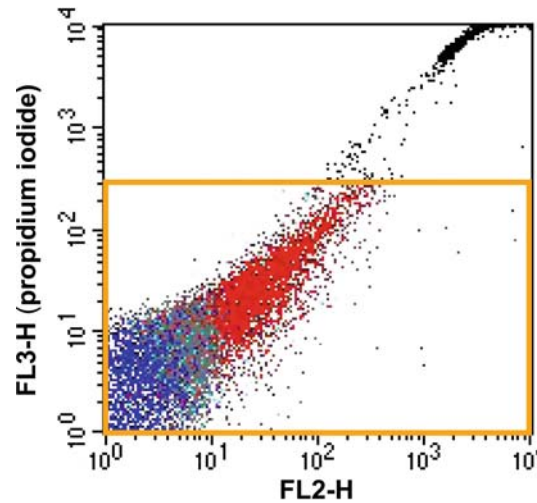


Fig. 1. FACS analysis of viable and dead C57BL/6 thioglycolate-elicited PECs. Prior to flow cytometric analysis PI was added to otherwise unstained PECs. The FL3 channel was used to detect PI fluorescence. $PI^+$ (i.e., dead) PECs are excluded from $PI^-$ cells (located in the *orange gate*) across the entire detection range of FL2 channel. The different colors of the $PI^-$ cells reflect the various subpopulations gated in the forward/side scatter analysis (*see* **Fig. 2b**). (*See Color Plates*)

Therefore, cells are first incubated with biotinylated mAbs, then washed and stained with fluorochrome-labeled streptavidin. It is recommended that only one of the mAbs used for three-color analyses is biotinylated, whereas the other two mAbs are directly conjugated with fluorochromes.

Each FACS analysis requires an unstained control to measure the autofluorescence of the MΦ, appropriate isotype controls for the different mAbs to control for specificity of binding, and individual staining with mAbs representative of each of the fluorochromes that are used to adjust compensation adequately.

1. Prior to staining and FACS analysis single-cell suspensions of MΦ have to be prepared. BMMΦ or PEC can be used directly for staining after harvesting them from Teflon® bags or the peritoneal cavity. Adherent MΦ which have been cultured in plates or dishes are detached from the plastic surface with Accutase (*see* **Subheading 3.3.5**).
2. The harvested MΦ are washed with FACS staining buffer (centrifugation at 300 × *g*, 5–10 min, 4°C) and then resuspended in the same buffer (50 µL per $1 \times 10^6$ MΦ). Visible clumps of cells have to be removed (e.g., by passing the cells through a nylon mesh (100 µm pore size) into a fresh tube).
3. To prevent nonspecific binding of fluorescence-labeled mAb to the Fc-receptors of MΦ, the suspended MΦ are incubated

with mouse Fc Block (1 μg mAb per 1 × $10^6$ cells) for 5–10 min at room temperature. After blocking, the cells are ready for staining without any further washing procedure.

4. During the blocking period prepare all reagents required for staining and label the FACS tubes according to the staining protocol.
5. Dispense 1 × $10^6$ MΦ (50 μL) into each tube. During the entire staining procedure MΦ are kept on ice. A cocktail of maximal three different fluorochrome-labeled mAbs or two different fluorochrome-labeled mAbs and one biotinylated mAb per test tube (or the respective controls; see earlier) is added in a volume of 5 μL at the appropriate dilution for efficient staining (*see* **Note 12**). The MΦ suspensions are gently mixed and incubated for 20–30 min in the dark.
6. Cells are washed by adding ca. 2 mL of cold staining buffer to each tube (centrifugation at 300 × *g*, 5–10 min, 4°C). The supernatant is discarded by rapid inversion of the tube.
7. For detection of biotinylated primary mAb the cells have to be stained with fluorochrome-labeled streptavidin followed by a washing step as described earlier.
8. MΦ are resuspended in 200–250 μL cold staining buffer after the last washing step and kept on ice in the dark until analysis by flow cytometry. If clumps of cells have formed, the MΦ suspensions have to be passed again through a 100-μm nylon mesh into a new tube. In order to detect dead cells 1 μL of PI solution is added to each tube prior to analysis (*see* **Note 13**).
9. The samples are analyzed on a BD FACS Calibur® applying the Cell Quest Pro® software. In order to get statistically reliable results for the staining of small subpopulations, at least 100,000 cells per tube should be acquired. Since the granularity of MΦ is very high, we also advise using the log scale for the side scatter. Examples of the forward/side scatter analyses of freshly isolated thioglycolate-elicited PECs or BM MΦ, respectively, are shown in **Figs. 2** and **3**. The gating of viable cells in FL-2 versus FL-3 dot plots is demonstrated with thioglycolate-elicited PECs in **Fig. 1**. The fluorescence parameters of the different stainings should be analyzed after exclusion of the PI positive (i.e., dead) cells (**Fig. 1**, orange gate = gate on viable cells). Both in PECs and BM MΦ different subpopulations (R1, R2, R3) can be defined by forward/side scatter analysis. These subpopulations also differ in the expression of myeloid and lymphoid cell surface markers (Figs. 2 and 3). Typical MΦ are mostly located within region R1, whereas lymphoid cells such as NK or T cells are exclusively found in region R3 *(29)*. Small

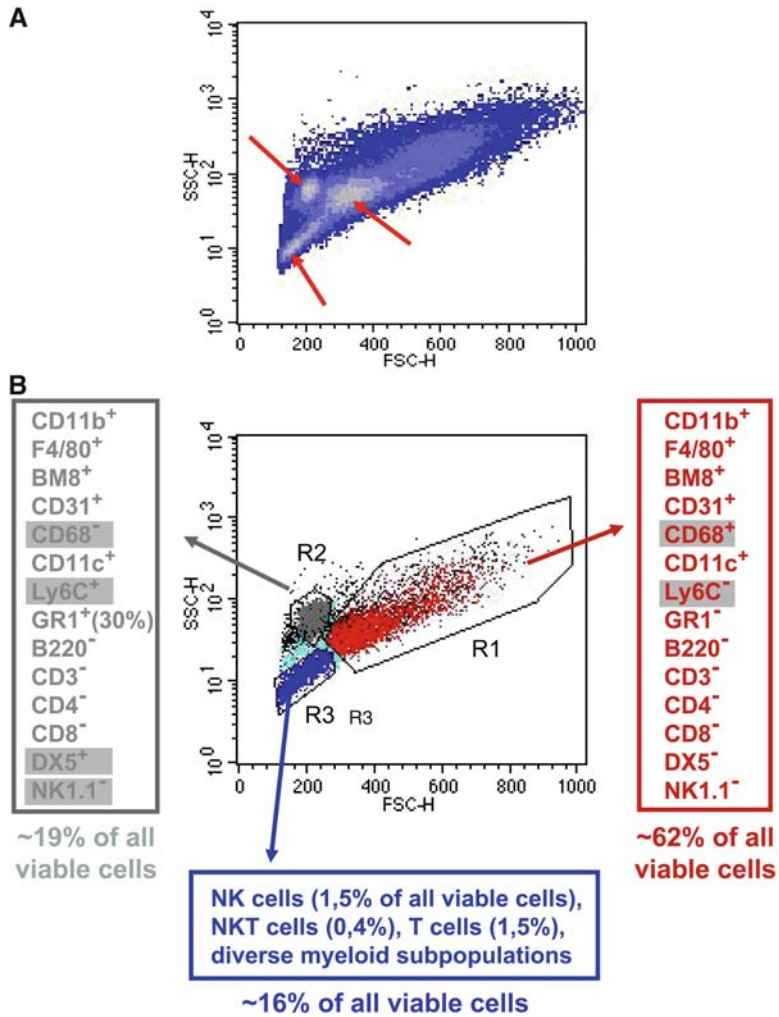


Fig. 2. FACS analysis of C57BL/6 thioglycolate-elicited PECs. (**A**) Forward/side scatter analysis of viable PECs shown as density plot. Three subpopulations within the high density area are distinguishable as indicated by the *red arrows*. (**B**) Forward/side scatter dot plot analysis of viable PECs. The three subpopulations shown in **Fig. 2a** were gated (R1, R2, R3) and analyzed for the expression of multiple myeloid (CD11b, F4/80, BM8, CD31, CD68, CD11c, Ly6C, GR1) and lymphoid (B220, CD3, CD4, CD8, DX5, NK1.1) surface markers. The positive or negative expression of the surface molecules for each subpopulation is listed in *boxes*. Typical MΦ – $CD11b^+F4/80^+BM8^+CD68^+$ – are mostly located within region R1, whereas lymphoid cells such as NK or T cells are found in region R3. (*See Color Plates*)

numbers of contaminant lymphoid cells are present in adherent PE-MΦ, resident peritoneal MΦ, as well as in BMMΦ *(29)*. Due to the high level of autofluorescence typical of MΦ as seen in R1 (*see* **Figs. 1** and **2**, red cells), the staining of contaminating lymphoid cells that are predominantly

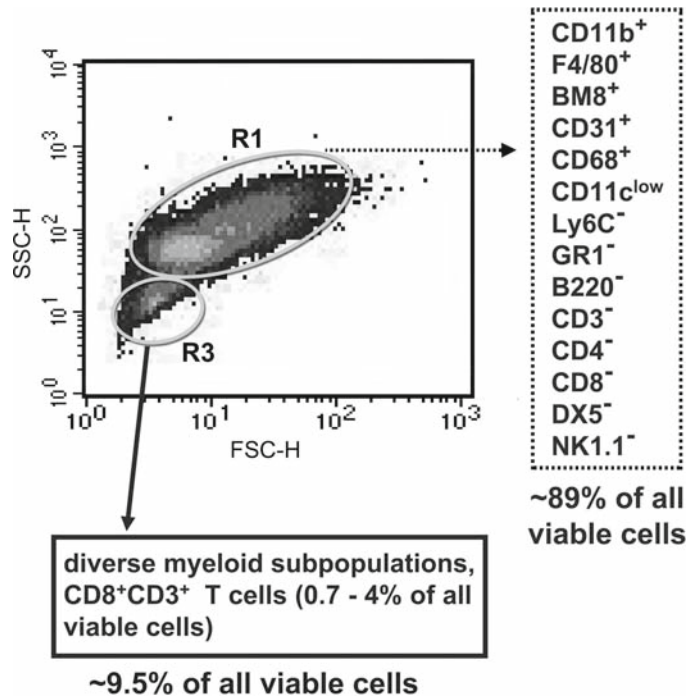


Fig. 3. FACS analysis of C57BL/6 BMMΦ. Forward/side scatter density plot analysis of viable BMMΦ is shown. Two subpopulations were discernible (R1, R3) and analyzed for the expression of multiple myeloid (CD11b, F4/80, BM8, CD31, CD68, CD11c, Ly6C, GR1) and lymphoid (B220, CD3, CD4, CD8, DX5, NK1.1) surface markers. The positive or negative expression of the surface molecules for each subpopulation is listed in *boxes*. Typical MΦ – $CD11b^+F4/80^+BM8^+CD68^+$ – are mostly located within region R1, whereas lymphoid T cells are found in region R3

located within region R3 (*see* **Figs. 1** and **2**, blue cells) may be not visible, if the whole MΦ population is analyzed. This is because the small population of positively stained lymphoid cells might shift toward the highly autofluorescent MΦ population. In order to detect any contaminating lymphoid cells, we advise gating of the subpopulations R1, R2, and R3 by forward/side scatter (*see* **Fig. 2**) followed by individual analysis of these gates for their respective stainings.

## 4. Notes

1. All solutions, buffers, and media that are used for the preparation, culture, or stimulation of MΦ have to be prepared with endotoxin (LPS)-free reagents and deionized water (minimum resistance of 18.2 MΩ × cm, total organic content of less than 5 ppm) and are subjected to sterile filtration

(0.2-μm pores). Alternatively, they are purchased as sterile ready-to-use products certified to be endotoxin-free. The endotoxin-free status of the reagents and the water is verified with the quantitative chromogenic limulus amebocyte lysate endpoint assay (QCL-1000; Lonza Inc., Verviers, Belgium; cat. no. US50-647U), which can be conveniently performed in an ELISA plate and has an exquisite sensitivity (5–10 pg LPS/mL). LPS is not only a potent activator of MΦ, but can also deactivate MΦ (notably at low concentrations) *(36)*.

2. The Bacto™ Brewer Thiogylocate Medium (BTM) from Difco (formula per liter: 500 g infusion from beef, 10 g proteose peptone, 5 g NaCl, 5 g Dextrose, 0.5 sodium thioglycolate, 0.5 g Bacto agar, 0.002 g methylene blue) is no longer available. A product with the same formula can be obtained from Sigma-Aldrich (cat. no. B-2551). The BBL™ Brewer Modified Thioglycolate Medium from BD Biosciences (cat. no. 21-1716; Heidelberg, Germany), in contrast, has a different composition, which has not been tested by us for its inflammatory activity. Independent of the source of the BTM it is strongly recommended to test the 4% (w/v) solution for its content of endotoxin. When thioglycollate medium absorbs oxygen, it will turn green, whereas the oxygen-free medium (autoclaved, stored without shaking) that should be used for injection has a golden-brown appearance.
3. Sera (e.g., FCS) used for the generation and culture of MΦ have to be carefully selected for (a) absent activating or inhibitory effects on MΦ, (b) low endotoxin and low hemoglobin levels (high levels would be a sign of infection of the donors, contamination during serum preparation or for hemolysis, respectively), and for (c) the sufficient support of the differentiation of BM MΦ (we routinely test at least 5–10 different batches from different suppliers). To exclude activating effects we recommend to measure the tumor cytotoxic activity of MΦ (cultured in various batches of sera) against Abelson 8.1 or EL-4 tumor target cells (the effector cell: target cell ratio should be 100:1). Unstimulated MΦ will not show any sign of tumor cell cytotoxicity. Additional tests for activating/inhibitory effects include the culture of MΦ with increasing amounts of FCS (e.g., 0.5–10%) in the absence or presence of suboptimal or optimal concentrations of known MΦ-stimulatory agents (e.g., IFN-γ, LPS, IFN-γ plus LPS); the 24 and 48 h supernatants of these cultures are tested for the accumulation of nitrite and/or MΦ cytokines (e.g., TNF). All sera are heat-inactivated for 30 min at 56°C. Aliquots of 25 or 50 mL are kept at 4°C (for daily use) or stored frozen at –20°C.

4. One side of the FEP-Teflon membrane is chemically etched to become hydrophilic, the other side of the membrane is hydrophobic. During storage the FEP membrane should be tightly packed to avoid contamination of the foil with dust. Teflon® cell culture bags are also commercially available (e.g., Miltenyi, Bergisch-Gladbach, Germany or Süd-Laborbedarf, Gauting, Germany).
5. Anprolene® must be stored in a cool place protected from sunlight (safety cabinet), because it is extremely flammable. Avoid breathing Anprolene® vapor and exposure to liquid Anprolene®, which can cause irritation of the skin and of the respiratory tract, headache, nausea, vomiting, or cardiac irregularities as well as neurotoxicity and mutagenesis during chronic exposure.
6. Fresh preparation of the FACS staining buffer with sterile PBS, FCS and EDTA is recommended for each experiment due to the fast contamination of the buffer. Residual buffer can be stored at 4°C and can be used as long as no bacterial or fungal contamination is visible. Buffer is kept on ice during staining procedures.
7. Cervical dislocation must be performed carefully. Avoid tearing the neck, which will lead to hemorrhages into the peritoneal cavity and subsequent deactivation of the peritoneal MΦ *(37, 38)*.
8. The yield of peritoneal cells increases with the age/size and varies with the mouse strain. Female CD1 outbred mice of 8–12 weeks of age or retired breeders are ideal. Do not use mice younger than 5–6 weeks of age (these mice frequently have an umbilical hernia, which might lead to loss of peritoneal lavage fluid during harvesting of the cells). The cellular composition of PECs depends on the eliciting agent and the length of the time period between i.p. injection and cell harvest. For example, after thioglycolate injection the PECs are neutrophil-rich within the first 18 h (maximum at 5–6 h) *(39)*, lymphocyte-rich at day 2 after injection, and macrophage-rich 4–5 days after injection *(30)*.
9. If the bags are used for the culture of BMMΦ, the hydrophobic side of the Teflon® foil is inside so that the MΦ do not strongly adhere to the membrane. In contrast, the bags that are used for the culture of L929 cells are hydrophilic inside to allow firm adhesion of the fibroblasts to the membrane. The hydrophobic and hydrophilic side of the FEB-Teflon membrane can be identified with the help of a waterproof permanent text marker (e.g., Edding™). It is possible to write on the hydrophilic side of the foil, whereas the textmarker ink fails to stick to the hydrophobic surface.

10. The yield of MΦ per Teflon® bag depends on the number of seeded macrophage precursor cells, the lot of L929 supernatant (SN), and/or the source of rmM-CSF. The concentration of L929 SN in the conditioned medium should be titrated to determine the optimal conditions for generation of BMMΦ. A recovery of more than $50 \times 10^6$ viable BMMΦ per bag is not possible, because in this case the nutrient supply in the culture medium would be insufficient and the cells die.

    In general the recovery of BMMΦ is higher with L929 SN compared to rmM-CSF. In our hands the quality of rmM-CSF varies strongly between different commercial sources. Thus, in case the yield of BMMΦ is poor, the testing of other preparations of rmM-CSF is recommended.

11. If self-made Teflon® bags are not available, BMMΦ can also be generated in commercially available bags (*see* **Note 4**) or in cell culture dishes (petriPERM®) with a gas-permeable base made of a hydrophobic plastic foil (bioFolie®) (VivaScience, Hannover, Germany). We do *not* recommend the use of bacteriological Petri dishes or tissue culture flasks for the generation of BMMΦ as described by others *(40)*. BMMΦ strongly attach to the surface of tissue culture plates and can only be detached with a cell scraper (*see* **Subheadings 2.3** and **3.3**), treatment with accutase (*see* **Subheading 3.3.5**), incubation with PBS/10 mM EDTA (10–15 min, 37°C; works poorly in our hands) or combination of these methods. All these methods may damage the cells or modify their activation status *(41)*.

12. If several different MΦ populations are to be stained with the same cocktail of mAbs, one master mix of fluorochrome-labeled mAbs should be prepared.

13. Do not fix the MΦ with paraformaldehyde or commercial FACS fixatives. First, PI can no longer be used for the differentiation of live and dead cells. Second, fixation impairs the separation of distinct subpopulations in the forward and side scatter.

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# Chapter 15

# Analyzing Classical and Alternative Macrophage Activation in Macrophage/Neutrophil-Specific IL-4 Receptor-Alpha-Deficient Mice

**Frank Brombacher, Berenice Arendse, Reagon Peterson, Alexandra Hölscher, and Christoph Hölscher**

## Summary

Macrophage activation can be divided into a classical and an alternative pathway. Interferon-gamma-induced, classically activated macrophages are indispensable for protective effector responses against intracellular pathogens. However, excessive inflammatory immune responses mediated by classical macrophage activation can also be detrimental to the host. In contrast, the IL-4 receptor-alpha-mediated alternative pathway of macrophage activation has been proposed as a mechanism to attenuate excessive inflammation. Indeed, the generation of macrophage/neutrophil-specific IL-4 receptor-alpha-deficient mice ($LysM^{cre}IL\text{-}4R\alpha^{-/lox}$) enables us now to evaluate the importance of this type of macrophage activation in vivo. Thus, the analysis of $LysM^{cre}IL\text{-}4R\alpha^{-/lox}$ mice and the phenotypic characterization of macrophage activation during inflammatory immune responses become of major importance for inflammation research, and useful markers have been identified that allow classically and alternatively activated macrophages to be distinguished. Inducible nitric oxide synthase and arginase-1 are not only prototypical markers of classical and alternative macrophage activation, but both enzymes are also strongly involved in regulating macrophage effector mechanisms and inflammatory immune responses. In this chapter, we describe the use of $LysM^{cre}IL\text{-}4R\alpha^{-/lox}$ mice and present experimental procedures to determine classical versus alternative macrophage activation by analyzing nitric oxide synthase and arginase-1 in vitro and in vivo in this murine model.

**Key words:** Inflammation, Classical macrophage activation, Interleukin-4 receptor-alpha, Alternative macrophage activation, Inducible nitric oxide synthase, Arginase-1.

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## 1. Introduction

The cell-mediated immune response is known to be critical in host defense against infection with intracellular pathogens such as mycobacteria. Interleukin (IL)-12 promotes the differentiation of T helper (TH)1 cells that play an important role in protective immune responses primarily by the production of interferon-gamma (IFN-γ). IFN-γ in turn stimulates the antimicrobial activity of infected macrophages, allowing intracellular bacterial killing through the production of reactive nitrogen intermediates (RNI), reactive oxygen intermediates, and other effector molecules. This inflammatory reaction causes impairment of tissue function, which is usually reversible following elimination of the invading pathogen. However, persistent chronic infection often leads to hyperinflammation and eventually to pathological tissue destruction. In order to control or terminate inflammation and to prevent uncontrolled release of mediators, multiple regulatory factors are operative in balancing the immune system mainly by modulating inflammatory macrophages.

Myeloid lineage cells such as dendritic cells and macrophages are capable of directing $CD4^+$ T cell polarization toward TH1 or TH2 differentiation *(1–3)*. TH1-dominated immune responses follow recognition of microbial products by innate immune cells that release IL-12 *(2)*. Although less well understood, there is also evidence to support a role for dendritic cells and macrophages in promoting TH2 responses *(1, 3)*. Hence, elucidation of the mechanisms by which this occurs is an area of intense study. Macrophage activation can be distinctly divided into a classical versus alternative pathway dependent on exposure to type 1 or type 2 cytokines, respectively *(1)*. Classically activated macrophages (caMϕ) are induced by IFN-γ and bacterial stimuli such as lipopolysaccharides (LPS). caMϕ produce tumor-necrosis-factor (TNF), IL-12, and RNI and are therefore indispensable effector cells of protective immune responses against intracellular pathogens, such as *Mycobacterium tuberculosis* and *Leishmania major (1, 4, 5)*. Despite this beneficial role in host defense, inflammatory immune responses can also be detrimental to host tissue; therefore, an alternative pathway of macrophage activation has been proposed as a mechanism for attenuation of excessive inflammation *(1, 3)*

Alternatively activated macrophages (aaMϕ) are induced by IL-4 and IL-13 via the IL-4 receptor-alpha chain (IL-4Rα), a crucial component that is common to their respective heterodimeric type 1 and type 2 IL-4R receptors. We recently established global ($IL\text{-}4R\alpha^{-/-}$) *(6)* and macrophage/neutrophil-specific IL-4Rα-deficient ($LysM^{cre}IL\text{-}4R\alpha^{-/lox}$) *(7)* mice as experimental model systems to characterize the phenotypic and functional properties

of aaMϕ as well as their potential role in human disease. Examination of these transgenic animal models using *Trypanosoma sp. (8–10)*, *Taenia crassips (11)*, or *Brugia malayi (12)* and subsequent gene expression profiling resulted in the identification of several marker genes for aaMϕ, such as the IL-4Rα, arginase1 (Arg-1), FIZZ1 (RELMα), YM1, the mouse mannose receptor (MMR), macrophage galactose-type C-type lectins (mMGLs), and 12/15 lipoxygenase (12/15-LOX) (**Table** 1). However, little is known about the actual functional role of these markers in aaMϕ. One of these, 12/15-LOX generates bioactive lipid mediators, which serve as ligands for the nuclear hormone receptor PPAR-α, also present on lymphocytes. Binding of aaMϕ-derived lipids to PPAR-α on activated T cells blocks cell proliferation by promoting inhibition of IL-2. This may account for the suppressive function of IL-4/IL-13-activated macrophages in *Taenia* infection by a heterogeneous $CD11b^+$ $Gr\text{-}1^+$ cell population, known as myeloid suppressor cells (MSC) *(11)*. IL-4/IL-13-activated MSC manifest 12/15-LOX activity, thereby secreting molecules that via PPAR-α activation, inhibit proliferation of T cells. Tumor-recruited $CD11b^+$ MSC are also known mediators of tumor-associated

**Table 1**
**Typical markers of caMϕ and aaMϕ and approaches to analysis**

| Marker | Classical activation | Alternative activation | In vitro analysis | In vivo analysis | References |
|---|---|---|---|---|---|
| IL-12 | ▲ | ▼ | RT-PCR, ELISA | RT-PCR, ELISA | *(7)* |
| TNF | ▲ | ▼ | RT-PCR, ELISA | RT-PCR, ELISA | *(7)* |
| FIZZ | ▼ | ▲ | RT-PCR | RT-PCR | *(28)* |
| YM-1 | ▼ | ▲ | RT-PCR | RT-PCR | *(28)* |
| IL-4Rα | ▼ | ▲ | RT-PCR, FACS | RT-PCR, FACS | *(7)* |
| Mouse mannose receptor (MMR) | ▼ | ▲ | RT-PCR, FACS | RT-PCR, FACS, immuno-histochemistry (IHC) | *(29)* |
| 12/15 Lipoxygenase (12/15-LOX) | ▼ | ▲ | RT-PCR | RT-PCR | *(11)* |
| Macrophage galactose-type C-type lectins (mMGLs) | ▼ | ▲ | RT-PCR, FACS | RT-PCR, FACS | *(9)* |
| WSX-1 | ▼ | ▲ | RT-PCR, FACS | RT-PCR, FACS | *(30)* |
| NOS2 | ▼ | ▲ | RT-PCR, Griess | RT-PCR, Griess, IHC | *(21)* |
| ARG-1 | ▼ | ▲ | RT-PCR, urea | RT-PCR, urea, IHC | *(31)* |

immune dysfunction, and recent studies *(12)* demonstrated that IL-4Rα is a marker of these cells. Indeed, IL-4Rα$^{+}$ CD11b$^{+}$ cells (but not IL-4Rα$^{-}$CD11b$^{+}$ cells) from tumor-bearing mice constitutively released IL-13 and IFN-γ and thereby suppressed the generation of tumor-specific cytotoxic T lymphocytes. This may be explained by a combination of NOS2 and Arg-1 activity resulting in reactive mediators able to suppress cytotoxic T lymphocytes *(12)*, this mechanism that was impaired in LysM$^{cre}$IL-4Rα$^{-/lox}$ mice, which were able to eject their tumors.

In contrast, *Schistosoma mansoni* infection studies demonstrated a beneficial and crucial role for IL-4Rα-bearing aaMφ in restricting egg-mediated intestinal immunopathology. This prevented the development of sepsis and subsequent mortality during acute schistosomiasis which otherwise was observed to occur in the absence of IL-4Rα-expressing macrophages in infected LysM$^{cre}$IL-4Rα$^{-/lox}$ mice *(7)*. Moreover, infected, myeloid-cell-specific IL-4Rα deficient LysM$^{cre}$IL-4Rα$^{-/lox}$ mice showed increased TH1 responses and TH1-dominant liver granuloma formation, but interestingly collagen deposition and fibrosis were present, suggesting that these responses were independent of aaMϕ *(13)*. Conversely, a detrimental role for aaMϕ was observed in *Leishmania major* infection as infected LysM$^{cre}$IL-4Rα$^{-/lox}$ mice showed strikingly delayed disease progression compared to nonhealer BALB/c mice *(14)*. Interestingly, in both schistosomiasis and in leishmaniasis increased TH1 responses were observed in LysM$^{cre}$IL-4Rα$^{-/lox}$ mice suggesting that immunomodulatory properties of aaMϕ are in part related to effects on T helper cell dichotomy *(13, 14)*. A similar type1 response was observed during proteoglycan-induced arthritis, which was exacerbated in LysM$^{cre}$IL-4Rα$^{-/lox}$ mice *(15)*. Together, the investigations of experimental disease models using a myeloid-cell-specific IL-4Rα-deficient mouse have demonstrated the involvement of aaMϕ in immunomodulation, immunosuppression, and immunopathology. These cells were also observed to promote tissue repair, and to influence innate and adaptive immune responses, and their ultimate impact on disease expression was dependent upon the specific model under examination.

Hence, the phenotypic characterization of macrophage activation during inflammatory immune responses is of major importance. As mentioned earlier, numerous markers have been defined that can be used for the experimental determination of classical and alternative macrophage activation in vitro and in vivo. Inducible nitric oxide synthase (NOS2) and Arg-1 are two such prototypic markers of classical and alternative macrophage activation, respectively. Classical macrophage activation plays an important role in combating infection with intracellular pathogens through IFN-γ-induced expression of the NOS2-dependent production of RNI *(16–18)*. However, uncontrolled macrophage

activation may also lead to immunopathology *(7)*. Arg-1 is an essential enzyme of the urea cycle and is expressed at high levels in hepatocytes. The enzyme hydrolyzes L-arginine to urea and L-ornithine; therefore, its main function in the liver is the detoxification of ammonia. Because L-ornithine is a necessary metabolite required for the production of prolines which in turn control collagen production, arginase activity is linked to tissue remodeling eventually leading to fibrosis *(19)*. The production of RNI is counter-regulated by IL-4Rα-dependent mechanisms, leading to Arg-1-expressing aaMϕ. Since NOS2 shares L-arginine as a substrate with arginase, substrate depletion by either enzyme is a key regulatory mechanism *(20, 21)*, and differential expression of NOS2 and the Arg-1 is important for regulating macrophage effector functions and immunopathology *(20, 22)* (**Fig. 1**).

As should be clear from the information reviewed earlier, NOS2 and Arg-1 are both prototypical markers of classical and alternative macrophage activation and also centrally involved in regulating inflammatory immune responses. Thus, analysis of both enzymes in vitro and in vivo is an important tool for evaluating differential macrophage activation during inflammatory immune responses. In this chapter, we describe the use of LysM$^{cre}$IL-4Rα$^{-/lox}$ mice and related experimental procedures to examine

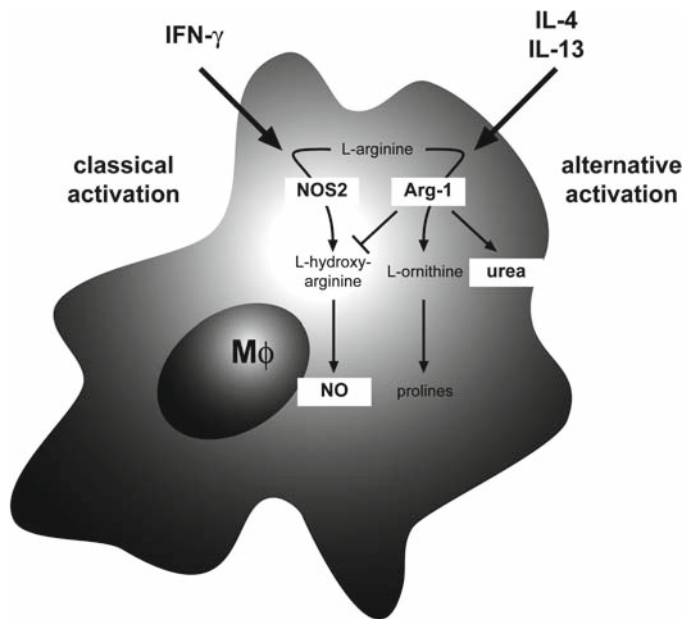


Fig. 1. L-Arginine metabolism in caMϕ and aaMϕ. In IFN-γ-induced caMϕ and IL-4/IL-13-generated aaMϕ, L-arginine is used as a substrate by both NOS2 and Arg-1. NOS2 metabolizes L-arginine to citrulline and NO, which is important for antimicrobial effector mechanisms in macrophages. Arg-1 is an essential enzyme of the urea cycle. The enzyme converts L-arginine to urea and L-ornithine, the latter being a necessary metabolite for the production of prolines which in turn are required for collagen production.

classical versus alternative macrophage activation in vitro and in vivo. Currently, this is the only cell-type specific animal model available to analyze differential macrophage activation in vivo.

## 2. Materials

### *2.1. Generation, Breeding Strategy, and Genotyping of Macrophage/ Neutrophil-Specific IL-4Rα-Deficient Mice*

1. Tail lysis buffer: 50 mM Tris–HCl, pH 8, 100 mM EDTA, 100 mM NaCl, 1% (w/v) SDS, proteinase K stock solution (10 mg/mL in $ddH_2O$; store aliquots at –20°C).
2. We recommend use of commercial double-distilled water ($ddH_2O$).

#### *2.1.1. DNA Extraction from Tail Biopsies*

#### *2.1.2. PCR*

Mice may be genotyped by conventional or real-time PCR the latter also allowing for quantitative analysis of Cre-mediated deletion efficiency. For both methods, we recommend use of commercial $ddH_2O$ and primer pairs at a working concentration of 6.25 µM in $ddH_2O$ (**Table** 2).

Conventional PCR

1. 200 µL PCR strips with lids.
2. Taq polymerase and 10× ThermoPol buffer (5 U/µL).
3. dNTPs (4 × 100 µmol, prepare stocks at 10 mM in $ddH_2O$).
4. $ddH_2O$. Appropriate aliquots are stored at –20°C.
5. 10× loading buffer: 1 mM sodium EDTA, 0.4% bromophenol blue, 50% (w/v) glycerol.
6. 10× TBE: 890 mM Tris, 890 mM boric acid, 20 mM EDTA, pH 8.0.
7. 1.6% agarose gel [1.6% (w/w) agarose in 0.5× TBE; microwave, cool to 60°C and add 10 mg/mL ethidium bromide (ETBR) to a final concentration of 0.45 µg/mL (*see* **Note 1**)]. Pour in gel trays and add gel combs.
8. 100-bp DNA ladder.

Real-Time PCR

1. 100 µL PCR tubes with caps.
2. 2× SensiMix DNA kit (50× SYBR Green, 2× Sensimix [reaction buffer, heat-activated Taq polymerase, 6 mM $MgCl_2$, internal reference and stabilizers], 50 mM $MgCl_2$).
3. RotorGene RG-3000A real-time cycler and RotorGene 6 version 6.1 software (Corbett Research).

**Table 2**
**Primer sequences, annealing temperatures, and elongation times for genotyping macrophage/neutrophil-specific LysM$^{cre}$IL-4R$\alpha^{-/lox}$ mice and quantitative real-time RT-PCR of NOS2 and Arg-1**

| | Forward primer | Reverse primer | Annealing | Elongation |
|---|---|---|---|---|
| Conventional PCR | | | [°C] | [s] |
| IL-4Rα wild type | TGACCTACAAG-GAACCCAGGC | CTCGGCGCACT-GACCCATCT | 58 | 60 |
| IL-4Rα deleted | GGCTGCTGACCT-GGAATAACC | CCTTTGAGAACT-GCGGGCT | 58 | 60 |
| IL-4Rα lox | CCCTTCCT-GGCCCTGAATTT | GTTTCCTCCTAC-CGCTGATT | 60 | 60 |
| LysMcre | CTTGGGCTGCCA-GAATTTCTC | CCCAGAAAT-GCCAGATTACG | 60 | 60 |
| Real-time PCR | | | [s] × [°C] | [s] |
| IL-4Rα wild type | TGACCTACAAG-GAACCCAGGC | CTCGGCGCACT-GACCCATCT | 20 × 58 | 60 |
| IL-4Rα deleted | GGCTGCTGACCT-GGAATAACC | CCTTTGAGAACT-GCGGGCT | 20 × 57 | 30 |
| LysMcre | CTTGGGCTGCCA-GAATTTCTC | CCCAGAAAT-GCCAGATTACG | 45 × 57 | 60 |
| Quantitative real-time RT-PCR | | | [°C] | [s] |
| NOS2 | AGCTCCTCCCAG-GACCACAC | ACGCTGAGTAC-CTCATTGGC | 60 | 10 |
| ARG-1 | CAGAAGAATGGAA-GAGTCAGA | CAGATATGCAG-GGAGTCACC | 63 | 10 |
| HPRT | GGCCATGAGGCT-GGATCTC | AACATTTGAATC-CTGCAGCCA | 60 | 10 |

### *2.2. Phenotyping Classical/Alternative Macrophage Activation In Vitro*

#### *2.2.1. In Vitro Generation of ca/aaMϕ*

1. 48-well tissue culture plates, bacterial-grade, and tissue-culture-grade petri dishes.
2. Disposable sterile cell scraper.
3. DMEM: Dulbecco's Modified Eagle's Medium (DMEM, high glucose; supplemented with 10% (v/v) FCS, 2 mM L-glutamine, 100 U/mL penicillin/100 µg/mL streptomycin, 1 mM sodium–pyruvate, 50 µM 2-mercaptoethanol.
4. PLUZNIK medium: DMEM (high glucose) supplemented with 10% (v/v) FCS, 5% (v/v) horse serum, 30% macrophage-colony-stimulating factor (M-CSF)-containing L929

supernatant (*see* **Note 2**), 2 mM L-glutamine, 100 U/mL penicillin/100 µg/mL streptomycin, 1 mM sodium–pyruvate, 50 µM 2-mercaptoethanol. Accutase.

5. For tissue culture purposes, we recommend use of commercial PBS w/o $Mg^{2+}$ and $Ca^{2+}$ (PAA).
6. Recombinant murine IFN-γ, IL-4, IL-13, and LPS are diluted in DMEM at the indicated final concentrations.

*2.2.2. Gene Expression Analysis of ca/aaMϕ*

11. RNAse-free 1.5-mL microtubes.
12. Lightcycler capillaries and adapters.
13. We recommend use of commercial PBS and double-distilled water ($ddH_2O$).
14. Primer pairs at a working concentration of 6.25 µM in $ddH_2O$ (**Table** 2).
15. RNA extraction:
    (a) Trifast.
    (b) 1-brom-3-chlorpropane.
    (c) GenElute linear polyacrylamide.
    (d) Isopropanol, 75% (v/v) ethanol in diethylpyrocarbonate water (*see* **Note 3**).
6. Reverse transcription (RT):
    (a) Oligo $p(dT)_{12–18}$ (25 U in 750 µL DEPC water).
    (b) dNTPs (100 µmol each, prepare stocks at 10 mM in DEPC water).
    (c) Ribolock ribonuclease inhibitor (40 U/µL).
    (d) Revertaid H minus M-muLV reverse transcriptase including 5× reaction buffer (10,000 U).
7. Quantitative real-time PCR:
    (a) $MgCl_2$ (50 mM $MgCl_2 \times 6H_2O$, autoclave).
    (b) 10× PCR buffer (200 mM Tris, 500 mM KCl, pH 8.4, sterile filtrate).
    (c) dNTPs (4 × 100 µmol, prepare stocks at 10 mM in $ddH_2O$).
    (d) 100× bovine serum albumin.
    (e) SYBR Green (dilute 1:1,000 in DMSO).
    (f) Taq polymerase (5 U/µL). Appropriate aliquots are stored at –20°C.

*2.2.3. Metabolic Analysis of ca/aaMϕ In Vitro*

1. Flat-bottomed 96-well microtiter plates.
2. We recommend use of commercial PBS w/o $Mg^{2+}$ and $Ca^{2+}$ and $ddH_2O$. For serial dilutions of the nitrite standard, DMEM is (*see* **Subheading 2.2.1**) used.

3. Griess reaction (detection of $NO_2$): 2 mM $NaNO_2$, 2.5% (v/v) $H_3PO_4$, Griess reagent #1 (1% sulfanilamide in 2.5% $H_3PO_4$), Griess reagent #2 (0.1% N-(1-naphthyl) ethylenediamine dihydrochloride in 2.5% $H_3PO_4$). All solutions are stored at 4 C in the dark.
4. Urea assay (detection of arginase activity): 0.1% (v/v) Triton X-100 in $ddH_2O$ supplemented with protease inhibitor cocktail, 25 mM Tris–HCl (pH 7.5), 10 mM $MnCl_2$, 0.5 M L-arginine (pH 9.7), 20 mg/mL urea, stop solution (1:3:7 [v/v/v] 96% $H_2SO_4$, 85% $H_3PO_4$ ($ddH_2O$), freshly prepared 9% (w/v) α-isonitrosopropiophenone (ISPF) dissolved in 100% ethanol.

### *2.3. Functional Analysis of Classical/ Alternative Macrophage Activation n Vivo*

#### *2.3.1. Histopathological and Immunohistochemical Analysis of ca/aaMϕ*

General Buffers and Chemicals

1. Xylol.
2. Different concentrations (100%, 70%, 40% [v/v]) of acetone in $ddH_2O$.
3. TBS (150 mM M NaCl, 10 mM Tris, pH 7.4).
4. 1% (v/v) $H_2O_2$ in TBS.
5. 3,3′-Diaminobenzidine (DAB) Sigma FAST Tablet set.
6. Kaiser's glycerol gelatin.
7. Gill's hematoxylin (20 mM hematoxylin, 3 mM sodiumiodate, 155 mM aluminum sulfate, 250 mL 25% (v/v) ethylene glycol, 6% (v/v) conc. acetic acid in tap water) (*see* **Note 4**).

NOS2 Staining

1. For antigen-retrieval, any pressure cooker could be used.
2. 10 mM citric acid monohydrate (pH 6.0).
3. 10% (v/v) FCS in TBS.
4. Rabbit antimouse NOS2 monoclonal antibody.
5. Mouse serum.
6. Goat antirabbit IgG peroxidase.
7. Rabbit antigoat IgG peroxidase.

Arg-1 Staining

1. 1% (w/v) SDS in TBS.
2. Avidin/Biotin-Blocking kit containing avidin D and biotin.
3. M.O.M. (*mouse on mouse*) Immunodetection kit containing mouse immunoglobulin (Ig)-blocking reagent.
4. M.O.M. protein concentrate.
5. M.O.M. biotinylated antimouse IgG reagent.
6. Vectastain ABC reagent A and reagent B.
7. Mouse antimouse Arg-1.

*2.3.2. Metabolic Analysis of ca/aaMϕ In Vivo*

1. Proteinase inhibitor cocktail (complete Mini EDTA-free tablets).
2. Nitrate determination:
   (a) Serum separator tubes.
   (b) Micron YM-30 centrifugal filter units.
   (c) Cayman Nitrate/Nitrite Colorimetric Assay kit containing nitrate reductase, enzyme cofactors, nitrate standard, nitrite standard.
   (d) Griess reagents #1 and #2.
   (e) 96-well microtiter plates.
3. Arginase activity:
   (a) Tissue homogenizator (e.g., Potter Elvehjem homogenizator).
   (b) For further material *see* also **Subheading 2.2.3**.

## 3. Methods

### *3.1. Generation, Breeding Strategy, and Genotyping of Macrophage/ Neutrophil-Specific IL-4Rα-Deficient Mice*

Functional studies on the role of aaMϕ in experimental mouse models for human diseases have been aided by the use of macrophage/neutrophil-specific IL-4Rα-deficient mice. These mice are created using homologous recombination in embryonic stem cells in combination with the Cre/loxP recombinase system. Gene targeting in BALB/c embryonic stem (ES) cells and Cre/loxP-specific site-specific recombination was performed to generate so called floxed IL-4Rα$^{lox/lox}$ BALB/c mice, flanking exon 7–9 of *IL4Rα (7)*. This results in a silent mutation of the IL-4Rα gene. Mice that specifically express the Cre recombinase in macrophages/neutrophils under control of the lysozyme M promoter (LysM$^{cre}$ mice) were generated by a *knock-in* approach *(23)*. LysM$^{cre}$ mice were first backcrossed to BALB/c for nine generations and then intercrossed with global IL-4Rα$^{-/-}$ BALB/c mice *(6)* to establish double transgenic LysM$^{cre}$IL-4Rα$^{-/-}$ BALB/c mice. These mice were further intercrossed with IL-4Rα$^{lox/lox}$ BALB/c mice to generate cell-type-specific LysM$^{cre}$IL-4Rα$^{-/lox}$ BALB/c mice *(7)* (**Fig. 2**). Efficient deletion of the IL-4Rα gene and subsequent impairment of IL-4Rα responsiveness in macrophages and neutrophils only was demonstrated *(7)*. In experiments, transgene negative littermates IL-4Rα$^{-/flox}$ are used as controls. This breeding strategy has the advantage of avoiding potential aberrant Cre expression. IL-4Rα hemizygosity ($^{-/lox}$) increases the probability of Cre-mediated deletion of the "floxed" allele *(7)*

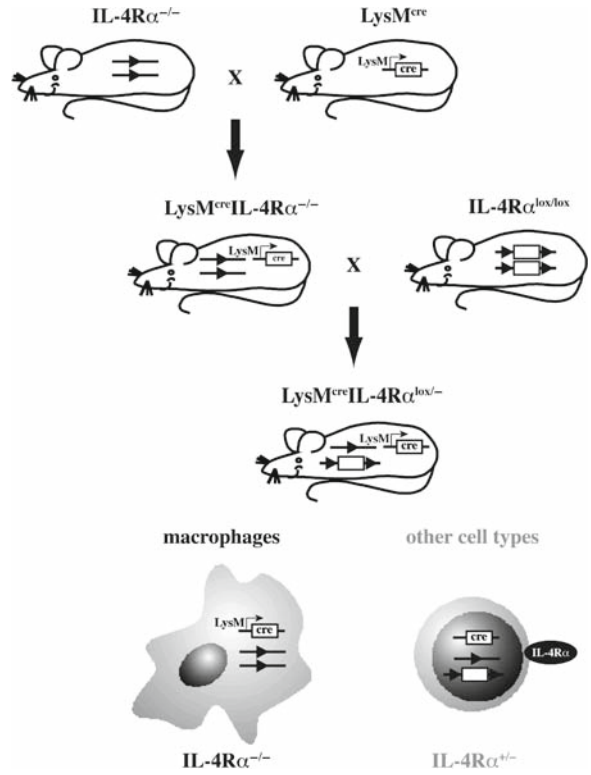


Fig. 2. Principle of macrophage/granulocyte specific deletion of the IL-4Rα. Conditional IL-4Rα$^{lox/-}$ mice are generated by homologous recombination. In these mice, exons of the IL-4Rα are flanked by two loxP sites (*triangles*) that are recognized by the Cre recombinase. Mice that specifically express the Cre recombinase in macrophages/neutrophils under control of the lysozyme M promoter (LysM$^{cre}$ mice) were first intercrossed with global IL-4Rα$^{-/-}$ mice to avoid non-Mendelian inheritance due to early and aberrant Cre activity (unpublished observation). To facilitate efficient Cre-mediated recombination, LysM$^{cre}$ IL-4Rα$^{-/-}$ mice were crossed with conditional IL-4Rα$^{lox/lox}$ mice. Because the Cre recombinase is only expressed in macrophages and neutrophils of hemizygote LysM$^{cre}$ IL-4Rα$^{lox/-}$ offspring, loxP-flanked exons of the IL-4Rα locus on one allele are deleted only in these cell types. Hemizygote mice are used to reduce the substrate for the enzyme and to increase efficiency of Cre-mediated deletion in macrophages/neutrophils. In other cell types, the enzyme is not active and a functional IL-4Rα is still expressed. In experiments, Cre-negative IL-4Rα$^{lox/-}$ littermates serve as controls for macrophage/neutrophil-specific LysM$^{cre}$ IL-4Rα$^{lox/-}$ mice.

Control and cell-type-specific LysM$^{cre}$IL-4Rα$^{-/lox}$ mice are routinely genotyped by either conventional or real-time PCR (**Fig. 3**). By real-time PCR, Cre-mediated deletion efficiency in cell-type-specific LysM$^{cre}$IL-4Rα$^{-/lox}$ mice was examined in purified macrophage cell suspensions. Nonmyeloid cells from cell-type-specific LysM$^{cre}$IL-4Rα$^{-/lox}$ mice may also serve as wild-type controls.

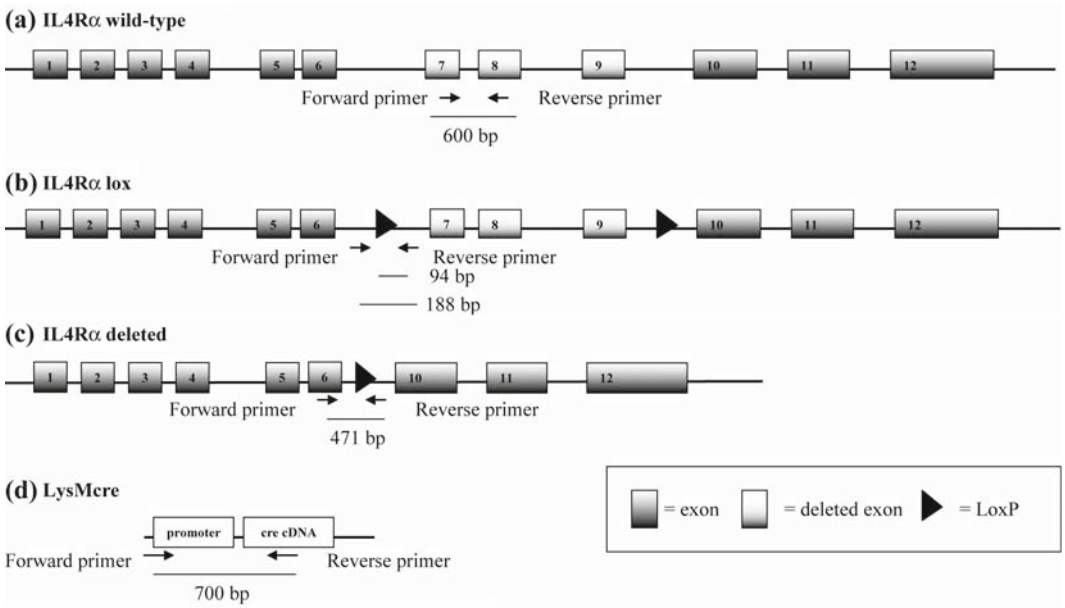


Fig. 3. Primer binding sites for genotyping of macrophage/granulocyte-specific LysM$^{cre}$IL-4Rα$^{lox/-}$ mice. IL-4Rα locus maps with primer binding sites for (**A**) IL-4Rα wild-type allele, the forward primer binds in exon 7 and the reverse primer in exon 8 resulting in the amplification of a 600-bp band. (**B**) IL4Rα lox PCR, the forward and reverse primer binds in intron 6 (3′ of exon 6 and 5′ of exon 10), flanking the lox p site, resulting in an amplified product of 188 bp (lox p specific) or 94 bp (wild type). **(C)** IL-4Rα deleted PCR, gene targeting, and Cre-mediated site-specific recombination at intron 6 and intron 9 results in the deletion of exons 7, 8, and 9 in the conditional IL4Rα$^{lox/-}$ mouse. The forward primer binds in exon 6 and the reverse primer in the intron 5′of exon 10 resulting in an amplification product of 471 bp. (**D**) LysM$^{cre}$ PCR, the forward primer binds upstream, within the *knocked-in* transgene, to the lysozyme M promoter and the reverse primer binds within the cre cDNA yielding an amplification product of 700 bp.

*3.1.1. DNA Extraction from Tail Biopsies*

1. Use a robust pair of surgical scissors to perform the tail biopsies.
2. Add 500 µL of lysis buffer containing 0.5 mg/mL proteinase K to a 1-cm mouse tail in a 1.5-mL microtube and incubate overnight at 56°C with rotation.
3. After lysis, agitate tube to make sure that digest is complete and centrifuge samples at 1,400 × *g* for 10 min.
4. Remove supernatant and add to 500 µL isopropanol in a fresh 1.5-mL microtube. Mix well to precipitate DNA and centrifuge at 14,000 × *g* for 10 min.
5. Carefully remove supernatant, leaving the DNA pellet. Wash pellet with 70% ethanol at 14,000 × *g* for 2 min.
6. Remove ethanol and allow excess ethanol to dry at room temperature. Add 500 µL ddH2O and dissolve pellet well. Use 2 µL per PCR reaction for mouse genotyping.

*3.1.2. PCR*

Conventional PCR

1. Keep all reagents and samples on ice. The Taq enzyme should be kept in a *cooling block* on the bench.
2. For preparation of PCR master mix, calculate the final amount of following components based on the number of sample plus 2. Per sample add 5 μL 10× PCR buffer, 4 μL dNTPs, 2 μL 6.25 μM forward primer, and 2 μL 6.25 μM reverse primer, 0.05 μL 5 U/μL Taq polymerase, 34.95 μL $ddH_2O$.
3. Aliquot 48 μL of master mix to each PCR tube, add 2 μL of sample DNA and firmly close tubes.
4. Set up the PCR program. The following parameters are the same for the different primer sets: 40 cycles of 94°C denaturation for 30 s, annealing (annealing temperature; **Table 2**) for 20 s, and extension at 72°C (for extension time see **Table 2**). There is usually an initial 60-s denaturation at 94°C for all primer sets.
5. After PCR, add 10 μL 6× loading buffer to 50 μL of the amplified product. Run 30 μL on a 1.6% agarose gel to which ETBR is added at 0.45 μg/mL using 0.5× TBE as running buffer (*see* **Note 1**). Use a DNA ladder to size products.
6. After gel electrophoresis, genotypes are evaluated according to the resulting band sizes (**Fig. 3**, **Table 3**).

Real-Time PCR

1. Keep all reagents and samples on ice.
2. For preparation of the PCR master mix, it is advisable to make up at least two extra volumes of the reaction mix. Calculate the final volume of the following components based

**Table 3**
**Genotyping of macrophage/neutrophil-specific $LysM^{cre}IL\text{-}4R\alpha^{-/lox}$ mice**

| | +/+ | +/– | –/– |
|---|---|---|---|
| Conventional PCR | Band size [bp] | | |
| IL-4Rα wild type | 600 | 600 | – |
| IL-4Rα deleted | – | 471 | 471 |
| IL-4Rα lox | 188 | 188/94 | 94 |
| LysMcre | – | 700 | 700 |
| Real-time PCR | Melting curve range [°C] | | |
| IL-4Rα wild type | 84.5–86.5 | 84.5–86.5 | – |
| IL-4Rα deleted | – | 86–88 | 86–88 |
| LysMcre | – | 85–87 | 85–87 |

on the number of sample plus the extra volume. Per sample add 10 µL 2× Sensimix, 0.5 µL 6.25 µM forward primer, and 0.5 µL 6.25 µM reverse primer, 0.4 µL 50× SYBR green, 6.6 µL dd$H_2O$.

3. Aliquot 18 µL of master mix to each PCR tube, add 2 µL of sample DNA, and firmly close tubes.
4. Set up the PCR program. The following parameters are the same for the different primer sets: 40 cycles of 94°C denaturation for 30 s, annealing at (annealing temperature; **Table 2**) for (annealing time; **Table 2**), and extension at 72°C for (extension time; **Table 2**). There is usually an initial 60-s denaturation at 94°C for all primer sets.
5. A melting range of 72–95°C is set up for all primer pairs, with an initial 45 s hold at the first temperature increment of 0.5°C, and a 5-s hold for each temperature increment thereafter.
6. On completion of the PCR reaction, genotypes can be evaluated according to the resulting melting curve range obtained for each primer pair (**Fig. 3**, **Table 3**).

### *3.2. Phenotyping Classical/Alternative Macrophage Activation In Vitro*

caMϕ are induced by IFN-γ/LPS whereas alternative macrophage activation is mediated by IL-4 and IL-13. The generation of both types of macrophages is observed in the presence of the respective stimuli.

#### *3.2.1. In Vitro Generation of ca/aaMϕ*

To analyze macrophage activation in vitro, different types of primary macrophages may be used. Here we describe the in vitro generation of macrophages from bone marrow precursor cells (BMMϕ) *(24)*.

##### Generation of BMMϕ

1. Euthanize mouse and fix the body on a cork board on a clean bench. Peel skin from the top of each hind leg and down over the foot. Remove excess muscle from legs by holding end of bone with forceps using scissors to push muscle downward away from forceps. Sever leg bones between joints and prepare femur and tibia.
2. Attach 10-mL syringe to 26-G needle and fill with DMEM. Insert needle into bone marrow cavity of femur and tibia. Flush bone cavities with DMEM medium until bones appear white. Collect flushed medium in a sterile 50-mL conical tube on ice.
3. Centrifuge cells at 320 × *g* for 10 min at 4 C. Resuspend cell pellet in 10 mL PLUZNIK medium per mouse and transfer to a tissue-culture-grade petri dish.
4. Incubate cells at 37°C and 5% $CO_2$ in a humidified incubator overnight.

5. Transfer nonadherent cells to 50-mL sterile conical tube and centrifuge cell suspension at 320 × *g* for 10 min at 4°C. Resuspend cell pellet in PLUZNIK medium and count cells.
6. Adjust concentration to $1.5 \times 10^6$ cells per mL and distribute every 10 mL of cell suspension to bacterial-grade petri dish ($1.5 \times 10^7$ cells). Incubate cells for 4 days at 37°C and 5% $CO_2$ in a humidified incubator (*see* **Note 5**).
7. Take off supernatant and add 10 mL fresh medium. Continue incubation for another 3 days.
8. Remove and discard culture supernatant. Wash remaining adherent cells with 15 mL prewarmed PBS.
9. Discard PBS wash and add 5 mL accutase to detach cells and incubate dishes 10 min at 37°C.
10. Gently remove cells by unidirectional scraping dish with a disposable sterile cell scraper (*see* **Note 6**).
11. Add 10 mL DMEM to dish and harvest detached cells. Wash remaining cells in dish with 10 mL DMEM, collect washes in 50-mL conical tubes, and centrifuge cell suspension at 320 × *g* for 10 min at 4°C. Resuspend cell pellet in DMEM and count cells.

### Induction of caM$\phi$ and aaM$\phi$ by Stimulation of BMM$\phi$ with IFN-γ/LPS or IL-4/IL-13

Classical or alternative activation of macrophages is facilitated by the presence of microbial stimuli or different cytokines. Whereas LPS and IFN-γ mediate the development of caMϕ, IL-4 and IL-13 induce aaMϕ. Hence, incubation of in vitro generated BMMϕ with either IFN-γ/LPS or IL-4/IL-13 will lead to classical or alternative activation, respectively *(7, 25)*.

1. Adjust cell concentration to $1.0 \times 10^6$ per μL in DMEM. Place 500 μL cell suspensions into wells of 48-well plates. Prepare enough samples for duplicate or triplicate sets to be processed under the following conditions:
   (a) Resting cells alone.
   (b) Cells treated with IFN-γ/LPS.
   (c) Cells treated with IL-4.
   (d) Cells treated with IL-13.
2. Incubate cells 4 h to allow macrophages to adhere, then gently wash wells two times with 500 μL DMEM.
3. To appropriate samples add:
   (a) DMEM.
   (b) IFN-γ/LPS to 10 U/mL and 10 ng/mL respectively, final concentrations.
   (c) IL-4 to 50 U/mL final concentration.
   (d) IL-13 to 500 U/mL final concentration.

4. Incubate cells for 24–72 h at 37°C and 5% $CO_2$ in a humidified incubator.
5. Supernatants and cell lysates are taken at 24, 48, and 72 h to analyze the expression of markers for classical and alternative macrophage activation (**Subheadings 3.2.2** and **3.2.3**).

*3.2.2. Gene Expression Analysis of ca/aaMϕ In Vitro*

We routinely perform quantitative analysis of NOS2 and Arg-1 gene expression in activated BMMϕ and tissue using a Light Cycler (Roche) *(4)* (*see* **Note 7**).

RNA Isolation

1. For each individual sample, prepare RNAse-free 1.5-mL microtubes and add 83 µL PBS to each.
2. After collecting culture supernatants for further analysis (*see* **Subheading 3.2.3** "**NOS2 Activity (Griess Reaction in Supernatants)**", **step 2**), add 250 µL Trifast to each well and resuspend cells. Check for efficient cell lysis under a microscope. Remove lysates and add to microtubes containing prealiquotted PBS and incubate for 10 min at room temperature (*see* **Note 8**).
3. Add 100 µL 1-brom-3-chlorpropane (BCP) per tube, shake well, and incubate at room temperature for 15 min.
4. For each individual sample, prepare fresh RNAse-free 1.5-mL microtubes and add 1 µL linear polyacrylamide (LPA). After centrifugation of samples at 14,000 × *g* for 20 min at 4°C, carefully transfer the upper aqueous phase to the tubes containing LPA. Add 250 µL isopropanol, mix by carefully turning tubes and incubate for 10 min at room temperature.
5. Centrifuge for 10 min at 14,000 × *g* at 4°C. Carefully remove supernatants, add 1 mL 75% ethanol, and vortex.
6. Centrifuge for 5 min at 9,000 × *g* at 4°C. Carefully remove supernatants, air dry pellet, and add 50 µL DEPC water. Store samples at –80°C.

Reverse Transcription

1. Unfreeze RNA on ice and preheat thermoblocks.
2. For each individual sample, prepare RNAse-free 1.5-mL microtubes and add 2 µL oligo dT.
3. Add 8 µL total RNA from samples to oligo dT-containing tubes, vortex, and incubate for 10 min at 65°C.
4. In the meantime, prepare RT master mix. Calculate the final amount of the following components based on the number of samples to be run plus 2. Per sample add 2 µL $ddH_2O$, 4 µL 5× reaction buffer, 2 µL dNTPs, 1 µL ribonuclease inhibitor, 1 µL reverse transcriptase to a single microtube and vortex.
5. After incubation, spin tubes briefly and place on ice. Add 10 µL RT master mix to each sample, vortex, and incubate for 90 min at 37°C. Stop transcription by increasing the temperature to 95°C and incubate for 2 min.

6. Quickly place samples on ice. After the samples are cooled, add 180 µL $ddH_2O$. Samples can be stored at 4°C. For long-term storage freeze samples at –80°C.

Quantitative Real-Time PCR of NOS2 and Arg-1

1. Set up the PCR program. The following parameters are the same for the different primer sets: 95°C denaturation for 3 s, annealing at (annealing temperature; **Table** 2) for 5 s, and extension at 72°C for (extension time; **Table** 2). There is usually an initial 30-s denaturation at 95°C for all primer sets.
2. Place Lightcycler capillaries in centrifuge adaptors and cool at 4°C.
3. Prepare dilutions of samples and standards (start with 3.73 ng/mL [$1 \times 10^6$ copies/µL], make up five dilutions 1:10 down to 10 copies/µL) in $ddH_2O$. Keep dilutions on ice.
4. For preparation of PCR master mix, calculate the final amount of following components based on the number of samples to be run plus 2. Per sample add 4.75 µL $ddH_2O$, 0.7 µL 50 mM $MgCl_2$, 1.0 µL 10× PCR buffer, 0.5 µL 10 mM dNTPs, 0.6 µL BSA, 0.4 µL 6.25 µM forward primer, and 0.4 µL 6.25 µM reverse primer. Before aliquotting master mix into capillaries, add 0.2 µL SYBR green and 0.1 µL 5 U/µL Taq polymerase.
5. Vortex master mix and add 9 µL to each capillary. Add 1 µL of each sample to capillaries and carefully place the cap on the capillary by gently applying firm pressure from directly above using the tip of a finger. Put cooled centrifuge adaptors with light cycler capillaries into the centrifuge. Quick spin at 380 × *g*. Remove capillaries and keep in cooling block until they are placed in the carousel of the light cycler (*see* **Note 9**).
6. Carefully transfer the capillaries to the carousel of the Lightcycler. Press down firmly using pressure from directly above the capillary and place the carousel into the Lightcycler.
7. Start the PCR program by clicking on the *RUN* button. When the run is finished the Lightcycler will automatically exit the *Running Screen* and display the collected data on the Lightcycler *Data Analysis* (*LCDA*) front screen. Select the *Melting Curve* program by clicking on the *Melting Curve* button. Check that the water controls are clear and that the product melting peaks are defined.
8. Use the Lightcycler Software to calculate the amount of target from the standard curve. You may use either the *Fit Points* or *Second Derivative Maximum* methods. Use both the *Comparative Threshold Cycle* (*CT*) *Method* and *Standard Curve Method* to compare the fold difference between samples.

### *3.2.3. Metabolic Analysis of ca/aaMϕ*

To analyze enzyme activity of NOS2 and Arg-1 in in vitro cultivated macrophages, specific metabolites of both enzymes are measured in either culture supernatants (NOS2) or cell lysates

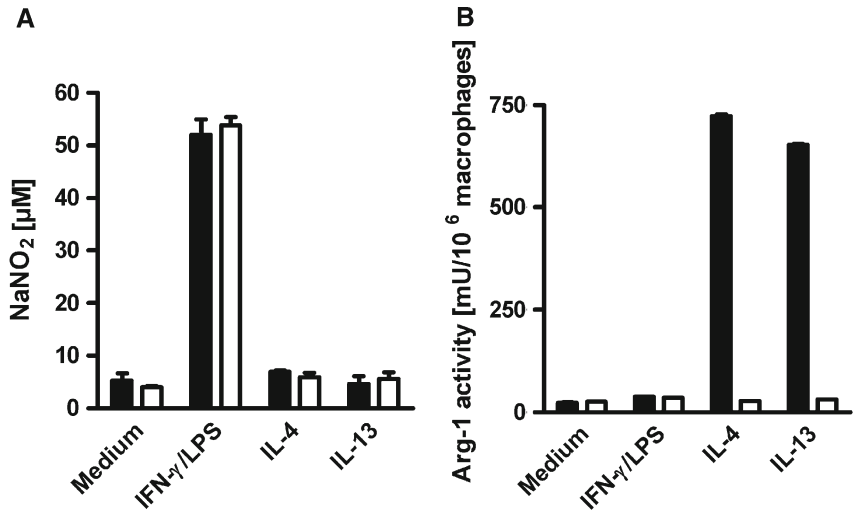


Fig. 4. Nitric oxide and urea production of caMϕ and aaMϕ in vitro. BMMϕ from control (*black bars*) and macrophage/neutrophil-specific LysM$^{cre}$IL-4Rα$^{-/lox}$ mice (*white bars*) were stimulated with medium, IFN-γ/LPS, IL-4, or IL-13. After 72 h, (**A**) $NaNO_2$ production in supernatants and (**B**) Arg-1 activity were measured using the Griess reaction and urea production, respectively. Whereas IFN-γ/LPS promote enzymatic activity of NOS2 and production of NO (classical activation), IL-4 and IL-13 induce Arg-1 expression and the release of urea (alternative macrophage activation).

(Arg-1) *(14)*. Whereas the Griess reaction measures the NOS2-mediated production of nitrite (**Fig. 4a)**, the urea assay measures the Arg-1-dependent production of urea (**Fig. 4b**).

### NOS2 Activity (Griess Reaction in Supernatants)

Nitric oxide undergoes a series of reactions with several molecules present in biological fluids. In supernatants of activated macrophages, the vast majority of NO is converted nitrite ($NO_2^-$). The amount of nitrite may be quantified using the Griess reaction, in which nitrite is converted into a deep purple azo compound. Photometric measurement of the absorbance due to this azo chromophore accurately determines nitrite concentration.

1. Add 50 µL of macrophage supernatants that have been collected after 24, 48, and 72 h of stimulation to wells of a 96-well microtiter plate (*see* **Note 10**).
2. In this plate, set up twofold serial dilutions in DMEM of nitrite solution (1 mM to 1 µM final concentration) in a final volume of 50 µL.
3. First, add 50 µL of Griess reagent #1 to nitrite standard and samples. Then add 50 µL Griess reagent #2 (*see* **Note 11**).
4. Measure absorbance at 550 nm in a microtiter plate reader (*see* **Note 12**).

### Arginase Activity (Urea Assay)

Urea is synthesized from l-arginine by Arg-1. To analyze enzyme activity in macrophages in vitro, exogenous L-arginine is added to cell lysates and urea turnover is measured *(14, 26)*.

1. Samples, controls, and urea standard are required for this assay as follows:
   (a) Cell lysates with exogenous arginine.
   (b) Cell lysates without exogenous arginine.
   (c) All reagents without cell lysates.
   (d) Serial dilutions of urea as standard.
2. Wash cells two times with 500 µL PBS (*see* **Note 13**).
3. Add 100 µL of 0.1% Triton X-100 containing a protease inhibitor cocktail to cells and incubate on a shaker for 30 min (*see* **Note 14**).
4. Add 100 µL of 25 mM Tris–HCl and place 100 µL of each lysates into two fresh 1.5-mL microtubes (a) and (b), add 10 µL of 10 mM $MnCl_2$ and activate Arg-1 activity by heating for 10 min at 56°C. Place also 100 µL of 0.1% Triton X-100 with protease inhibitor cocktail and 100 µL of 25 mM Tris–HCl in a separate tube (c).
5. Add to one lysate 100 µL of 0.5 M L-arginine (a), add 100 µL $ddH_2O$ to control lysates (b). Incubate lysates at 37°C for 15–20 min to conduct arginine hydrolysis (*see* **Note 15**).
6. In the meantime, set up 200 µL twofold serial dilutions of urea standard (1 mg/mL to 1 µg/mL final concentration in $ddH_2O$) (d).
7. Add 800 µL of stop solution to all tubes and add 40 µL α-isonitrosopropiophenone solution; vortex tubes thoroughly until phases are mixed, and heat at 95°C for 30 min (*see* **Note 16**).
8. Add 200 µL of samples (a), controls (b, c), and urea standard (d) to wells of a 96-well microtiter plate and measure the urea concentration at 540 nm in a microtiter plate reader (*see* **Note 17**).

### 3.3. Functional Analysis of Classical/Alternative Macrophage Activation In Vivo

Classical and alternative macrophage activation may also be analyzed in murine tissue and serum, e.g., after infection with various pathogens *(7, 14, 26)*. In contrast to in vitro studies of pure macrophage cultures, it is difficult to correlate markers of caMϕ and aaMϕ such as the production of RNI or urea, respectively, in vivo to a single cell type because these metabolites are also produced by other cells. In contrast, flow cytometric analysis, e.g., of MMR or IL-4Rα expression on gated macrophages can identify aaMϕ in vivo. Alternatively, immunohistochemical analysis of NOS2 and Arg-1 expression may also be used to identify caMϕ and aaMϕ, respectively. Gene expression analysis or determination of enzymatic activity of NOS2 and Arg-1 in whole tissue may also classify classical and alternative macrophage activation. Here we present immunohistochemical and enzymatic methods to detect NOS2 and Arg-1 in murine tissue or serum.

*3.3.1. Histopathological and Immunohistochemical Analysis of ca/aaMϕ*

Both enzymes can be detected in formalin-fixed and paraffin-embedded tissue *(7, 27)*.

Tissue Processing

After removing organs from mice, tissue specimens are fixed and placed in 10% neutral buffered formalin. The purpose of fixation is to preserve tissues permanently in as life-like a state as possible. Fixation should be carried out as soon as possible after removal of the tissues to prevent autolysis. Once the tissue has been fixed, it has to be dehydrated and infiltrated with paraffin. The tissue, placed in cassettes, is processed using an automated tissue processor. The automation process, used to cope with large volumes of routine samples, moves the tissues through the various reagents on a preset time frame. Once processed, the tissue is removed from the cassettes manually and placed in a block over which molten paraffin is poured. This *embedding* process is very important, as the tissue must be aligned, or oriented, correctly in the block of paraffin. Once the tissues have been embedded, they are cut into 2–10 μm sections using a microtome. The cut sections are floated on a warm water bath that helps to remove "wrinkles" in the section and thereafter picked up on a coated glass microscopic slide. The glass slides are placed on a warm plate for approximately 3 h to facilitate drying and adherence of the section to the slide.

1. The embedding process must be reversed in order to get the paraffin wax out of the tissue and allow water soluble dyes to penetrate the sections. Therefore, before any staining can be done, the slides are "deparaffinized" by running them through xylol to acetone to water. Once placed in a slide rack, slides can be easily transferred to different staining dishes. By this means, slides are deparaffinized by consecutive incubation steps for 10 min each in xylol, 100% acetone, 70% acetone, 40% acetone. Slides are then rinsed with $ddH_2O$.
2. Place slides three times in different trays filled with TBS. Before staining, leave slides in TBS.

Formalin or other aldehyde fixation forms protein crosslinks that mask the antigenic sites in tissue specimens, thereby giving weak or false negative staining for immunohistochemical detection of certain proteins such as NOS2 and Arg-1. Depending on the antigen, different antigen retrieval methods have to be evaluated to obtain optimal staining results.

NOS2 Expression

1. For immunohistochemical staining of NOS2 (**Fig. 5a, b**), a citrate-based buffer is used for antigen retrieval to break the protein crosslinks, thereby unmasking the epitope in the formalin-fixed and paraffin-embedded tissue sections. This enhances staining intensity of the anti-NOS2 antibodies. Boil citrate buffer in a pressure cooker, add rack with tissue

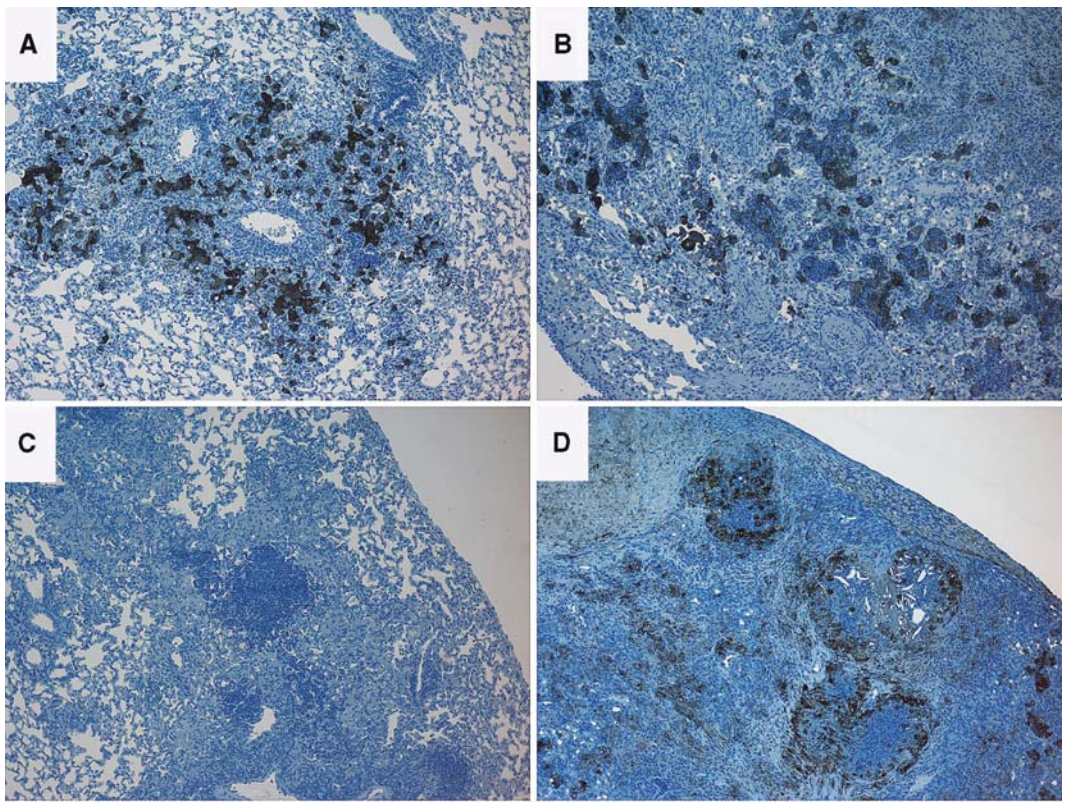


Fig. 5. NOS2 and Arg-1 expression in murine tissue in vivo. (**A**, **C**) C57BL/6 and (**B**, **D**) IL-13-transgenic mice were infected with 100 CFU of M. tuberculosis via the aerosol route. After 63 days, the expression of (**A**, **B**) NOS2 and (**C**, **D**) Arg-1 was determined in formalin-fixed and paraffin-embedded lung tissue by immunohistochemistry (notice brown stainings). Whereas IFN-γ production in both *M. tuberculosis*-infected C57BL/6 and IL-13-transgenic mice leads to NOS2 expression (classical macrophage activation), overexpression of IL-13 also induces and is required for Arg-1 expression (alternative activation).

sections, and heat slides for another 2 min. Afterward, rinse slides three times in different staining dishes filled with TBS. Block endogenous peroxidase by incubating slides in freshly prepared 1% $H_2O_2$/TBS for 20 min in the dark. Wash slides three times in TBS.

2. Drain slides and wipe excess buffer. For staining, add 200 µL rabbit anti-NOS antibody diluted 1:800 in TBS/10% FCS and place cover slips on slides to evenly disperse solution. Incubate slides in a humid chamber for 45 min at room temperature.
3. Discard cover slips and wash slides three times with TBS. Drain slides and wipe excess buffer. Add 200 µL secondary antibody solution containing goat antirabbit IgG peroxidase conjugate (1:30 in TBS/10% FCS) and mouse serum (1:10 in TBS/10% FCS). Place cover slips on slides and incubate in a humid chamber for 45 min at room temperature.

4. Discard cover slips and wash slides three times with TBS. Drain slides and wipe excess buffer. Add 200 µL rabbit anti-goat IgG peroxidase conjugate diluted 1:50 in TBS/10% FCS. Place cover slips on slide and incubate in a humid chamber for 45 min at room temperature.
5. Fifteen minutes before incubation is finished, prepare DAB substrate solution by mixing 1 DAB and 1 urea tablet in 5 mL dd$H_2O$.
6. Discard cover slips and wash slides three times with TBS. Drain slides and wipe excess buffer. Add 200 µL DAB solution and incubate without cover slip for 5 min in the dark. Staining should be controlled under the microscope. Stop enzymatic reaction by washing slides with TBS.
7. Counterstain in Gill's hematoxylin for 3 min. Wash in running tap water for 10 min until nuclei are blue.
8. Mount slides in 70 µL Kaiser's glycerol gelatin that has been prewarmed at 37°C.

Arg-1 Expression

1. For immunohistochemical staining of Arg-1 (**Fig. 5c, d**), SDS is used for antigen retrieval to increase the staining intensity by Arg-1 immunohistochemistry. Place tissue section in a staining dish containing 1% SDS in TBS and incubate for 5 min at room temperature. Afterward, rinse slides three times in different staining dishes filled with TBS. Quench endogenous peroxidase by incubating slides in freshly prepared 1% $H_2O_2$/TBS for 20 min in the dark. Wash slides three times in TBS. Drain slides and wipe excess buffer.
2. To block endogenous biotin, add 200 µL avidin D-blocking reagent, add cover slips to evenly disperse the solution, and incubate in a humid chamber for 15 min at room temperature. Remove cover slip after incubation and discard. Rinse slides three times with TBS, drain, and wipe excess buffer. Add 200 µL biotin-blocking reagent. Add cover slips and incubate for 15 min in humid chamber at room temperature.
3. Remove cover slip and discard. Rinse slides three times for 2 min each with TBS, drain, and wipe excess buffer. Add 200 µL M.O.M. Ig-blocking reagent (two drops of reagent in 2.5 mL TBS) to block unspecific binding of endogenous immunoglobulins. Add cover slips and incubate in humid chamber for 1 h at room temperature. Remove cover slip after incubation and discard. Rinse slides three times with TBS for 2 min each, drain, and wipe excess buffer. Add 200 µL M.O.M. diluent (600 µL protein concentrate in 7.5 mL TBS) and incubate in humid chamber for 5 min at room temperature. Drip off excess M.O.M. diluent after incubation (*see* **Note 18**).

4. For staining of Arg-1, add 200 µL mouse anti-Arg-1 antibody diluted 1:250 in M.O.M. diluent to slides. Add cover slips on slides and incubate in a humid chamber for 45 min at room temperature.
5. Remove cover slip after incubation and discard. Rinse slides three times for 2 min each with TBS, drain, and wipe excess buffer. Add 200 µL biotinylated antimouse IgG reagent in M.O.M. (10 µL antibody solution in 2.5 mL M.O.M. diluent). Add cover slips and incubate in a humid chamber for 10 min at room temperature.
6. In the meantime, prepare Vectastain ABC reagent (add two drops of reagent A in 2.5 mL TBS and mix; add two drops of reagent B to this solution and mix; ABC reagent should be prepared 15 min before use).
7. Remove cover slip after incubation and discard. Rinse slides three times for 2 min each with TBS, drain, and wipe excess buffer. Add 200 µL Vectastain ABC reagent. Add cover slips and incubate in a humid chamber for 5 min at room temperature.
8. Rinse slides three times for 5 min each with TBS, drain, and wipe excess buffer. Add 200 µL DAB solution and incubate without cover slip for 5 min in the dark. Staining should be monitored microscopically. After sufficient staining (**Fig. 5**), stop enzymatic reaction by washing slides with TBS.
9. Counterstain in Gill's hematoxylin for 3 min. Wash in running tap water for 10 min until nuclei are blue.
10. Mount slides in 70 µL Kaiser's glycerol gelatin that has been prewarmed at 37°C.

### *3.3.2. Metabolic Analysis of ca/aaMϕ*

#### Determination of Nitrate

The final products of NO in vivo are both nitrite and nitrate ($NO_3^-$). Because nitrate is not detected in the Griess reaction, it has to be reduced to nitrite first. To measure NO production in murine tissue or serum, the first step is the conversion of nitrate to nitrite utilizing the nitrate reductase. This conversion can be measured using the Griess reaction as described in **Subheading 3.2.3 "NOS2 Activity (Griess Reaction in supernatants)"**. However, to facilitate the conversion of nitrate to nitrite we utilize a commercial nitrate/nitrite colorimetric assay. To obtain reasonable results, samples taken from murine tissue have to be deproteinized first.

1. For determination of nitrate in murine tissue, prepare pieces of organs of approximately 1 $cm^2$. Determine tissue weight (*see* **Note 19**) and place pieces in 1.5-mL microtubes containing 1 mL of a proteinase inhibitor cocktail in PBS. Homogenize organs and centrifuge at 14,000 × *g* for 20 min at 4°C. Transfer supernatants into fresh 1.5-mL microtube.

2. Place YM-30 filters into these new 1.5-mL microtubes. Equilibrate filters by adding 100 µL dd$H_2O$ and centrifuge at 14,000 × *g* for 5 min at 4°C. Discard filtrate and place washed filters in new tubes (*see* **Note 20**).
3. Transfer 300 µl homogenate supernatants into washed filters. Centrifuge at 14,000 × *g* for 20 min at 4°C, discard filter, and use filtrate for assay (*see* **Note 21**).
4. For determination of nitrate in serum, add freshly isolated murine blood to serum separator tubes on ice. Centrifuge at 4,000 × *g* for 10 min at 4°C. Use supernatant for assay (*see* **Note 22**).
5. To prepare a nitrate standard, place 0.9 mL assay buffer in a 1.5-mL microtube. Add 0.1 mL of reconstituted nitrate and vortex. The concentration of this stock is 200 µM. In a 96-well microtiter plate, set up twofold serial dilutions of this nitrate stock solution (100 µM to 0.8 µM final concentration) in a final volume of 80 µL.
6. Samples containing nitrate are assayed by the addition of up to 80 µL of sample per well to the plate. When using less than 80 µL of sample, the volume must be adjusted to 80 µL by addition of the appropriate volume assay buffer. When necessary, dilution of samples should be done using the assay buffer. Serum should be diluted 1:2 in assay buffer.
7. Add 200 µL of water or assay buffer to the microtiter plate as blank. Do not add any other reagents to these wells.
8. Add 10 µL of the enzyme cofactor mixture to each well. Add 10 µL of the nitrate reductase mixture to each well. Seal the plate and incubate at room temperature for 3 h.
9. Add 50 µL of Griess reagent #1 to each well. Immediately, add 50 µL of Griess reagent #2 to each well. Allow the color to develop for 10 min at room temperature.
10. Measure absorbance at 550 nm in a microtiter plate reader (*see* **Note 23**).

Arg-1 Activity

To analyze enzyme activity of Arg-1 in murine tissue ex vivo, exogenous L-arginine is added to tissue homogenates and urea turnover is measured as described in **Subheading 3.2.3 "Arginase Activity (Urea Assay)"** *(7, 26)*

1. To determine arginase activity in murine tissue, weighed pieces of organs are placed in 1.5-mL microtubes. Add 100 µL of 0.1% Triton X-100 containing a protease inhibitor cocktail. Homogenize organs and determine arginase activity ex vivo using 1–100 µL of homogenates following the method described in **Subheading 3.2.3 "Arginase Activity (Urea Assay)"** (*see* **Note 24**).

## 4. Notes

1. Protective gloves must be worn at all times when handling ETBR as well as eye and skin protection. ETBR and ETBR-contaminated debris should be treated as hazardous waste.
2. M-CSF-containing supernatants are produced using L929 fibroblasts (ATCC [CCL-1], Rockville, MD USA). In numerous 175-$cm^2$ tissue culture flasks (Nunc), $2 \times 10^7$ cells are cultured at 37°C and 10% $CO_2$ in 100 mL DMEM medium (Biochrom) supplemented with 10% (v/v) FCS (Biochrom), 2 mM L-glutamine (PAA), 100 U/mL/100 µg/mL streptomycin (Biochrom). After 7 days, collect supernatants and centrifuge at 2,000 × *g* for 10 min at room temperature. Pool supernatants and store aliquots at –80°C.
3. DEPC inactivates RNAses. To prepare RNAse-inactivated DEPC water, dilute DEPC (Sigma) 1: 1,000 in $ddH_2O$ (B. Braun). Shake well until no more bubbles rise, leave for 24 h, and autoclave.
4. Dissolve all the solids in tap water first. Then add ethylene glycol and acetic acid. Stir at least for 3 h and filter solution before use.
5. The yield of bone marrow cells obtained per mouse varies with the strain. However, a yield of $3–10 \times 10^6$ cells per mouse can be expected.
6. Use of a cell scraper limits destruction of cells. To limit damage further, scrape in one direction only.
7. Gene expression of caMϕ and aaMϕ could also be determined in vivo in murine tissue.
8. After this step aliquots may be stored at –80°C.
9. Be careful when handling the capillaries as they break easily. Do not centrifuge capillaries with speeds higher than 500 × *g*.
10. $NO_2^-$ synthesis begins 4–6 h after treatment with IFN-γ and LPS and is linear for up to 72 h.
11. For optimal results, add sulfanilamide before naphthylenethylenediamine.
12. Production of nitrite can be expressed as total concentration of $NO_2^-$ in µM or calculated as moles $NO_2^-$ on the basis of cell number. The results of the standard dilution should give a standard curve that is linear between 0 and 125 µM nitrite. The detection threshold for this method is about 1 µM $NO_2^-$.
13. It is important to wash macrophages in PBS so as to deplete the cells of arginine-containing medium.

14. At this step, plates can be stored at –80°C.
15. Depending on the amount of Arg-1, incubation time can vary between 15 and 120 min.
16. Secure caps on microtubes to ensure caps do not pop at this high temperature (e.g., by placing a weight on the tubes once in the heating block)
17. One unit of enzyme activity is defined as the amount of enzyme that catalyzes the formation of 1 μmol urea/min; arginase activity of macrophages is calculated as units of enzyme activity on the basis of cell number.
18. After addition of M.O.M. diluent, do not add cover slip. After incubation do not wash with TBS; only dip off excess buffer.
19. Concentration of nitrate/nitrate and arginase activity in murine tissue will be calculated on the basis of tissue weight.
20. Turbidity and proteins in tissue homogenates could interfere with absorbance readings. Therefore, samples are filtrated before the assay.
21. Samples could be stored at –80° until further use.
22. Serum could be stored in separator tubes at –80°C. For assay, use only samples that are not hemolytic.
23. Production of nitrite and nitrate can be expressed as total concentration of $NO_2^-$ and $NO_3^-$ in μM or calculated as moles $NO_2^-$ and $NO_3^-$ on the basis of weight tissue. The absorbance of the samples should be between 0.05 and 1.2 absorbance units. Higher absorbance values imply higher nitrate levels. Under these conditions, however, there may be incomplete conversion of nitrate to nitrite. The detection limit of the assay is approximately 1 μM $NO_2^-$.
24. One unit of enzyme activity is defined as the amount of enzyme that catalyzes the formation of 1 μmol urea/min. Arginase activity in organ homogenates is calculated as units of enzyme activity on the basis of weight tissue.

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"Arginase-1 mediated modulation of granuloma necrosis and immune defense against mycobacterial infections"; Nation-wide Collaborative Grant: "Pulmonary Tuberculosis – host and pathogen determinants of resistance and disease progression." Workpackages E (Animal models).

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# Chapter 16

# Analysis of Matrix Metalloproteinase Secretion by Macrophages

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## Summary

Matrix metalloproteinases (MMPs) are zinc-dependent proteases whose physiological roles include control of leukocyte migration. They are implicated in tissue destruction in inflammatory and infectious diseases. MMPs are not only capable of degrading all components of the extracellular matrix, but they also can modulate the immune response by cleaving cytokines and chemokines to alter their activity. Macrophages secrete a broad range of MMPs and represent a key source of MMPs in inflammatory lesions such as granulomas. Zymography is substrate-based gel electrophoresis that allows direct visualization of MMP activity. Here we describe measurement of MMP secretion from macrophages focusing on quantitative zymography. We also discuss complementary methods that should be used in parallel with zymography. The ability to analyze and quantify MMP secretion by macrophages offers an additional window through which to understand the contributions of macrophages to a wide variety of infectious, inflammatory, and immunologic disorders.

**Key words:** Macrophage, Matrix metalloproteinase, Extracellular matrix, Zymography.

## 1. Introduction

Inflammatory diseases are frequently characterized by excessive degradation of the extracellular matrix (ECM). Matrix metalloproteinases (MMPs) are zinc-dependent proteases that collectively can degrade all components of the ECM *(1)*. They also perform an immunomodulatory role by cleaving cytokines and chemokines to either enhance or reduce their activity *(2)*. Excess MMP activity has been implicated in diverse diseases including atherosclerosis, emphysema, inflammatory arthritis, cancer progression, and, more recently, infectious diseases *(3, 4)*. MMP

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activity is tightly regulated at the level of gene transcription, by secretion as proenzymes that require proteolytic cleavage for activation, by compartmentalization and the secretion of specific tissue inhibitors of metalloproteinases (TIMPs) *(5)*.

Macrophages, as central effector cells of the innate immune response, secrete a broad array of MMPs. The number and the quantity of MMPs secreted by macrophages increase during differentiation from monocytes *(6)*. Zymography is a functional assay that involves electrophoretic separation of proteins followed by degradation of enzyme substrate embedded into the gel, and therefore identifies MMPs with potential functional activity secreted by cells *(7)*. The benefits of the technique include being able to identify multiple MMPs with the same substrate specificity on a single gel, the possibility of identifying both the proenzyme and active form (i.e., MMPs with functional activity secreted by cells), as well as a relative lack of expense. However, zymography is not adequate in isolation, and it is often useful to identify total immunoreactive MMP concentrations. Therefore, in this chapter we shall also briefly consider additional approaches to analysis of MMP secretion which complement zymography. Complex functional and migration assays (which do not usually distinguish between specific MMPs) are beyond the scope of this chapter.

## 2. Materials

Source of materials is shown but where no specific reference is made, supplies are readily obtainable from Sigma.

### 2.1. Cell Culture

1. RPMI supplemented with 200 mM glutamine, ±10% fetal bovine serum. Antibiotics can be added if required.
2. Macrophage-colony-stimulating factor (M-CSF). Reconstitute to a final concentration of 10 mg/mL and store in 250 mL aliquots at –80°C.
3. Ficoll Paque. Store in the dark.

### 2.2. Gelatin Zymography

1. Gelatin solution: Make a 1.2% (w/v) solution by dissolving 1.2 g gelatin in 100 mL water in a water bath set at 65°C for 10 min. Make 5 mL aliquots and store at 4°C.
2. MMP-9 standard. Make aliquots to a final concentration of 1 ng/mL in buffer of 60 mM Tris, 15 mM $CaCl_2$, 80 mM NaCl, and 0.1% (w/v) bovine serum albumin (BSA) and store at –20°C.
3. 5× sample loading buffer: 0.25 M Tris–HCl, pH 6.8, 50% (w/v) glycerol, 5% (w/v) sodium dodecyl sulfate (SDS), 0.01 g bromophenol blue. Make 10 mL and store in 1 mL aliquots at room temperature.

4. 40% acrylamide (29:1 acrylamide:bis-acrylamide ratio) and 1.44 M Tris–HCl, 0.384% SDS, pH 8.8 running gel buffer. For stacking gel buffer use 0.5 M Tris–HCl, 0.4% SDS, pH 6.8.
5. 10% (w/v) ammonium persulfate: 0.1 g in 1 mL $ddH_2O$, make up fresh on each occasion.
6. *N,N,N,N*-Tetramethylethylenediamine (TEMED).
7. Running buffer: 25 mM Tris base, 190 mM glycine, 0.1% (w/v) SDS. Make up 10× stock by dissolving 30.3 g Tris base and 144.1 g glycine in 1 L $ddH_2O$. Store at room temperature. Make working solution by adding 100 mL 10× stock and 10 mL 10% (w/v) SDS and $ddH_2O$ up to a final volume of 1 L.
8. 2.5% (v/v) Triton X-100: 25 mL Triton X-100 in 1 L $ddH_2O$, prepare in advance on stirrer since Triton-X takes time to dissolve. Store at room temperature.
9. Low-salt collagenase buffer: 55 mM Tris–HCl, 200 mM sodium chloride, 5 mM calcium chloride, 0.02% Brij, pH 7.6. Dissolve 60.6 g Tris, 117 g NaCl, and 5.5 g $CaCl_2$ in 900 mL $ddH_2O$, adjust pH to 7.6 with concentrated HCl, make up to 1 L, and store at 4°C. To make 1× working solution, make 100 mL 10× stock up to 1 L with $ddH_2O$, add 670 mL Brij-35, then recheck pH and correct to 7.6 if necessary.
10. 0.2% Coomassie blue stock solution: Dissolve one tablet PhastGel-Blue (Pharmacia, Sweden) in 80 mL $ddH_2O$ and stir for 10 min. Add 120 mL methanol; then filter at 4°C through Whatman number 1 filter paper. Store at 4°C.
11. Destain solution: 200 mL glacial acetic acid, 600 mL methanol, 1,200 mL $ddH_2O$ to give proportion 1:3:6. Store at room temperature.
12. Single-step stain–destain solution: 5 mL 0.2% Coomassie blue stock with 95 mL destain solution per gel.

### 2.3. Casein Zymography

1. Novex precast 12% Casein gels (Invitrogen, Paisley, UK). These need to be run using the X-Cell *Surelock* Minigel system (Invitrogen).
2. MMP-1 standard. Aliquot 1 µL neat MMP-1, store at –20°C.
3. MMP-1 standard buffer: 50 mM Tris base, 5 mM $CaCl_2$, 0.05% (v/v) Brij 35. 0.6 g Tris base, 0.55 g $CaCl_2$ with 0.05 mL Brij 35 made up to 100 mL, freeze in 0.5 mL aliquots at –20°C.
4. Running buffers, sample loading buffer, low-salt collagenase buffer and destain solutions are as for gelatin zymography (**Subheading 3, item 2**).

5. 0.1% stain solution: 20 mL stock 0.2% Coomassie blue (see earlier), 16 mL dd$H_2O$, 4 mL acetic acid.

## 3. Methods

### 3.1. Cell Culture

MMP secretion increases with monocyte maturation into monocyte-derived macrophages (MDMs). However, the precise conditions of maturation will influence the MMP secretion phenotype. For example MDMs matured in granulocyte-macrophage-colony-stimulating factor (GM-CSF) are likely to have a reduced inflammatory secretion profile producing less MMPs when compared with MDMs matured in M-CSF. We briefly outline our macrophage maturation protocol below, highlighting the key steps with regard to analysis of MMP secretion.

1. Obtain leukocytes either by venesection using a 50-mL syringe or by obtaining buffy coat (leukocyte-rich) residues from blood transfusion services if available. 50 mL human blood will provide ~$1 \times 10^7$ monocytes which will coat a 24-well plate at 250,000 cells/$cm^2$. A buffy coat from 500 mL donated blood will usually produce $1 \times 10^8$ monocytes, sufficient to plate a total of 400 $cm^2$ of tissue culture plastic.
2. Isolate monocytes by density centrifugation through Ficoll-Paque in 50-mL falcon tubes followed by five wash steps by resuspending the PBMC layer in HBSS and centrifuging at 308RCF.
3. Accurate quantification of monocyte numbers prior to adhesion is critical in generating a confluent monolayer that will secrete sufficient MMPs for detection by zymography. Take 20 μL from the 50-mL cell suspension and place in a Neubauer counting chamber. If a buffy coat from 500 mL blood is being used, a 1 in 25 dilution is necessary. Place the counting chamber in a tissue culture incubator at 37°C for 5 min while performing penultimate wash. When final wash has started, remove the counting chamber and examine under phase II of an inverted light microscope. Monocytes will have adhered to the counting chamber, appear gray, and show pseudopodia. Lymphocytes will not adhere and appear brighter, rounder, and smaller.
4. After the final wash step, resuspend the cells in warmed RPMI, with no FCS, since this reduces cell adhesion. Plate the cells at 250,000 monocytes per $cm^2$ and incubate for 1 h at 37°C. During cell adhesion, prepare the RPMI with FCS and M-CSF.

5. After adhesion, wash monolayer three times with HBSS to remove nonadherent cells. Finally add RPMI with FCS and M-CSF 100 ng/ml and incubate for 4 days at 37°C in a 5% $CO_2$ tissue culture incubator.
6. On day 4 (after 96 h in M-CSF) aspirate the RPMI/FCS/M-CSF cell culture supernatant and replace with RPMI/FCS only. Then rest the cells for 24 h.
7. On day 5, the cells are ready for stimulation; this is experiment day 0. Prior to commencing the experiment, the RPMI with FCS is aspirated, cells are washed once more with warmed HBSS, then stimulated in serum-free RPMI.

### 3.2. Gelatin Zymography

MMPs are secreted as proenzymes that, in vivo, require proteolytic cleavage by the release of the cysteine switch *(8)*. Zymography activates proenzyme MMPs by releasing the cysteine switch in a nonproteolytic manner, so that substrate degradation allows the detection of both proenzymes and active cleaved forms of each MMP being analyzed. Consequently, zymography provides data on all the potentially active MMP present. The molecular weight of each band will give an indication of which MMP is causing proteolysis in the gel, but the exact identification needs to be confirmed by running a recombinant MMP standard, performing ethylenediamine tetraacetic acid (EDTA) inhibition (see later) and by a second method such as Western blotting.

This protocol can be applied to any gel system. Our routine practice is to use the Hoeffer full-size plates (15 × 18 cm) since larger plates gives more crisp resolution of the gelatinolytic bands. However, this protocol could easily be amended for minigel systems.

1. Set up plates for gel pouring.
2. Melt gelatin in water bath at 65°C for 10 min (*see* **Note 1**).
3. Prepare 11% polyacrylamide gel by mixing 22 mL 40% acrylamide, 20 mL running gel buffer, 30 mL $ddH_2O$, 8 mL 1.2% gelatin stock, 160 mL TEMED, and 320 mL APS. This volume is sufficient for two full-size plates. Pour gel using a 25-mL disposable serological pipette to level approximately 5 mm below where the comb will be and then overlay with 1 mL isopropanol.
4. Once gel has set (approximately 20 min), pour off the isopropanol, insert combs, and pour stacking gel. Remove combs once the stacking gel has set. Once made up, gelatin gels can be stored overnight at 4°C.
5. Prepare samples and standards by mixing each sample with 5× sample loading buffer to make a final volume of 20 mL (4 mL loading buffer with 16 mL sample). Standards should be diluted to a final concentration of 0.1 ng/mL (e.g., 56 mL

MMP-9 standard buffer, 16 mL sample loading buffer, 8 mL standard at a concentration of 1 ng/mL). Load 20 mL of standard into lane one on each gel and 20 mL of each sample into subsequent wells (*see* **Note 2**).

6. Run gel at 180 V until marker dye reaches bottom of plate, usually 3–4 h (*see* **Note 3**).
7. Once the gel has run, dismantle the electrophoresis tank. Remove the spacers from each side of the gel and lift the upper plate off carefully. Use a gel cutter to remove the stacking gel and then cut a triangle off the gel in the top left hand corner for orientation.
8. Place the gel over a plastic container containing 200 mL 2.5% Triton-X 100. Gently lever off the gel with a spatula so that it falls into the Triton. The gels are fairly robust, but tears have been known to occur at this stage. Incubate gel in Triton for one hour with gentle shaking at 30 rpm on a rocker, thus washing the SDS off the gels.
9. Pour off the Triton, rinse gel in 150 mL of collagenase buffer twice, and then add 200 mL collagenase buffer for incubation (*see* **Note 4**). Place in incubator at 37°C overnight. Minimum incubation time should be 16 h; we routinely incubate for between 16 and 20 h.
10. Pour off collagenase buffer and rinse gel briefly in $ddH_2O$.
11. Add 100 mL single-step stain–destain solution to container. Bands of gelatinolytic activity will be visible within about 2 min, but the optimal length of staining is for 1 h. Gels can be left staining for up to 4 h, though this does not improve resolution further. Use standard band as an indication of when to stop staining.
12. Rinse off stain solution and wash gel once with water. Place on transparency film and wrap in cling-film. Acquire image using a light box with digital camera.
13. For densitometric analysis, we use Scion Image software, which can be downloaded free of charge from http://www.scioncorp.com/pages/scion_image_windows.htm. This was designed for the analysis of black on white bands, such as western blots, and so we invert the image using Adobe Photoshop before analysis. This analysis gives a linear standard curve between 10 and 500 pg MMP-9 (**Fig. 1**).
14. For comparison between different gels, in particular if they have been run on different days, each sample must be standardized by dividing the densitometric value generated by that of the MMP-9 standard. This controls for gel-to-gel variability in proteolysis.

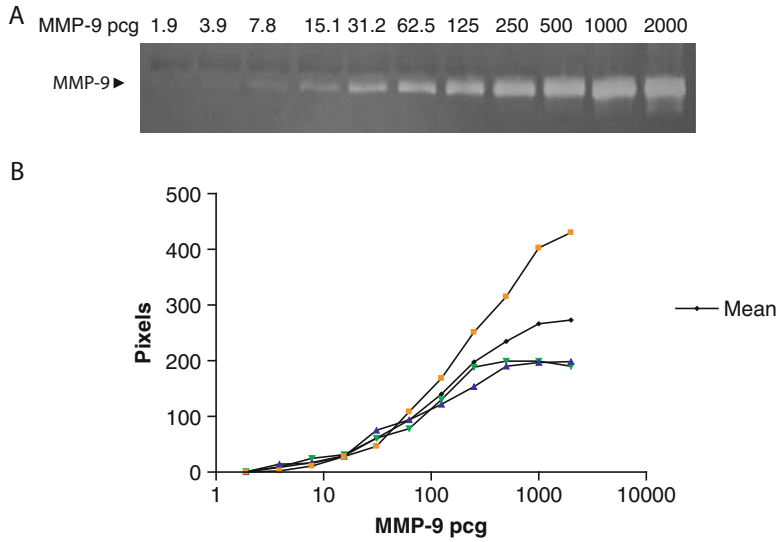


Fig. 1. (**A**) Recombinant MMP-9 was serially diluted to generate a standard curve and analyzed by gelatin zymography. (**B**) Densitometry of three separate gelatin zymograms demonstrating relationship between pixels versus pg MMP-9 loaded per lane, analyzed by NIH Image. Black squares indicate mean of three values.

### *3.3. Casein Zymography*

The technique of casein zymography has many similarities to gelatin zymography, though there are subtle differences that affect sensitivity which are highlighted later. MMP-9 can degrade casein, though not as rapidly as gelatin, and so a band of proteolysis at 90 kDa is seen. However, MMP-1, the major human collagenase, degrades casein relatively slowly, and so prolonged incubation of the gel is needed (*see* **Note 5**). Serial dilutions of recombinant MMP-1 analyzed by casein zymography demonstrate that the lower level of detection is 0.1 ng MMP-1 (**Fig. 2**). Therefore casein zymography is approximately 2 log units less sensitive for MMP-1 than gelatin zymography is for MMP-9. MMP-3 is not usually secreted by cells of the monocyte lineage in sufficient quantities to be detected by casein zymography, but since MMP-3 has a similar molecular weight to MMP-1, the identity of bands must be confirmed by Western blotting for MMP-1 and -3.

MMP-7 also degrades casein, and so a third band of proteolysis occurs at 28 kDa, caused by pro-MMP-7 (**Fig. 3A**). If active MMP-7 is present, further bands representing proteolytically cleaved components of the enzyme may be visualized, though this may require concentration by lyophilization (**Fig. 3B**). Therefore, casein zymography can give multiple readouts identifying several MMPs secreted by macrophages.

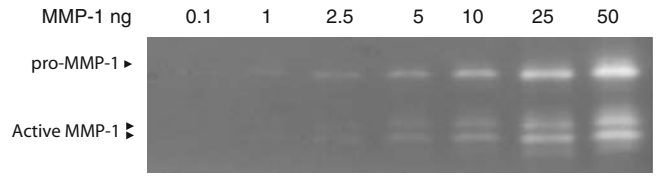


Fig. 2. Recombinant MMP-1 was serially diluted and analyzed by casein zymography.

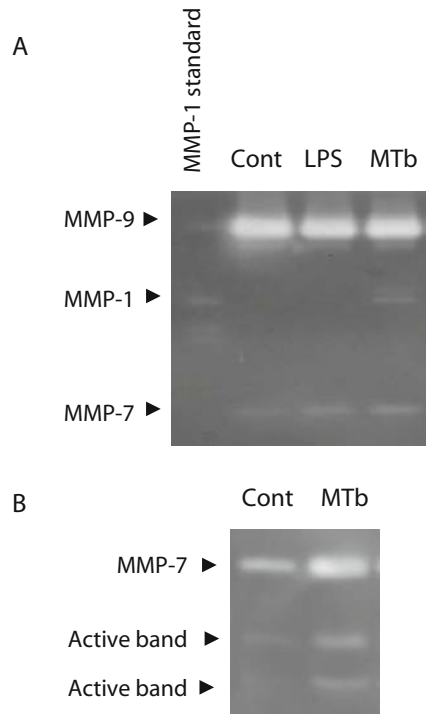


Fig. 3. (**A**) Identification of multiple MMPs on casein zymography. MDMs were left unstimulated or stimulated with LPS or by infection with live *M. tuberculosis* (MTb). Supernatants were harvested at 24 h, sterile filtered, and then analyzed by casein zymography. MMP-9 activity is detected at 92 kDa, MMP-1 at 50 kDa -only in MTb infected cells- and MMP-7 activity at 28 kDa. (**B**) MDMs were infected with live MTb or unstimulated. MMP activity was analyzed in cell culture medium at 72 h after tenfold concentration by lyophilization followed by casein zymography.

1. Remove casein gels from plastic packet and rinse each well three times with 100 µL running buffer (*see* **Note 6**).
2. Assemble minigel tank and fill with running buffer.
3. Prepare standards and samples. 5 ng MMP-1 standard should be loaded. For example, if undiluted standard is 48 µg/mL, 0.52 µL standard mixed with 80 µL MMP-1 standard buffer and 20 µL loading buffer, will give 5 ng standard per well. Add 16 µL of each sample to 4 µL loading buffer.

4. Load 20 µL standard or sample per well and run at 125 V until blue marker reaches end of gel. 2.5 h is usually sufficient (*see* **Note 7**).
5. When gel has run, pour off running buffer, and remove the gels. Extracting the gel from the casing can easily cause tears, so this should be done with care. First, using a metal palette (which is provided with the X-cell Electophoresis module), crack open all three sides of the casing, with the front of the gel casing downward (*see* **Note 8**). A quick, sharp movement is most effective. Then, lift off the back of the casing, ensuring that the gel remains adhered on the front part of the case. Use the palette to cut around the gel, removing the comb indent and freeing the bottom of the gel, and cut a triangle off the top left side for orientation.
6. Place the gel over a plastic container measuring approximately 9 × 15 cm (we use large pipette tip box lids) containing 100 mL 2.5% Triton-X 100. Gently lever the gel off with a spatula so that the gel falls into the Triton. The gel is fragile and so care is needed. Incubate at room temperature for 1 h with gentle agitation.
7. Pour off the Triton and rinse the gel once in 75 mL low-salt collagenase buffer, then incubate in 75 mL low-salt collagenase buffer for 30 min at room temperature with agitation.
8. Place the gel in 100 mL fresh collagenase buffer, cover lid with cling film, and incubate the gel for ~40 h (two nights) at 37°C (*see* **Note 5**).
9. Pour off collagenase buffer and rinse gel briefly with $ddH_2O$. Stain gel with 0.1% Coomassie blue solution for one hour with agitation.

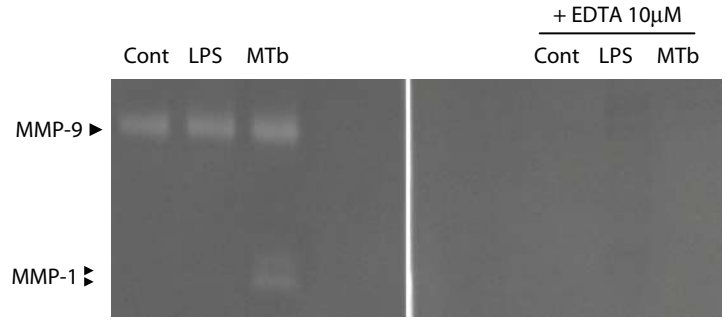


Fig. 4. Cell culture medium from control, LPS-treated, or MTb-infected MDMs was run in duplicate on a casein zymogram. The gel was cut into half and one half developed in collagenase buffer containing 10 mM EDTA. Proteolysis was completely inhibited by divalent cation sequestration, confirming that the bands are caused by MMPs.

10. Pour off stain solution, rinse briefly in water, and then add 100 mL destain solution. Incubate at room temperature with agitation.
11. Usually 30–45 min destain is sufficient. Since this is not a single-step stain–destain solution, the timing of stopping destaining is more critical than for gelatin zymography (*see* **Note 9**).
12. When sufficient destaining has occurred (judged by watching the standard), pour off destain solution and rinse briefly with $ddH_2O$. Place on transparency film, wrap in cling film and acquire image using a lightbox with digital camera.
13. As with MMP-9, standardize between gels by dividing value for samples by MMP-1 standard. MMP-1 appears as a couplet at 50 kDa, while the MMP-1 standard appears as a single band at 50 kDa, with two bands at MW of ~40 kDa (*see* **Note 10**).
14. The identity of proteolytic bands as MMPs should be confirmed by EDTA inhibition (*see* **Note 11** and **Fig. 4**).

### *3.4. Measuring Immunoreactive MMP and TIMP Concentrations; Western Analysis, ELISAs and Luminex Bead Assays*

Zymography is an important method for analysis of MMP secretion since it identifies proteolytic activity, although it may be limited in sensitivity as described earlier. Therefore, we also employ complementary techniques to confirm the identity of the proteolytic bands and to improve sensitivity for MMPs that are secreted at a low level. These complementary approaches involve antibody detection of MMPs and therefore will detect all the immunoreactive MMP being studied, irrespective of whether it has proteolytic potential.

#### *3.4.1. Western Blotting*

This is relatively straightforward, but the sensitivity for MMP-9 is significantly less than for gelatin zymography. Western analysis of MMP-1 has an equivalent sensitivity to casein zymography. A full description of Western analysis may be found in any major methods textbook.

#### *3.4.2. Elisa*

Several commercially produced ELISAs for MMP analysis are available. Although some paired antibody kits are available, many only come as individual precoated plates and therefore are relatively expensive for the analysis of numerous samples.

#### *3.4.3. Multiplexing*

More recently, multiplex analysis of MMPs by bead array technology has become available. This requires a Luminex machine and commercial MMP kits. The technique is straightforward and allows analysis of multiple MMPs in a single 50-μL sample. It is particularly useful for the analysis of precious clinical specimens and low volumes from animal experiments. However, it is less useful for the analysis of single MMPs when the cost means that it

offers no advantage over a single ELISA plate. Some success has been achieved by conjugating antibody to prepurchased beads, which can reduce costs somewhat, but needs quality control. It is not possible to purchase labeled beads for flow cytometric multiplex analysis as are available for cytokine measurements. We understand that some companies are in the process of optimizing this methodology for MMP analysis.

*3.4.4. Sample Sterilization*

MMPs are implicated in the immunopathology of many infectious diseases, and therefore the sterilization of samples to remove pathogens may be necessary. Filtration through a 0.2-μM filter will remove bacteria, but the choice of filter membrane is critical if MMP concentrations in the filtrate are to be analyzed. We have found that MMPs bind to the Anopore membrane, and therefore MMPs are only detectable in the filtrate if present in very high concentrations in the original sample *(9)*. Similarly, polypropylene membranes remove MMPs from the filtrate, which may lead to false negative results. At present, such artifacts in the analysis of MMP activity in infectious diseases models are frequently not recognized. Currently, we use the Durapore 0.2-μM membrane for filtration of *Mycobacterium tuberculosis*-infected samples, as we have found that this membrane displays minimal MMP binding. We recommend that any candidate filtration membrane first be checked for MMP binding activity in a specific pathogen-based culture system before use in sterilizing samples for MMP analysis.

## 4. Notes

1. Melting gelatin without overheating improves reliability of gelatin zymography. Though gelatin can be melted in a microwave, we find that warming in a water bath at 65°C for 10 min produces more consistent results.
2. Macrophages secrete very high quantities of MMP-9. For gelatin zymography, dilution of cell culture supernatant by 1 in 100 is needed, so that the gelatinolytic activity is not above the linear part of the standard curve. In contrast, it is not normally necessary to dilute monocyte supernatants.
3. Since the gelatinases MMP-9 and MMP-2 have MWs of 90 kDa and 72 kDa, respectively, a longer running time is acceptable and may be useful if attempting to separate pro- and active forms of MMPs.
4. The pH of the collagenase buffer is critical for sensitive zymography.

5. The sensitivity of casein zymography can be increased by prolonged incubation in collagenase buffer. However, this leads to less precise bands of proteolysis due to diffusion of MMPs within the gel and if a sample has high levels of either MMP-9 or MMP-7, these may cause widespread proteolysis which may affect other bands that are less intense. This can be circumvented by excising the upper and lower thirds of the gel, leaving only the area of 50 kDa around MMP-1 remaining**.** If MMP-7 is being analyzed and is present at high concentrations, incubation for 16 h may be optimal to prevent excessive caseinolysis.
6. We have attempted to make casein zymograms "in house" but have never been successful in making gels with equivalent sensitivity to those commercially available from Invitrogen. However, these commercially available gels do go off with time, and their sensitivity falls as they approach their sell by date. Therefore it is advisable to try to perform zymography in batches using fresh gels.
7. Excessive electrophoresis time may lead to MMP-7 running off the end of the gel.
8. The precast gels have an opening at the front about 1 cm from the bottom of the gel, where the gel emerges. We refer to this side as the front, while the side without the opening is described as the back.
9. Experience is needed to decide when sufficient destaining has been done, but often a slightly longer destain improves sensitivity. One approach we have used for training is to remove the gel after half an hour, take a digital image, and then destain further, repeat image acquisition, and compare the results, continuing until the gel is excessively destained.
10. The two bands of MMP-1 at 50 kDa represent glycosylated and nonglycosylated forms of pro-MMP-1. The recombinant MMP-1 standard does not have a glycosylated form, but has two characteristic bands at a lower MW due to autoactivation and proteolytic cleavage of the pro-MMP-1.
11. To confirm that a band of proteolysis is caused by MMPs, divalent cations can be chelated by adding 10 mM EDTA to the collagenase buffer. Samples should be loaded in duplicate on either side of a zymography gel, and after it has been run, the gel should be divided down the middle. One half is developed normally, while the other is developed in collagenase buffer containing 10 mM EDTA. The disappearance of bands in the presence of EDTA confirms that they are caused by a metalloproteinase (**Fig. 4**).
12. MMP activity is stable through at least nine freeze–thaw cycles (unpublished data).

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# Chapter 17

# Identification of Pathogen Receptors on Dendritic Cells to Understand Their Function and to Identify New Drug Targets

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## Summary

Dendritic cells (DCs) are crucial in the defence against invading pathogens. These professional antigen-presenting cells express a diversity of pattern recognition receptors to recognize pathogens and to induce adaptive immune responses. However, pathogens have also developed several mechanisms to suppress or modulate DC function through specific receptors, thereby ensuring pathogen survival and dissemination. In this chapter, we will discuss techniques to identify and functionally characterize pathogen receptors on DCs and to determine whether DCs elicit protective immune responses or whether pathogens subvert these responses to escape immunity.

**Key words:** Pathogens, Dendritic cells, C-type lectins, Binding, Transmission.

## 1. Introduction

Defining molecular mechanisms involved in pathogen recognition is important in order to understand elicited immune responses against pathogens and to eventually develop new antimicrobial therapies. With their unique function of forming a bridge between pathogen invasion and initiation of adaptive immune responses, dendritic cells (DCs) are targets for pathogens (**Fig. 1**). Extensive research on interactions of DCs with pathogens has revealed that DCs can contribute to both immunity and disease, depending on the nature of the specific pathogen interactions *(1, 2)*.

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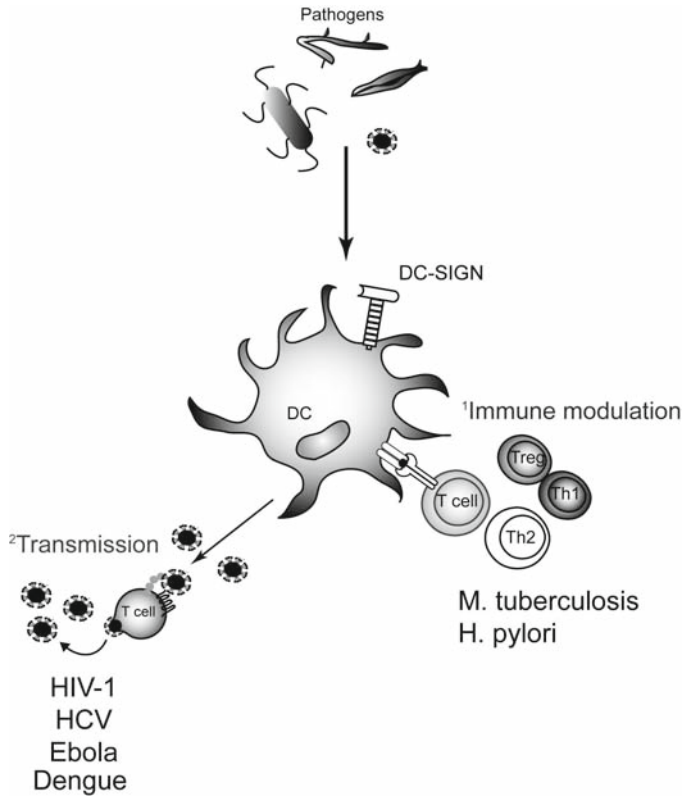


Fig. 1. Pathogens interfere with DC functions to infect the host. DCs interact with different pathogens such as viruses and bacteria. Some of these pathogens have evolved to suppress DC function to escape immune surveillance[1], or subvert DC function for infection and dissemination[2].

Here, we discuss a variety of approaches that are available in DC research to identify pathogen receptors. First, we will briefly review the current concepts in the field, and subsequently we will discuss different DC models and techniques, which can be used to identify novel pathogen receptors and to characterize DC–pathogen interactions.

### *1.1. Dendritic Cells Express Receptors for Antigen Uptake*

Immature DCs express specialized pathogen receptors on their cell–surface that facilitate pathogen capture. Binding of a pathogen to these receptors activates processes required for antigen presentation, which include pathogen binding, internalization, degradation, and MHC class I and II antigen presentation *(3)*. Moreover, triggering of these receptors by the pathogen is necessary for DC maturation, which results in morphological changes, up-regulation of co-stimulatory molecules, migration, and production of pro- and anti-inflammatory cytokines (**Table 1**). The transformation of a resting immature DC into a mature DC is a

**Table 1**
**A basic overview of DC activation and methods to test different parameters**

| DC function | Immature state | Mature state | Method to measure maturation |
|---|---|---|---|
| Antigen capture | High | Low | Down-regulation pathogen receptors<br>Decreased pathogen binding |
| Antigen uptake | High | Low | Uptake ligands (e.g. Dextran-FITC) |
| Morphology | Small dendrites | Large dendrites, cell clustering | Light microscopy<br>Increased side scatter in flow cytometry |
| Migration | Low | High | Migration assay<br>Up-regulation chemokine receptors |
| Co-stimulatory molecules | Low | High | Up-regulation CD80,CD83,CD86, HLA-DR |
| Cytokine production | Low | High | Up-regulation cytokine protein or mRNA levels |
| T-cell activation | Low | High | Mixed Leucocyte reaction (MLR)<br>Specific T-cell stimulation |
| T-cell differentiation | T reg | TH1/TH2/TH17 | T-cell stimulation assays |

complex multi-step process that must be carefully controlled to avoid auto-immunity and allergy *(4)*.

### *1.2. Dendritic Cells Express Pattern Recognition Receptors*

DCs express different classes of pathogen receptors (**Table 2**), including the specialized pathogen recognizing receptors, which are called pattern recognition receptors (PRRs). PRRs recognize pathogen-associated molecular patterns (PAMPs) *(5)*. Main families of cell-surface PRRs are the Toll-like receptors (TLRs), the C-type lectins, and the scavenger receptors (SRs). PRRs recognize pathogenic structures that are shared between different pathogens, such as the bacterial cell wall component LPS. The best known PRRs are the TLRs that signal via conserved pathways to induce the activation of several transcription factors, of which nuclear factor-κB (NF-κB) appears pivotal to DC maturation as well as cytokine production**.** C-type lectins recognize carbohydrate structures on pathogens, which facilitate pathogen capture and internalization *(6)*. This receptor family is not only important in antigen presentation, but it is now becoming evident

**Table 2**
**Different pathogen receptors expressed by DCs**

| Receptor | Ligands | Examples | Mediates |
|---|---|---|---|
| Toll-like receptors (TLRs) | For example, CpG, LPS, LTA | TLR1 to 11 | DC activation and antigen processing |
| C-type lectins | Carbohydrates | DC-SIGN, Mannose receptor, Dectin | Pathogen-binding, uptake, DC activation |
| Scavenger receptors (SRs) | Polyanionic ligands, low-density lipoproteins | Marco, SR-A | Pathogen binding and uptake |
| Pathogen-specific receptors | Proteins, carbohydrates, lipids | CD4/CCR5; CD150; syndecans | Pathogen binding, entry, internalization, activation |

that these receptors can induce DC activation *(7, 8)*. In addition to PRRs, other specific cell-surface molecules act as receptors for pathogens to either facilitate or mediate infection of DCs, such as HIV-1 receptor CD4, measles virus receptor CD150, and heparan sulphates that serve as attachment receptor for several viruses, such as herpes simplex virus. These receptors do not function as PRRs, but have other cellular functions and are subverted by viruses to infect the host.

### *1.3. Pathogens Bind Specific Receptors to Subvert Dendritic Cell Function*

Within the host, there is a constant battle between the host's immune system and invading pathogens. Pathogens have developed a variety of immune evasive strategies to enable survival and replication in the host (**Fig. 1**). DCs are crucial in the initiation of adaptive immune responses, and it is becoming evident that pathogens can subvert the function of DCs. For example, the C-type lectin DC-SIGN is an important receptor on DCs that functions as an adhesion receptor mediating early DC–T cell interaction and DC migration *(9)*. Besides recognizing cellular proteins, DC-SIGN also functions as a broad-spectrum pathogen receptor. It recognizes a great variety of pathogens, including viruses, mycobacteria, bacteria, and parasites *(8)*. Strikingly, the immune responses initiated by the interaction of DC-SIGN with these various pathogens differ between pathogens. Viruses target DC-SIGN for viral transmission, whereas DC-SIGN interactions with mycobacteria and bacteria may result in either DC activation or inactivation and in the case of the latter ultimately immune suppression *(10, 11)*. Thus, various pathogens have evolved different strategies to subvert DC function, and most of the underlying mechanisms remain to be elucidated. These mechanisms can be important or even essential for pathogen dissemination

and survival. Therefore, DCs are promising targets for developing preventive or curative treatments.

## 2. Materials

### 2.1. Pathogen-Binding Assay Using FITC Labelling

1. DCs: Culture monocyte-derived DCs as described by Sallusto et al. *(12)*. Use the cells between day 5 and 7 (*see* **Note 1**).
2. Phosphate-buffered saline (PBS) (10×) 1.37 M NaCl, 27 mM KCl, 100 mM $Na_2HPO_4$, 18 mM $KH_2PO_4$, pH 7.4, autoclave before storage at room temperature. Prepare a fresh 1× working solution by 1:10 dilution in MilliQ water.
3. Pathogen: Yeast or bacteria stocks in PBS (store in freezer or fridge).
4. Fresh FITC solution: Dissolve FITC (Fluorescein 5′-IsoThioCyanate isomer I $C_{21}H_{11}NO_5S$) in DMSO (Dimethyl sulphoxide) at a concentration of 10 mg/mL and protect the solution from light.
5. TSM buffer (10×) 200 mM Tris–HCl, 1,500 mM NaCl, 10 mM $CaCl_2$, 20 mM $MgCl_2$, pH 7.4, autoclave before storage at room temperature. Prepare a fresh 1× working solution by a 1:10 dilution in MilliQ water.
6. Bovine serum albumin (BSA).
7. TSA: Prepare a solution of 1× TSM containing 0.5% BSA, prepare fresh.
8. V-bottom 96-well plate.
9. Flow cytometer.

### 2.2. Bead Adhesion Assay

1. Fluorescent Beads: transfluorospheres 488/645; 1 μm; 2% solids in water containing 2 mM sodium azide, store at 4°C, protected from light.
2. MES buffer: 50 mM MES, pH 6.0, store at 4°C.
3. Streptavidin stock: Dissolve streptavidin at a concentration of 5.0 mg/mL in MES buffer, snap freeze aliquots in Eppendorf tubes, and store at –80°C.
4. EDAC solution: Dissolve EDAC (1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide, store stock at –20°C) in MES buffer at a concentration of 1.33 mg/mL, do not re-use the EDAC after opening the vial.
5. Glycine buffer: 1.1 M glycine.
6. PBA: Prepare a solution of PBS, containing 0.5% BSA and 0.02% Sodium azide (use gloves, very toxic); store at 4°C.

7. Biotinylated antibody: Biotin-SP-Affinipure F(ab′)$_2$ fragment goat anti-human or -mouse IgG Fc (gamma) fragment; prepare a stock at 0.5 mg/mL; store in aliquots at –20°C.
8. V-bottom 96-well plate.
9. Streptavidin-coated beads: store at 4°C, protected from light.
10. TSA: Prepare a solution of TSM containing 0.5% BSA, prepare fresh for every experiment.
11. 0.5 M EDTA stock store at 4°C.
12. Mannan stock 50 mg/mL; store at –20°C.
13. Blocking antibodies, store as recommended.
14. Blocking reagent, working dilution: Prepare a 4× blocking solution in TSA (*see* **Note 4**).
15. Flow cytometer.

### 2.3. Generation of Hybridomas

1. BALB/c mice.
2. Complete and incomplete Freund's adjuvant.
3. Dulbecco's Modified Eagle's Medium (DMEM).
4. Foetal calf serum (FCS).
5. Penicillin, streptomycin, and glutamine 100× stock solution.
6. Complete DMEM: DMEM supplemented with 10% FCS heat-inactivated, 100 units/mL penicillin, and 100 mg/mL streptomycin; store at 4°C.
7. Hybridoma medium: DMEM supplemented with 15% FCS not heat-inactivated, 5% Hybridoma Cloning Factor, 1× HAT medium (hypoxanthine/aminopterin/thymidine), 100 units/mL penicillin, and 100 mg/mL streptomycin; store at 4°C.
8. SP2/0 cells cultured in complete DMEM.
9. 100-μm cell strainer and syringe plunger.
10. Polyethylene Glycol (PEG), store at 4°C.
11. 96-well flat-bottom plates.

### 2.4. Detection of DC Maturation and Cytokine Secretion

1. Plates: 96-well round-bottom plate (*see* **Note 15**), 96-well V-bottom plate.
2. DC culture medium: RPMI-1640 supplemented with 10% FCS heat-inactivated, 100 units/mL penicillin, 100 mg/mL streptomycin and 2 mM glutamine, 500 units/mL IL-4, and 800 units/mL GM-CSF.
3. Cytokine ELISA kits.
4. Antibodies against DC maturation markers: anti-CD83, anti-CD80, and anti-CD86; store at 4°C.
5. PBA (*see* **Subheading 2.2**).

### 2.5. Viral Infection of DCs and Transmission to T Cells

1. Plates: 96-well round-bottom plate, 96-well V-bottom plate (*see* **Note 15**).
2. DC culture medium (*see* **Subheading 2.4**)
3. RPMI complete: RPMI-1640 supplemented with 10% FCS heat-inactivated, 100 units/mL penicillin, 100 μg/mL streptomycin, and 2 mM glutamine.
4. Blocking antibody or blocking reagent: Prepare a fresh 10× dilution in DC medium, and if necessary sterilize the solution using a spin filter and centrifugation.
5. Virus and Mock control preparations (stocks stored at –80°C): Prepare your virus dilutions in DC culture medium on ice, just before adding it to your cells.
6. Activated $CD4^+$ T cells: Stimulate peripheral blood mononuclear cells (PBMCs) with phytohaemagglutinin (PHA) 2 μg/mL in RPMI complete for 3 days. Isolate the $CD4^+$ T cells using the $CD4^+$ T-cell isolation kit (MACS) according to the manufacturer's protocol. Culture the isolated $CD4^+$ T cells in RPMI complete, supplemented with IL-2 (100 units/mL) and use the cells after 2–5 days.
7. Antibodies against T-cell or DC marker: anti-CD3, anti-HLA-DR, respectively.

## 3. Methods

### 3.1. Pathogen-Binding Assays

In order to identify a pathogen receptor, it is essential to develop and validate a pathogen-binding assay, which can be either a cellular method or a molecular method. Particular conditions will depend upon the type of pathogen and the research question. For cellular-binding methods, it is necessary to label the pathogen in order to measure binding of the pathogen by either flow cytometry or microscopy. Cellular methods have the advantage that binding is measured on a single cell basis. Moreover, using flow cytometry the binding is quickly quantified. Here we will describe two cellular methods that are suitable to demonstrate that C-type lectins such as DC-SIGN are receptors for viruses and mycobacteria.

#### 3.1.1. FITC Labelling of Bacteria, Yeast, or Parasites

This binding method is simple and broadly applicable to characterize binding of both bacteria and yeast to cells. A drawback of this method is that the FITC-labelling method can interfere with the binding, since it might alter the structure of the bacterial surface proteins. In that case, other labelling techniques such as recombinant GFP expressing bacteria are preferable.

1. Harvest the pathogen in an Eppendorf tube, wash with PBS, and resuspend in PBS at a concentration between $1 \times 10^8$ and $1 \times 10^{10}$/mL. This depends on the size of the pathogen; larger pathogens should be at lower concentrations.
2. Prepare the FITC solution, and incubate the pathogen with 1% FITC/DMSO (final concentration: 0.1 mg/mL FITC) at room temperature for 1 h, gently shaking. Protect all FITC-labelled solutions from light.
3. To remove all free FITC, wash 3–6 times extensively with PBS using centrifugation between 2,000 and 4,000 rpm (depending on the size of the pathogen) for 5 min (*see* **Note 2**), and subsequently incubate with PBS/4%BSA to crosslink any free FITC to BSA for 15 min at room temperature.
4. Wash extensively with PBS as described in **step 3** and store up to 2 weeks at 4°C (*see* **Note 3**) or proceed with adhesion assay.
5. Add 50.000 DCs per well in a 96-well V-bottom plate. Wash DCs by centrifuging for 2 min at 450 g and discard supernatant.
6. Resuspend the cells in TSA (*see* **Note 4**).
7. Optional: add blocking reagent to block receptors at 37°C for 30 min (*see* **Note 5**).
8. Determine the concentration of the FITC-labelled pathogen, since the multiple washing steps will result in a decreased concentration.
9. Add different concentration of FITC-labelled pathogens at 37°C for 45 min in a total volume of 40 µL (*see* **Note 6**).
10. Wash cells two times by centrifuging 2 min at 450 g with 100 µL TSA. Resuspend in 100 µL TSA and measure the binding using flow cytometry. Use cells without the addition of FITC labelled pathogen as negative control.

*3.1.2. Fluorescent Bead Adhesion Assay*

The fluorescent bead adhesion assay is very effective to measure binding of purified glycoproteins, virus-like particles *(13)*, or proteins derived from bacteria and parasites *(8, 14)* to cells (**Fig. 2**). Fluorescent beads are coated with streptavidin and subsequently with biotin-labelled antibodies specific for the protein or carbohydrate *(15, 16)*. These antibody-coated beads are used to capture protein or glycoprotein of interest for particle coating. Moreover, it is possible to coat beads with proteins present in a crude lysate by incubating the lysate with antibody-coated beads. The assay has the advantage that the size of the beads prevents internalization, and therefore the interaction of beads with cells is a direct reflection of ligand binding to the DC surface (**Fig. 2**).

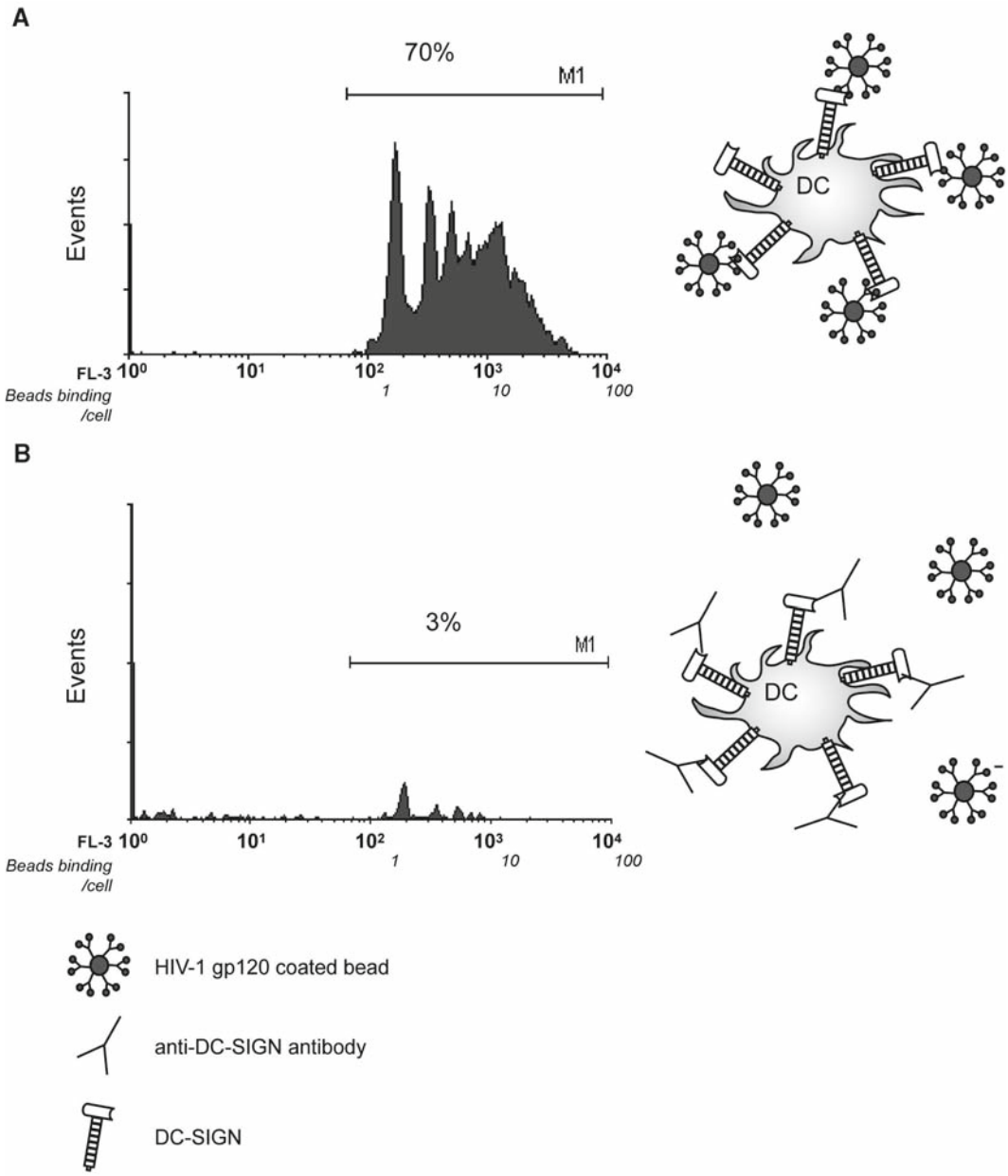


Fig. 2. The fluorescent bead adhesion assay is useful to determine pathogen binding to host cells. (**A**) DCs interact with HIV-1 gp120-coated fluorescent beads. DCs are incubated with gp120-coated fluorescent beads for 30 min at 37°C, washed and the binding is determined by flow cytometry. (**B**) Pre-incubation of the DCs with blocking antibodies against DC-SIGN abrogate the bead binding.

1. Covalent coupling of streptavidin: Incubate 50 µL beads with 20 µL streptavidin (5.0 mg/mL in MES buffer) in an Eppendorf tube at room temperature for 15 min.

2. Dissolve EDAC in MES Buffer (1.33 mg/mL), and incubate beads with 30 µL fresh EDAC solution, adjust pH to 6.5, and incubate for 2 h at room temperature while shaking.
3. Stop reaction using 10 µL 1.1 M glycine and incubate for 30 min at room temperature.
4. Wash beads extensively with PBS by centrifuging in an Eppendorf centrifuge for 2 min at 12,000 g. Remove the supernatant using a 1-mL pipette, resuspend the pellet in 100 µL of PBS by pipetting up and down, and add 900 µL PBS. Repeat the washing steps two times using PBS and one time using PBA.
5. Resuspend beads in 150 µL PBA (*see* **Note 7**). Store the beads at 4°C or proceed with the coupling of monoclonal antibodies.
6. Incubate 15 µL streptavidin-coated beads with 20 µL biotinylated antibody (0.5 mg/mL) at 37°C for 3 h in a shaker at 450 g.
7. Wash extensively using PBA by centrifuging at 12,000 g for 2 min, and incubate with the specific antibody (5 µg in 300 µL PBA) for 48 h at 4°C while shaking.
8. Wash extensively as described in **step 7**; resuspend beads in 100 µL PBA and store at 4°C (*see* **Note 8**) or proceed with the adhesion assay.
9. Wash 50.000 DCs per well in TSA by centrifuging 2 min at 450 g in a V-bottom 96-well plate. Resuspend the cells in 20 µL TSA (*see* **Note 4**).
10. Optional: Add blocking antibody or blocking reagents for 30 min at 37°C (*see* **Note 5**).
11. Add 2 µL beads per well in a total volume of 40 µL TSA and incubate 45 min at 37°C. Different concentrations beads can be used to optimize the assay dependent on ligand–receptor interaction.
12. Wash cells with 100 µL TSA and measure fluorescence at 645 nm by flow cytometry. Beads without cells should be used to determine the optimal settings for the fluorescent beads. The setting we use most is when one bead has a mean fluorescence of 100 in a $10^0$–$10^4$ range.

### *3.2. Receptor Identification Using Hybridoma Screening*

After the development of a binding assay, specific approaches can be used to identify the DC receptor(s) responsible for binding to either the pathogen or the pathogen ligand. The first and easiest approach to identify the receptor is to use specific inhibitors to known receptors on DCs and to measure the ability of these inhibitors to reduce the signal in the binding assays.

Different inhibitors can be used, either blocking antibodies against specific receptors or high concentrations of a competitive ligand (*see* **Note 5**).

A potential problem with receptor-specific antibodies is that not all of these will function as blocking antibodies and thus do not interfere with ligand binding. Moreover, it is possible that a receptor that interacts with a pathogen is unknown. The hybridoma antibody screening method can be used to generate antibodies that inhibit pathogen–cell interaction and are directed against receptors involved *(10)*. Although this method is time consuming, it has the great advantage of directly isolating a receptor-specific antibody that blocks the interaction. These antibodies are extremely useful in identifying unknown receptors through immune precipitation, but they are also indispensable in functional assays.

*3.2.1. Immunization*

1. Day 1: Immunization of BALB/c mice with DC lysate ($5 \times 10^6$ cells) in complete Freund's adjuvant (CFA) intraperitoneally (*see* **Note 9**, **10**).
2. Day 21/35/49 booster: ($5 \times 10^6$ DCs) in incomplete Freund's adjuvant (IFA) (0.1 mL) subcutaneously.
3. Day 52: Test serum for the presence of antibodies against DCs by flow cytometry. If yes, continue with fusion.

*3.2.2. Fusion and Screening*

4. Day –2: Split SP2/0 cells 1:4, make >10 T-175 flasks (*see* **Note 11**).
5. Day 0: sacrify mouse, isolate spleen. Generate a single cell suspension of the spleen using a 100-μm cell strainer and a syringe plunger.Wash the SP2/0 and spleen cells three times with plain DMEM (*see* **Note 12**).
6. Count the spleen cells and mix them 1:1 with SP2/0 cells.
7. Wash once in plain DMEM (10 min at 130 g).
8. Dispose the supernatant using a pipette and loosen the palette gently.
9. Add 1.5 mL Polyethylene Glycol (PEG) during 1 min (one drop at the time).
10. Shake the tube gently in the 37°C water bath for 1.5 min.
11. Add plain DMEM with the following interval, 1 mL in 30 s, 4 mL in 1 min, 15 mL in 1 min.
12. Incubate mixture for 1 h in 37°C water bath, shake gently every 15 min.
13. Centrifuge cells for 10 min at 130 g.
14. Dispose the supernatant using a pipette and loosen the palette gently.

15. Resuspend the cells in Hybridoma medium.
16. Plate: $5 \times 10^6$ spleen cells per 96-well plate; 200 µL per well in a 96-well flat-bottom plate (*see* **Note 13**).
17. When medium turns yellow harvest supernatants (usually after 10–15 days).
18. Pre-incubate the DCs with 50 µL supernatant for 30 min.
19. Measure the binding using your specific pathogen-binding assay as described in the previous section.

### 3.3. DC Activation by Pathogens

The effect of a pathogen on DC maturation can be determined by measuring the cell-surface expression of co-stimulatory molecules and MHC Class I and II by flow cytometry. Most notable maturation markers are CD83 and the co-stimulatory molecules CD80 and CD86, which are expressed at low levels on immature and high levels on mature DCs. In addition, MHC class I and II expressions are also upregulated on mature DCs (**Table1**). Pathogen receptors such as C-type lectins and scavenger receptors are usually down-regulated upon maturation *(15, 17)*.

In addition to analysis of maturation markers, which only determines immune activation of DCs, analysis of cytokine production also gives insight into the type of immune response that occurs, such as pro- or anti-inflammatory immune responses. Moreover, the DC cytokine profile is indicative of what can be expected in terms of driving T-cell differentiation. For example, IL-12 and IL-23 are inducers of a T-helper 1 response, whereas IL-4 drives the T-cell response towards T-helper 2 *(18)*. In addition, the anti-inflammatory cytokine IL-10 can be involved in suppression of the immune response. However, DC cytokine response profiles are only a guide, and it is essential to investigate directly the type of T-cell response that ensues. Cytokine production can be determined by using the supernatant of the stimulated DCs in an ELISA.

1. Plate 100.000 DCs in a round-bottom 96-well plate in 100 µL DC medium.
2. Add pathogen to DCs in a total volume of 200 µL of DC medium (*see* **Note 14**, **15**).
3. After 24 h, transfer to a V-bottom 96-well plate and centrifuge for 2 min at 450 g. Collect supernatant for cytokine ELISA and analyse cells for expression of maturation markers by flow cytometry. Store supernatant at –20°C until analysis.
4. For the analysis of maturation markers, pipette 50.000 DCs in a V-bottom 96-well plate.
5. Wash DCs in PBA by centrifuging 2 min at 450 g. Vortex briefly afterwards.
6. Add 10 µg/mL primary antibody at 4°C for 30 min in a total volume of 25 µL.

7. Wash cells once with 100 µL PBA.
8. Incubate with secondary antibody at 4°C for 30 min in a total volume of 25 µL.
9. Wash cells once with 100 µL PBA.
10. Determine expression of maturation markers by flow cytometry (*see* **Note 16**).

### 3.4. Identification of an Entry Receptor

Pathogens can also use immune cells for replication to produce progeny or as a vehicle to reach their target cell (**Fig. 3**). To investigate whether a receptor is involved in infection of DCs, blocking antibodies can be used. When the receptor is involved in infection, these blocking antibodies prevent or decrease infection. Read-out of infection is dependent on the pathogen being studied, but may include measuring pathogen proteins by flow cytometry or specific CFU assays. This method is especially suitable for viruses, since they can replicate intracellular, and host cells will display viral glycoproteins on their surface that can be detected by flow cytometry. This method can also be used for intracellular bacteria, such as *Mycobacteria tuberculosis*. However, since extracellular pathogens do not infect DCs, they will primarily affect DCs on the level of immune modulation as described in the previous section. Therefore, for these extracellular pathogens, identification of binding receptors and immune modulation are of greater importance.

It is important to keep in mind that although a pathogen may bind to a specific receptor, this may not be essential for infection of DCs. Strikingly, some receptors do not function as entry receptors but are able to facilitate attachment of pathogen to the target cell. These receptors facilitate infection in cis *(19)* (**Fig. 3**). This process is dependent on the first interaction of pathogen with the attachment receptors, and the second step involves interaction of

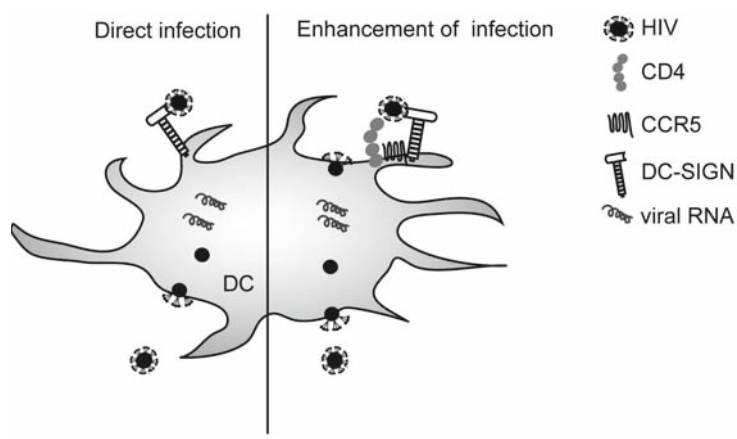


Fig. 3. Viruses interact with receptors to infect DCs. DCs express receptors that are involved in viral infection or enhance infection in cis.

the pathogen with the actual entry receptors. For example, receptor A may capture a pathogen. This will not lead to infection of the DCs, but instead it facilitates the interaction of a pathogen with receptor B. Receptor B, in turn, is an entry receptor and binding of the pathogen to receptor B will lead to infection of the DCs. This is an example in which receptor A facilitates infection in cis, while receptor B acts as an entry receptor.

When binding to either receptor A or receptor B is inhibited by using blocking antibodies, a decrease in infection will be observed. Thus care must be taken to not conclude from inhibition experiments that a receptor is an actual entry receptor.

1. Add 100.000 DCs per well in 100 µL 96-well round-bottom plate in DC medium.
2. Optional: Pre-incubate DCs with a blocking antibody (20 µg/mL) or blocking reagent for 1 h at 37°C.
3. Add virus and mock in different concentrations (*see* **Note 17**, **18**).
4. After different time point, harvest cells or supernatant and read out virus infection by flow cytometry or ELISA (*see* **Note 19**).

### *3.5. Identification of Receptors Involved in Viral Transmission*

In case of viral transmission, pathogens use immune cells as a vehicle to increase viral dissemination. Several studies have demonstrated that DCs capture virus, but that the virus is protected and efficiently transmitted to other target cells *(10, 20, 21)*. Viral transmission can be independent of viral infection of DCs, although it is possible that viral infection also facilitates transmission *(21)*. Here we describe HIV-1 transmission to $CD4^+$ T cells.

1. Seed 50.000 DCs in a 96-well V-bottom plate in 50 µL DC medium.
2. Optional: pre-incubate DCs with blocking antibody (20 µg/mL) for 1 h at 37°C to determine receptor involvement.
3. Add different concentrations of HIV-1 (e.g. Tissue Culture Inflammatory Dose 50 ($TCID_{50}$) 10 and 100) in 50 µL of DC medium.
4. Incubate for 2 h at 37°C.
5. Wash three times with 200 µL warm DC medium, centrifuge for 2 min at 450 g.
6. Add DCs in different ratios to PHA/IL2-activated CD4 T cells in a final volume of 200 µL (e.g. 1:10–1:200 DC-T cells) in a 96-well flat-bottom plate (*see* **Note 20**).

#### *3.5.1. Detection of Infection by ELISA*

7. Harvest 100 µL supernatant and refresh with 120 µL DC medium (to compensate for evaporation) every 2 or 3 days, store supernatant for HIV-1 p24 capsid ELISA at –20°C. Follow infection for 14 days.
8. Test supernatant for HIV-1 p24 content by ELISA.

*3.5.2. Detection of Infection by Flow Cytometry*

9. Harvest cell samples every 2 or 3 days.
10. Fix and permeabilize cells. Stain for intracellular HIV-1 p24.
11. Double stain using a T-cell and DC marker (e.g. CD3 or HLA-DR, respectively).
12. Follow infection for 14 days.

### *3.6. Identification of Receptors Involved in Enhancement of Viral Infection*

The formation of the infectious synapse between DCs and T cells provides a scaffold that enhances interaction of virus with T-cell surface and presumably the receptors involved in infection. This process facilitates entry of virus in the target cell and thus DCs enhance infection. Using a similar assay as the transmission assay, it is possible to determine whether DCs are able to enhance infection of target cells. In this assay, HIV-1 infection of T cells is examined in the presence and absence of DCs. This assay is subtly different from the transmission assay since sub-optimal virus concentrations are used as well as the absence of a washing step to remove virus. In every condition, the amount of virus is the same and independent of the amount captured by DCs. Thus, the ability of DCs to enhance infection is determined, and the involvement of a specific receptor on DCs can be investigated using blocking antibodies.

*3.6.1. HIV-1 Enhancement Assay*

1. Seed 50.000 DCs in a 96-well flat-bottom plate.
2. Optional: pre-incubate DCs with blocking antibody (20 μg/mL) for 1 h at 37°C to determine receptor involvement.
3. Add different concentrations of virus (HIV-1 TCID 0.5, 1, 10, and 100).
4. Incubate for 2 h at 37°C.
5. Importantly, do not wash! Add 100.000 PHA/IL-2-activated $CD4^+$ T cells to DCs in a final volume of 200 μL.
6. Follow infection for 14 days as described for the HIV-1 transmission assay, by either flow cytometry or ELISA on different days (*see* **Note 21**).

### *3.7. Concluding Remarks*

Protection against pathogens is a major challenge for the immune system and DCs are a vital component of this ingenious system. DCs operate at the interface of innate and acquired immunity by detecting pathogens, which are internalized, processed, and presented on MHC class I and II molecules to T cells to initiate pathogen-specific immune responses. The efficacy of DCs in activating and directing immune responses is attributed to their particular array of receptors that mediate pathogen recognition and induce appropriate immune responses. However, many pathogens target the same receptors to escape immune surveillance and to persist within the host. Identification and functional characterization of these pathogen receptors is therefore of vital importance in understanding the role of DCs in these infections.

In this chapter, we have described a number of strategies that can be used to characterize pathogen receptors on DCs. These included a novel approach to functionally identify receptors involved in pathogen recognition, using an unbiased, rapid, and high-throughput screen of antibody libraries to isolate antibodies that inhibit defined DC-pathogen interactions. These inhibitory antibodies are pivotal not only in the identification of receptors, but also in the characterization of receptor function. Together with soluble inhibitors, these antibodies enable to examine the role of DC receptors in the generation of immune responses to pathogens. Importantly, they are useful tools to determine whether DCs elicit protective immune responses or whether their functions are subverted by the pathogen to escape immunity.

Besides studying DCs cultured in vitro, it is essential to investigate pathogen interactions with DC subsets isolated directly ex vivo, which will provide insight into the complex functions of DC subsets in pathogen recognition in vivo. The in vivo role of DC subsets and pathogen receptors can be further determined by investigating the localization, receptor-expression, and infection of DC subsets in infected human tissues.

## 4. Notes

1. Always analyse the phenotype of the immature monocyte-derived DCs. Microscopically one can observe that the DCs are in suspension and have an irregular cell-membrane-containing dendrites. Moreover, the immature DCs should express CD80, CD86, HLA-DR, and DC-SIGN, and low levels of CD83, which can be analysed by flow cytometry.
2. All free FITC should be carefully washed away; check the supernatant of your last washing for free FITC by incubating the same volume of supernatant as pathogens with the cells.
3. Check your labelling efficiency using fluorescence microscopy or flow cytometry. Optimize your labelling efficiency by varying pathogen concentration, FITC concentration, and labelling time. Labelled pathogens should be kept away from light since this will strongly diminish the fluorescence.
4. Always use a buffer or medium that contains calcium, since C-type lectins require calcium for their function.
5. For competitive blocking, DCs should be pre-incubated with inhibitors before the addition of the ligand (without washing). C-type lectins interact with pathogens through recognition of carbohydrate structures *(17)*. Therefore, carbohydrates are often used at high concentrations as

competitive inhibitors of C-type lectins *(22)*. For blocking antibodies use a final concentration between 10 and 50 μg/mL, and for competitive ligands, such as the carbohydrate mannan concentrations between 50 μg/mL and 1 mg/mL. EDTA is a calcium and magnesium chelator and can be used as a blocking reagent for receptors that are calcium and magnesium dependent (for example C-type lectins). Standard concentration for blocking is 10 mM.

6. A typical range of the amount of pathogens per well to start the first experiment is: $5 \times 10^4$ (MOI 1), $1.5 \times 10^5$ (MOI3), $5 \times 10^5$ (MOI 10), $1.5 \times 10^6$ (MOI 30), $5 \times 10^6$ (MOI 100).
7. The fluorescent beads should be kept away from light at all times; beads should be stored at 4°C and not be frozen.
8. Here a streptavidin–biotin step is included; however, the ligand can also be coated onto the beads directly.
9. The best results are obtained using primary cells.
10. If possible, immunize two mice and choose the best responder at day 52.
11. Cells must be in log phase of growth.
12. After the generation of a single cell suspension spleen cells are very vulnerable, discard the supernatant carefully. Centrifuge at low speed (200 g, 15 min). Resuspend the cells gently by using a pipette.
13. Many cells (non-fused cells) should die during the first night.
14. Make sure lab safety levels are taken into consideration when live pathogens are used. To avoid this, heat-killed or otherwise inactivated pathogens can be used.
15. DCs are in optimal conditions when they have contact with each other, and therefore functional DC experiments are carried out in 96-well round-bottom plates. However, for washing 96-well V-bottom plates are recommended to minimize loss of cells during multiple washing steps.
16. Cytokine secretion can be measured using cytokine specific kits according to the manufacturer's instructions.
17. An optimal concentration needs to be determined for each virus.
18. After 2 h of incubation virus can also be washed away by using three centrifugation steps (2 min 450 g in a 96-well V-bottom plate) in warm medium to remove dead and unbound virus.
19. An optimal time to read out infection needs to be determined for each virus.

20. To infect T cells efficiently, the cells should be in log phase. Ideally, the cells should be cultured at a concentration between 1 and 2 million cells/mL.
21. Enhancement of infection will have occurred when the T cells in the DC/T cell co-culture are efficiently infected at the sub-optimal virus concentration that is not enough to cause infection of T cells alone.

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# Chapter 18

# Stable Lentiviral Vector-Mediated Gene Silencing in Human Monocytic Cell Lines

**Jimmy S. Lee and Neil E. Reiner**

## Summary

A major obstacle in studying monocyte cell biology has been the resistance of these cells to genetic manipulation, particularly when using nonviral methods. In the approach outlined in this chapter, we describe a strategy for stable gene silencing of monocytic cells. Using a vesicular stomatitis virus (VSV)-pseudotyped lentiviral vector expressing short hairpin RNA (shRNA), stable silencing of the p110α isoform of PI3-kinase in the human monocytic cell lines THP-1 and U-937 was achieved.

**Key words:** Monocyte, PI3-kinase, Lentiviral vector, Transduction, siRNA, shRNA, RNAi.

## 1. Introduction

Studying mononuclear phagocyte cell biology through genetic manipulation by nonviral transfection methods has been challenging due to the dual problems of low transfection efficiency and the difficulty in obtaining stable transfection. To overcome this problem, we developed a system for mediating RNA interference (RNAi) in monocytic cells. The p110α isoform of phosphoinositide 3-kinases (PI3K) was silenced using a lentiviral vector expressing short hairpin RNA (shRNA). This resulted in the generation of stable THP-1 and U-937 monocytic cell lines deficient in p110α.

RNA interference is a sequence-specific, post-transcriptional gene silencing mechanism initiated by the introduction of double-stranded RNA (dsRNA) into target cells through either viral infection or experimental manipulation *(1)*. The RNAi pathway begins by processing dsRNA into short (<30 bp) dsRNA

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duplexes, termed small-interfering RNA (siRNA), by a host RNAse Dicer. The siRNA then becomes incorporated into a multicomponent nuclease complex called the RNA-induced silencing complex (RISC). RISC then uses the siRNA sequence as a guide to recognize cognate mRNAs for degradation.

Delivery of siRNAs into mammalian cells by transfection of siRNA or DNA vectors expressing shRNA has been shown to mediate RNAi successfully *(2–5)*. Transfection of siRNA is transient, lasting only for a week or so *(6)*, although DNA-based vectors may last longer with drug selection *(4)*. In contrast, viral vectors have also been used to deliver siRNA successfully, and these methods tend to provide more stable gene silencing *(7–10)*.

This chapter describes how human monocytic cell lines can be effectively transduced using a lentiviral vector to stably silence an endogenous lipid kinase, PI3K. This involves three major steps: construction of shRNA sequences targeting the gene of interest, production of viral vectors using packaging cells, and transduction of monocytic cells.

## 2. Materials

### 2.1. Reagents and Antibodies

RPMI 1640, Dulbecco's Modified Eagle Medium (DMEM), Hanks' balanced salt solution (HBSS), penicillin/streptomycin and 1 M HEPES solution, fetal bovine serum (FBS).

1. Polybrene, cell dissociation solution, and poly-L-lysine (Sigma-Aldrich, Oakville, ON).
2. Antibodies to human p110δ (Calbiochem, San Diego, CA). Antibody to human p110α (clone 19) (BD Biosciences, Mississauga, ON). Antibodies to human p110β and actin (Santa Cruz Biotechnology, Santa Cruz, CA). Anti-p85-N-SH3 antibody (Upstate Biotechnology, Lake Placid, NY).

### 2.2. Cell Culture

The promonocytic cell lines THP-1 and U-937 were cultured in RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, penicillin (100 U/mL), and streptomycin (100 μg/mL). Cultures were maintained without exceeding $0.5 \times 10^6$ cells/mL. 293T human embryonic kidney (HEK) cells were also from ATCC and were cultured in DMEM, supplemented with 10% FBS, 2 mM L-glutamine, penicillin (100 U/mL), and streptomycin (100 μg/mL), and 20 mM HEPES.

### 2.3. Lentiviral Vector Construction and Transduction of Monocytic Cells

1. *Constructs* – The U6 promoter vector pSHAG-1 (**Fig. 1A**) was a gift from Dr. G.J. Hannon (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) *(3)*. pSHAG-1 contains the *att*L1/L2 transposition elements that are compatible with Gateway Cloning Technology (Invitrogen Canada Inc., Burlington, ON).

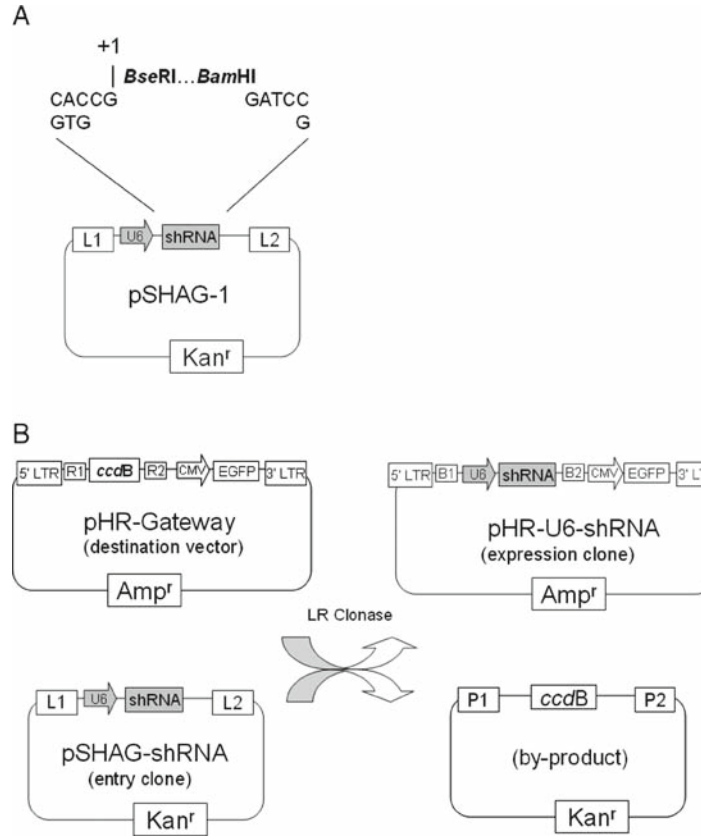


Fig. 1. pSHAG-1 plasmid and LR Clonase-mediated transposition. (**A**) pSHAG-1 vector. Schematic diagram of pSHAG-1 showing U6 promoter, the *att*L1/L2 transposition elements that are compatible with Gateway Cloning Technology, and directional insertion sites for shRNA encoding fragment, generated by double digestion with *Bse*RI and *Bam*HI. (**B**) Generation of pHR-U6-shRNA vector. The Gateway LR Clonase enzyme mix (Invitrogen) catalyzes the *in vitro* recombination between an entry clone (containing a gene of interest flanked by *att*L sites) and a destination vector (containing *att*R sites) to generate an expression clone. The resulting transposition elements are labeled *att*P1/P2, and *att*B1/B2. The pSHAG vector containing the shRNA sequence serves as the entry clone, while the pHR-Gateway is the destination vector. The expression clone is selected on LB plates containing ampicillin.

2. Antisense to p110α mRNA was targeted to two nucleotide segments: 5′-ATATACATTCCTGATCTTCCTCGTGCTG-3′ (nucleotide positions 1171–1198, referred to as α3) and 5′-CAAGACCATCATCAGGTGAACTGTGGGG-3′ (nucleotide positions 8–35, referred to as α1). The hairpin-containing sequence was created as described *(8)*, using a protocol available at http://katahdin.cshl.org:9331/homepage/portal/scripts/main2.pl (*see* **Note 1**).
3. Oligonucleotides p110α1 and p110α3 listed in **Table 1** were synthesized by Qiagen Inc. (Valencia, CA). All the sequences contained a *Hind* III site in the hairpin region, a site that is not

**Table 1**
**Short hairpin RNA encoding sequences targeting human p110α mRNA**

| Construct | 5′-Antisense sequence (28 nt) haripin loop (8 nt) Sense sequence (28 nt) Termination (6 T's)-3′ |
|---|---|
| p110α1 | CCCCACAGTTCACCTGATGATGGTCTTG-*GAAGCTTG*CGAGACCGTCATCGGGTGAGCT-GTGGGGCATTTTTTT |
| | GATC AAAAAAATGCCCCACAGCTCACCCGAT-GACGGTCTCG *CAAGCTTC* CAAGACCATCAT-CAGGTGAACTGTGGGG CG |
| p110α3 | CAGCACGAGGAAGATCAGGAATGTATAT-*GAAGCTTG* ATATGCATTCCTGGTCTTCTTCGT-GTTGCTCTTTTTT |
| | *GATC* AAAAAAGAGCAACACGAAGAAGACCAG-GAATGCATAT *CAAGCTTC* ATATACATTCCT-GATCTTCCTCGTGCTG CG |

*nt* nucleotide(s)
Hairpin sequences are shown with the *Hind*III site underlined and italicized. For each construct, the two strands of DNA were annealed and ligated into the pSHAG-1 vector. The first strand is an example of "oligo A," while the second strand is "oligo B" (*see* **Note 1**). The underlined sequences at the 5′ and 3′ ends are for directional cloning into pSHAG-1, which was cut with *Bse*RI and *Bam*H1 (reproduced from **ref.***10* with permissions from American Society for Biochemistry and Molecular Biology)

present in the native pSHAG-1 vector, and *Bam*HI and *Bse*RI ends to enable directional cloning. Competent DH5α *E. coli* (Invitrogen) were transformed with pSHAG-1 containing the sequences of interest and clones were screened by *Hind*III digestion.

4. Construction of the lentiviral transducing plasmid, pHR-CMV-EGFP (9401 bp), the packaging vector pCMV R8.2 (13.4 kb), and the VSV envelope vector pMD.G (6.01 kb) (*see* **Note 2**) has been described elsewhere *(11, 12)*, and the vectors were obtained from Dr. Alice Mui (Department of Surgery, Faculty of Medicine, University of British Columbia). All three vectors contain an ampicillin resistance gene. Purified pSHAG-1, pSHAG-p110α1, and pSHAG-p110α3 served as entry clones. The lentiviral transducing vector pHR-CMV-EGFP was modified by inserting the Gateway vector conversion cassette (Invitrogen) into the *Cla*I site, that is located downstream of the 5′LTR, but upstream of the CMV promoter (**Fig. 1b**). The resulting pHR-Gateway served as a destination vector since it contained *att*R1/2 sites (*see* **Note 3**). pHR-Gateway was also obtained from Dr. Alice Mui

(Department of Surgery, University of British Columbia). The various entry clones were transposed to the pHR-Gateway by Gateway LR Clonase Enzyme Mix (Invitrogen). Positive clones were then isolated and the plasmids (pHR-U6, pHR-p110α1, pHR-p110α3) purified. All plasmid purifications were carried out using Qiagen Endofree Plasmid kits.

### 2.4. Flow Cytometry Analysis

1. Binding buffer: HBSS, 1% FCS, and 0.1% $NaN_3$. Also prepare another binding buffer stock with 1.85% paraformaldehyde prior to use.
2. HRPO conjugated antirabbit, antimouse, and antigoat secondary antibodies.

## 3. Methods

### 3.1. Construction of VSV-Pseudotyped Lentiviral Vector Expressing shRNA (*see* Note 1)

1. Reconstitute the synthesized sense and antisense oligonucleotides encoding the shRNA (**Table 1**) in TE buffer at 100 μM.
2. Anneal the oligonucleotide pairs at 1:1 by heating the mixture in a heating block at 94°C for 15 min, followed by cooling at room temperature for 40 min. Use 5 μL of each oligonucleotide, plus 90 μL nuclease-free water. The mixture will give 5 μM of duplex DNA in 100 μL total volume.
3. Ligate the duplex into pSHAG-1 via the *Bam*HI/*Bse*RI site using ligase at 16°C overnight. Use a 3:1 insert to vector ratio, 1 μL ligase, and add appropriate volumes of 5× buffer and water to make up a total reaction volume of 10 μL.
4. Transform competent *E. coli* and plate overnight without selection.
5. Select several colonies from each plate, and add each to a 4-mL LB broth plus kanamycin (50 μg/mL). Incubate overnight on a shaker at 37°C.
6. Purify plasmids using a Miniprep kit (Qiagen) (*see* **Note 4**). Plasmids containing an insert will be cleaved by *Hind*III digestion and will migrate slower (~2,500–3,000 bp band) on 1% agarose in TAE when compared to pSHAG-1 without insert which will not be digested.
7. Purify the positive clones and transpose the U6-shRNA element into the viral vector pHR-Gateway (**Fig. 1B**) using Invitrogen LR Clonase kit (a mix of lambda recombination proteins that mediate the *att*L × *att*R recombination reaction); carefully following instructions provided with the kit. The resulting plasmid – pHR-U6-shRNA – is then ready for

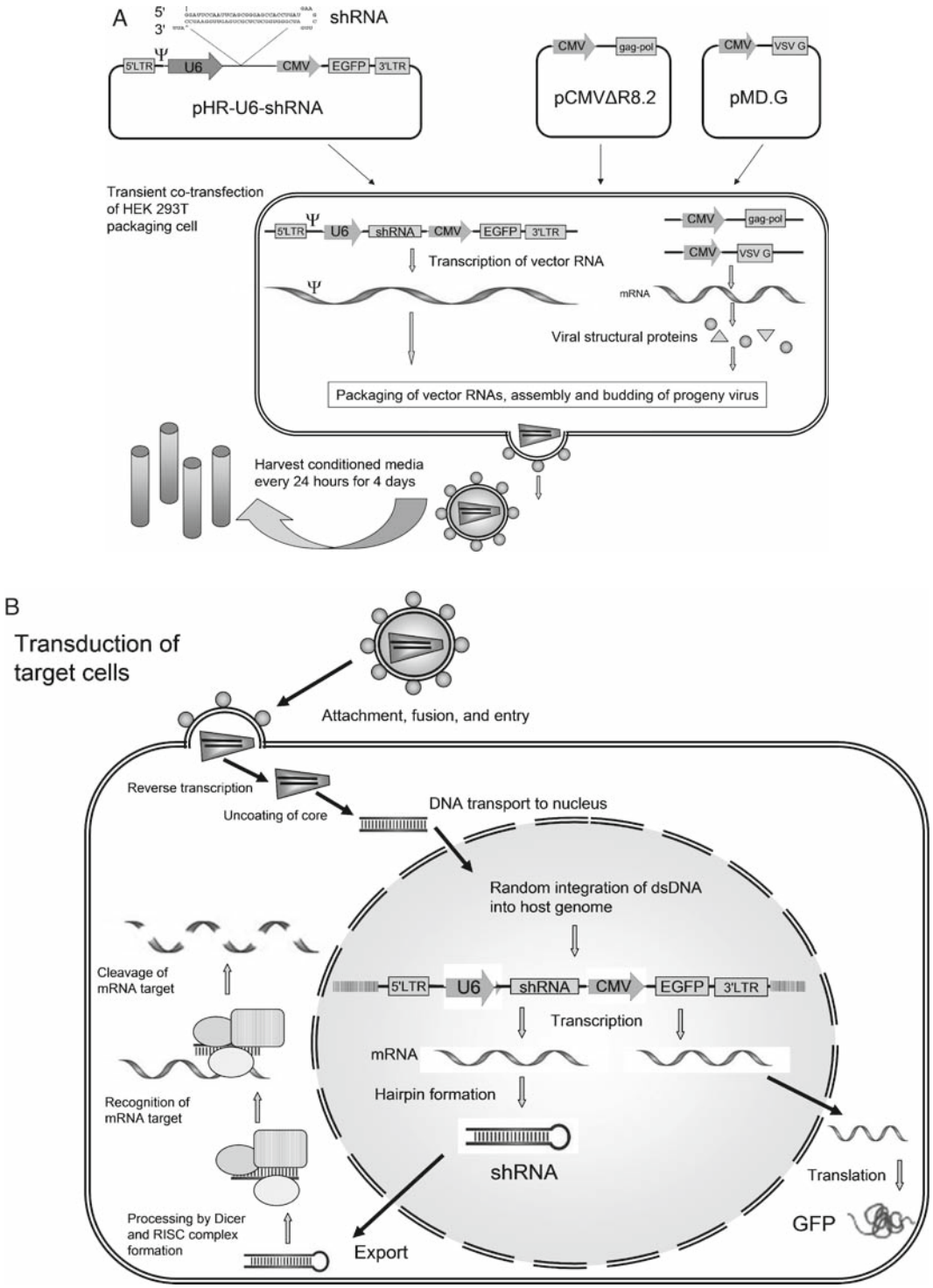


Fig. 2. Construction of a lentiviral vector for transduction of shRNA into target cells. (**A**) To produce the recombinant lentiviral vectors, the packaging cell line HEK 293T was cotransfected by the vector plasmid (pHR-U6-shRNA), helper

use for transient transfection of the packaging cell line HEK 293T (**Fig. 2A**).

### 3.2. Transient Transfection of HEK 293T Packaging Cells

1. The three vectors pHR-U6-shRNA (hairpin vector), pCMVΔR8.2 (helper plasmid), and pMD.G (envelop plasmid) should all be prepared using an endotoxin-free plasmid purification kit (e.g., Qiagen Endofree Maxi kit). Concentration and purity should be checked by both OD reading and gel electrophoresis. Purified plasmids should be dissolved in endotoxin-free TE buffer.
2. The day before transfection, coat 10 cm cell culture plates with poly-L-lysine (*see* **Note 5**) by adding 3 mL of a 0.01% solution to one plate. Then remove and transfer to the next plate, making sure that each plate is completely coated before transferring to the next. Keep repeating the procedure until all the plates are coated. Then seed $1.6 \times 10^6$ HEK 293T cells per plate, in complete DMEM. Incubate in a 5% $CO_2$ incubator at 37°C for 12 h overnight.
3. On the next day, add the necessary volumes of the three plasmids to Opti-MEM I Reduced Serum Media in an Eppendorf tube, final volume 1.5 mL. Label this "mix A." The amounts of plasmids to use are: 10 μg pHR-U6-shRNA, 7.5 μg of pCMVΔR8.2, and 2.5 μg of pMD.G (*see* **Note 5**). In another vial, labeled "mix B," add 60 μL of Lipofectamine 2000 with 1.5 mL Opti-MEM for 5 min at room temperature. Then combine "mix A" and "mix B" and incubate for 20 min at

Fig. 2. (continued) plasmid (pCMVΔR8.2), and envelope plasmid (pMD.G). The general strategy in the production of lentiviral vector-delivered siRNA is to segregate the *trans*-acting sequences that encode for viral proteins from the *cis*-acting sequences (nonencoding regions recognized by viral proteins) involved in the transfer of vector sequences encoding the shRNA [reviewed in **ref.**15]. The vector plasmid contained a U6 promoter-driven shRNA coding sequence, followed by a CMV-driven enhanced green fluorescent protein (EGFP) reporter. The shRNA nucleotide sequence shown is not specific and is only intended to illustrate a generic shRNA. These elements were flanked by long terminal repeats (LTR), and also contained *cis*-acting sequences that allowed the vector RNA to be packaged and subsequently, to be reverse transcribed and integrated in the target cell. The packaging sequence (Ψ) was only present in the vector plasmid, and not in the other two plasmids. Following transfection, the plasmids pCMVΔR8.2 and pMD.G were transcribed downstream of CMV promoters, and they provided the viral structural proteins in *trans.* These included viral integrase, protease, reverse transcriptase, capsid and matrix proteins, and vesicular stomatitis virus G protein. Together, these proteins act to *trans*-complement the vector by assembling the progeny viral particles, which are limited to a single round of infection. Vector proteins were produced as well, so transfected cells were GFP positive. Conditioned medium was then harvested, concentrated by ultracentrifugation, and stored at −70°C. (**B**) Transduction of target cells was done at a MOI of 10:1. Virions attach at the cell surface via VSV G proteins, fuse with the cell membrane, and release the viral core. Reverse transcription and uncoating of the viral core occurs in the cytoplasm. The dsDNA is then transported into the nucleus where it integrates randomly into the target cell genome. Following integration, the U6 and CMV promoters transcribe their respective genes, and this results in shRNA production and mRNA for the GFP reporter. The shRNA and mRNAs are exported to the cytoplasm. GFP is then translated, and the shRNA is processed by Dicer and then incorporated into the RNA-induced silencing complex (RISC) [reviewed in **ref.**8]. RISC then targets and degrades cognate mRNAs (reproduced from **ref.***10* with permissions from American Society for Biochemistry and Molecular Biology).

room temperature. The total amount of mix A + B required will be determined by the number of plates of 293T cells to be transfected.

4. Remove the media from the plates containing 293T cells and replace with 15 mL of fresh complete DMEM.
5. Add the 3 mL of combined mix A + B solution to each plate, to give a final volume of 18 mL per plate. Swirl plate gently, and incubate overnight at 37°C.
6. On the next day, remove the media and add 6 mL of complete DMEM to each plate (*see* **Note 6**). Plates can be checked for transfection by examining by fluorescent microscopy. Most of the cells should be strongly GFP positive at this time point. Incubate overnight again at 37°C.
7. Roughly 48 h post-transfection, begin the first supernatant collection. Collect the entire 6 mL from each plate and replace with 6 mL of fresh complete DMEM.
8. The collected conditioned media is cleared of debris by low-speed centrifugation (2,500 × g for 5 min, 4°C) and filtered through a 0.45 μm (*see* **Note 7**) filter. Label this tube "Day 1," and store the filtered collection at –70°C immediately.
9. Repeat this collection procedure every 12 or 24 h, daily for three more days (*see* **Note 8**).
10. Thaw the conditioned media from the four individual collections on ice (*see* **Note 9**), and pool these setting aside 500 μL for titering purposes. Label this 500 μL "Before concentration." Ultracentrifuge the remainder at 100,000 × *g*, at 4°C for 2 h.
11. Aspirate the supernatant carefully without disturbing the pellet, and keep 2 mL for titering purposes (see later). Label this 2 mL "Undiluted supernatant." Resuspend the pellet in 500 μL of complete DMEM (chilled on ice), and place on a nutator or flat bed shaker at 4°C overnight. Pipette the resuspended pellet a few times while it is shaking.
12. After shaking the resuspended virus, transfer it to a microcentrifuge tube and spin at 3,000 rpm for 10 min to remove any remaining debris. Remove 6 μL for titering purposes (see later) and label this sample "Concentrated." Store the 6 μL and the remainder in aliquots (25–200 μL) at –70°C (*see* **Note 10**).
13. Prepare the dilutions shown in **Table 2** for titering purposes (*see* **Note 11**).
14. Add 1 mL of each diluted sample plus 20 μL polybrene (8 μg/mL final) to 1 × $10^5$ HEK 293T cells plated the night before in a 6-well plate (*see* **Note 12**). Plate an additional three wells to count at the time of titration to get an average cell number per well. Incubate overnight at 37°C.

**Table 2**
**Volumes and dilutions for titering virus concentrations on HEK 293T cells**

| Sample | Dilution | Volumes to use |
|---|---|---|
| Before concentration | 10 | 222.2 µL supernatant + 2 mL complete DMEM |
| | 100 | 222.2 µL of 1/10 dilution + 2 mL complete DMEM |
| | 1,000 | 222.2 µL of 1/100 dilution + 2 mL complete DMEM |
| After concentration | Undiluted supernatant | Use 1 mL undiluted supernatant |
| | 10 | 222.2 µL of supernatant + 2 mL complete DMEM |
| | 100 | 222.2 µL of 1/10 dilution + 2 mL complete DMEM |
| Concentrated | 500 | 4.01 µL concentrated supernatant + 2 mL complete DMEM |
| | 5,000 | 222.2 µL of 1/500 dilution + 2 mL complete DMEM |
| | 50,000 | 222.2 µL of 1/5,000 dilution + 2 mL complete DMEM |

15. Change the medium the next day, and incubate overnight once again.
16. Remove medium and rinse the wells with PBS. Remove cells with cell dissociation solution and spin down the cells. Wash once with binding buffer (HBSS, 1% FCS, and 0.1% $NaN_3$) and spin the cells down once again. Resuspended in 1 mL of binding buffer containing 1.85% paraformaldehyde. Analyze at least 10,000 cells in flow cytometer and measure the percent GFP positive cells using nontransduced cells as controls. Calculate the titer by the following formula: transduction units/mL = (average cell number at the time of titration × % of GFP positive cells)/100 × dilution factor. Example: 30% positive at 1:100 dilution of $8 \times 10^5$ cells = $0.30 \times 100 \times 8 \times 10^5 = 2.4 \times 10^7$ transduction units/mL. Expect $1 \times 10^8$ to $1 \times 10^9$ transduction units/mL in the concentrated samples.

### *3.3. Transduction of Human Monocytic Cell Lines*

1. Seed fresh cultures of $1 \times 10^5$ THP-1 or U-937 cells in each well of a 12-well plate in 500 µL of complete RPMI.
2. Based on the viral titer calculations, add enough lentiviral vectors for a multiplicity of infection of 10:1 (*see* **Note 13**). Transduction should be carried out in the presence of polybrene (8 µg/mL). Premix the viral stock with polybrene before adding to the cells.

3. Incubate the transduced cells at 37°C for 6 days. On day 3–4, cells may be examined by immunofluorescent microscopy to monitor GFP expression. If cells proliferate rapidly during the 6 days, add enough complete medium to keep the cells from exhausting the medium.
4. Analyze the transduced cells by flow cytometry after 6 days for % GFP positive. Expect greater than 90% GFP positivity (**Fig. 3A**).
5. Allow cells to proliferate under optimal conditions (do not exceed $1 \times 10^6$ cells/mL) until enough cells are obtained for Western blot analysis and storage in liquid nitrogen.
6. Western blot analysis of the targeted protein should reveal marked reduction relative to appropriate controls (**Fig. 3B, C**).
7. Freeze positive clones and control cells in liquid nitrogen for future use (*see* **Note 14**).

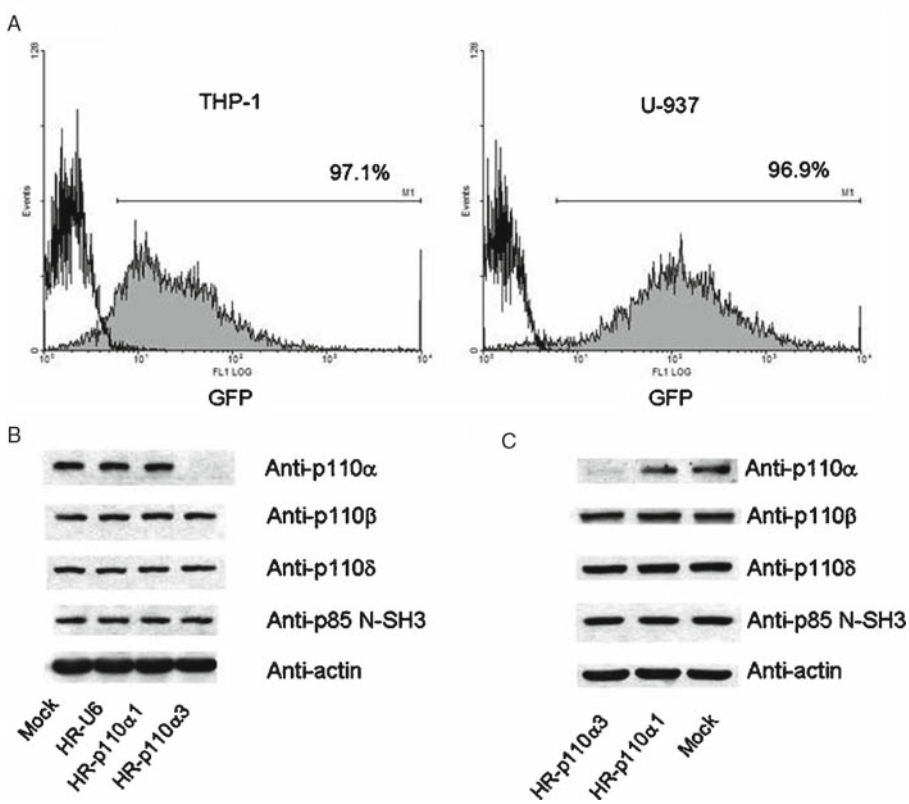


Fig. 3. Transduction of monocytic cell lines by lentiviral vectors is efficient and generates stable cell lines deficient in p110α. (**A**) Flow cytometry analysis of transduced (solid histogram) or mock transduced cells (clear histogram) was performed 6 days after viral transduction. 10,000 cells were analyzed, and GFP fluorescence intensity was measured on the FL1 channel. Approximately 97% of the THP-1 and U-937 cells were GFP positive. The mean fluorescence intensity (MFI) for mock-infected cells was 8.5 for THP-1 and 6.6 for U-937. Transduced cells had MFIs of 38.3 for THP-1 and 132.3 for U-937. (**B**) and (**C**) Western blot analysis of class IA PI3K p110 catalytic subunit isoforms (α, β, and δ), and p85 regulatory subunit in THP-1 cells and U-937 cells. Actin was used as protein loading control (reproduced from **ref.** *10* with permissions from American Society for Biochemistry and Molecular Biology).

## 4. Notes

1. The protocol for designing oligonucleotides for insertion into *Bse*RI *Bam*HI cut pSHAG-1 vector is as follows (from Dr. G. Hannon, Cold Spring Harbor Laboratory):

   Note that N1, N2…N30, N31 are numbered positions for any nucleotide, N.

   (a) Using the sense coding sequence for the gene of interest to be targeted, find 5′-N1 NNNNN….NNNNNNNNNN C N30 N31–3′ such that the oligo must end in a "C" so that pol III, which initiates at a "G" in the U6 promoter, will initiate precisely at the first base of the antisense strand.

   (b) Get the reverse complement of the sequence in step (a) which is:

   5′- N31′ N30′ G N′N′N′N′N′N′…..N′N′N′N′N′N′ N′N′N′N1′-3′

   Note that N31′ N30′…N1′ are complementary to N31, N30…N1, respectively.

   (c) Remove N30′ N31′ from sequence in step (b) to get *5′- G* N′N′N′N′N′N′N′N′…..N′N′N′N′N′N′N′N1′-3′

   (d) Add 5′-GAAGCTTG-3′ to 3′END of the sequence in step (c) to get 5′- G N′N′N′N′N′N′N′N′N′… ..N′N′N′N′N′N′N1′*GAAGCTTG -3′*

   (e) Optional (to reduce hairpin formation by DNA oligonucleotides prior to ligation)

From sequence in step (a), in positions N2 through N28 convert every third base possible from A to G or from C to T such that:

- Adjacent bases are not changed
- More than four bases are not changed
- Homopolymeric runs greater than six bases in length do not result

   (f) To the 3′END of the sequence in step (d), add the sequence in step (e) to get

   5′- G N′N′N′N′N′N′N′N′N′….N′N′N′N′N′N′N1′GAAGC TTG *N1 NNNNNNN…..NNNNNNNN C N30 N31*

   (g) Add pol III terminator TTTTTT to the sequence in step (f) to get

   5′- G N′N′N′N′N′N′N′N′N′….N′N′N′N′N′N′N1′GAAGCTTG N1

   NNNNNNN…..NNNNNNNN C N30 N31 *TTTTTT*

   The inclusion of the HindIII site allows for rapid identification of clones containing a hairpin.

   (h) Drop the 5′- G in the sequence in step (g) to get

*5′-N′*N′N′N′N′N′N′N′N′….N′N′N′N′N′N′N1′GAAGC TTG N1

NNNNNNNN….NNNNNNN C N30 N31 TTTTTT

This sequence is now herein after referred to as "oligo A."

(i) Get reverse complement of the sequence in step (h).

(j) To the sequence in step (i), Add GATC to 5′ end.

(k) To the sequence in step (j), add CG to 3′ end.

This yields what is herein after referred to as "oligo B."

Examples of oligo A and B are shown in **Table 1**. Note that with the exception of the criteria stated in step (a), the target sequences chosen are randomly selected. Check the uniqueness of the sequences using GenBank. Prepare at least three target sequences. More sophisticated methods are now available through commercial venders or through free web sites such as: http://katahdin.cshl.org:9331/homepage/siRNA/RNAi.cgi?type=siRNA. See **ref.** *13* for more information on rationale siRNA design. Scrambled sequences may be used for control purposes. On the other hand, editorial boards of certain journals have explicit guidelines for the types of controls required for RNAi gene silencing experiments and authors are urged to consult the journals for specific details.

2. The vesicular stomatitis virus (VSV) glycoprotein G is a substitute for the lentiviral gp120/gp41 as the viral coat protein since it broadens the range of cell types that can be infected by the virus and also helps to stabilize the virion, yielding higher titers of the virus *(11)*. Furthermore, by stabilizing the virion, VSV G protein also allows the viral particles to be concentrated by ultracentrifugation, thereby providing higher titers for transduction *(11, 14)*.
3. DB3.1-competent cells are required to propagate the destination pHR-Gateway vector because these cells are resistant to the effects of the *ccd*B gene product produced by the Gateway conversion cassette, which is also present on the pHR-Gateway. The *ccd*B gene encodes a protein that interferes with *E. coli* DNA gyrase. When recombination occurs between the destination vector (e.g., pHR-Gateway) and an entry clone (e.g., pSHAG-shRNA), the *ccdB* gene on the destination vector is replaced by the U6-shRNA element from the pSHAG-shRNA. Cells that take up unreacted pHR-Gateway will fail to grow in non-DB3.1 cells.
4. A different commercial kit will suffice. Follow the manufacturer's instructions for optimal results.
5. Use early passage HEK 293T cells for transfection to get better results. Poly-L-lysine enhances adherence of the cells to the plate during the transfection protocol. The 4:3:1 ratio for pHR-shRNA, pCMV R8.2, and pMD.G, respectively, may be varied to achieve optimal viral production.
6. Lentiviral vectors should be prepared in a BSL-2 facility at a minimum. Check with your local authorities if in doubt.

7. A 0.2-μm filter maybe used without losing any viral particles. However, some clogging may occur. You may need to filter through a 0.8-μm and a 0.45-μm filter first.
8. 12-h collection yields the most virus, but collections can be done at 24 h intervals for convenience.
9. Keep everything on ice to maximize viral recovery. Using clear centrifuge bottles will facilitate pellet identification.
10. As an optional step, viral stocks can also be assayed for the p24 core antigen using commercial HIV-1 Antigen ELISA kits. This will give an approximation of how many viral particles are present; however, it does not directly indicate how many effective virions were made. This may be a helpful step for troubleshooting when viral titers are low.
11. All the samples used for titering should be frozen to –70°C and thawed at least once before titering. This will result in a more accurate estimation of effective transduction units after thawing the viral stocks for use in monocyte transduction. The three different samples allow for troubleshooting if viral yield is low in the "Concentrated" sample. The undiluted supernatant from the "After concentration" sample should not contain many transduction units, if at all. If it does, it indicates that the ultracentrifugation step or removable of the supernatant, was not performed optimally.
12. Polybrene acts as a transduction adjuvant to promote viral attachment to cells.
13. The multiplicity of infection may be varied to achieve optimal silencing. It is recommended that the minimal amount of virus to achieve silencing should be used in order to minimize the chance of nonspecific effects from random integration events.
14. When cells are thawed, perform FACS analysis to verify that the percent GFP positivity of the transduced cells is above 85–90%. Since there is no drug selection, FACS sorting maybe performed to enrich GFP positive cells. Always do Western blot analysis on the targeted protein of interest to verify silencing prior to experimentation.

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# Chapter 19

# Use of Fluorescent Probes to Detect Lipid Signaling Intermediates in Macrophages

**Vivian Kwok, Eric Vachon, and Gregory P. Downey**

## Summary

To fulfill their function in host defense, professional phagocytes such as neutrophils and macrophages exhibit the ability to ingest (phagocytose), kill, and dispose of pathogenic microorganisms. Recent studies have provided strong evidence for the importance of membrane lipids such as polyphosphoinositides in these processes. In turn, reversible phosphorylation events, involving protein and lipid kinases and phosphatases, regulate signaling pathways involving metabolism of membrane lipids. Our ability to study lipid signaling events has been greatly facilitated by the development of fluorescent molecular imaging techniques. In particular, the expression of recombinant fusions of derivatives of the jellyfish-derived green fluorescent proteins (GFP) coupled to reporter molecules enables real-time monitoring of signaling events in live cells. Here, we discuss methods to monitor alterations in membrane polyphosphoinositides involved in signaling events regulating phagocytosis. To illustrate the use of this technology, we will focus on the role of protein tyrosine phosphatase MEG2 in phagocytosis and its modulation by phosphatidylinositol-3,4,5-triphosphate ($PIP_3$). This approach enables investigators to ascertain the involvement of lipid intermediates in diverse signaling pathways.

**Key words:** Fluorescent probes, Green fluorescent protein (GFP), Macrophages, Phagocytosis, Lipids, Phosphoinositides, Protein tyrosine phosphatases (PTP), Signal transduction, Confocal microscopy.

## 1. Introduction

Alterations in membrane lipids are integral to diverse cellular processes such as proliferation and programmed cell death (apoptosis), motility, and uptake of fluid (endocytosis) and particles (phagocytosis). The latter is a process by which cells engulf large (>3 μm diameter) particles. In unicellular eukaryotes, phagocytosis is crucial for nutrition. In higher eukaryotes including mammals, phagocytosis is pivotal in innate and adaptive immune responses

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and in clearance of dead cells and debris *(1, 2)*. Phagocytosis is initiated by the binding of ligands displayed on the particle surface to specific receptors such as the Fcγ and complement (CR3) receptors on the surface of the phagocyte. This triggers signaling pathways that lead to rearrangement of the actin cytoskeleton and membrane fusion events that facilitate closure and maturation of the phagosome *(3, 4)*. Among many signaling intermediaries, protein and lipid kinases and phosphatases and their substrates appear to be particularly important in regulating phagocytosis. In this chapter, we focus on a subset of these reactions involving reversible tyrosine phosphorylation-dependent events regulated by the reciprocal actions of protein tyrosine kinases (PTK) and protein tyrosine phosphatases (PTP) *(5–9)*.

PTP-MEG2, first identified in megakarocytes and endothelial cells *(10)*, has recently been reported to interact specifically with polyphosphoinositides $PIP_2$ and $PIP_3$ through its N-terminal lipid-binding domain *(11, 12)*. Dynamic alterations in $PIP_2$ and $PIP_3$ occur at the phagocytic cup, and these are believed to play central roles in recruiting actin-associated proteins enabling phagocytosis to proceed *(13–15)*. Given the presence of PTP-MEG2 in the phagosome and its affinity to bind $PIP_3$ in vitro *(11, 12)*, we considered the possibility that regulation of PTP-MEG2 during phagocytosis is modulated by these interactions. We have recently shown that $PIP_3$ colocalizes with PTP-MEG2 during phagocytosis *(16)*, possibly triggering downstream signaling pathways regulating phagocytosis.

Fluorescent probes are widely used as reporters to monitor dynamic processes in living cells. Fluorescent tags can be engineered to fuse to signaling proteins thus allowing subcellular localization of these proteins and enabling researchers to track their movement and dynamic interactions in response to different agonists or other stimuli in live cells *(17)*. Transfection of fluorescently tagged molecules that bind specifically and with high affinity to specific lipids enables real-time visualization of dynamic alterations in lipid intermediates using confocal fluorescence microscopy. For example, the pleckstrin homology (PH) domain of Akt (Akt-PH) binds specifically to $PIP_3$, the primary product of phosphoinositide-3 kinase (PI3K) while the C2 domain of PKCδ binds to $PIP_2$. In the case of phagocytosis, fluorescent particles (e.g., beads, bacteria) coated with immunoglobulins serve as phagocytic prey and can be visualized simultaneously by fluorescence microscopy. Additionally, the use of paramagnetic beads enables isolation of phagosomal components -including lipids and proteins- by magnetic sorting (MACS) which can then be identified by biochemical techniques such as western blotting.

To illustrate the utility of these approaches, we describe their use to study the role of PTP-MEG2 in the dynamic turnover of membrane lipids during phagocytosis. These imaging techniques can be combined with molecular techniques to generate point mutant and/or truncated recombinant MEG2 proteins that

allow characterization of the functional importance of the various domains of PTP-MEG2. Finally, the ability to reconstitute some membrane events in vitro using mixed liposomes, the composition of which can be varied, enables the study of the role of specific lipids in regulation of PTP-MEG2.

## 2. Materials

### 2.1. Cell Culture and Lysis

1. High Glucose Dulbecco's Modified Eagle's Medium (High Glucose DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, and 100 μg/mL streptomycin (*see* **Note 1**).
2. Sterile Polyethylene Cell Lifters.
3. Phosphate-buffered saline (PBS). Autoclave before storage at 4°C.
4. Sterile Polyethylene Cell Scrapers.
5. SDS lysis buffer: For a 40 mL aliquot, combine 5 mL of 0.5 M Tris–HCl pH 6.8, 8 mL 100% SDS, 4 mL 100% glycerol, and QS with water. For every 4.875 mL of SDS lysis buffer, add 125 μL of 1 M Dithiothreitol (DTT) before use (*see* **Notes 2** and **3**).
6. 10× Laemmli sample buffer for cell lysis: 60 mM Tris–HCl, pH 6.8, 100 mM DTT, 2% (w/v) SDS, 10% (v/v) glycerol, 0.001% (w/v) bromophenol blue. Store in aliquots at –20°C.

### 2.2. Cell Transfection

1. Plasmid DNA. The following plasmid constructions were used for transfection. Green fluorescent protein-tagged pleckstrin homology domain of AKT (Akt-PH). The recombinant fusions of PTP-MEG2 were generated using either restriction digests or site-directed mutagenesis (using the Quick-Change™ site-directed mutagenesis kit, Stratagene, La Jolla, CA) were cloned into pcDNA3.0. Full length wild-type PTP-MEG2; the N-terminal domain of PTP-MEG2 fused to 6 histidine (polyHis) epitope tags; the catalytically inactive mutant of PTP-MEG2 (C515S MEG2); and lipid-binding domain mutants (K55M, K184M, and K55M/K184M MEG2). For clarification of the lipid-binding domain mutants, lysines at positions 55 and 184 in MEG2, $K^{55}$ and $K^{184}$, were mutated individually or in combination to methionine residues (M), giving rise to K55M, K184M single mutants and the K55M/K184M double mutant. Mutations were confirmed by DNA sequencing. A schematic diagram of all the PTP-MEG2 recombinant DNAs utilized is illustrated in **Fig. 1**.
2. Plasmid DNA is diluted to a concentration of 1 μg/μL such that accurate amounts can be used for transfection. Freeze at –80°C for long-term storage to avoid degradation (*see* **Note 4**).

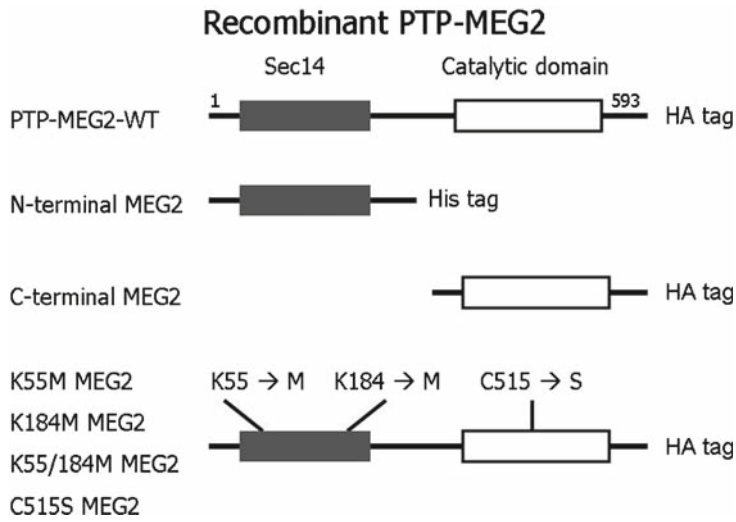



Fig. 1. Schematic diagrams of recombinant PTP-MEG2 proteins. Open and solid bars represent the catalytic domain and the Sec14-like lipid-binding domain of PTP-MEG2, respectively. PTP-MEG2 wild type is referred to as PTP-MEG2-WT. The N-terminal MEG2 has a poly His-tag and all other constructs are tagged with hemagglutinin (HA). Point mutations in the Sec14 domain (K55M, K184M, and K55/184M MEG2) all impair the ability of MEG2 to bind lipids. Lysines at positions 55 and 184 in MEG2, $K^{55}$ and $K^{184}$, were mutated individually or in combination to methionine residues (M), giving rise to K55M, K184M, and the K55M/K184M double mutant. The catalytically inactive domain mutant and the C-terminal domain are referred to as C515S MEG2 and C-terminal MEG2, respectively (reproduced from **ref**. *16)*.

3. SuperFect Transfection Reagent (*see* **Note 5**).
4. Sterile serum-free DMEM. Prewarm DMEM at 37°C and equilibrate with 5% $CO_2$ before use for cell culture (*see* **Note 6**).
5. Phosphate-buffered saline (PBS): 1× solution. Prewarm at 37°C before use.
6. 12-well polystyrene cell culture plates.
7. 18-mm-diameter circular glass microscope coverslips, 0.25 mm thick.

### *2.3. Confocal Immunofluorescence for PTP-MEG2*

1. 18-mm-diameter circular microscope glass coverslips, 0.25 mm thick.
2. Microscope slides.
3. Phosphate-buffered saline (PBS): 1× solution at room temperature.
4. 16% Paraformaldehyde Solution (PFA): Prepare 4% (v/v) solution in PBS fresh for each experiment. (PFA is a rapid fixative. Avoid contact with the body. Wear protective clothing. PFA is also flammable. Do not breathe vapors) (*see* **Note 7**).
5. Permeabilization solution: 0.1% (v/v) Triton X-100 in PBS.
6. Blocking and antibody dilution buffer: 5% (v/v) goat serum and 3% (w/v) BSA in PBS.

7. Primary antibodies: Rabbit polyclonal anti-HA or mouse monoclonal anti-KDEL. Anti-HA antibody stains for recombinant HA-tagged PTP-MEG2. KDEL is a motif located at the carboxy terminal of endoplasmic reticulum luminal proteins; it enables precise sorting of these proteins along the secretory pathway. KDEL serves as an endoplasmic reticulum (ER)-specific marker *(18)*.
8. Secondary antibodies: donkey-antirabbit FITC or donkey-antimouse Texas Red.
9. Mounting medium: DAKO.

### 2.4. Liposome Preparation

1. Lipids: phosphatidylinositol-4,5-bisphosphate ($PIP_2$), phosphatidylinositol-3,4,5-triphosphate ($PIP_3$), and phosphatidylserine (PS). Keep under inert gas (e.g., argon) and seal to avoid oxidation.
2. Chloroform (Chloroform evaporates easily into the air. Breathing chloroform may cause dizziness, fatigue, headache, and loss of consciousness. Work in a well-ventilated hood to avoid unnecessary exposure to chloroform. Chloroform is difficult to pipette because it leaks from the tip after aspirating. Do not use plastic containers for chloroform. It must be stored and handled in glass containers).
3. Dioleoyl-phosphatidylcholine (DOPC).
4. Rotary evaporator and vials.
5. Liposome buffer: 30 mM Hepes, pH 7.2, 100 mM NaCl, and 1 mL EDTA.
6. 22-gauge and 27-gauge needles.
7. Ethanol and dry ice. (To perform freeze–thaw cycles).
8. Extruding chamber with 400-nm filter.

### 2.5. Phosphatase Assays

1. Recombinant bacterially expressed PTP-MEG2 (30 μg/mL) proteins were synthesized from recombinant PTP-MEG2 DNAs as described in **Subheading 2.2.1** the PTP-MEG2 recombinant DNAs used are summarized in **Fig.** 1. Briefly, full length wild-type and mutant PTP-MEG2 were subcloned into the prokaryotic expression vector pGEX-4T-1 (PTP-MEG2-WT, C515S MEG2, C and N-terminal) or pGEX-6T-1 for lipid-binding domain mutants (K55M, K184M, and K55/184M MEG2). Fusion proteins were expressed in *Escherichia coli* and purified using glutathione-sepharose beads *(12)*. The glutathione-S-transferase (GST) was removed by cleavage with thrombin (Sigma-Aldrich Ltd).
2. Phosphatase buffer: 40 mM Hepes pH 7.2, 100 mM NaCl, 1 mM EDTA, 10 mM DTT, 0.025% Triton X-100, and 0.02% Nonidet P-40.
3. 10 mg/mL *p*-nitrophenyl phosphate (*p*NPP) and 10 mM DTT. *p*NPP is a phosphate ester used as a substrate for protein

phosphatases. When it interacts with phosphatases, it is hydrolyzed yielding a soluble end product that is yellow in color and can be quantified by absorbance at 405 nm. The intensity of the yellow color corresponds to the catalytic activity of a particular phosphatase. Dithiothreitol (DTT) protects the proteins with cysteine residues such as PTP from oxidative modification that can compromise their catalytic activity.

4. Microplate reader.

### 2.6. Phagocytosis

1. Three micrometer latex beads in 0.2% solution. Vortex well before use.
2. Purified lyophilized human IgG. Human IgG is utilized to coat the latex beads for Fcγ receptor (FcγR)-specific phagocytosis.
3. Prewarmed complete DMEM.
4. Phosphate-buffered saline (PBS): 1× solution. Keep one aliquot at 4°C and another at room temperature.
5. Ice.

### 2.7. Confocal Immunofluorescence for Localization of PTP-MEG2 and Akt-PH During Phagocytosis

1. The materials and reagents required for confocal immunofluorescence are the same as described earlier (*see* **Subheading 2.3**). Primary and secondary antibodies used are listed as follows.
2. Primary antibodies: Rabbit polyclonal anti-HA.
3. Secondary antibody: donkey-antirabbit Texas Red.

### 2.8. Confocal Immunofluorescence of PTP-MEG2 at Nascent Phagosomes

1. Materials and reagents required for confocal immunofluorescence and phagocytosis are described earlier (*see* **Subheadings 2.3** and **2.6**
2. TRITC conjugated goat-antihuman IgG. TRITC conjugated antihuman IgG antibody is used to stain noninternalized portion of the prey.

### 2.9. Phagosome Isolation

1. Polystyrene and sterile tissue culture dish, 60 × 15 mm.
2. Three micrometer magnetic beads. These beads serve as the particles to be ingested by the phagocytic cells. The beads containing phagosomal elements can be easily retrieved using a magnet.
3. Purified human IgG is utilized to opsonize the magnetic beads to facilitate the uptake of particles via Fcγ receptors.
4. Latrunculin B and Wortmannin. Latrunculin B is an actin polymerization inhibitor and Wortmannin is a phosphatidyl inositol 3 kinase inhibitor. They are used to block phagocytosis and these samples serve as negative controls.
5. Phosphate-buffered saline (PBS): 1× solution. Keep one aliquot at 4°C and another at room temperature.

6. Hanks Balanced Salt Solution (HBSS++) with 0.5 mM of $Ca^{2+}$ and 0.5 mM of $Mg^{2+}$.
7. Ice.
8. Sterile Polyethylene Cell Scrapers.
9. Cytoskeletal Extraction Buffer (CSKB): 0.5% Triton X-100, 50 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 10 mM PIPES, 20 µg/mL aprotinin, 1 µg/mL pepstatin, 1 mM PMSF. Aprotinin, pepstatin, and PMSF are protease inhibitors; they should be added just before the buffer is used. The buffer without the protease inhibitors can be prepared ahead of time and stored at 4°C. Buffer should be chilled.
10. Protease inhibitor cocktail tablet. The tablet contains a mixture of protease inhibitors that inhibit many proteases including serine-cysteine- and metalloproteases. When dissolved in aqueous solution, the tablet offers extensive protection of the proteins in cell lysates.
11. Potter homogenizer and sonicator. These instruments are used briefly to break down the cell membranes, allowing the recovery of components within the cells.
12. Capture-Tec™ stand (Invitrogen Canada Inc.). The stand facilitates the isolation and recovery of the magnetic beads within the cell lysates solution. Prechill the stand on ice before use.

### *2.10. SDS–Polyacrylamide Gel Electrophoresis (SDS–PAGE)*

1. 0.5 M Tris–HCl, pH 6.8, for stacking gel. Store at 4°C.
2. 1.5 M Tris–HCl, pH 8.8, for separating gel. Store at 4°C.
3. 30% acrylamide/bis solution (37.5:1 with 2.6% C) (This is a neurotoxin and it may cause cancer and heritable genetic damage. Avoid exposure by wearing lab coat, goggles, and gloves).
4. Sodium dodecyl (lauryl) sulfate (SDS): prepare 10% solution in water and store room temperature (*see* **Notes 8** and **9**).
5. 1,2-Di-(Dimethylamino)Ethane (TEMED) (TEMED is highly flammable in liquid and vapor. It should be used and stored away from any heat source) (*see* **Note 10**).
6. Ammonium persulfate: prepare 10% solution in water and immediately freeze in 500 µL aliquots at –20°C (*see* **Note 11**).
7. Sterile water.
8. Water-saturated isobutanol. Shake equal volumes of water and isobutanol in a glass bottle and allow to separate. Use the top layer only. Store at room temperature.
9. Running buffer: For 1 L, prepare 10× stock by combining 30.3 g Tris, 144 g glycine, and 100 mL 10% SDS. QS with water. Mix thoroughly (do not adjust pH). Store at 4°C. Dilute 100 mL 10× running buffer stock with 900 mL water for use.

10. Prestained molecular weight marker: PageRuler Prestained Protein Ladder Plus (Fermentas, EU) (*see* **Note 12**).

### 2.11. Western Blotting for Phagosomal Proteins

1. Transfer buffer (10×): For 1 L, prepare 10× stock by combining 30.3 g Tris, 144 g glycine. QS with water. Mix and store at 4°C (1×: 25 mM Tris, 192 mM glycine, 20%, v/v methanol).
2. Mix 100 mL 10×transfer buffer, 200 mL methanol, and 700 mL water for use. Store at 4°C. Prepare buffer in advance and refrigerate. It is important that buffer temperature is 4°C at the start of transfer. Buffer preparation is extremely important. Do not adjust transfer buffer pH by addition of acid or base unless indicated in the instructions. Improperly made buffer will cause excess heat generation and potential safety hazards.
3. Nitrocellulose membrane and Whatman blotting paper. Cut them so they are slightly larger than the gel.
4. Fill Bio-Ice cooling unit with water and store it in –20°C in advance. The ice block serves as a heat sink for the heat generated during an electrophoretic transfer.
5. Tris-buffered saline with Tween (TBS-T): Prepare 1.5 L of 10× stock by mixing 36.3 g Tris and 120 g NaCl in water. Dilute 100 mL 10× stock with 900 mL water and add in 1 mL of Tween-20. Mix well before use.
6. Blocking buffer: 5% (w/v) nonfat dry milk in TBS-T.
7. Ponceau S solution (0.1% Ponceau S w/v in 5% acetic acid (v/v): (*see* **Note 13**) Dilute 5 mL Ponceau S with 45 mL water. Store at room temperature in dark container.
8. Primary antibody dilution buffer: TBS-T supplemented with 5% (w/v) albumin bovine serum fraction V (BSA). Store at 4°C.
9. Primary antibody: rabbit anti-PTP-MEG2.
10. Secondary antibodies: ECL™ antimouse IgG, horse radish peroxidase linked whole antibody (from sheep) or ECL™ antirabbit IgG, horse radish peroxidase linked whole antibody (from donkey). Dilute in 5% (w/v) milk in 1× TBS-T.
11. Enhanced chemiluminescence (ECL) reagents. Mix equal parts of Luminol and Oxidizing Reagent together and store in tinted container at 4°C until use.
12. Bioflex Econo Film.

### 2.12. Stripping and Reprobing

1. Stripping buffer: 64 mM Tris–HCl pH 6.8, 2% (w/v) SDS. For every 10 mL stripping buffer, add 79.7 µL of mercaptoethanol and mix well before use (*see* **Note 14**).
2. Water bath at 50°C.
3. Wash buffer: 1× TBS-T.

4. Primary antibodies: mouse antilysosome-associated-membrane-protein 1 (LAMP1), rabbit anticytochrome C.
5. Secondary antibodies: ECL™ antimouse IgG, horse radish peroxidase linked whole antibody (from sheep) or ECL™ antirabbit IgG, horse radish peroxidase linked whole antibody (from donkey). Dilute in 5% (w/v) milk in 1× TBS-T.

## 3. Methods

To determine the subcellular localization of PTP-MEG2, wild-type PTP-MEG2 cDNA was introduced by transfection into RAW264.7 murine macrophages and visualized using immunofluorescence microscopy and antibodies to the epitope tag (hemagglutinin; HA). The distribution of PTP-MEG2 was compared to that of known organelle markers to assist in determination of its localization to specific intracellular compartments. Demonstrated previously to bind to phosphoinositides $PIP_2$ and $PIP_3$ *(12)*, regulation of PTP-MEG2 phosphatase activity by these purified lipids in liposomes was tested using recombinant PTP-MEG2. A colorimetric phosphatase assay using *p*NPP *(19)* was used to determine if the different lipids were able to modulate the catalytic activity of PTP-MEG2. The domain responsible for this lipid-mediated regulation can be identified and confirmed by in vitro assays using bacterially expressed recombinant mutants of PTP-MEG2.

To ascertain the role of PTP-MEG2 in phagocytosis, fluorescently tagged PTP-MEG2 was transfected into RAW264.7 macrophages along with the pleckstrin homology domain of Akt that binds specifically to $PIP_3$ *(20)*. IgG-coated beads were then fed to the macrophages as prey to initiate Fc receptor-specific phagocytosis *(21)*. The subsequent recruitment of these fluorescent proteins to the nascent phagosome was tracked using confocal fluorescence microscopy. Recruitment of these molecules to the phagosome was verified by magnetic phagosome isolation *(22)* and western blot analyses of the phagosome-associated proteins.

### 3.1. Cell Culture

1. Murine macrophages RAW 264.7 are cultured in complete DMEM. When at 80% confluence, cells are removed from the surface of the flask by gentle scraping with a cell lifter.
2. The cells are kept under exponential growth conditions at 37°C with 5% $CO_2$.

### 3.2. Cell Transfection

1. All the transfections are performed according to the general protocols given later. Specific details for transfection for each

experiment, including the types of plasmid DNAs utilized are highlighted in the subsequent subheadings.

2. One day prior to transfection, $2.5 \times 10^5$ cells are seeded on each 18-mm-diameter glass coverslip in a 12-well plate (in 1 mL complete DMEM).
3. For transfection the following day, combine 1.5 µg of plasmid DNA with 7.5 µg of SuperFect Reagent in a final volume of 75 µL of serum-free DMEM in a labeled tube (*see* **Note 4**).
4. Mix by pipetting up and down five times or by vortexing for 10 s.
5. Incubate the samples at room temperature for 20 min to allow transfection-complex formation.
6. While complex formation takes place, gently aspirate growth medium from the plate and wash cells once with 1 mL PBS.
7. Add complete DMEM to the transfection mixture to a total volume of 1 mL.
8. Immediately transfer the total volume to cells adhered on coverslips in 12-well plate (*see* **Note 15**).
9. Incubate cells with transfection complex for at least 6 h under normal growth conditions (*see* **Note 16**).
10. Double transfections can be performed using the same procedures, except 1.5 µg of each vector and 15 µL of SuperFect Reagent are used per transfection.

### *3.3. Confocal Immunofluorescence for PTP-MEG2*

1. Transfection of RAW cells with HA-tagged PTP-MEG2-WT is performed according to the procedures described in **Subheading 3.2.** As illustrated in **Fig. 1**, PTP-MEG2-WT consists of an N-terminal lipid-binding domain homologous to yeast Sec14, *(23)*, as well as a catalytic domain at its C-terminus.
2. Post-transfection (6–24 h), fix cells with 1 mL of 4% paraformaldehyde for 20 min at room temperature (*see* **Note 17**). Remove fixative by washing cells twice with PBS. Leaving samples in fixative for long periods of time ("overfixation") will lead to degradation of the immunofluorescence signal likely due to epitope modification.
3. Permeabilize cell membranes with 1 mL of 0.1% Triton X-100 in PBS at room temperature for 20 min. Rinse cells twice with PBS after permeabilization.
4. Block nonspecific binding sites by incubating samples at room temperature with blocking buffer (5% goat serum and 3% BSA) for 30 min.

5. Dilute primary antibodies in blocking buffer: rabbit anti-HA (1:500) or mouse anti-KDEL (1:250). These antibodies can be prepared ahead of time and stored at 4°C until use. For cotransfected samples, combine each antibody at the appropriate dilution.
6. Carefully aspirate the blocking buffer from the wells and coverslips (*see* **Note 18**).
7. Add 250 μL of primary antibody solution to the sample of interest. Ensure that the glass coverslip is evenly covered with the antibody. Incubate samples with antibody for 1 h at room temperature.
8. Remove primary antibody by two washes with PBS.
9. Prepare secondary antibodies in blocking buffer at the following dilutions: donkey antirabbit FITC (1:500), and/or donkey antimouse Texas Red (1:500). Dispense 200–250 μL of secondary antibody solution in the same way as for primary antibody. Incubate samples for 1 h at room temperature.
10. Discard secondary antibody by rinsing cells twice with PBS for 20 min. Samples are ready to be mounted on slides.
11. Dispense a drop of mounting medium on a labeled microscope slide. Using a pair of forceps with fine tips, carefully lift and invert coverslips on microscope slide making certain that there are no bubbles (these can be removed by gently tapping the slide with the forceps) (*see* **Note 19**).
12. Store and dry slides flat in the dark at room temperature overnight (in slide box or covered with aluminum foil).
13. Samples can be viewed with a microscope when the mounting medium is completely dry. Slides can be stored flat in the dark temporary at 4°C or long-term at –20°C.
14. Fluorescent images are captured with a confocal microscope (ZEISS LSM510) or with a conventional fluorescence microscope (Leica DMRA2) equipped with a cooled CCD camera (QImaging). Excitation at 484 nm induces the FITC (green emission 515 nm) for HA-tagged PTP-MEG2-WT, while excitation at 568 nm induces Texas Red (red emission) to detect endogenous KDEL. Fluorescent images can be overlayed using imaging software (OpenLab by Improvision or Adobe Photoshop). Examples of fluorescent images demonstrating the distribution of PTP-MEG2 and its colocalization with an ER marker, KDEL, are shown in **Fig. 2**.

### *3.4. Liposome Preparation*

1. Each lyophilized lipid is dissolved in chloroform at a concentration of approximately 1 mg/mL and transferred to glass tubes or vials. Seal tubes with parafilm.

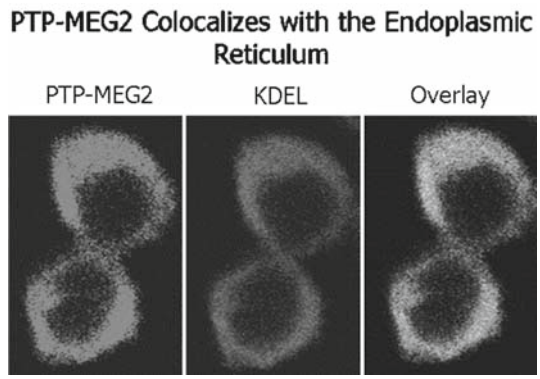



Fig. 2. PTP-MEG2 wild type colocalizes with the endoplasmic reticulum in resting macrophages. RAW264.7 murine macrophages grown on glass coverslips were transfected with PTP-MEG2-WT. Cells were fixed, permeabilized, and stained with anti-HA and anti-KDEL as described in methods. (a) PTP-MEG2-WT is shown in green, (b) the endoplasmic reticulum-specific marker is in red, and (c) colocalization of PTP-MEG2-WT in endoplasmic reticulum appears in yellow (overlay) (reproduced from **ref**. *16)*.

2. In a rotary-evaporator vial, mix DOPC solution with test lipid solution in a 19:1 ratio to form mixed liposomes. The bottom of the vial is immersed in a 37°C water bath, while the top of the vial is connected to a rotary evaporator.
3. The rotary evaporator rotates the vial in the water bath and applies suction that facilitates evaporation of the chloroform. The remaining lipid mixture will appear as a film on the side of the vial. When completely evaporated, there should be no residual odor of chloroform.
4. Dissolve lipid film in buffer to a final concentration of 1 mM. Resuspend lipid by tapping the vial and by pipetting up and down through a needle (first use a 22-gauge and then a 27-gauge needle).
5. Transfer the sample to a plastic microfuge tube and warm to 37°C. Plastic can be used from this point on because there is no chloroform remaining.
6. Subject the solution to three cycles of freeze–thawing to disrupt very large vesicles and generate a suspension of more uniform size. For each cycle, samples are frozen in a container of ethanol and dry ice for 5 min and then warm to 37°C for 5 min.
7. Incubate the samples at room temperature for 20 min and then vortex gently. Pass samples through an extruding chamber containing a 400-nm filter. Collect the filtered samples in the same plastic tubes and keep at 37°C until all samples have been prepared. Samples may then be stored at 4°C until use.

### 3.5. Phosphatase Assays

1. Incubate 30 μg/mL of recombinant PTP-MEG2 protein for 40 min at 37°C with or without liposomes (300 μM) in phosphatase buffer.
2. Following the incubation, add 40 μL of phosphatase buffer containing 10 mg/mL para-nitrophenylphosphate (*p*NPP) and 10 mM DTT to each sample.
3. Incubate samples for an additional 40 min at 37°C with agitation. Measure absorbance at 405 nm on a microplate reader. **Table 1** summarizes the absorbance readings obtained from the recombinant PTP-MEG2 proteins in both the presence and the absence of the various test lipids; the values shown represent the means ± standard deviation (SD) and the 95% confidence interval of six experiments. Test lipids were considered "present" in liposomes containing 5% $PIP_2$, $PIP_3$, or PS (in addition to 95% DOPC) and "absent" in liposomes containing only DOPC. DOPC is regarded as a vehicle control in these experiments. The phosphatase activities of the recombinant PTP-MEG2 proteins, in the absence of any test lipids (i.e., only DOPC was present), are shown in **Fig. 3A**. Percentage of phosphatase activity is expressed

**Table 1**
**Phosphatase activity of recombinant PTP-MEG2 is modulated by lipids. The phosphatase activity of PTP-MEG2-WT is modulated by $PIP_3$ and $PIP_2$, but not by PS. The N-terminal mutants (K55M, K184M, K55/184M) with impaired lipid-binding ability displayed much less phosphatase activity compared to that of the PTP-MEG2-WT/DOPC activity. Other PTP-MEG2 constructs are not affected significantly by lipids. We speculate that the enhancement in MEG2 phosphatase activity may occur by a conformational change in the molecule that unfolds the lipid-binding domain from the catalytic domain, leading to enhanced activity. Values represent the means ± SD and the 95% confidence interval of $n = 6$ experiments (reproduced from ref. *16*)**

| Means ± SD CI [95%] | PTP MEG2-WT | C-truncated MEG2 | K55M MEG2 | K184M MEG2 | K55/184M MEG2 | C515S MEG2 | N-truncated MEG2 |
|---|---|---|---|---|---|---|---|
| DOPC | 100 | 146 ± 101 | 17 ± 20 | 23 ± 20 | 9 ± 10 | 0 ± 0 | 0 ± 0 |
| | | [67–227] | [1–33] | [7–39] | [1–17] | | |
| $PIP_2$ | 136 ± 29 | 123 ± 112 | 20 ± 15 | 37 ± 24 | 12 ± 11 | 0 ± 0 | 0 ± 0 |
| | [112–160] | [32–213] | [8–32] | [17–57] | [4–20] | | |
| $PIP_3$ | 158 ± 37 | 138 ± 103 | 26 ± 20 | 40 ± 20 | 13 ± 15 | 0 ± 0 | 0 ± 0 |
| | [125–191] | [56–220] | [0–54] | [24–56] | [1–25] | | |
| PS | 95 ± 42 | 93 ± 79 | 30 ± 28 | 50 ± 38 | 18 ± 26 | 0 ± 0 | 0 ± 0 |
| | [54–136] | [17–169] | [3–57] | [23–77] | [0–43] | | |

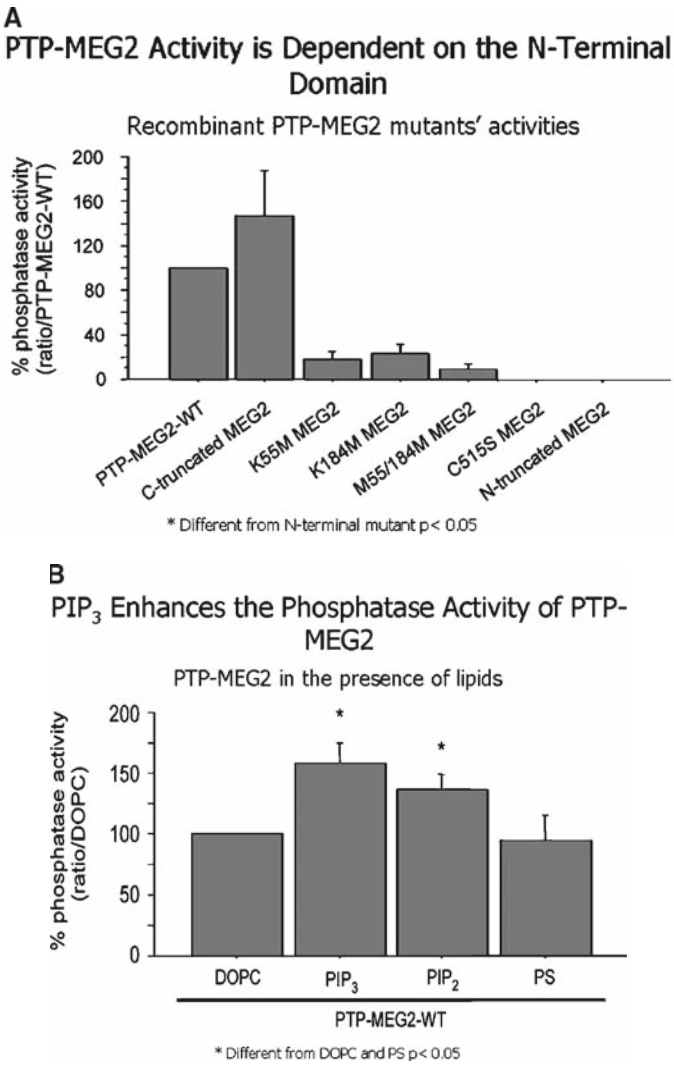



Fig. 3. The phosphatase activity of recombinant PTP-MEG2 is dependent on the integrity of its N-domain. (**A**) Recombinant proteins were mixed with liposomes containing only DOPC for 40 min. followed by addition of the substrate, *p*NPP substrate. Samples were incubated for 40 min at 37°C and absorbance was measured at 405 nm. The phosphatase activity is reported as percentage of PTP-MEG2-WT activity. Values represent the means ± SD and the 95% confidence interval of six experiments. In the presence of only DOPC, the C-terminal domain of MEG2 (i.e., N terminal truncated) has the highest phosphatase activity, compared to the full length PTP-MEG2-WT (100%). C515S MEG2 and N-terminal MEG2 possessed no phosphatase activity due to loss of a key cysteine residue and lack of the phosphatase domain, respectively. The lipid-binding mutants (K55M, K184M, and K55/184M) which retain their C-terminal catalytic domain had much lower phosphatase activity compared to PTP-MEG2-WT. These findings imply that the lysine-to-methionine point mutations of the N-terminal Sec14 homology domain affect the phosphatase activity of PTP-MEG2. This suggests an intact Sec14 domain may serve as a regulatory domain that controls the magnitude and extent of PTP-MEG2 activation, and it may be dependent upon its ability to associate with certain lipids in determining the tertiary structure of the molecule (reproduced from **ref.** *16)*. (**B**) Phosphatase activity of recombinant PTP-MEG2 wild type is modulated by lipids. The recombinant protein was mixed with various test lipid-containing liposomes for 40 min at 37°C followed by addition of *p*NPP Samples were incubated for an additional 40 min at 37°C. Absorbance was measured at 405 nm. Phosphatase activities were reported as a percentage of PTP-MEG2-WT/DOPC activity. Values represent the means ± SD and the 95% confidence interval of six experiments. The asterisk (*) represents significant difference from PS, $P$ 0.05. Mixed liposomes containing 5% $PIP_3$ enhanced the phosphatase activity of PTP-MEG2-WT, compared to DOPC alone (100%). $PIP_2$ also increased the phosphatase activity of PTP-MEG2, but the effect was of a smaller magnitude (reproduced from **ref.** *16)*.

relative to PTP-MEG2-WT (PTP-MEG2 WT as 100%). The phosphatase activities of PTP-MEG2-WT in the presence of different test lipids are illustrated in **Fig. 3b**. These data suggest that the N-terminal domain exerts a negative regulatory influence on MEG2 catalytic activity because when it is deleted, the activity increases. However, the catalytic activity of the Sec14 domain point mutants is dramatically decreased compared to WT control. One possible explanation for these observations is that the mutations in these residues result in a conformational change in the molecule that prevents access of the substrate to the enzymatic site. The percent phosphatase activity is calculated relative to the value obtained from DOPC which was set at 100%.

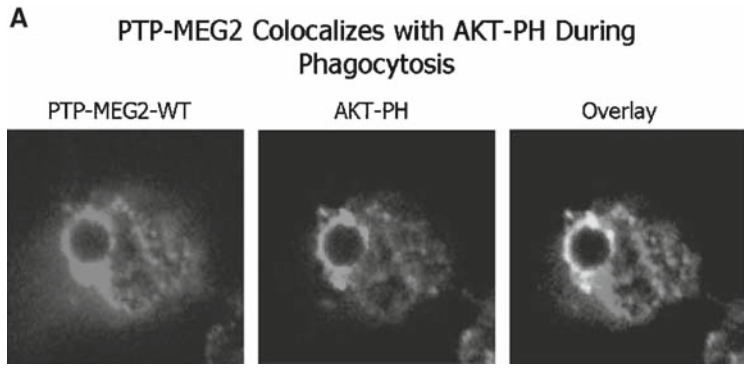
### *3.6. Phagocytosis*

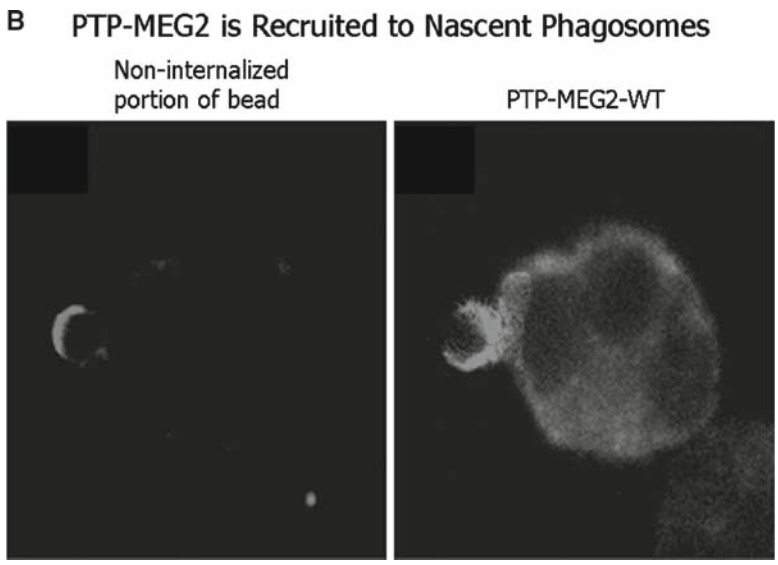
1. Double transfections of HA-tagged PTP-MEG2-WT and GFP-AKT-PH into RAW macrophages are performed according to the procedures outlined in **Subheading 3.2.**
2. Proceed with the phagocytosis assay the following day.
3. Sediment 100 μL latex beads by brief centrifugation at 2,000 g in a microfuge.
4. Wash beads twice with 1 mL PBS. Carefully aspirate PBS to avoid loss of beads.
5. Coat beads with 1 mg/mL human IgG in PBS for 1 h at 37°C with slow rotation.
6. Centrifuge beads briefly and wash twice with 1 mL PBS. Keep on ice until use.
7. Following DNA transfection, wash cells once with 1 mL chilled PBS. Cool cells by placing culture plate on ice.
8. For each sample, add 1 mL human IgG-coated latex beads solution to cells (*see* **Note 20**).
9. Incubate the samples 20 min on ice. During this period, the beads will settle and bind to the cells. Phagocytosis of the beads by the cells is restrained by the low temperature. This enables the onset of the phagocytic process to be accurately timed and synchronized. Contact of the cells and beads can be hastened by brief centrifugation at 800 g for 1 min at 4°C.
10. Remove excess unbound beads by washing coverslips once with 1 mL cold PBS. Check and note the extent of bead binding to the cells. Aspirate all the PBS from wells.
11. Initiate phagocytosis by adding 1 mL prewarmed (37°C) complete DMEM to each sample. Incubate cells at 37°C for 5 min with 5% $CO_2$.
12. Wash cells twice with chilled PBS to terminate the process of phagocytosis. Samples are ready to be examined by immunofluorescence.

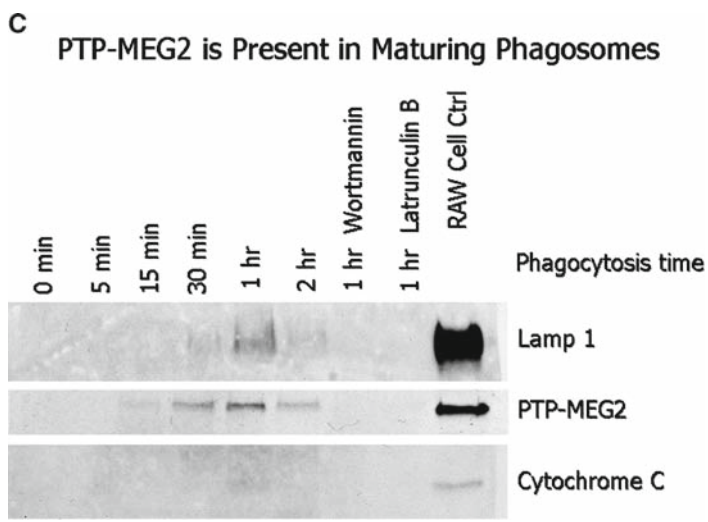
### 3.7. Confocal Immunofluorescence for Localization of PTP-MEG2 and Akt-PH During Phagocytosis

1. The procedures for immunofluorescence are outlined in **Subheading 3.3, steps 1–4**.
2. Dilute primary antibody in blocking buffer: rabbit anti-HA (1:500). 250 µL of primary antibody solution is added carefully and evenly to each sample. Incubate samples with antibody for 1 h at room temperature.
3. Remove primary antibody by two washes of PBS.
4. Secondary antibody is prepared with blocking buffer: donkey antirabbit Texas Red (1:500). Dispense 200–250 µL of secondary antibody solution in the same way as with primary antibody. Incubate samples for 1 h at room temperature.
5. Wash and mount coverslips onto microscope slides as described in **Subheading 3.3, steps 10–13**.
6. Fluorescent images are captured with a confocal microscope (ZEISS LSM510) or with a conventional fluorescence microscope (Leica DMRA2) equipped with a cooled CCD camera (QIImaging). Excitation at 484 nm induces the FITC (green emission: 515 nm) for GFP-AKT-PH domain, while excitation at 568 nm induces Texas Red (red emission) to detect HA-tagged PTP-MEG2. Fluorescent images can be overlaid using imaging software (OpenLab by Improvision or Adobe Photoshop). **Fig. 4a, b** are examples of the fluorescent images of GFP-AKT-PH and PTP-MEG2-HA. It is evident that during phagocytosis, these two molecules are recruited to the nascent phagosome enclosing the IgG-opsonized latex bead within. **Fig. 4c** represents a western blot of phagosome-associated proteins illustrating recruitment of the phagosomal protein LAMP1 along with PTP-MEG2 to the phagosome. Cytochrome C is a cytosolic protein included as a negative control.

Fig. 4 . (**A**) PTP-MEG2-WT colocalizes with Akt-PH domain in nascent phagosomes. RAW cells grown on glass coverslips were cotransfected with PTP-MEG2-WT and GFP-Akt-PH domains. Cells were fed human IgG-coated latex beads on ice for 20 min and rewarmed for 5 min to allow phagocytosis to proceed. Cells were then fixed and immunostained with the anti-HA antibody. PTP-MEG2-WT is shown in red and GFP-Akt-PH in green. Colocalization of PTP-MEG2 and Akt-PH domain appears in yellow (overlay). Images were acquired using a confocal microscope (reproduced from **ref.***16)*. (**B**) PTP-MEG2 is recruited at early times to the phagosome. RAW macrophages cultured on glass coverslips were transfected with PTP-MEG2-WT. Cells were incubated with human IgG-coated latex beads on ice for 20 min and rewarmed for 5 min to allow phagocytosis to proceed. Cells were then fixed for immunofluorescence with anti-HA antibody. The external part of the beads was labeled in red with TRITC conjugated anti-IgG antibody prior to permeabilization. PTP-MEG2-WT appears in green. Images were captured using a confocal microscope as described in the methods (reproduced from ref. *16)*(**C**) PTP-MEG2 is recruited to the nascent phagosome. RAW macrophages grown on 60 × 15 mm tissue culture plates were fed with IgG-coated magnetic beads (3 µm) for incubation times ranging from 0 min to 2 h. Following phagocytosis, phagosomes were isolated by MACS and separated by SDS–PAGE. Western analysis of phagosome-associated proteins showed maximal accumulation of MEG2 1 h after phagocytosis was initiated. Treatment of RAW macrophages with phagocytosis inhibitors wortmannin or latrunculin B as well as LAMP1 and cytochrome C immunoblotting confirmed the efficiency and specificity of the phagosome isolation technique (reproduced from **ref.** *16)*.


A
PTP-MEG2 Colocalizes with AKT-PH During Phagocytosis
PTP-MEG2-WT
AKT-PH
Overlay


B
PTP-MEG2 is Recruited to Nascent Phagosomes
Non-internalized portion of bead
PTP-MEG2-WT


C
PTP-MEG2 is Present in Maturing Phagosomes
0 min
5 min
15 min
30 min
1 hr
2 hr
1 hr Wortmannin
1 hr Latrunculin B
RAW Cell Ctrl
Phagocytosis time
Lamp 1
PTP-MEG2
Cytochrome C

### 3.8. Confocal Immunofluorescence of PTP-MEG2 at Nascent Phagosome

1. Transfection of RAW cells with HA-tagged PTP-MEG2-WT is same as before (*see* **Subheading 3.2**).
2. Next day post-transfection, proceed with phagocytosis assay as outlined in **Subheading 3.3**, **steps 3–12**.
3. Continue on to the immunofluorescence procedures in **steps 1–4**, **Subheading 3.3.** In addition, prior to permeabilization, stain the noningested portion of the bead first with TRITC-conjugated goat-antihuman IgG (1:500) to delineate the noninternalized portion of the prey.
4. Prepare primary antibody in blocking buffer: rabbit anti-HA (1:500). 250 μL of primary antibody solution is added to each sample. Incubate samples with antibody at room temperature for 1 h. Wash samples twice with PBS to remove primary antibody afterward.
5. Dilute secondary antibody in blocking buffer: donkey anti-rabbit FITC (1:500). Dispense 200–250 μL of secondary antibody solution in the same way as for primary antibody. Incubate samples for 1 h at room temperature.
6. Proceed to washing and mounting coverslips onto microscope slides as described in **Subheading 3.3**, **steps 10–13**.
7. Fluorescent images are captured and overlaid as earlier (*see* **Subheading 3.3**, **step 14** and **Subheading 3.7**, **step 6**). Excitation at 484 nm induces the FITC for HA-tagged PTP-MEG2-WT, and excitation at 568 nm induces Texas Red to detect the noninternalized portion of the latex bead that is opsonized with human IgG. Examples of the fluorescent images demonstrating the recruitment and enrichment of PTP-MEG2 to the nascent phagosome are illustrated in **Fig. 4A**, **B**.

### 3.9. Phagosome Isolation

1. To determine the recruitment of various proteins to the nascent phagosome, the duration of phagocytosis can be set at different time points: 0, 5, 15, 30, 60, and 120 min. For negative control purposes, cells are pretreated for 1 h at 37°C 5% $CO_2$ with either 1 μM latrunculin B or 100 nm wortmannin (pharmacological agents that prevent phagocytosis from proceeding). Always collect a sample of total cell lysate for analysis by SDS–PAGE and Western blotting to confirm the Mr of the particular protein to be studied.
2. Plate RAW267.4 macrophages (phagocytes) in a total volume of 4 mL of complete DMEM on each of nine 60 × 15 mm cell culture plates. Culture cells at 37°C with 5% $CO_2$ until near confluence. Each plate is used for one time point for phagocytosis or control condition. For control purposes, prepare an additional plate under the same conditions for collection of total cell lysates.

3. Coat 1 mL of the 3-μm magnetic beads (i.e., phagocytic prey) with 50 μg/mL human IgG for 30 min rotating at 37°C (in PBS/HBSS). After incubation, wash the beads twice with PBS. Resuspend beads to a total volume of 1 mL. Sonicate beads for 10 s with a probe sonicator to minimize clumping.
4. Aspirate the culture medium from the macrophages and dispense 4 mL of HBSS++ buffer for each plate. The phagocytic assay will be conducted in HBSS++ buffer.
5. Add 100 μL of the human IgG-coated beads to each sample of macrophages. Swirl gently to distribute beads evenly.
6. Incubate cells on ice for 20 min. Beads will sediment and bind to the cells without being ingested because the low temperature inhibits phagocytosis. In this manner, the duration of phagocytosis can be accurately timed and synchronized among all the samples (*see* **Note 20**).
7. After incubation on ice, place the dishes in a 37-C incubator with 5% $CO_2$ to initiate phagocytosis. Allow phagocytosis to proceed for different time periods (from 0 min to 2 h). Incubate cells with beads for 1 h with latrunculin B or wortmannin as negative controls.
8. Following phagocytosis, place the samples immediately on ice to stop phagocytosis. Wash cells three times with cold PBS to remove unbound beads.
9. Dissolve one tablet of protease inhibitor cocktail tablet in 10 mL of chilled CSKB buffer. Mix thoroughly before dispensing. Place CSKB buffer on ice.
10. Collect cells using a cell scraper from each dish in 1 mL CSKB containing protease inhibitors. Put samples into labeled tubes on ice.
11. With samples submerged in ice, disrupt the cells using a Potter homogenizer (20 strokes) and sonicator (10 s). Phagosomes containing internalized magnetic beads are released during this process and can be retrieved.
12. Place the tubes into the prechilled Capture-Tec™ stand for the magnetic isolation of the phagosomes containing the magnetic beads. The magnetic beads will be sedimented by the magnet.
13. With the tubes remaining in the stand, carefully aspirate the excess CSKB buffer without disturbing the magnetic beads. Wash the beads 2–3 more times with 1 mL CSKB. Sediment the beads using the magnetic stand and aspirate the CSKB.
14. To each sample of phagosomes containing magnetic beads, add 30–100 μL of 10× Laemmli sample buffer to elute the phagosomal proteins from the beads. The amount of Laemmli

sample buffer to be used depends primarily on the amount of phagosomal components retrieved (i.e., internalized beads) (*see* **Note 21**).

15. For control purposes, lyse the macrophages in the additional plate prepared in 1 ml SDS lysis buffer using a cell scraper (*see* **Subheading 3.9**, **step 2,** and **Note 22**). Collect total cell lysates in a labeled tube and add in 100 µL 10× Laemmli sample buffer.
16. Close tubes tightly and secure lids with plastic guards. Boil samples for 10 min; then vortex briefly and centrifuge at 2,000 g for 30 s in a microfuge to sediment the magnetic beads.
17. Samples are ready for separation by SDS–PAGE once they cool to room temperature. Avoid loading of the magnetic beads.
18. Store the leftover phagosome lysates at –20°C for further analyses. Boil, vortex, and centrifuge before use.

### *3.10. SDS–Polyacrylamide Gel Electrophoresis (SDS–PAGE)*

1. The following directions are for the use of Bio-Rad Mini-Protean 3 Cell System. The system consists of a gel casting stand and glass plates with built-in spacers to eliminate leaking during hand casting.
2. The spacer plate is the taller glass plate with gel spacers permanently set. Various thicknesses of the spacer plates and combs are available: 1.5-mm thick spacer plates and a 15-well comb are used in this SDS–PAGE protocol.
3. Before use, wash spacer plate, short plate, and comb with detergent and rinse extensively with distilled water. Dry with Kim-Wipe and remove residual moisture with 70% ethanol.
4. Align spacer and short plates, with short plate facing front. Slide them into the casting frame. Make certain that both plates are perpendicular to the level surface to avoid leaking. Lock the pressure cams to secure the glass plates in place. This forms the gel cassette assembly.
5. Engage the spring-loaded lever and place cassette assembly on the gray casting stand. The lever pushes the spacer plate down against the gray rubber gaskets.
6. Prepare a 10% separating gel by mixing together 3.3 mL 30% acrylamide, 2.5 mL 1.5 M Tris–HCl of pH 8.8, 4.1 mL of water, 100 µL 10% SDS solution, 5 µL TEMED, and 50 µL 10% ammonium persulfate solution.
7. With a transfer pipette, transfer the solution into the space between the spacer and short plates, leaving about 2 cm of space for the stacking gel. Overlay with water-saturated isobutanol to prevent evaporation and bubble formation. The separating gel should polymerize in about 30 min (*see* **Note 23**).

8. Pour off isobutanol and rinse the top of the gel with water until there is no residual odor of isobutanol.
9. Prepare 4% stacking gel by mixing together 1.3 mL 30% acrylamide, 2.5 mL 0.5 M Tris–HCl of pH 6.8, 6.1 mL of water, 100 μL 10% SDS solution, 20 μL TEMED, and 100 μL 10% ammonium persulfate solution.
10. Pour the 4% gel on top of the polymerized separating gel. Avoid bubbles. Insert the comb in between the two glass plates. The stacking gel should polymerize within 10 min. When the stacking gel is set, remove the gel cassette sandwich from the casting frame (*see* **Notes 24** and **25**).
11. Place the gel cassette sandwich into the slot at the bottom of each side of the electrode assembly with short plate facing inward toward the notches of the U-shaped gaskets. If only one gel is to be run, use the one-piece molded buffer dam for the other side of the electrode assembly. The electrode assembly houses the sealing gasket, upper and lower electrodes, and the connecting banana plugs.
12. Slide the gel cassette sandwiches and electrode assembly into the clamping frame. Press down the electrode assembly while closing the two cam levers of the clamping frame. This holds the gel cassette sandwiches and electrode assembly in place and insures a proper seal of the Short Plate against the notch on the U-shape gasket. This forms the inner buffer chamber.
13. Lower the inner chamber into the mini tank.
14. Prepare 1× running buffer from 10× stock with water. Remove comb from gel cassette sandwiches and carefully pour approximately 125 mL 1× running buffer into the inner chamber. Excess buffer will cause siphoning of buffer into the lower chamber which can result in buffer loss and interruption of electrophoresis.
15. Add approximately 200 mL of 1× running buffer to mini tank (lower buffer chamber).
16. With a gel-loading tip, wash wells with running buffer.
17. Load 20 μL of each sample in a well. Load samples slowly to allow them to settle evenly on the bottom of the well. Be careful not to puncture the well bottom with pipette tip. Reserve one well for prestained molecular weight markers.
18. Place the lid on the mini tank. Make sure color-coded banana plugs and jacks are correctly oriented and insert properly into the power supply. The anode is red and the cathode is black.
19. The gel is first run at 75 V for 15 min or until dye front is through the stacking gel, and the voltage is then increased to 150 V through the separating gel. Depending on the Mr of the proteins of interest, the dye front can be run off the

gel to maximize protein separation to study large molecular weight proteins. Alternatively, the electrophoresis can be stopped early to enable detection of low molecular weight proteins.

### 3.11. Western Blotting for Phagosomal Proteins

1. The samples separated by SDS–PAGE are transferred to nitrocellulose membranes electrophoretically. These instructions are for the use of the Mini Trans-Blot Electrophoretic Transfer Cell from Bio-Rad.
2. To avoid membrane contamination with human skin proteins, always handle nitrocellulose membrane with forceps or wear gloves.
3. Label the membrane with a permanent marker to identify the gel and orientation. In separate trays, soak the membrane, six precut pieces of Whatman blotting paper and two fiber pads in cold 1× transfer buffer for 30 min before use. Complete wetting of all these components is important for proper binding. Insufficient wetting can cause entrapment of air bubbles in the matrix and block transfer of protein molecules.
4. Following electrophoresis, remove gel carefully from spacer and short plates. Cut away the stacking gel with a sharp spatula. Rinse gel in 1× transfer buffer prior to blotting to remove running buffer salts and detergents. If salts are not removed, they will increase the conductivity of the transfer buffer and the heat generated during the transfer.
5. Slide and lift the latch to open gel holder cassette. One panel of the cassette is clear and the other is gray. The clear panel is the anode (+) and the gray is the cathode (–). For the electrode module, the cathode is the black electrode panel and the anode is the red panel located in the center of the buffer tank. Insert the gel cassette such that the gray plastic faces the black plastic of the cathode electrode.
6. Lay the gray panel side of the cassette flat down on a surface. Place a presoaked fiber pad on top of the gray panel. All components should be centered such that they do not extend past the edge of the cassette. Otherwise, they will catch on the guide rails and distort the blot-gel contact and transfer pattern.
7. Place three pieces of soaked blotting paper on top of fiber pad and then the rinsed gel on top. Make sure the gel is aligned at the center of the cassette and make certain that there are no air bubbles trapped between the gel and the blotting papers.
8. Hold membrane at opposite ends, allow center portion to contact gel first, and gradually lower the ends. Place another three pieces of soaked blotting paper on top of the membrane.

9. With a plastic pipette or test tube, roll over gel and membrane sandwich with steady pressure to exclude all bubbles and ensure good gel and membrane contact. Finish the assembly by placing another piece of saturated fiber pad on top of the blotting paper.
10. Close the cassette by holding it firmly and secure the latch. Place it into the buffer tank so that the gray panel of the holder is facing the black cathode electrode panel. This orientation is crucial; otherwise, the proteins will be lost from the gel into the blotting paper rather than transferred to the membrane.
11. Place a magnetic stir bar at the bottom of the tank. Place in Bio-Ice cooling unit. Fill the tank with 1× transfer buffer just above the level of the gel holder cassette. Set tank on top of magnetic stirrer and secure the lid in place (*see* **Note 26**).
12. Ensure that the electrode wires on the lid are attached to the appropriate pins of the electrode module (i.e., black wire to cathode and red wire to anode). Connect transfer unit to power supply. Turn on power supply and magnetic stirrer to initiate transfer and circulating bath.
13. The transfer is usually run at 100 V for 2 h. To maintain the temperature of the transfer buffer around 10–15°C, the Bio-Ice cooling unit should be changed every 30 min to avoid overheating and protein degradation.
14. When the transfer is complete, carefully take out gel holder cassette and disassemble the gel-blot sandwich. Remove the first layer of fiber pad and blotting papers. Slowly peel the membrane off the gel and rinse it briefly with water. The membrane should be kept moist at all times.
15. Decant water and stain the membrane with diluted Ponceau S solution for 2–3 min on rocking platform at room temperature. Ponceau S solution stains the protein bands transferred from gel to membrane red. Decant the Ponceau S solution and wash the membrane with 1× TBS-T until bands are visible. The transfer pattern and quantification of proteins can be quickly noted and, if necessary, an image captured. Excess membrane can be trimmed away at this time.
16. Reverse Ponceau S stain with several TBS-T washes on rocking platform. When all the dye is removed, block the membrane with 10–15 mL of blocking buffer for 1 h at room temperature on a rocking platform (*see* **Note 27**).
17. Discard the blocking buffer and quickly rinse the membrane once with TBS-T prior to addition of 1:2,000 dilution of rabbit anti-PTP-MEG2 antibody. Incubate the membrane with antibody overnight at 4°C on rocking platform.

18. Remove the primary antibody and wash the membrane three times for 15 min with TBS-T (*see* **Note 28**).
19. Prepare secondary antibody fresh for every experiment at the recommended (e.g., 1:10,000-fold) dilution in blocking buffer. Add to membrane for 40 min at room temperature on a rocking platform. Prolonged incubation with the secondary antibody will result in a high background.
20. Pour off the secondary antibody solution and wash the membrane three times for 20 min with TBS-T. Incubate the blot with premixed ECL solution on shaker for 1 min. Line the X-ray film cassette in advance with an acetate sheet protector. Place the blot between the leaves of the acetate sheet. In a darkroom, expose the film for a suitable amount of time, usually ranging from 30 s to 2 min. **Fig. 4C** is an example demonstrating the increased presence of PTP-MEG2 as phagosomes mature.

### *3.12. Stripping and Reprobing*

1. When a suitable exposure for the first protein to be detected is obtained, the membrane is ready to be stripped to remove the first antibody and can then be reprobed with another antibody that recognizes another protein of interest. For example, PTP-MEG2 can be visualized first and after stripping; other proteins such as lysosome-associated-protein 1 (LAMP1; a phagosomal protein)) or cytochrome C (a cytosolic protein) can be visualized **(Fig. 4C**).
2. Prior to stripping, wash membrane with TBS-T for 10 min to remove the ECL solution.
3. In a container with a lid, add to the blot 10 mL of stripping buffer with mercaptoethanol. Close the lid tightly and incubate blot in a 50°C water bath for 30 min with agitation.
4. When blot is stripped, wash it three times with a large volume of TBS-T; each wash is for 15 min. Block blot again in blocking buffer.
5. Reprobe the blot with mouse anti-LAMP1 (1:1,000 in TBS-T/5% BSA) with washes, antimouse secondary antibody, and ECL detection as discussed earlier. Lysosome-associated-membrane-protein 1, LAMP-1, is a well-established marker that is recruited to phagosomes as they fuse with the lysosomes during the maturation process. This provides them with the degradative properties required to destroy ingested materials *(24)*. The purpose of LAMP-1 immunoblotting is to demonstrate the specificity of the phagosome isolation, to show kinetics of phagosome maturation, and to validate the isolation techniques. An example of the result produced is shown in **(Fig. 4C**).

6. Repeat stripping and reprobing steps for detection of cytochrome C (1:500 in TBS-T/5% BSA). Cytochrome C immunoblotting serves as a negative control marker as it is found exclusively in the mitochondria of healthy cells *(25)*. Therefore, no detection of cytochrome C is expected from the samples containing only the purified phagosomal isolates **(Fig. 4c**).
7. If needed for further analyses, freeze blot at –20°C.
8. Primary antibody is removed from the blot during the stripping procedure; however, some target proteins are dislodged from the blot as well. Hence, avoid excessive stripping of the blot and consider running identical samples in two gels so that two blots can be utilized simultaneously.

## 4. Notes

1. Medium prepared with all these components is referred as the "complete DMEM" or "complete medium."
2. Unless stated otherwise, all solutions should be prepared using Milli Q water that has a resistivity of 18.2 MΩ-cm and total organic content of less than five parts per billion. It is by this standard that "water" is referred to in this text.
3. Dithiothreitol (DTT) is widely used for disruption of protein disulfide bonds in SDS–PAGE. It is not stable in solution. Make 1 M DTT solution and immediately freeze 500 µL aliquots at –20°C. Thaw and add appropriate amount of DTT to SDS lysis buffer just before use.
4. Plasmid DNA is usually diluted in water or TE buffer. When the dilution is done with cell growth medium, ensure that the medium does not contain any serum, proteins, or antibiotics. Serum and antibiotics present will interfere with complex formation and will significantly decrease transfection efficiency.
5. For every new plasmid and/or new cell line used, the amount of plasmid DNA needed for transfection should be adjusted to optimize transfection efficiency and protein expression levels. Some factors to consider are the transfection reagent/ DNA ratio, cell density, growth phase, and optimal time to measurement of reporter gene activity.
6. Save aliquots of sterile serum-free culture medium when preparing for the complete medium. It is useful for transfection.
7. Store unused 4 or 16% paraformaldehyde solution at 4°C in a shaded container (no longer than 1 week). To avoid oxidation, displace oxygen in the container with nitrogen or argon gas.

8. Fine powder of SDS is irritating to eyes, respiratory system, and skin. Wear suitable protective clothing when handling SDS and measure in a hood.
9. Do not put SDS on ice; it will precipitate.
10. Buy small bottle of TEMED as it may degrade after opening and as a result it will take longer for gels to polymerize.
11. Add APS last when making gels as it will cause gels to polymerize rapidly.
12. We have found that the PageRuler Prestained Protein Ladder transfers completely from gel to nitrocellulose membrane. It contains two orange reference bands so that proper orientation can be easily identified.
13. Ponceau S solution is a light-sensitive reagent. Stock and diluted Ponceau S solution should be store in shaded containers. Ponceau S solution can stain protein bands on nitrocellulose membrane. Its red color makes it easy to quantify protein loading and transfer efficiency. Staining is reversible by washing the nitrocellulose membrane in TBS-T. Proceed to the blocking step once the red stain is removed.
14. Mercaptoethanol gives off an extremely unpleasant odor (rotten eggs). When membranes need to be stripped, mix and dispense stripping buffer in a well-ventilated hood and use containers with tight-fitting lids.
15. In contrast to many liposomal transfection reagents, the presence of serum and antibiotics during transfection with SuperFect Reagent enhances transfection efficiency. Use complete medium with serum that the cells have adapted in normal culture condition during incubations with transfection complexes afterward to optimize expression of the transgene. However, it is not recommended to use serum during complex formation between SuperFect Reagent and plasmid DNA because it inhibits complex formation.
16. If the plasmid DNA is conjugated with a fluorescent reporter, the samples can be viewed under the fluorescent microscope with the appropriate filter to ascertain expression of the introduced gene and percentage of cells transfected.
17. If proteins expressed are fluorescently labeled, cover sample with aluminum foil to prevent unnecessary bleaching of the signal.
18. Before primary antibody incubation, use a Pasteur glass pipette to aspirate all PBS, especially around the rim of the coverslips. This prevents spilling of the antibody when it is added on top of the coverslips. By this method, less antibody is used for economy. If the antibody is very scarce, consider chamber slides. Avoid touching or scratching the surface of the coverslips.

19. To minimize bubbles, first tilt the coverslip at an angle and carefully lay it down onto microscope slide. Avoid using excess mounting medium.
20. Usually excess phagocytic prey is added to the phagocytes. The usual ratio of prey to phagocyte ranges between 5:1 and 20:1.
21. Adjust the volume of 10× Laemmli sample buffer for the samples accordingly. Determine the amount needed for gel loading and always prepare extra for future analyses. Do not overdilute the samples.
22. The cell lysate will be very viscous at this stage because of the release of DNA. It is very hard to pipette. Cut pipette tips to increase the size of the opening; autoclave pipette tips before use to collect lysate. The viscosity of cell lysates will reduce during boiling due to the destruction of DNA.
23. Keep leftover gel mixture and use it as an indicator of gel polymerization. Do not disturb cast gel. It will distort the boundary between stacking and separating gels and affect running pattern.
24. Be careful with splashing of unpolymerized gel mix, especially during the insertion of the comb. Wear protective gloves, lab coat, and goggles. Ensure comb thickness corresponds to thickness of the Spacer Plate. Stacking gel polymerizes much faster than separating gel.
25. It is best to prepare the gel 1 day prior to use for SDS–PAGE. Remove Gel Cassette Sandwich from Casting Frame. Wrap it with a moist paper towel and plastic wrap. Store the gel with combs at 4°C. This makes the gel compact and firm such that it can be handled without tearing or fracturing.
26. Transfer buffer at 1× working concentration can be reused for up to five transfers as long as constant voltage is maintained for each run (the current will increase each time). To prevent heat-induced damage to the apparatus and the experimental samples, ensure that the buffer is cooled by using a refrigerated/circulating bath with ice.
27. Ensure 5% milk solution is not spoiled before use for blocking.
28. Primary antibody in 5% BSA solution may be saved for other experiments and can be frozen at –20°C for long-term storage. Thaw antibody in a 37°C water bath. This is especially useful when primary antibody is scarce. Antibodies will be depleted over many usages, so the length of exposure and type of film (normal vs. sensitive) may need to be adjusted.

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# Chapter 20

# Large-Scale Phagosome Preparation


## Adrien F. Vinet and Albert Descoteaux



## Summary

Phagocytosis is the process by which cells engulf and destroy large particles such as pathogens or apoptotic cells. In this way, macrophages play a pivotal role in the resolution of microbial infections. However, many microorganisms have evolved efficient strategies to preempt the weaponry of macrophages. A better understanding of the components engaged in the phagosome formation and maturation is necessary to devise novel approaches aimed at counteracting these microbial strategies. Recently, large-scale approaches have been used to improve our understanding of phagosome functional properties by the identification of hundreds of proteins and by studying each of them.

Presently, purification of pathogen-containing phagosomes presents several technical challenges, whereas the use of latex beads to isolate phagosomes presents many advantages because this system can mimic host–pathogen interactions during phagocytosis. This system thus remains the best approach to advance our knowledge of phagosome biology, notably when used in conjunction with functional approaches. In this chapter, we outline an approach for the isolation of large-scale phagosome preparations with high degrees of purity.



**Key words:** Phagosome, Phagocytosis, Proteome, Isolation.


## 1. Introduction

Phagocytosis by specialized cells is an efficient process for the uptake and degradation of particles larger than 0.5 μm. Newly formed phagosomes, whose membranes originate from the plasma membrane and from internal vesicles *(1–3)*, undergo compositional modifications through a maturation process. Acquisition of microbicidal properties by maturing phagosomes requires highly regulated sequential interactions with endosomes and lysosomes, allowing for the recruitment of molecules conferring new functions to these maturing phagosomes *(4, 5)*. Despite recent major


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advances in the identification of phagosomal proteins, many remain to be identified and their roles remain largely unknown.

To study recruitment kinetics to phagosomes of specific effectors, a method for isolation of phagosomes was needed. A method introduced by Wetzel and Korn *(6)* and "re-discovered" by Desjardins and colleagues allowed new advances in phagosome biology. Thus latex-bead-containing phagosomes can be isolated by taking advantage of the low density of the beads, thereby permitting subsequent immunochemistry or biochemical assays. These phagosome preparations, which are devoid of major contaminants from other organelles *(7, 8)*, possess several of the functional properties required to generate a microbicidal phagolysosome *(8, 9)*, as they mature into phagolysosomes *(10)* and display degradative molecules such as hydrolases *(11)*, reflecting the high level of complexity of the cellular processes involved in phagolysosome biogenesis *(7, 8)*.

Using this approach, we previously showed that PKC-α plays a role during phagosome maturation by regulating the acquisition of molecules associated with microbicidal properties *(12)*. Phagosome preparations can be used to identify new proteins not previously known to be associated with these organelles and to demonstrate their role in regulating phagosome biology *(12, 13)*. Multiprotein complexes can also be isolated by immunoprecipitation and analyzed subsequently by 2D-gel electrophoresis. Ultimately, data obtained with these approaches need to be validated by current immunochemical or biochemical methods such as western blot or confocal immunofluorescence. In this chapter, we describe methods for the large-scale isolation of phagosome preparations with high degrees of purity.

## 2. Materials

### 2.1. Cell Culture

1. Dulbecco's modified Eagle's medium with glutamine (DMEM).
2. DMEM is supplemented with 10% heat-inactivated (56°C, 30 min) fetal bovine serum.
3. 1% Streptamycin/penicillin from Invitrogen and 10% 10 mM HEPES, pH 7.3, from Bioshop are added.
4. Cells are grown in 100 × 15 mm dishes in a 37-°C incubator with 5% $CO_2$.

### 2.2. Phagosome Isolation and Immunoprecipitation

1. Deionized, distilled water should be used throughout these protocols ($ddH_2O$).
2. Tissue culture dishes of 150 × 20 mm.

3. Latex beads: 0.8 μm diameter, 10% suspension, blue dyed.
4. Protease Inhibitor Cocktail Tablets. Prepare a 25× solution by dissolving one tablet in 2 mL $ddH_2O$. Store at –20°C.
5. Sucrose solutions: 8.55%, 10%, 25%, 35%, and 62% (w/v), add Imidazol to each solution at a final concentration of 3 mM and 1× protease inhibitor solution. Solutions can be heated to dissolve sucrose crystals. Adjust pH to 7.4 and filter on Millipor 0.45 μm (except the 62% sucrose solution). Store at 4°C.
6. 1% NP-40 lysis buffer: 150 mM NaCl, 20 mM Tris–HCl, EDTA 10 mM, 1% (v/v) NP-40 and without protease and phosphatase inhibitors. Complete with $ddH_2O$ and adjust pH to 7.5.
7. 1% NP-40 lysis buffer containing protease and phosphatase inhibitors: NP-40 lysis buffer with the same composition as in **item 6** but with the addition of 50 mM NaF, 10 mM $Na_4P_2O_7$, 1 mM $Na_3VO_4$, 1.5 mM EGTA, and 1× protease inhibitor.
8. 0.1% NP-40 lysis buffer without protease and phosphatase inhibitors: Tris–HCl, EDTA 10 mM, 0.1% (v/v) NP-40. Complete with $ddH_2O$ and adjust pH to 7.5.
9. Immobilized Protein A IPA300.

### *2.3. SDS–Polyacrylamide Gel Electrophoresis (SDS–PAGE)*

1. 30.8% acrylamide/bis solution: 30% (w/v) acrylamide, 0.8% (w/v) bisacrylamide, adjust with $ddH_2O$. Store at 4°C.
2. 10% (w/v) SDS in $ddH_2O$.
3. Separating gel solution (4×): Tris–HCl 1.5 M, pH 8.8, 0.4% SDS. Complete with $ddH_2O$. Store at room temperature.
4. Stacking buffer (4×): 0.5 Tris–HCl M, pH 6.8, 0.4% SDS. Complete with $ddH_2O$. Store at room temperature.
5. Ammonium persulfate: prepare 10% (w/v) solution in $ddH_2O$ aliquot, and freeze at –20°C. APS can be stored at 4°C several weeks.
6. N,N,N,N′-Tetramethyl-ethylenediamine (TEMED).
7. Running buffer (5×): 1.5% (w/v) Tris base, 7.2% (w/v) glycine, 0.5% SDS. Complete with $ddH_2O$. Store at room temperature.
8. 2× SDS-loading buffer: 60 mM Tris–HCl, pH 7.5, 2 mM EDTA, 0.2 M DTT, 20% glycerol (v/v), 2% SDS (v/v from a 10% SDS stock w/v in $ddH_2O$), and 0.1 mg/mL bromophenol blue (The dye allows the investigator to track the progress of the electrophoresis). Complete with $ddH_2O$.

### *2.4. Western Blotting*

1. Transfer buffer: 25 mM Tris, 250 mM glycine, 20% methanol. Complete with $ddH_2O$. Store at room temperature.

2. Nitrocellulose membrane, 3-MM paper.
3. Ponceau S solution: 0.5% (v/v) Ponceau S, 1% (v/v) acetic acid, adjusts with $ddH_2O$. Store at room temperature.
4. Phosphate-buffered saline (PBS) 10×: 1.37 mM NaCl, 27 mM KCl, 100 mM $Na_2HPO_4$, 15 mM $KH_2PO_4$ (adjust to pH 7.4 if necessary). Autoclave before storage at room temperature. Working solution is prepared by diluting one part with nine parts $ddH_2O$.
5. Enhanced chemiluminescence (ECL) reagents.

***2.5. Two-Dimension Gels***

1. Rehydration/lysis buffer: 8 M urea, 2 M thiourea, 4% (w/v) CHAPS, 40 mM DTE, 20 mM Tris, 2% (v/v) IPG buffer, 0.0025% bromophenol blue, complete with $ddH_2O$.
2. Agarose solution 1%: 0.188 M Tris–HCl, pH 8.8, 0.1% (w/v) SDS, 9% (v/v) glycerol, 1% (w/v) agarose, 0.005% (v/v) bromophenol blue, complete with ddH2O.
3. Equilibration buffer: 6 M urea, 2% (w/v) SDS, 30% (v/v) glycerol, and 0.1 M Tris–HCl, pH 8.8.
4. Equilibration buffer I: Equilibration buffer, 13 mM DTE.
5. Equilibration buffer II: Equilibration buffer, 2.5% (w/v) iodoacetamide.
6. After silver staining, the gel is scanned, and the protein pattern is analyzed with the Image Master 2D software (Amersham–Pharmacia Biotech).

***2.6. Synchronized Phagocytosis***

1. Microscope coverslips (Circles No. 1 – 0.13–0.17 mm thick; Size: 12 mm) and microscope slides (25 × 75 × 1 mm).
2. 3 μm polybeads dyed microparticles. Prepare a diluted solution by adding 0.5 mL of the stock solution in 4.5 mL of cold PBS, NaN3 0.1%. Particle number can be counted to determine concentration. Store at 4°C for several months.
3. Zymosan. Prepare a diluted solution by adding 4 mL of the stock solution in 36 mL of cold PBS 0.1% NaN3. Particle number can be counted to determine concentration. Store at 4°C during several month.
4. Sheep Red Blood Cells. Store at 4°C during 1 month. Prepare a diluted solution by adding 1 mL of the stock solution in 9 mL of cold PBS. Particle concentration should be determined. Store at 4°C up to several days.

***2.7. Confocal Immunofluorescence***

1. Paraformaldehyde: prepare a fresh 2% (v/v) solution in PBS for each experiment. Store at room temperature before use.
2. Blocking/permeabilization solution: 0.1% (v/v) Triton X-100, 1% (w/v) BSA, and 20% (v/v) normal goat serum,

6% (w/v) milk, 50% (v/v) fetal bovine serum. This solution must be freshly prepared for each experiment.

3. Anti-mouse or Anti-rabbit AlexaFluor 488 or 568. DRAQ-5 5 mM.
4. Fluoromount-G.
5. Detailed analysis of protein localization on phagosomes is performed using an oil immersion Nikon Plan Apo 100X (N.A. 1.4) objective mounted on a Nikon Eclipse E800 microscope equipped with a Bio-Rad Radiance 2000 confocal imaging system.
6. Fluorochrome excitation is achieved using a 10-mW Argon-Krypton laser for 488 nm (Alexa488) and 568 nm (Alexa568), and a 10-mW diode laser for 638 nm (Alexa647). Images are obtained using appropriate filters, through the sequential scanning mode of the LaserSharp software (Bio-Rad Laboratories) with a Kalman filter of at least 4, and converted using MRCtoM 1.9.3 and AdobePhotoshop 6.0.

## 3. Methods

A simple technique can be used to isolate pure preparations of phagosomes from macrophage cell lines such as RAW264.7 or J774 cells (**Fig. 1**). Enrichment of specific phagosome components associated with the microbicidal activity of phagosomes, such as hydrolases (e.g., cathepsin D and S) or LAMP-1, can easily be assessed by Western blot (**Fig. 2**). Phagosome isolation has been used to identify new phagosome-associated proteins as well as to study protein modifications during phagosome maturation *(14)*. After phagosome isolation and lysis, components can be separated by two-dimension gel electrophoresis, enabling the identification of proteins according to their isoelectric point on immobilized pH gradients 4–7 and their molecular weight (standard SDS–PAGE).

A global view of the compositional changes that occur during phagolysosome biogenesis can be obtained by comparing proteomic patterns of samples from phagosomes isolated at various time points. More specific studies on protein complex formation can be achieved by immunoprecipitation of a known component of the phagosome proteome. Obviously, these methods are not sufficient to clearly demonstrate the presence of these newly identified components, and other methods (e.g., Western blotting and confocal microscopy) must be used to confirm and validate the proteomic data. Ultimately, it is important to

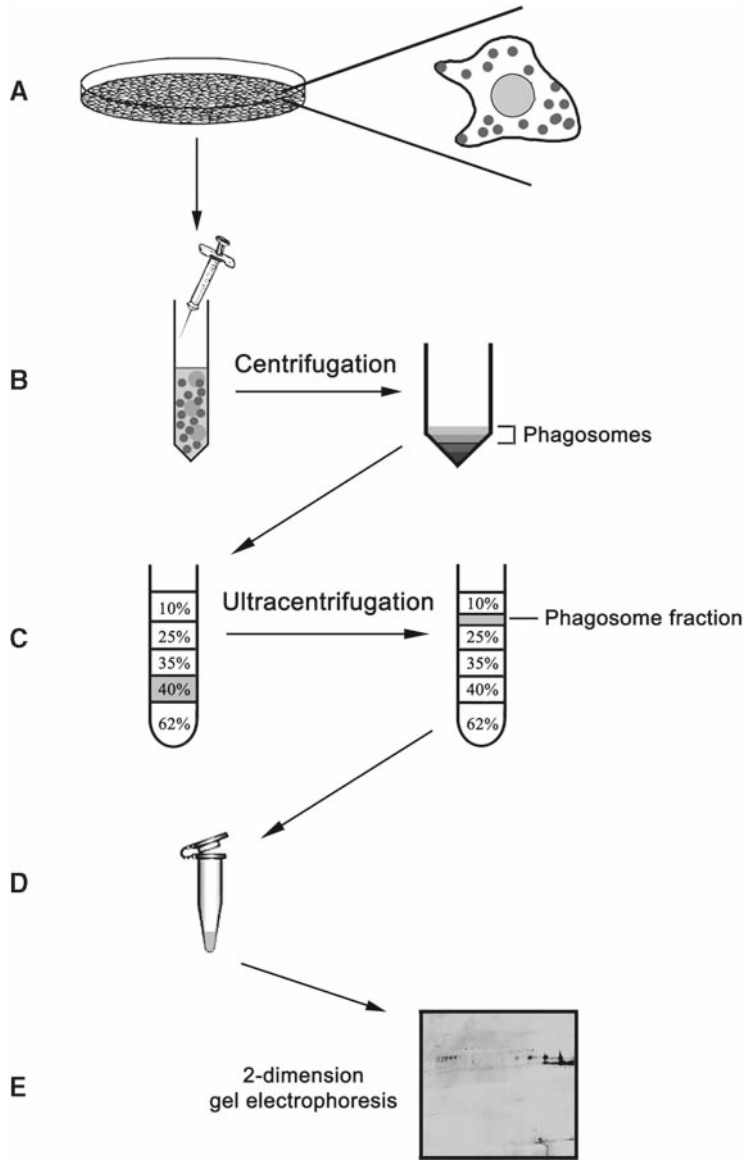


Fig. 1. Scheme of phagosome purification. (**A**) *Phagocytosis*. Macrophages are allowed to internalize a large amount of latex beads. (**B**) *Homogenization*. Cells are scrapped in PBS and homogenized by vigorously ejecting the cell suspension through a needle on the wall of the tube. After centrifugation, the phagosome layer corresponding to the two clearer phases is mixed with 62% sucrose solution in order to obtain a 40% sucrose solution containing phagosomes. (**C**) *Purification*. The 40% sucrose solution containing phagosomes is laid down on a 62% sucrose solution and covered up by the sucrose solutions as shown. Ultracentrifugation allows purification of latex-beads-containing phagosomes by migration according to beads density, at the interface between the 10 and the 25% sucrose solution. (**D**) *Lysis*. Phagosome solution is then mixed with PBS and ultracentrifugation allows phagosome precipitation at the bottom of the tube. Phagosomes can be lysed. (**E**) *Analysis*. Immunoprecipitation of a protein known to be involved in complex formation is possible on phagosome lysates and separation of protein complexes can be realized by two-dimension gel electrophoresis.

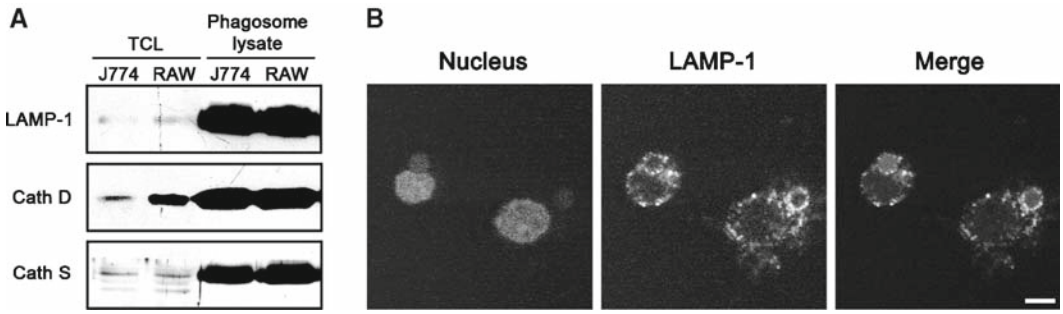


Fig. 2. Enrichment of proteins in the phagosomal compartment. The lysosomal marker LAMP-1 and the hydrolases Cathepsin D and S are massively acquired during the late steps of phagosomal maturation. (**A**) Phagosomes were obtained after a 90-min phagocytosis. 15 μg of total cell lysate (TCL) and 30 μg of phagosome lysate from RAW264.7 and J774 cell lines were loaded on a 10% acrylamide gel, transferred, and blotted with an anti-LAMP-1, an anti-Cath D, and an anti-Cath S antibody. (**B**) Immunofluorescence analysis by confocal microscopy shows recruitment of LAMP-1 on phagosomes. RAW264.7 cells were allowed to internalize fluorescent latex beads for 90 min before fixation. Cells were stained with an anti-LAMP-1 antibody and an anti-rat coupled with a 488 fluorochrome (Molecular Probes). Nuclei were stained using DRAQ-5 reagent (Biostatus, Leicestershire). Bar, 3 μm.

determine the functions of these components during phagocytosis and phagolysosome biogenesis.

### *3.1. Phagosome Isolation*

1. Adherent macrophages ($5 \times 10^7$ per 150 × 20 mm tissue culture dish) are incubated 24 h, reaching confluence between 70 and 80%.
2. Vortex latex beads vigorously before use to disrupt aggregates. Prior to initiating internalization, medium is gently removed and cells are incubated at 4°C for 10 min in the presence of 0.8 μm-diameter latex beads diluted 1:50 in 10 mL of complete medium, to allow beads to make contact with the cell monolayer. Cells and beads are then transferred to 37°C and incubated for 90 min to allow internalization to take place (*see* **Note 1**).
3. Gently wash cells three times with warm medium to remove non-internalized beads. During washes, dishes are put on a shaker. After this step, cells can be observed by light microscopy to verify the presence of a high number of bead containing phagosomes (black points in cells).
4. To obtain mature phagolysosomes, cells are further incubated for a chase period of 60 min in fresh medium at 37°C. Cells are then washed (3 × 5 min) in PBS at 4°C on a shaker, scraped with a rubber policeman in 4 mL of ice-cold PBS, and transferred to a 15-mL Falcon tube (one Falcon tube can accommodate cells from two dishes).
5. Cells are washed twice in 8 mL ice-cold PBS (centrifugation at 1,500 rpm for 3 min) to remove noninternalized beads.

A third wash is performed in 2 mL of homogenization buffer (8.5% sucrose).

6. Cells are pelleted in 1 mL homogenization buffer and homogenized on ice with a 1 mL syringe using a 22-G needle (12–15 strokes) in the 15-mL Falcon tube.
7. Homogenization is carried out until about 90% of cells are broken without major breakage of nuclei, as monitored by light microscopy.
8. Cell extracts are pelleted in a 15-mL Falcon tube at 2,000 rpm for 5 min at 4°C. The supernatant (about 1 mL) contains the phagosomes (**Fig.** 1). The phagosome suspension is carefully removed and is brought to 40% sucrose by adding an equal volume of a 62% sucrose solution in a 2-mL microfuge tube. Mix thoroughly with a pipet.
9. This 40% sucrose phagosome suspension is gently loaded on top of a 3-mL cushion of 62% sucrose, followed by the addition of solutions of 2 mL of 35% sucrose, 2 mL of 25% sucrose, and 2 mL of 10% sucrose.
10. Centrifugation is performed at 4°C in a swinging bucket rotor (SW41; Beckman Instruments) for 1 h at 100,000 × *g*.
11. Phagosomes (a band colored in blue) are carefully collected at the interface of the 10% and 25% sucrose layers and resuspended in 12 mL cold PBS containing 1× protease inhibitors. Phagosomes from two tubes can be pooled for the next centrifugation.
12. The phagosomes are pelleted at 4°C by a 15-min centrifugation at 40,000 × *g* in an SW41 rotor.
13. Supernatant is removed and the phagosome pellet is resuspended in 40–50 µL of ice-cold 1% NP-40 lysis buffer containing protease and phosphatase inhibitors (*see* **Note 2**). It is important to dry out the wall of the tube before adding lysis buffer to remove PBS 1×. Place on ice for 10 min and store at –20°C. The protein concentration of the phagosome preparations can be determined using the Pierce BCA protein assay reagent or any other standardized protein assay system.

### *3.2. Immunoprecipitation*

1. Aliquot 50 µL of immobilized Protein A into four separate 1.5-mL microfuge tubes (tubes 1, 2, 3, and 4) and equilibrate three times with 500 µL 1% NP-40 lysis buffer without protease and phosphatase inhibitors (i.e., add lysis buffer; mix gently, spin down, and remove supernatant).
2. Tubes 1 and 2 are used for antibody binding: add 500 µL of 1% NP-40 lysis buffer without protease and phosphatase inhibitors and 5 µg of the antibody. Incubate for 3–4 h at 4°C in a rotary shaker.

3. Tubes 3 and 4 are used for preclearing phagosome extracts: add 700 µg per tube of phagosome extract in 0.5 mL total (adjust with 1% NP-40 lysis buffer with protease and phosphatase inhibitors if necessary) to equilibrate beads and incubate for 1 h at 4°C in a rotary shaker in order to eliminate non-specific binding.
4. After incubation with the antibody, beads from tubes 1 and 2 are washed three times in 500 µL of 1% NP-40 lysis buffer without protease and phosphatase inhibitors by mixing gently, spinning down, and removing supernatant.
5. Transfer precleared phagosome extracts from tubes 3 and 4 into tubes 1 and 2 containing antibody linked to beads. Incubate overnight at 4°C in a rotary shaker.
6. Spin tubes 1 and 2 down and remove supernatants; wash the beads twice with 1% NP-40 lysis buffer without protease and phosphatase inhibitors and twice with lysis buffer 0.1% NP-40 without protease and phosphatase inhibitors.
7. Resuspend the beads in a small volume of rehydration/lysis buffer (cf. **Subheading 2.5**, **step 1**), pool the contents of the two microfuge tubes, spin down and resuspend beads in 450 µL of rehydration/lysis buffer.
8. Vortex 1 h at room temperature before loading the samples for two-dimensional gel electrophoresis.

### *3.3. Two-Dimensional Gel Electrophoresis*

1. Proteins are first separated according to their isoelectric points on 24-cm linear immobilized pH-gradient strips. Remove a dry strip of pH 4–7 from –20°C and allow to equilibrate at room temperature for 15 min.
2. Load the sample into the rehydration tray, leave about 1 cm at each end.
3. Note the basic end of the strip and position it at the left side of the tray.
4. Place the IPG strip gel-side down on the top of the sample; verify that there are no bubbles.
5. Completely overlay with mineral oil, cover the tray with lid, and leave at room temperature overnight.
6. Cut two electrode papers of 1.5 cm and wet with $ddH_2O$. Mop up until the papers become semi-dry.
7. Take out IPG strip and lay it down on the filter paper gel-side at top to remove oil excess.
8. Place the IPG strip in a strip holder gel-side on top and the acid side (+) at the positive side. The strip must be placed between the electrodes.
9. Lay down electrode papers lengthwise at 0.5 cm of the gel end for both sides, and gently lay down electrodes on the last

0.5 cm of the electrode paper in order that they are aligned properly.

10. Place strip holder cover and close the apparatus to test the voltage for 500 V. If voltage fails, verify that electrode papers are on the strip and that the electrode papers are not too dry.
11. Remove cap and completely cover the strip with mineral oil.
12. First dimension separation according to the isoelectric point.
13. Replace strip holder cover, close the apparatus, and proceed with the migration. At this step, voltage time volt-hours (Vh) will total approximately 106,000 Vh according to the following program:

| 500 V | 15 min |
|---|---|
| 1,000 V | 30 min |
| 2,000 V | 30 min |
| 3,000 V | 30 min |
| 4,000 V | 30 min |
| 5,000 V | 60 min |
| 6,000 V | 60 min |
| 8,000 V | Overnight |

14. The strip is then rapidly taken out and after excess oil is drained out on filter paper, is placed in a glass tube containing Equilibration Buffer I. Incubate 10 min on a shaker at room temperature.
15. Remove the strip and place it in a glass tube containing Equilibration buffer II for 5 min at room temperature on a shaker. During this step, melt agarose solution which can be stored in a boiling hot beaker to keep it liquid.
16. Second dimension separation according to molecular weight: Prepare a 10% SDS–polyacrylamide gel (cf. **Subheading 3.5**) before the equilibration steps so that it can polymerize during the equilibration steps. Mix 7.9 mL $H_2O$, 6.7 mL 30.8% acrylamide/bis, 5 mL 1.5 M Tris–HCl, pH 8.8, 200 µL 10% SDS, 200 µL 10% ammonium persulfate, and 10 µL TEMED. Leave about 1.5 cm from the top for the strip.
17. Overlay with 0.1% SDS to avoid the drying of the gel and the gel should polymerize in about 20–30 min.
18. Remove the 0.1% SDS solution and rinse the top of the gel twice with $ddH_2O$. Mop up remaining water with a filter paper.
19. Place the strip on the top of the gel, leaving space for agarose. Place the acid side on the left. Add two small filter papers saturated with molecular weight marker at both ends of the gel.

20. Add the agarose solution and with care, quickly push the strip in until it touches the polyacrylamide separation gel. Make sure that the plastic-side of the gel remains pressed on the glass. Avoid bubbles between the strip and the separation gel. Let the agarose solidify.
21. Add running buffer and proceed with the migration by running the gel at 200 V. This step takes several hours.
22. Stop the migration as soon as the colorimetric marker leaves the gel. Disassemble the migration system, remove the agarose and the strip, take the gel out, and identify the pH by piercing at both ends.

### *3.4. Coomassie Blue Coloration, Nonfixing Silver Stain, and Protein Extraction*

#### *3.4.1. Coomassie Blue (see Note 3)*

1. Wash the gel three times with $ddH_2O$ (5 min each).
2. Proceed as described for the Colloidal Blue Staining Kit.
3. Non-fixing silver stain.
4. Always use freshly prepared solutions and perform all steps at room temperature with constant gentle agitation. Place the polyacrylamide gel in a plastic container.
5. Incubate the gel for 30 min in fixation solution consisting of 125 mL ethanol, 12.5 mL glacial acetic acid, and 112.5 mL $ddH_2O$. For a double fixation, repeat this step for an additional 30 min.
6. Pour out fixing solution and wash the gel for 10 min in a mixed solution of 125 mL ethanol and 125 mL $ddH_2O$. Then wash twice with $ddH_2O$ (10 min each wash).
7. Incubate the gel for 5 min in a sensitizing solution: 0.02 g sodium thiosulfate in 250 mL $ddH_2O$.
8. Pour out sensitizing solution and wash the gel three times with $ddH_2O$ (5 min each wash).
9. For the silver reaction, incubate the gel for 30 min in silver solution: 0.25 g silver nitrate and $ddH_2O$ to a volume of 250 mL.
10. Pour out silver solution and wash the gel once with $ddH_2O$ for 1 min.
11. Incubate the gel in developing solution: 5 g sodium carbonate, 0.25 mL formaldehyde (37% w/v) and $ddH_2O$ until 250 mL. Do not allow the gel to overstain, and once the gel spots become visible, stop the reaction.
12. Briefly wash the gel in $ddH_2O$ and stop the reaction by incubating the gel in a solution of 12.5 mL acetic acid and 237.5 mL $ddH_2O$ for 5 min. The gel can be stored in a solution of 2.5 mL acetic acid and 247.5 mL $ddH_2O$ for several days.
13. Extraction of protein spots. For the following steps, it is absolutely necessary to work with latex gloves, bonnet, mask,

and any additional protection to prevent keratin contamination of the samples. Wash all components with ethanol.

14. Put the gel between two acetate sheets wetted with ultrapure water.
15. Scan the gel and number the protein spots.
16. Lay down the gel between acetate sheets on a plexiglass tray.
17. Add ultrapure water on the gel.
18. Extract each protein spot with a scalpel. Wash the scalpel between each extraction with ethanol and ultrapure $H_2O$. Place pieces of gel containing proteins in wells of a 96-well plate. The plate should be prepared beforehand by filling wells with 0.2 mL of ultrapure $H_2O$, 1% acetic acid.
19. Cover and seal the plate with parafilm. Samples can be stored at 4°C for several days.
20. The samples are now suitable to be analyzed by matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry.

### *3.5. SDS–Polyacrylamide Mini-Gel Electrophoresis (SDS–PAGE)*

1. Prepare a 10% separating gel by mixing 4 mL $H_2O$, 3.3 mL 30.8% acrylamide/bis, 2.5 mL 4× separating gel solution, 100 µL 10% ammonium persulfate, and 5 µL TEMED. Let about 1.5 cm from the top for the stacking gel.
2. Overlay with 0.1% SDS to avoid the drying of the gel which should polymerize in 20–30 min.
3. Remove the 0.1% SDS solution and rinse the top of the gel twice with $ddH_2O$.
4. Prepare the stacking gel by mixing 3 mL $H_2O$, 650 µL 30.8% acrylamide/bis, 1.25 mL 4× Tris stacking buffer, 25 µL 10% ammonium persulfate, and 5 µL TEMED. Rinse the top of the separating gel a final time with 0.5 mL of the stacking gel solution, then add the stacking solution over the separating gel, and insert the comb. The gel should polymerize in about 20–30 min.
5. Prepare the running buffer by diluting 160 mL of the 5× running buffer with 640 mL $H_2O$ and mix.
6. When the stacking gel has set, remove the comb and assemble the gel unit.
7. Add running buffer. It is important to carefully wash twice the wells with running buffer by using a 25-G needle before loading the samples.
8. The protein concentration of each sample has to be known and adjusted to have the same amount of protein in all samples. Adjustment may be done by adding lysis buffer. Be careful not to add too much lysis buffer because the protein

sample is subsequently diluted 1:1 (v/v) with 2× SDS-loading buffer, and the final volume should not exceed 15 µL for loading.

9. Incubate samples for 5 min at 100°C, perform a quick spin, mix and load 15 µL of each sample in a well. One well must include molecular weight markers prepared according to the supplier's instructions.
10. Complete the assembly of the gel unit and connect to a power supply. The gel can be run at 100 V.
11. When the dye front reaches the bottom of the gel, transfer to the nitrocellulose membrane may be carried out (the dye fronts can be run off the gel if desired).

### 3.6. Western Blotting

1. Make sure that all parts of the apparatus are clean.
2. Cut three pieces of Whatman 3-MM filter paper barely larger than the gel and pre-wet with transfer buffer. Lay down the three pieces on the transfer apparatus. A sheet of the nitrocellulose barely larger than the separating gel size is laid on the surface of the 3-MM paper to wet the membrane. Pour transfer buffer onto the membrane and remove any air bubbles.
3. After the samples have been separated by SDS–PAGE, the gel unit is disassembled. The stacking gel is removed and one corner of the separating gel is cut to track its orientation. The separating gel is then laid on the top of the nitrocellulose membrane and the gel surface is moistened with transfer buffer.
4. Three additional pieces of 3-MM paper of the appropriate size are submerged in the transfer buffer and carefully laid on top of the gel. It is also extremely important to make sure of the proper orientation of the resulting sandwich, or proteins will be lost into the buffer (components must be placed in the following order: three pieces of 3-MM paper, nitrocellulose membrane, polyacrylamide gel, and finally three other pieces of 3-MM paper. Remove any bubbles after each step). Close the transfer system.
5. Set the power supply at 15 V for 30 min.
6. After the transfer, the gel and the membrane are disassembled from the apparatus and the membrane is promptly rinsed with $ddH_2O$.
7. The membrane is then stained with Ponceau S for 1 min at room temperature to visualize the sample proteins and to assess the efficiency of the transfer. Red protein bands should become visible after removing excess dye with $ddH_2O$. The positions of the lanes and the markers are marked by gently pricking the membrane with the point of a needle.

8. Destain the membrane completely by rinsing in PBS on a shaker for about 10 min (*see* **Note 4**).
9. Block with blocking buffer (PBS-0.1% Tween, 5% BSA) for 2 h at room temperature or overnight at 4°C on a shaker (*see* **Note 5**).
10. Wash with PBS-0.1% Tween four times for 15 min each on a shaker.
11. Incubate the membrane with primary antibody diluted in 15 mL PBS + 5%BSA for 3 h at room temperature on a shaker or overnight at 4°C.
12. Wash with PBS-0.1% Tween four times for 15 min each on a shaker.
13. Incubate the membrane with the secondary antibody diluted in 15 mL PBS + 5% BSA for 45 min at room temperature on a shaker.
14. Wash with PBS-0.1% Tween four times for 15 min each on a shaker.
15. Following the instructions provided with the ECL kit, mix 5 mL Solution 1 and 5 mL Solution 2 immediately before use and incubate the blot for exactly 1 min (two blots can successively be developed with the same solution) which is rotated by hand to ensure the entire coverage of the membrane (*see* **Note 6**).
16. The blot is placed on an acetate sheet and promptly covered up with Saran wrap to avoid drying of the membrane. Keep out of light.
17. In the darkroom, the acetate-containing membrane is placed immediately in an autoradiography cassette with a medical film for a suitable exposure time (start from 30-s exposures to 45 min).

### *3.7. Preparation of Samples for Immunofluorescence*

#### *3.7.1. Synchronized Phagocytosis*

1. Place round coverslips into wells of a 24-well tissue culture plate. The coverslips are sterilized or kept in alcohol until required. Coverslips kept in alcohol are dried in a sterile environment and dropped into each well with sterile forceps.
2. Harvest and resuspend macrophages in complete DMEM at 1–4 × $10^5$ cells/mL. Seed 0.5 mL into each well of the 24-well plate and incubate at least 2 h or overnight at 37°C in 5% CO2 to allow cells to adhere to the coverslip.
3. During cell adherence, particles to be internalized are opsonized if necessary. Usually, the particle-to-cell ratio is 10:1 or 20:1.
4. Zymosan particles are laid down in a microfuge tube, spun 1 min at 14,000 × *g*, and resuspended in cold, sterile PBS 1× solution twice for washing (aspirate the supernatant carefully

using a 26-G needle). Particles can be opsonized in 150 µL of pure mouse serum or in 0.3–0.5 mL of diluted mouse serum in sterile PBS 1× solution. Do not dilute the serum to less than 10%. Incubate 40 min to 1 h at 37°C for complement opsonization. The tube is shaken by inversion three or four times during incubation to allow optimal particle opsonization.

5. For IgG opsonization, sheep red blood cells (SRBC) or beads can be used.
6. SRBC are laid down in a 15-mL Falcon tube and centrifugated at 200 × *g* for 5 min at 4°C and resuspended twice in cold sterile PBS for washing. After the last wash, SRBC are suspended in 7 mL sterile cold PBS and 7 µL of SRBC-specific IgG at 1 µg/µL (from mouse or rabbit) is added. Incubate SRBC 30 min in a rotary shaker at 4°C.
7. Beads are added to a microfuge tube, centrifugated 1 min at 14,000 × *g*, and resuspended in sterile cold PBS twice for washing (aspirate the supernatant carefully with a 26-G needle). After the last wash, beads are suspended in 1 mL 50 mM Tris–HCl, pH 9, and 1 µl of IgG at 1 µg/µL (from mouse or rabbit) is added. Incubate beads for 40 min in a rotary shaker at 4°C.
8. After opsonization, wash three times with cold sterile PBS 1× solution. For SRBC, gently shake the tube after centrifugation to avoid cell lysis. For zymosan and latex beads, particles can be vigorously shaken after centrifugation to avoid aggregate formation. Resuspend particles in an appropriate volume of complete DMEM and keep at 4°C until phagocytosis (keeping in mind that a volume of 300 µL will be added to each well for phagocytosis).
9. Once macrophages are ready for phagocytosis, remove the medium by gently aspirating the contents of each well. Wash cells twice with 0.5 mL cold DMEM. Keep plates at 4°C for 5 min before adding particles.
10. Remove medium and gently add 300 µL of the cold particle suspension. Keep the plates at 4°C during 10 min to allow particle deposition and their binding to the cells.
11. Remove unbound particles by three washes with cold DMEM and incubate cells at 37°C for 90 min for internalization.
12. Coverslips are then washed three times with PBS to eliminate non-internalized particles and cells are fixed in PBS 1×+2% paraformaldehyde for 10 min at room temperature.
13. Wash coverslips twice with PBS 1× and incubate at room temperature for 10 min before a final wash (see **Note 8**).

14. Plates containing coverslips can be stored at 4°C for 1 month before immunofluorescence staining. Be careful that coverslips are always covered with cold PBS.

*3.7.2. Confocal Immunofluorescence*

1. For each subsequent step involving incubation of coverslips containing adherent cells (i.e., blocking/permeabilization, first and second antibody incubation), place a piece of parafilm in the bottom of a 150 × 20 mm dish. The outside of the dish (both top and bottom) must be fully covered beforehand with aluminium foil. Place 50–80 μL of the incubation solution (e.g., blocking/permeabilization solution, first antibody solution, or second antibody solution) into the dish, and using forceps gently place each coverslip with the cells face down in the desired solution. Do not press on coverslips. After incubation, carefully remove coverslips with forceps, and immerse them in wells containing PBS 1× solution. Keep the cell-side face up.
2. To permeabilize cells and block nonspecific surface Fcγ-receptors, promptly drain excess PBS 1× and incubate in the blocking/permeabilization buffer at room temperature for 15–20 min.
3. Coverslips are then dipped in PBS 1× and washed three times. Washes have to be gentle to maintain cell attachment.
4. Excess PBS is promptly drained from the coverslips, and the primary antibody solution (usually at a dilution of 1/1,000 to 1/100 in PBS) is added for 1 h at room temperature.
5. Cells are gently washed three times in PBS 1×.
6. Coverslips are promptly drained for excess PBS and incubated with a solution containing the secondary antibody (1/500 dilution) coupled to an Alexa fluorochrome (Alexa 488, 568, or 647 for example) for 30 min at room temperature. A nuclear stain such as DRAQ 5 can be used and is diluted at 1/400 in the secondary antibody solution. From this step, be careful to protect samples from direct light to avoid bleaching.
7. Samples are gently washed three times and are ready to be mounted.
8. Coverslips are promptly drained for excess PBS and are mounted on glass slides with Fluoromount-G. Drops of Fluoromount-G are placed on glass slides and coverslips are laid down cell-face in contact with fluoromount. Excess fluoromount is removed and slides are then sealed with nail polish on the edges of coverslips.
9. Freshly mounted slides must be dried at room temperature and protected from light for 24 h before use. Slides can be stored for up to a month at 4°C in the dark.

## 4. Notes

1. Depending on the experiments, various internalization time points are possible during phagosome isolation, and the chase period must be adapted to the protein component of interest, which may be recruited to phagosomes at early or later time points after initiation of phagocytosis. Internalization between 30 and 120 min is often used, and the chase period can cover a period from 30 min to more than 2 h. For example, to observe the early recruitment of a specific protein on phagosomes, cells and medium containing beads can be kept at 4°C for 20 min and then placed at 37°C for 30 min before phagosome isolation.
2. Phagosomes can be resuspended in ice-cold lysis buffer containing 150 mM NaCl, 20 mM Tris–HCl, pH 7.5, and 1% NP-40, but 1% Triton X-100 can be used instead of NP-40.
3. Coomassie blue staining is preferable if proteins are visible on acrylamide gel before sample isolation and mass spectrometry identification.
4. TBS can be used instead of PBS according to the manufacturer providing the first antibody.
5. Nitrocellulose membranes can be blocked with blocking buffer containing milk (PBS 0.1% Tween, 5% (w/v) milk) for 2 h at room temperature or overnight at 4°C on a shaker.
6. If the signal is too strong during exposure, the ECL solutions can be diluted (from 1/2 to 1/20) in $ddH_2O$ as long as solutions 1 and 2 remain at the ratio of 1:1.
7. If the silver stain is not sufficient, the gel can be incubated in the silver solution overnight at room temperature with agitation.
8. During preparation of coverslips for immunofluorescence, observation by light microscopy after washing is advised in order to verify that cells have not detached during washing. Do not expose samples to light after incubation with the secondary antibody.

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# Chapter 21

# Coordinate Regulation of Sphingosine Kinase and Actin Dynamics

**Shankar S. Iyer and David J. Kusner**

## Summary

The activation of lipid-modifying enzymes generally involves a physical change in their interactions with the membrane substrate. For sphingosine kinase, a predominantly cytosolic enzyme in resting cells, activation is accompanied by translocation to specific subsets of cellular membranes where catalysis occurs. As all eukaryotic membranes have a tightly associated filamentous actin skeleton, we investigated potential regulatory interactions between sphingosine kinase and actin. Sphingosine kinase 1 (SK1) exhibited constitutive- and stimulus-enhanced association with actin filaments and F-actin-enriched membrane fractions in both intact macrophages and an in vitro reconstitution assay, whereas SK1 bound G-actin only under stimulated conditions. Actin inhibitors disrupted SK1 localization and increased its enzymatic activity. Both the localization and the activity of SK1 were coordinately regulated with the actin cytoskeleton. The association of enzymes with the actin cytoskeleton and how this regulates their activities and functions are subjects of intense interest. Here, we describe the approach we used to investigate regulation of SK1. This provides general methods that can be used to examine the role of actin in regulating enzyme activity in macrophages and other myeloid cells.

**Key words:** Enzyme, Lipid, Signal transduction, Cytoskeleton, Phosphorylation.

## 1. Introduction

Sphingosine kinases (SK), constitute an evolutionarily conserved enzyme family that regulate critical cellular functions, including motility, proliferation, and differentiation *(1–6)*. The two mammalian isoforms, SK1 and SK2, exhibit approximately 45% identity and 80% similarity *(7)*. SK activity is stimulated by cytokines, growth factors, immune complexes, complement components, and phorbol esters *(6, 8)*. SK catalyzes the phosphorylation of sphingosine to sphingosine-1-phosphate (S1P), and its critical

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roles in health and disease are due to the dynamic interconversion of these two signaling lipids *(6, 8)*. S1P transduces biologic signals both extracellularly as well as intracellularly. The broad responsiveness of diverse cell types to S1P argues for precise spatiotemporal regulation of SK1 activity. Furthermore, since agonists induce only modest increases (1.5- to 3-fold) in the specific activity of SK1, localization must be critical to its regulation. As the majority of SK activity is localized to the cytosol *(3, 5, 6, 8–10)* stimulation involves translocation to the lipid substrate of the membrane.

Several lipid-modifying enzymes, including phospholipases C and D, are known to interact with the membrane-associated actin cytoskeleton *(11–16)*. These physical interactions have important bidirectional functional effects, including modulation of lipase activity as well as actin dynamics. In the case of SK, binding to actin may serve as a determinant of its localization to specific cellular membranes. In addition, actin is critical to several cellular functions to which SK has been linked, including motility *(17–20)*, vesicle trafficking *(21–23)*, and secretion *(21, 24)*. To characterize the physical interactions between SK and the actin cytoskeleton, it is necessary to consider both monomeric G-actin and filamentous, polymeric F-actin. Confocal microscopy is a valuable method for assessing the association of proteins with actin filaments. Biochemical methods serve as complementary approaches to define binding interactions and to characterize regulatory factors. In terms of functional effects, both the enzymatic activity of SK and its subcellular localization are modulated by interactions with actin. The approach described later to investigate the regulation of SK1 based upon its association with actin, provides general methods that may be applied to study actin-dependent regulation of enzyme activity in macrophages and other myeloid cells.

## 2. Materials

1. H/$K^{++}$ buffer: 25 mM HEPES, pH 7.4, 100 mM KCl, 3 mM NaCl, 5 mM $MgCl_2$, 1 mM EGTA.
2. 2 mM leupeptin, 0.5 mM phenylmethylsulfonylfluoride (PMSF), 1 mM dithiothreitol (DTT).
3. Sphingosine Kinase (SK) Assay Buffer: 0.2 M Tris, pH 7.4; 0.9 mM $CaCl_2$, 1 mM EGTA, 40 mM β-glycerophosphate, 0.5 mM deoxypyridoxine, 15 mM NaF, 1 mM sodium vanadate, 10 μg/mL each of the protease inhibitors leupeptin, aprotinin, and soybean trypsin inhibitor, 1 mM PMSF, 10% glycerol, and 0.01% 2- mercaptoethanol.

4. PMA: 1 mM stock in DMSO; store at –20°C.
5. Phalloidin: 10 mM stock in methanol, store at –20°C.
6. Zymosan.
7. Triton X-100.
8. PVDF membrane.
9. Polystyrene beads: 3 μm diameter.
10. EZ View Protein G-agarose.
11. DNase 1, bovine pancreas.
12. Polyclonal rabbit anti-SK1 antibody was a gift from Dr. Lina Obeid, MUSC, South Carolina.
13. Polyclonal rabbit anti-SK1 antibody.
14. Murine antiactin monoclonal antibody (Ab-1).

## 3. Methods

Characterization of the physical interactions between SK1 and actin involves microscopic definition of the enzyme's association with actin filaments and biochemical evaluation of the binding of SK1 to both F- and G-actin. Examination of the functional effects of these physical interactions requires assay of lipid kinase activity and microscopic and biochemical assessment of actin cytoskeletal dynamics.

### 3.1. Characterization of the Association of SK1 and Actin Filaments by Confocal Microscopy

1. The RAW 264.7 murine macrophage cell line and human THP-1 promonocytic leukemia cells were cultured in RPMI containing 10% fetal bovine serum and 1% penicillin/streptomycin (P/S).
2. Plasmids encoding enhanced green fluorescent protein (EGFP) fusion proteins of wild-type SK1, catalytically inactive $SK1^{G82D}$ *(25–27)*, phosphorylation-defective $SK1^{S225A}$ *(28)*, and/or red fluorescent protein (RFP)-actin *(29, 30)* were transfected into RAW 264.7 or THP-1 cells using FuGENE6. Cells were loaded onto 25-mm glass coverslips and allowed to adhere. The next day, cells were transfected with 1 μg of plasmid DNA. Confocal microscopy was performed 24 h after transfection.
3. Primary human monocyte-derived macrophages (MDM) were isolated from venous blood from healthy adult volunteers in accordance with the human subjects' guidelines approved by the University of Iowa Institutional Review Board *(31)*. Blood was diluted 1:1 with sterile 0.9 M NaCl, layered over Ficoll-Hypaque density gradient medium, and

centrifuged at 300 × *g* for 45 min at 25°C. The peripheral blood mononuclear cells (PBMC) were removed and cultured in RPMI containing 20% autologous serum in Teflon wells for 5 days at 37°C, 5% $CO_2$ to enable differentiation of monocytes to macrophages. MDM were purified by adherence to glass chamber slides for 2 h at 37°C in RPMI, 20 mM HEPES, pH 7.4, 10% autologous serum. MDM monolayers were washed three times and then incubated in RPMI containing 1% bovine serum albumin, without antibiotics, for use in experiments (*see* **Note 1**).

4. Preparation of phagocytic particles. IgG-coated polystyrene beads and complement-opsonized zymosan (COZ) were used as model phagocytic particles. IgG-coated beads: latex beads (3 μm diameter) were rotated overnight in 2% bovine serum albumin (BSA) at room temperature and opsonized by incubating with 1:200 dilution of rabbit anti-BSA IgG for 2 h at 37°C *(32)*. Beads were then washed and resuspended in RPMI containing 1% bovine serum albumin. IgG opsonization was verified by indirect immunofluorescence utilizing Oregon Green-conjugated antirabbit IgG. COZ: zymosan A was boiled for 15 min at 100°C, washed with PBS, and opsonized in 50% fresh human serum for 30 min at 37°C *(33)*. COZ was then washed three times with PBS and stored on ice until used (*see* **Note 2**).
5. Confocal microscopy – For fixed cell imaging, MDM or RAW 264.7 macrophages were adhered in 8-chamber glass culture slides at approximately 50% confluence and incubated with appropriate stimuli for the indicated times. Cells were fixed in 3.75% paraformaldehyde for 15 min and permeabilized in ice-cold methanol: acetone (1:1) or acetone alone (for experiments using phalloidin) (*see* **Note 3**) *(34)*. Samples were sequentially incubated with blocking buffer (PBS, 5% BSA, 10% horse serum), Texas Red-phalloidin (0.17 μM), rabbit polyclonal anti-SK1 Ab (1:100 dilution), and Oregon Green-conjugated goat antirabbit IgG 2° Ab, all for 1 h at 25°C. In select experiments, cells were preincubated with 100 nM PMA (in 0.1% DMSO) for 5–30 min. The 0.1% DMSO solvent control exhibited no differences in viability or SK1 localization, compared to cells incubated in buffer alone. Analysis was performed using a Zeiss 510 laser scanning confocal microscope. Fifty cells were analyzed per sample and duplicate samples were run for each experimental condition.
6. For live-cell confocal imaging, RAW 264.7 cells were cultured and transfected as described earlier. On the day of analysis, the coverslips were placed in a heated stage at 37°C, and analyzed at rest, during phagocytosis of model particles,

or after incubation with various pharmacologic agents, using the Zeiss 510 laser scanning microscope and the associated LSM time series imaging software. Z-series confocal images were acquired every 30–45 s for 45 min. Loss of fluorescence at later time points due to photobleaching was compensated for by increasing the gain of the photomultiplier tube. Quantification of differences in fluorescent staining between samples, including the degree of colocalization, was performed using ImageJ software.

### 3.2. Definition of the Interaction of SK1 with Actin-Enriched Membrane Fractions of Intact Cells

To further define the interaction of SK1 with actin filaments, we characterized the detergent solubility of SK1 in resting and stimulated macrophages. For this purpose we utilized the murine macrophage cell line RAW 264.7.

1. RAW cells were cultured in RPMI 1640 with 10% FBS and 1% P/S. Freshly cultured cells adherent to tissue culture flasks were scraped, washed, and resuspended in $H/K^{++}$ buffer at $3 \times 10^7$ cells/2 mL. A 2 mL aliquot of the cell suspension was used as control cells. Another 2 mL aliquot was stimulated with 2 μM PMA (*see* **Note 4**) for 30 min at 37°C. Poststimulation, the cells were washed and resuspended in 2 mL of buffer.
2. Control and PMA-stimulated cells were disrupted by nitrogen cavitation (450 psi, 25 min, 4°C) (*see* **Note 5**). The undisrupted cells and nuclei were removed by centrifugation at $900 \times g$ for 8 min at 4°C to yield the postnuclear supernatant. Membranes and cytosol were isolated a second time by ultracentrifugation at $150{,}000 \times g$ for 60 min at 4°C. Membranes were washed with $H/K^{++}$ and the cytosol clarified by re-centrifugation as mentioned earlier. Washed membranes were suspended in 2 mL $H/K^{++}$ buffer and homogenized with a Tenbroeck tissue grinder.
3. Membranes from control and PMA-treated cells were subjected to detergent extraction by incubating them in 1% Triton X-100 in H/K buffer for 60 min on ice. The detergent-soluble (TxS) and detergent-insoluble pellets (TxI) were separated by centrifugation at $14{,}000 \times g$ for 15 min at 4°C. The insoluble pellets (TxI) were washed twice in H/K buffer with 1% Triton X-100 and resuspended in H/K buffer without detergent.
4. The TxS and TxI fractions obtained by extraction of membranes from control and PMA-treated cells were subjected to 9% SDS–PAGE using the Laemmli buffer and transferred to polyvinylidene difluoride (PVDF) membrane.
5. The PVDF membrane was blocked with 5% nonfat dry milk and probed with a polyclonal rabbit antibody to sphingosine

kinase 1 (anti-SK1, 1:100 dilution) followed by immunodetection using horseradish-peroxidase-coupled secondary antibody and enhanced chemiluminescence (ECL).

6. The majority of SK1 in membranes from resting macrophages were detected in the Triton X-100 insoluble fraction (**Fig. 1A,** major protein doublet at ~43 kDa). The additional protein bands at higher molecular weights likely represent post-translational modifications of SK1.

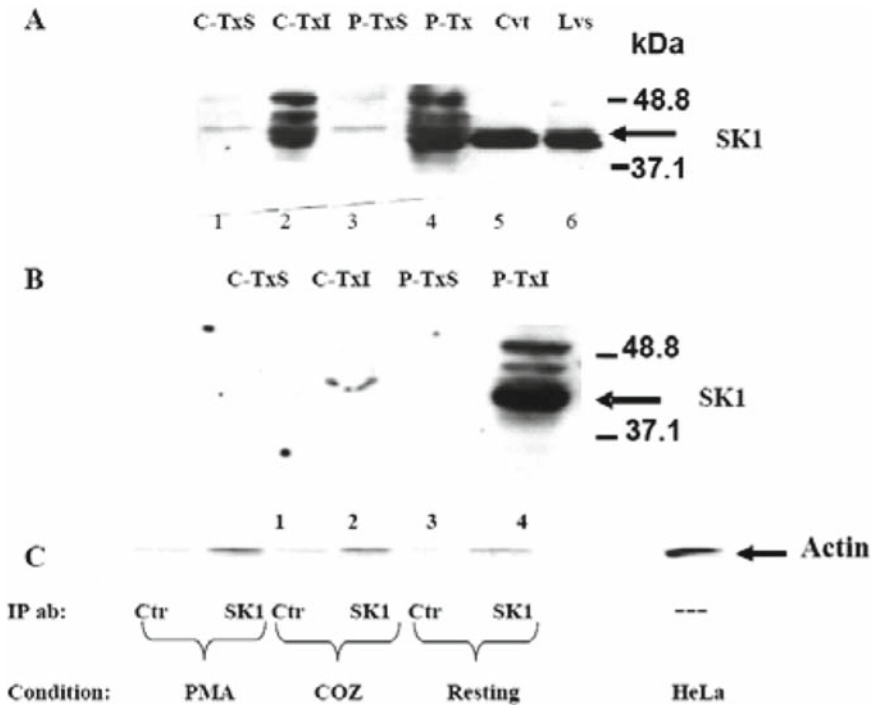


Fig. 1. SK1 exhibits constitutive and PMA-stimulated association with detergent-resistant membrane domains. (**A**) RAW 264.7 cells were incubated in the presence of PMA (2 μM) or buffer for 30 min at 37°C. Cells were disrupted by nitrogen cavitation and membrane, and cytosol fractions were separated by differential centrifugation. Membranes were extracted with lysis buffer containing 1% Triton X-100, and the detergent-insoluble membrane fraction was reisolated and washed. Membrane-derived fractions were subjected to SDS–PAGE followed by immunoblotting for SK1. Lanes are as follows: (1) control, Triton X-100 soluble (C-TxS); (2) control, Triton X-100 insoluble (C-TxI); (3) PMA-stimulated, Triton X-100 soluble (P-TxS); (4) PMA-stimulated, Triton X-100 insoluble (P-TxI). Positive controls included: (5) cytosol from U937 cells, and (6) HeLa cell lysate. (**B**) Cell-free assay: Membrane and cytosol fractions from resting RAW 264.7 cells were incubated with PMA (2 μM) or buffer for 15 min at 37°C. The membrane fraction was reisolated by centrifugation, washed, and extracted with 1% Triton X-100 in lysis buffer. Detergent-soluble and -insoluble fractions were subjected to SDS–PAGE followed by immunoblotting for SK1. Lane designations are as follows: (1) control, Triton X-100 soluble (C-TxS); (2) control, Triton X-100 insoluble (C-TxI); (3) PMA-stimulated, Triton X-100 soluble (P-TxS); (4) PMA-stimulated, Triton X-100 insoluble (P-TxI). (**C**) Coimmunoprecipitation assay: Lysates from resting, PMA-stimulated or COZ-stimulated RAW 264.7 cells were prepared by nitrogen cavitation. These were precleared and then immunoprecipitated using anti-SK1 or control rabbit IgG. Immunoprecipitates were washed extensively in a RIPA-like lysis buffer and subjected to SDS–PAGE followed by Western blotting with an antiactin antibody. HeLa lysate was used as a control. Reprinted with permission from **ref.***35*.

7. In membranes isolated from cells after PMA stimulation, there was increased detection of SK1 in the Triton X-100-insoluble fraction. The level of SK-1 in the Triton X-100 soluble fractions was low and did not change significantly after PMA stimulation.

### 3.3. Characterization of the Association of SK1 with Actin Filaments in a Reconstituted Cell-Free Assay System

Cell-free assay systems have contributed significant insights into the interactions between actin filaments and phospholipases, sphingomyelinases, and lipid kinases/phosphatases. Our working model is that activation of SK1 leads to its stable association with actin filaments, rendering SK1 detergent insoluble. We hypothesized that these SK1-actin interactions could be mimicked in a broken cell preparation.

1. Membranes and cytosol from resting RAW 264.7 cells were prepared essentially as detailed earlier (*see* **Subheading 3.2.2**). For the assay, a volume corresponding to $3 \times 10^6$ cell equivalents of membranes was incubated with a volume corresponding to $1 \times 10^6$ cell equivalents of cytosol in an $H/K^{++}$ buffer, in the absence and presence of 2 μM PMA for 15 min at 37°C. Membranes were isolated a second time by centrifugation (150,000 × *g*, 60 min, 4°C), washed with buffer, and extracted with 1% Triton X-100 for 60 min on ice. The detergent-soluble and -insoluble fractions were separated and washed as described earlier (*see* **Subheading 3.2.3**) and subjected to SDS–PAGE and Western blotting.
2. Control membranes, derived from incubation with cytosol in buffer, contained very low level of SK1 in the Triton X-100-insoluble fraction (**Fig. 1B**). Likewise, there was no detectable SK1 in the detergent-soluble fraction.
3. On the other hand, the Triton X-100-insoluble fraction derived from activated membranes, prepared by incubating membranes with cytosol in the presence of PMA, contained approximately 56-fold greater level of SK1 than the corresponding control (**Fig. 1B**). Again, there was no detectable SK1 in the detergent-soluble fraction.

### 3.4. Coimmunoprecipitation of SK1 and Actin

To directly evaluate the proposed physical interaction of SK1 with actin, a coimmunoprecipitation assay was undertaken.

1. RAW cells were stimulated with 500 nM PMA, COZ (particle:cell ratio of 10:1), or buffer control for 20 min at 37°C and then washed in RPMI. Cells were scraped, pelleted, and resuspended in a RIPA-like lysis buffer (H/K buffer containing 1% Triton X- 100, 1% octylglucoside, 1% deoxycholate, 0.05% SDS with protease inhibitors), passed through an 18-gauge needle 10 times, and incubated on ice for 60 min.
2. Insoluble material was removed by centrifugation at 14,000 × *g* for 15 min at 4°C.

3. The lysates were precleared by incubation with a 50-µL aliquot of a 50% suspension of EZ View Protein G-agarose (Sigma) for 2 h at 4°C.
4. Control antirabbit IgG or anti-SK1 IgG were preadsorbed to the Protein G-agarose by incubating with the beads for 2 h at 4°C. The beads were extensively washed with lysis buffer to remove unadsorbed IgG.
5. Precleared lysates were incubated with the preadsorbed control IgG or anti-SK1 IgG by incubation for 3 h at 4°C. The immunoprecipitates were washed extensively with lysis buffer and then boiled in SDS sample buffer and subjected to 9% SDS–PAGE.
6. After transfer to PVDF membrane, blots were blocked in 5% nonfat dry milk, washed, and probed for actin by incubating overnight in antiactin antibody (monoclonal IgM) at 4°C. Detection involved the use of horseradish-peroxidase-coupled secondary antibody and ECL.
7. No actin was detected in immunoprecipitates (IPs) obtained with control, irrelevant antibody (**Fig. 1C**). In contrast, each of the anti-SK1 IPs contained actin, and the level of immunoprecipitated actin was greater in PMA- and COZ-treated samples (**Fig. 1c**) compared with buffer-treated controls.

### *3.5. Determination of the Binding of SK1 to Actin Filaments by Velocity Sedimentation Density Gradient Centrifugation*

Velocity sedimentation density gradient centrifugation is a useful method to examine the physical form of actin and to analyze actin-associated proteins. An advantage of this technique over differential centrifugation is that it avoids artifactual copelleting in a centrifugal field. The method can be used to identify microfilament-associated proteins in the presence of the actin filament-stabilizing bicyclic peptide toxin, phalloidin (*see* **Note 6**). Stabilization of microfilaments by phalloidin increases the size of microfilament cores, hence shifting the filament peak in velocity sedimentation density gradient centrifugation to fractions of greater density. This is referred to as the characteristic "phalloidin-shift."

1. Membranes and cytosol were freshly isolated from RAW cells as described earlier (**Subheading 3.2.2**).
2. For the experiment, membranes and cytosol from $2 \times 10^8$ cells were incubated with PMA (100 nM) for 30 min at 37°C. In select samples, phalloidin was added at 10 µM for the final 15 min of the incubation.
3. Post incubation, membranes were reisolated by centrifugation at 17,500 × *g* for 30 min at 4°C and washed in H/$K^{++}$ buffer.
4. Washed membranes were solubilized in 500 µL of TEB (1% Triton X-100 in H/K buffer) for 60 min on ice and loaded

on top of a 4-mL linear sucrose gradient (20–55%) prepared in TEB. A 0.5-mL sucrose cushion (80%) was placed at the bottom of each tube.

5. Samples were centrifuged at 150,000 × *g* for 16 h at 8°C using an Optima XL ultracentrifuge (Beckman) with an SW55Ti rotor. Fractions (400 μL) were collected from the top of the gradient.
6. Fractions were subjected to 9% SDS–PAGE and after transfer to PVDF membrane; blots were blocked in 5% nonfat dry milk, washed, and probed for actin by incubating overnight in antiactin antibody (monoclonal IgM) at 4°C. The blots were also probed for SK1 using the anti-SK1 rabbit polyclonal antibody. Detection utilized horseradish-peroxidase-coupled secondary antibody and ECL.
7. In the absence of phalloidin, SK1 was detected primarily in fractions 4–6 (**Fig. 2A)**. These fractions also contained a significant amount of actin.
8. In the presence of phalloidin, however, SK1 exhibited a "density shift" and became localized to more dense fractions, 7–9 (**Fig. 2A**). There was a similar shift in the localization of actin to fractions of greater density.

### *3.6. Analysis of SK1 Binding to Actin Filaments by Cosedimentation*

A second method to biochemically assess the binding of SK1 to actin filaments involves cosedimentation. Cytosolic G-actin can be efficiently converted to F-actin by the addition of GTPγS. The GTPγS-induced polymerization of G-actin is stabilized by the addition of jasplakinolide. After polymerization and/or stabilization, actin filaments were sedimented in a low centrifugal field and washed. Proteins that are physically associated with F-actin cosediment with the actin filaments.

1. Cytosol isolated from murine macrophages (RAW cells) or the human promonocytic line (THP-1 cells) as described earlier (**Subheading 3.2.2**) was precleared by ultracentrifugation (150,000 × *g*, 30 min at 4°C).
2. Aliquots of cytosol (100 μg) in H/K buffer were incubated with GTPγS (100 μM) to induce actin polymerization, in the absence or presence of jasplakinolide, added at a final concentration of 1 μM to stabilize the formed actin filaments.
3. Actin filaments were pelleted by centrifugation at 17,500 × *g* for 30 min at 4°C and the supernatants saved. Pellets were washed extensively in H/K buffer.
4. One-tenth aliquots of the supernatants and all the pellets were analyzed on 9% SDS–PAGE gels and probed for SK1 and actin as noted earlier. Results obtained with the pellet fractions are shown in **Fig. 2B**.

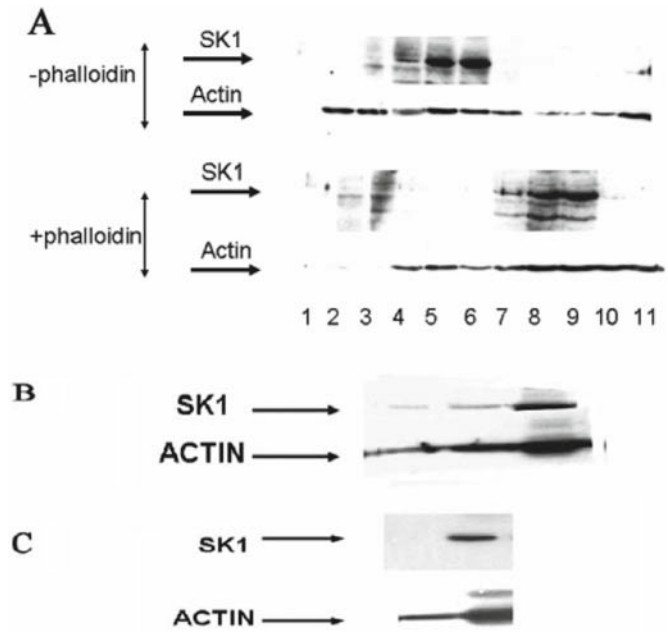


Fig. 2. SK1 binds to actin filaments and G-actin. (**A**) Effect of phalloidin on velocity sedimentation on sucrose gradients. Membranes and cytosol from RAW 264.7 (or THP-1) cells were incubated with PMA (100 nM) at 37°C for 30 min. Membranes were then reisolated by centrifugation at 17,500 × *g*, washed, and phalloidin (10 μM) was added to select samples for the final 15 min of the incubation. Membranes were washed and solubilized in 1% Triton X-100 buffer and then loaded on top of a 4-mL linear gradient of sucrose (20–55%). Samples were centrifuged at 150,000 × *g* for 16 h at 8°C, and fractions (400 μL) collected from the top of gradient were subjected to SDS–PAGE followed by Western blotting for SK1 and actin. (**B**) Cosedimentation assay. Cytosol from $10^8$ THP-1 cells was precleared by ultracentrifugation (150,000 × *g*, 30 min, 4°C). Aliquots (100 μg) were incubated with GTPγS (100 μM) (*lanes 2 and 3*) to induce actin polymerization, and jasplakinolide was added at a final concentration of 1 μM (*lane 3*) to stabilize actin filaments. Actin filaments were pelleted by centrifugation at 17,500 × *g* for 30 min at 4°C, washed extensively, and the pellet fractions analyzed by 10% SDS–PAGE gels and probed for SK1 and actin by immunoblotting. (**C**) Coisolation of SK1 with G-actin by binding to immobilized DNase1. Cytosol from $10^8$ THP-1 cells was dialyzed extensively against actin monomer buffer (AMB), precleared by centrifugation, and then incubated with (*lane 2*) or without (*lane 1*) PMA (100 nM) for 30 min at 37°C. An equal aliquot of immobilized DNase1 was added to each fraction and incubated for 4 h at 4°C. The DNase-bound material was pelleted and washed extensively with AMB before being subjected to SDS–PAGE and immunoblotting for SK1 and actin. Reprinted with permission from **ref.***35*.

5. Incubation with GTPγS alone resulted in the recovery of low amount of SK1 in the pellet (**Fig. 2B**). Addition of jasplakinolide resulted in a much greater recovery of both actin and SK1 in the pellet fraction (*see* **Note 7**).
6. The results of the velocity sedimentation and cosedimentation assays confirm the coimmunoprecipitation and confocal

microscopy studies to support the hypothesis that SK1 associates with actin filaments and/or globular G-actin.

### 3.7. Determination of SK1 Binding to G-Actin by Coisolation with DNase 1

DNase 1 is a well-characterized G-actin binding protein that forms a stable 1:1 complex with G-actin. We utilized immobilized DNase 1 to pull down G-actin and, in the process, hypothesized that any other protein associated with G-actin would also be coisolated.

1. Cytosol from RAW or THP-1 cells was isolated as described earlier (**Subheading 3.2.2**) and dialyzed extensively against actin monomer buffer (AMB, 5 mM Tris–HCl, pH 8.0, 0.2 mM $CaCl_2$ with 0.2 mM ATP, 0.5 mM dithiothreitol) and then precleared by ultracentrifugation.
2. Aliquots of clarified cytosol (100 μg) were incubated with or without PMA (100 nM) for 30 min at 37°C.
3. Immobilized DNase 1 (*see* **Note 8**) was added to each fraction and incubated for 4 h at 4°C.
4. The DNase 1-bound material was pelleted at low speed (2000 × *g*, 5 min at 4°C) and washed extensively with AMB buffer.
5. The pelleted agarose beads were subjected to 9% SDS–PAGE after addition of SDS sample buffer and heating for 5 min at 90°C.
6. The proteins were transferred to PVDF membrane; blots were blocked in 5% nonfat dry milk, washed, and probed for actin by incubating overnight in antiactin antibody (monoclonal IgM) at 4°C. The blots were also probed for SK1 using the anti-SK1 rabbit polyclonal antibody. Detection utilized horseradish-peroxidase-coupled secondary antibody and ECL.
7. In the absence of PMA, no SK1 was detected in the G-actin–DNase 1 complex (*lane 1*, **Fig. 2C**). However, in the presence of PMA (*lane 2*, **Fig. 2C**), SK1 was detected in association with the DNase1 pellet, supporting the hypothesis that SK1 binds G-actin under stimulated, but not basal, conditions.

### 3.8. Assessment of the Effects of Modulation of Actin Polymerization on SK1 Activity

We evaluated whether modulation of actin dynamics would affect the enzymatic activity of SK1. Sphingosine kinase activity was determined in control RAW cells and cells preincubated with the known actin cytoskeleton modifiers, jasplakinolide and latrunculin B. Jasplakinolide promotes actin polymerization by increasing the rate of monomer nucleation and via stabilization of actin filaments *(35)*. Latrunculin B binds G-actin monomers with high affinity resulting in progressive depolymerization of actin filaments.

1. RAW 264.7 cells were incubated with jasplakinolide (1 μM, 15 min), latrunculin B (20 μg/mL, 10 min), or buffer control at 37°C.

2. PMA (100 nM, final concentration) was added to select samples and incubated for 30 min.
3. Cells were washed once with ice-cold phosphate-buffered saline and scraped into ice-cold SK Assay Buffer.
4. Cells were disrupted by sonication on ice and assessed for cell lysis by microscopy.
5. Aliquots of each whole cell lysate were used for determination of enzymatic activity by incubation with the substrate, sphingosine (1 mM sphingosine prepared in 5% Triton X-100, final concentrations: 0.25% Triton X-100, 50 μM sphingosine). The reaction was started by the addition of [$\gamma$-$^{32}$P]ATP (0.025 μCi/sample) and was terminated after 10 min at 37°C.
6. $^{32}$P-labeled sphingosine 1-phosphate formed during the reaction was isolated by thin layer chromatography using the solvent system, 80 butanol: 20 ethanol: 10 acetic acid: 20 water.
7. The radioactivity in the sphingosine 1-phosphate band was quantified using the Typhoon PhosphorImager (GE Healthcare).
8. SK activity of the samples was expressed as the percent of control cells incubated in buffer alone (mean ± range).
9. The positive control, PMA, increased SK activity by 77% (range: 66–88%, $p < 0.02$, $n = 5$). Jasplakinolide caused an 85% increase in SK activity, compared to buffer-treated control cells (range: 79–91%, $p < 0.01$). Latrunculin B resulted in a modest 38% increase in SK activity (range: 25–51%, $p < 0.02$). The fact that both jasplakinolide, which increases actin polymerization, and latrunculin, which decreases polymerization, have similar positive effects on SK activity suggests that it is their common physiologic effect – that of decreasing the concentration of G-actin – which is responsible for enhancement of SK activity. In this model, G-actin inhibits SK activity, and effectors which decrease the concentration of G-actin cause an increase in the activity of SK.
10. These results are consistent with the hypothesis that the enzymatic activity of SK1 is modulated by changes in actin dynamics and/or the state of actin polymerization.

## 4. Notes

1. Effects of experimental manipulations on MDM viability were assessed by exclusion of trypan blue, and monolayer density was determined by nuclei counting with naphthol blue-black

stain *(33)*. Purity and viability of MDMs were >95% by Wright staining and trypan blue exclusion, respectively.

2. COZ contains the complement component, C3bi, as its active surface ligand. Since serum contains factors which degrade C3bi, it is imperative that COZ be washed carefully to remove traces of serum. Maintaining COZ on ice will prolong its stability.
3. For experiments in which phalloidin is used to image actin filaments, methanol is omitted from the permeabilization media due to its interference with phalloidin.
4. PMA is the commonly used phorbol ester. Phorbol 12-myristate 13-acetate is a potent tumor promoter. Stock solution (10 mM) was prepared in absolute ethanol or DMSO and stored at –20°C.
5. $N_2$ cavitation: Cell disruption is performed by the nitrogen decompression method in the Parr Cell Disruption Bomb precooled at 4°C. The cells are pressurized with $N_2$ and left on ice for equilibration with the gas. The $N_2$ pressure and the duration of equilibration depend on the cell type and the number of cells. Efficiency of cell disruption can be evaluated by monitoring release of cytosolic constituents, such as lactate dehydrogenase, or by microscopic examination. Controlled cell disruption occurs when the pressure in the chamber is released by opening the discharge valve and collecting the homogenate.
6. Phalloidin is a bicyclic toxin from the mushroom *Amanita phalloides*. Care must be exercised in its handling. Stock solution at 10 mg/mL is prepared in anhydrous methanol and stored at –20°C.
7. Because intact cytosol was utilized, the cosedimentation of SK1 with actin does not indicate direct binding to F-actin. The association of SK1 with actin filaments may be indirect, via actin-binding proteins.
8. Immobilized deoxyribonuclease 1 (DNase 1) was prepared essentially according to published protocol *(36)*. 200 mg of DNase 1 (Bovine pancreas) was dissolved in 10 mL of coupling buffer (100 mM HEPES, pH 7.2, 80 mM $CaCl_2$. 1 mM PMSF) and dialyzed overnight against the same buffer at 4°C. It was mixed with 10 mL of washed Affi-Gel 10 matrix and incubated for 4 h at 4°C. Excess reactive sites were blocked with 10 mM ethanolamine (pH 7.0) for 1 h at 4°C. Coupling efficiency was determined from protein amounts before and after coupling. For column chromatography, we coupled DNase 1 to HiTrap-NHS- activated matrix (GE Healthcare Biosciences, NJ) following the manufacturer's protocol.

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