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Leishmania major: Recruitment of Gr-1+ cells into draining lymph nodes during infection is important for early IL-12 and IFNγ production

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Index Descriptors and Abbreviations:
Leishmania
Gr-1 antigen
PMN, polymorphonuclear cells
MNC, mononuclear cells
DC, dendritic cells
Th, T-helper
NK, natural killer
IL-12, interleukin-12
IFNγ, interferon-γ
FCS, fetal calf serum
PBS, phosphate-buffered saline
ELISA, enzyme-linked immunosorbent assay

ABSTRACT

The production of interleukin-12 and interferon-γ is a key event for controlling leishmaniasis. Here, we tested the hypothesis that after murine infection with *Leishmania major*, cell migration into draining lymph nodes is crucial for early production of those cytokines. We showed that inflammatory cells carrying the marker of recently migrated cells, the Gr-1 antigen, including polymorphonuclear and mononuclear cells, migrate rapidly into the site of promastigote infection and, subsequently, into draining lymph nodes. Treatment with RB6-8C5 monoclonal antibody reduced local inflammation and migration of Gr-1+ cells into the draining lymph nodes. This reduction was associated with a decrease of interleukin-12 production by draining lymph node cells from BALB/c mice but not C57BL/6 mice. Additionally, interferon-γ was also reduced in both mouse strains after depletion of Gr-1+ cells, suggesting that these cells are important for early interleukin-12 and interferon-γ production. Our findings suggest that recently migrated myeloid cells, more than resident cells, are the major source of the early IL-12 production after *L. major* infection.

1. Introduction

Leishmaniasis is a zoonotic disease caused by the protozoa of the genus *Leishmania* sp. (Desjeux, 2004). In experimental leishmaniasis, infective parasites are inoculated into animal tissue such as the murine footpad. At the site of inoculum, parasite compounds activate resident cells and attract cells of the innate immune system from the blood. These include PMN, mainly neutrophils, which are recruited to the tissue as a first response after infection, and MNC comprising monocytes as precursors of macrophages and DC, and NK cells, which are recruited later and drained into lymph nodes (Bajenoff et al., 2006; Beil et al., 1992; Muller, 2001).

During the first hours of *Leishmania* infection, the parasites also gain access to the draining lymph nodes where interactions among the lymphocytes and resident or recently migrated parasite-loaded antigen presenting cells of the innate immune system determine the outcome of the disease. It has been demonstrated that the early production of IL-12 and IFNγ by antigen presenting cells and NK cells, respectively, drives the immune response towards a protective profile against intracellular protozoa (Bajenoff et al., 2006; Gazzinelli et al., 1998; Scharton and Scott, 1993). In murine *Leishmania major* infection it has been clearly defined that NK cells are present already at an early phase in the draining lymph nodes, and they are the prime source of the initially produced IFNγ (Bajenoff et al., 2006; Scharton and Scott, 1993). In contrast, the initial source of IL-12 after *in vivo* infection of mice with *L. major* has not yet been identified. The infective promastigote forms of *L. major* barely induce IL-12 after *in vitro* stimulation of macrophages (Oliveira et al., 2000; Reiner et al., 1994; Vieira et al., 1994). Amastigote as well as promastigote forms do induce IL-12 production in DC *in vitro* (Bennett et al., 2001; Konecny et al., 1999; von Stebut et al., 1998). However, the amount of IL-12 detected in the DC-culture supernatants after infection with promastigotes is very low compared with that obtained by *ex vivo* lymph node-cell cultures after early *in vivo* infection (Bennett et al., 2001; Konecny et al., 1999; Vieira et al., 1994). These data suggest that cell migration into draining lymph nodes may be necessary to the *in vivo* IL-12 production. We demonstrated an increased IL-12 production by macrophages in immature stages of development stimulated with

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L. major promastigotes, suggesting that the source of IL-12 *in vivo* could be a myeloid mononuclear cell that recently migrated into the lymph node after infection rather than resident cells in final stages of maturation (Oliveira et al., 2005). However, this hypothesis was not tested *in vivo*.

Cells that are potential candidates to participate in early IL-12 production during *L. major* infection are the myeloid Gr-1+ (Ly6G/C) cells. This notion arose from the observation that leukocytes migrating into the peritoneal cavity 3 h after thioglycollate injection include granulocytes as well as monocytes, both expressing Gr-1 antigen (Henderson et al., 2003). After 24 h of inflammation, the numbers of immigrating Gr-1+ monocytes and neutrophils were equivalent. The Gr-1 antigen as a marker of recently migrated cells to inflammatory site was also confirmed by the studies showing that blood Gr-1^{high} monocytes migrate to inflamed sites whereas Gr-1^{low} monocytes migrate to non-inflamed tissues (Geissmann et al., 2003). At inflamed sites, the Gr-1+ monocytes can differentiate into DC or macrophages (Geissmann et al., 2003; Tacke and Randolph, 2006).

In some acute infectious inflammation models, the presence of Gr-1+ cells has been demonstrated (Iezzi et al., 2006; Mordue and Sibley, 2003; Robben et al., 2005; Scharton and Scott, 1993; Sunderkotter et al., 2004; Tam and Wick, 2006). In *Toxoplasma gondii* infection, neutrophils as well as Gr-1+ monocytes produce IL-12 leading to resistance to the infection (Mordue and Sibley, 2003). In *L. major* infection, PMN infiltrate into the site of infection and draining lymph nodes (Lima et al., 1998; Tacchini-Cottier et al., 2000) but they are unable to produce IL-12 (Chen et al., 2005).

To investigate whether recently migrated cells are important for early IL-12 production induced by *L. major*-promastigote infection, we evaluated the dependence of lymph node cytokine production on the recruitment of Gr-1+ cells. We also investigated whether these cells contribute for IFNγ production at the early phase of infection.

2. Materials and methods

2.1. Animals

Male BALB/c or C57BL/6 mice were obtained from the animal facilities of the Tropical Pathology and Public Health Institute, Federal University of Goiás, Brazil, and from the animal facilities of the Department of Pathology, University of Brasília, Brazil as a gift of Dr. Raimundo Antônio Teixeira. Mice were used at 45–60 days of age and up to five mice per cage were maintained in specific pathogen-free conditions with water and food ad libitum. All procedures with the animals were in accordance with the ethical principles of the Colégio Brasileiro de Experimentação Animal (COBEA).

2.2. Parasite cultures

Promastigotes of *L. major* (MHOM/IL/80/Friedlin) were kindly provided by Dr. Leda Quercia Vieira (Federal University of Minas Gerais, Brazil). The parasites were grown in 24-well plates (Costar, Cambridge, MA, USA) in Grace's insect medium (Sigma Chemical Co., St. Louis, MO) supplemented with 20% FCS (Gibco–BRL, Argentine), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin, at 26 °C. The cultures started with 1×10^5 promastigotes/ml and were replicated in growth logarithmic phase. Parasites in stationary phase were used to infect the mice.

2.3. Antibodies

The monoclonal antibody to Gr-1 (clone RB6-8C5, rat IgG2b) was kindly provided by Dr. Glória Maria Collet de Araújo Lima (University of São Paulo, Brazil). The hybridoma producing monoclonal antibodies to IL-12p40 (clones C17.8 and C15.6) were donated by Dr. Giorgio Trinchieri (National Cancer Institute, Frederick, MD, USA) and those to IFNγ (clones XMG 1.2 and ASN-18) were provided by Dr. Ises Abrahamsohn (University of São Paulo, Brazil). Monoclonal antibodies obtained from cultures, and control IgG from rat serum, were purified in G protein columns (Pierce, IL, USA), according to the manufacturer's instructions.

2.4. Mouse infection and treatments

Mice were inoculated into both footpads with 1×10^6 promastigote forms, in the absence or in the presence of control IgG (50 μg/footpad) or anti-Gr-1 antibodies (50 μg/footpad) in 50 μl of PBS. Non-infected mice were treated with control IgG (50 μg/footpad) or anti-Gr-1 antibodies (50 μg/footpad). In each experiment, the groups comprised at least three mice in one to five experiments performed as indicated in the legends of the figures.

2.5. Lymph node-cell cultures and cell characterization

After 6, 12, 24, or 48 h of infection, mice were sacrificed (CO_2 gas chamber) and popliteal (draining) and axillary (control) lymph nodes were collected. The organs were disrupted and lymph node cells were obtained in RPMI 1640 medium (Sigma), supplemented with 10% FCS, 10 mM Hepes, 100 U/ml penicillin, and 100 μg/ml streptomycin. All supplements were purchased from Sigma. Cytospins were prepared using 5×10^5 cells in 200 μl of PBS containing 2% FCS for morphological analysis or immunocytochemistry. The cytospins were stained by commercial kit Instant Prov (Newprov, Pinhais, PR, Brazil) for morphological analysis. Under light microscopy, 1000 cells in each cytospin were analyzed to obtain the frequency of PMN. For immunocytochemistry analysis, the cytospins were fixed with 1% paraformaldehyde (Merck Research Laboratories, Rahway, NJ, USA) for 20 min at room temperature (RT) and then treated twice with 10% hydrogen peroxide for 10 min (RT) to block endogenous peroxidase. The cytospins were washed five times with PBS–Tween 20 (0.04% PBS–Tween) and treated with blocking solution (2% FCS in PBS) for 15 min. Antibodies to Gr-1 or control IgG were added (50 μg/ml in blocking solution) and the slides were incubated for 1 h at 4 °C in a humidified chamber. Then, the cytospins were washed five times with PBS–Tween and biotinylated anti-rat IgG was added. After 2 h of incubation at 4 °C in a humidified chamber, the slides were washed five times with PBS–Tween and streptavidin peroxidase conjugate (Sigma) was added (1/500 in blocking solution). After 40 min of incubation at 4 °C in a humidified chamber, cytospins were washed again (five times with PBS–Tween) and the substrate solution was added (17.5 ml of PBS containing 2.5 mg diaminobenzidine (DAB, Sigma) plus 5 μl of hydrogen peroxide 30%). After 10 min (RT) in darkness, the cytospins were washed with water and stained with methylene blue 1/20 (Instant Prov kit). Coverslides were fixed with Entelan (Merck) and the cells were analyzed under light microscopy (1000×). The frequency of Gr-1+ cells was determined in 1000 cells evaluated. To analyze cytokine production, after 48 h of infection, lymph node cells were collected and cultured (2×10^6 cells/200 μl) in 96-well plates (Costar) for 48 h at 37 °C in a humidified atmosphere containing 5% of CO_2. The supernatants were collected to analyze IL-12 and IFNγ production as described below.

2.6. Detection of IL-12p40 and IFNγ

IL-12p40 and IFNγ in the culture supernatants were measured by sandwich ELISA. The following monoclonal antibody pairs were used of which the second cited was biotinylated: IL-12p40, C17.15, and C15.6; IFNγ, XMG1.2, and ASN-18. Standard

curves were obtained with recombinant mouse cytokines (R&D Systems, Minneapolis, MN, USA). The reaction was developed with peroxidase-conjugated streptavidin (Sigma) followed by the substrate mixture containing hydrogen peroxide and orthophenylenediamine (OPD, Sigma). The optical density (OD) was detected in an ELISA reader Thermo Labsystems using a filter of 492 nm and a 620 nm filter as reference. The minimal detectable concentration in each test was 300 pg/ml for both cytokines.

2.7. Peripheral blood leukocyte analyses

After 6, 24, or 48 h of infection, mice were bled through caudal vein and the blood was diluted in Turk's solution to quantify leukocytes using a hemocytometer. The results were expressed as leukocytes $\times 10^6$/ml. Blood smears, in duplicates, were stained using the Instant Prov kit. The percentages of MNC and PMN were determined in 200–300 cells evaluated in each microscope slide, under light microscopy (400×). The absolute numbers of cells were expressed as MNC $\times 10^6$/ml and PMN $\times 10^6$/ml.

2.8. Histological analyses

To analyze the local inflammatory reaction the footpads of *L. major*-infected mice were collected and saved in 10% formalin. Decalcification was performed by immersion of the footpads in 5% nitric acid plus 5% paraformaldehyde (Merck) in distilled water for 3 days. Then, the material was processed to obtain 5-μm tissue sections which were stained using hematoxylin–eosin.

2.9. Enrichment of Gr-1+ cells by magnetic columns

Selection of Gr-1+ cells was made using a paramagnetic microbeads system (MiniMacs, Miltenyi BiotecGmbH, Bergisch Gladbach, Germany). The cell population obtained from infected-mouse draining lymph node was pre-incubated at 4 °C, for 20 min, with 10 μg/ml of RB6-8C5 antibody added to 10^7 cells in PBS containing 2% FCS. The cells were washed twice and further incubated with beads bound to goat anti-rat IgG (Miltenyi Biotec) and separated on a magnetic column as indicated by the manufacturer. The efficiency of the selection was checked by immunocytochemistry. There was not any positive cell on negative population and enriched population was not higher than 20% of Gr-1+ cells.

2.10. Statistical analysis

Data represent means ± standard deviation and were analyzed by ANOVA/Bonferroni's test for multiple comparisons or by *t* test for two groups, using Graph-Pad Prism Software 4.0 (San Diego, CA, USA). The differences were considered significant when $p < 0.05$.

3. Results

3.1. Leishmania major infection induces early, transient recruitment of Gr-1+ cells into draining lymph nodes

To assess the putative contribution of Gr-1+ cells to IL-12 production in skin-draining lymph nodes early after *L. major* infection, we first determined the presence of these cells in popliteal lymph node of BALB/c mice. To that end, cytospins of lymph node-cell sus-

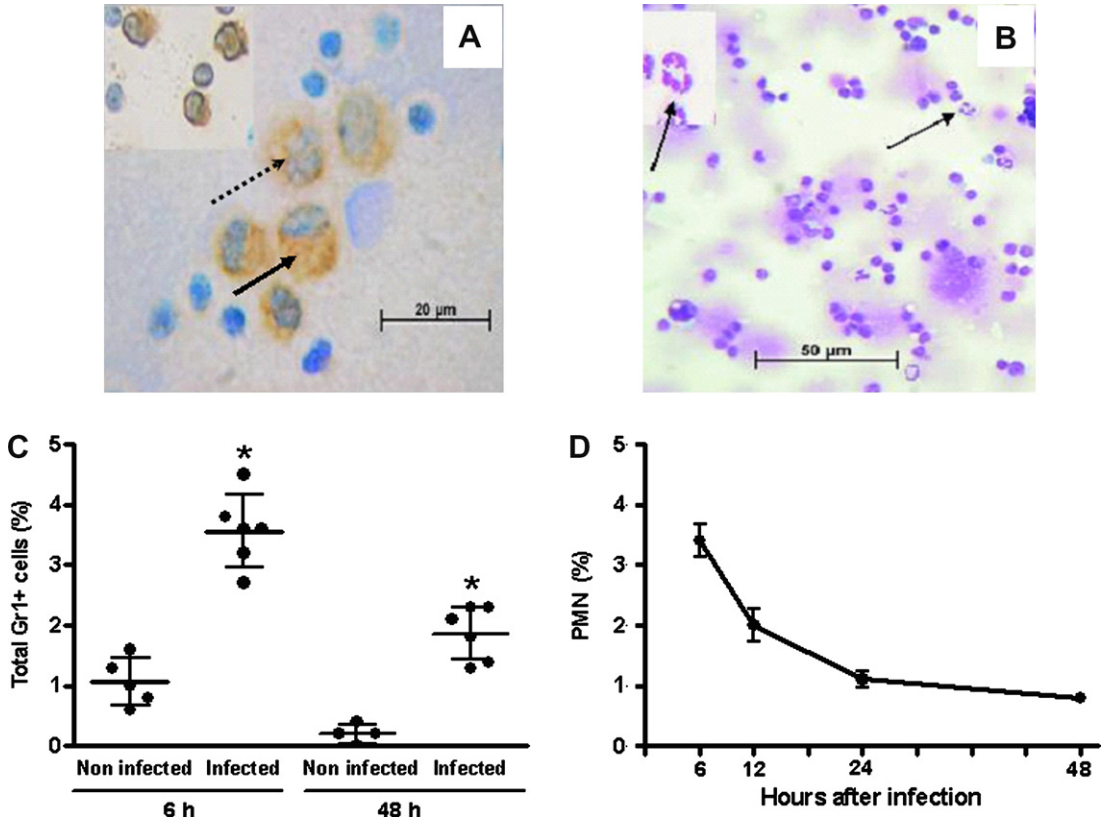

Fig. 1. *Leishmania major* infection leads to an early recruitment of Gr-1+ cells into draining lymph nodes. Promastigote forms of *L. major* (1×10^6/footpad) were injected in the presence of control IgG antibodies (50 μg/footpad) into BALB/c mice. Control animals were injected with control IgG antibodies only. After different times of infection, draining lymph node cells were collected to prepare cytospins. (A) Cells were stained using immunocytochemistry, arrows indicate Gr-1+ PMN (dotted arrow) or Gr-1+ MNC (straight arrow). (B) Cytochemical staining, arrows indicate PMN. (C) Frequency of Gr-1+ cells determined in 1000 cells. (D) Frequency of PMN determined in 1000 cells. In (C and D) means ± SD (n = 3–6 mice) of three experiments are shown. $^*p < 0.05$. In (A and B) the horizontal lines indicate 20 and 50 μm, respectively. Naïve lymph nodes contain 0.1–0.9% of Gr-1+ cells and PMN are not detected.

pensions were analyzed by immunocytochemistry. In naïve mice, a low frequency of Gr-1+ cells was detected (0.1–0.9%). As Gr-1+ cells comprise both PMN and MNC and in immunocytochemistry preparations the morphology is not determined with assurance (Fig. 1A), the cytospins of lymph node cells were also analyzed under light microscopy after cytochemical staining. In naïve lymph nodes, we were unable to detect PMN, leading to the conclusion that all detected Gr-1+ cells presented MNC morphology.

After injection of control rat IgG antibodies, a small frequency of Gr-1+ cells was detected into lymph nodes (1.1 ± 0.4%), however, infection with *L. major* promastigotes led to a significant increase of Gr-1+ cell recruitment into draining lymph nodes (3.6 ± 0.6%, Fig. 1B, Fig. 1C). The maximal frequency of PMN (3.4 ± 0.4%) was coincident with the peak of Gr-1+ cells detected 6 h post-infection (Fig. 1C and D). However, at 48 h after infection the frequency of Gr-1+ cells was still elevated in infected mice (1.9 ± 0.4%, Fig. 1C), whereas the frequency of PMN had significantly decreased (0.8 ± 0.1%, Fig. 1D).

3.2. Treatment with anti-Gr-1 antibodies causes transient decrease of blood PMN and MNC numbers, local inflammation, and recruitment of Gr-1+ cells into draining lymph nodes

To evaluate the migration of Gr-1+ cells during the early phase of *L. major* infection, mice were infected with promastigote forms of *L. major* into the footpads in the presence of anti-Gr-1 antibodies or control IgG. The draining lymph nodes were evaluated at 6 h post-infection. The treatment with anti-Gr-1 antibodies significantly decreased the frequency of PMN (2.6 ± 1.0 vs 0.3 ± 0.1%, $p < 0.05$, Fig. 2A) as well as the frequency of total Gr-1+ cells (3.5 ± 0.6 vs 0.6 ± 0.4%, $p < 0.05$; Fig. 2B). In parallel, after 6 h of infection there was a moderate inflammatory reaction in the dermis comprising mainly PMN (Fig. 3A and C), which almost completely disappeared after anti-Gr-1 treatment (Fig. 3B and C).

The decrease of infiltrating inflammatory cells at the site of simultaneous injection of promastigotes and anti-Gr-1 antibodies suggested that the antibody treatment could cause systemic cell depletion. To clarify this question, the peripheral blood leukocyte composition was also evaluated after infection in the presence or in the absence of anti-Gr-1 antibodies. The treatment decreased the number of leukocytes in non-infected as well as in infected mice (non-infected mice: 10.8 ± 1.6 vs 6.1 ± 1.0 × 10^6 cell/ml, Fig. 4A; infected mice: 12.9 ± 1.9 vs 6.5 ± 1.7 × 10^6 cell/ml, $p < 0.05$, Fig. 4D). In addition to the strong reduction of PMN numbers (non-infected mice: 2.1 ± 0.9 vs 0.2 ± 0.1 × 10^6 PMN/ml, $p < 0.05$, Fig. 4B; infected mice: 3.8 ± 1.4 vs 0.4 ± 0.2 × 10^6 PMN/ml, $p < 0.05$, Fig. 4E), also MNC numbers decreased when mice were infected simultaneously with anti-Gr-1 treatment (non-infected mice: 8.7 ± 2.0 vs 5.9 ± 1.0 × 10^6 MNC/ml, Fig. 4C; infected mice: 9.1 ± 2.1 vs 6.1 ± 1.7 × 10^6 MNC/ml, $p < 0.05$, Fig. 4D). The effect of the treatment with anti-Gr-1 antibodies on peripheral blood leukocyte composition was transient since no differences were detected after 48 h between infected or non-infected mice treated with control IgG or anti-Gr-1 antibodies (data not shown).

3.3. In vivo depletion of Gr-1+ cells decreases IL-12 and IFNγ production at the early phase of L. major infection

As we demonstrated a rapid recruitment of Gr-1+ cells into draining lymph nodes after *L. major* infection, we further investigated a possible contribution of Gr-1+ cells to the early IL-12 production. Popliteal lymph node cells from BALB/c mice infected with promastigotes and injected with control IgG or anti-Gr-1 antibodies were collected after the first 48 h of the treatment. The supernatants of 48-h cell cultures were assayed for IL-12 concentration. As shown in Fig. 5A, IL-12 was significantly increased in cultures of lymph node cells from *L. major*-infected mice (<300 vs 1406 ± 422 pg/ml, $p < 0.05$). The production of IL-12 was significantly reduced with the anti-Gr-1 antibody treatment (1406 ± 422 vs 416 ± 72 pg/ml, $p < 0.05$, Fig. 5A). A new set of experiment was performed to compare anti-Gr-1 treatment in BALB/c and C57Bl/6 mice and, in that case, there was not significant effect on the IL-12 production in C57Bl/6 treated mice (Fig. 5A, insert).

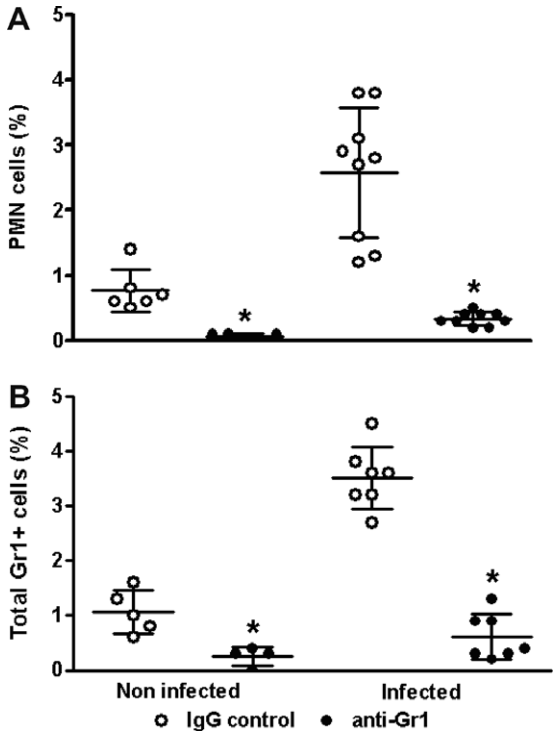

Fig. 2. *In vivo* treatment of BALB/c mice with antibodies to Gr-1 simultaneously to the injection with *Leishmania major* decreases Gr-1+ cell recruitment into draining lymph nodes. Mice were infected with promastigote forms (1 × 10^6/footpad) in the presence of control IgG antibodies or antibodies to Gr-1 antigen (50 μg/footpad). Also non-infected mice were treated with control IgG antibodies or antibodies to Gr-1 antigen (50 μg/footpad) as controls. After 6 h of infection, draining lymph node cells on cytospins were stained to determine PMN frequency (A) and to determine the frequency of total Gr-1+ cells (in 1000 cells, B), respectively. Data represent individual values and means ± SD ($n = 4$–9 mice) of three experiments. $^*p < 0.05$ (non-infected/control IgG vs non-infected/anti-Gr-1; infected/control IgG vs infected/anti-Gr-1).

As IFNγ is also produced early in *L. major* infection and it is dependent on IL-12, the levels of IFNγ were also evaluated. *L. major* infection induced a high amount of IFNγ (13.3 ± 5.6 vs 0.3 ± 0.1 ng/ml, $p < 0.05$, Fig. 5B), which was significantly decreased after depletion of Gr-1+ cells in BALB/c mice (13.3 ± 5.6 vs 3.4 ± 4.4 ng/ml, $p < 0.05$, Fig. 5B). When experiments were performed to compare anti-Gr-1 treatment in BALB/c and C57Bl/6 mice it was observed that the IFNγ production was significantly reduced in both mouse strains, although the reduction of IFNγ in culture supernatants from anti-Gr-1-treated BALB/c mice was more accentuated than in antibody treated-C57Bl/6 mice (reduction of 54% vs 32%; Fig. 5B, insert).

3.4. Ex vivo production of IL-12 and IFNγ by draining lymph node cells from infected BALB/c mice is dependent on the presence of Gr-1+ cells

To further investigate the contribution of Gr-1+ cells to the IL-12 and IFNγ production during *ex vivo* cultures, Gr-1+ cells from 48 h-infected BALB/c mice draining lymph nodes were negatively or positively selected by using paramagnetic beads. In the absence

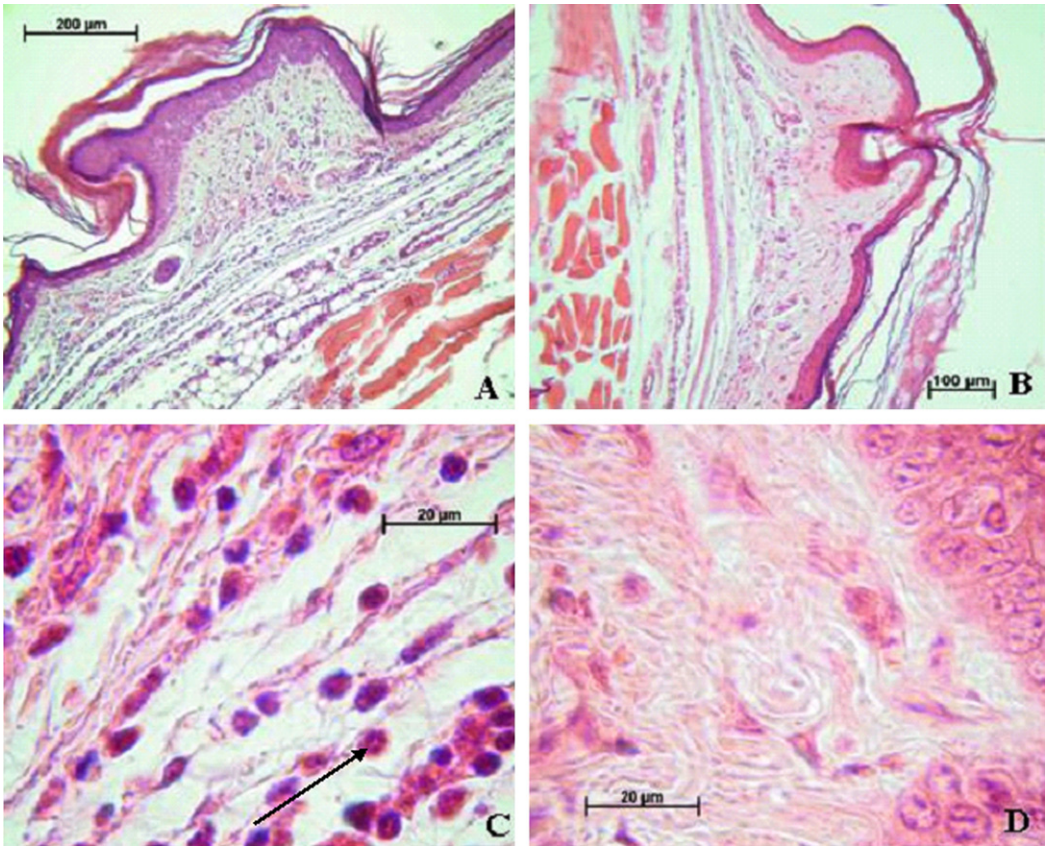

Fig. 3. Decrease of cellular infiltration at the inflammatory site of *Leishmania major* infection after *in vivo* treatment with antibodies to Gr-1 antigen. BALB/c mice were infected with promastigote forms (1×10^6/footpad) in the presence of control IgG antibodies (A and C) or antibodies to Gr-1 antigen ((B and D) 50 µg/footpad). Six hours after infection, the footpads were collected and processed for histological analysis and HE staining. (A and C) A moderate inflammatory reaction is observed in the dermis comprising mainly PMN ((C) the arrow points one PMN). (B and D) The cellular infiltration almost disappeared upon anti-Gr-1 treatment. The horizontal bars represent in (A) 200 µm; in (B) 100 µm; in (C and D) 20 µm.

of Gr-1+ positive cells the productions of IL-12 (772 ± 306 vs 227 ± 68 pg/ml, $p < 0.05$, Fig. 6A) and IFNγ (6.9 ± 4.4 vs 3.4 ± 1.9 ng/ml, $p < 0.05$, Fig. 6B) were significantly decreased. By the contrary, enrichment of Gr-1+ cells increased the production of these cytokines, although this increase in IL-12 production did not reach significance in statistical analyses (IL-12: 772 ± 306 vs 1227 ± 470 pg/ml, Fig. 6A; IFNγ: 6.9 ± 4.4 vs 18.5 ± 7.7 ng/ml, $p < 0.05$, Fig. 6B).

4. Discussion

In the present study, we showed the necessity of recently migrated Gr-1+ cells, to the initial IL-12 and IFNγ production in response to *L. major* infection in BALB/c mice. We have shown that non-infected popliteal lymph nodes contain about 0.1–0.9% of Gr-1+ cells, which are mononuclear in nature. This cell population in naïve popliteal lymph nodes probably corresponds to Gr-1+ plasmacytoid DC previously described before (Asselin-Paturel et al., 2003; Nakano et al., 2001). After footpad infection with *L. major*, the frequency of Gr-1+ cells in popliteal lymph nodes increased significantly, reaching a maximum at 6 h after infection. Notably, the peak of cells with PMN morphology was also at the same time point. However, after 48 h of infection, when the frequency of PMN strongly decreased, the frequency of Gr-1+ cells was still significantly elevated. Together, these data indicate that Gr-1+ PMN as well as Gr-1+ MNC are recruited into the draining lymph nodes after infection with *L. major*. These results are supported by previous reports (Lima et al., 1998; Tacchini-Cottier et al., 2000) showing the presence of PMN in draining lymph nodes of *L. major*-infected mice, and a recent report describing the presence of Gr-1+ DC within lymph nodes after 24 h of *L. major* infection (Iezzi et al., 2006).

In our experiments, treatment of *Leishmania* promastigote-infected mice with antibodies to Gr-1 antigen drastically reduced the frequency of Gr-1+ cells in the draining lymph nodes. This decrease was a consequence of a transient depletion of both PMN as well as MNC in peripheral blood. The latter indicates a depletion of Gr-1+ monocytes. Since PMN and Gr-1+ but not Gr-1− monocytes migrate to sites of peripheral inflammation (Geissmann et al., 2003; Lima et al., 1998; Tacke and Randolph, 2006), and in the current study they were decreased in the blood, it was not a surprise that we also observed a reduced inflammatory reaction at the site of *L. major* injection in mice treated with anti-Gr-1 antibodies. The increase of Gr-1+ cells within popliteal lymph nodes after *L. major* infection suggested that these cells may be involved in the early response to promastigotes. Indeed, the *ex vivo* production of IL-12 and IFNγ by lymph node cells was significantly decreased after *in vivo* depletion of Gr-1+ cells. Since resident phagocytes such as macrophages or DC in the dermis or epidermis do not carry Gr-1 antigen, we suggest that Gr-1+ cells that are important for the early IL-12 production represent recently migrated cells from blood into the lesion site and then into the lymph nodes or Gr-1+ cells migrated from blood directly into the draining lymph nodes of BALB/c mice. *In vitro* depletion of Gr-1+ cells in lymph node-cell preparations almost abolished the IL-12 production although the production of IFNγ was partially decreased. These data suggest that

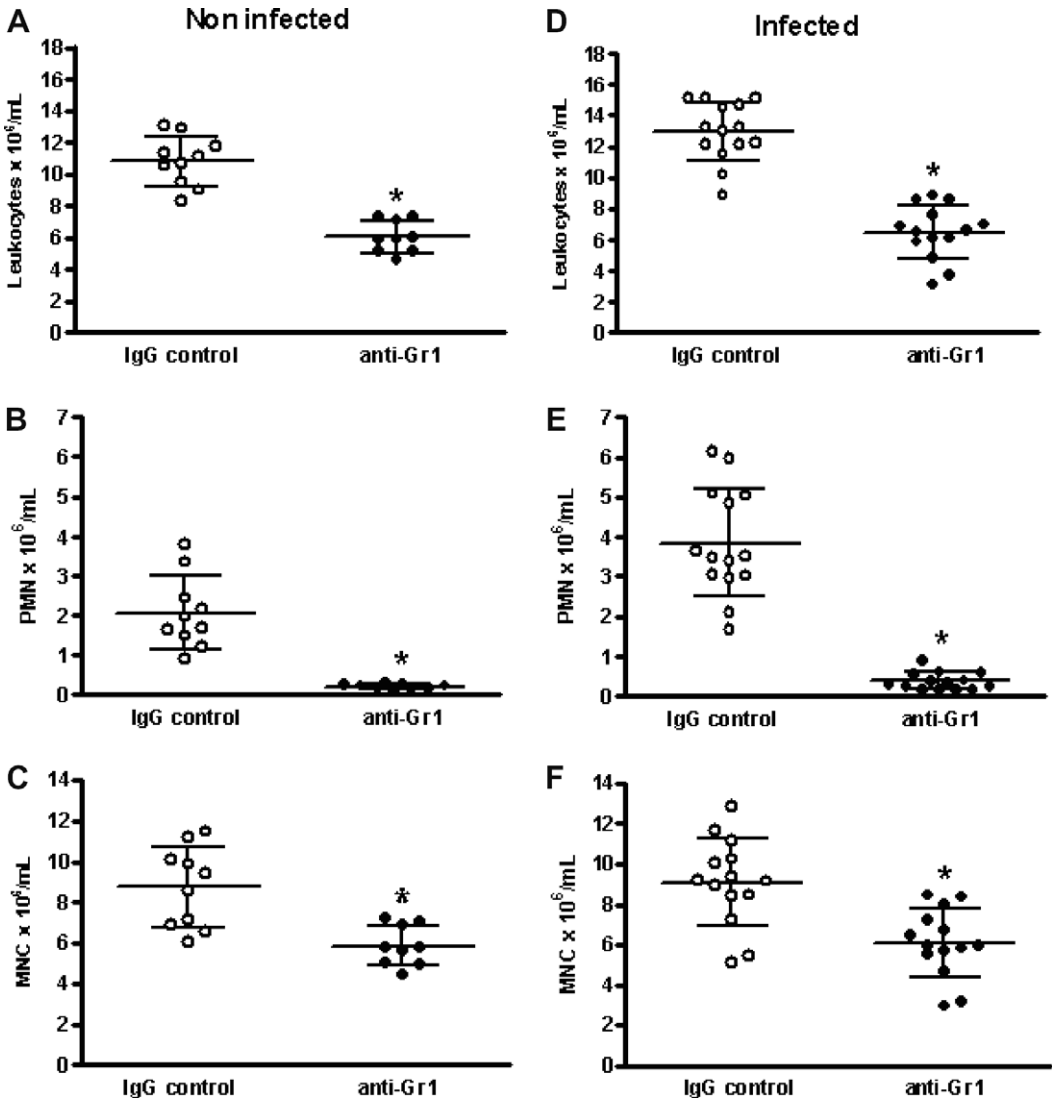

Fig. 4. *In vivo* treatment with antibodies to Gr-1 causes leucopenia in BALB/c mice. Non-infected mice (A–C) or mice infected with promastigote forms of *Leishmania major* (1×10^6/footpad (D–F)) were treated with control IgG or anti-Gr-1 (50 μg/footpad). After 6 h, the peripheral blood was evaluated for total leukocyte number (A and D) as well as for PMN and MNC numbers (PMN (B and E); MNC (C and F)). Data represent individual values and means ± SD (n = 9–14) of five experiments. $^{*}p < 0.05$ (Control IgG vs anti-Gr-1).

Gr-1+ cells are source of early IL-12 which increases the production of IFNγ by other cells, probably NK cells (Scharton and Scott, 1993).

Immature myeloid cells are probably the most important cells involved in the early IL-12 production during *L. major* infection. It was demonstrated that IL-12 was produced at highest levels when mononuclear phagocytes in intermediate stages of development, i.e., post-monocytic but not fully differentiated, were stimulated with *L. major* promastigotes (Oliveira et al., 2005). Indeed, immature bone-marrow derived myeloid DC $CD86^{low}$- produce high amount of IL-12p40 after stimulation with *Leishmania infantum* or *Leishmania braziliensis in vitro* (Schleicher et al., 2007), confirming the immature stage of IL-12p40-producing cells. Thus, it is tempting to speculate that blood Gr-1+ monocytes that migrate to the inflammatory site of infection with *L. major*-promastigote forms and further into the draining lymph nodes are source of early IL-12 production. In agreement with this notion, immature Gr-1+ macrophages produce high amounts of IL-12 early in *T. gondii* infection (Mordue and Sibley, 2003). Besides, Gr-1+ monocytes can give rise to Gr-1+ DC that produce IL-12, as suggested in *Listeria* infection (Tam and Wick, 2006). This idea is in agreement with the results obtained by Schleicher et al., who abrogated initial IFNγ production by *L. infantum* infected mice by depleting CD11c+ cells of the myeloid lineage (Schleicher et al., 2007). Since depletion of Gr-1+ cells decreases IL-12 and IFNγ production in our experiments, we believe that the treatment used by these authors was able to deplete recently migrated Gr-1+ cells committed to DC population, which seems to be the population responsible for early IL-12 production in *Leishmania* infection as demonstrated in *Listeria* infection (Tam and Wick, 2006).

Neutrophils, that also express Gr-1, are known as IL-12-producing cells (Cassatella et al., 1995) and the production of IL-12 early in infection with *T. gondii* is dependent on the presence of neutrophils at the inflammatory site (Bliss et al., 2000). However, it was recently shown that neutrophils from BALB/c mice do not produce IL-12 after infection with *L. major* (Chen et al., 2005), then we regard it is less likely that neutrophils themselves are the source of the early IL-12 produced after *L. major* infection. Alternatively, it is possible that neutrophils contribute indirectly to IL-12 production via their immunomodulatory functions, for instance by inducing DC maturation (van Gisbergen et al., 2005) and production of IFNγ by macrophages (Venuprasad et al., 2002).

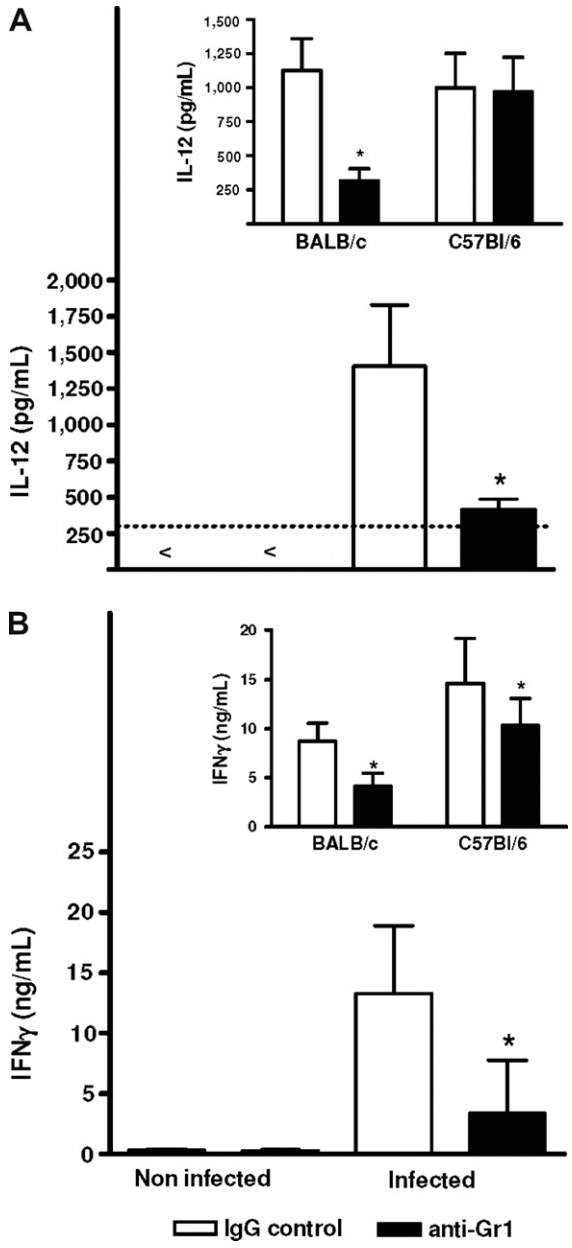

Fig. 5. Gr-1+ cells recruited early into draining lymph nodes during *Leishmania major* infection are important for IL-12 and IFNγ production in BALB/c mouse strain. BALB/c and C57B1/6 mice were infected with promastigote forms (1×10^6/footpad) in the presence of control IgG antibodies or antibodies to Gr-1 antigen (50 μg/footpad). After 48 h of infection, draining lymph node cells were cultured (2×10^6 cells/200 μl, 48 h) and IL-12 (A) and IFNγ (B) were measured by ELISA in the cell supernatants. Insert: BALB/c and C57BL6 mice were infected as described above and IL-12 (insert A) and IFNγ (insert B) measured by ELISA. Cells from control lymph nodes were cultured at the same conditions. Data represent means ± SD ($n = 3$–4 mice) at least three experiments. $^*p < 0.05$. In (A) < below the detection limit, and dotted line represents the detection limit of the assay.

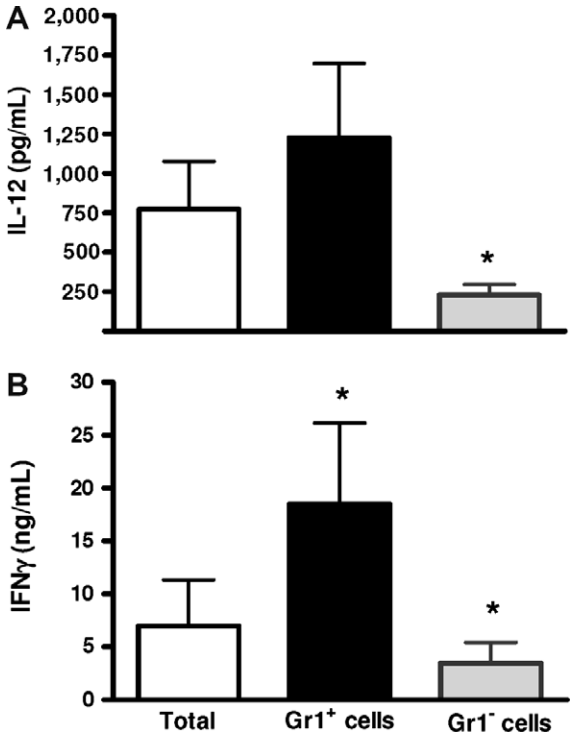

Fig. 6. Absence of Gr-1+ cells in infected-draining lymph node-cell cultures is associated to the decrease of production of IL-12 and IFNγ. BALB/c mice were infected with promastigote forms of *L. major* (1×10^6/footpad). After 48 h of infection, draining lymph node cells were collected and cells were positively or negatively selected according to Gr-1 expression by using paramagnetic microbeads. Negative population or positive enriched population was cultured (2×106 cells/200 μl, 48 h) and IL-12 (A) and IFNγ (B) were measured by ELISA in the cell supernatants. Data represent means ± SD ($n = 3$ mice) of four experiments. $^*p < 0.05$ (total lymph node vs enriched Gr-1+ or Gr-1− population).

It is known that Gr-1+ plasmacytoid DC migrate from blood into the inflamed lymph nodes (Colonna et al., 2004) and they are present in *L. major*-infected-draining lymph nodes (Iezzi et al., 2006). However, it has been shown that in *Leishmania* infection plasmacytoid DC is not an important source of IL-12 (Schleicher et al., 2007), and besides that we do not have evidence that these cells are relevant in our model.

Our experiments showed that recently migrated Gr-1+ cells are early source of IL-12 and collaborate to the production of IFNγ mainly in susceptible BALB/c mice infected with *L. major*, since we did not observe decrease of IL-12 production, and there was small decrease of IFNγ production when C57BL/6 mice were treated with anti-Gr-1 antibodies. The profile of the initial inflammatory infiltrate present in the lesions of susceptible BALB/c mice is different from that observed in C57BL/6 mice. The first one has, e.g., higher amount of PMN and immature MNC (Beil et al., 1992; Sunderkotter et al., 1993). Based on the fact that both PMN and immature MNC are the major cells carrying the Gr-1 antigen (van der Loo et al., 1995; de Bruijn et al., 1998), it is not surprise that the changes observed in BALB/c mice due to the treatment with anti-Gr-1 antibodies was more expressive. Our data point out that whereas in susceptible BALB/c mice the lymph node influx of Gr-1+ cells is crucial for the early IL-12 production, in resistant C57Bl/6 mice it seems to be not relevant.

The present work showed only the production of IL-12p40, but this molecule can be presented in different forms, as monomers (p40), homodimers (p80), and heterodimers IL-12p35p40 (IL-12p70) or IL-12p19p40 (IL-23) (Abdi, 2002). The IL-12p70 is pivotal to induce Th1 differentiation in *L. major* infection (Mattner et al., 1996), however, the amount of IL-12p40 can be 1000 times higher than IL-12p70 (Oliveira et al., 2005), what could justify that we were unable to detect this protein in supernatant of our cultures (data not shown). It was shown that Gr-1+ cells were important for IL-12p40 production by BALB/c mice, but not for C57BL/6 mice in our experiments. It is possible that different concentrations of distinct p40 forms can drive the immune response for susceptibility or resistance to *L. major* infection. As previously reported, early production of IL-12p70 is need for Th1 differentiation in resistant C57BL/6 mice (Mattner et al., 1996), nevertheless the pro-

duction of p40 homodimers is higher in BALB/c- than in C57BL/6-mouse DC cultures, and it inhibits Th1 differentiation (Nigg et al., 2007). Thus, the determination of the contribution of Gr-1+ cells on the production of different p40 homodimers or heterodimers should enable us to better understand the role of these cells and IL-12p40 in the outcome of leishmaniasis.

The present study demonstrated that after injection of *L. major*-promastigote develops an inflammatory reaction in the skin, with rapid recruitment of Gr-1+ cells from blood to tissue and into lymph nodes. That process is required for the early production of IL-12 and IFNγ mainly in BALB/c mice. Moreover; the data favor the view that the recently migrated Gr-1+ monocytes are the main players on early IL-12 production. These cells could give rise to inflammatory macrophages or DC expressing Gr-1 antigen during the first hours of *L. major* infection, as has been demonstrated by others (Iezzi et al., 2006). The relative contribution of each Gr-1+ cell subsets to IL-12 production and its consequences on the adaptive immune response on susceptible and resistant mouse strains deserves further investigation. Beyond doubt, the recruitment and continuous differentiation of recently migrated cells *in situ* interfere with the IL-12 production and that may have important implications on acquired immunity and control of the disease.

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