# Protective Effect on *Leishmania major* Infection of Migration Inhibitory Factor, TNF-$\alpha$, and IFN-$\gamma$ Administered Orally via Attenuated *Salmonella typhimurium*[1]

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**The genes encoding murine macrophage migration inhibitory factor (MIF), IL-2, IFN-$\gamma$ or TNF-$\alpha$ were cloned individually into an expression plasmid under the control of the inducible promoter *nirB* and transfected into the aroA$^-$aroD$^-$ deletion mutant strain of *Salmonella typhimurium* (BRD509). These *S. typhimurium* derivatives (henceforward called constructs and termed GIDMIF, GIDIL2, GIDIFN and GIDTNF) expressed their respective cytokines in vitro under anaerobic conditions and stably colonized BALB/c mice up to 14 days after oral administration. The highly susceptible BALB/c mice that had received the constructs orally and that had been subsequently infected via the footpad with *Leishmania major*, developed significantly reduced disease compared with control mice administered the untransfected *Salmonella* strain (BRD509). Importantly, a combination of GIDMIF, GIDIFN, and GIDTNF administered orally after *L. major* infection was able to significantly limit lesion development and reduced parasite loads by up to three orders of magnitude. Spleen and lymph node cells of mice administered this combination expressed markedly higher levels of inducible nitric oxide synthase (iNOS) compared with those from mice receiving an equivalent dose of the control strain of *Salmonella* (BRD509). These data therefore demonstrate the feasibility of therapeutic treatment in an infectious disease model using cytokines delivered by attenuated *Salmonella*. The protective effect observed correlates with the induction of inducible nitric oxide synthase in vivo.** ***The Journal of Immunology*, 1998, 160: 1285–1289.**

The therapeutic potential of cytokine administration has been demonstrated in a number of disease states including spontaneous and induced autoimmunity, tumor models, and infectious diseases. The in vivo administration of cytokines has been conducted using a variety of techniques, the simplest of which is injection of recombinant or purified material. This approach is often hampered by the short half-life of cytokines in vivo (1), reducing the time of exposure and cytokine concentration such that high and multiple doses of injected cytokines are required to have a biologic effect. We have previously demonstrated the potential of attenuated *Salmonella typhimurium* for the in vivo oral delivery of sustained biologically active recombinant IL-1$\beta$ (2).

There is currently much interest in attenuated *Salmonella* constructs as live heterologous carriers for vaccination (3–5) because they provide a relatively safe and efficient means of administering Ags via the oral route. Infection of susceptible BALB/c mice with *Leishmania major* results in uncontrolled lesion development, followed by disseminated infection and death (6, 7). Cure from experimental leishmaniasis in resistant mouse strains depends on the generation of a Th1 response and the production of IFN-$\gamma$ (8–10), required for the induction of inducible nitric oxide synthase (iNOS)[3] in infected macrophages (11–13). However, treatment of susceptible mice with recombinant IFN-$\gamma$ in vivo has not demonstrated significant effects (14). This could be attributed to a lack of sustained provision of the delivered cytokine in vivo.

We report here the construction of four *Salmonella typhimurium* strains that express cytokines known to be involved in the Th1 responses or the activation of macrophages and assess their therapeutic potential in treating *L. major* infection. IFN-$\gamma$, TNF-$\alpha$, and MIF are potent activators of macrophages, while IL-2 is a principal T cell growth factor. We demonstrate here that each construct administered to susceptible mice before infection markedly reduced disease development. Furthermore, a combination of strains expressing MIF, TNF-$\alpha$, and IFN-$\gamma$ given orally after infection significantly reduced lesion development and parasite burdens. The effect of these constructs correlated with the induction of iNOS expression.

## Materials and Methods

### *Mice*

Inbred BALB/c mice were obtained from Harlan Olac (Bicester, U.K.). Female mice 6 to 8 wk old were used.

### *Parasites*

The *L. major* isolate MRHO/SU/59/P (LV39) was used. The maintenance, cultivation, and isolation of *L. major* promastigotes have been described in detail elsewhere (15).

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[3] Abbreviations used in this paper: iNOS, inducible nitric oxide synthase; MIF, migration inhibitory factor; NO, nitric oxide; MLN, mesenteric lymph node.



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### *Construction of the pnirB/cytokine plasmids*

The expression plasmid pTETnir15 has been described previously (16) and modified to include unique restriction enzyme sites downstream of the promoter as previously described (17). The cDNA for IL-2, IFN-$\gamma$, and TNF-$\alpha$ were provided by DNAX (Palo Alto, CA) and MIF cDNA was reported previously (18). cDNA were modified by PCR to include *Nde*I and *Bam*HI sequences at the 5′ and 3′ ends respectively. The oligonucleotides used for PCR (synthesized by Genosys Biotechnologies, Inc., U.K.) were as follows: IL-2, 5′-GGATCCATATGGCACCCACTTCAAGCTCC-3′ and 3′-GGATCCAAGACTAGTAGTAGTTAC-3′; MIF, 5′-GGTCCTT CTGCCCATATGCCGATGTTC-3′ and 5′-GGATCCCTGCGGCTCT TAGGC-3′; IFN-$\gamma$, 5′-GAATTCATATGCACGGCACAGTCAT-TGAA-3′ and 3′-GGATCCCCACCCCGAATCAGCAGCG-5′; TNF-$\alpha$, 5′-GGATCC ATATGCTCAGATCATCTTCTCAA-3′ and 3′-GGATCCCATTCCCT TCACAG-5′.

### *Construction of recombinant* S. typhimurium

The aroA$^-$aroD$^-$ vaccine strain of *S. typhimurium*, BRD509, has been described in detail elsewhere (19). Bacteria were routinely cultured on L-agar or in L-broth (LB) with or without 100 $\mu$g/ml ampicillin. Plasmids were transformed into LB5010 and bacteria selected for resistance to ampicillin. Plasmids were transduced to BRD509 using p22 transduction. Ampicillin-resistant clones were named GIDIL2 (pnirB/IL-2), GIDMIF (pnirB/MIF), GIDIFN (pnirB/IFN-$\gamma$), and GIDTNF (pnirB/TNF-$\alpha$).

### *Induction and detection of cytokines in vitro.*

Induction of cytokine expression was conducted as previously described (17). Briefly, bacterial colonies were grown overnight at 37°C in L-broth containing ampicillin and then diluted 1:100 with L-broth containing ampicillin and 4 mg/ml glucose into a closed screw-cap container. Bacteria were incubated for 4 to 6 h at 37°C before screening. Bacterial lysates were analyzed on SDS polyacrylamide gels, transferred to nitrocellulose (Bio-Rad Laboratories, Richmond, CA) and proteins were detected with the following Abs: monoclonal rat anti-mouse IL-2 (Genzyme), monoclonals anti-murine IFN-$\gamma$ (R46A2) or anti-murine TNF-$\alpha$ (XT22.11) (both provided by DNAX), and goat anti-human MIF (18). The relevant horseradish peroxidase-conjugated secondary Ab (Bio-Rad, Hertfordshire, U.K.) was then applied, and the blots were developed with horseradish peroxidase color-developing reagent (Bio-Rad).

### *Electron microscopy*

Bacteria were fixed in 4% paraformaldehyde in PBS for 30 min, pelleted, and then encapsulated in 10% gelatin in PBS at 37°C, followed by solidifying the gelatin at 4°C. Pieces of the encapsulated pellet were brought to 2.3 M sucrose in cold PBS for 45 min after which each was mounted in a droplet on cryospecimen pins (Leica, Wein, Austria) and plunge-frozen in liquid nitrogen. Specimens were cryosectioned using a Reichert Ultracut E/FC4D cryoultramicrotome (Leica) at −110°C, and 100-mm sections were collected onto carbon-coated support grids. Sections were immunostained with rabbit anti-MIF Ab or normal rabbit serum at 1/20 dilution in PBS/1% BSA for 1 h, followed by addition of protein A conjugated to 5 nm gold at 1/100 in the same buffer using washing and blocking procedures described by Griffiths (20). Sections were finally stained and embedded in methyl cellulose (21) and examined in a Zeiss 902 EFTEM electron microscope (Hertfordshire, U.K.) by zero-loss imaging to enhance contrast.

### *Cytokine biologic assays*

IL-2 activity was measured (22) by its ability to sustain the proliferation of the T cell line, CTLL (American Type Culture Collection (ATCC)). TNF-$\alpha$ activity was assayed (22) by its cytotoxicity on the fibroblast cell line, L929 (ATCC). IFN-$\gamma$ activity was determined (23) by its ability to induce NO synthesis by the murine macrophage cell line, J774 (ATCC) in the presence of LPS (10 ng/ml). MIF activity was examined using the tautomerase assay as previously described (18). In each case, the appropriate recombinant cytokine was used as standard for constructing a standard curve.

### *Plasmid stability in vivo*

Determination of plasmid stability was conducted as previously described (17). Homogenates of spleen, liver, and mesenteric lymph nodes (MLN) from mice previously administered with $1 \times 10^{10}$ bacteria were plated onto L-agar plates in the presence or absence of ampicillin to determine the number of colonies that had maintained plasmid.

### *Treatment of mice with cytokine constructs*

Immediately before administration of *Salmonella* or PBS, mice were given 100 $\mu$l of 5% $Na_2CO_3$ orally. Mice were then administered orally with an overnight culture of $1 \times 10^{10}$ (in 0.4 ml PBS) GIDIL2, GIDMIF, GIDIFN, GIDTNF, or the control strain, BRD509. The inoculum dose was verified by plating dilutions of each culture on L-agar plates with or without ampicillin.

### *Competitive PCR for iNOS*

The plasmid containing murine iNOS cDNA was kindly provided by Dr. Ian Charles (The Cruciform Project, University of London, U.K). To construct the iNOS competitor, the plasmid was digested with *Eco*RI and *Bam*HI to release the 500-bp iNOS cDNA fragment, which was ligated into the plasmid pBluscript. This plasmid was digested with *Pst*I to cause a 300-bp deletion in the middle of the insert before religation with a 134-bp *Pst*I fragment from *L. major* gp63. The resultant plasmid yielded a PCR product of 334 bp after amplification with iNOS primers (see below). This was gel purified, quantitated by spectrophotometry, and stored in aliquots. The competitive PCR method has been described in detail previously (24). Equal loading was confirmed by PCR using $\beta$-actin primers (5′-CTCTT TGATGTCACGCACGATTTC-3′ and 5′-GTGGGCCGCTCTAGGCAC CAA-3′). For quantification, an equal amount of cDNA was diluted and 1 to 10 $\mu$l of the diluted cDNA was used for amplification of target iNOS cDNA with iNOS primers (5′-AGCTCCTCCCAGGACCACAC-3′ and 5′-ACGCTGAGTACCTCATTGGC-3′). A constant amount of diluted cDNA was placed in seven tubes for PCR along with varying amounts of competitor template. After the PCR reaction, products were electrophoresed on an ethidium bromide-stained agarose gel. The point of equivalence in intensity of competitor (334 bp) and wild-type bands (500 bp) was then designated the concentration of the experimental cDNA for iNOS.

### *Challenge with* L. major.

Mice were challenged by s.c. injection in the footpad with $1 \times 10^5$ stationary phase *L. major* promastigotes in 50 $\mu$l of PBS. Lesion development was measured as an increase in footpad thickness, as described previously (17).

### *Quantification of* L. major *in infected footpads.*

Mice were killed by cervical dislocation. Footpads were removed above the ankle and diced into small pieces and then homogenized. Serial dilutions of the homogenate were made in culture medium on a 96-well tissue culture plate that was then incubated at 28°C. Wells with viable *Leishmania* were scored daily, and the parasite loads were calculated as described previously (8).

### *Statistical analysis*

Statistical significance was analyzed using Student's *t* test. $p < 0.05$ was considered significant.

## Results and Discussion

Attenuated *Salmonella* strain (AroA$^-$AroD$^-$) transfected with the plasmid (pnirB) encoding MIF, IL-2, IFN-$\gamma$, or TNF-$\alpha$ produced the respective cytokines under anaerobic conditions as demonstrated by Western blot. A single band of the appropriate size was detected in the bacteria (Fig. 1). This was also visualized by immunogold-electron microscopy (data not shown). The cytokines produced were biologically active (U/$10^6$ organisms): IL-2 (in GIDIL2), 4,400; IFN-$\gamma$ (in GIDIFN), 8,000; TNF-$\alpha$ (in GIDTNF), 1,500. MIF (in GIDMIF) activity was not detectable by the currently available biologic assay, which has a sensitivity limit of $>1$ $\mu$g/ml. MIF was produced at 20 to 25 ng/ml/$10^6$ organisms by ELISA.

Analysis of plasmid stability in vivo (Fig. 2) demonstrated that all four constructs effectively colonized the MLN, spleen, and liver of BALB/c mice for up to 14 days after oral administration. They maintained plasmid stably throughout the period of colonization in the MLN and spleens although some loss of plasmids from GIDIFN and GIDTNF was recorded in the liver. These data indicate that cytokine expression would be expected to occur at systemic sites for 1 to 2 wk after administration. However, there was

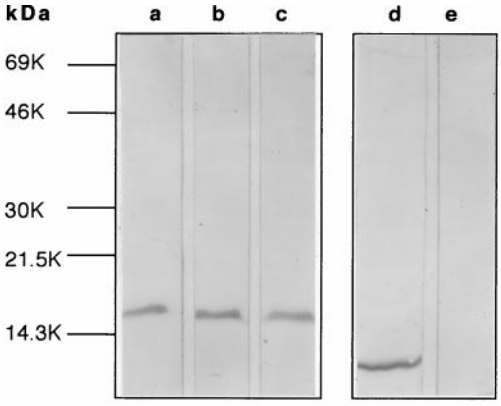


**FIGURE 1.** Western blot analysis of IL-2, IFN-γ, TNF-α, and MIF expression in transduced BRD509. Bacteria were cultured, induced, lysed, and blotted as described in *Materials and Methods*. *Lane a*, GIDIL2; *b*, GIDIFN; *c*, GIDTNF; *d*, GIDMIF; and *e*, BRD509.

no detectable cytokine in the serum of administered mice (data not shown). Spleen and MLN cells from all the mice produced similar levels of the cytokines when stimulated with a range of concentrations of Con A (data not shown). This indicates either that cytokines produced are at too low a level to be detected in the circulation or that they are are confined to the site of colonization. All the mice remained in good health, with no apparent gross side effect, gaining weight at the normal rate, the same as that of control mice administered with saline alone (data not shown).

The period of colonization by these strains is less than that reported previously for the IL-1β-expressing strain, which persisted in vivo for up to 5 wk (2), but is significantly greater than that of the IL-4-expressing strain SL7207 (pOmpAmIL-4) which had less

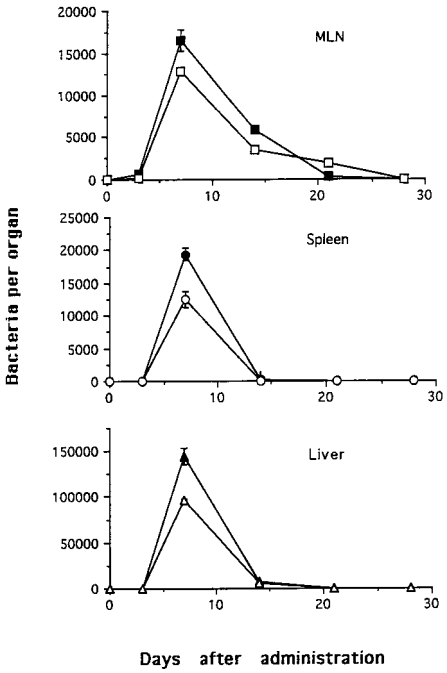


**FIGURE 2.** Plasmid stability in vivo. Groups of BALB/c mice were administered orally with $1 \times 10^{10}$ organisms of GIDMIF/mouse. Spleen, liver, and MLN were removed at the times indicated, and colony-forming units were assayed on plates with (filled symbol) or without (open symbol) ampicillin. Each point represents the geometric mean ± SEM of three mice. Results are representative of two experiments. Similar results were obtained with the transductants GIDIL2, GIDTNF, and GIDIFN.

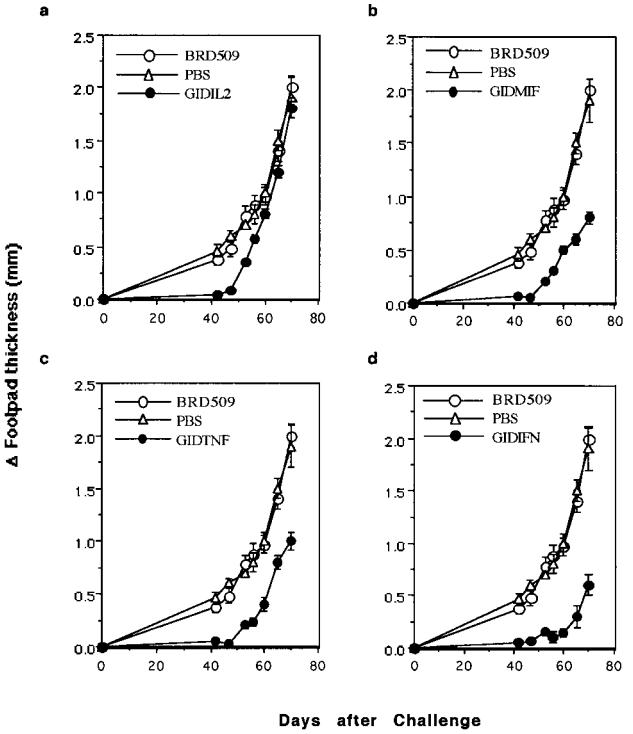


**FIGURE 3.** Prophylactic effect of the cytokine constructs. Mice were administered orally with a single dose of either control BRD509, or (*a*) GIDIL2, (*b*) GIDMIF, (*c*) GIDTNF, or (*d*) GIDIFN ($1 \times 10^{10}$ organisms/mouse). They were challenged 1 wk later in the footpad with $1 \times 10^5$ *L. major* promastigotes. Lesion development was measured at regular intervals as increase in footpad thickness (subtracting the thickness of the uninfected footpad from that of the infected footpad). Data are mean ± SEM, $n = 6$, and are representative of two experiments. Control mice administered with PBS alone developed disease indistinguishable from those administered with BRD509. In accordance with the current U.K. guidelines for animal experimentation, experiments were terminated by day 42 when control mice (BRD509) developed necrotic lesions in the footpad.

than 100 bacteria per organ after oral inoculation (25). The reason for this difference is unclear and could be due to macrophages activation by locally produced cytokines.

Pretreatment of BALB/c mice with a single oral dose of $1 \times 10^{10}$ organisms of any of the four constructs 1 wk before a challenge infection with *L. major* significantly delayed disease progression (Fig. 3), with GIDIFN having the greatest effect. Administration of the constructs 1 day before or on the same day as the infection was less protective (data not shown). These results demonstrate the capacity of all four constructs to deliver bioactive cytokine in vivo and further indicate that each of these cytokines when administered orally can have a beneficial prophylactic effect upon subsequent infection.

To investigate the potential therapeutic effect of these constructs, BALB/c mice were infected with *L. major* and administered orally with the constructs 7 days after infection. A modest reduction in lesion size was observed with only one construct, GIDIFN (Fig. 4*a*). This was accompanied by a reduction of two orders of magnitude of parasite load in the footpad (Table I). The other three constructs provided no significant protection from infection. These data indicate that oral cytokine is less effective in controlling *L. major* infection therapeutically than prophylactically. We then investigated whether a combination of cytokines would be more effective than a single treatment. From a combination of all possible pairings among the four constructs (data not

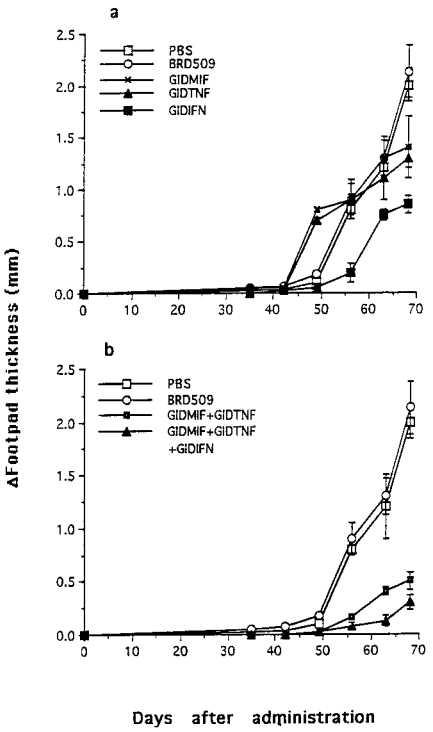


**FIGURE 4.** Therapeutic effect of the cytokine constructs. Groups of mice were infected in the footpads with $1 \times 10^5$ *L. major* promastigotes and at 7 days postinfection were given orally with (*a*) PBS, BRD509, GIDMIF, GIDTNF, or GIDIFN ($1 \times 10^{10}$ organisms/mouse), or (*b*) a combination of GIDMIF and GIDTNF (1:1 mixture, total of $1 \times 10^{10}$ organisms/mouse), or a combination of GIDMIF, GIDTNF, and GIDIFN (1:1:1 mixture, total $1 \times 10^{10}$ organisms/mouse). This administration was repeated on days 14 and 28 postinfection. Lesion development was measured at regular intervals as increase in footpad thickness. Data are mean ± SEM, $n = 6$, and are representative of two experiments. Experiments were terminated by day 42 when control mice (BRD509) developed necrotic lesions in the footpad in accordance with the U.K. guidelines for animal experimentation.

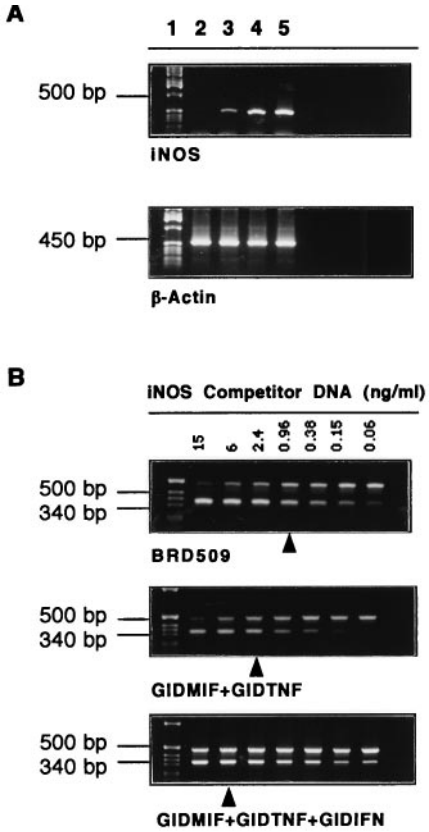


**FIGURE 5.** Competitive PCR of iNOS expression in spleens and MLN. Mice were given BRD509 orally ($1 \times 10^{10}$ organisms/mouse), or a combination of GIDMIF, GIDTNF, and GIDIFN as described in Figure 4. Spleen and MLN cells were harvested 14 days later and cDNA prepared as described in *Materials and Methods*. *A*, *Lane 1*, 1-Kb DNA ladder. *Lanes 2–5*, mice given PBS, BRD509, GIDMIF + GIDTNF, and GIDMIF + GIDTNF + GIDIFN, respectively. *B*, cDNA was then amplified in a PCR reaction in the presence of varying concentrations of an iNOS competitor. Arrows indicate equivalent points. Results are representative of 3 experiments.

shown), GIDMIF plus GIDTNF conferred the best therapeutic effect, and this was modestly but consistently enhanced by the addition of GIDIFN (Fig. 4*b* and Table I). The therapeutic effect waned and lesion began to increase 3 to 4 wk after the withdrawal of treatment (data not shown).

NO produced by macrophages activated with immunologic stimuli is a major effector molecule against *Leishmania* and other intracellular parasites (11–13, 26–29). We therefore investigated whether the therapeutic effect of the constructs was accompanied by an elevation of iNOS expression in vivo. Spleen and MLN cells from mice administered orally 2 wk previously with the various constructs were collected, and the expression of iNOS was detected by RT-PCR (Fig. 5*A*). The relative expression was determined by semiquantitative RT-PCR (Fig. 5*B*). Cells from mice administered with GIDMIF + GIDTNF or GIDMIF + GIDTNF + GIDIFN expressed markedly more iNOS message compared with those from control mice administered with PBS or BRD509 alone. These results suggest that the therapeutic effects of these constructs are likely to be associated with the induction of iNOS expression (13) and, hence, NO synthesis in the treated mice.

The essential role of GIDMIF in the therapeutic effect in the present experimental system is also consistent with the demonstrated functions of MIF as a macrophage activator (30) and mediator of delayed-type hypersensitivity (DTH) (31). Data presented here demonstrate that MIF synergizes with TNF-$\alpha$ in limiting the replication of *Leishmania* in vivo, most likely via the induction of NO synthesis. TNF-$\alpha$ and IFN-$\gamma$ are known to induce NO synthesis synergistically (32) with potent leishmanicidal activities (33, 34). It is of interest that the combined oral delivery of TNF-$\alpha$ with MIF conferred stronger therapeutic effect than that induced by

Table I. *Quantification of parasite loads in the infected footpad*[a]

| Treatment of Mice | Viable Parasite per Footpad ($Log_{10}$ Mean ± SEM) |
|---|---|
| PBS | 5.59 ± 0.2 |
| BRD509 | 5.90 ± 0.4 |
| GIDMIF | 4.89 ± 0.0 |
| GIDIFN | 3.19 ± 0.6* |
| GIDTNF | 4.89 ± 0.9 |
| GIDMIF + GIDTNF | 2.79 ± 0.0** |
| GIDMIF + GIDTNF + GIDIFN | 2.49 ± 0.4** |

[a] Groups of mice were infected with *L. major* and subsequently administered orally with the cytokine constructs as in Figure 4. Footpads were removed from the mice on day 42 after infection and tissues were homogenized. Serial dilutions of the homogenates were cultured in medium as described in *Materials and Methods*. Data show $Log_{10}$ ± SEM of the last dilution containing viable *L. major* from triplicate cultures, $n = 5$. Results are representative of three experiments.

* $p < 0.01$, ** $p < 0.001$ compared with control PBS group.

TNF-$\alpha$ with IFN-$\gamma$. It is unlikely that this is due to insufficient IFN-$\gamma$ being produced, since GIDIFN alone conferred the best prophylactic effect among the constructs tested (Fig. 3). It may be that the synergistic therapeutic effect of MIF and TNF-$\alpha$ involves mechanisms additional to NO.

In conclusion, we report here the therapeutic effect of attenuated *Salmonella* constructs expressing cytokines, particularly MIF, in an infectious disease model. The lack of toxicity of the attenuated *Salmonella* and the continuous but relatively short duration of cytokine delivery suggest that these and similar constructs may be useful as potential immunotherapeutic agents against clinical infectious and autoimmune diseases.

## References


1. Cantell, K., S. Hirvonen, L. Pyhala, A. de Reus, and H. Schellekens. 1983. Circulating interferon in rabbits and monkeys after administration of human gamma interferon by different routes. *J. Gen. Virol. 64:1823.*
2. Carrier, M. J., S. N. Chatfield, G. Dougan, U. T. A. Nowicka, D. O'Callaghan, J. E. Beesley, S. Milano, E. Cillari, and F. Y. Liew. 1992. Expression of human IL-1$\beta$ in *Salmonella typhimurium*: a model system for the delivery of recombinant therapeutic proteins in vivo. *J. Immunol. 148:1176.*
3. Dougan, G., C. E. Hormaeche, and D. J. Maskell. 1987. Live oral *Salmonella* vaccines: potential use of attenuated strains as carriers of heterologous antigens to the immune system. *Parasite Immunol. 9:151.*
4. Hormaeche, C. E. 1991. Live attenuated *Salmonella* vaccines and their potential as oral combined vaccines carrying heterologous antigens. *J. Immunol. Methods 142:113.*
5. Tacket, C. O., M. B. Sztein, G. A. Losonsky, S. S. Wasserman, J. P. Nataro, R. Edelman, D. Pickard, G. Dougan, S. N. Chatfield, and M. M. Levine. 1997. Safety of live oral *Salmonella typhi* vaccine strains with deletions in htrA and aroC aroD and immune response in humans. *Infect. Immun. 65:452.*
6. Liew, F. Y., and C. A. O'Donnell. 1993. Immunology of leishmaniasis. *Adv. Parasitol. 32:161.*
7. Reiner, S. L., and R. M. Locksley. 1995. The regulation of immunity to *Leishmania major*. *Annu. Rev. Immunol. 13:151.*
8. Scott, P., P. Natovitz, R. L. Coffman, E. Pearce, and A. Sher. 1988. Immunoregulation of cutaneous leishmaniasis: T cell lines that transfer protective immunity or exacerbation belong to different T helper subsets and respond to distinct parasite antigens. *J. Exp. Med. 168:1675.*
9. Heinzel, F. P., M. D. Sadick, B. J. Holaday, R. L. Coffman, and R. M. Locksley. 1989. Reciprocal expression of interferon-gamma or interleukin-4 during the resolution or progression of murine leishmaniasis: evidence for expansion of distinct helper T cell subsets. *J. Exp. Med. 169:59.*
10. Swihart, K., U. Fruth, N. Messmer, K. Hug, R. Behin, S. Huang, G. Del Giudice, M. Aguet, and J. A. Louis. 1995. Mice from a genetically resistant background lacking the interferon $\gamma$ receptor are susceptible to infection with *Leishmania major* but mount a polarised T helper cell 1-type $CD4^+$ T cell response. *J. Exp. Med. 181:961.*
11. Green, S. J., M. S. Meltzer, J. B. Hibbs, and C. A. Nacy. 1990. Activated macrophages destroy intracellular *Leishmania major* amastigotes by an L-arginine-dependent killing mechanism. *J. Immunol. 144:278.*
12. Liew, F. Y., S. Millott, C. Parkinson, R. M. J. Palmer, S. Moncada. 1990. Macrophage killing of *Leishmania* parasites in vivo is mediated by nitric oxide from L-arginine. *J. Immunol. 144:4794.*
13. Stenger, S., H. Thuring, M. Rollinghoff, and C. Bogdan. 1994. Tissue expression of inducible nitric oxide synthase is closely associated with resistance to *Leishmania major*. *J. Exp. Med. 180:783.*
14. Scott, P. 1991. IFN-$\gamma$ modulates the early development of Th1 and Th2 responses in a murine model of cutaneous leishmaniasis. *J. Immunol. 147:3149.*
15. Liew, F. Y., J. G. Howard, and C. Hale. 1984. Prophylactic immunization against experimental leishmaniasis. III. Protection against fatal *Leishmania tropica* infection induced by irradiated promastigotes involves Lyt-$1^+2^-$ T cells that do not mediate cutaneous DTH. *J. Immunol. 132:456.*
16. Chatfield, S. N., I. G. Charles, A. J. Makoff, M. D. Oxer, G. Dougan, D. Pickard, D. Slater, and N. F. Fairweather. 1992. Use of the *nirB* promoter to direct the stable expression of heterologous antigens in *Salmonella* oral vaccine strains: development of a single-dose oral tetanus vaccine. *Biotechnology 10:888.*
17. McSorley, S. J., D. Xu, and F. Y. Liew. 1997. The efficacy of *Salmonella* strains expressing gp63 using different promoters. *Infect. Immun. 65:171.*
18. Weiser, W. Y., P. A. Temple, J. S. Witek-Giannotti, H. G. Remold, C. Clark, and J. R. David. 1989. Molecular cloning of a cDNA encoding a human macrophage migration inhibitory factor. *Proc. Natl. Acad. Sci. USA 86:7522.*
19. Strugnell, R., G. Dougan, S. Chatfield, I. Charles, N. Fairweather, J. P. Tite, J. L. Li, J. Beesley, and M. Roberts. 1992. Characterization of a *Salmonella typhimurium* aro vaccine strain expressing the P.69 antigen of *Bordetella pertussis. Infect. Immun. 60:3994.*
20. Griffiths, G. 1993. *Fine Structure Immunocytochemistry*. Springer-Verlag, Heidelberg.
21. Tokuyasu, K. T. 1986. Application of cryoultramicrotomy to immunocytochemistry. *J. Microscopy 143:139.*
22. Clemens, M. J., A. G. Morris, and A. J. H. Gearing. 1987. *Lymphokines and Interferons: A Practical Approach*. IRL Press, Oxford.
23. Liew, F. Y., Y. Li, D. Moss, C. Parkinson, M. V. Rogers, and S. Moncada. 1991. Resistance to *Leishmania major* infection correlates with the induction of nitric oxide synthase in murine macrophages. *Eur. J. Immunol. 21:3009.*
24. Li, B., P. K. Sehajpal, A. Khanna, H. Vlassara, A. Cerami, K. H. Stenzel, and M. Suthanthiran. 1991. Differential regulation of transforming growth factor $\beta$ and interleukin 2 genes in human T cells: demonstration by usage of novel competitor DNA constructs in the quantitative polymerase chain reaction. *J. Exp. Med. 174:1259.*
25. Denich, K., P. Borlin, P. D. O'Hanley, M. Howard, and A. W. Heath. 1993. Expression of the murine interleukin-4 gene in an attenuated aroA strain of *Salmonella typhimurium*: persistence and immune response in BALB/c mice and susceptibility to macrophage killing. *Infect. Immun. 61:4818.*
26. Nathan, C. F., and J. B. Hibbs, Jr. 1991. Role of nitric oxide synthesis in macrophage antimicrobial activity. *Curr. Opin. Immunol. 3:65.*
27. Green, S. J., and C. A. Nacy. 1993. Antimicrobial and immuopathologic effects of cytokine-induced nitric oxide synthesis. *Curr. Opin. Infect. Dis. 6:384.*
28. Wei, X., I. G. Charles, A. Smith, J. Ure, G. Feng, F. Huang, D. Xu, W. Muller, S. Moncada, and F. Y. Liew. 1995. Altered immune responses in mice lacking inducible nitric oxide synthase. *Nature 375:408.*
29. Vouldoukis, I., V. Riveros-Moreno, B. Dugas, F. Quaaz, P. Becherel, P. Debre, S. Moncada, and M. D. Mossalayi. 1995. The killing of *Leishmania major* by human macrophages is mediated by nitric oxide induced after ligation of the FcεRII/CD23 surface antigen. *Proc. Natl. Acad. Sci. USA 92:7804.*
30. Calandra, T., J. Bernhagen, R. A Mitchell, and R. Bucala. 1994. The macrophage is an important and previously unrecognised source of macrophage migration inhibitory factor. *J. Exp. Med. 179:1895.*
31. Bernhagen, J., M. Bacher, T. Calandra, C. N. Metz, S. B. Doty, T. Donnelly, and R. Bucala. 1996. An essential role for macrophage migration inhibitory factor in the tuberculin delayed-type hypersensitivity reaction. *J. Exp. Med. 183:277.*
32. Ding, A. H., C. F. Nathan, and D. J. Stuehr. 1988. Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritoneal macrophages: comparison of activating cytokines and evidence for independent production. *J. Immunol. 141:2407.*
33. Bogdan, C., H. Moll, W. Solbach, and M. Rollinghoff. 1990. Tumor necrosis factor-$\alpha$ in combination with interferon-gamma, but not with interleukin-4 activates murine macrophages for elimination of *Leishmania major* amastigotes. *Eur. J. Immunol. 20:1131.*
34. Liew, F. Y., Y. Li, S. Millott. 1990. Tumor necrosis factor alpha synergizes with IFN-$\gamma$ in mediating killing of *Leishmania major* through the induction of nitric oxide. *J. Immunol. 145:4306.*