

## Pretreatment of Macrophages with the Combination of IFN- $\gamma$ and IL-12 Induces Resistance to *Leishmania major* at the Early Phase of Infection

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**ABSTRACT.** Interferon (IFN)- $\gamma$  is essential but not sufficient to control leishmaniasis. It is known that IFN- $\gamma$  is one of the major macrophage-activating cytokines, and the activated macrophages are a principal source of interleukin (IL)-12, which induces autocrine macrophage activation. In this study, the combined effect of IFN- $\gamma$  and IL-12 on the susceptibility of macrophages to *Leishmania major* infection was evaluated. Macrophages pretreated with IFN- $\gamma$  and/or IL-12 were infected with the parasites. Four hr post-infection (p.i.), the levels of infection and parasite load in the macrophages treated with the combination of IFN- $\gamma$  and IL-12 (IFN- $\gamma$ /IL-12) were significantly lower than those in the nontreated cells. However, the macrophages treated with either IFN- $\gamma$  or IL-12 did not show resistance to *L. major* infection. In addition, 72 hr p.i., the IFN- $\gamma$ /IL-12-treated and IFN- $\gamma$ -treated macrophages showed significantly lower levels of infection and parasite load than the nontreated cells, and higher levels of resistance was observed in the IFN- $\gamma$ /IL-12-treated macrophages than in the IFN- $\gamma$ -treated macrophages. Although IFN- $\gamma$ /IL-12 treatment of macrophages prior to the infection led to the induction of resistance, as described above, this resistance was not induced when these cytokines and the parasites were added simultaneously to the macrophage culture. These results suggest that IFN- $\gamma$ /IL-12 treatment prior to the infection restricts the early phase of the infection.

**KEY WORDS:** interferon- $\gamma$ , interleukin-12, *Leishmania*, macrophage.

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Studies on *Leishmania* as well as *Toxoplasma* and *Mycobacterium* have been largely responsible for the characterization of the Th1/Th2 paradigm of healing/nonhealing associated with intracellular infection [3, 4, 17]. Interferon (IFN)- $\gamma$  was identified as a cytokine required for not only Th1 cell differentiation [18] but also macrophage activation to suppress the activity of *Leishmania* infecting the macrophages [5]. In an experimental murine leishmaniasis model, it was reported that the susceptibility of mice to *Leishmania* infection is due to the lack of Th1 responses related to IFN- $\gamma$  production and the development of interleukin (IL)-4-dominated Th2 responses [6]. However, despite the importance of IFN- $\gamma$  in the development of resistance to *Leishmania* infection, it was reported that the administration of exogenous IFN- $\gamma$  does not protect susceptible BALB/c mice from *L. major* infection [15]. In addition, IFN- $\gamma$  mRNA was detected in the infected skin lesions of not only resistant C57BL/6 mice but also susceptible BALB/c mice [21]. These reports suggest that IFN- $\gamma$  alone is not sufficient to control *L. major* and that additional factor(s) would be required for the development of protective immunity to *L. major*. IL-12 is known as a cytokine involved in Th1 cell differentiation, and it stimulates NK cells to produce IFN- $\gamma$  [8, 19, 20]. Moreover, IL-12 induces autocrine macrophage

activation and consequent IL-12 and IFN- $\gamma$  production from the activated macrophages [10, 12]. Previously, it was reported that continuous treatment of *L. major*-infected mice with IL-12 suppressed disease progression [7], and that IL-12-deficient mice were more susceptible to *L. major* infection than wild-type control mice [11]. These results indicate the importance of IL-12 in the development of resistance to *L. major* infection.

*Leishmania* infects phagocytic cells such as macrophages and dendritic cells in the mammalian host [2]. IFN- $\gamma$  is one of the principle macrophage-activating cytokines, and the activated macrophages are one of the principal sources of IL-12 [14]. Therefore, it is expected that IFN- $\gamma$ /IL-12 are directly related to the behavior of *Leishmania* in infected macrophages.

In this study, we focused on the effect of IFN- $\gamma$  and IL-12 on *L. major* infection in macrophages and demonstrated that these cytokines restrict the *L. major* infection during the early phase, i.e., before inception of parasite proliferation.

### MATERIALS AND METHODS

**Mice:** Six-week-old female BALB/c mice were obtained from Japan SLC, Inc. (Shizuoka, Japan). Animal experiments were carried out under the strict guidance of the Animal Research Committee in accordance with the Guidelines on Animal Experiments at The University of Tokyo.

**Parasites:** The *L. major* PM2 strain (MHOM/UZ/91/PM2) was kindly provided by Dr. Margarita Strelkova

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(Martsinovskiy Institute of Medical Parasitology and Tropical Medicine, Moscow, Russia). Promastigotes were cultured in 199 medium (Nissui Pharmaceutical Co., Ltd, Tokyo, Japan) containing 10% heat-inactivated fetal calf serum (FCS; Thermo Electron Corp., Melbourne, Australia) and 20 mM HEPES (MP Biomedicals Inc., Aurora, OH) at 25°C. Infectious-stage metacyclic promastigotes were used for this study.

**Macrophages:** Resident mouse peritoneal macrophages were harvested from the peritoneal cavity by lavage with 5 ml of ice-cold phosphate-buffered saline (PBS). For each experiment, cells isolated from three or four mice were pooled. Macrophages were centrifuged and resuspended in ice-cold PBS. After additional 2 washes with ice-cold PBS, the cells were suspended at a density of  $2.5 \times 10^5$ /ml in RPMI 1640 medium (Sigma) containing 10% heat-inactivated FCS and 20  $\mu$ g/ml gentamicin (Schering-Plough, Kenilworth, NJ). The cells were then cultured on a 16-well chamber slide (Nalge Nunc, Rochester, NY) at 37°C with 5% CO<sub>2</sub> in air. After incubation for 2 hr, the slides were washed three times in the medium to remove the unattached cells, and the remaining (attached) cells were used as the macrophage monolayer.

**In vitro infection:** Prior to inducing the infection, the macrophage monolayer, prepared as described above, was cultured for 15 hr in the presence of 1 ng/ml recombinant IFN- $\gamma$  (rIFN- $\gamma$ ) (BD Bioscience, San Diego, CA) and/or 1 ng/ml rIL-12 (R&D systems, Minneapolis, MN) at 37°C with 5% CO<sub>2</sub> in air. *L. major* promastigotes were suspended at a concentration of  $5 \times 10^6$  cells/ml in the media containing the same cytokines used for the pretreatment of the macrophage monolayer. The suspension of *L. major* promastigotes was then added to the macrophage monolayer in each well at a ratio of five parasites per macrophage. Four hr postinfection (p.i.), the cultures were washed to remove the unbound parasites, and fresh media containing the same cytokines that were used during the preculture and infection procedures were added into each well. As a control condition, macrophages were cultured and infected with promastigotes by following the process described above except that the cells were cultured in a cytokine-deficient medium for all the procedures. Four or 72 hr p.i., the wells were washed with PBS, and the cells were fixed with 100% methanol and stained with Giemsa solution. Using light microscopy, 300 macrophages per well were observed, and the number of infected macrophages and the number of intracellular parasites were counted. To examine the effect of cytokines added to the culture after the parasitic infection, the harvested macrophages were incubated for 15 hr in a cytokine-deficient medium and infected with only promastigotes, using the above-described procedure. After washing out the unbound parasites, fresh media with or without 1 ng/ml rIFN- $\gamma$  and/or 1 ng/ml rIL-12 were added to each well. After 4 or 72 hr p.i., the number of infected macrophages and the number of intracellular parasites were counted as described above. The following three evaluation criteria were examined in the present study: (1) infection rate, calcu-

lated as the percentage of infected macrophages to all macrophages; (2) the number of parasites per 100 macrophages; and (3) parasite load, which is the number of parasites per infected macrophage. The results are representative of more than three separate experiments.

**Statistical analysis:** Data are expressed as mean  $\pm$  S.D. One-way ANOVA with post hoc analysis with the Turkey-Kramer test was used to compare groups.

## RESULTS

**Effect of IFN- $\gamma$  and IL-12 on the susceptibility of macrophages to *L. major* infection:** To investigate the effect of IFN- $\gamma$  and IL-12 on the susceptibility of macrophages to *L. major* infection, peritoneal macrophages pretreated with IFN- $\gamma$  and/or IL-12 were infected with the parasites.

Four hr p.i., the levels of infection rate and the number of parasites per 100 macrophages in the macrophages treated with IFN- $\gamma$ /IL-12 were significantly lower than those in the nontreated cells (Fig. 1A and 1B). The number of macrophages infected with more than one parasite was lower in the treated macrophages than in the nontreated cells (Fig. 1C). However, when the macrophages were treated with either IFN- $\gamma$  or IL-12, the cells did not exhibit resistance to the infection (Fig. 1).

At the late phase of infection, i.e., 72 hr p.i., the IFN- $\gamma$ /IL-

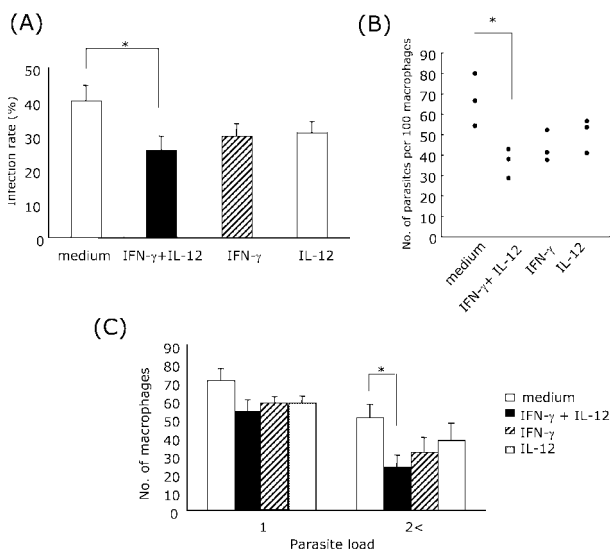


Fig. 1. Induction of resistance to *L. major* at the early phase of infection in cytokine-treated macrophages. The effect of cytokine to induce resistance to *L. major* in macrophages was determined by the infection rate (A), the number of parasites per 100 macrophages (B), and the parasite load (C). Prior to the infection, the macrophages were cultured in the cytokine-deficient medium (open), medium containing IFN- $\gamma$ /IL-12 (1 ng/ml each, filled), 1 ng/ml IFN- $\gamma$  (hatched), or 1 ng/ml IL-12 (grey), as described in the Materials and Methods. Four hr after the addition of the parasites, the numbers of infected macrophages and intracellular parasites were evaluated. All data are presented as mean  $\pm$  S.D. \*  $p < 0.05$ .

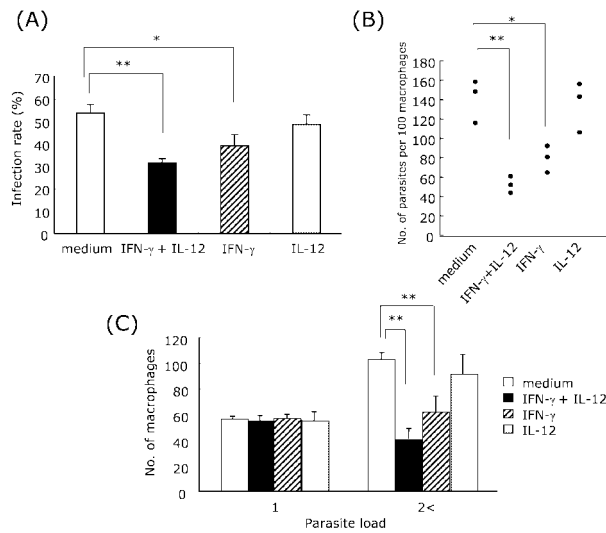


Fig. 2. Induction of resistance to *L. major* at the late phase of infection by cytokine treatment of macrophages. The effect of cytokines to induce resistance to *L. major* in macrophages was determined by the infection rate (A), the number of parasites per 100 macrophages (B), and the parasite load (C). Prior to the infection, the macrophages were cultured in the cytokine-deficient medium (open), medium containing IFN- $\gamma$ /IL-12 (1 ng/ml each, filled), 1 ng/ml IFN- $\gamma$  (hatched), or 1 ng/ml IL-12 (grey), as described in the Materials and Methods. Seventy-two hr after the infection, the numbers of infected macrophages and intracellular parasites were evaluated. All data are presented as mean  $\pm$  S.D. \*  $p < 0.05$ , \*\*  $P < 0.01$ .

IL-12-treated macrophages showed significantly lower *L. major* infection rates and numbers of parasites per 100 macrophages than the nontreated control cells (Fig. 2A and 2B). The number of macrophages infected with more than 1 parasite was also lower in the treated cells than in the nontreated cells. Consistent with a previous report [5], the IFN- $\gamma$ -treated macrophages also showed considerably lower infection levels and parasite load than the nontreated cells; however, the resistance was marked in the IFN- $\gamma$ /IL-12-treated macrophages (Fig. 2). The macrophages treated with IL-12 alone did not display any resistance to the parasite infection (Fig. 2). These results suggest that in the macrophages, IFN- $\gamma$ /IL-12 induced the resistance to *L. major* infection at the early phase of infection.

*The optimum time for IFN- $\gamma$ /IL-12 treatment of macrophages for the control of L. major infection:* The fact that IFN- $\gamma$ /IL-12 treatment of the macrophages induced resistance to *L. major* infection at the early phase of infection (Figs. 1 and 2) prompted us to examine whether treatment with these cytokines prior to infection induces resistance to the infection. As shown in Fig. 3, the treatment of macrophages with IFN- $\gamma$ /IL-12 prior to infection significantly reduced the infection rate and the number of parasites per 100 macrophages 4 hr p.i. (Fig. 3A and 3B). On the other hand, when the treatment was commenced immediately after the addition of parasites, induction of the resistance to *L. major* infection

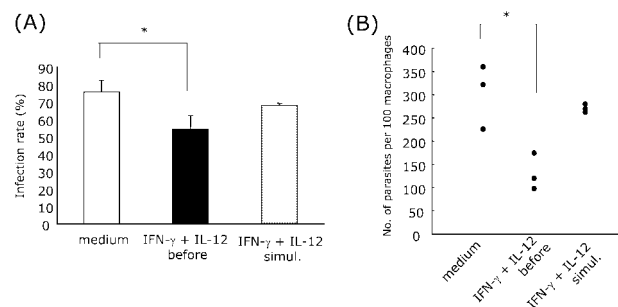


Fig. 3. Inhibition of *L. major* infection at the early phase of infection by IFN- $\gamma$ /IL-12 treatment prior to the infection. The effect of IFN- $\gamma$ /IL-12 to induce resistance to *L. major* in macrophages was determined by the infection rate (A) and the number of parasites per 100 macrophages (B). The macrophages were treated with IFN- $\gamma$ /IL-12 prior to the infection (1 ng/ml, each; filled) or simultaneously with the addition of the parasites (grey), as described in the Materials and Methods. As a control condition, the macrophages were cultured in the cytokine-deficient medium (open) and infected with the parasites. Four hr after the infection, the numbers of infected macrophages and intracellular parasites were evaluated. All data are presented as mean  $\pm$  S.D. \*  $p < 0.05$ .

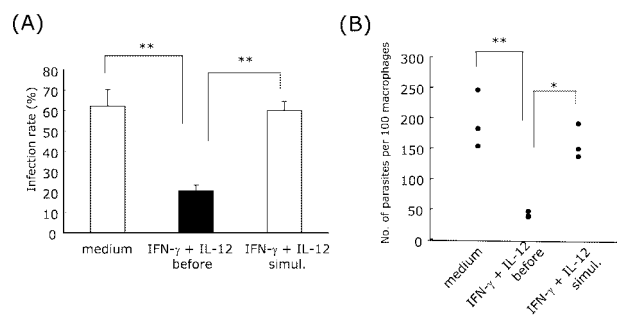


Fig. 4. Inhibition of *L. major* infection at the late phase of infection by IFN- $\gamma$ /IL-12 treatment prior to the infection. The effect of IFN- $\gamma$ /IL-12 to induce resistance to *L. major* in macrophages was determined by the infection rate (A) and the number of parasites per 100 macrophages (B). The macrophages were treated with IFN- $\gamma$ /IL-12 prior to the infection (1 ng/ml, each; filled) or simultaneously with the addition of the parasites (grey), as described in the Materials and Methods. As a control condition, the macrophages were cultured in a cytokine-deficient medium (open) and infected with the parasites. Seventy-two hr after the infection, the numbers of infected macrophages and intracellular parasites were evaluated. All data are presented as mean  $\pm$  S.D. \*  $p < 0.05$ , \*\*  $P < 0.01$ .

was not observed (Fig. 3). There was no reduction in the infection rate and the number of intracellular parasites at 72 hr p.i. when the macrophages were treated with IFN- $\gamma$ /IL-12 and the parasite simultaneously (Fig. 4).

DISCUSSION

In this study, we investigated the effect of IFN- $\gamma$  and IL-12 treatment of the macrophages on the resistance to *L. major* infection. Four hr p.i., statistically significant reduc-

tion in both infection rate and parasite load was observed when the macrophages were treated with IFN- $\gamma$ /IL-12 prior to the infection (Figs. 1 and 3). The importance of pretreatment with IFN- $\gamma$ /IL-12 in reducing the intracellular parasites suggests that the effect is mediated by the induction of the production of several proteins and/or modulation of their expression levels. Green *et al.* demonstrated that nitrogen oxide (NO) produced by the action of inducible NO synthetase (iNOS), which is upregulated by IFN- $\gamma$  treatment to macrophages, functions to reduce the number of infected *L. major* 72 hr p.i. [5]. It is thus possible that NO also plays an important role in reducing the parasite number during an infection. However, there was no difference in the NO levels between the IFN- $\gamma$ /IL-12-treated and nontreated macrophages 4 hr p.i. (unpublished observation). Based on this and other observations, it is suggested that IFN- $\gamma$ /IL-12 treatment of the macrophages prior to the infection induced resistance to *L. major* infection at the early phase in an NO-independent manner.

The proliferation of *L. major* in the infected macrophages might have not initiated until 4 hr p.i. Thus, IFN- $\gamma$ /IL-12 probably induces some inhibition of parasite binding to host macrophages or alteration in invasion as the resistant mechanisms. It has been reported that several cytokines, including IFN- $\gamma$ , regulate the expression levels of macrophage surface receptor molecules such as mannose receptor [9] and complement receptor 3 [16], which function as macrophage receptors for *Leishmania* [1, 12]. It is possible that IFN- $\gamma$ /IL-12 also regulates the expressions of these (or other) receptor molecules on macrophages. Moreover, IFN- $\gamma$ /IL-12 treatment to macrophages prior to the infection may affect their intracellular functions such as establishment of parasitophorous vacuole or fusion of the vacuole and lysosome. The results of this study indicate the need and importance of a further study on the possible IFN- $\gamma$ /IL-12-induced regulation underlying the binding or invasion of *Leishmania* to macrophages.

Treatment with IFN- $\gamma$ /IL-12 induced considerably higher levels of resistance in the macrophages than the treatment with IFN- $\gamma$  alone (Figs. 2 and 4). Interestingly, when the cells were treated with the cytokines prior to the infection, the IFN- $\gamma$ /IL-12-treated macrophages produced greater levels of NO than IFN- $\gamma$ -treated macrophages 72 hr p.i. (unpublished observation). It is certain that NO plays an important role in controlling the intracellular parasite. However, the results indicated the involvement of additional mechanisms in inducing resistance to *L. major* infection in macrophages.

In the animals vaccinated against or resistant to *Leishmania* infection, tissue macrophages around the infected cells might be exposed to IFN- $\gamma$  and IL-12 secreted from the activated macrophages and probably acquire resistance to parasite binding or invasion at the site of infection. Further, we believe that the parasites that could not infect the macrophages are killed by the complement-mediated pathway, and that resistance to parasite binding or invasion is one of the important defense mechanisms.

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