

# Mice from a Genetically Resistant Background Lacking the Interferon $\gamma$ Receptor are Susceptible to Infection with *Leishmania major* but Mount a Polarized T Helper Cell 1-type CD4<sup>+</sup> T Cell Response

By Kristin Swihart,\* Ulrich Fruth,\* Nathalie Messmer,\* Katharina Hug,\* Reza Behin,\* Sui Huang,† Giuseppe Del Giudice,\* Michel Aguet,† and Jacques A. Louis\*

From the \*World Health Organization Immunology Research and Training Center, Institute of Biochemistry, University of Lausanne, 1066 Epalinges; and the †Institute of Molecular Biology I, University of Zurich, 8093 Zurich, Switzerland

## Summary

Mice with homologous disruption of the gene coding for the ligand-binding chain of the interferon (IFN)  $\gamma$  receptor and derived from a strain genetically resistant to infection with *Leishmania major* have been used to study further the role of this cytokine in the differentiation of functional CD4<sup>+</sup> T cell subsets in vivo and resistance to infection. Wild-type 129/Sv/Ev mice are resistant to infection with this parasite, developing only small lesions, which resolve spontaneously within 6 wk. In contrast, mice lacking the IFN- $\gamma$  receptor develop large, progressing lesions. After infection, lymph nodes (LN) and spleens from both wild-type and knockout mice showed an expansion of CD4<sup>+</sup> cells producing IFN- $\gamma$  as revealed by measuring IFN- $\gamma$  in supernatants of specifically stimulated CD4<sup>+</sup> T cells, by enumerating IFN- $\gamma$ -producing T cells, and by Northern blot analysis of IFN- $\gamma$  transcripts. No biologically active interleukin (IL) 4 was detected in supernatants of in vitro-stimulated LN or spleen cells from infected wild-type or deficient mice. Reverse transcription polymerase chain reaction analysis with primers specific for IL-4 showed similar IL-4 message levels in LN from both types of mice. The IL-4 message levels observed were comparable to those found in similarly infected C57BL/6 mice and significantly lower than the levels found in BALB/c mice. Anti-IFN- $\gamma$  treatment of both types of mice failed to alter the pattern of cytokines produced after infection. These data show that even in the absence of IFN- $\gamma$  receptors, T helper cell (Th) 1-type responses still develop in genetically resistant mice with no evidence for the expansion of Th2 cells.

Infection of mice with the protozoan parasite *Leishmania major* has been used as a model system for the study of the effector functions of IFN- $\gamma$  and other lymphokines against intracellular microorganisms. Several findings have already clearly demonstrated the importance of IFN- $\gamma$  for the elimination of *L. major* by infected hosts. Results from several laboratories have documented that, when treated with anti-IFN- $\gamma$  neutralizing antibodies, mice normally resistant to infection with *L. major* are not able to control this infection (1, 2). In addition, the destruction of intracellular leishmania by murine macrophages was shown to result from the IFN- $\gamma$ -induced production of nitric oxide (NO) by these cells (3, 4).

After infection of mice from various inbred strains with *L. major*, the parasite burden and the resulting pattern of disease correlates with the expansion of CD4<sup>+</sup> T cells from a particular functional subset. Resistance to infection with

*L. major*, which characterizes mice from several inbred strains (CBA, C57BL/6, etc.) has been shown to correlate with the selective triggering of a Th1 CD4<sup>+</sup> T cell response and results at least in part from the activity of these cells. In contrast, the susceptibility of BALB/c mice results from the preferential expansion and activity of Th2-type T cells (5, 6).

Thus, the mouse model of infection with *L. major* has been instrumental in the elucidation of some of the mechanisms controlling the development of polarized Th1 versus Th2 responses during antigenic stimulation. In this context, studies have already underlined the importance that cytokines, present at the early stage of infection with *L. major*, could have on the differentiation of CD4<sup>+</sup> T cell precursors toward one or the other functional phenotype. Administration of anti-IFN- $\gamma$  mAb to genetically resistant mice was shown to interfere with the development of a Th1-type response and to promote the

differentiation of a Th2-type response (7). Similarly, treatment of susceptible BALB/c mice with anti-IL-4 mAbs at an early stage of infection hampers the expansion of Th2 cells, allowing the development of a Th1-type response (8). Administration of exogenous IFN- $\gamma$  at the initiation of infection transiently reversed the Th2-type response normally displayed by T lymphocytes from susceptible mice (7). Similarly, the injection of recombinant IL-4 to resistant mice at the time of infection led to the transient development of a Th2-type response (9). Taken together, these data indicate that IFN- $\gamma$  and IL-4 play a pivotal role in the development of polarized Th1 and Th2 responses, respectively, in this model of infection. The role of IL-4 in the generation of a Th2-type response is also supported by several observations made both in vitro and in vivo in mitogen- or nominal antigen-driven responses (10–13). However, the data concerning the importance of IFN- $\gamma$  in promoting the expansion of Th1 cells and inhibiting the development of Th2-type response are still conflicting (10, 12–15).

Recently, transgenic mice that lack the IFN- $\gamma$ R have been produced, offering the possibility to elucidate further the effector and the regulatory functions of IFN- $\gamma$  (16). In this study, we investigated the course of disease and the functional characteristics of the CD4<sup>+</sup> T cell responses triggered after infection with *L. major* of mice from a genetically resistant background lacking the IFN- $\gamma$ R.

## Materials and Methods

**Mice.** 129/Sv/Ev mice of either sex homozygous for disrupted IFN- $\gamma$ R gene (IFN- $\gamma$ R<sup>-/-</sup>) and for null mutation (IFN- $\gamma$ R<sup>+/+</sup>) were produced as described (16) and maintained under pathogen-free conditions until use at 6–10 wk of age. BALB/c and C57BL/6 mice of the same age (IFFA Credo, Saint-Germain-sur-l'Abresle, France) were used in some experiments.

**Parasites and Infection of Mice.** *L. major* LV 39 (MRHO/SU/59/P-strain) were maintained in vivo and grown in vitro as described (17). Mice were infected subcutaneously into one hind foot pad with 2 × 10<sup>6</sup> *L. major* stationary-phase promastigotes in a final volume of 50  $\mu$ l. The development of lesions was monitored by measuring, with a vernier caliper, the increase in foot pad thickness compared with the uninfected contralateral foot pad. At selected times, the number of viable parasites in infected tissues was also measured by use of a parasite-limiting dilution assay previously described (18). In some experiments, mice were treated intraperitoneally with 2 mg XMG 1.2 anti-IFN- $\gamma$  mAb (19) 2 d before infection and with 320  $\mu$ g of mAb weekly for 3 wk after infection.

**Lymphocyte Cultures.** LN and spleen cell suspensions were stimulated, at the indicated cell numbers, with 4 × 10<sup>6</sup> UV-irradiated *L. major* promastigotes in a final volume of 1 ml at 37°C and an atmosphere of 7% CO<sub>2</sub>. Cells were cultured in DMEM supplemented with 5% heat-inactivated FCS, L-glutamine (216 mg/ml), 5 × 10<sup>-5</sup> M 2-ME, and 10 mM Hepes. Culture supernatants were collected after 18–72 h of stimulation and stored at -20°C until use. Similar experiments were also carried out with CD4<sup>+</sup> T cell-depleted populations. Depletion of CD4<sup>+</sup> cells was accomplished by treatment of 5 × 10<sup>7</sup> cells with an anti-CD4 IgM mAb (RL-172.4 [20], a gift of H. R. MacDonald, Ludwig Institute for Cancer Research, Epalinges, Switzerland) and rabbit complement (Low-Tox; Cederlane, Hornby, Canada).

**Detection of IFN- $\gamma$  and IL-4 in Culture Supernatant.** IFN- $\gamma$  was measured by ELISA as previously described (21). IL-4 was measured by a bioassay using the CT.4S cell line as described (22). Recombinant mouse IFN- $\gamma$  expressed in L1210 cells (gift of Y. Watanabe, Kyoto University, Kyoto, Japan) and recombinant mouse IL-4 expressed in X63Ag-653 cells (gift of F. Melchers, Basel Institute of Immunology, Basel, Switzerland) were used as standards. The limit of detection of these assays was 10 U/ml for IFN- $\gamma$  and 0.3 ng/ml for IL-4.

**Enumeration of IFN- $\gamma$ -producing Cells.** Cells producing IFN- $\gamma$  were enumerated by use of a modification of an ELISPOT assay previously reported (23). Briefly, 96-well flat-bottom microtiter plates (Nunc, Roskilde, Denmark) were coated with purified anti-IFN- $\gamma$  mAb R4-6A2 (24) (5  $\mu$ g/ml) and incubated at 4°C for 14–16 h. Free binding sites were blocked with PBS containing 1% BSA for 2 h at 37°C. Wells were washed three times with sterile PBS containing 0.5% Tween-20. Serial twofold dilutions of lymphocyte suspensions (100  $\mu$ l/well) from spleen or LN cell cultures that had been stimulated in vitro with *L. major* for 18 h were then added and incubated for 4 h. After three washes, biotinylated anti-IFN- $\gamma$  mAb AN 18.17.24 (gift of S. Landolfo, University of Turin, Turin, Italy) diluted in PBS containing 1% BSA, was added (2.5  $\mu$ g/ml). After 14 h of incubation at 4°C, plates were washed three times before the addition of alkaline phosphatase–streptavidin conjugate (diluted 1:1,000 in PBS) and further incubated for 2 h at 37°C before washing and the addition of substrate. The substrate, consisting of 5-bromo-4-chloro-3-indolyl phosphate (BCIP), diluted in 2-amino-2-methyl-1-propanol (AMP) (both from Sigma, Buchs, Switzerland) buffer, was incubated at 37°C for 45 min before addition to wells. After 2 h of incubation with the substrate, the blue spots were counted by use of an inverted microscope.

**Treatment of LN Cells with Recombinant IFN- $\gamma$  and Flow Cytometry.** LN cells from IFN- $\gamma$ R<sup>-/-</sup> and IFN- $\gamma$ R<sup>+/+</sup> mice were cultured in vitro (4 × 10<sup>6</sup> cells/ml) in the presence or absence of 50–1,000 U/ml recombinant mouse IFN- $\gamma$  (Genentech, South San Francisco, CA). After 24, 48, or 72 h, cells were harvested, washed with PBS containing 5% FCS, and assayed by flow cytometry for the expression of the IFN- $\gamma$ -inducible Ly6A/E surface marker (25). Staining was performed with 0.25  $\mu$ g FITC-conjugated rat IgG2a anti-Ly6A/E mAb D7 (26) (Pharmingen, Lugano, Switzerland) in the presence of 1  $\mu$ g rat IgG2b anti-mouse Fc $\gamma$ RII mAb 24G2 (kind gift of Dr. H. R. MacDonald) to inhibit nonspecific binding of Abs to the Fc $\gamma$ RII. Analysis of stained cells was performed by use of a FACScan<sup>®</sup> (Becton Dickinson & Co., Mountain View, CA).

**Northern Blot Analysis.** Total RNA was isolated from spleen and LN cell populations depleted or not of CD4<sup>+</sup> cells. After washing in PBS, cells were resuspended in guanidine thiocyanate denaturing solution (4 M GCN, 0.75 M sodium citrate, 0.5% sarkosyl, 0.1 M  $\beta$ -ME). The mixture was acidified with one-tenth volume 2 M sodium acetate, pH 4.5, and extracted with phenol-chloroform. After precipitation with one-tenth vol 7.5 M NH<sub>4</sub> acetate and 2 vol ethanol, the RNA was resuspended in water. 5  $\mu$ g total RNA was denatured at 65°C in sample buffer (50% formamide, 0.66 M formaldehyde, 0.02 M 3-[N-morpholino] propane-sulfonic acid [MOPS], 5 mM sodium acetate, 1 mM EDTA, 0.5% glycerol). 1  $\mu$ l of 1 mg/ml ethidium bromide was added to each sample before electrophoresis through a 1.1% agarose gel containing 0.66 M formaldehyde, 0.02 M MOPS, 5 mM sodium acetate, and 1 mM EDTA. RNA was transferred to a membrane (Hybond; Amersham, Bucks, UK) by capillary action, and RNA was fixed to the membrane by use of a UV Stratilinker (Stratagene Inc., La Jolla, CA). Filters were prehybridized in 50% formamide, 5 $\times$  SSC, 2 $\times$  Denhardt's solution (0.04% Ficoll 400, 0.04% polyvinyl pyr-

rolidone K30, 0.04% BSA), 1 mM EDTA, 0.2% SDS, 0.25 mg/ml salmon sperm DNA, and 0.5 mg/ml yeast tRNA at 65°C for 4 h before addition of the probe. Antisense RNA probes specific for IFN- $\gamma$  or IL-4 (27, 28) were generated by use of SP6 RNA polymerase. Transcripts were treated with DNase and unincorporated nucleotides removed by passage over a TE (10 mM Tris, 1 mM EDTA)-equilibrated Sephadex G-75 column (Pharmacia, Uppsala, Sweden). After overnight hybridization at 65°C, filters were washed four times for 30 min with 0.1 $\times$  SSC, 0.1% SDS, at 65°C.

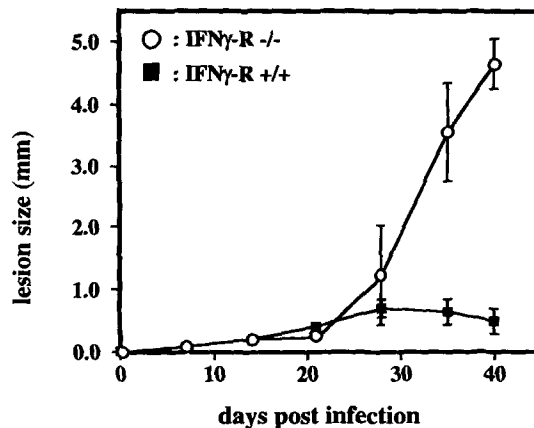
**PCR Analysis.** First-strand cDNA synthesis was performed on 1  $\mu$ g total RNA by use of a first-strand cDNA kit (Pharmacia). PCR was performed directly on the first-strand cDNA by use of Taq DNA polymerase (Boehringer Mannheim, Mannheim, Germany) according to the manufacturers specifications with IL-4- and GAPDH-specific primers described previously (29). The level of IL-4 message was normalized between samples with the level of GAPDH message used as a control.

## Results

**Development of Lesions Induced by *L. major* in IFN- $\gamma$   $^{-/-}$  and IFN- $\gamma$   $^{+/+}$  Mice.** IFN- $\gamma$   $^{-/-}$  mice and syngeneic control mice (IFN- $\gamma$   $^{+/+}$ ) were infected with *L. major* subcutaneously in one hind footpad, and the development of lesions was monitored for 40 d. In Fig. 1, representative results from one of three experiments show that control IFN- $\gamma$   $^{+/+}$  mice were resistant to infection since only small lesions developed and then resolved spontaneously within 40 d. In contrast, the IFN- $\gamma$   $^{-/-}$  mice developed large lesions, which were still progressing at the time of killing. Estimation by a limiting dilution assay of the numbers of parasites in lesions 40 d after infection showed a 15-fold increase in the number of *L. major* in foot pads of IFN- $\gamma$   $^{-/-}$  mice. Additionally, LN draining the site of lesions and spleens of IFN- $\gamma$   $^{-/-}$  mice contained significantly higher numbers of parasites compared with IFN- $\gamma$   $^{+/+}$  mice (Table 1). It is noteworthy that for the first 20 d of infection, the numbers of parasites found in lesions and lymphoid organs were similar in IFN- $\gamma$   $^{+/+}$  and IFN- $\gamma$   $^{-/-}$  mice (data not shown). These results confirm the crucial role of IFN- $\gamma$  in the elimination of *L. major* by infected hosts.

**Functional Characteristics of the CD4<sup>+</sup> T Cells Triggered in IFN- $\gamma$   $^{-/-}$  Mice Infected with *L. major*.** The susceptibility of BALB/c mice to infection with *L. major* has clearly been related to the expansion of Th2-type cells, whereas resistant mice developed a Th1-type response (5). Since IFN- $\gamma$  has been shown to inhibit the proliferation of Th2 cells and favor the differentiation of T cell precursors toward a Th1 phenotype (30), we assessed whether *L. major* infection of IFN- $\gamma$   $^{-/-}$  mice, derived from a strain resistant to infection with this parasite, would lead to a polarized Th2 response.

To this end, the production of IFN- $\gamma$  and IL-4 by spleen and LN cells obtained from IFN- $\gamma$   $^{-/-}$  and IFN- $\gamma$   $^{+/+}$  mice at various times after infection with *L. major* was measured after stimulation in vitro with UV-irradiated parasites as a source of specific antigen. Results from a representative experiment show that, during the first 20 d of infection, the production of IFN- $\gamma$  by specifically stimulated LN and spleen cells was comparable in both IFN- $\gamma$   $^{-/-}$  and IFN- $\gamma$   $^{+/+}$



**Figure 1.** Course of *L. major* infection in IFN- $\gamma$   $^{-/-}$  and IFN- $\gamma$   $^{+/+}$  mice. Mice (eight per group) were infected by injecting  $3 \times 10^6$  stationary-phase parasites subcutaneously into the left hind foot pad. Foot pad swelling was monitored by use of a caliper, and lesion size was determined by subtracting the thickness of the uninfected foot pad from the thickness of the infected foot pad. The mean and SEM are shown from one of three experiments that gave similar results.

mice (Table 2). On day 40, significantly larger amounts of IFN- $\gamma$  were produced by both LN and spleen cells from IFN- $\gamma$   $^{-/-}$  mice. The IFN- $\gamma$  was produced by CD4<sup>+</sup> T cells since depletion of these T cells from both LN and spleen cell populations abrogated the production of IFN- $\gamma$  upon specific stimulation (Table 2).

The release of high amounts of IFN- $\gamma$  by specifically stimulated CD4<sup>+</sup> T cells from IFN- $\gamma$   $^{-/-}$  mice 40 d after infection could not only be the result of an increased frequency of these cells, but could also be a consequence of the inability of cells from these mice to absorb some of the IFN- $\gamma$  produced in cultures. Therefore, the number of CD4<sup>+</sup> T cells producing IFN- $\gamma$  upon specific stimulation in vitro was compared in the spleen and draining LN of infected IFN- $\gamma$   $^{-/-}$  and IFN- $\gamma$   $^{+/+}$  mice by use of a quantitative ELISPOT assay that allows the detection of individual IFN- $\gamma$ -producing

**Table 1.** Parasite Load in Lesions and Lymphoid Tissues of Mice Lacking IFN- $\gamma$  Receptors Infected with *L. Major*

| Organ   | Number of parasites<br>40 d after infection* |                              |
|---|--|------------------------------|
|   | IFN- $\gamma$ $^{-/-}$                       | IFN- $\gamma$ $^{+/+}$       |
| Foot pad ( <i>L. major</i> /footpad) <sup>†</sup>       | 10 <sup>7</sup>                              | 4.5 $\times$ 10 <sup>5</sup> |
| LN ( <i>L. major</i> /10 <sup>6</sup> LNC) <sup>§</sup> | 6 $\times$ 10 <sup>3</sup>                   | 11                           |
| Spleen ( <i>L. major</i> /10 <sup>6</sup> SC)           | 4.1  | 0.2                          |

\* Data represent the number of viable parasites as determined by limiting dilution assay (18). Results are from one of three experiments that gave similar results.

<sup>†</sup> Foot pads from two mice were used for these determinations.

<sup>§</sup> LN cells (LNC) or spleen cells (SC) from four to eight mice were used for these determinations.

**Table 2.** *IFN- $\gamma$  Production In Vitro by Specifically Stimulated Spleen and Lymph Node Cells from Mice Lacking IFN- $\gamma$  Receptors after Infection with L. major*

| Organ  | In vitro depletion with anti-CD4 mAb | IFN- $\gamma$ production (U/ml) at different times after infection* |                    |        |       |        |       |
|--------|--------------------------------------|---|--------------------|--------|-------|--------|-------|
|        |                                      | Day 12  |                    | Day 20 |       | Day 40 |       |
|        |                                      | - / - <sup>†</sup>  | + / + <sup>‡</sup> | - / -  | + / + | - / -  | + / + |
| Spleen | -                                    | nt  | nt                 | 17     | 12    | 823    | 36    |
|        | +                                    | nt  | nt                 | <10    | <10   | <10    | <10   |
| LN     | -                                    | 10  | 12                 | 28     | 38    | 800    | 64    |
|        | +                                    | nt  | nt                 | <10    | <10   | <10    | <10   |

\* IFN- $\gamma$  production by cells stimulated in vitro for 4 h with UV irradiated parasites was measured by ELISA as described in Materials and Methods. Results are from one of three experiments with similar results.

<sup>†</sup> IFN- $\gamma$ R<sup>-/-</sup> mice.

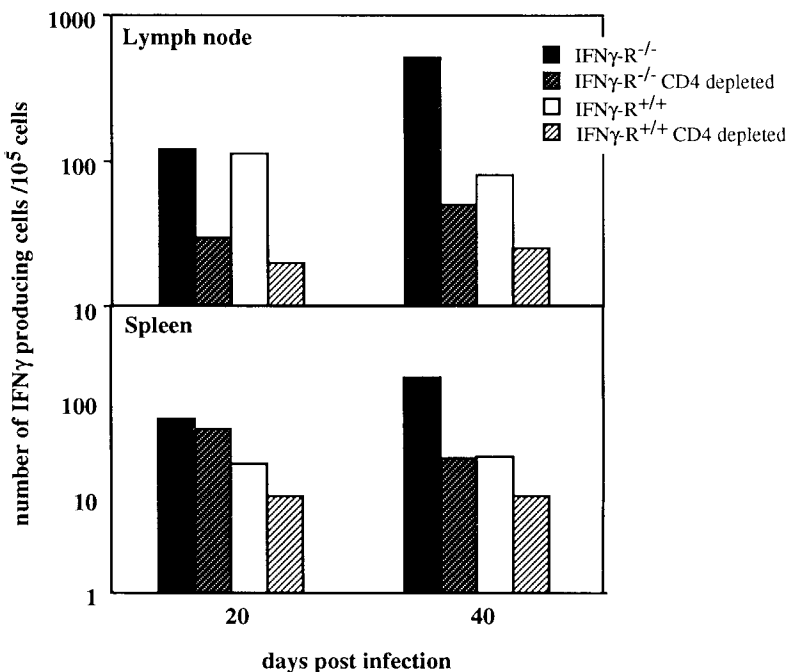
<sup>‡</sup> IFN- $\gamma$ R<sup>+/+</sup> mice.

nt, not tested.

cells. Compared to similarly infected IFN- $\gamma$ R<sup>+/+</sup> mice, the number of CD4<sup>+</sup> T cells producing IFN- $\gamma$  in response to stimulation with *L. major* in vitro was greatly increased in the spleen and draining LN of IFN- $\gamma$ R<sup>-/-</sup> mice 40 d after infection (Fig. 2). It is noteworthy that these cells were not detectable in noninfected mice (data not shown) and that their numbers were similar in IFN- $\gamma$ R<sup>-/-</sup> and IFN- $\gamma$ R<sup>+/+</sup> mice 20 d after infection (Fig. 2). These results show that the increased IFN- $\gamma$  production by CD4<sup>+</sup> T cells from IFN- $\gamma$ R<sup>-/-</sup> mice results from an increased frequency of responding cells.

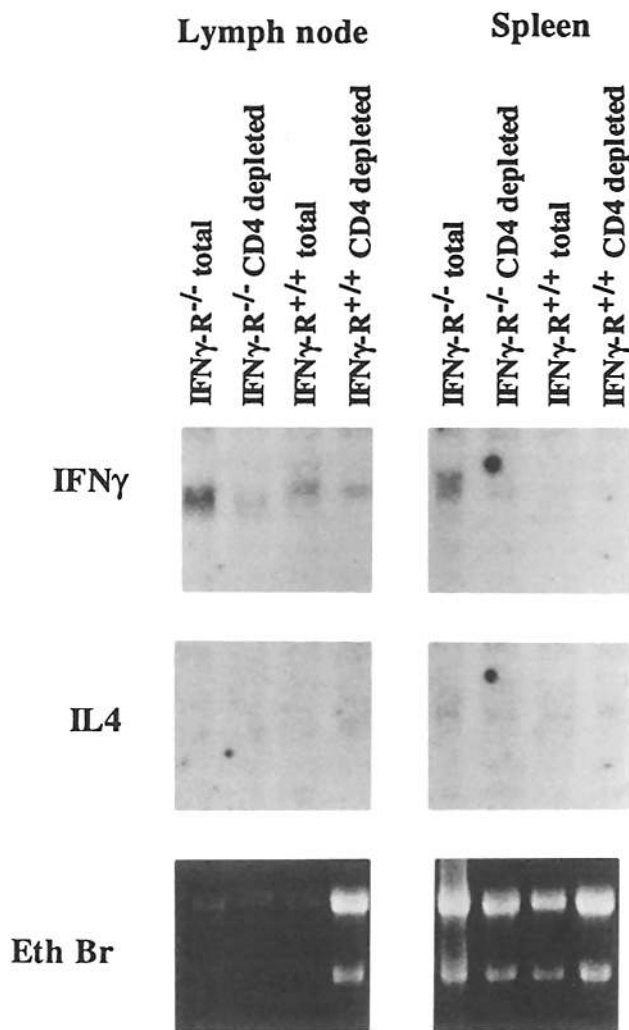
We then investigated whether IFN- $\gamma$ R<sup>-/-</sup> mice exhibited an expansion of Th2-type CD4<sup>+</sup> T cells after infection with *L. major* by measuring biologically active IL-4 in supernatants of LN and spleen cells specifically stimulated with *L. major*. Results have consistently shown that IL-4 was not detectable (<0.3 ng/ml) in supernatants of specifically stimulated CD4<sup>+</sup> T cells from both IFN- $\gamma$ R<sup>-/-</sup> and IFN- $\gamma$ R<sup>+/+</sup> mice. Supernatants of similarly stimulated lymphocytes from infected genetically susceptible BALB/c mice contained biologically active IL-4 ( $\leq 2$  ng/ml).

To evaluate whether the type of CD4<sup>+</sup> T cell responses



**Figure 2.** Enumeration of IFN- $\gamma$ -producing spleen and LN cells from *L. major*-infected mice. Total or CD4<sup>+</sup> depleted cell populations from eight infected mice were stimulated in vitro for 18 h with UV-irradiated parasites as described in Materials and Methods. Serial twofold dilutions were then tested in duplicate for cells producing IFN- $\gamma$  by ELISPOT. Data are presented as the number of IFN- $\gamma$ -producing cells per 10<sup>5</sup> cells. These results are from one of three experiments with similar results.

seen in vitro reflected the type of responses induced by infection in vivo, the RNA isolated from spleen and LN of IFN- $\gamma$ R<sup>-/-</sup> and IFN- $\gamma$ R<sup>+/+</sup> mice 20 d after infection was examined for IFN- $\gamma$  and IL-4 transcripts. Results presented in Fig. 3 show higher levels of IFN- $\gamma$  mRNA in cells obtained from IFN- $\gamma$ R<sup>-/-</sup> compared with IFN- $\gamma$ R<sup>+/+</sup> mice. Cell populations depleted of CD4<sup>+</sup> T cells had very low levels of IFN- $\gamma$  mRNA, indicating that CD4<sup>+</sup> T cells were the main source of IFN- $\gamma$  in these mice. It is noteworthy that no detectable expression of IFN- $\gamma$  mRNA was found in either noninfected IFN- $\gamma$ R<sup>-/-</sup> or IFN- $\gamma$ R<sup>+/+</sup> mice (data not shown). A low level of IL-4 message was present in spleen cells from both types of infected mice (Fig. 3), which, however did not differ from the levels of expression in normal uninfected mice (data not shown). Interestingly, similar low



**Figure 3.** Cytokine mRNA levels in draining LN and spleen cells from mice 20 d after infection with *L. major*. Total RNA was purified from LN and spleen cells from infected mice (eight per group) and was analyzed by Northern blot for presence of cytokine messages by probing with IFN- $\gamma$ - or IL-4-specific probes. A photo for the ethidium bromide-stained gel (*Eth Br*; bottom) allows comparison of relative amounts of RNA loaded in each lane. Similar results were obtained in three separate experiments.

amounts of IL-4 mRNA were observed in cell populations depleted of CD4<sup>+</sup> T cells. Similar results were obtained with RNA isolated from mice 40 d after infection (data not shown).

Taken together, these results show that *L. major* infection leads, in both IFN- $\gamma$ R<sup>-/-</sup> and IFN- $\gamma$ R<sup>+/+</sup> mice, to the expansion of Th1-type CD4<sup>+</sup> T cell populations that produce IFN- $\gamma$  without evidence for a concomitant expansion of IL-4-producing Th2-type CD4<sup>+</sup> T cells.

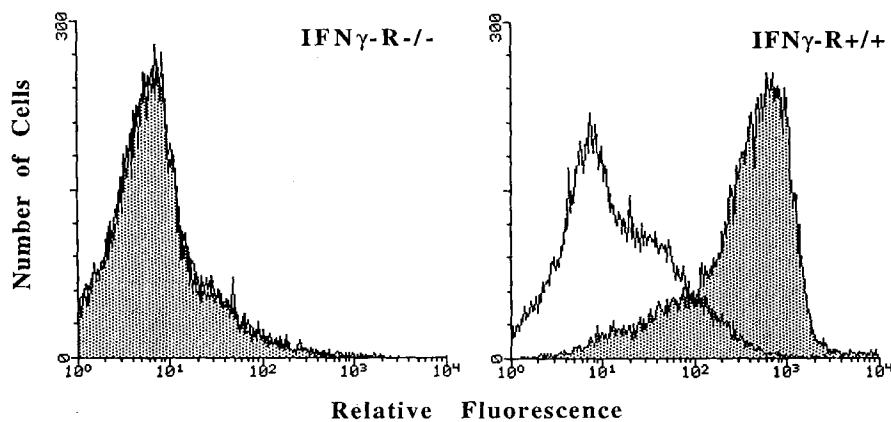
**Effect of Treatment of IFN- $\gamma$ R<sup>-/-</sup> Mice with Anti-IFN- $\gamma$  mAb on the Course of *L. major* Infection and the Functional Characteristics of the CD4<sup>+</sup> T Cell Response.** Since IFN- $\gamma$ R<sup>-/-</sup> mice lack the ligand-binding chain of the IFN- $\gamma$  (16), it is highly unlikely that the Th1-type CD4<sup>+</sup> T cell response observed in these mice after *L. major* infection is mediated through signals delivered by IFN- $\gamma$  through its receptor. In fact, the increased expression of the IFN- $\gamma$ -inducible Ly6A/E marker at the surface of IFN- $\gamma$ R<sup>+/+</sup> LN cells stimulated in vitro with recombinant IFN- $\gamma$  (Fig. 4, right) was never observed when LN cells from IFN- $\gamma$ R<sup>-/-</sup> mice were stimulated in the same manner at doses of IFN- $\gamma$  as high as 1,000 U/ml (Fig. 4, left).

To further rule out the remote possibility that the IFN- $\gamma$  produced in IFN- $\gamma$ R<sup>-/-</sup> mice is driving the differentiation of CD4<sup>+</sup> T cell precursors toward a Th1 functional phenotype through a pathway not requiring the ligand-binding chain of its receptor, we studied the effect of administration of anti-IFN- $\gamma$  mAb to infected IFN- $\gamma$ R<sup>-/-</sup> mice, at the time of initiation of infection, on the functional type of CD4<sup>+</sup> T cells triggered. Anti-IFN- $\gamma$  mAb was administered following a protocol shown to result in the modulation of the functional phenotype of CD4<sup>+</sup> T cells triggered in resistant C3H mice (7).

As shown in Fig. 5, treatment with anti-IFN- $\gamma$  mAb significantly increased both lesion size and parasite burden of IFN- $\gamma$ R<sup>+/+</sup> mice to levels similar to those seen in IFN- $\gamma$ R<sup>-/-</sup> mice not treated with antibodies. Although there was a slight, not significant, increase in lesion size in IFN- $\gamma$ R<sup>-/-</sup> mice treated with anti-IFN- $\gamma$  antibodies compared with untreated IFN- $\gamma$ R<sup>-/-</sup> controls, there was no increase in parasite burden.

Enumeration of IFN- $\gamma$ -producing cells by ELISPOT showed that anti-IFN- $\gamma$  mAb treatment of both infected IFN- $\gamma$ R<sup>-/-</sup> and IFN- $\gamma$ R<sup>+/+</sup> mice did not result in a decrease in the number of spleen or LN cells capable of producing IFN- $\gamma$  upon specific stimulation in vitro (Fig. 6). Similarly, Northern blot analysis of RNA from spleen and LN cells confirmed those results since no decrease in IFN- $\gamma$  mRNA was observed after treatment of either IFN- $\gamma$ R<sup>-/-</sup> or IFN- $\gamma$ R<sup>+/+</sup> mice with anti-IFN- $\gamma$  mAb (Fig. 7).

This treatment with anti-IFN- $\gamma$  mAb did not lead to the expansion of IL-4-producing T cells since biologically active IL-4 could not be detected in supernatants of spleen or LN cells from infected IFN- $\gamma$ R<sup>-/-</sup> and IFN- $\gamma$ R<sup>+/+</sup> mice after specific stimulation in vitro. Northern blot analysis shows that treatment of either IFN- $\gamma$ R<sup>-/-</sup> or IFN- $\gamma$ R<sup>+/+</sup> mice with anti-IFN- $\gamma$  mAb did not have any effect on the level of expression of IL-4 mRNA (data not shown). As the level



**Figure 4.** IFN- $\gamma$  treatment in vitro does not enhance Ly6A/E expression in IFN- $\gamma$ R<sup>-/-</sup> LN cells. In the experiment shown, LN cells ( $4 \times 10^6$ /ml) from IFN- $\gamma$ R<sup>-/-</sup> (left) and IFN- $\gamma$ R<sup>+/+</sup> mice (right) were cultured for 72 h with (shaded areas) or without (white areas) 200 U/ml of recombinant mouse IFN- $\gamma$ . The expression of the Ly6A/E molecule was assayed by flow cytometry with the FITC-conjugated anti-Ly6A/E D7 mAb as described in Materials and Methods. Identical results were also obtained when either the IFN- $\gamma$  concentration (50 or 1,000 U/ml) or the cell culture time (24 or 48 h) was varied.

of IL-4 mRNA was always extremely low, we used a more sensitive RT-PCR method in an attempt to detect IL-4 mRNA in uninfected and *L. major*-infected IFN- $\gamma$ R<sup>-/-</sup> and IFN- $\gamma$ R<sup>+/+</sup> mice. RNA from infected BALB/c and C57BL/6 mice were used as reference controls. The level of IL-4 message in infected IFN- $\gamma$ R<sup>-/-</sup> and IFN- $\gamma$ R<sup>+/+</sup> mice was similar to that seen in resistant C57BL/6 mice and significantly lower than that observed in BALB/c mice infected for the same period of time (Fig. 8). Additionally, use of this technique confirmed that treatment with anti-IFN- $\gamma$  mAb did not significantly modify the IL-4 mRNA levels in infected IFN- $\gamma$ R<sup>-/-</sup> mice. Similarly, it is noteworthy that this treatment did not subsequently lead to enhanced levels of IL-4 message in IFN- $\gamma$ R<sup>+/+</sup> mice.

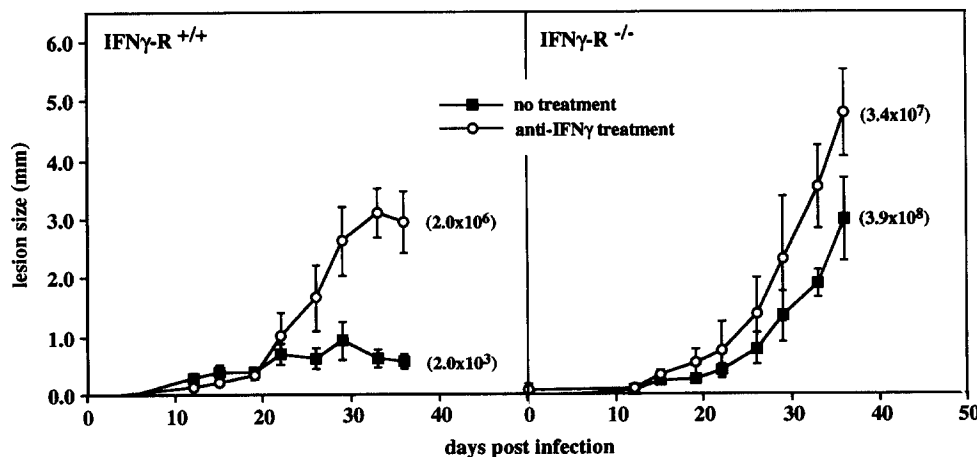
## Discussion

The results presented in this report confirm the crucial role of IFN- $\gamma$  in the elimination of *L. major* by infected hosts. Indeed, macrophages are the only mammalian cells that support the growth of these parasites, and IFN- $\gamma$  has clearly been shown to be the main lymphokine that activates macrophages to a parasitocidal state (2, 31). The IFN- $\gamma$ -mediated destruction of intracellular *L. major* by macrophages has been demon-

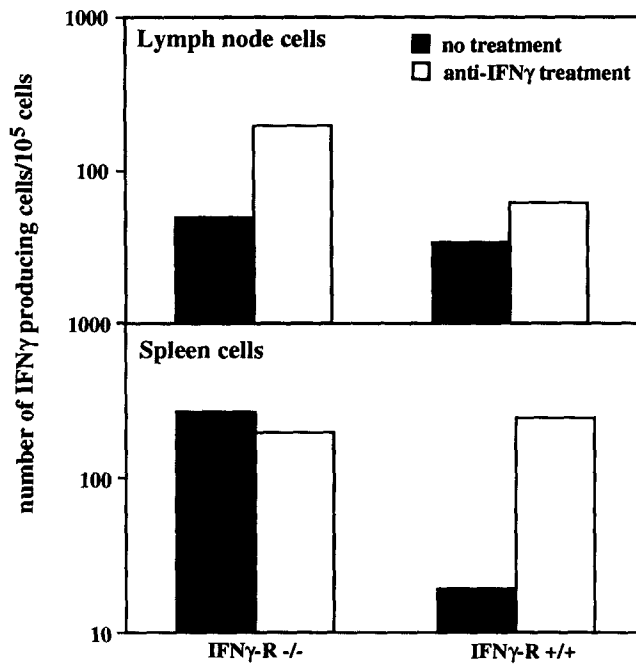
strated, in the mouse, to depend on the L-arginine-dependent production of NO by these cells (3, 4). Thus, the severity of infection observed in IFN- $\gamma$ R<sup>-/-</sup> mice is consistent with previous results showing that macrophages from IFN- $\gamma$ R<sup>-/-</sup> mice did not release nitrites upon stimulation in vitro with IFN- $\gamma$  either in the absence or the presence of LPS (16).

Understanding of the mechanisms operating in the initiation of Th1 or Th2 CD4<sup>+</sup> T cell development from naive precursors is an important issue currently under investigation in several laboratories. Evidence, derived from in vitro and in vivo studies with various antigenic systems, exists for an important role of cytokines present at the initiation of primary CD4<sup>+</sup> T cell stimulation on the differentiation of naive CD4<sup>+</sup> T cells toward one or the other functional phenotype.

Experimental infection of mice with *L. major* represents one of the first and best models of infection in which resistance and susceptibility were shown to correlate with the expansion and result from the activity of functionally distinct CD4<sup>+</sup> T cell subsets (5, 6). Furthermore, by use of this murine model of infection, it has also been suggested that cytokines present at the initiation of infection could direct the differentiation of CD4<sup>+</sup> T cell precursors toward one or

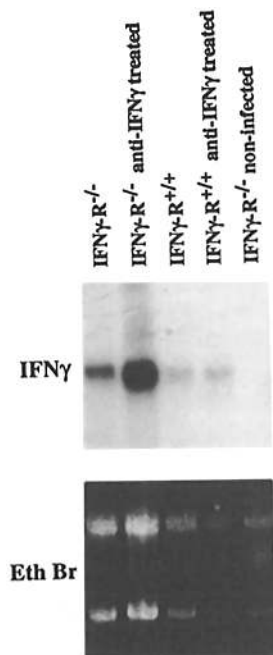


**Figure 5.** Effect of anti-IFN- $\gamma$  treatment on the course of *L. major* infection in IFN- $\gamma$ R<sup>+/+</sup> and IFN- $\gamma$ R<sup>-/-</sup> mice. Mice (four in each group) were treated with 2 mg anti-IFN- $\gamma$  mAb 2 d before infection and with three additional weekly injections of 320  $\mu$ g mAb. Numbers in parentheses indicate the number of parasites per lesion as estimated by limiting dilution assay. These results are from one of two experiments with similar results.

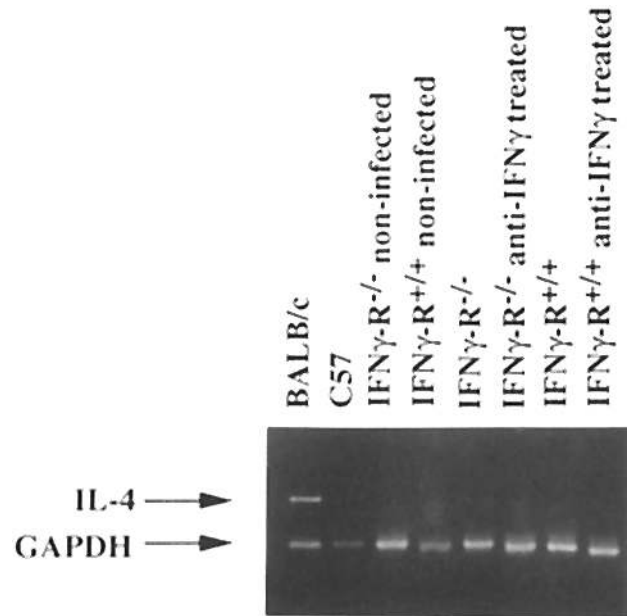


**Figure 6.** Effect of treatment with anti-IFN- $\gamma$  mAb on the number of IFN- $\gamma$ -producing cells. Spleen cells of four mice were isolated 40 d after infection and stimulated in vitro for 18 h with UV-irradiated parasites. Serial twofold dilutions were tested in duplicate by ELISPOT to detect IFN- $\gamma$ -producing cells. Data presented show the number of IFN- $\gamma$ -producing cells per  $10^5$  cells and derive from one of two independent experiments that gave similar results.

the other functional phenotype. Although there is a consensus that IL-4 and IL-12 play a role in the respective development of polarized Th2 and Th1 responses after infection with *L. major*, the data concerning the role of IFN- $\gamma$  for a sus-



**Figure 7.** Effect of treatment with anti-IFN- $\gamma$  mAb on IFN- $\gamma$  message levels in mice 40 d after infection. Total RNA was purified from LN and spleen cells from infected mice (four per group) and was analyzed by Northern blotting for the presence of IFN- $\gamma$  messages. A photo of the ethidium bromide-stained gel (from one of two separate experiments with similar results) (*Eth Br*; bottom) allows comparison of relative amounts of RNA loaded in each lane.



**Figure 8.** Comparison of IL-4 message levels by RT-PCR. This is a photograph (from one of two separate experiments with similar results) of an ethidium bromide-stained gel showing PCR products obtained by use of LN cell RNA from various strains of *L. major*-infected mice 40 d after infection. The bands corresponding to IL-4 and GAPDH are marked.

tained expansion of Th1-type CD4<sup>+</sup> T cells are still conflicting.

The observations reported here show that mice lacking the ligand-binding chain of the IFN- $\gamma$ R still mount a polarized IFN- $\gamma$ -producing CD4<sup>+</sup> T cell response without evidence for the expansion of IL-4-producing CD4<sup>+</sup> cells, indicating that IFN- $\gamma$ -mediated signals are not necessary for the differentiation of CD4<sup>+</sup> T cell precursors toward a Th1 phenotype. Furthermore the present results also indicate that the lack of IFN- $\gamma$ -mediated signals does not lead to the expansion of IL-4-producing CD4<sup>+</sup> T cells.

For the first 20 d after infection, both parasite burden and IFN- $\gamma$  levels were similar in IFN- $\gamma$ R<sup>+/+</sup> and IFN- $\gamma$ R<sup>-/-</sup> mice. However, compared with wild-type mice, significantly higher numbers of IFN- $\gamma$ -producing CD4<sup>+</sup> T cells were observed in lymphoid tissues of mice lacking the IFN- $\gamma$ R 40 d after infection. Since at this time the parasite burden was considerable in IFN- $\gamma$ R<sup>-/-</sup> mice, it is likely that this increase in IFN- $\gamma$  production resulted in part from a strong and sustained antigenic stimulation. Recent evidence strongly indicates that *L. major* amastigotes are able to induce IL-12 p40 mRNA in macrophages (32). Thus, it is likely that substantial amounts of IL-12 are generated as a result of the important number of amastigotes in IFN- $\gamma$ R<sup>-/-</sup> mice and could account for the important production of IFN- $\gamma$  observed in these mice at later stages of infection with *L. major*, since IL-12 has been shown to stimulate IFN- $\gamma$  production by T and NK cells (33). Production of substantial amounts of IL-12 in IFN- $\gamma$ R<sup>-/-</sup> mice could also account for the suppression of an IL-4 response in these mice, since recent results have clearly shown that IL-12 strongly suppresses both base

line and induced IL-4 gene expression (34). However, one cannot completely rule out that a lack of appropriate regulation of IFN- $\gamma$  production in mice unable to respond to this cytokine could also account, at least in part, for this increased production of IFN- $\gamma$  observed in IFN- $\gamma$ R<sup>-/-</sup> mice after various stimuli.

Our results are in line with observations showing that administration of recombinant IFN- $\gamma$  to infected susceptible mice neither allowed the development of sustained Th1-type responses nor inhibited the expansion of Th2 cells (35). In addition, infection of BALB/c mice with parasites, into which the murine IFN- $\gamma$  gene was introduced and expressed, led to the development of severe lesions and did not switch the polarized Th2-type response (36). Furthermore, these results are in agreement with recent results showing that IFN- $\gamma$ R<sup>-/-</sup> mice infected with pseudorabies virus develop a Th1-characteristic cytokine response (37).

In contrast to these results, evidence for a role of IFN- $\gamma$  on the differentiation of Th1-type CD4<sup>+</sup> T cells in the murine model of infection with *L. major* also exists. Administration of anti-IFN- $\gamma$  mAb at the time of parasite inoculation not only abrogated the natural resistance of C3H mice (2), but also ablated the Th1-type CD4<sup>+</sup> T cell response normally seen in these mice and promoted the development of a Th2-type response (7). NK cells have been shown to be the major source of IFN- $\gamma$ , produced early during infection of mice from some genetically resistant strains, possibly responsible for the expansion of Th1-type CD4<sup>+</sup> T cells (38). Finally, recent observations made in IFN- $\gamma$ -deficient C57BL/6 mice have revealed that these mice, in contrast to wild-type control mice, develop a Th2-type response after infection with *L. major*, without evidence of a Th1-type response (39).

The reasons for the difference in the type of CD4<sup>+</sup> T cell response triggered in IFN- $\gamma$ R<sup>-/-</sup> (this study) and IFN- $\gamma$  knockout mice (39) after infection with *L. major* are not known. Although unlikely, it might be argued that, in IFN- $\gamma$ R<sup>-/-</sup> mice, either IFN- $\gamma$  could interact with cells through a pathway not requiring the ligand-binding chain of the IFN- $\gamma$ R, or that an IFN- $\gamma$ -binding receptor still exists on CD4<sup>+</sup> T cell precursors in IFN- $\gamma$ R<sup>-/-</sup> mice, thus accounting for their differentiation toward the Th1 pathway after infection with *L. major*. However, these hypotheses are very unlikely since no evidence exists for any residual response to IFN- $\gamma$  of cells from IFN- $\gamma$ R<sup>-/-</sup> mice. In fact, treatment of cells lacking the IFN- $\gamma$ R  $\alpha$  chain with doses of IFN- $\gamma$  as high as 10<sup>5</sup> U did not lead to enhanced expression of MHC molecules (our unpublished observations). In addition, results presented here show that in vitro treatment of IFN- $\gamma$ R<sup>-/-</sup> LN cells with IFN- $\gamma$  does not induce the expression of the Ly6A/E molecule (Fig. 4), an IFN- $\gamma$ -inducible surface marker (34). In contrast, such treatment strongly induced the expression of this marker on LN cells from wild-type IFN- $\gamma$ R<sup>+/+</sup> mice. Furthermore, early treatment of IFN- $\gamma$ R<sup>-/-</sup> mice with anti-IFN- $\gamma$  mAb failed to result in a switch of the CD4<sup>+</sup> T cell response toward the Th2 pathway. Treatment with anti-IFN- $\gamma$  mAb was initiated before infection

with *L. major*, following a regimen previously shown to result in the modulation of the functional phenotype of the CD4<sup>+</sup> T cells triggered in resistant C3H mice, consequently reversing their normal resistance to infection (7). This effect was critically dependent on the timing of administration of anti-IFN- $\gamma$  mAb, since treatment after infection was unable to alter the course of infection (2), strongly suggesting that the level of IFN- $\gamma$  at the initiation of infection was crucial in determining whether a Th1 or Th2 cytokine pattern subsequently developed (7). It is important to emphasize that no detectable IFN- $\gamma$  was produced by either IFN- $\gamma$ R<sup>-/-</sup> or IFN- $\gamma$ R<sup>+/+</sup> mice at the time of infection. Furthermore, even during the early stages of infection, no evidence was obtained for the production of larger amounts of IFN- $\gamma$  in IFN- $\gamma$ R<sup>-/-</sup> mice. Thus, it is likely that neutralization of IFN- $\gamma$  by anti-IFN- $\gamma$  mAb was similar, during the early stages of infection, in both IFN- $\gamma$ R<sup>-/-</sup> and IFN- $\gamma$ R<sup>+/+</sup> mice. Taken together, these findings strongly suggest that the Th1-type response observed in the present system is not mediated by IFN- $\gamma$ .

The implication of IFN- $\gamma$  in Th1 cell development is based mainly on observations showing impairment of Th1 responses in the absence of IFN- $\gamma$  rather than on experiments demonstrating a promotion of sustained expansion of Th1 cells by exogenous IFN- $\gamma$ . In the absence of IFN- $\gamma$ , the remote possibility that other lymphokines could bind to the IFN- $\gamma$ R and transmit signals leading to the differentiation of CD4<sup>+</sup> T cell precursors towards the Th2 pathway could be considered. Although the evidence for the specificity of the binding of IFN- $\gamma$  to its receptor is strong, a structural homology between the IFN- $\gamma$ R and the receptor for IL-10 has recently been described (40). Thus, it would be of interest to study the differentiations of T cell subsets after infection with *L. major* in transgenic mice that lack both the IFN- $\gamma$  and the IFN- $\gamma$ R genes. Finally, since the IFN- $\gamma$  (39) and IFN- $\gamma$ R knockout mice (this study) were derived on genetically different backgrounds, it is possible that the influence of IFN- $\gamma$  on the differentiation of T cell subsets may vary in different mouse strains. Such genetic differences in the importance of IFN- $\gamma$  for Th1-type response development could also account for the presently reported failure of anti-IFN- $\gamma$  mAb, administered at the initiation of infection with *L. major*, to result in the differentiation of CD4<sup>+</sup> T cell precursors toward the Th2 functional pathway, even in resistant wild-type IFN- $\gamma$ R<sup>+/+</sup> 129/Sv/Ev mice. Such treatment has indeed been shown to result in a shift in the T cell response from a Th1 to a Th2-type response in resistant C3H/HeN mice infected with *L. major* (7).

The importance of IL-12, a cytokine produced by cells with accessory or antigen-presenting function (41), for the development of Th1-type CD4<sup>+</sup> T cell responses after infection with *L. major*, has recently been recognized (42, 43). Although the effect of IL-12 on Th1 cell development in IFN- $\gamma$ R<sup>-/-</sup> mice after infection with *L. major* has not yet been directly assessed, the results reported here suggest that such an effect of IL-12 would not be dependent on IFN- $\gamma$ . These data are in agreement with other observations showing, on the one



hand, the failure of IFN- $\gamma$  to modify the course of infection with *L. major* in susceptible mice (35) and, on the other hand, the capacity of IL-12 to cure these mice (42). Moreover, in other antigenic systems the effect of IL-12 on the differentiation of Th1-type cells was unaffected by treatment with anti-IFN- $\gamma$  antibody (44). Considering this important role of IL-12 in Th1 cell development, it would be of great interest to compare its production after infection with *L. major* in IFN- $\gamma$ R<sup>-/-</sup> and IFN- $\gamma$  knockout mice.

In other antigenic systems, the use of CD4<sup>+</sup> T cells obtained from TCR- $\alpha/\beta$  transgenic mice has facilitated the study of the parameters influencing short-term differentiation of CD4<sup>+</sup> T cells after activation by specific antigen *in vitro*. The dominant effect of IL-4 in promoting the development of Th2-type responses was clearly demonstrated by observations showing that the addition of IL-4 during specific priming of CD4<sup>+</sup> T cells from TCR- $\alpha/\beta$ -transgenic mice inhibited the development of Th1-type responses and led to the differentiation of Th2-type responses (12, 13). Addition of IL-12 during specific priming of CD4<sup>+</sup> T cells from TCR- $\alpha/\beta$ -transgenic mice led to the development of Th1-type responses (45, 46).

In contrast to the well-accepted role of IL-4 and IL-12 on the development of Th2 and Th1 responses, respectively, the data concerning the assessment of the importance of IFN- $\gamma$  in promoting the expansion of Th1 cells are still unclear. By use of differentiated T cell clones, IFN- $\gamma$  has been demonstrated to selectively inhibit the proliferation of Th2 cells *in vitro* (30). Using TCR- $\alpha/\beta$ -transgenic mice, some studies have shown a requirement for IFN- $\gamma$  in the development of CD4<sup>+</sup> T cells producing high amounts of IFN- $\gamma$  by use of neutralizing antibodies against this cytokine, but have been unable to drive Th1-type cell development by addition of IFN- $\gamma$  to the priming cultures (47). In these studies, CD4<sup>+</sup> T cells from TCR- $\alpha/\beta$ -transgenic mice on a BALB/c background were used. The requirement for IFN- $\gamma$  in Th1-type T cell development was, however, not shown in other studies

with CD4<sup>+</sup> T cells from other TCR- $\alpha/\beta$ -transgenic mice on a B10 background, where IFN- $\gamma$  itself did not enhance priming for IFN- $\gamma$  production, nor did anti-IFN- $\gamma$  reduce such priming (13). Furthermore, even though evidence for an IFN- $\gamma$  dependence of the effect of IL-12 on the development of Th1-type response has been obtained in one TCR- $\alpha/\beta$ -transgenic system (47), the IL-12-mediated enhancement of priming for IFN- $\gamma$  production, also using an accessory cell-dependent antigenic system with CD4<sup>+</sup> T cells from other TCR- $\alpha/\beta$ -transgenic mice, was not blocked by anti-IFN- $\gamma$  mAb (45). However, in this study, upon stimulation of CD4<sup>+</sup> T cells in an accessory cell-independent system, the ability of IL-12 to enhance production of IFN- $\gamma$  was substantially diminished in the presence of anti-IFN- $\gamma$  antibodies (45). As CD4<sup>+</sup> T cells from mice with different genetic backgrounds were used in these two studies, these results could reflect genetic differences in the importance of IFN- $\gamma$  in the regulation of T cell subset differentiation. Furthermore, they indicate that the requirements for priming in accessory cell-dependent and -independent systems may be different. In humans, IL-12 was also recently shown to promote Th1-type response in an IFN- $\gamma$ -independent manner (48).

In conclusion, the data presented here do not support a critical role for IFN- $\gamma$  in Th1 cell development *in vivo*. They are in agreement with observations made *in vitro* that have led to the hypothesis that, in the absence of sufficient amounts of IL-4, the development of an IFN- $\gamma$ -producing CD4<sup>+</sup> T cell response could represent the "default" pathway of differentiation of CD4<sup>+</sup> T cell precursors (13). Thus, it is possible that the concentrations of IL-4 at the site of the developing lesion in IFN- $\gamma$ R<sup>-/-</sup> mice are not sufficient to either initiate priming for IL-4-producing T cell response or inhibit the differentiation of IFN- $\gamma$ -producing CD4<sup>+</sup> T cells. Our results also underline the importance of mice genetically deficient in critical cytokine receptors for a better understanding of the factors regulating the T cell response to infectious pathogens.

---

The authors thank P. Kropf and R. Lee for technical assistance, Dr. E. Shevach for the kind gift of the anti-Ly6A/E mAb, Dr. J.-P. Rosat for technical advice, and Drs. H. R. MacDonald and J.-C. Cerottini for suggestions and critical reading of the manuscript.

This work was supported by the Swiss National Science Foundation and the World Health Organization.

Address correspondence to Jacques A. Louis, WHO-IRTC, Institute of Biochemistry, University of Lausanne, Chemin des Boveresses, CH-1066 Epalinges, Switzerland. M. Aguet's present address is Genentech, South San Francisco, CA 94080.

Received for publication 15 June 1994 and in revised form 30 September 1994.

## References

1. Müller, I., T. Pedrazzini, J.P. Farrell, and J.A. Louis. 1989. T cell responses and immunity to experimental infection with *Leishmania major*. *Annu. Rev. Immunol.* 7:561-578.
2. Belosevic, M., D.S. Finbloom, P.H. Van der Meide, M.V. Slayter, and C.A. Nacy. 1989. Administration of monoclonal anti-IFN- $\gamma$  antibodies *in vivo* abrogates natural resistance of

- C3H/HeN mice to infection with *Leishmania major*. *J. Immunol.* 143:266–274.
3. Green, S.J., C.A. Nacy, and M.S. Meltzer. 1991. Cytokine-induced synthesis of nitrogen oxides in macrophages: a protective host response to *Leishmania* and other intracellular pathogens. *J. Leukoc. Biol.* 50:93–103.
  4. Mauël, J., S. Betz-Corradin, and Y. Buchmüller-Rouiller. 1991. Nitrogen and oxygen metabolites and the killing of *Leishmania* by activated murine macrophages. *Res. Immunol.* 142:577–580.
  5. Heinzel, F.P., M.D. Sadick, S. Mutha, and R.M. Locksley. 1991. Production of interferon  $\gamma$ , interleukin 2, interleukin 4, and interleukin 10 by CD4<sup>+</sup> lymphocytes *in vivo* during healing and progressive murine leishmaniasis. *Proc. Natl. Acad. Sci. USA.* 88:7011–7015.
  6. Holaday, B.J., M.D. Sadick, Z. Wang, S.L. Reiner, F.P. Heinzel, T.G. Parslow, and R.M. Locksley. 1991. Reconstitution of *Leishmania* immunity in severe combined immunodeficient mice using Th1- and Th2-like cell lines. *J. Immunol.* 147:1653–1658.
  7. Scott, P. 1991. IFN- $\gamma$  modulates the early development of Th1 and Th2 responses in a murine model of cutaneous leishmaniasis. *J. Immunol.* 147:3149–3155.
  8. 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. *J. Exp. Med.* 169:59–72.
  9. Chatelain, R., K. Varkila, and R.L. Coffman. 1992. IL-4 induces a Th2 response in *Leishmania major*-infected mice. *J. Immunol.* 148:1182–1187.
  10. Le Gros, G., S.Z. Ben-Sasson, R. Seder, F.D. Finkelman, and W.E. Paul. 1990. Generation of interleukin 4 (IL-4)-producing cells *in vivo* and *in vitro*: IL-2 and IL-4 are required for *in vitro* generation of IL-4-producing cells. *J. Exp. Med.* 172:921–929.
  11. Abeshira-Amar, O., M. Gibert, M. Jolij, J. Thèze, and D.L.J. Jankovic. 1992. IL-4 plays a dominant role in the differential development of Th0 into Th1 and Th2 cells. *J. Immunol.* 148:3820–3829.
  12. Hsieh, C.-S., A.B. Heimberger, J.S. Gold, A. O'Garra, and K.M. Murphy. 1992. Differential regulation of T helper phenotype development by interleukins 4 and 10 in an  $\alpha\beta$  T-cell receptor transgenic system. *Proc. Natl. Acad. Sci. USA.* 89:6065–6069.
  13. Seder, R.A., W.E. Paul, M.M. David, and B. Fazekas de St. Groth. 1992. The presence of interleukin 4 during *in vitro* priming determines the lymphokine-producing potential of CD4<sup>+</sup> T cells from T cell receptor transgenic mice. *J. Exp. Med.* 176:1091–1098.
  14. Fitch, F.W., M.D. McKisic, D.W. Lancki, and T.F. Gajewski. 1993. Differential regulation of murine T lymphocyte subsets. *Annu. Rev. Immunol.* 11:29–48.
  15. Maggi, E., R. Parronchi, R. Manetti, C. Simonelli, M.P. Piccinni, F.S. Rugin, M. De Carli, M. Ricci, and S. Romagnani. 1992. Reciprocal regulatory effects of IFN- $\gamma$  and IL-4 on the *in vitro* development of human Th1 and Th2 clones. *J. Immunol.* 148:2142–2147.
  16. Huang, S., W. Hendriks, A. Althage, S. Hemmi, H. Bluethmann, R. Kamijo, J. Vilcek, R.M. Zinkernagel, and M. Aguet. 1993. Immune response in mice that lack the interferon- $\gamma$  receptor. *Science (Wash. DC).* 259:1742–1745.
  17. Louis, J.A., E. Moedder, R. Behin, and H.D. Engers. 1979. Recognition of protozoan parasite antigens by murine T lymphocytes. I. Induction of specific T lymphocyte dependent proliferative response to *Leishmania tropica*. *Eur. J. Immunol.* 9:841–847.
  18. Titus, R.G., M. Marchand, T. Boon, and J.A. Louis. 1985. A limiting dilution assay for quantifying *Leishmania major* in tissues of infected mice. *Parasite Immunol.* 7:545–555.
  19. Chervinski, H.M., J.H. Schumacher, K.D. Brown, and T.R. Mosmann. 1987. Two types of mouse helper T cell clone. III. Further differences in lymphokine synthesis between Th1 and Th2 clones revealed by RNA hybridization, functionally monospecific bioassays, and monoclonal antibodies. *J. Exp. Med.* 166:1229–1244.
  20. Ceredig, R., J.W. Lowenthal, M. Nabholz, and R.H. MacDonald. 1985. Expression of interleukin-2 receptors as a differentiation marker on intrathymic stem cells. *Nature (Lond.)* 314:98–100.
  21. Sade, S.S., and J. Langhorne. 1989. Production of interferon- $\gamma$  during infection of mice with *Plasmodium chabaudi*. *Immunobiology.* 179:353–365.
  22. Hu-Li, J., J. Ohara, C. Watson, W. Tsang, and W.E. Paul. 1989. Derivation of a T cell line that is highly responsive to IL-4 and IL-2 (CT.4R) and of an IL-2 hyporesponsive mutant of that line (CT.4S). *J. Immunol.* 142:800–807.
  23. Skidmore, B.J., S.A. Stammes, K. Townsend, A.L. Glacebrook, K.C.F. Sheehan, R.V. Schreiber, and J.M. Chiller. 1989. Enumeration of cytokine-secreting cells at the single-cell level. *Eur. J. Immunol.* 19:1591–1597.
  24. Spiltany, G.L., and E.A. Havell. 1984. Monoclonal antibody to murine interferon- $\gamma$  inhibits lymphokine-induced antiviral and macrophage tumoricidal activities. *J. Exp. Med.* 159:1560–1565.
  25. Dumont, F.J., R.G.E. Palfree, and L.Z. Coker. 1986. Phenotypic changes induced by interferon in resting T cells: major enhancement of Ly6 antigen expression. *J. Immunol.* 137:201–210.
  26. Cross, D., and J.C. Cambier. 1990. Transforming growth factor  $\beta$ 1 has differential effects on B cell differentiation and activation antigen expression. *J. Immunol.* 144:432–439.
  27. Grau, Z.W., and D.V. Goeddel. 1983. Cloning and expression of murine immune interferon cDNA. *Proc. Natl. Acad. Sci. USA.* 80:5842–5846.
  28. Noma, Y., P. Sideras, T. Naito, S. Bergstedt-Linquist, C. Azuma, E. Severinson, T. Tanabe, T. Kinashi, F. Matsuda, Y. Yaoita, et al. 1986. Cloning of cDNA encoding the murine IgG1 induction factor by a novel strategy using SP6 promoter. *Nature (Lond.)* 319:640–646.
  29. Kramar, G., T. Aguado, J. Louis, G. Del Giudice, and P.H. Lambert. 1993. Characterization of host CD4<sup>+</sup> T lymphocytes in mice neonatally tolerized to alloantigens. *Eur. J. Immunol.* 23:2551–2556.
  30. Gajewski, T.F., J. Joyce, and F.W. Fitch. 1989. Anti-proliferative effect of IFN- $\gamma$  in immune regulation. III. Differential selection of Th1 and Th2 murine helper T lymphocyte clones using recombinant IL-2 and recombinant IFN- $\gamma$ . *J. Immunol.* 143:15–22.
  31. Murray, H.W., B.Y. Rubin, and C.D. Rothermel. 1983. Killing of intracellular *Leishmania donovani* by lymphokine-stimulated human monocytes. *J. Clin. Invest.* 72:1506–1510.
  32. Reiner, S.L., S. Zeng, Z.-E. Wang, L. Stowring, and R.M. Locksley. 1994. *Leishmania* promastigotes evade interleukin 12 (IL-12) induction by macrophages and stimulate a broad range of cytokines from CD4<sup>+</sup> T cells during initiation of infection. *J. Exp. Med.* 179:447–456.
  33. Chan, S.H., B. Perussia, J.W. Gupta, M. Kobayashi, M. Pospisil, H.A. Young, S.F. Wolf, D. Young, S.C. Clark, and G. Trinchieri. 1991. Induction of interferon  $\gamma$  production by natural

- killer cell stimulatory factor: characterization of the responder cells and synergy with other inducers. *J. Exp. Med.* 173:869–879.
34. Morris, S.C., K.B. Madden, J.J. Adamovicz, W.C. Gause, B.R. Hubbard, M.K. Gately, and F.D. Finkelman. 1994. Effects of IL-12 *in vivo* cytokine gene expression and Ig isotype selection. *J. Immunol.* 152:1047–1056.
  35. Sadick, M.D., F.P. Heinzel, B.J. Holaday, R.T. Pu, R.S. Dawkins, and R.M. Locksley. 1990. Cure of murine *Leishmaniasis* with anti-interleukin 4 monoclonal antibody. Evidence for a T cell–dependent, interferon  $\gamma$ –independent mechanism. *J. Exp. Med.* 171:115–127.
  36. Tobin, J.F., S.L. Reiner, F. Hatam, S. Zheng, C.L. Leptak, D.F. Wirth, and R.M. Locksley. 1993. Transfected *Leishmania* expressing biologically active IFN- $\gamma$ . *J. Immunol.* 150:5059–5069.
  37. Schijns, V.E.C.J., B.L. Haagmans, E.O. Rijke, S. Huang, M. Aguet, and M.C. Horzinek. 1994. IFN- $\gamma$  receptor-deficient mice generate antiviral Th1-characteristic cytokine profiles but altered antibody responses. *J. Immunol.* 153:2029–2037.
  38. Sharton, T.M., and P. Scott. 1993. Natural killer cells are a source of interferon  $\gamma$  that drives differentiation of CD4<sup>+</sup> T cell subsets and induces early resistance to *Leishmania major* in mice. *J. Exp. Med.* 178:567–577.
  39. Wang, Z.-E., S.L. Reiner, S. Zheng, D.K. Dalton, and R.M. Locksley. 1994. CD4<sup>+</sup> effector cells default to the Th2 pathway in interferon- $\gamma$ –deficient mice infected with *Leishmania major*. *J. Exp. Med.* 179:1367–1371.
  40. Ho, A.S.Y., Y. Liu, T.A. Khan, D.-H. Hsu, J.F. Bazan, and K.W. Moore. 1993. A receptor for interleukin 10 is related to interferon receptors. *Proc. Natl. Acad. Sci. USA.* 90:11267–11271.
  41. Trinchieri, G. 1993. Interleukin-12 and its role in the generation of Th1 cells. *Immunol. Today.* 14:335–338.
  42. Heinzel, F.P., D.S. Schoenhaut, R.M. Rerko, L.E. Rosser, and M.K. Gately. 1993. Recombinant interleukin 12 cures mice infected with *Leishmania major*. *J. Exp. Med.* 177:1505–1509.
  43. Sypek, J.P., C.L. Chung, S.E.H. Mayor, J.M. Subramanyam, S.J. Goldman, D.S. Sieburth, S.F. Wolf, and R.G. Schaub. 1993. Resolution of cutaneous leishmaniasis: interleukin 12 initiates a protective T helper type 1 immune response. *J. Exp. Med.* 177:1797–1802.
  44. McKnight, A.J., G.J. Zimmer, I. Fogelman, S.F. Wolf, and A.K. Abbas. 1994. Effects of IL-12 on helper T cell-dependent immune responses *in vivo*. *J. Immunol.* 152:2172–2179.
  45. Seder, R.A., R. Gazzinelli, A. Sher, and W. Paul. 1993. Interleukin 12 acts directly on CD4<sup>+</sup> T cells to enhance priming for interferon  $\gamma$  production and diminishes interleukin 4 inhibition of such priming. *Proc. Natl. Acad. Sci. USA.* 90:10188–10192.
  46. Hsieh, C.S., S.E. Macatonia, C.S. Tripp, S.F. Wolf, A. O'Garra, and K.M. Murphy. 1993. Development of Th1 CD4<sup>+</sup> T cells through IL-12 produced by *Listeria*-induced macrophages. *Science (Wash. DC).* 260:547–549.
  47. Macatonia, S.E., C.S. Hsieh, K.M. Murphy, and A. O'Garra. 1993. Dendritic cells and macrophages are required for Th1 development of CD4<sup>+</sup> T cells from  $\alpha\beta$  TCR transgenic mice: IL-12 substitution for macrophages to stimulate IFN- $\gamma$  production is IFN- $\gamma$ -dependent. *Int. Immunol.* 5:1119–1128.
  48. Manetti, R., P. Parronchi, M.G. Giudizi, M.-P. Piccinni, E. Maggi, G. Trinchieri, and S. Romagnani. 1993. Natural killer cell stimulatory factor interleukin 12 (IL-12) induces T helper type 1 (Th1)–specific immune responses and inhibits the development of IL-4–producing Th cells. *J. Exp. Med.* 177:1199–1204.