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# The IL-4 Rapidly Produced in BALB/c Mice After Infection with *Leishmania major* Down-Regulates IL-12 Receptor $\beta$ 2-Chain Expression on CD4<sup>+</sup> T Cells Resulting in a State of Unresponsiveness to IL-12<sup>1</sup>

Hayo Himmelrich, Carlos Parra-Lopez, Fabienne Tacchini-Cottier, Jacques A. Louis,<sup>2</sup> and Pascal Launois<sup>3</sup>

Within 1 day of infection with *Leishmania major*, susceptible BALB/c mice produce a burst of IL-4 in their draining lymph nodes, resulting in a state of unresponsiveness to IL-12 in parasite-specific CD4<sup>+</sup> T cells within 48 h. In this report we examined the molecular mechanism underlying this IL-12 unresponsiveness. Extinction of IL-12 signaling in BALB/c mice is due to a rapid down-regulation of IL-12R  $\beta$ 2-chain mRNA expression in CD4<sup>+</sup> T cells. In contrast, IL-12R  $\beta$ 2-chain mRNA expression was maintained on CD4<sup>+</sup> T cells from resistant C57BL/6 mice. The down-regulation of the IL-12R  $\beta$ 2-chain mRNA expression in BALB/c CD4<sup>+</sup> T cells is a consequence of the early IL-4 production. In this murine model of infection, a strict correlation is shown in vivo between expression of the IL-12R  $\beta$ 2-chain in CD4<sup>+</sup> T cells and the development of a Th1 response and down-regulation of the mRNA  $\beta$ 2-chain expression and the maturation of a Th2 response. Treatment of BALB/c mice with IFN- $\gamma$ , even when IL-4 has been produced for 48 h, resulted in maintenance of IL-12R  $\beta$ 2-chain mRNA expression and IL-12 responsiveness. The data presented here support the hypothesis that the genetically determined susceptibility of BALB/c mice to infection with *L. major* is primarily based on an up-regulation of IL-4 production, which secondarily induces extinction of IL-12 signaling. *The Journal of Immunology*, 1998, 161: 6156–6163.

Mice from the majority of inbred strains (e.g., B10D2, C57BL/6, CBA, C3H, etc.) spontaneously heal cutaneous lesions resulting from infection with *Leishmania major*, an obligate intracellular protozoan parasite of mononuclear phagocytes in mammalian hosts. In contrast, mice from BALB strains are susceptible to infection, developing severe progressing lesions. Genetically determined resistance and susceptibility to infection with this parasite have clearly been shown to result from the development of parasite-specific CD4<sup>+</sup> Th1 or Th2 responses, respectively (1).

The initial demonstration of the dominant role of cytokines during the early stage of antigenic stimulation in directing the functional differentiation of CD4<sup>+</sup> T cell precursors from TCR  $\alpha\beta$  transgenic mice in vitro (2–4) has been largely confirmed in vivo using the murine model of infection with *L. major*. Thus, during the initial period of infection, IL-12 and IL-4 have been demonstrated to play critical roles in mediating the differentiation of Th1 and Th2 subsets as well as the ability to contain or the failure to contain lesion development, respectively (5–8).

The aberrant Th2 response developing in BALB/c mice after infection with *L. major*, resulting in progressive disease, critically depends on the production of IL-4 early after infection. This contention is supported by previous studies from this laboratory, which have shown that BALB/c mice, in contrast to mice from resistant strains, exhibit a burst of IL-4 transcripts in CD4<sup>+</sup> T cells in draining lymph nodes within 16 h of infection (9). This burst of IL-4 occurred during the period in which neutralizing IL-4 redirected protective Th1 cell development in BALB/c mice (8).

Comparing the intrinsic tendencies of CD4<sup>+</sup> T cells from TCR  $\alpha\beta$  transgenic mice from different genetic backgrounds to mature into either Th1 or Th2 effector cells in vitro, naive CD4<sup>+</sup> T cells from BALB/c mice were shown to preferentially differentiate toward the Th2 phenotype after priming under otherwise neutral conditions, i.e., in the absence of exogenous cytokines (10). This biased Th2 cell development has been recently proposed to proceed from a rapid loss of responsiveness to IL-12 by BALB/c CD4<sup>+</sup> T cells during priming in vitro (11). The IL-12R comprises two components, the IL-12R  $\beta$ 1 and the IL-12R  $\beta$ 2 subunits (12), which are expressed only on T cells having engaged their TCR (13). Recent data have clearly shown that extinction of IL-12 signaling during priming in vitro of naive CD4<sup>+</sup> T cells from TCR  $\alpha\beta$  transgenic BALB/c mice results from a selective loss of expression the IL-12R  $\beta$ 2-chain (13), which is necessary for IL-12 signaling through the JAK/STAT pathway (14). The presence of IL-4 during priming in vitro strongly inhibited the expression of the IL-12R  $\beta$ 2-chain by activated T cells, an effect that was over-riden by IFN- $\gamma$  (13).

In this context we have recently shown that from 48 h after infection with *L. major*, administration of exogenous IL-12 to BALB/c mice becomes ineffective in preventing the development of a Th2 response (15). At this time, CD4<sup>+</sup> T cells from infected BALB/c mice no longer responded to IL-12 in terms of IFN- $\gamma$

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production in vitro. Based on these findings, we initiated studies aimed at analyzing the molecular basis of this state of unresponsiveness to IL-12 rapidly induced in vivo following infection of BALB/c mice with *L. major* and its regulation by other cytokines.

## Materials and Methods

### Mice

Female BALB/c and C57BL/6 mice were purchased from IFFA Credo (St. Germain sur l'Abresle, France). BALB/c mice homozygous for disrupted IFN- $\gamma$ R  $\alpha$ -chain gene (IFN- $\gamma$ R<sup>-/-</sup>) (16) were derived from IFN- $\gamma$ R<sup>-/-</sup> mice on the Sv129 genetic background (17) by seven serial backcrosses to BALB/c mice. All mice were used at 6–8 wk of age.

### Parasites, infection, and treatment of mice

*L. major* LV 39 (MRHO/Sv/59/P strain) were maintained in vivo and grown in vitro as previously described (18). Mice were infected s.c. in the hind footpads with  $3 \times 10^6$  stationary phase *L. major* promastigotes in a final volume of 50  $\mu$ l. At various times following infection, mice were sacrificed, and their popliteal lymph nodes were removed for further analysis.

Recombinant murine IL-12 was provided by Hoffmann-La Roche (Nutley, NJ). Recombinant IFN- $\gamma$  was provided by Genentech (South San Francisco, CA). BALB/c mice were treated i.p. with one injection of 1  $\mu$ g of IL-12 or  $10^5$  U of IFN- $\gamma$  at various times with regard to infection. Antimurine IL-4 and anti-murine IFN- $\gamma$  mAb were produced from the 11B11 rat hybridoma (19) or the rat XMG 1.2 hybridoma (20), respectively, and purified as previously described (15). Mice were treated with 1 mg of the corresponding Ab at various time points with regard to infection.

### RNA extraction, and competitive and qualitative PCR

Total RNA was isolated from popliteal lymph node cells as previously described (21). First-strand cDNA synthesis was performed on total RNA using a first-strand cDNA synthesis kit (Pharmacia, Uppsala, Sweden). The semiquantitative PCR developed by Reiner et al. (22) was performed using the competitor construct PQRS containing sequences for multiple cytokines. Primer sequences (hypoxanthine guanine phosphoribosyl transferase (HPRT)<sup>4</sup> and IL-4) and PCR conditions were used as described by the authors. The first-strand cDNA was used directly as a template in the presence of fivefold serial dilutions of the competitor plasmid. After separation of the PCR products by electrophoresis in agarose gel containing ethidium bromide, the ratio of the relative concentration of the IL-4 gene to the relative concentration of HPRT was calculated (22). Results are expressed as the fold increase in mRNA expression in mice infected with *L. major* compared with that in noninfected mice.

Expression of IL-12R  $\beta$ 1- and  $\beta$ 2-chain mRNA was monitored using a qualitative PCR assay. HPRT levels for all samples of a given experiment were assessed by the PCR method described by Reiner et al. (22). All samples were normalized with respect to their HPRT content. Using normalized samples and control plasmids containing the respective cDNA (12, 23), IL-12R  $\beta$ 1- and  $\beta$ 2-specific PCR reactions were performed using the following primers:  $\beta$ 1 3', 5'-ATT CTT GGG GTT CTT GGA GGC-3';  $\beta$ 1 5', 5'-GCC AAG ATT AAG TTC TTG GTG-3';  $\beta$ 2 3', 5'-GCG TCG GTA CTG AAT TTC GCA-3'; and  $\beta$ 2 5', 5'-GGG AGT ACA TAG TGG AAT GGA-3'. The PCR conditions used for the amplification of the IL-12R  $\beta$ 1- and  $\beta$ 2-chain cDNA were similar to those used for HPRT cDNA, except for the annealing temperature, which was 58°C. PCR products were visualized on 1% agarose gels stained with ethidium bromide and scored for expression or lack of expression. All reactions were repeated two or three times to ensure reproducibility.

### Lymphocyte cultures and detection of IFN- $\gamma$ in supernatants

CD4<sup>+</sup> T cells were purified from the draining popliteal lymph nodes by magnetic cell sorting (Miltenyi Biotech, Bergish-Gladbach, Germany) according to the manufacturer's procedure. Cells ( $5 \times 10^5$ ) were stimulated with UV-irradiated *L. major* promastigotes ( $1 \times 10^6$ ) in the presence of  $5 \times 10^6$  irradiated (3000 rad) spleen cells from normal mice in the presence or the absence of IL-12 (10 ng/ml) in a final volume of 1 ml. Cells were cultured in DMEM supplemented with 5% heat-inactivated FCS, L-glutamine (216 mg/ml),  $5 \times 10^{-5}$  M 2-ME, and 10 mM HEPES in an atmosphere of 7% CO<sub>2</sub>. Culture supernatants were collected after 72 h of stimulation and were stored at -20°C until use. IFN- $\gamma$  was measured in

supernatants by ELISA as previously described (24). Mouse rIFN- $\gamma$  (supernatant of L1210 cells transfected with the murine IFN- $\gamma$  gene, gift from Y. Wanabe, Kyoto University, Kyoto, Japan) was used as standard. The limit of the detection of the assay was 10 U/ml.

### Statistics

Data were analyzed statistically by Student's *t* test.

## Results

### The responsiveness to IL-12 of CD4<sup>+</sup> T cells from mice infected with *L. major* correlates with the expression of the IL-12R $\beta$ 2-chain

In a previous study we observed that from 48 h after infection with *L. major*, treatment of BALB/c mice with exogenous IL-12 was no longer capable of inhibiting the development of a CD4<sup>+</sup> Th2 response, reflected by increased IL-4 mRNA expression from day 5 of infection, or enhancing IFN- $\gamma$  mRNA expression. This loss of responsiveness to IL-12 in vivo was corroborated by results showing that 3 days after infection, CD4<sup>+</sup> T cells in draining lymph nodes of BALB/c mice were no longer responsive to IL-12 in terms of IFN- $\gamma$  production following specific activation in vitro (15). This state of unresponsiveness was prevented by neutralization of the IL-4 produced during the first days of infection.

Experiments were designed to assess whether the unresponsiveness to IL-12 rapidly induced in CD4<sup>+</sup> T cells of BALB/c mice following infection with *L. major* proceeded from a loss of transcription of the IL-12R  $\beta$ 2-chain gene. The results in Fig. 1 confirm that 5 days after infection with *L. major*, CD4<sup>+</sup> T cells from draining lymph nodes of BALB/c mice specifically stimulated with *L. major* in vitro do not respond to IL-12 in terms of enhanced IFN- $\gamma$  production. RT-PCR analysis revealed that the IL-12R  $\beta$ 2 subunit mRNA was completely absent in CD4<sup>+</sup> lymph node T cells of BALB/c mice 5 days after infection with *L. major* (Fig. 2). In contrast, similar analysis in resistant C57BL/6 mice showed that CD4<sup>+</sup> T cells were responsive to IL-12 and expressed IL-12R  $\beta$ 2 mRNA at high levels. The CD4<sup>+</sup> T cells from BALB/c mice treated with anti-IL-4 mAbs at the initiation of infection also expressed high levels of the IL-12R  $\beta$ 2 mRNA (Fig. 2) and produced increased amounts of IFN- $\gamma$  upon specific activation in vitro in the presence of IL-12 (Fig. 1).

Kinetic analysis of IL-12R  $\beta$ 1 and IL-12R  $\beta$ 2 mRNA expression in CD4<sup>+</sup> T cells from C57BL/6 and BALB/c mice showed that 16 and 24 h after infection CD4<sup>+</sup> T cells from mice from both strains expressed both IL-12R  $\beta$ 1- and IL-12R  $\beta$ 2-chain mRNA (Fig. 3). On days 2 and 5, although IL-12R  $\beta$ 1 mRNA was maintained in CD4<sup>+</sup> T cells from both C57BL/6 and BALB/c mice, only C57BL/6 cells expressed IL-12R  $\beta$ 2 mRNA, which was no longer detectable in BALB/c cells. Neither IL-12R  $\beta$ 1 nor IL-12R  $\beta$ 2 mRNA was detectable in lymph node cells from noninfected mice (Fig. 3). Similar results were obtained 7 days after infection (data not shown).

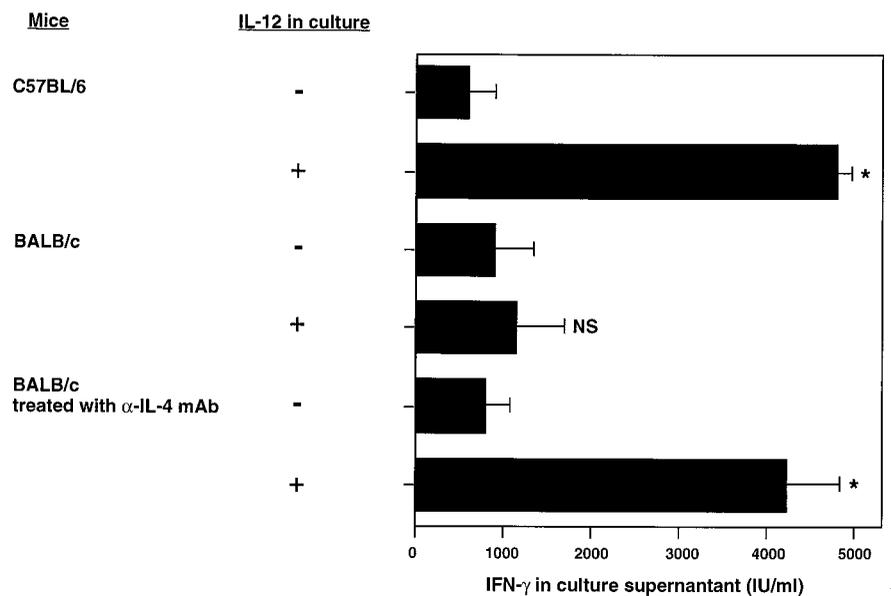
Taken together, these results strongly suggest that 1) expression of the IL-12R  $\beta$ 2 subunit by CD4<sup>+</sup> T cells is necessary for IL-12 signaling in vivo; and b) the IL-4 produced rapidly in BALB/c mice following infection with *L. major* down-regulates expression of the IL-12R  $\beta$ 2-chain in CD4<sup>+</sup> T cells.

### Effect of treatment of BALB/c mice with exogenous IL-12 or IFN- $\gamma$ on maintenance of responsiveness to IL-12 and transcription of the IL-12R $\beta$ 2 subunit

It has been shown that the presence of IL-12 or IFN- $\gamma$  during priming in vitro of TCR  $\alpha\beta$  transgenic BALB/c CD4<sup>+</sup> T cells resulted in maintenance of IL-12R  $\beta$ 2 subunit mRNA expression and prevented the inhibition of IL-12 signaling, even when IL-4 was also present in cultures (13). We therefore tested whether

<sup>4</sup> Abbreviation used in this paper: HPRT, hypoxanthine guanine phosphoribosyl transferase.

**FIGURE 1.** Responsiveness to IL-12 in vitro of CD4<sup>+</sup> T cells purified from the popliteal lymph nodes of infected mice. CD4<sup>+</sup> T cells ( $5 \times 10^5$ ) obtained 5 days after infection from either C57BL/6 or BALB/c mice (four mice per group) treated or not with 1 mg of anti-IL-4 mAb 16 h before infection with  $3 \times 10^6$  *L. major* were stimulated with  $10^6$  UV-irradiated parasites in the presence of  $5 \times 10^6$  irradiated (3000 rad) spleen cells from syngeneic normal mice in the absence or the presence of exogenous IL-12 (10 ng/ml). After 72 h of culture, IFN- $\gamma$  production in supernatants was measured by ELISA as described in *Materials and Methods*. For each determination, background levels of IFN- $\gamma$  detected in cultures without *L. major* were subtracted. Bars represent the mean and SD of triplicate determinations. Similar results were obtained in three individual experiments. Statistical significance for cultures containing IL-12 vs control cultures is indicated as follows: NS, not significant; \*,  $p < 0.05$ .



treatment of BALB/c mice with exogenous IL-12 or IFN- $\gamma$  at various times after inoculation of parasites resulted in maintenance of both responsiveness to IL-12 and IL-12R  $\beta$ 2 mRNA expression.

The results in Figs. 4 and 5 show that 5 days after infection, CD4<sup>+</sup> T cells from the draining lymph nodes of BALB/c mice given IFN- $\gamma$  at the initiation of infection or 16 or 48 h later still responded to IL-12 in terms of enhanced production of IFN- $\gamma$  in vitro and expressed IL-12R  $\beta$ 2 subunit mRNA. Administration of 1  $\mu$ g of IL-12 to BALB/c mice at the time of infection or 16 h later also resulted in maintenance of IL-12 responsiveness (Fig. 4) and IL-12R  $\beta$ 2-chain mRNA expression (Fig. 5). In contrast, when given 2 days after initiation of infection, IL-12 was no longer able to either reverse the IL-12 unresponsiveness of CD4<sup>+</sup> T cells (Fig. 5) or rescue the IL-12R  $\beta$ 2 subunit mRNA expression (Fig. 4). These results show that IFN- $\gamma$ , in contrast to IL-12, is effective in

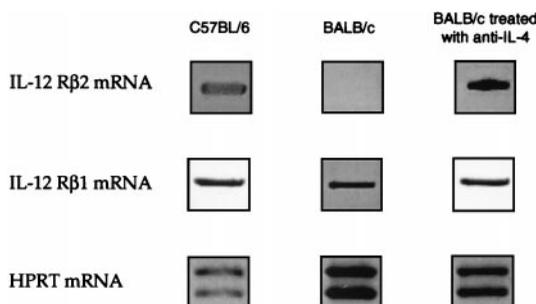
maintaining IL-12 signaling in CD4<sup>+</sup> T cells in vivo even if administered when IL-4 has been produced during 48 h.

*The effects of exogenous IL-12 on the maintenance of IL-12 responsiveness and on the expression of the IL-12R  $\beta$ 2 subunit are IFN- $\gamma$  dependent*

The effect of IL-12 on the differentiation of TCR  $\alpha\beta$  transgenic CD4<sup>+</sup> T cells toward the Th1 phenotype in vitro has been shown to require IFN- $\gamma$  (25). Furthermore, we have recently shown that exogenous IL-12 given to BALB/c mice during the first day of infection significantly enhanced IFN- $\gamma$  mRNA expression in draining lymph node cells (15). Thus, it was relevant to assess whether the capacity of treatment with IL-12 during the first day of infection to maintain IL-12 responsiveness and IL-12R  $\beta$ 2 subunit mRNA expression in draining lymph node CD4<sup>+</sup> T cells was the consequence of the IFN- $\gamma$  produced in vivo.

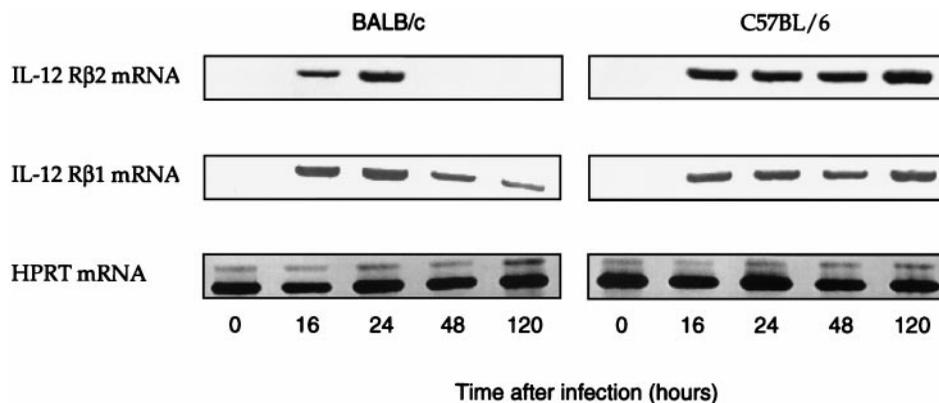
The results in Fig. 6 confirm that treatment of BALB/c mice with exogenous IL-12 during the first day of infection results in maintenance of responsiveness to IL-12 of specifically stimulated CD4<sup>+</sup> T cells obtained 5 days after infection. However, when the IFN- $\gamma$  produced in vivo in response to treatment with IL-12 was neutralized by administration of a specific mAb, the CD4<sup>+</sup> T cells became unresponsive to IL-12 in vitro in terms of enhanced IFN- $\gamma$  production (Fig. 6). The strict correlation between the IL-12 responsiveness and the expression of IL-12R  $\beta$ 2-chain mRNA is exemplified further by results showing that the maintenance of IL-12R  $\beta$ 2-chain mRNA expression by CD4<sup>+</sup> T cells from BALB/c mice as a result of administration of exogenous IL-12 during the first day of infection is abrogated following neutralization of the IFN- $\gamma$  produced in vivo (Fig. 7). To confirm the IFN- $\gamma$  dependence of the effect of treatment of BALB/c mice with IL-12 on the maintenance of responsiveness to IL-12 by CD4<sup>+</sup> T cells, this effect of IL-12 was investigated in IFN- $\gamma$ R<sup>-/-</sup> BALB/c mice.

The results in Fig. 6 clearly show that 5 days after infection, CD4<sup>+</sup> T cells from BALB/c IFN- $\gamma$ R<sup>-/-</sup> mice, treated or not with exogenous IL-12 at the initiation of infection, did not produce increased amounts of IFN- $\gamma$  when specifically stimulated in vitro in the presence of IL-12. These mice developed a polarized Th2 response (data not shown).



**FIGURE 2.** Expression of the IL-12R  $\beta$ 1- and IL-12R  $\beta$ 2-chains mRNA in CD4<sup>+</sup> T cells from draining lymph nodes of mice 5 days after infection with *L. major*. Five days after infection, RNA was extracted from CD4<sup>+</sup> T cells purified from the popliteal lymph nodes of C57BL/6 or BALB/c mice (four mice per group) treated or not with 1 mg anti-IL-4 mAb 16 h before infection. Expression of IL-12R  $\beta$ 1- and IL-12R  $\beta$ 2-chain mRNA was monitored by RT-PCR as described in *Materials and Methods*. All samples were normalized with respect to HPRT content. Results are from one of three experiments that gave similar results.

**FIGURE 3.** Kinetics of IL-12R  $\beta$ 1- and IL-12R  $\beta$ 2-chain mRNA expression in CD4<sup>+</sup> lymph node T cells of BALB/c and C57BL/6 mice following infection with *L. major*. At different times after infection, RNA was extracted from the popliteal lymph node CD4<sup>+</sup> T cells of C57BL/6 and BALB/c mice (four mice per group). Expression of IL-12R  $\beta$ 1- and IL-12R  $\beta$ 2-chain mRNA was monitored by RT-PCR as described in *Materials and Methods*. All samples were normalized with respect to their HPRT content. Results are from one of two experiments that gave similar results.



*Inhibition of IL-12R  $\beta$ 2-chain mRNA expression pertains to the development of a polarized Th2 response in BALB/c mice infected with *L. major**

Loss of IL-12 responsiveness could represent an early step in the engagement of CD4<sup>+</sup> T cells to the Th2 pathway of differentiation. Therefore, experiments were designed to assess, in BALB/c mice, whether the differentiation of disparate Th subsets following infection with *L. major* correlates with the maintenance or inhibition of expression of IL-12R  $\beta$ 2-chain mRNA in CD4<sup>+</sup> T cells, leading to the maintenance or loss of IL-12 signaling, respectively.

Following the initial burst of IL-4 mRNA expression within 16 h in CD4<sup>+</sup> T cells in draining lymph nodes of BALB/c mice in response to *L. major*, we have documented a second wave of IL-4 transcripts in CD4<sup>+</sup> T cells from day 5 of infection onward, reflecting the development of a polarized Th2 response (15). We have shown above that treatment of BALB/c mice with either exogenous IFN- $\gamma$  or IL-12 during the first day of infection resulted in the maintenance of IL-12 responsiveness and IL-12R  $\beta$ 2-chain mRNA expression in draining lymph node CD4<sup>+</sup> T cells 5 days after infection. Thus, we further studied the effects of these treatments on the IL-4 mRNA expression normally seen in control infected BALB/c mice on day 5. The results depicted in Fig. 8 show that administration of either IFN- $\gamma$  or IL-12 to BALB/c mice at the time of parasite inoculation or 16 h later resulted in a clear inhibition of the IL-4 mRNA expression normally seen in CD4<sup>+</sup> T cells from control infected mice.

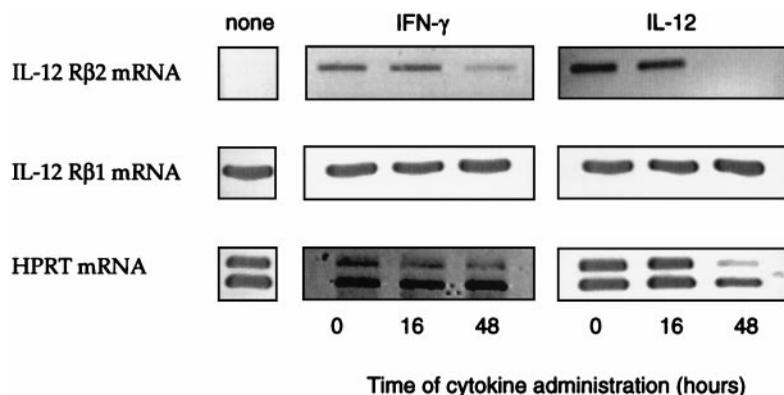
The inhibitory effect of IL-12 on IL-4 mRNA expression was abrogated by administration of neutralizing anti-IFN- $\gamma$  Abs (Fig. 5). Although inhibition of IL-4 mRNA expression was still observed when IFN- $\gamma$  was given 48 h after infection, mice given IL-12 48 h after infection exhibited increased IL-4 mRNA levels similar to those seen in control infected mice (Fig. 8). Identical

results were observed when levels of IL-4 mRNA were determined 10 days after infection (data not shown), indicating that these treatments did not simply delay IL-4 mRNA expression. Together, these data show that 1) the loss of IL-12R  $\beta$ 2-chain mRNA expression and IL-12 signaling in CD4<sup>+</sup> T cells from BALB/c mice infected with *L. major* correlates with Th2 cell development; and b) the maintenance of IL-12R  $\beta$ 2-chain mRNA expression and IL-12 signaling in vivo following administration of exogenous cytokines is clearly related to an inhibition of Th2 cell development.

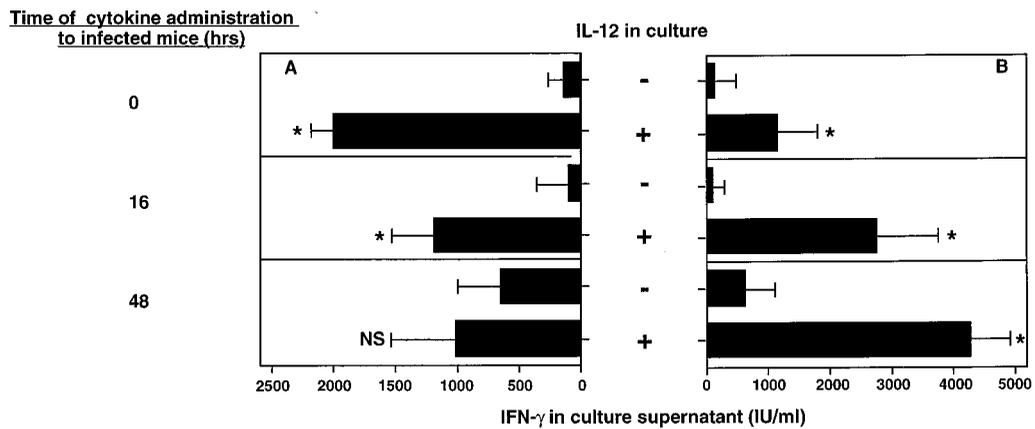
## Discussion

The data presented in this report provide the molecular basis for, on the one hand, the rapid induction of a state of unresponsiveness to IL-12 in CD4<sup>+</sup> T cells from susceptible BALB/c mice (15) and, on the other hand, the maintenance of responsiveness to IL-12 in CD4<sup>+</sup> T cells from resistant C57BL/6 mice following infection with *L. major*. We show that CD4<sup>+</sup> T cells obtained from both C57BL/6 and BALB/c mice express both chains of the IL-12R as soon as 16 h after infection with *L. major*. However, although IL-12R  $\beta$ 2-chain expression is maintained on CD4<sup>+</sup> T cells from C57BL/6 mice at least up to 5 days after infection, IL-12R  $\beta$ 2 subunit mRNA expression is readily down-regulated on BALB/c CD4<sup>+</sup> T cells from day 2 on.

The expression of the IL-12R  $\beta$ 1-chain was maintained on CD4<sup>+</sup> cells from mice from both strains. The results obtained demonstrate that maintenance or loss of IL-12R  $\beta$ 2-chain mRNA expression by CD4<sup>+</sup> T cells in vivo strictly correlates with their responsiveness or lack of responsiveness to IL-12 in vitro, respectively. Thus, expression of the IL-12R  $\beta$ 1-chain by itself does not appear crucial for IL-12 signaling (13). Neither chain of the IL-12R was expressed on CD4<sup>+</sup> T cells from noninfected C57BL/6 or BALB/c mice, indicating that in vivo TCR engagement is also



**FIGURE 4.** Effect of the administration of IL-12 or IFN- $\gamma$  to BALB/c mice on the expression of IL-12R  $\beta$ 2-chain mRNA in CD4<sup>+</sup> lymph nodes T cells 5 days after infection with *L. major*. One microgram of IL-12 or 10<sup>5</sup> U of IFN- $\gamma$  was administered i.p. at the time of infection with 3  $\times$  10<sup>6</sup> *L. major* (0) or 16 or 48 h later. Similarly infected BALB/c mice not treated with IL-12 or IFN- $\gamma$  were used as controls. Five days after infection, mice from these groups (four mice per group) were killed, RNA was extracted from their popliteal lymph node CD4<sup>+</sup> T cells, and expression of IL-12R  $\beta$ 1- and IL-12R  $\beta$ 2-chain mRNA was monitored by RT-PCR as described in *Materials and Methods*. All samples were normalized with respect to HPRT content. Results are from one of two experiments that gave similar results.

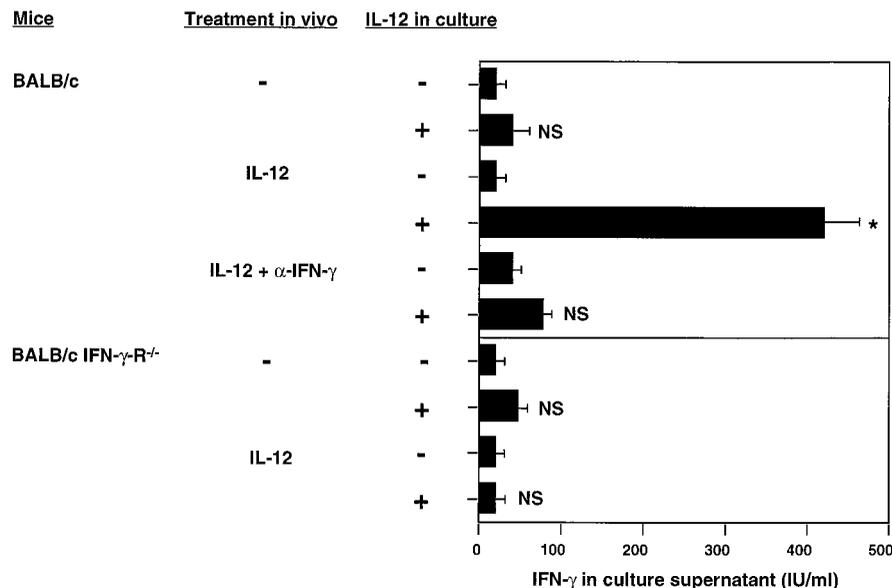


**FIGURE 5.** Effect of administration of IL-12 or IFN- $\gamma$  to BALB/c mice on the responsiveness to IL-12 in vitro of CD4<sup>+</sup> lymph node cells isolated 5 days after infection with *L. major*. One microgram of IL-12 (A) or 10<sup>5</sup> U of IFN- $\gamma$  (B) was administered i.p. at the time of infection with  $3 \times 10^6$  *L. major* (0) or 16 or 48 h later. Five days after infection,  $5 \times 10^5$  popliteal lymph node CD4<sup>+</sup> T cells from mice of each group (four mice per group) were stimulated in vitro with 10<sup>6</sup> UV-irradiated parasites in the presence of  $5 \times 10^6$  irradiated (3000 rad) spleen cells and in the absence or the presence of exogenous IL-12 (10 ng/ml). After 72 h of culture, IFN- $\gamma$  production in supernatants was measured by ELISA as described in *Materials and Methods*. For each determination, background levels of IFN- $\gamma$  detected in cultures without *L. major* were subtracted. Bars represent the mean and SD of triplicate determinations. Similar results were obtained in two separate experiments. Statistical significance for cultures containing IL-12 vs control cultures is indicated as follows: NS, not significant; \*,  $p < 0.05$ .

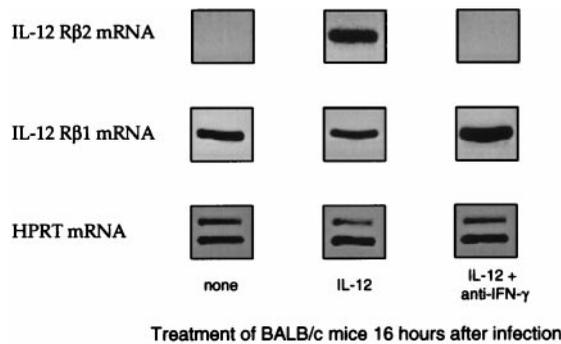
necessary for the expression of IL-12R. Together, these results demonstrate the importance of the IL-12R  $\beta 2$  subunit for IL-12 signaling in vivo. A similar conclusion was reached from the results of experiments performed in vitro, using human and murine cell lines and TCR  $\alpha\beta$  transgenic CD4<sup>+</sup> T cells (13, 26).

Down-regulation of IL-12R  $\beta 2$ -chain expression on CD4<sup>+</sup> T cells from BALB/c mice was prevented by neutralization of the IL-4 produced as a result of the early IL-4 transcriptional burst

previously shown to occur in draining lymph nodes within 16 h after the s.c. injection of *L. major* (9). Neutralization of this IL-4 at the initiation of infection has been demonstrated to be capable of redirecting protective Th1 cell development in BALB/c mice (8, 15). Furthermore, previous results from our laboratory have shown that the production of IL-4 is necessary only during a short period of time (<48 h after infection) for irreversible Th2 cell development to occur in BALB/c mice (15). Interestingly, we show here



**FIGURE 6.** Maintenance of responsiveness to IL-12 in vitro of CD4<sup>+</sup> T cells from BALB/c mice 5 days after infection with *L. major* as a result of treatment with exogenous IL-12 during the first day of infection is IFN- $\gamma$  dependent. Sixteen hours after infection with  $3 \times 10^6$  *L. major*, wild-type and IFN- $\gamma$ R<sup>-/-</sup> BALB/c mice were given 1  $\mu$ g of IL-12 i.p., and some wild-type BALB/c mice were also treated with 1 mg of anti-IFN- $\gamma$  mAb. Similarly infected wild-type BALB/c mice, not treated with IL-12 and anti-IFN- $\gamma$  mAb, and IFN- $\gamma$ R<sup>-/-</sup> BALB/c mice not treated with IL-12 were used as controls. Five days after infection CD4<sup>+</sup> T cells obtained from mice from these groups (four mice per group) were stimulated in vitro with 10<sup>6</sup> UV-irradiated parasites in the presence of  $5 \times 10^6$  irradiated (3000 rad) spleen cells and in the absence or the presence of exogenous IL-12 (10 ng/ml). After 72 h of culture, IFN- $\gamma$  production in supernatants of cultures was measured by ELISA as described in *Materials and Methods*. For each determination, background levels of IFN- $\gamma$  detected in cultures without *L. major* were subtracted. Bars represent the mean and SD of triplicate determinations. Similar results were obtained in two separate experiments. Statistical significance for cultures containing IL-12 vs control cultures is indicated as follows: NS, not significant; \*,  $p < 0.05$ .



**FIGURE 7.** The ability of exogenous IL-12 given to BALB/c mice during the first day of infection with *L. major* to rescue IL-12R  $\beta$ -chain mRNA expression is IFN- $\gamma$  dependent. Sixteen hours after infection with  $3 \times 10^6$  *L. major*, BALB/c mice were given 1  $\mu$ g of IL-12 i.p. and treated or not with 1 mg of anti-IFN- $\gamma$  mAb. Similarly infected but not treated BALB/c mice were used as controls. Five days after infection, mice from these groups (four mice per group) were killed, RNA was extracted from their lymph node CD4<sup>+</sup> T cells, and expression of IL-12R  $\beta$ 1- and IL-12R  $\beta$ 2-chain mRNA was monitored by RT-PCR as described in *Materials and Methods*. All samples were normalized with respect to their HPRT content. Results are from one of two experiments that gave similar results.

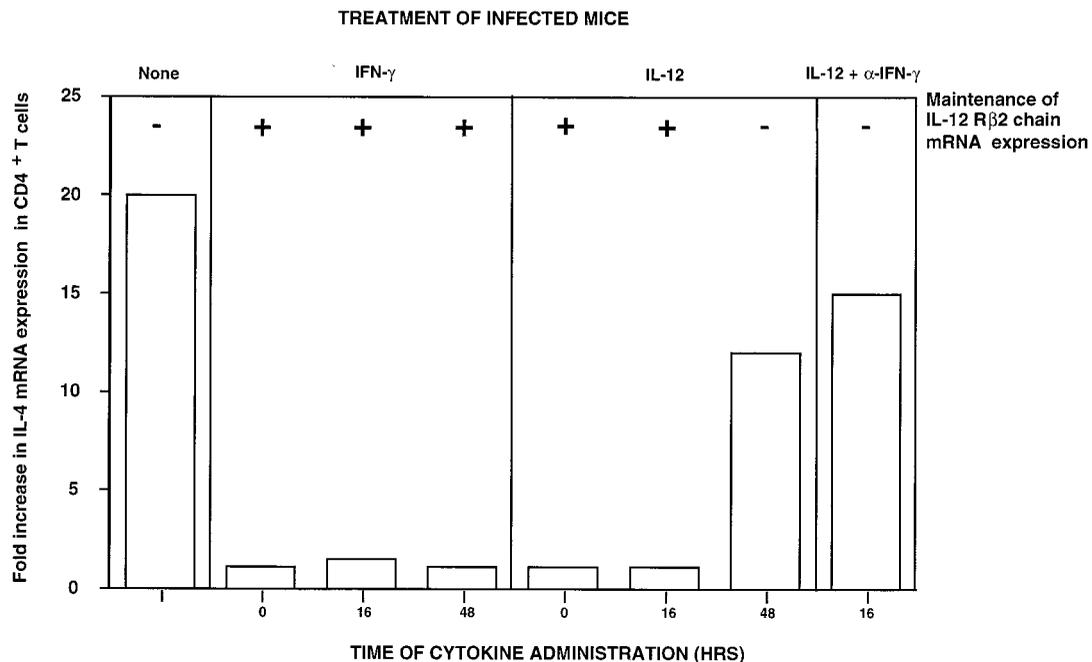
that the IL-4-mediated inhibition of the IL-12R  $\beta$ 2 chain expression in CD4<sup>+</sup> T cells from BALB/c mice also occurs 48 h after infection. In contrast, CD4<sup>+</sup> T cells from resistant C57BL/6 mice, in which Th1 cell maturation does occur, maintain IL-12R  $\beta$ 2-chain expression and IL-12 responsiveness at least up to the time when the functional differentiation of CD4<sup>+</sup> T cells starts.

Together, these results strongly suggest that in vivo the maintenance or loss of IL-12R  $\beta$ 2-chain expression and the resulting responsiveness or lack of responsiveness to IL-12 represent early

and important steps in the stable commitment of naive CD4<sup>+</sup> T cells to the Th1 or Th2 pathway of differentiation, respectively. This conclusion is supported by our recent results, which have shown that the inhibition of Th2 cell development and the striking redirection of the resulting effector cells to the Th1 phenotype observed in BALB/c mice following treatment with anti-IL-4 mAb at the onset of infection with *L. major* require IL-12 (15). By losing the expression of the IL-12R  $\beta$ 2 subunit, CD4<sup>+</sup> T cells induced by IL-4 to differentiate to the Th2 pathway rapidly become refractory to the Th1-differentiating signals of IL-12.

The observations reported here reinforce the critical importance of the IL-4 produced in BALB/c mice during the first day of infection for the subsequent differentiation of parasite-specific CD4<sup>+</sup> T cell precursors toward the Th2 phenotype. In this context we have recently observed that the early burst of IL-4 mRNA expression seen in draining lymph nodes of BALB/c mice within 16 h of infection occurs within CD4<sup>+</sup> T cells that express the V $\beta$ 4 V $\alpha$ 8 TCR chains and recognize the LACK Ag of *L. major* (27). This rapidly produced IL-4 has an essential role in instructing subsequent Th2 cell differentiation and consequently susceptibility to *L. major*, since BALB/c mice lacking CD4<sup>+</sup> T cells expressing the V $\beta$ 4 TCR chain do not exhibit an early IL-4 response to *L. major*, subsequently develop a Th1 response, and are fully resistant to infection (27). Interestingly, CD4<sup>+</sup> T cells from these V $\beta$ 4 CD4<sup>+</sup> T cell-deficient BALB/c mice maintained their expression of the IL-12R  $\beta$ 2-chain at least up to 8 days after infection with *L. major* (H. Himmelrich et al., in preparation).

These results strongly suggest that in the absence of IL-4, differentiating CD4<sup>+</sup> T cells maintain expression of the IL-12R  $\beta$ 2 subunit and default to the Th1 pathway. Thus, it is possible that the presently observed maintenance of the IL-12R  $\beta$ 2-chain expression on CD4<sup>+</sup> T cells from C57BL/6 mice following infection



**FIGURE 8.** Down-regulation of IL-12R  $\beta$ 2-chain mRNA expression resulting in IL-12 unresponsiveness in CD4<sup>+</sup> T cells correlates with the development of a Th2 response. One microgram of IL-12 or  $10^5$  U of IFN- $\gamma$  was administered i.p. to BALB/c mice at the time of infection with  $3 \times 10^6$  *L. major* (0) or 16 or 48 h later. Similarly infected, but not treated, BALB/c mice were used as controls. On day 5 postinfection, mice from these groups (three mice per group) were killed, RNA was extracted from their pooled lymph node CD4<sup>+</sup> T cells, and the relative levels of IL-4 mRNA were determined by semiquantitative competitive RT-PCR as described in *Materials and Methods*. For untreated mice, the results are expressed as the fold increase in IL-4 mRNA in mice infected with *L. major* compared with that in noninfected mice. For treated mice, results are expressed as the increase in infected mice treated with IL-12 or IFN- $\gamma$  compared with that in similarly treated, noninfected mice. Results are from one of two experiments that gave similar results.

with *L. major* results from the fact that these mice do not produce sufficient amounts of IL-4 in response to infection. It is noteworthy that we never observed a significant increase in IL-4 mRNA expression in draining lymph nodes of C57BL/6 mice following infection with *L. major* (9). Similarly, the preferential differentiation of TCR  $\alpha\beta$  transgenic CD4<sup>+</sup> T cells toward the Th2 phenotype when specifically primed in vitro in the presence of IL-4 has been extensively demonstrated (4, 27). This effect has been recently correlated with a selective loss of IL-12R  $\beta$ 2-chain expression and IL-12 signaling (13). The genetic tendency of naive CD4<sup>+</sup> T cells from BALB/c mice to differentiate to the Th2 pathway in vitro (10) has been proposed to be the consequence of a loss of IL-12 signaling rather than an overproduction of IL-4 (11). Nonetheless, neutralization of IL-4 with anti-IL-4 mAb during priming in vitro of TCR  $\alpha\beta$  transgenic CD4<sup>+</sup> BALB/c T cells resulted in maturation of a Th1 response (4), confirming the critical role of the small amount of IL-4 produced during priming on Th2 cell development. These data combined with our results showing that the IL-4 rapidly produced in BALB/c mice in response to *L. major* readily down-regulates the expression of the IL-12R  $\beta$ 2 subunit on CD4<sup>+</sup> T cells, rather, suggest that the genetically determined tendency to develop a Th2 response, resulting in susceptibility to infection with *L. major*, is primarily based on an up-regulation of IL-4 production that secondarily induces extinction of IL-12 signaling.

The role of IL-4 in Th2 cell development and susceptibility of BALB/c mice to infection with *L. major* has been questioned by results that have shown that IL-4 knockout BALB/c mice neither heal their cutaneous lesions nor switch their CD4<sup>+</sup> T cell response toward the Th1 phenotype (28). Even though other studies performed using BALB/c mice with homologous disruption of the IL-4 gene have led to conflicting results (29), it is possible that the commitment of CD4<sup>+</sup> T cells, developing in the IL-4 free environment of IL-4 knockout mice, to the Th2 pathway is driven by a factor(s) different from the IL-4 normally directing the Th2 maturation of CD4<sup>+</sup> T cells in wild-type BALB/c mice. This hypothesis deserves experimental testing.

Treatment of BALB/c mice with either IFN- $\gamma$  or IL-12 at the initiation of infection resulted in the maintenance of IL-12R  $\beta$ 2-chain expression induced on CD4<sup>+</sup> T cells following TCR engagement and responsiveness to IL-12. This effect cannot simply be attributed to the ability of these cytokines to down-regulate the early burst of IL-4 transcripts occurring in these mice following infection with *L. major* (9), since exogenous IFN- $\gamma$  was still effective in maintaining IL-12R  $\beta$ 2-chain expression and IL-12 signaling even if given when IL-4 had been produced for 48 h. The effect of exogenous IL-12 was clearly the result of the IFN- $\gamma$  produced in vivo following this treatment, since it was abrogated by neutralization of IFN- $\gamma$ . Thus, the lack of effect of IL-12 when administered 48 h after infection, i.e., at a time when CD4<sup>+</sup> T cells from infected BALB/c mice have lost expression of the IL-12R  $\beta$ 2 subunit, probably results from its inability to signal and induce IFN- $\gamma$  production. These results imply that IL-12 by itself does not up-regulate the expression of the  $\beta$ 2-chain of its receptor. They also confirm previous data obtained in vitro (13), which have shown that IFN- $\gamma$  is capable of superseding the inhibition of IL-12R  $\beta$ 2-chain expression caused by IL-4.

As already suggested, this effect of IFN- $\gamma$  could explain the requirement for this cytokine, observed in some experimental systems, in the IL-12-generated development of Th1 cell in vitro (25). Indeed, under conditions when IL-4 is produced during priming in vitro, the presence of IFN- $\gamma$ , by its ability to maintain IL-12R  $\beta$ 2 subunit mRNA expression, will permit the IL-12 signaling necessary for Th1 cell maturation (13). In this context, it has been shown that 3 days after infection with *L. major*, lymph nodes from

BALB/c mice treated with IFN- $\gamma$  produce, upon specific stimulation in vitro, substantially less IL-4 and more IFN- $\gamma$  than lymph nodes from similarly infected mice not administered IFN- $\gamma$  (30). Furthermore, treatment with IFN- $\gamma$  of BALB/c mice infected with *L. major*, although not significantly altering the course of disease, results in significant reduction of the serum IgE levels, which are normally elevated in these mice following infection as a result of sustained IL-4 production (8). When IL-4 is not produced during priming in vitro, IL-12R  $\beta$ 2-chain expression is maintained on activated CD4<sup>+</sup> T cells, allowing IL-12 to induce Th1 cell development independently of IFN- $\gamma$  (13). This contention is supported by results obtained in vivo showing that administration of exogenous IL-12 at the onset of infection to resistant C57BL/6 mice with disruption of the IFN- $\gamma$  gene markedly suppressed the IL-4 mRNA expression otherwise seen in these mice 5 days after infection (31). Furthermore, we have recently shown that IFN- $\gamma$ R<sup>-/-</sup> mice on a resistant 129/Sv/Ev background, although susceptible to infection as a result of the inability of their macrophages to receive the IFN- $\gamma$ -mediated activating signals, develop a polarized Th1 response, in contrast to BALB/c or BALB/c IFN- $\gamma$ R<sup>-/-</sup> mice (21). In contrast to BALB/c IFN- $\gamma$ R<sup>-/-</sup> mice, Sv129 IFN- $\gamma$ R<sup>-/-</sup> mice do not produce an early burst of IL-4 mRNA expression in their draining lymph nodes in response to *L. major* and maintain IL-12R  $\beta$ 2-chain mRNA expression on their CD4<sup>+</sup> T cells at least up to 6 days after infection (P.L. and J.A.L., unpublished results). Thus, it is likely that in the absence of IL-4, activated CD4<sup>+</sup> T cells do not require IFN- $\gamma$  signaling for the maintenance of IL-12R  $\beta$ 2-chain expression and IL-12 signaling in vivo.

It has been recently shown that treatment of BALB/c mice with a soluble protein before priming with this protein Ag in CFA diverted their CD4<sup>+</sup> T cell response toward the Th2 phenotype (32). Interestingly, administration of IL-12 together with the soluble protein Ag redirected the response to the Th1 pathway, an effect that was attributed to the expression of IL-12R  $\beta$ 2-chain mRNA expression on responding CD4<sup>+</sup> cells (32). Even though the IFN- $\gamma$  dependence of this effect of IL-12 was not studied, the injection of IFN- $\gamma$  together with the soluble protein Ag failed to redirect the response to the Th1 pathway (32). Results from other studies have clearly shown that the IL-12-induced Th1 cell development from naive precursor CD4<sup>+</sup> T cells required IFN- $\gamma$ , which by itself was not sufficient to induce Th1 cell maturation (25, 33). Thus, it is possible that treatment of BALB/c mice with soluble Ag with or without IFN- $\gamma$  would not lead to the production of IL-12 in amounts sufficient for inducing Th1 cell maturation.

In summary, this study indicates that following infection of BALB/c mice with *L. major*, the IL-4-mediated down-regulation of IL-12R  $\beta$ 2-chain expression represents an important step in the development of a Th2 cell response in vivo. In-depth understanding of the factors capable of overriding this suppression of IL-12 signaling might be instrumental for the design of strategies to prevent the induction of Th2 responses detrimental to the host following infection with intracellular micro-organisms.

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