

## **CD4<sup>+</sup> Effector Cells Default to the Th2 Pathway in Interferon $\gamma$ -deficient Mice Infected with *Leishmania major***

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### **Summary**

Mice with homologous disruption of the interferon  $\gamma$  (IFN- $\gamma$ ) gene on the C57BL/6 background were infected with *Leishmania major* and the immune response assessed. In contrast to wild-type or heterozygous knockout mice, deficient animals were unable to restrict growth of the parasite and suffered lethal infection over 6–8 wk. Although wild-type and heterozygous littermates developed CD4<sup>+</sup> cells that contained transcripts for IFN- $\gamma$  and lymphotoxin, typical of T helper type 1 (Th1) cells, the knockout mice developed CD4<sup>+</sup> cells that contained transcripts for interleukin 4 (IL-4), IL-5, and IL-13, typical of Th2 cells. ELISPOT assays confirmed the reciprocal patterns of IFN- $\gamma$  or IL-4 production by T cells in similar frequencies in the respective groups of mice, and antibody analysis confirmed the presence of Th2-mediated isotype switching in the knockout mice. These data suggest that CD4<sup>+</sup> T cells that normally respond to antigens by differentiation to Th1 cells default to the Th2 pathway in the absence of endogenous IFN- $\gamma$ .

Murine Th clones are divided into Th1 and Th2 subsets based upon the types of cytokines produced (1). Th1 cells produce IFN- $\gamma$ , lymphotoxin, and IL-2, and mediate delayed-type hypersensitivity reactions, whereas Th2 cells produce IL-4, -5, -6, -9, -10, and -13, and stimulate antibody production. Reciprocal regulation occurs between these two subsets: IFN- $\gamma$  restricts the proliferation of Th2 clones and IL-4 and IL-10 interfere with IFN- $\gamma$  production by Th1 clones. Studies using limiting dilution analysis (2) and transgenic mice (3, 4) suggest that individual naive T cells have the capacity to differentiate into either Th1 and Th2 cells, and intense interest has focused on the role of distinct cytokines in mediating the development of these effector subsets.

Experimental leishmaniasis due to *Leishmania major* comprises a well-characterized model for examining the developmental regulation of Th1 and Th2 cell differentiation in vivo (5). Th1 cells are required for protection and differentiate naturally in genetically resistant strains of mice, such as C57BL/6, whereas Th2 cells are nonprotective and differentiate naturally in genetically susceptible strains of mice, such as BALB/c. Prior studies using mice made deficient in IL-4 through gene disruption have documented the abrogation of Th2 responses in such animals after challenge with helminths, potent stimuli for Th2-biased reactions (6). We used genetically resistant mice with homologous disruption of the IFN- $\gamma$  gene to assess whether such mice develop programmed Th1 responses when challenged with *L. major*, or whether CD4<sup>+</sup> effector cells

default into the Th2 pathway in the absence of endogenous IFN- $\gamma$ .

### **Materials and Methods**

**Animals and Parasites.** Mice with targeted disruption of the IFN- $\gamma$  gene (IFN- $\gamma^{0/0}$ ) were developed and screened as described (7), backcrossed seven generations onto the CD57BL/6 background, and maintained in the University of California at San Francisco (UCSF) Transgenic Facility. 6–8-wk-old BALB/c and C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and housed in the UCSF animal facility. Groups of five to six mice were infected in the hind footpads with 10<sup>6</sup> stationary-phase promastigotes of *L. major* (strain WHOM/IR/-/173) and the course of infection monitored as described (5). Designated BALB/c mice were treated intraperitoneally with 1 mg of anti-IL-4 mAb 11B11 at the time of inoculation of the parasites in order to induce a healer phenotype as described (8).

**Collection of LN Tissues.** For analysis of cytokine transcripts, the popliteal LN were collected at designated times and flash frozen in liquid nitrogen, pulverized to a fine powder, and placed in RNazol (Biotecx, Houston, TX). For analysis of cell subsets, popliteal LN were excised, teased to a single-cell suspension, and incubated with mAb against CD4<sup>+</sup> (GK1.5; Becton Dickinson & Co., Mountain View, CA). Cells were selected in a magnetic field using secondary antibody coupled to ferrous beads (Advanced Magnetix, Inc., Cambridge, MA) as described (9). Isolations were carried out entirely at 4°C. The purity of the selected CD4<sup>+</sup> population exceeded 90% as assessed by FACS<sup>®</sup> analysis (Becton Dickinson & Co.) in select experiments. Selection by this method

resulted in no induction of cytokine transcripts in resting lymphocyte populations. Single-cell suspensions from the popliteal LN were used for the ELISPOT assays without further modification.

**Competitive RT-PCR.** Total RNA was extracted in RNazol according to the manufacturer's directions. Reverse transcription was performed using murine Moloney leukemia virus reverse transcriptase (GIBCO BRL, Bethesda, MD) and random hexamer primers (Promega, Madison, WI) as described (10). PCR was performed using a multiple cytokine-containing competitor construct as described in detail elsewhere (10). Briefly, aliquots of cDNA were assayed for levels of the constitutively expressed mRNA, hypoxanthine-guanine phosphoribosyltransferase (HPRT), by placing equal concentrations of the competitor construct in each reaction and examining the ratio of competitor to the wild-type band intensity after amplification using HPRT-specific primers. After separation on a 2.5% ethidium bromide-stained agarose gel, this visual inspection can discern differences as small as 1.25-fold (10). After adjusting the amount of experimental cDNA in order to standardize the HPRT levels to comparable intensities relative to a constant competitor concentration, cytokine transcript levels were quantitated using the competitor in the presence of cytokine-specific primers. The primers used for HPRT, IL-4, IL-5, and IFN- $\gamma$  were as described (10). Additional primers included those for lymphotoxin (5'-ATGACACTGCTCGGCCGCTCTCCA; 3'-CACTGGTGGGATCAGGAGGGAG; [11]) and IL-13 (5'-CTTGCTTGCCTTGGTGGTCTCGC, 3'-GCAGTTTGTATATAAAGTGGGCT; [12]). All analyses were repeated in three independent experiments. HPRT levels were reestablished at the end of each experiment to confirm their reproducibility. Photographic negatives of the gels were scanned with an imaging densitometer (BioRad Laboratories, Inc., Hercules, CA) in order to quantitate band intensities.

**ELISPOT Assays.** Cytokine production by individual lymphocytes was assessed by a modification of described methods (13). Single-cell suspensions were prepared from popliteal LN and distributed in duplicate aliquots containing  $10^6$  cells in  $100 \mu\text{l}$  RPMI with 5% fetal bovine serum (FBS) and antibiotics to 96-well plates (Immulon II; Dynatech, Chantilly, VA) that had been precoated with either mAb BVD4-1D11.2 against IL-4 or mAb R46A2 against IFN- $\gamma$ . In designated experiments, cells were first selected for expression of CD4<sup>+</sup> using magnetic beads. Serial threefold dilutions of cells were prepared and the plates were incubated undisturbed without further stimulation for 8 h at 37°C in a 5% CO<sub>2</sub> atmosphere. Wells were washed with PBS to remove cells and further incubated with biotinylated secondary antibodies, BVD6-24G2.3 and XMG-6, against IL-4 or IFN- $\gamma$ , respectively. After 1 h, wells were washed and  $100 \mu\text{l}$  streptavidin alkaline phosphatase (Jackson Immuno Research Laboratories, West Grove, PA) in PBS with .05% Tween 20 and 5% FBS were added for an additional hour. Color was developed using 5-bromo-4-chloro-3-indolyl phosphate in 0.1 M 2-amino-2-methyl-1-propanol buffer (both from Sigma Chemical Co., St. Louis, MO) suspended in agarose (SeaPlaque; FMC Bioproducts, Rockland, ME) and, after solidification of the agar, individual blue spots were counted using inverted microscopy.

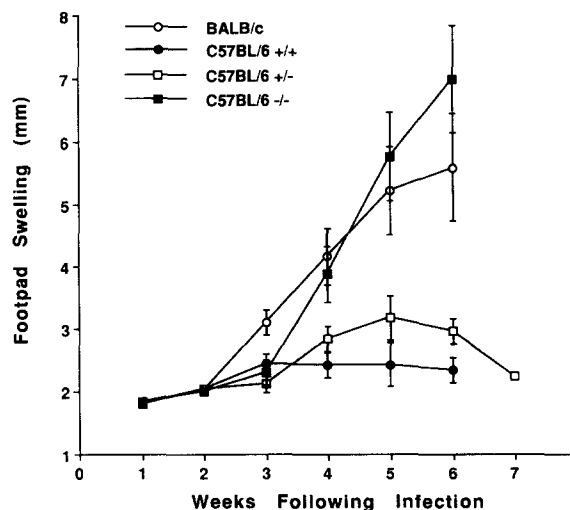
**Antibody Isotypes.** Total serum IgE was quantitated using ELISA as described (8). Leishmania-specific IgG isotypes were quantitated by incubating sera in wells that had been previously coated with  $50 \mu\text{g}$ /well soluble *L. major* antigen (8). After washing with PBS containing 0.05% Tween 20, wells were incubated with biotinylated murine anti-Ig isotype-specific mAbs (Caltag Laboratories, San Francisco, CA), washed, developed using horseradish peroxidase-streptavidin (Zymed Laboratories, San Francisco, CA) with

*o*-phenylenediamine (Sigma Chemical Co.), and quantitated using a spectrophotometer.

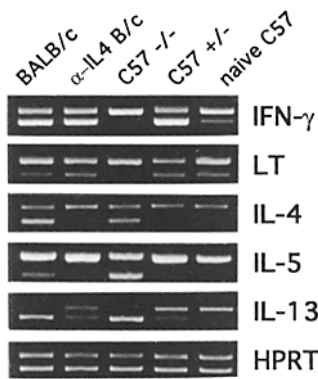
## Results

**IFN- $\gamma^{0/0}$  Mice Are Unable to Restrict the Progression of *L. major* Infection.** Mice with homozygous disruption of the IFN- $\gamma$  gene on the resistant C57BL/6 background developed progressive infection with *L. major* characterized by large lesions at the site of inoculation (Fig. 1) and sudden death from 5 to 7 wk after infection. Necropsy revealed tremendous hypertrophy of all LN and the spleen; cultures of lymph tissues and footpads revealed parasites at substantially increased numbers as compared with control animals (IFN- $\gamma^{0/0}$ :  $>10^8$  parasites per 0.1 g footpad tissue or per  $5 \times 10^6$  spleen cells; C57BL/6 heterozygote and wild type:  $<10^2$  parasites per footpad tissue or spleen). Control mice included wild-type C57BL/6 and heterozygous IFN- $\gamma$ -deficient littermates, both of which were able to restrict the progression of infection. Disease in IFN- $\gamma^{0/0}$  mice was more fulminant than in susceptible BALB/c mice, none of which died during the first 7 wk after infection (Fig. 1).

**CD4<sup>+</sup> T Cells Default to the Th2 Pathway in IFN- $\gamma^{0/0}$  Mice.** CD4<sup>+</sup> cells were purified using magnetic bead selection from LN tissues of designated groups of mice 4 wk after infection. After RNA purification and reverse transcription, cDNA was analyzed for cytokine transcripts and standardized to HPRT using a competitive PCR construct (10). Heterozygous IFN- $\gamma$ -deficient mice had transcripts for IFN- $\gamma$  and lymphotoxin that were induced 7.7- and 2.1-fold, respectively, above those in uninfected animals (Fig. 2). Similar induction of the Th1 transcripts occurred in infected wild-type C57BL/6



**Figure 1.** *L. major* infection in IFN- $\gamma$  knockout mice. Groups of five mice were infected in the hind footpads with promastigotes of *L. major* and the course of disease monitored using metric calipers to quantitate the swelling at the site of inoculation. Infected mice included susceptible BALB/c and resistant wild-type (C57BL/6 +/+), heterozygous IFN- $\gamma$  knockout mice (C57BL/6 +/-) and homozygous knockout mice (C57BL/6 -/-). Results represent mean and SE from one of three representative experiments.

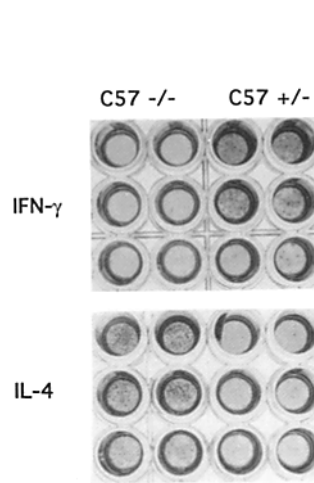


**Figure 2.** Cytokine transcripts in CD4<sup>+</sup> T cells after infection with *L. major*. CD4<sup>+</sup> cells were purified from LN from groups of four infected BALB/c, BALB/c treated with anti-IL4, C57BL/6 homozygous knockout mice (C57<sup>-/-</sup>), C57BL/6 heterozygous knockout mice (C57<sup>+/-</sup>), and uninfected C57BL/6 (naive) mice. RNA was purified and reverse transcribed, and the cDNA used for PCR-mediated amplification of the designated cytokines. Concentrations of input cDNA were standardized by equalizing ampli-

fication of the constitutively expressed HPRT transcript by use of a competitor construct containing tandemly arrayed amplification products that are larger and migrate more slowly in agarose gels. After ethidium bromide staining, quantitation of transcripts was accomplished by comparing amplification of the wild-type cDNA (lower band for each paired amplification product) with the construct product (upper band for each paired amplification product) using imaging densitometry of the photographic negative. Results are representative of three independent experiments assaying interferon- $\gamma$  (IFN- $\gamma$ ), lymphotoxin (LT), and IL-4, -5, and -13.

mice (9; data not shown). Further, Th2 cytokine transcripts, including IL-4, IL-5, and IL-13, were minimally expressed by these cells. Susceptible BALB/c mice had detectable IFN- $\gamma$  transcripts, but significantly less induction of lymphotoxin (greater than a twofold decrease) and substantial induction of IL-4, IL-5, and IL-13. Similarly, IFN- $\gamma^{0/0}$  mice had undetectable levels of lymphotoxin and substantial levels of mRNA for IL-4, IL-5, and IL-13, consistent with the appearance of Th2 cells.

Two assays were performed to confirm that biologically active IL-4 was being produced in the setting of induced IL-4 transcripts. First, ELISPOT assays, which detect focused cytokine production by lymphocytes (13), were carried out for both IL-4 and IFN- $\gamma$ . In these assays, lymphocytes isolated from infected mice were incubated over plates coated with mAb to the designated cytokine without further intervention for 8 h. After washing, cytokine trapped on the plate was identified using a second mAb before colorimetric development. Lymphocytes producing IFN- $\gamma$ , but not IL-4, were readily identified from heterozygous IFN- $\gamma$ -deficient mice, whereas the reciprocal pattern, lymphocytes producing IL-4 but not IFN- $\gamma$ , was evident in IFN- $\gamma^{0/0}$  mice (Fig. 3). The frequency of cytokine-producing cells was comparable in the two groups of mice. Similar results were obtained if CD4<sup>+</sup> lymphocytes were first enriched using magnetic bead selection before plating in the ELISPOT assay (data not shown), consistent with the prior identification of CD4<sup>+</sup> cells as the primary source of these cytokines in leishmaniasis (9). Lymphocytes from uninfected animals that generated IFN- $\gamma$  or IL-4 were <8 and 1%, respectively, than the number recovered from infected mice. Second, antibody isotypes were quantitated in serum from infected mice as an indirect measure of the ability of IL-4 to promote IgG1 and IgE and of IFN- $\gamma$  to promote IgG2a and IgG3 (14). As anticipated,



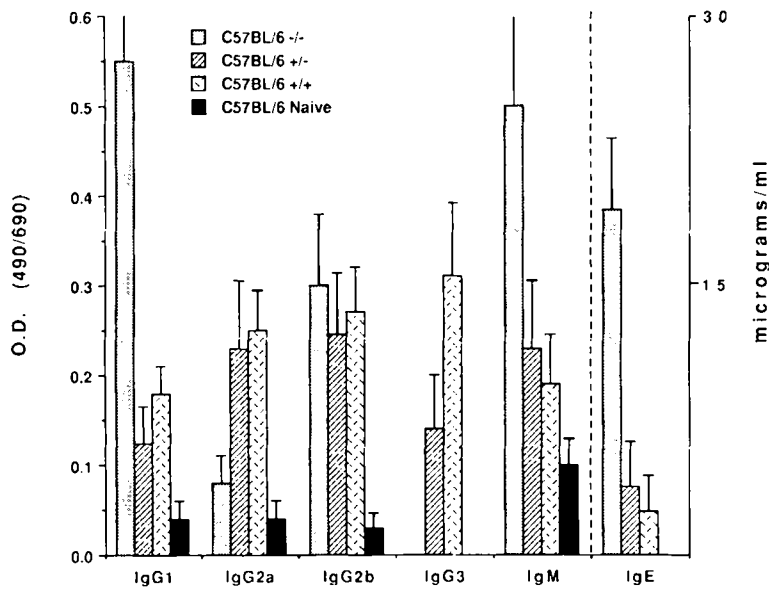
**Figure 3.** Cytokine production by LN cells after infection with *L. major*. Popliteal LN cells draining the site of infection were incubated over wells that had been precoated with mAbs against IFN- $\gamma$  (top) or IL-4 (bottom). Cells were diluted threefold in duplicate from the top to the bottom of each panel. After undisturbed incubation for 8 h, cells were washed away and the captured cytokine developed using a second mAb and colorimetric reagent in agar. (C57<sup>-/-</sup>) Cells from a homozygous knockout mouse; (C57<sup>+/-</sup>) cells from a heterozygous littermate. Results represent one of two comparable experiments.

IFN- $\gamma^{0/0}$  mice had substantially lower levels of IgG2a and IgG3, and, consistent with the in vivo activation of IL-4-producing cells, enhanced levels of IgG1 and IgE, as compared with phenotypically resistant animals (Fig. 4).

## Discussion

The development of Th1 and Th2 cells from naive precursors is of much interest in understanding the nature of the immune response, including determinants of disease susceptibility and the evaluation of vaccines. IL-4 has been demonstrated to enhance Th2 development in vitro (3, 4, 15) and in vivo (15), and mice with homologous disruption of the IL-4 gene have impaired ability to generate Th2 responses (6). Conversely, IL-12, a heterodimeric cytokine produced by macrophages (and B cell lines) (16), has been demonstrated to induce Th1 cell responses in several in vitro systems (17, 18). IL-12 induces the production of IFN- $\gamma$  from both T lymphocytes and NK cells. It is also a growth factor for activated T cells and promotes the development of Th1 effector cells. It is unclear whether the Th1-promoting effects of IL-12 are indirectly mediated by IFN- $\gamma$ , however. Evidence suggests that IFN- $\gamma$  cannot itself prime for Th1 cell development in vitro (4, 19) and cannot mediate sustained Th1 cell development in vivo in the *L. major* system in susceptible BALB/c mice (8). Conflicting results have been described for the capacity of anti-IFN- $\gamma$  to abrogate Th1 cell development in vitro (18–20), although anti-IFN- $\gamma$  can ablate the ability of genetically resistant mice to control infection due to *L. major* (21). Detailed evaluation of the latter mice, however, has not been reported to suggest whether default occurs into the Th2 pathway. The experiments detailed here further establish the requirement for IFN- $\gamma$  in the control of Leishmania.

The *L. major* system provides a mechanism for providing antigens that normally trigger Th1 cell development to animals deficient in IFN- $\gamma$ . If genetic resistance alone constituted the basis for the Th1/Th2 switch in CD4<sup>+</sup> T cells, the expected



**Figure 4.** Antibody isotypes after infection with *L. major*. Groups of designated mice were infected with *L. major* for 5 wk and serum prepared for analysis. Uninfected wild-type C57BL/6 are shown for comparison (C57BL/6 naive). IgG isotypes and IgM were measured by incubating serum over wells precoated with *L. major* antigens, washing, and quantitating the captured antibody using an isotype-specific mAb and colorimetric development. Results are expressed as mean  $\pm$  SEM of the optical density units (O.D.). Total IgE was quantitated using a double mAb antibody-based sandwich ELISA and is expressed as mean  $\pm$  SEM  $\mu$ g/ml as determined using a standardized control. Levels of IgE were not different between uninfected C57BL/6 heterozygous and homozygous knockout mice ( $<1 \mu$ g/ml in both).

result would have been CD4<sup>+</sup> T cells containing transcripts for lymphotoxin, but not the Th2-type transcripts, IL-4, IL-5, and IL-13. In contrast, these mice uniformly developed CD4<sup>+</sup> T cells containing low levels of lymphotoxin and high levels of Th2-type transcripts, consistent with default of these cells into a Th2 effector pathway. Although either outcome would be predicted to lead to failure to control disease due to the absence of the macrophage-activating capacity of IFN- $\gamma$ , the mRNA and ELISPOT studies readily demonstrated the differences in CD4<sup>+</sup> subset differentiation. The methods used required minimal manipulation of tissues in vitro, such as restimulation with antigens or mitogens, and accurately reflected the antibody isotypes quantitated in serum. Taken together, the results are consistent with the interpretation that, at least for this well-characterized system, IFN- $\gamma$  is required for the development of Th1 cells in vivo. The possibility that IFN- $\gamma$  derived from NK cells might play a role in Th1 cell differentiation in the response to *L. major* has been suggested (22), and would be consistent with the results obtained here. Challenge of these mice or mice deficient in the IFN- $\gamma$  receptor with additional organisms, including *Listeria* and mycobacteria, also demonstrated inability to control infection, although detailed analysis of the immune response was not reported (7, 23, 24).

The mechanism by which naive T cells default into the

Th2 pathway may reflect the occurrence of unopposed IL-4 generation at the time of T cell activation by antigen. The addition of IL-4 during priming constitutes a strong stimulus for development of Th2 cells (3, 4), and neutralization of even the small amounts of IL-4 generated during the initial activation of naive cells results in default into the Th1 pathway (3). The recent report that IL-4 suppresses IL-2 and IFN- $\gamma$  production by naive cells during their initial activation by APCs (25) suggests that unopposed IL-4 would be detrimental for diseases requiring Th1 responses, like leishmaniasis. Finally, IFN- $\gamma$  itself can inhibit proliferation of Th2 cells (26), and loss of this cytokine would be expected to favor sustained expansion of this population. It seems unlikely that these results reflect outgrowth of a small population of cells responding to a unique antigen, however, in view of the extensive analysis of the CD4<sup>+</sup> cell response in these mice, suggesting that Th1 and Th2 cells arise from common precursors (27). Such findings are in agreement with studies using cells from TCR transgenic mice demonstrating the capacity to drive these cells to both the Th1 and Th2 phenotypes (3, 4). The data presented suggest an important role for IFN- $\gamma$  in Th1 development in vivo and emphasize the useful role genetically modified mice play in elucidating critical factors involved in the immune response to infectious organisms.

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