Interferon γ Derived from CD4$^+$ T Cells Is Sufficient to Mediate T Helper Cell Type 1 Development

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Summary

Interferon γ (IFN-γ) has been implicated in T helper type 1 (Th1) cell development through its ability to optimize interleukin 12 (IL-12) production from macrophages and IL-12 receptor expression on activated T cells. Various systems have suggested a role for IFN-γ derived from the innate immune system, particularly natural killer (NK) cells, in mediating Th1 differentiation in vivo. We tested this requirement by reconstituting T cell and IFN-γ doubly deficient mice with wild-type CD4$^+$ T cells and challenging the mice with pathogens that elicited either minimal or robust IL-12 in vivo (*Leishmania major* or *Listeria monocytogenes*, respectively). Th1 cells developed under both conditions, and this was unaffected by the presence or absence of IFN-γ in non-T cells. Reconstitution with IFN-γ–deficient CD4$^+$ T cells could not reestablish control over *L. major*, even in the presence of IFN-γ from the NK compartment. These data demonstrate that activated T cells can maintain responsiveness to IL-12 through elaboration of endogenous IFN-γ without requirement for an exogenous source of this cytokine.

Key words: T helper type 1 cells • interferon γ • natural killer cells • *Leishmania* • *Listeria*

The development of CD4$^+$ T cell subsets remains an important topic in understanding mechanisms by which protective or detrimental T effector cell populations arise (1, 2). Critical cytokines that promote the appearance of Th1 cells include IL-12 and IL-18, which provide important growth and survival signals for this subset of cells (3–5). The canonical Th1 cytokine, IFN-γ, may also contribute to Th1 development, since mice deficient in IFN-γ (IFN-γ$^{-/-}$) aberrantly developed Th2 cells when confronted with pathogens that normally engender Th1 responses (6–8).

IFN-γ can influence Th1 development by several mechanisms. IFN-γ mediates IL-12Rβ2 chain expression (9) and promotes IL-12 secretion from macrophages (10). Since both IL-12 and a functional IL-12 receptor are required for the development of fully competent Th1 cells (3, 11, 12), the requirement for IFN-γ may be indirect. Because naive T cells do not produce IFN-γ until some time after activation, it is possible that IFN-γ derived upon activation of NK cells (13) may prime Th1 development through its ability to confer a competent IL-12 response to the T cells. IFN-γ derived from Th1 cells can also negatively regulate the growth of Th2 cells (14). Such studies predict that T cells conditioned in vivo in the absence of a source of exogenous IFN-γ might display defective Th1 development.

To examine the relative contributions of NK cell– and T cell–derived IFN-γ in Th1 development, we have generated mice with a selective deficiency of IFN-γ in either the NK cell or T cell compartments. The mice were challenged with pathogens that in the early stages of infection elicit either little (e.g., *Leishmania major*; reference 15) or robust (e.g., *Listeria monocytogenes*; reference 16) amounts of IL-12 in vivo, and were assayed for their capacity to develop Th1 cells.

Materials and Methods

Mice. C57BL/6 mice, and IFN-γ$^{-/-}$ (17) and recombinase activating gene 1–deficient (RAG1$^{-/-}$; reference 18) mice (all 6–8 wk old) on the C57BL/6 background were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were bred in the UCSF transgenic facility to generate doubly deficient mice (IFN-γ$^{-/-}$RAG1$^{-/-}$). The RAG1$^{-/-}$ phenotype was confirmed by flow cytometric demonstration of complete blood T and B cell deficiency and the IFN-γ$^{-/-}$ genotype was confirmed by PCR amplification of the targeted alleles from genomic DNA for the

[1]*Abbreviation used in this paper:* RAG-1$^{-/-}$, recombinase activating gene–deficient mice.

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presence of the introduced construct and the absence of the wild-type gene as described (17).

CD4$^+$ T Cell Purification and Adoptive Transfer. Lymph nodes and spleens were collected from designated mice and dispersed through a 0.75-μm nylon mesh filter to produce single cell suspensions. After washing and counting, suspensions were depleted of B, dendritic, and CD8$^+$ T cells by treatment with mAbs against heat-stable antigen (J11d; American Type Culture Collection [ATCC], Rockville, MD), MHC class II (BP107; ATCC), and CD8 (3.155; ATCC), followed by treatment with rabbit and guinea pig complement (Cedarlane Labs., Hornby, Ontario, Canada). After removal of dead cells over a Ficoll gradient, cells (routinely 75–85% CD4$^+$ T cells) were labeled with FITC-conjugated anti-Thy 1.2 (5a-8; Caltag Labs., S. San Francisco, CA), PE-conjugated anti-NK1.1 (PK136; PharMingen, San Diego, CA), and TriColor-conjugated anti-CD4 mAbs (CT-CD4; Caltag Labs.), and then sorted using flow cytometry (FACStar PLUS®, Becton Dickinson, Mountain View, CA) to collect CD4$^+$Thy1.2$^+$NK1.1$^-$ cells (>99% purity). Designated cohorts of recipient mice were reconstituted intraperitoneally with 10^6 cells in 400 μl PBS with 1% FCS.

Leishmania Infection. *L. major* (strain WHOM/IR/-/173) was grown in M199 medium supplemented with 30% FCS and antibiotics. For infection, metacyclic promastigotes were purified from stationary phase cultures by negative selection with peanut lectin–coated beads (Sigma Chemical Co., St. Louis, MO) as previously described (15). Designated groups of four to eight mice were inoculated with 4×10^5 organisms in each hind footpad. Footpad swelling was quantitated weekly using a metric caliper. At various times, mice were killed and the footpads and spleens were collected for quantitation of parasites as previously described (11). In brief, single cell suspensions were prepared and diluted serially 10-fold in triplicate microtiter wells in M199 medium with 30% FCS and antibiotics. After incubation for 2 wk at 26°C, motile promastigotes were identified using inverted microscopy.

Listeria Infection. *L. monocytogenes* (provided by D. Portnoy, U.C. Berkeley, Berkeley, CA) was maintained as frozen stock and grown in Luria-Bertani medium. Mice were infected intraperitoneally with 10^4 organisms in 100 μl of PBS. Mice were killed after 8 d and the spleens were collected for analysis of cytokines (see below).

ELISPOT Assays for Cytokine Production. Spleens from mice infected with *L. major* or *Listeria* were dispersed into single cell suspensions in RPMI with 10% FCS and antibiotics (culture medium). Production of IL-4 and IFN-γ by individual cells was determined using an ELISPOT assay as previously described (7). In brief, duplicate aliquots (containing between 2.5×10^5 and 1×10^6 cells in various experimental groups) were diluted twofold across 96-well microtiter plates (Immulon II; Dynatech, Chantilly, VA) that had been precoated with either mAb 11B11 against IL-4 or mAb R46A2 against IFN-γ. Plates were incubated overnight at 37°C in 5% CO_2-air. The wells were washed and incubated for 1 h with biotinylated secondary antibodies BVD6-24G2.3 and XMG-6 for IL-4 and IFN-γ, respectively. Wells were washed and incubated with streptavidin alkaline phosphatase (Jackson ImmunoResearch Labs., West Grove, PA) and developed colorimetrically with 5-bromo-4-chloro-3-indolyl-phosphate in 0.1 M 2-amino-2-methyl-1-propanol buffer (Sigma Chemical Co.) suspended in 0.6% agarose (SeaPlaque; FMC Bioproducts, Rockland, ME). After solidification, blue spots were counted by inverted microscopy.

Flow Cytometric Analysis and Intracellular Cytokine Detection. Spleen cells collected from designated mice were analyzed for the numbers of CD4$^+$TCR$^+$NK1.1$^-$ and NK1.1$^+$TCR$^-$ cells using FITC-conjugated anti-CD4 (Caltag Labs.), PE-conjugated anti-NK1.1 (PharMingen), and TriColor-conjugated anti–TCR-β (Caltag Labs.) mAbs, using a Becton Dickinson Vantage flow cytometer with Lysis II software (Becton Dickinson).

Intracellular cytokine detection was performed as previously described (19). In brief, 3×10^6 cells in 1 ml culture medium were incubated in duplicate in 24-well plates that had been precoated with anti–TCR-β mAb (H57.597; 10 μg/ml) and anti-CD28 mAb (37N51.1; 5 μg/ml). After 16 h at 37°C in 5% CO_2, cells were washed twice in PBS/1% FCS, incubated for 30 min with FITC-conjugated anti-CD4 mAb, washed twice, and then fixed with 4% formaldehyde for 10 min at room temperature. After washing twice in PBS/1% FCS, cells were resuspended in 0.5% saponin (Sigma Chemical Co.) in PBS for 5–10 min. After further washing, cells were incubated for 30 min with PE-conjugated 11B11 or PE-conjugated XMG1.2 (mAbs for IL-4 and IFN-γ, respectively), or PE-conjugated isotype control mAbs. Cells were washed twice in 0.5% saponin/PBS and resuspended in 200–500 μl of PBS/1% FCS for flow cytometric analysis.

Serum IgE Analysis. Serum prepared at the time of death was quantitated for total IgE using a mAb-based sandwich ELISA as previously described (7).

NK Cytolytic Assay. YAC-1 tumor targets (10^6 cells) were labeled at 37°C for 1 h with 200 μCi ^{51}Cr-sodium chromate (Amersham Pharmacia Biotech, Arlington Heights, IL) in RPMI 1640 supplemented with 10% FCS, antibiotics, 5×10^{-5} M ME (complete RPMI). Cells were washed twice in complete RPMI and resuspended at 10^5 cells/ml, and 10^4 cells were added to triplicate microtiter wells of a 96-well plate. Serially diluted spleen effector cells were added to each well at the indicated E/T ratios. After 4 h at 37°C, the plates were centrifuged (200 *g* for 5 min) and 100 μl supernatant from each well was counted in a gamma counter. Percentage of cytotoxicity was calculated as previously described (20). For each assay, the SE of each point was <5%.

Results

NK Cell-derived IFN-γ Is Not Required for Control of L. major Infection. Prior studies of murine *L. major* infection, including in C57BL/6 mice, have suggested a role for IFN-γ derived from NK cells in establishing Th1 cytokine responses necessary for control of disease (21, 22). To assess this directly, T cell–deficient mice were created on the IFN-γ$^{-/-}$ background by crossing and selecting C57BL/6 mice doubly deficient for RAG-1 and IFN-γ. As expected, these mice, and singly deficient RAG-1$^{-/-}$ mice, expressed no B or T lymphocytes. Analysis of spleens from such mice revealed that the NK compartment, as assessed by NK1.1$^+$ TCR-α/β, -γ/δ$^-$ cells, comprised ~$2.6 \pm 0.4 \times 10^6$ cells in Rag-1$^{-/-}$ mice and $2.8 \pm 0.7 \times 10^6$ cells in RAG-1$^{-/-}$ IFN-γ$^{-/-}$ mice, as compared with $2.5 \pm 0.3 \times 10^6$ splenic NK cells in wild-type C57BL/6 mice. Thus, despite the substantial decrease in spleen size in RAG-1$^{-/-}$ and RAG-1$^{-/-}$IFN-γ$^{-/-}$ mice, the numbers of NK cells were comparable to wild-type mice.

The capacity of CD4$^+$ T cells to reconstitute host responses to *L. major* in immunodeficient mice (23) prompted us to reconstitute such doubly deficient mice with 10^6 highly purified CD4$^+$ T cells from wild-type C57BL/6 an-

imals. These adoptive transfers thus establish mice in which the IFN-γ–competent T cell compartment is donor derived and the IFN-$\gamma^{-/-}$ NK cell compartment is recipient derived. Mice were infected 2 d after reconstitution. In three independent experiments, all reconstituted mice were capable of controlling infection with *L. major*, as assessed by lesion size over time and by the numbers of parasites recovered from the footpads and spleens after 8 wk (Fig. 1 *A*). In contrast, nonreconstituted mice were highly susceptible to *L. major* infection ($\log_{10}$ parasites in footpads $\geq 10^{12}$ in both and in spleens, $10^{7.1}$ and $10^{6.6}$ in RAG-$1^{-/-}$ and doubly deficient mice, respectively), as expected based on prior studies in T cell–deficient and IFN-$\gamma^{-/-}$ mice (7, 11, 23). Analysis of the reconstituted mice at the conclusion of the experiment confirmed the purity of the $CD4^+$ T cell reconstitution; $CD8^+$ T cells (<1%), γ/δ T cells (<1%), and serum IgE (<1.2 μg/ml) were at or below the levels of detection in nonreconstituted RAG-$1^{-/-}$ mice. The cytolytic capacity of NK cells taken from the infected T cell–reconstituted IFN-$\gamma^{+/+}$ or $^{-/-}$ mice was comparable, as assessed by the ability to kill labeled YAC-1 targets in vitro (Fig. 2). Thus, wild-type $CD4^+$ T cells conferred a phenotypically

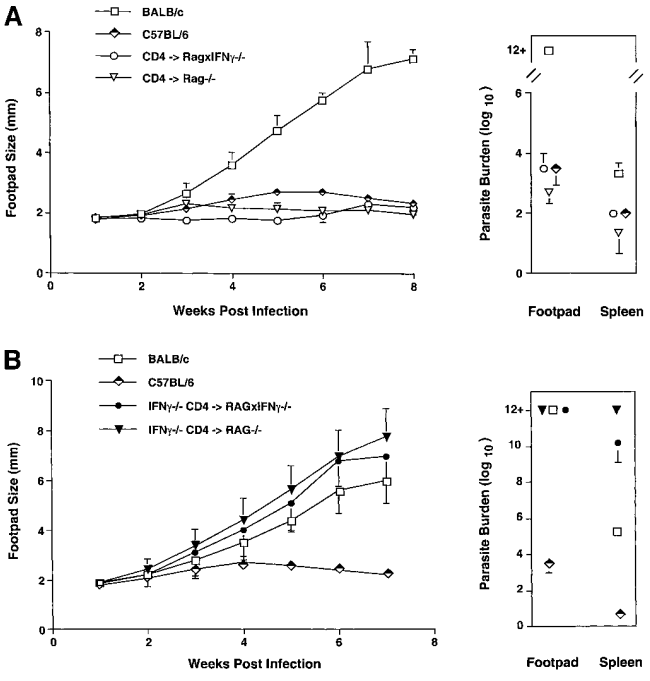

Figure 1. *L. major* infection in $CD4^+$ T cell–reconstituted mice. (*A*) Groups of five to eight C57BL/6 RAG-$1^{-/-}$ or C57BL/6 RAG-$1^{-/-}$ IFN-$\gamma^{-/-}$ mice were reconstituted with 10^6 wild-type C57BL/6 $CD4^+$ T cells 2 d before infection in the hind footpads with *L. major*. Cohorts of wild-type resistant C57BL/6 and susceptible BALB/c mice were infected concurrently. The course of infection was monitored by measurements of local footpad swelling using a metric caliper. Bars represent SEM. Box to right indicates log recovered parasites from footpads and spleens at the conclusion of the experiment. (*B*) Same experimental groups as depicted in *A* except that C57BL/6 RAG-$1^{-/-}$ and C57BL/6 RAG-$1^{-/-}$IFN-$\gamma^{-/-}$ mice were reconstituted with 10^6 $CD4^+$ T cells from C57BL/6 IFN-$\gamma^{-/-}$ mice 2 d before infection with *L. major*. Bars represent SEM. Box to right indicates tissue parasite burdens.

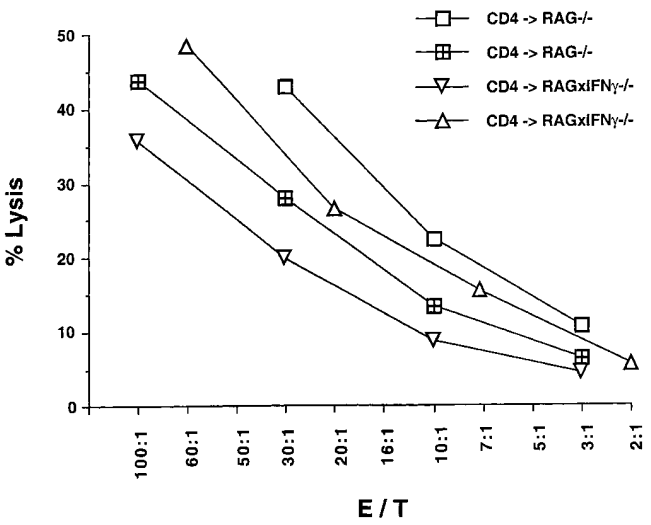

Figure 2. NK lytic activity from reconstituted mice. Spleen cells were recovered at the conclusion of *L. major* infection from two individual RAG-$1^{-/-}$ or RAG-$1^{-/-}$IFN-$\gamma^{-/-}$ mice that had been reconstituted with wild-type $CD4^+$ T cells. Cells were diluted at the designated E/T ratios into cultures with labeled YAC-1 target cells. Percentage of lysis was calculated by the proportion of label released into the supernatant as a percentage of the total label released by nonspecific lysis of the cells with detergent.

normal healer response to *L. major* in mice containing an NK compartment unable to contribute the cytokine IFN-γ.

L. major Infection Is Unaffected by the Presence of NK Cell–derived IFN-γ in the Absence of T Cell–derived IFN-γ. To assess whether IFN-γ contributed from NK cells could impart any control over *L. major*, we reconstituted either RAG-$1^{-/-}$ or doubly deficient mice with highly purified $CD4^+$ T cells from IFN-$\gamma^{-/-}$ mice. Reconstituted RAG-$1^{-/-}$ mice have intact IFN-γ production from the NK cell compartment, unlike the doubly deficient mice, which allows us to compare the course of infection in the two cohorts of animals. In two separate experiments, both reconstituted groups of mice were unable to control *L. major*, and no significant differences were apparent among the groups in either lesion size or parasite burdens (Fig. 1 *B*). Indeed, susceptible BALB/c mice controlled infection better than either cohort of reconstituted mice. Thus, IFN-γ derived from the NK cell compartment alone could not sustain control of *L. major* in the absence of $CD4^+$ T cell–derived IFN-γ.

Th1 Cell Development Occurs in the Absence of NK Cell IFN-γ. Control of *L. major* among healer mice, including C57BL/6 mice, is dependent upon the development of Th1 cells that produce IFN-γ. As assessed by both ELISPOT assays (data not shown) and intracellular cytokine determinations (Fig. 3 *A*) in individual $CD4^+$ T cells, Th1, but not Th2, cells developed effectively in doubly deficient mice reconstituted with wild-type $CD4^+$ T cells. In contrast, mice reconstituted with IFN-$\gamma^{-/-}$ $CD4^+$ T cells developed IL-4–producing cells, and such cells developed in both RAG-$1^{-/-}$ mice (990 ± 95 IL-4–producing cells per 10^6

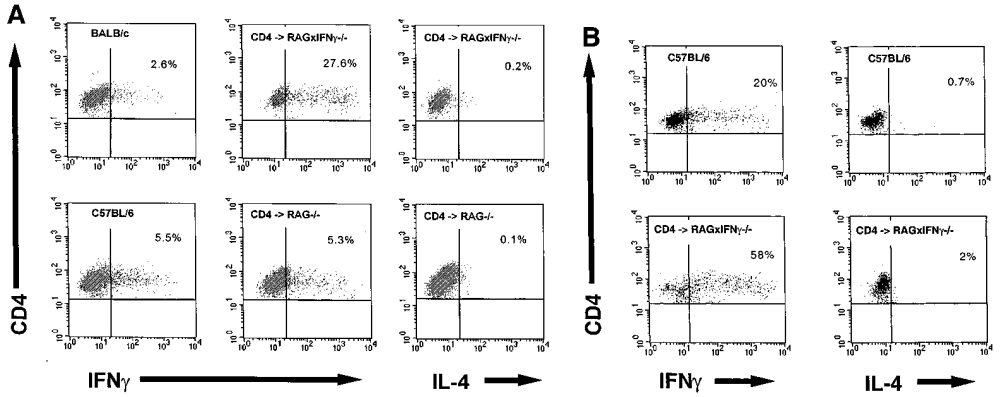

Figure 3. Intracellular cytokine production by $CD4^+$ T cells. (*A*) $CD4^+$ T cells from draining lymph nodes were examined for intracellular IFN-γ or IL-4 using flow cytometry (see Materials and Methods) after 8 wk of infection with *L. major*. Representative panels from resistant C57BL/6, susceptible BALB/c, and wild-type $CD4^+$ T cell–reconstituted RAG-$1^{-/-}$ IFN-$\gamma^{-/-}$ and RAG-$1^{-/-}$ mice are shown. Results were comparable in exams of four to six mice under each set of conditions in two separate experiments. $CD4^+$ T cells from uninfected, age-matched, C57BL/6 mice revealed <0.7% IFN-γ–producing cells and <0.2% IL-4–producing cells under the same experimental conditions. (*B*) Intracellular cytokine staining after infection with *Listeria*. Spleen $CD4^+$ T cells were collected 8 d after infection from wild-type C57BL/6 or RAG$^{-/-}$ IFN-$\gamma^{-/-}$ C57BL/6 mice that had been reconstituted with wild-type $CD4^+$ T cells 2 d before infection and examined for intracellular presence of IFN-γ and IL-4. Comparable results were obtained on three to four mice under each set of conditions in two separate experiments.

spleen cells) or doubly deficient mice (2,050 ± 960 IL-4–producing cells per 10^6 spleen cells) in numbers comparable to infected susceptible BALB/c mice (1,250 ± 170/10^6 spleen cells) and greater than those in resistant C57BL/6 mice (225 ± 10/10^6 spleen cells). Thus, under these conditions, Th1 cell development was unimpeded and Th2 development was not apparent in mice containing IFN-$\gamma^{-/-}$ NK cells. Conversely, development of IL-4–producing cells occurred when IFN-$\gamma^{-/-}$ $CD4^+$ T cells were used in the reconstitutions despite the absence or presence of IFN-γ in non-T cells.

L. major is a relatively subacute infection that develops over weeks. It is possible that some kinetic deficit in Th1 development was missed in the doubly deficient mice due to the relatively slow course of infection. To address this issue, we compared the development of Th1 cells in doubly deficient mice reconstituted with wild-type $CD4^+$ T cells and then inoculated with *L. monocytogenes*, a gram-positive bacterium that is capable of inducing strong Th1 responses (16). As assessed by both ELISPOT (data not shown) and intracellular cytokine determinations (Fig. 3 *B*), Th1 cells also developed in these mice, indicating that the results seen with *L. major* were not limited to that organism.

Discussion

These studies demonstrate that Th1 cells can differentiate in vivo without exposure to IFN-γ from other cells, such as NK cells. Differentiated cells were functionally competent, as assessed by their capacity to maintain control over a parasite, *L. major*, which is crucially dependent upon Th1 development (24). Finally, we were unable, at least under these conditions, to define a role for IFN-γ derived from NK cells in modulating, either positively or negatively, Th1 effector differentiation. These data suggest that IFN-γ derived from $CD4^+$ T cells can autonomously modulate responsiveness to IL-12 and Th1 competence.

The role of IFN-γ in priming Th1 cell development has been demonstrated using transgenic T cells in vitro (25–28), and presumably reflects the capacity of IFN-γ to promote IL-12Rβ2 chain expression and the appearance of competent IL-12 receptors on activated $CD4^+$ lymphocytes (9, 29). IFN-γ also induces IL-12 p40 production by macrophages (10), thus establishing an autocrine loop for the amplification of type 1 immunity. The importance of this pathway was revealed by the profound Th1 deficiency in mice with genetic deletion of members of the IL-12 signaling cascade, including IL-12, IL-12Rβ1, and stat4 (3, 12, 30, 31). Interestingly, IFN-γ has also been implicated in Th1 development in vivo in some systems (6–8, 32), consistent with effects mediated indirectly through IL-12. The exquisite sensitivity of BALB/c mice to *L. major* infection has been proposed to develop due to premature downregulation of competent IL-12 receptors on activated $CD4^+$ T cells (33, 34). Such a defect might be secondary to the decreased IFN-γ produced after activation of BALB/c $CD4^+$ T cells as compared with cells from other inbred mouse strains (35).

The earliest expression of IFN-γ after infection with *L. major* was shown to arise not in $CD4^+$ T cells, but in the NK cell population (15, 21, 22, 36). Despite these observations, the studies here suggest that IFN-γ derived from the NK cell population was neither required for Th1 cell differentiation nor for the control of *L. major*. Conversely, we could discern no control of infection through IFN-γ derived from the NK cell population in the absence of $CD4^+$ T cell–derived IFN-γ, although this may reflect the smaller contribution made by NK cells in C57BL/6 mice (21). The functional consequences of NK cell-depletion or activation that have been described in murine experimental leishmaniasis may reflect contributions from NK cells that are independent of IFN-γ (21, 22). These findings contrast with those in some viral systems, in which a critical role of NK cell-derived IFN-γ has been demonstrated (37), and may reflect differences among $CD4^+$ and $CD8^+$ effector cells. Further studies will be required to elucidate how applicable such studies are to other classes of pathogens. We could discern no impact on Th1 development in the ab-

sence of NK cell IFN-γ after challenge with Listeria, a more acute infectious disease than Leishmania. In *L. major* infection, the earliest IFN-γ and NK cell responses were shown to be dependent on the induction of interferon α/β by the parasite and the consequent induction of type 2 nitric oxide synthase (38). As shown here in mice with intact interferon α/β and type 2 nitric oxide synthase genes, these early responses were unable to establish protective immunity in the absence of IFN-γ from $CD4^+$ T cells. This endogenous source of IFN-γ is presumably necessary and sufficient to downregulate TGF-β activation that was felt to contribute to early and rapid dissemination of the parasite (38, 39). However, the consistently higher proportion of Th1 cells that developed in the reconstituted doubly deficient mice as compared with wild-type or reconstituted $RAG^{-/-}$ mice (Fig. 3, *A* and *B*) suggests that the total amounts of IFN-γ required for a given biologic response may be regulated, suggesting that IFN-γ derived from non-T cells may have functional activity independent of effects on Th development.

These results suggest that IFN-γ, in a cell-autonomous manner, is required and sufficient to enable $CD4^+$ T cells to respond to exogenous signals, such as IL-12 and IL-18, to develop into functional Th1 effector cells. Similar conclusions have been reached regarding Th2 development, in which autocrine IL-4 derived from T cells themselves was sufficient to regulate this differentiative process (40–42). It will be important to establish whether signals exogenous to the T cells are required for Th2 development, akin to the roles for IL-12 and IL-18 in Th1 development (3, 4). Such insights will be critical in gaining understanding over the final pathways that regulate these differentiative pathways in vivo during allergic and inflammatory diseases, so that cell autonomous and cell extrinsic regulatory molecules can be efficiently targeted during immune intervention.

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