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Interferon γ Enhances Both In Vitro and In Vivo Priming of $CD4^+$ T Cells for IL-4 Production

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Abstract

Classical studies have demonstrated that in vitro priming of naive CD4 T cells to become T helper (Th)2 cells is strikingly dependent on interleukin (IL)-4, whereas priming for interferon (IFN)γ production is IL-12/IFNγ-dependent. Therefore, it was quite surprising when we noted that priming of naive C57BL/6 $CD4^+$ cells to become IL-4 producers was substantially inhibited by the addition of anti-IFNγ antibodies. This was true using immobilized anti-CD3 and anti-CD28 antibodies or soluble anti-CD3/anti-CD28 and antigen-presenting cells in the presence or absence of added IL-4. Priming of CD4 T cells from $IFN\gamma^{-/-}$ C57BL/6 mice with immobilized anti-CD3 and anti-CD28 resulted in limited production of IL-4, even with the addition of 1,000 U/ml of IL-4. Titrating IFNγ into such cultures showed a striking increase in the proportion of T cells that secreted IL-4 upon challenge; this effect was completely IL-4–dependent in that it was blocked with anti–IL-4 antibody. Thus, IFNγ plays an unanticipated but substantial role in Th2 priming, although it is an important Th1 cytokine, and under certain circumstances a Th1 inducer.

Key words: T cell activation • cell differentiation • T lymphocyte subsets • cytokine • T-bet

Introduction

T lymphocytes undergo complicated patterns of differentiation from uncommitted precursors to highly competent effector cells of at least two distinct subsets, Th1 and Th2, defined both by their functions and their cytokine profiles. Th1 cells secrete IFNγ and TNFβ and govern cellular immunity against intracellular pathogens, whereas Th2 cells produce IL-4, IL-5, IL-6, and IL-13 and promote antihelminthic immunity and allergic inflammation (1, 2). IFNγ and IL-4 are prototypic Th1 and Th2 cytokines, respectively, influencing Th1/Th2 cell differentiation, IgG subclass switching, IgE production, macrophage stimulation, and modulation of MHC molecule expression (3). The mechanisms that induce or influence the process of differentiation of naive Th cells into the two respective subtypes have been intensely studied. The cytokine milieu during and after the process of the antigen recognition has been shown to be a critical determinant (3). It is well documented that IL-12 and IL-4, acting via signal transducer and activator of transcription 4 (STAT4) and STAT6, respectively, are major determinants of the Th commitment process (4, 5). Two transcriptional factors, T-bet and GATA3, play crucial roles in this process (6, 7). Significant efforts have been concentrated on studies to understand the hierarchy of regulatory elements involved in Th1 and Th2 intracellular regulatory pathways. The roles that IFNγ plays remain somewhat controversial. The addition of IFNγ to cultures of early developing Th2 cells maintains their IL-12R β 2 expression and the ability of these cells to functionally respond to IL-12, which would be otherwise lost due to IL-4. However, it does not inhibit the cells' ability to produce IL-4 (8). In several rounds of in vitro priming of CD4 T cells, IFNγ was found to be required to stabilize the phenotype of Th1 cells (9). In other experiments, IFNγ was sufficient to induce Th1 polarization of C57BL/6 CD4 cells independently of IL-12 (10), but failed to optimally do so in BALB/c mice (11). IFNγ was shown to induce T-bet (12), and retroviral expression of T-bet in Th2 cells suppressed IL-4 production and induced secretion of IFNγ (6).

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Abbreviations used in this paper: CFSE, carboxyfluorescein diacetate succinimidyl ester; STAT, signal transducer and activator of transcription.

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In contrast, retroviral expression of GATA3 has been shown to halt Th1 development by down-regulation of STAT4 (13).

In vivo studies using control of *Leishmania major* infection as a model of a Th1 response revealed that absence of IFNγ results in a failure to successfully control the infection (14, 15) and that CD4 cells may default to the Th2 pathway (16). However, when animals with a disrupted receptor for IFNγ were similarly infected, Th1 responses still developed with no evidence for the expansion of Th2 cells (17). Other studies using experimental autoimmune diseases such as myocarditis showed that the Th1 cytokine IL-12 was essential for development of the disease, whereas IFNγ was surprisingly protective (18). Lafaille et al. has demonstrated that the presence of IFNγ during in vitro Th2 priming of TCR transgenic T cells produces Th2 subpopulations with enhanced IL-5 expression (19).

In this paper, we present data that strongly indicate that the role of T-bet and IFNγ in the polarization of naive Th cells is even more complex than previously regarded. Complete neutralization of IFNγ in priming cultures of C57BL/6 $CD4^+$ T cells resulted in suboptimal Th2 differentiation, even in the presence of exogenous IL-4. This was not limited to a single mouse strain; we were able to reproduce it in several others. In an effort to better understand this unexpected activity of IFNγ in Th2 differentiation, we used IFNγ-deficient C57BL/6 CD4 T cells and tested the effect of adding IFNγ to their priming cultures. We show that IFNγ synergized with endogenous IL-4 to enhance the proportion of cells that could be stimulated to produce IL-4 at the end of the priming cultures. This was even more striking when exogenous IL-4 was added. Using quantitative PCR and intracellular staining, we showed that in spite of inducing T-bet, IFNγ synergized with IL-4 in promoting IL-4 production by CD4 cells and their Th2 differentiation. This was not due to enhancement of levels of GATA3. IFNγ enhanced IL-4 mRNA and protein levels, whereas, in the very same cells, T-bet was strongly up-regulated and GATA-3 levels were unchanged. A portion of the IFNγ effect appears to be due to preferential survival of IL-4 producers. Finally, in vivo priming for IL-4 production in IFNγ-deficient hosts reveals that complete absence of IFNγ during the priming leads to less than optimal Th2 differentiation. Addition of IFNγ during the priming enhanced Th2 polarization to levels significantly higher than in the wild-type animals.

Materials and Methods

Mice. C57BL/6 mice were obtained from the Division of Cancer Treatment (National Institutes of Health), and IFNγ knockout on a C57BL/6 background (C57BL/6 $IFN\gamma^{-/-}$) and DO11.10 transgenic RAG2-deficient BALB/c mice were obtained from Taconic Farms. BALB/c, BALB/c IFNγ-deficient (BALB/c $IFN\gamma^{-/-}$), B10.A, and 129S6/SvEv were purchased from The Jackson Laboratory. Unless specified, the mice were used at 6–8 wk of age.

Monoclonal Antibodies. Anti–IL-4 (11B11), anti-IFNγ (XMG1.2), and anti–IL-12 (C17.8) were purchased from Harlan Biosciences and used at 10 μg/ml. Mouse anti-GATA3 (HG3-31) was purchased from Santa Cruz Biotechnology, Inc. Mouse isotype control IgG1κ was obtained from BD Biosciences. Cy-5 donkey F(ab′)2 anti–mouse IgG (Multiple Labeling) was purchased from Jackson ImmunoResearch Laboratories. Anti–T-bet 4B10 mAb was a gift from L. Glimcher (Harvard Medical School, Boston, MA).

In Vitro Stimulation of Naive T Cells. LN-derived cells were depleted of $CD8^+$, $B220^+$, CD16, $I\text{-}A^{b+}$, and $NK1.1^+$ cells by negative selection using FITC-labeled specific mAbs, anti-FITC magnetic beads, and an AutoMACS magnetic bead column (Miltenyi Biotec). Upon purification, the cell populations contained 98–99% of $CD4^+$ cells and 1–2% of non-CD4, non-CD8, $CD3^+$, TCR^+, and $Thy1.2^+$ T cells. More than 95% were $CD44^{low}$ $CD4^+$ cells as detected by FACS® analysis. No $FITC^+$ cells were detected. For experiments in Fig. 2 A, cells were further sorted for naive phenotype ($CD44^{low}$, $CD62L^{bright}$, and $CD4^+$) to virtually 100% purity. Primary stimulation was performed either by culturing $CD4^+$ cells (3×10^5 cells in 2 ml of media per well of 24-well plates) in plates precoated with 5 μg/ml anti-CD3 and anti-CD28 mAbs or by coculture with irradiated, T cell–depleted APCs (in a CD4/APC ratio of 1:5) and soluble anti-CD3 and anti-CD28 mAbs (5 μg/ml each) for 4 d. In both cases, 10 U/ml IL-2 was added to the culture media. In experiments evaluating the proliferative rates, the CD4 T cells were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes) at a final concentration of 1.25 μM before their priming. Primed cells were washed and restimulated for 6 h by incubation with plate-bound anti-CD3 and anti-CD28 mAbs (5 μg/ml), and for the last 3 h in the presence of 2 μM monensin (BD Biosciences).

Intracellular Cytokine and Transcription Factor Staining. Restimulated $CD4^+$ cells were washed, harvested, and fixed with 4% paraformaldehyde for 10 min at room temperature. They were permeabilized with 0.1% Triton X-100 and 0.5% BSA in PBS before incubation with 2.4G2 mAb anti–FcγRII-III (Harlan Biosciences) to block nonspecific binding. They were stained with FITC-labeled anti-IFNγ, PE-anti–IL-4, and APC–anti-CD4 (all reagents obtained from BD Biosciences). When cells were examined for transcription factor contents, CyCR anti-CD4 was used, and anti-GATA3, anti–T-bet, or mouse isotype control IgG1κ mAbs were used as primary antibodies and detected with Cy-5 donkey F(ab′)2 anti–mouse IgG. Samples were analyzed using a FACScalibur™ (Becton Dickinson); 10,000 events were collected.

Real-Time PCR. LN-derived $CD4^+$ T cells from either C57BL/6 $IFN\gamma^{-/-}$ or C57BL/6 were cultured in plates precoated with 5 μg/ml anti-CD3 and anti-CD28 mAbs in the presence of 10 U/ml IL-2 for 24 or 48 h. The cells were collected, total RNA was prepared using SV Total RNA isolation system (Promega), and 1 μg was reverse transcribed with random primer hexamers and SuperScript First-Strand synthesis system (Invitrogen). The amounts of cDNAs specific for GATA3 T-bet and IL-4 were determined using the ABI Prism 7700 Sequence Detection System (Applied Biosystems). The individual samples were normalized using murine 18S rRNA housekeeping gene expression (Applied Biosystems).

In Vivo Priming of CD4 T Cells for IL-4 Production. Groups of $IFN\gamma^{-/-}$ BALB/c mice ($n = 4$) were implanted with mini-osmotic pumps (Alzet 2001; 1 μl/h; Durect Corp.) containing 1,000 μg OVA (Sigma-Aldrich) in PBS and indicated amounts of recombinant IFNγ (0, 0.1, 1.0, and 10 μg; BD Biosciences). In parallel, wild-type BALB/c mice received pumps containing OVA

only. The next day, the mice received intraperitoneally naive LN-derived $CD4^+$ cells from anti-$OVA_{323-339}$/I-A^d TCR transgenic DO11.10 · $RAG2^{-/-}$ donors (3 × 10^6 cells/animal) purified by negative selection (depleted of $CD8^+$, $B220^+$, CD16, I-A^{b+}, and $DX5^+$). After 4.5 d, mice were killed, and draining LNs (axillary, brachial, and inguinal) were harvested. Pooled LN cells from each animal were restimulated with 3 μM $OVA_{323-339}$ peptide (American Peptide Company) and 3 μg/ml of soluble anti-CD28 for 8 h; monensin was added for the last 4 h. IFNγ and IL-4 cytokine contents were determined in clonotypic KJ1-26^+CD4^+ cells by flow cytometry as aforementioned. The statistical differences among the groups were analyzed using Student's *t* test.

Results

Priming of $CD4^+$ T Cells for IL-4 Production Is Partially IFNγ Dependent. We observed that when C57BL/6 LN-derived $CD4^+$ T cells were stimulated with plate-bound anti-CD3 and anti-CD28, neutralization of IFNγ resulted not only in lower proportions of IFNγ producers but also surprisingly in a lower proportion of IL-4 producers upon restimulation of the cells. This was observed both in absence or presence of exogenous IL-4 (Fig. 1 A). The same observations were made when the cells were stimulated with soluble anti-CD3 and anti-CD28 and irradiated T cell–depleted splenocytes (Fig. 1 B). Addition of anti-IFNγ mAb reduced the relative proportion of IL-4 producers by 30–70% in various experiments.

To analyze further the role of IFNγ in the early stages of Th2 polarization, we used IFNγ-deficient C57BL/6 (20) mice as a source of CD4 cells. The CD4 cells were purified as described in Materials and Methods and primed by plate-bound anti-CD3 and anti-CD28 with or without IL-4. Defined amounts of IFNγ were added to the priming cultures. Interestingly, the addition of IFNγ dramatically enhanced the proportion of IL-4–producing T cells (Fig. 2 A). This enhancement was entirely IL-4 dependent; neutralization of endogenous IL-4 by addition of anti–IL-4 prevented priming for IL-4 production (Fig. 2 A, left). Sorting CD4 T cells for naive phenotype (namely $CD4^+$, $CD44^{dull}$, $CD62L^{bright}$, and $NK1.1^-$) resulted in no "background" IL-4 priming,

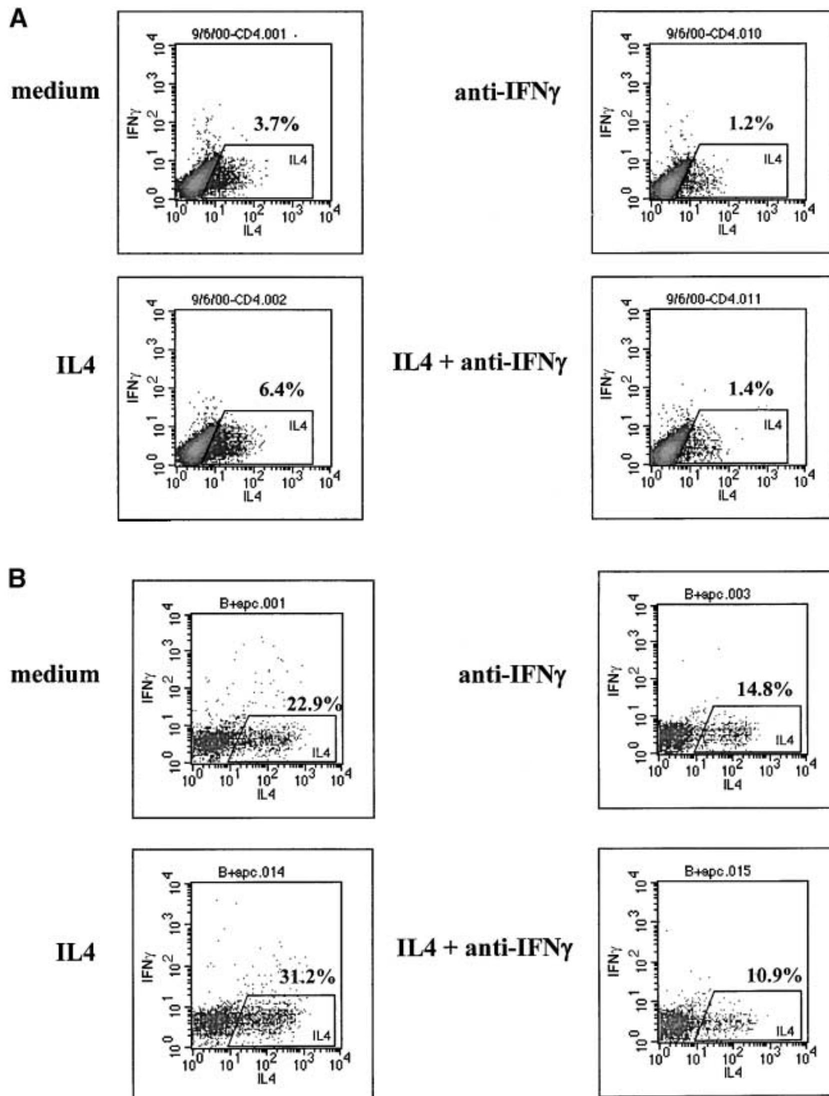


Figure 1. Priming of $CD4^+$ T cells for IL-4 production under neutral conditions is at least partially IFNγ dependent. C57BL/6 LN-derived $CD4^+$ T cells (purified by depletion of $CD8^+$, $B220^+$, CD16, I-A^{b+}, and $NK1.1^+$ cells as described in Materials and Methods) were stimulated for 4 d with 5 μg/ml plate-bound anti-CD3 and anti-CD28 (A) or with 5 μg/ml soluble ones and T cell–depleted irradiated splenocytes (B). In both cases, 10 U/ml IL-2 was added to the media. Blocking mAbs were added at 10 μg/ml, and IL-4 was added at 1,000 U/ml. Cells were restimulated for 6 h with 5 μg/ml plate-bound anti-CD3 and anti-CD28. Percentages of IL-4 and IFNγ producers in the cultures were evaluated by intracellular staining of fixed permeabilized cells for content of respective cytokines by flow cytometry. The conditions during priming and the relative proportion of IL-4–producing cells are indicated.

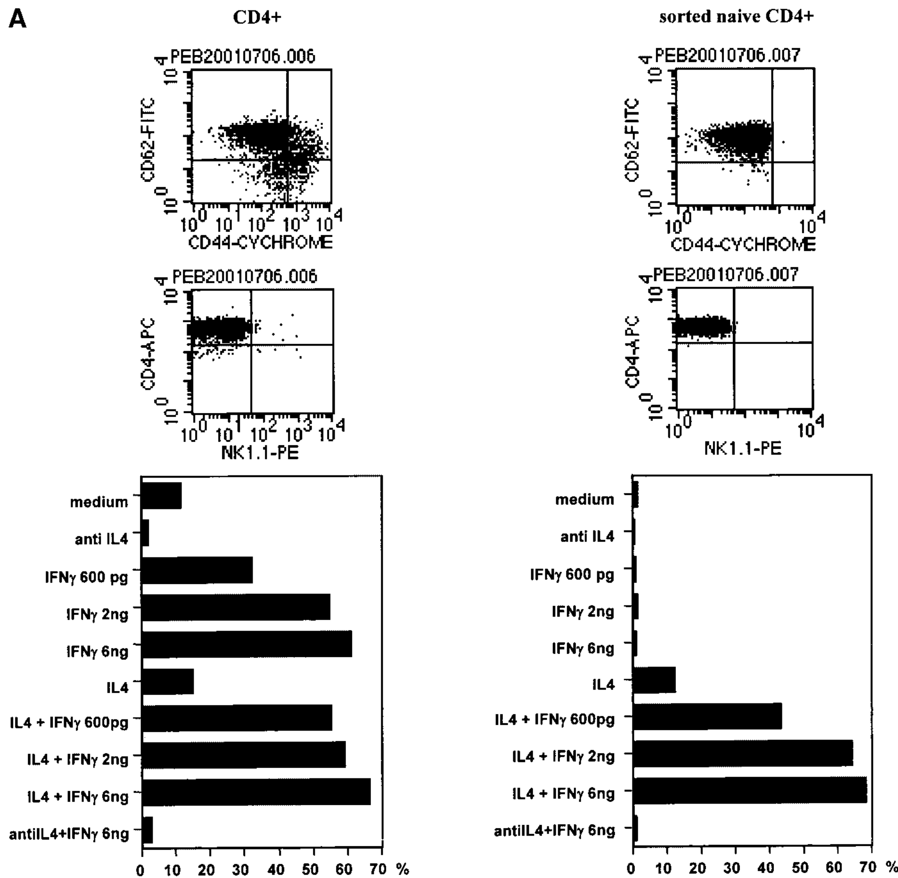


Figure 2. Addition of IFNγ to priming cultures of C57BL/6 IFN$\gamma^{-/-}$ CD4^{+} T cells enhances the proportion of IL-4–producing T cells in an IL-4–dependent manner. C57BL/6 IFN$\gamma^{-/-}$ CD4^{+} cells were derived from LNs by depletion of CD8^{+}, B220^{+}, CD16, I-Ab^{+}, and NK1.1^{+} cells (left). A subpopulation was further purified using an automatic cell sorter collecting only CD4^{+} cells of naive phenotype, namely CD4^{+}, CD44^{dull}, CD62L^{bright}, and NK1.1-PE (right). Each group was stimulated under the indicated conditions (IL-4, 1,000 U/ml and anti–IL-4, 10 μg/ml). The cells were restimulated, and proportions of IL-4 producers were analyzed by intracellular staining and FACS®.

presumably because it removed cells that produced the IL-4 needed for priming (Fig. 2 A, right). Such cells did not show any induction of IL-4–producing capacity upon the addition of IFNγ, unless IL-4 was also added. In either culture, the combination of exogenous IFNγ and IL-4 resulted in a significantly higher relative proportion of Th2 cells than that induced by IL-4 alone.

We have further investigated how the addition of IFNγ to C57BL/6 IFN$\gamma^{-/-}$ CD4^{+} T cell priming cultures influences other cytokines made by Th2 cells, namely IL-5, IL-13, IL-10, and TNFα. Adding IFNγ to C57BL/6 IFN$\gamma^{-/-}$ CD4^{+} T cell priming cultures enhanced not only IL-4 but also IL-10 and IL-13. The enhancement was completely IL-4 dependent because it was blocked by the addition of anti–IL-4 (Fig. 2 B). Levels of TNFα were not significantly influenced by the addition of IFNγ, and overall levels of IL-5 were too low to allow meaningful analysis (unpublished data). This is consistent with our observations that Th2 cells require typically more than one round of priming to produce higher levels of IL-5.

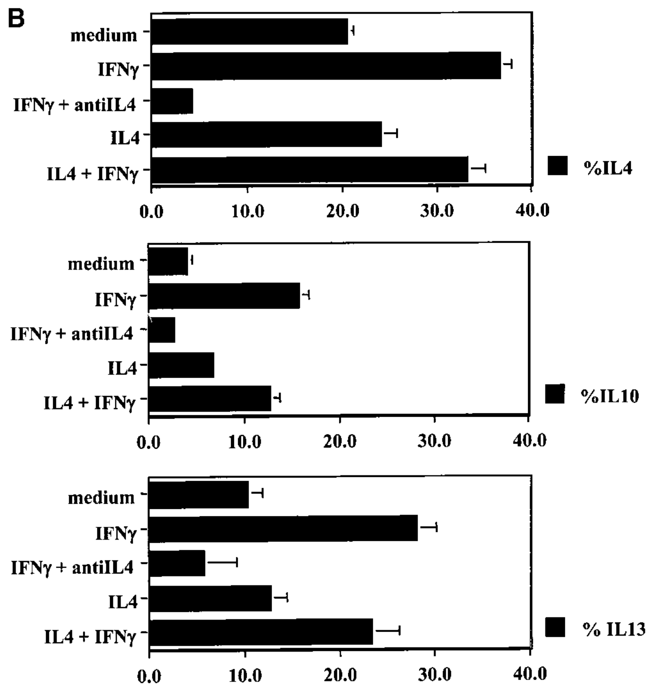
Quantitative PCR measurements showed that IFNγ-enhanced IL-4 mRNA expression was not observed until 48 h of culture. CD4 cells derived either from C57BL/6 or C57BL/6 IFN$\gamma^{-/-}$ animals were incubated with plate-bound anti-CD3 and anti-CD28 for 24 or 48 h, and amounts of IL-4–specific mRNA were determined by quantitative PCR. There were only minimal changes observed at 24 h. However, at 48 h, neutralization of IFNγ in C57BL/6 CD4 cultures decreased IL-4 mRNA particularly in the presence of IL-4. Similarly, IFNγ dramatically increased IL-4 mRNA in C57BL/6 IFN$\gamma^{-/-}$ CD4^{+} cells cultured with IL-4 (Fig. 2 C).

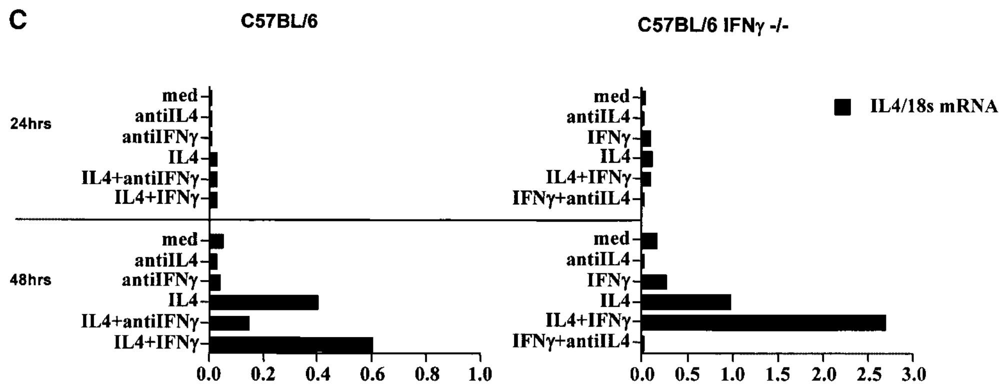
The proportion of IL-4–producing cells was diminished by neutralization of IFNγ in the Th2 priming cultures of not only C57BL/6 CD4 T cells but also in other mouse strains

Table I. *Effect of IFNγ Neutralization on IL-4 Production in Several Mouse Strains*

Strain	C57BL/6	129S6/SvEv	B10.A	C57BL/6 IFN$\gamma^{-/-}$
Priming				
IL-4	34.5	26.3	23.7	13.5
IL-4 and anti-IFNγ	21.1	9.2	15.4	13.9

LN-derived CD4^{+} T cells derived from indicated mouse strains were stimulated for 4 d with 5 μg/ml of plate-bound anti-CD3^{+} anti-CD28 in the presence of 10 U/ml IL-2 and IL-4 at 1,000 U/ml without or with 10 μg/ml IFNγ-blocking mAbs. Cells were restimulated for 6 h with 5 μg/ml plate-bound anti-CD3 and anti-CD28. Percentages of IL-4 in the cultures were evaluated by intracellular staining and FACS®. Cultures of CD4 cells derived from the IFNγ-deficient strain served as a control.





(B) The exogenous IFNγ in C57BL/6 IFNγ$^{-/-}$ CD4^{+} T cell priming cultures enhances numbers of not only IL-4, but also IL-10 and IL-13–producing cells. C57BL/6 IFNγ$^{-/-}$ CD4^{+} cells were stimulated as aforementioned. Fixed and permeabilized cells were analyzed for intracellular contents of IL-4, IL-10, and IL-13 by flow cytometry gating on CD4^{+} cells. The means and standard deviations of triplicate experiments are presented. (C) IFNγ and IL-4 dramatically increased IL-4 mRNA above the level induced by IL-4 alone between 24 and 48 h. Neutralization of IFNγ in CD4 priming cultures (wild-type C57BL/6 strain) decreases levels of IL-4 mRNA. Relative ratios of mRNA specific for IL-4 and housekeeping 18S rRNA were determined by real-time PCR at 24 and 48 h in either C57BL/6 or C57BL/6 IFNγ$^{-/-}$ CD4^{+} T cells primed by plate-bound anti-CD3 and anti-CD28.

(Table I). As a control to show that the effects of anti-IFNγ were specific, we show that it has no effect on IL-4 production by CD4 T cells from C57BL/6 IFNγ$^{-/-}$ mice.

T-bet But Not GATA3 Expression Is Dependent on IFNγ. T-bet and GATA3 are key regulatory elements involved in polarization of T helper cells into Th1 and Th2 phenotypes, respectively (6, 7). Therefore, we have examined the effects of IFNγ neutralization in priming cultures of C57BL/6 CD4 cells or of the addition of exogenous IFNγ to C57BL/6 IFNγ$^{-/-}$ on the levels of the two respective transcription factors. Purified LN CD4 cells derived from C57BL/6 and C57BL/6 IFNγ$^{-/-}$ strains were stimulated with or without addition of anti-IFNγ or IFNγ, respectively (as described in Materials and Methods), for 24 or 48 h. The cells were harvested, and the amounts of mRNA specific for GATA3 and T-bet were determined by quantitative RT-PCR (Fig. 3). Levels of GATA3 were enhanced by IL-4 and not influenced by neutralization of IFNγ in C57BL/6 CD4 T cells. In C57BL/6 IFNγ$^{-/-}$ CD4 cells, GATA3 mRNA was also up-regulated by IL-4, significantly suppressed by anti–IL-4 and modestly diminished by addition of IFNγ. Thus, the up-regulation of IL-4 mRNA and ultimately of the proportion of IL-4–producing cells is not due to the effect of IFNγ on GATA3. T-bet expression was strongly induced by IFNγ both in C57BL/6 and C57BL/6 IFNγ$^{-/-}$ CD4^{+} T cells, and it was diminished by IFNγ neutralization in C57BL/6 CD4 T cells priming cultures.

IFNγ Up-Regulates T-bet in Both IL-4 Negative and IL-4 Positive Cells. Overexpression of T-bet via infection of Th2 cells with a retroviral vector has been reported to suppress their IL-4 production and to induce a Th1 phenotype (6). Therefore, we have concentrated on the IFNγ$^{-/-}$ cells where background levels of T-bet were negligible and where T-bet was highly induced by IFNγ, and asked whether the endogenous T-bet is up-regulated in both IL-4 positive and negative cells. IFNγ$^{-/-}$ CD4^{+} cells were primed as aforementioned for 96 h in the presence of IFNγ, IL-4, or IFNγ and IL-4, and restimulated for 6 h. Intracellular T-bet and GATA3 were measured by flow cytometry, gating separately on IL-4 positive or negative cells (Fig. 4).

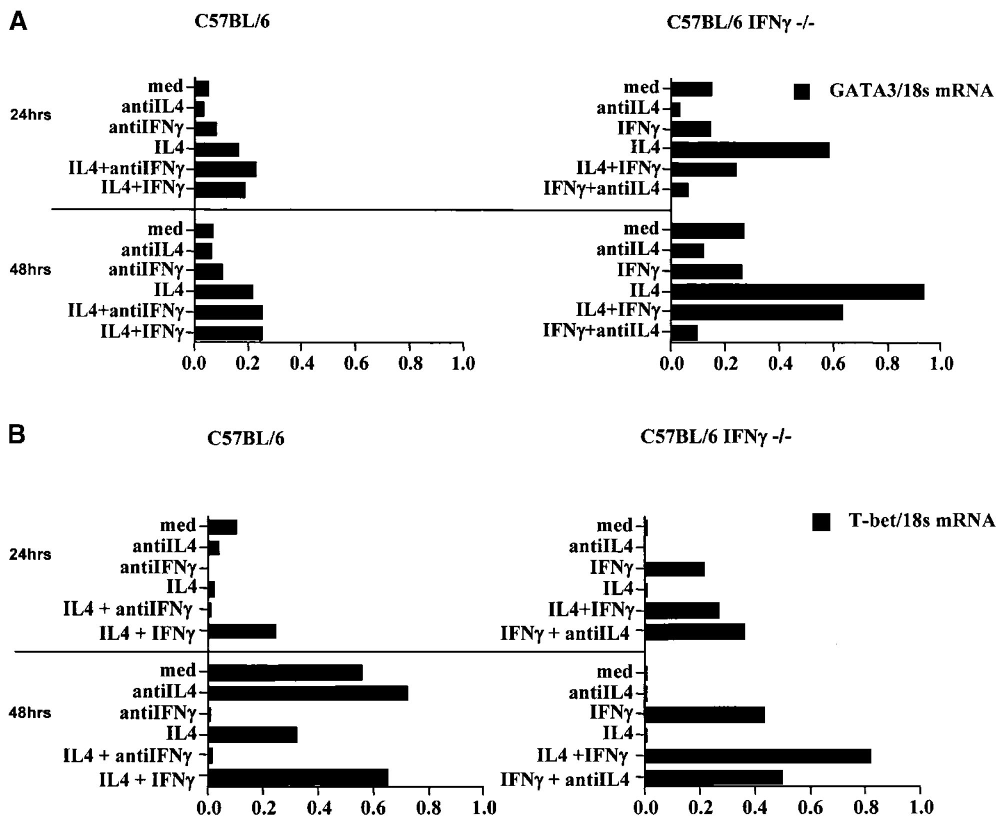


Figure 3. The effect of IFNγ on the mRNA levels of GATA3 and T-bet were studied in C57BL/6 and C57BL/6 IFN$\gamma^{-/-}$ strains by real-time PCR. Relative ratios of mRNA specific for GATA3 (A), T-bet (B), and housekeeping 18S rRNA were determined by real-time PCR at 24 and 48 h in respective strain CD4$^+$ T cells primed by plate-bound anti-CD3 and anti-CD28 (5 μg/ml each). Addition or neutralization of IFNγ in priming cultures of C57B/6 IFN$\gamma^{-/-}$ or C57B/6 CD4$^+$ T cells, respectively, do not seem to significantly influence specific mRNA levels (A), whereas the T-bet expression is dependent on IFNγ and is strongly induced by it both in C57B/6 and C57B/6 IFN$\gamma^{-/-}$ CD4$^+$ T cells (B).

Consistent with our previous experiments, IFNγ increased the proportions of IL-4 producers. T-bet was not detected in cells cultured in the absence of IFNγ but was induced to an equivalent degree by IFNγ in IL-4$^+$ and IL-4$^-$ cells. IL-4 did not suppress levels of T-bet. The levels of GATA3 were slightly higher in IL-4–producing cells; GATA3 was not at all suppressed by IFNγ at the dose we used.

IFNγ Diminishes Numbers of IL-4 Negative CD4$^+$ Cells and, Thus, Increases Proportion of IL-4 Producers. Consistent with the antiproliferative/proapoptotic properties of IFNγ, we observed decreased cell yields in C57BL/6 IFN$\gamma^{-/-}$ CD4$^+$ priming cultures whenever IFNγ was added. This led us to determine the numbers of IL-4$^+$ and IL-4$^-$ cells emerging from these cultures. LN-derived C57BL/6 IFN$\gamma^{-/-}$ CD4$^+$ cells were primed with plate-bound anti-CD3 and anti-CD28 in the presence or absence of IFNγ and/or IL-4 for 4 d. Upon restimulation, the cells were enumerated, and IL-4 content was determined as described in Fig. 4. Under the influence of IFNγ, CD4 cell numbers were diminished (Fig. 5 A), but the proportion of IL-4$^+$ was significantly enhanced (Fig. 5 B). Absolute numbers of CD4$^+$ IL-4$^+$ cells were also increased (Fig. 5 C), but less so than their proportion. The numbers of CD4$^+$ IL-4$^-$ were dramatically diminished (Fig. 5 D). Thus, it appears that the IFNγ-induced increase in the proportion of IL-4$^+$ CD4$^+$ cells is at least partially due to their preferential survival or to the preferential death of the IL-4$^-$ CD4$^+$ T cells in the presence of IFNγ. CFSE-labeling studies revealed no difference in the proliferation history of IL-4 producers and nonproducers in the presence or absence of IFNγ, suggesting that the observed differences in cell yield cannot be accounted for by differences in proliferative rate (Fig. 6).

Exogenous IFNγ Enhances In Vivo Th2 Priming in an IFNγ-deficient Host. Continuous administration of soluble proteins by a mini-osmotic pump was shown previously to result in the selective development of Th2 helper cells in BALB/c mice (21). To test the role played by IFNγ during Th2 priming in vivo, $OVA_{323-339}$/I-A^d-specific DO11.10 TCR transgenic Rag2$^{-/-}$ cells were transferred (22) into groups of IFN$\gamma^{-/-}$ BALB/c mice implanted previously with a mini-osmotic pump containing OVA and titrated amounts of recombinant IFNγ. After 4.5 d of priming, cells from draining LNs were stimulated with OVA peptide and anti-CD28 mAb for 8 h. Cellular cytokine content of transferred OVA-specific T cells was measured by flow cytometry after intracellular staining. Results are presented in Fig. 7. The proportions and absolute numbers of IL-4–producing KJ1-26$^+$CD4$^+$ cells were lower in IFN$\gamma^{-/-}$ BALB/c mice (primed with OVA only) than in wild-type mice. Th2 priming in IFN$\gamma^{-/-}$ BALB/c animals was enhanced dramatically by the addition of recombinant IFNγ during the priming process. The IFN$\gamma^{-/-}$ animals that received 1 and 10 μg of IFNγ during priming had significantly higher proportions of IL-4 producers even compared with the wild-type mice. These results strongly suggest that efficient in vivo Th2 priming requires small amounts of IFNγ, whose origin remains to be defined. It should be pointed out that

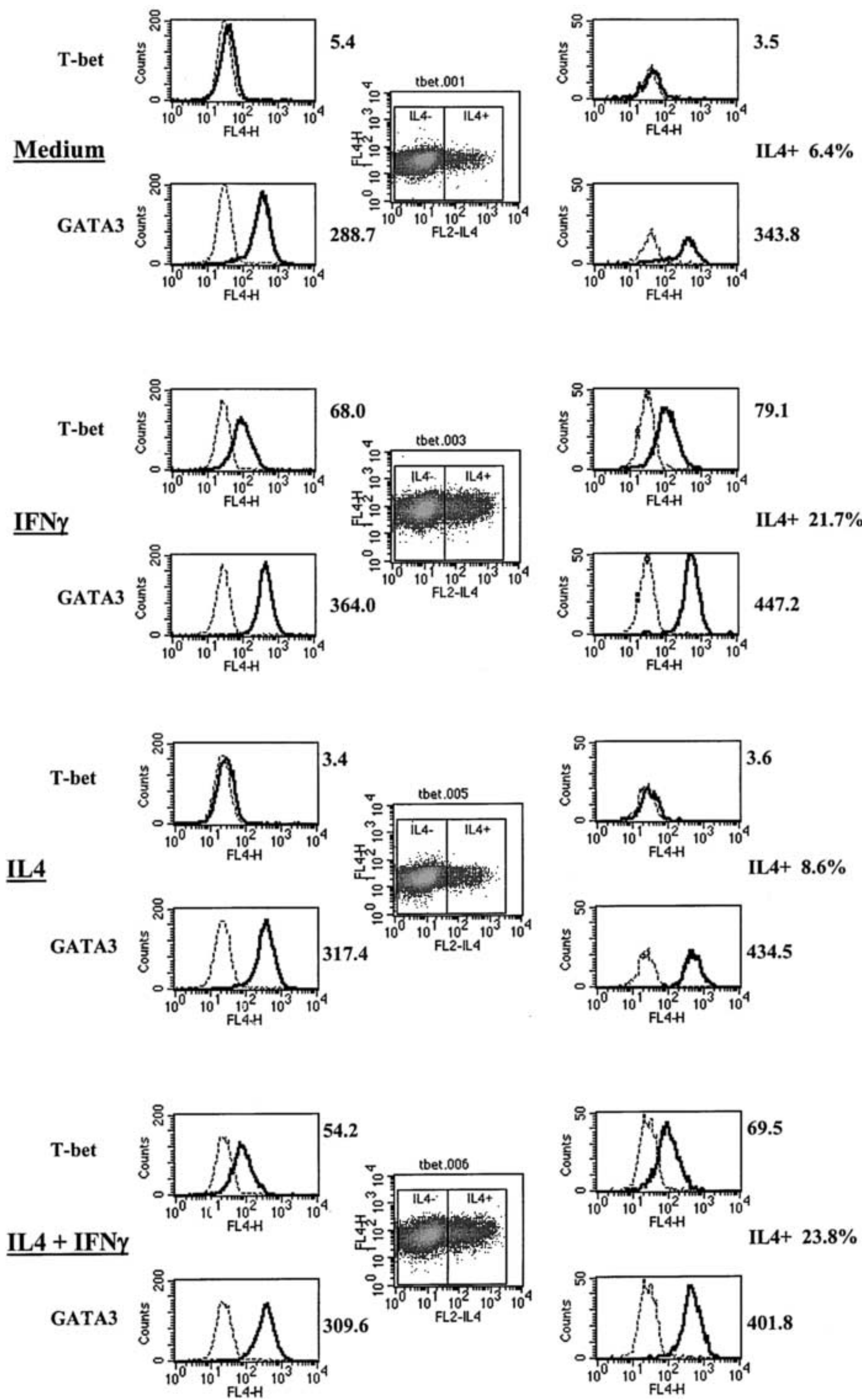


Figure 4. Addition of IFNγ to priming cultures of C57BL/IFNγ$^{-/-}$ CD4^{+} T cells increases the proportion of IL-4 producers and up-regulates T-bet in both IL-4 negative and IL-4 positive cells, whereas levels of GATA3 are not diminished. LN-derived C57BL/6 IFNγ$^{-/-}$ CD4^{+} cells (purified by depletion of CD8^{+}, B220^{+}, CD16, I-A^{b+}, and NK1.1^{+} cells as described in Materials and Methods) were primed with plate-bound anti-CD3 and anti-CD28 (5 μg/ml each) and 10 U/ml IL-2 in the presence of indicated cytokines (IFNγ, 6 ng/ml and IL-4, 1,000 U/ml) followed by 6 h of restimulation. Cells were fixed and permeabilized, and the content of IL-4, T-bet, and GATA3 was determined by flow cytometry as described in Materials and Methods. Priming conditions are underlined, and the proportions of IL-4 producers are indicated on the right. Relative content of intracellular T-bet and GATA3 are presented by a difference between specific mean fluorescence index (solid lines) and that of isotype matched antibody (dashed lines) gating separately on population of IL-4 negative cells (histograms, left) and IL-4 positive cells (histograms, right). The net difference in mean fluorescence intensity is presented for each histogram.

the administration of IFNγ to the IFNγ$^{-/-}$ mice resulted in an increased yield of KJ1-26^{+}CD4^{+} cells, particularly in the 10-μg IFNγ group; the reason for this increase is unclear. In addition, the numbers of IL-4–nonproducing KJ1-26^{+}CD4^{+} cells was not diminished by administration of IFNγ so that the in vivo mechanism of increase in the proportion of IL-4 producers is not likely due to preferential cell death of the IL-4 nonproducers (Table II).

Discussion

The "classical' view of Th1/Th2 polarization holds that the presence of IL12 and/or IFNγ during activation of naive CD4 T cells induces the Th1 phenotype, whereas IL-4 plays a central role in Th2 induction. It is now clear that this concept is far more complex and intricate. BALB/c mice with disrupted IL-4 loci remain surprisingly susceptible to *L. major* infection and fail to mount a Th1 response

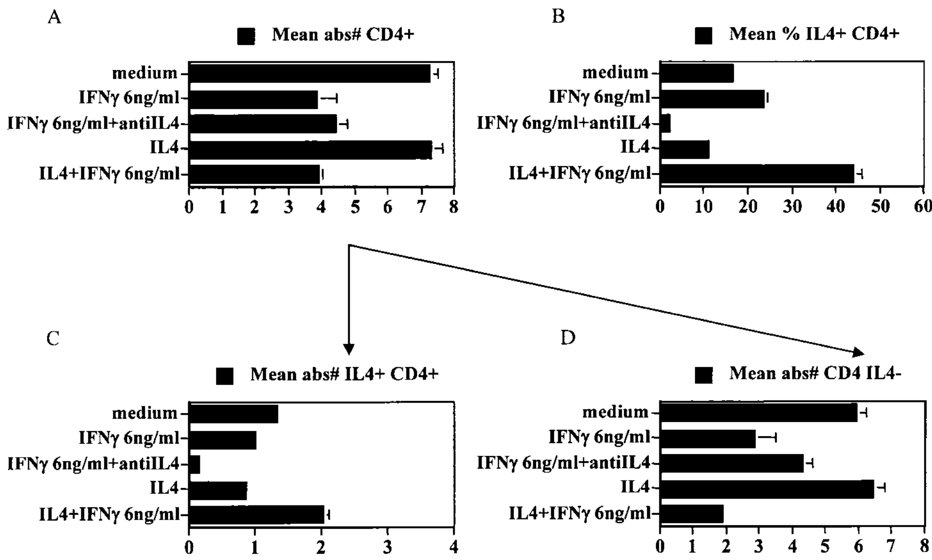


Figure 5. IFNγ added to priming cultures of C57BL/6 IFN$\gamma^{-/-}$ CD4$^+$ T cells diminishes cell numbers by preferentially inducing cell death of IL-4 negative cells and, thus, increases proportion of IL-4 producers. LN-derived C57BL/6 IFN$\gamma^{-/-}$ CD4$^+$ cells (purified by depletion of CD8$^+$, B220$^+$, CD16, I-A^{b+}, and NK1.1$^+$ cells as described in Materials and Methods) were primed with plate-bound anti-CD3 and anti-CD28 (5 μg/ml each) and 10 U/ml IL-2 in the presence of indicated cytokines or antibodies (IFNγ, 6 ng/ml; IL-4, 1,000 U/ml; and anti–IL-4, 10 μg/ml) followed by 6 h of restimulation. Cells were enumerated, fixed, and permeabilized; and the content of IL-4 was determined by FACS®. Under the influence of IFNγ, cell numbers are diminished (A), but the proportion of IL-4$^+$ is significantly enhanced (B). Absolute numbers of CD4$^+$ IL-4$^+$ cells may be also increased (C), whereas those of CD4$^+$ IL-4$^-$ are dramatically decreased (D). The means and standard deviations of triplicate experiments are presented.

(23). More recent experiments showed that IL-4 enhances the development of the Th1 phenotype and helps in the initiation of delayed type hypersensitivity, in the development of cytotoxic T lymphocyte–mediated tumor immunity (24–27), and in the induction of long-term survival of cytotoxic antigen-specific CD8 T cells (28). At least some of these findings may be explained by IL-4 action on APCs. Hochrein et al. reported that IL-4 was important in the differentiation of dendritic cells that produced IL12 p70 (29). *L. major*–infected BALB/c mice were rendered resistant to the parasite by injection of IL-4 during the period of APC activation, causing them to secrete IL-12. When IL-4 was present during the T cell priming period, a Th2 response developed and the animals were susceptible to the parasite (30).

Thus far, IFNγ has been considered mainly as a Th1 inducer and as a down-regulator of Th2 priming and responses. Indeed, IFNγ has been reported to stabilize the Th1 phenotype; C57BL/6 IFN$\gamma^{-/-}$ CD4 cells undergoing several rounds of in vitro priming in the presence of anti–IL-4 can still be induced to develop into IL-4 producers (9).

Studying the initial stages of in vitro Th1/Th2 polarization based on cytokine milieu, we observed that complete neutralization of IFNγ in priming cultures results in subop-

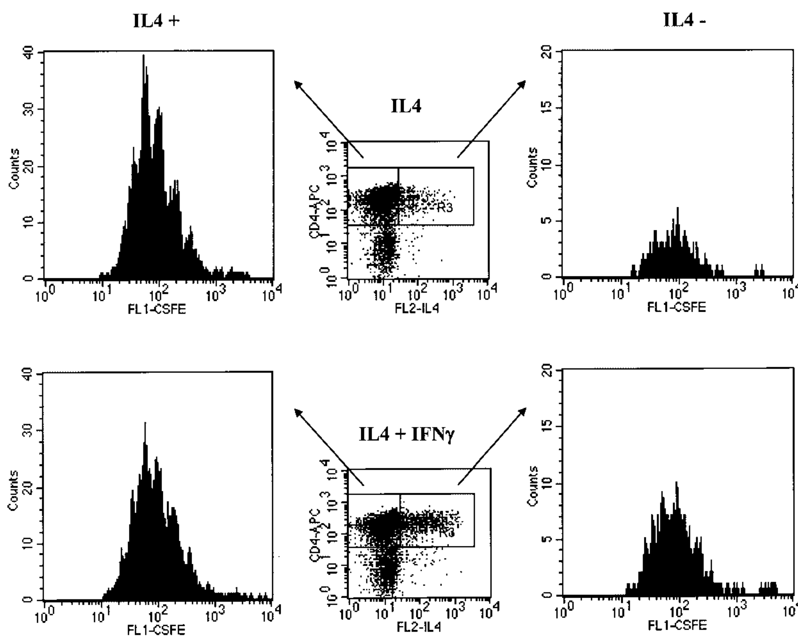


Figure 6. The proliferative rates of CD4$^+$ IL-4 producers and nonproducers in the presence of IFNγ are similar. LN-derived C57BL/6 IFN$\gamma^{-/-}$ CD4$^+$ cells (purified by depletion of CD8$^+$, B220$^+$, CD16, I-A^{b+}, and NK1.1$^+$ cells as described in Materials and Methods) were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) and primed with plate-bound anti-CD3 and anti-CD28 (5 μg/ml each) and 10 U/ml IL-2 in the presence of 6 ng/ml IFNγ and 1,000 U/ml IL-4 or IL-4 only followed by 6 h of restimulation. CSFE dilution profiles were examined by flow cytometry gating on IL-4 positive (histograms, left) or IL-4 negative CD4$^+$ T cells (histograms, right).

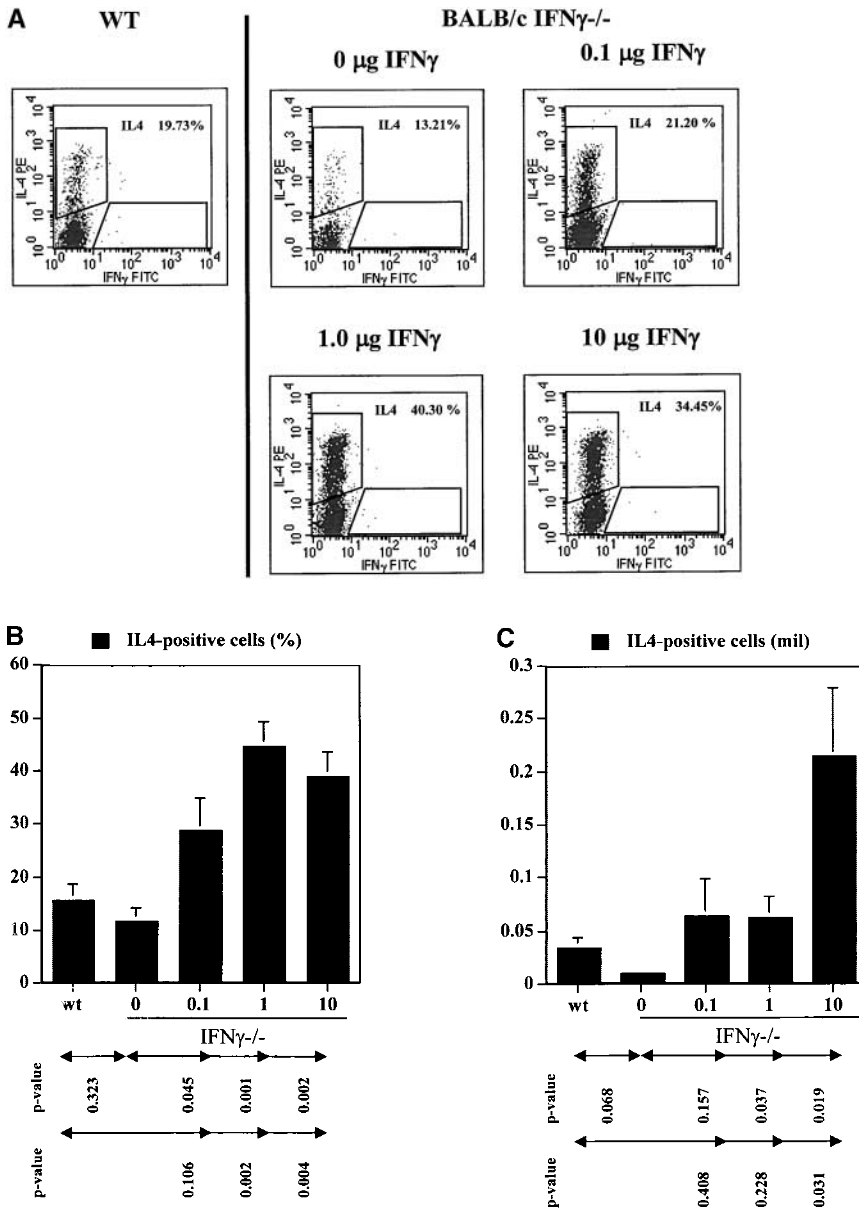


Figure 7. IFNγ enhances in vivo Th2 priming. Wild-type and IFN$\gamma^{-/-}$ BALB/c mice were implanted with a mini-osmotic pump containing OVA (1 mg/animal) in PBS and recombinant IFNγ as indicated. The next day, LN-derived naive $CD4^+$ cells (purified by depletion of $CD8^+$, $B220^+$, CD16, I-A^{b+}, and $DX5^+$ cells as described in Materials and Methods) (3×10^6 cells/mouse) from RAG2-deficient DO11.10 transgenic donors were transferred by i.p. route. After 4.5 d, animals were killed, and LN cells were restimulated with 10 μM $OVA_{323-339}$ peptide and 5 μg/ml anti-CD28 for 7 h. Cytokine contents for IL-4 and IFNγ in transferred KJ1-26^+CD4^+ T cells were measured by flow cytometry as presented for one representative animal in A. The means and standard errors of relative (B) and absolute (C) numbers of IL-4–producing KJ1-26^+CD4^+ T cells for each group are presented. The p-values of the Student's *t* test comparison between IFN$\gamma^{-/-}$ BALB/c that received OVA only and a specific group are indicated (top); lower p-values are representative of comparison between wild-type and respective groups.

timal Th2 differentiation, marked by lower proportions of IL-4– and IL-13–producing CD4 cells. This was observed when the CD4 cells were primed with either plate-bound or soluble anti-CD3 and anti-CD28. This observation was reproduced in several mouse strains. It was not due to a nonspecific effect of the monoclonal anti-IFNγ antibody because the antibody had no effect on IL-4 production in an IFNγ-deficient strain.

We have further used IFN$\gamma^{-/-}$ mice to study the observed phenomenon in greater detail. The addition of IFNγ to C57BL/6 IFN$\gamma^{-/-}$ CD4 T cells significantly enhanced the proportion of cells making IL-4, IL-10, and IL-13, but not TNFα. Neutralization of endogenous IL-4 by anti–IL-4 prevented the Th2 priming, indicating that the IFNγ-induced Th2 priming enhancement was IL-4 dependent. Indeed, when CD4 T cells were first sorted for naive phenotype, no spontaneous IL-4 production was observed. This is presumably because sorting removed "preactivated" cells that produced the IL-4 needed for priming. Also, naive CD4 T cells only make IL-4 in response to stimulation with "low affinity" peptides (31) or with low concentrations of cognate peptide (32), and, as we show here, IFNγ did not induce "early" IL-4 production by naive CD4 T cells. However, when exogenous IL-4 was also added, IFNγ dramatically enhanced the proportion of Th2 cells. Interestingly, it has been reported recently that LPS classically induces Th1 responses by stimulating Toll-like receptors or can enhance Th2 APC priming. This Th2-inducing effect of LPS was reported to be due to its action on APCs (33). Our observations indicate that IFNγ can act directly on undifferentiated CD4 cells to enhance Th2 priming. The most persuasive argument in favor of this is that IFNγ

Table II. *Total Yields of KJ1-26⁺ IL-4 Negative Cells after In Vivo Priming in the Presence or Absence of IFNγ*[a]

	IFNγ KO recipients							
	Total KJ	IL-4neg KJ	Total KJ	IL-4neg KJ	Total KJ	IL-4neg KJ	Total KJ	IL-4neg KJ
IFNγ dose	0		0.1		1		10	
	0.130	0.113	0.078	0.066	0.229	0.113	0.452	0.296
	0.062	0.058	0.386	0.230	0.094	0.056	0.221	0.156
	0.055	0.044	0.187	0.116	0.130	0.060	0.608	0.316
	0.150	0.138	0.090	0.071	0.086	0.056	0.764	0.420
Mean (cells $\times 10^6$)	0.099	0.088	0.185	0.120	0.135	0.071	0.512	0.297

[a]In millions.

enhances IL-4 production in a system in which highly purified sorted naive $CD4^+$ T cells are primed with plate-bound anti-CD3 and anti-CD28; no other cell types are present in the culture.

Using a quantitative PCR procedure, we determined that IFNγ began to up-regulate IL-4 mRNA in C57BL/6 $IFN\gamma^{-/-}$ CD4 T cells between 24 and 48 h of culture. It also strongly induced mRNA for T-bet but it did not diminish GATA3 mRNA. The expression of T-bet mRNA in C57BL/6 CD4 T cells was significantly diminished by the removal of IFNγ. The latter findings are consistent with a previous report suggesting an IFNγ/T-bet "autocrine" loop in Th1 differentiation (12).

The overexpression of retroviral T-bet in Th2 cells has been reported to suppress IL-4 production and induce IFNγ through an IFNγ-independent mechanism (6). However, we observed that in $IFN\gamma^{-/-}$ $CD4^+$ T cells primed for 4 d in the presence of IFNγ or IFNγ and IL-4, T-bet expression was equally enhanced in IL-4 producers and IL-4 nonproducers, as determined by flow cytometric analysis of cells stained for intracellular IL-4 and T-bet content. T-bet was not detected in cells cultured in the absence of IFNγ and addition of IL-4 did not suppress levels of T-bet in cells cultured with added IFNγ. The difference between our results and those aforementioned that used retroviral T-bet may have several explanations. In our experimental system, IFNγ is present from the outset of CD4 priming cultures, causing several intracellular events beyond induction of T-bet, whereas retroviral infection requires that cells first be activated, and only T-bet is induced. In addition, one can expect that retroviral overexpression will produce sustained levels of intracellular T-bet that are in excess of those induced in response to IFNγ action on resting or recently activated CD4 T cells.

In our effort to understand the mechanism by which IFNγ enhances IL-4 production, we examined its effects on intracellular levels of phosphorylated STAT6 during Th2 differentiation. Consistent with previous reports (34), we did not observe any significant changes (unpublished data). Furthermore, IFNγ did not enhance surface expression of IL-4Rα nor did increasing the concentration of IL-4 used for priming diminish the capacity of IFNγ to enhance IL-4 priming (unpublished data). Thus, it is unlikely that IFNγ acts by increasing the efficiency of IL-4 signaling.

The antiproliferative/proapoptotic properties of IFNγ have been well established (20). Early studies examining proliferation of T cell clones producing IL-4 versus IFNγ indicated the ability of recombinant IFNγ to inhibit proliferation of the IL-4–producing clones (35). A recent paper indicated that IFNγ is required for activation-induced cell death of T cells and suggested that Th2 cells may be less sensitive to this effect (36). We observed decreased cell yields in $IFN\gamma^{-/-}$ $CD4^+$ priming cultures in the presence of IFNγ. The higher proportion of IL-4 producers induced by IFNγ could be due to their preferential survival or the preferential death of IL-4 nonproducers. Indeed, in the presence of IFNγ, the numbers of $CD4^+$ $IL\text{-}4^-$ cells diminished dramatically; however, the absolute number of IL-4 producers still increased. Thus, although a survival advantage of IL-4–producing cells may partially explain the effect of IFNγ in increasing priming for IL-4 production, it cannot fully account for the observed effect.

To determine whether IFNγ enhanced IL-4 priming in vivo, we adapted an experimental system using continuous administration of soluble protein by implanted mini-osmotic pumps in BALB/c mice. This system has been shown previously to result in selective induction of antigen-specific Th2 cells (21). $IFN\gamma^{-/-}$ BALB/c mice, which had received DO11.10 TCR transgenic T cells and had been implanted with mini-osmotic pumps containing OVA, developed somewhat fewer cells that produced IL-4 upon subsequent in vitro challenge with peptide than did similarly primed wild-type BALB/c recipients. Administration of IFNγ in the mini-osmotic pumps that contained OVA significantly increased priming for IL-4 production in the $IFN\gamma^{-/-}$ mice; indeed, these animals developed even higher proportions of IL-4 producers than did the primed wild-type mice. Administration of IFNγ also increased the yield of TCR transgenic cells. Furthermore, the yield of IL-4–nonproducing TCR transgenic cells was similar or greater in the groups that received IFNγ, supporting the argument that in vivo the principal mechanism through

which IFNγ influenced IL-4 production was not by preferentially killing IL-4 nonproducers.

These results suggest that optimal in vivo Th2 priming requires small amounts of IFNγ. However, higher concentrations of IFNγ may well inhibit the Th2 polarization and induce Th1 cell development, as we observed that the frequency of IFNγ-producing cells correlated with the amount of IFNγ present during priming (unpublished data). These results emphasize the complexity of the Th1/Th2 polarization process and argue that the significance of IFNγ to a cell undergoing priming will be quite different depending on whether IL-4 is or is not present. One may hypothesize that the enhancing effect of IFNγ on Th2 polarization we have observed may represent a certain regulatory measure to prevent extreme polarization of the Th effectors toward Th1 phenotype. Whether the induction of T-bet in IFNγ-treated cells is key to the enhancement of IL-4 production or one of the myriad other factors induced by this cytokine is unknown. In preliminary experiments, we observed that infection of $IFN\gamma^{-/-}$ cells with a T-bet retrovirus enhances the proportion of cells that produced IL-4, suggesting that T-bet may be critically involved in the increased expression of IL-4.

These observations indicate that cytokine regulation of polarization of CD4 T cells to the Th1 or Th2 phenotype is substantially more complex than originally envisioned in studies that examined the dominant roles of IL-12/IFNγ on the one hand and IL-4 on the other. It now seems clear that precise timing and precise control of amounts of cytokine may be key to whether the dominant effect of IFNγ is to enhance or suppress Th2 priming and, similarly, with regard to IL-4, whether its dominant effect is to increase the potency of dendritic cells biasing priming to the Th1 phenotype or, through its direct action on T cells, to bias priming to the Th2 phenotype. These experiments emphasize the need for detailed analysis of in vivo priming conditions if one is to be able to predict the outcome of a given immunization protocol.

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