# Interleukin 21 Is a T Helper (Th) Cell 2 Cytokine that Specifically Inhibits the Differentiation of Naive Th Cells into Interferon γ–producing Th1 Cells

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## Abstract

The cytokine potential of developing T helper (Th) cells is directly shaped both positively and negatively by the cytokines expressed by the effector Th cell subsets. Here we find that the recently identified cytokine, interleukin (IL)-21, is preferentially expressed by Th2 cells when compared with Th1 cells generated in vitro and in vivo. Exposure of naive Th precursors to IL-21 inhibits interferon (IFN)-γ production from developing Th1 cells. The repression of IFN-γ production is specific in that the expression of other Th1 and Th2 cytokines is unaffected. IL-21 decreases the IL-12 responsiveness of developing Th cells by specifically reducing both signal transducer and activator of transcription 4 protein and mRNA expression. These results suggest that Th2 cell-derived IL-21 regulates the development of IFN-γ–producing Th1 cells which could serve to amplify a Th2 response.

Key words: cytokines • T lymphocytes helper inducer • cell differentiation • immunosuppression • interleukins

## Introduction

Th subsets are distinguished by their ability to produce distinct cytokine patterns and promote specific immune responses (1–3). Th1 cells produce IFN-γ and promote cell–mediated immunity directed toward intracellular pathogens. In contrast, Th2 cells produce cytokines such as IL-4, IL-5, and IL-13, activate mast cells and eosinophils, and direct B cell responses against extracellular pathogens. Dysregulation of Th cell responses can result in immunopathology in that aberrant Th1 responses can be responsible for organ-specific autoimmunity and exaggerated Th2 responses have been associated with allergic diseases. Consequently, understanding the regulation of balanced Th cell immune responses has been an area of intense investigation in recent years.

The specific cytokines produced by polarized Th1 and Th2 cells are the primary effectors that promote the differentiation of precursor Th cells, but these cytokines also cross-regulate the other subset's functional activity (3). For example, IL-4 is the most potent factor described to promote the differentiation of Th precursor (Thp)* cells to Th2 effectors but IL-4 also antagonizes the production of IFN-γ by Th1 cells (4). IL-10, another cytokine produced by Th2 cells, has also been described to inhibit Th1 development and IFN-γ–induced macrophage function (5). Conversely, the IFN-γ–produced by Th1 cells amplifies Th1 development and inhibits the expansion of Th2 cells (4, 6). The ability of these cytokines to promote the development of specific Th cell subsets while simultaneously inhibiting the alternate developmental fate results in a progressively polarized immune response.

IL-21 is a recently described cytokine produced by activated $CD4^+$ T cells that is capable of costimulating T and B cell proliferation and regulating natural killer (NK) cell activation and expansion (7, 8). IL-21 shares sequence homology with the cytokines IL-2, IL-15, and IL-4 and mediates its effects through a novel class I cytokine receptor, IL-21R, associated with the common γ cytokine receptor chain (7–9). IL-21R, which is similar to the IL-2/IL-15Rβ

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*Abbreviations used in this paper: DTH, delayed-type hypersensitivity; NK, natural killer; STAT, signal transducer and activator of transcription; Thp, Th precursor.

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chain and IL-4R α chain, is widely expressed among lymphoid tissues (7, 10). Although IL-21 was shown to be expressed exclusively by $CD4^+$ T cells, the expression of IL-21 within Th cell subsets has not been reported (7). Additionally, the potential role of IL-21 in Th cell differentiation has not been explored.

In this report, we demonstrate that IL-21 is preferentially expressed in Th2 cells generated in vitro and in vivo. Exposure of differentiating Th cells to IL-21 specifically inhibits the production of IFN-γ but not other Th1 cytokines such as IL-2 or TNF-α. IL-21 treatment reduces the ability of developing Th cells to respond to IL-12 by decreasing the total amount of cellular signal transducer and activator of transcription (STAT)-4 resulting in diminished IFN-γ production by Th1 cells. These findings suggest that IL-21 is a cytokine produced by previously derived Th2 effector cells that serves to modulate the development of IFN-γ–producing Th1 cells and consequently amplify a Th2 response.

## Materials and Methods

*Mice.* Unless indicated otherwise, C57BL/6 mice of 6–8 wk of age were used in all experiments. STAT-6–deficient mice on C57BL/6 background were generated as described previously (11). IL-21R–deficient mice backcrossed on the C57BL/6 background were generated as described previously (12).

*Lymphocyte Preparation and Culture.* Lymphocytes were cultured in RPMI 1640 supplemented as described previously (11). Naive Thp cells were purified from lymph node and spleens by cell sorting using anti-CD4 and anti-CD62L (BD PharMingen) to 95–98% purity.

*Antibodies and Cytokines.* T-bet–specific antiserum was a gift from L. Glimcher and S. Szabo, Harvard School of Public Health, Boston, MA (13). Antibodies specific for STAT-4, STAT-1, and actin were obtained from Santa Cruz Biotechnology, Inc. Antibody specific for phosphorylated STAT-4 was obtained from Zymed Laboratories. The antibodies to anti-CD3, anti-CD28, IL-4, and IFN-γ used in Th cell differentiation cultures were obtained from BD PharMingen. Recombinant IL-4 was obtained from Peprotech. Recombinant IL-2 was provided by Chiron. Recombinant IL-12 was provided by Hoffmann-LaRoche. Mouse IL-21 expressed in COS cell supernatants was prepared and concentrated as described previously (12). Mock transfected COS supernatant, prepared and concentrated in parallel with IL-21, was used as a control.

*In Vitro Th Cell Differentiation.* Naive Thp cells were plated onto 1 μg/ml anti-CD3, 2 μg/ml anti-CD28–coated plates at $1–2 \times 10^6$ cells per milliliter in the presence of 10 ng/ml IL-4, 10 μg/ml anti–IFN-γ (Th2 conditions), or 1 ng/ml IL-12 and 10 μg/ml anti–IL-4 (Th1 conditions). 100 U/ml IL-2 was added 24 h later. Cultures were expanded in 100 U/ml IL-2 3 d after initial culture. After 1 wk in culture, the cells were stimulated with PMA/ionomycin and cytokine production was determined by intracellular cytokine staining as described previously (14).

*RNA Analysis.* Total RNA was isolated using RNeasy (QIAGEN). For Northern blot analysis the RNA was separated on a 1.5% agarose/6% formaldehyde gel and transferred to GeneScreen (NEN Life BioSciences) membrane. The membrane was hybridized with radiolabeled cDNA probes for IL-21, IL-4, IFN-γ, and γ-actin. For RealTime PCR, 1 μg of RNA was primed with oligo (dT) and converted to cDNA using Superscript (Invitrogen Life Technologies). 1/40th of the resulting cDNA was used as template in PCR reactions using SYBR Green 2× or TaqMan 2× PCR mix (Applied Biosystems) and analyzed in the ABI Prism 7700 Sequence Detector (Applied Biosystems) using the following primers: IL-21 forward 5′ AAGATTCCTGAGGATCCGAGAAG 3′; IL-21 reverse 5′ GCATTCGTGAGCGTCTATAGTGTC 3′; IL-21 TaqMan probe 5′ TTCCCGAGGACTGAGGAGACGCC 3′; IL-12Rβ2 forward 5′ TTTCCATTTTTGCATCAAGTTCTC 3′; IL-12Rβ2 reverse 5′ CCGATCTAGAGTCAGCCGCT 3′; STAT-4 forward 5′ AAACCTGAGCCCAACGACAA 3′; STAT-4 reverse 5′ AGTGTCCGTTTGCACCGTC 3′. Primers and TaqMan probes for IL-4, IFN-γ, and GAPDH have been published previously (15).

*Immunoblot Analysis.* Whole cell extracts were prepared by lysing cells in 50 mM Tris, 0.5% NP40, 5 mM EDTA, 50 mM NaCl, and clearing the lysates by centrifugation. Protein extracts were separated on an 8–10% polyacrylamide gel and transferred to an Optitran membrane (Schleicher and Schuell). The immunoblots were blocked for 1 h at room temperature in 5% milk in TBST (50 mM Tris, pH 7.5,100 mM NaCl, and 0.03% Tween 20) and incubated with the indicated antibody overnight at 4°C. The blots were washed with TBST and incubated with anti–rabbit HRP-conjugated antibody (Zymed Laboratories) at room temperature. After washing the blots with TBST, detection was performed using enhanced chemiluminescence (Amersham Pharmacia Biotech) according to manufacturer's instructions.

*Leishmania Infections.* Eight 4–5-wk-old BALB/c and C57BL/6 mice were infected in the hind right foodpad with $2 \times 10^6$ stationary-phase *Leishmania major* promastigotes (LV39) as described previously (16).

*Delayed-type Hypersensitivity Reaction.* 8-wk-old male mice were immunized subcutaneously with 100 μg TNP-KLH (Biosearch Technologies) emulsified in CFA at the tailbase. After 6 d mice were challenged with 50 μg of TNP-KLH in one hind footpad and PBS in the contralateral hind footpad. Footpad thickness was measured 24 h after challenge. Specific swelling was determined by subtracting nonspecific swelling in the PBS-injected foot from antigen-induced swelling. Purified $CD4^+$ T cells from draining lymph nodes were removed and stimulated in vitro with 250 μg/ml TNP-KLH and irradiated APCs. The resulting supernatants were assessed for IFN-γ production by ELISA 72 h later.

## Results

*IL-21 Is a Th2 Cytokine.* Although IL-21 has been reported to have a wide range of effects on a number of cell types, the only reported source of IL-21 thus far is activated $CD4^+$ T cells (7). To determine if IL-21 is expressed exclusively within Th cell subsets, Northern blot analysis was performed on mRNA from naive Thp cells differentiated into Th1 or Th2 cells for 1 wk and restimulated for 4 h to induce cytokine production. IL-21 mRNA was undectable in Th1 cell cultures, but induced in Th2 cells suggesting that IL-21 is a Th2 cytokine (Fig. 1 A).

IL-21 could be regulated like other Th2 cytokines in that the potential to produce IL-21 increases as the cells develop along the Th2 pathway. Alternatively, the preferential expression of IL-21 in Th2 cells could be due to an extinction

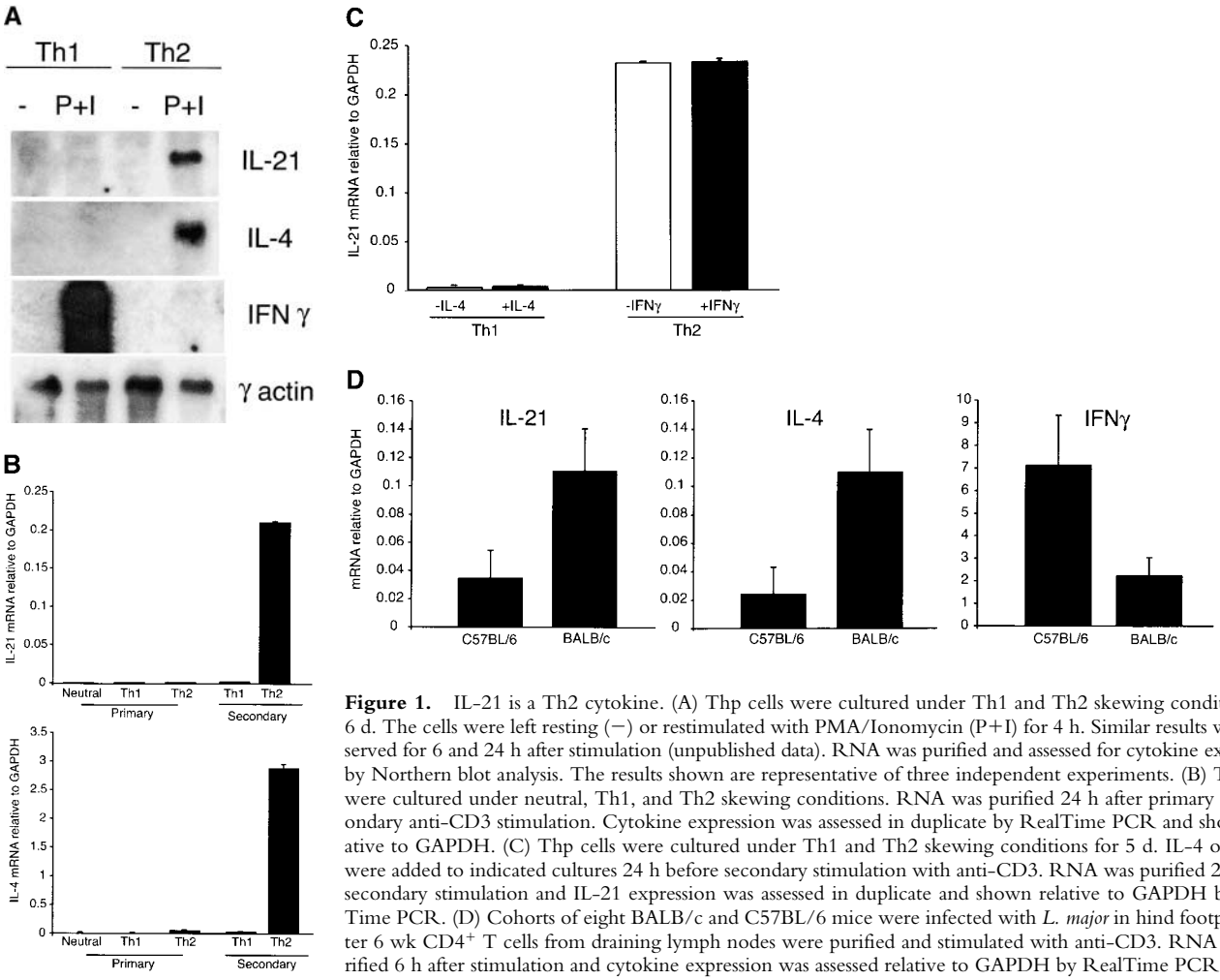


**Figure 1.** IL-21 is a Th2 cytokine. (A) Thp cells were cultured under Th1 and Th2 skewing conditions for 6 d. The cells were left resting (−) or restimulated with PMA/Ionomycin (P+I) for 4 h. Similar results were observed for 6 and 24 h after stimulation (unpublished data). RNA was purified and assessed for cytokine expression by Northern blot analysis. The results shown are representative of three independent experiments. (B) Thp cells were cultured under neutral, Th1, and Th2 skewing conditions. RNA was purified 24 h after primary and secondary anti-CD3 stimulation. Cytokine expression was assessed in duplicate by RealTime PCR and shown relative to GAPDH. (C) Thp cells were cultured under Th1 and Th2 skewing conditions for 5 d. IL-4 or IFN-γ were added to indicated cultures 24 h before secondary stimulation with anti-CD3. RNA was purified 24 h after secondary stimulation and IL-21 expression was assessed in duplicate and shown relative to GAPDH by RealTime PCR. (D) Cohorts of eight BALB/c and C57BL/6 mice were infected with *L. major* in hind footpads. After 6 wk $CD4^+$ T cells from draining lymph nodes were purified and stimulated with anti-CD3. RNA was purified 6 h after stimulation and cytokine expression was assessed relative to GAPDH by RealTime PCR.

of already high IL-21 production in Thp cells as the cells develop into Th1 effectors. To address this we compared IL-21 message expression in primary stimulated Thp cells and secondary stimulated Th2 cells. We found that IL-21 message, like IL-4, is relatively low but detectable in Thp cells after primary stimulation and is markedly induced after the cells are allowed to differentiate along the Th2 pathway (Fig. 1 B). These results suggest that IL-21 gene expression is regulated similarly to other Th2-specific cytokines.

Next, we determined if IL-21 expression in differentiated cells could be directly affected by the cytokine milieu. To address this, we cultured Th1 and Th2 cells in the presence of IL-4 and IFN-γ, respectively, before secondary stimulation. Cells were responsive to these cytokines as evidenced by the activation of STAT-6 and STAT-1 by IL-4 and IFN-γ, respectively (unpublished data). We found that the addition of IL-4 to Th1 cells had no effect on IL-21 expression (Fig. 1 C). Moreover, the addition of IFN-γ to the Th2 cultures had no inhibitory effect on the expression of IL-21. These results suggest that the expression of IL-21 in Th2 cells appears to be fixed early in Th2 differentiation and not modulated directly by IL-4 or IFN-γ.

The experiments described above examined IL-21 expression during in vitro Th2 differentiation. We wished to determine if IL-21 expression by Th2 cells is also reflected during a Th2 immune response in vivo. Infection with the protozoan *Leishmania major* provides a well characterized model for studying the in vivo response of Th cells (17). Inbred mouse strains such as C57BL/6 infected with *L. major* mount a protective Th1 response against the pathogen that provides resistance to the disease. Conversely, BALB/c mouse strains develop a predominantly Th2 response and fail to clear the infection. We infected both C57BL/6 and BALB/c mice with *L. major* and assessed the cytokine expression in $CD4^+$ cells purified from draining lymph nodes from infected animals. As expected, $CD4^+$ T cells from C57BL/6 infected mice expressed more IFN-γ than cells from BALB/c infected mice (Fig. 1 D). Corresponding with a predominantly Th2 response, $CD4^+$ T cells from infected BALB/c mice made significantly more IL-4 than T cells from C57BL/6 mice (Fig. 1 D). Similar to that observed under in vitro Th2 skewing conditions, IL-21 was also preferentially expressed during an in vivo Th2 response in BALB/c mice (Fig. 1 D). These results,

combined with our in vitro findings, strongly suggest that IL-21 is a Th2 cytokine.

*IL-21 Specifically Inhibits the Production of IFN-γ in Developing Th Cells.* Because IL-21 shares a similar expression pattern in Th cells as well as structural similarity to IL-4, we wished to determine if IL-21, like IL-4, could influence Th cell differentiation directly. To address this, purified naive Thp cells were primed under neutral, Th1 and Th2 skewing conditions in the presence and absence of IL-21. The cytokine potential of these cells was assessed by intracellular cytokine staining after 1 wk in culture. Like IL-4, the inclusion of IL-21 to neutral and Th1 cultures resulted in a marked reduction in the number of IFN-γ–producing cells (neutral and Th1 conditions, Fig. 2 A). The decreased number of IFN-γ–producing cells in Th1 cultures was confirmed by ELISPOT analysis and observed by restimulating with either PMA/Ionomycin or anti-CD3 (unpublished data). Additionally, the defect in IFN-γ production in Th1 cultures could not be overcome by the inclusion of IL-18 (unpublished data). Unlike IL-4, however, IL-21 itself was unable to potentiate the production of IL-4–producing Th2 cells (neutral conditions, Fig. 2 A). IL-21 treatment also had no stimulating or inhibitory effect on the generation of IL-4–producing cells under Th2 skewing conditions (Th2, Fig. 2 A). Inclusion of IL-21 in Th cell cultures had no detrimental effect on cell viability as assessed by Trypan blue exclusion.

As we observed reduced numbers of IFN-γ–producing Th1 effector cells 1 wk after IL-21 was included in the primary stimulation, we wished to determine if IL-21 also affected the IFN-γ produced from recently stimulated Thp cells. To assess this, purified naive Thp cells were cultured under neutral and Th1 conditions in the presence or absence of IL-21 for 48 h. The resulting culture supernatants were assessed for IFN-γ production by ELISA. We found that IL-21 reduced the amount of IFN-γ produced early in differentiation cultures (Fig. 2 B). Therefore, the presence of IL-21 during Th cell priming affects the ability of differentiating Th1 cells to produce IFN-γ.

*IL-21 Does Not Directly Inhibit IFN-γ Production from Th1 Effector Cells.* The experiments described above assessed the effects of IL-21 on the development of IFN-γ–producing cells when included during the initial priming of Thp cells. This raises the question of whether IL-21 is directly repressing the production of IFN-γ or affecting the differentiation of IFN-γ–producing Th1 cells. To address this issue, purified naive Thp cells were cultured under Th1 skewing conditions for 5 d and IL-21 was subsequently added 24 h before secondary stimulation. Unlike what was observed when IL-21 was added at the initiation of culture, the addition of IL-21 to Th1 cultures at the end of the differentiation period had no effect on IFN-γ production (Fig. 3 A) even though Th1 cells express IL-21R (unpublished data). This finding suggests that IL-21 impairs the ability of Thp cells to differentiate into IFN-γ–producing Th1 cells but does not directly inhibit IFN-γ production from Th1 effector cells.

*IL-21 Inhibition of IFN-γ Is Independent of IL-4 Signaling.* IL-4 is known to inhibit the differentiation of Th1 cells and effects of IL-4 on Th cell differentiation largely depend on the expression of STAT-6 (11, 18, 19). To determine if IL-21–mediated inhibition of IFN-γ production is mediated indirectly through the action of IL-4 we assessed the ability of IL-21 to influence Th1 differentiation in STAT-6–deficient Thp cells. In the absence of STAT-6, IL-21 was just as effective in preventing the generation of IFN-γ–producing cells (Fig. 3 B). These results suggest that the IL-21 induced repression of IFN-γ is not mediated indirectly through the action of IL-4. Additionally, unlike IL-4, IL-21 does not depend on STAT-6 signaling to prevent the generation of IFN-γ–producing Th1 cells.

*IL-21 Does Not Inhibit Other Th1 Cytokines.* The experiments thus far evaluated the ability of IL-21–treated Th1 cells to produce IFN-γ. To determine if IL-21 treatment affects other aspects of Th1 development we assessed the ability of IL-21–treated Thp cells to produce other cytokines preferentially expressed by Th1 cells. Thp cells were cultured under Th1 skewing conditions in the pres-

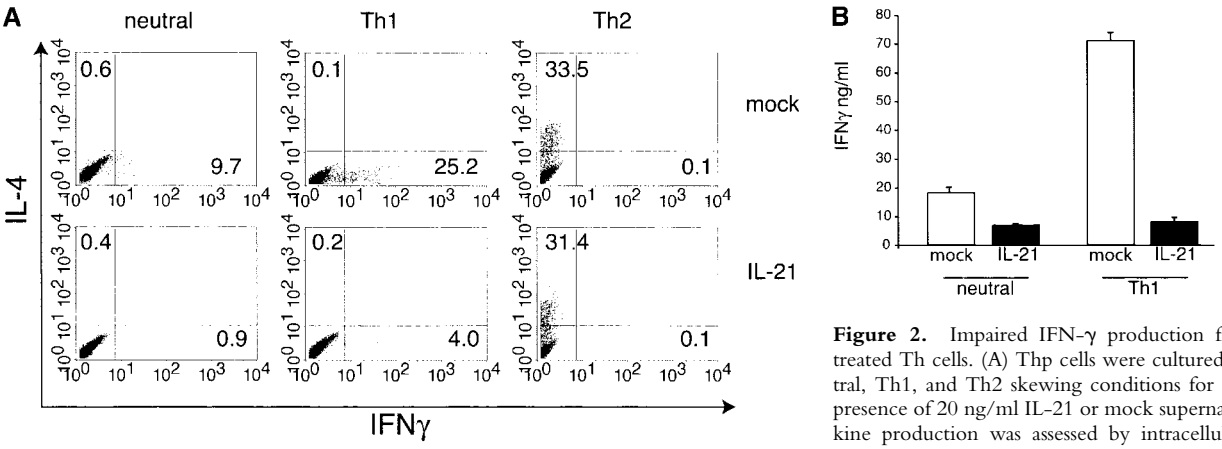


**Figure 2.** Impaired IFN-γ production from IL-21–treated Th cells. (A) Thp cells were cultured under neutral, Th1, and Th2 skewing conditions for 1 wk in the presence of 20 ng/ml IL-21 or mock supernatants. Cytokine production was assessed by intracellular cytokine staining 4 h after restimulation with PMA/Ionomycin. Results are representative of at least 10 experiments. (B) Thp cells were cultured in the presence of 20 ng/ml IL-21 or mock supernatants under neutral and Th1 conditions for 48 h. Culture supernatants were assessed for IFN-γ production by ELISA.

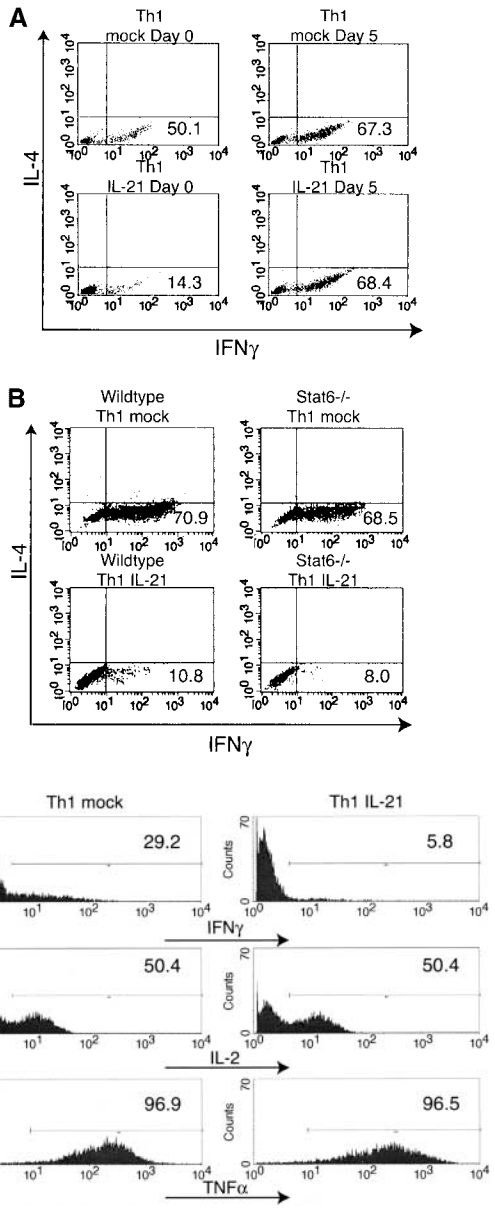


**Figure 3.** IL-21 specifically inhibits IFN-γ production from developing Th1 cells. (A) Thp cells were cultured under Th1 skewing conditions. 20 ng/ml IL-21 or mock supernatant was added either at the beginning of culture (day 0) or 24 h before restimulation and analysis (day 5). Cytokine production was assessed by intracellular cytokine staining. (B) Thp cells purified from wild-type or STAT-6–deficient mice were cultured for 1 wk under Th1 skewing conditions in the presence of IL-21 or mock supernatant. Cytokine production was assessed by intracellular cytokine staining. (C) Thp cells were cultured under Th1 skewing conditions in the presence of IL-21 or mock supernatant. Cytokine expression was assessed by intracellular cytokine staining.

ence and absence of IL-21 and assessed for cytokine production by intracellular cytokine staining after secondary stimulation. Surprisingly, although the number of IFN-γ–producing cells is significantly reduced when IL-21 is included in the priming conditions, the same cell population has normal numbers of IL-2 and TNF-α–producing cells as well as normal message levels for LTα (Fig. 3 C and unpublished data). Additionally, IL-21–treated and untreated Th1 cells make similar, high levels of IL-3 and GM-CSF, two cytokines that are made only by differentiated but not naive T cells, thus suggesting that IL-21–treated Th1 cells have differentiated beyond the naive state (unpublished data). These results imply that although IL-21 efficiently suppresses the ability of Th1 cells to produce IFN-γ, the same cells maintain the capacity to produce other Th1 cytokines and do not default to produce Th2 cytokines (Fig. 1).

*T-bet Expression Is Unaffected by IL-21.* T-bet is a recently identified transcription factor specifically expressed in differentiating Th1 cells that is capable of potently inducing IFN-γ (13). Because of its important role in IFN-γ production in Th1 cells, T-bet is an attractive potential target for IL-21. To determine if IL-21 treatment of differentiating Th1 cells affects the induction of T-bet expression in Th1 cells, naive Thp cells were cultured under Th1 skewing conditions in the presence and absence of IL-21 for 48 h. Protein extracts from these cells were analyzed for T-bet expression by Western blot analysis. As expected, T-bet expression was induced in Th1 cultures and remained low under Th2 conditions (Fig. 4 A). Addition of IL-21 had no effect on T-bet expression in Th1 cells. This result indicates that IL-21–mediated suppression of IFN-γ production is not a result of reduced T-bet expression.

*IL-21 Inhibits IL-12 Signaling.* IL-12 signaling plays a critical role in the development of Th1 cells. Th cells lacking IL-12R are severely compromised in their ability to produce IFN-γ (20). Additionally, IL-12Rβ2 chain expression is specifically extinguished in developing Th2 cells, an effect mediated by IL-4 (21). To determine if IL-21, like IL-4, affects the expression of IL-12Rβ2 chain in Th1 cells, mRNA from naive Thp cells cultured under Th1 skewing conditions in the presence and absence of IL-21 was analyzed for IL-12Rβ2 expression by RealTime PCR. As expected, IL-12Rβ2 expression was high in Th1 cells when compared with Th2 cells. However, addition of IL-21 to the Th1 cultures did not affect IL-12Rβ2 expression (Fig. 4 B). Therefore, unlike that reported for IL-4, IL-21 treatment did not result in decreased expression of IL-12Rβ2. Moreover, the high IL-12Rβ2 expression coupled with high TNF-α and IL-2 levels suggests that IL-21–treated Th1 cells maintain many Th1 characteristics with the distinct exception of decreased IFN-γ production.

STAT-4 is specifically activated by IL-12 and is a critical signaling mediator for the generation of IFN-γ–producing Th1 cells (22). To determine if IL-21 affects the ability of IL-12 to activate STAT-4, naive Thp cells were activated for 48 h in the presence or absence of IL-21. The cells were subsequently stimulated with IL-12 and the extent of STAT-4 phosphorylation was determined by Western blot analysis. Although IL-21–treated cells express normal levels of IL-12Rβ2, STAT-4 phosphorylation in response to IL-12 stimulation was reduced in IL-21–treated cells (Fig. 4 C). The decreased level of phosphorylated STAT-4 is also reflected by a decrease in total STAT-4 protein levels (Fig. 4 C). As a comparison, STAT-1 protein expression is unaffected by IL-21 treatment (Fig. 4 C). The IL-21–induced

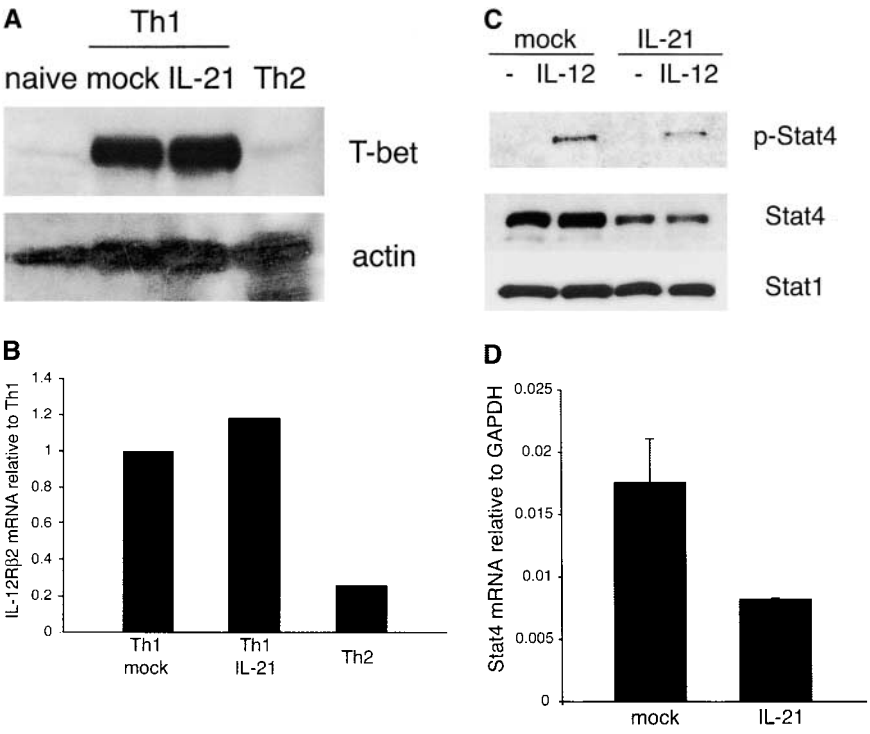


**Figure 4.** IL-21 inhibits STAT-4 signaling. (A) Thp cells were cultured under Th1 or Th2 skewing conditions. Protein extracts were harvested at the beginning (naive) and 48 h after culture (Th1 and Th2). During which 20 ng/ml IL-21 or mock supernatants were included in the indicated cultures. T-bet and actin expression were determined by Western blot analysis. (B) Thp cells were cultured under Th1 or Th2 skewing conditions for 1 wk. 20 ng/ml IL-21 or mock supernatant was included in indicated cultures. RNA was harvested 24 h after secondary stimulation with anti-CD3 and assessed for IL-12Rβ2 expression by RealTime PCR. The results are representative of three independent experiments. (C) Thp cells were stimulated with anti-CD3 for 48 h in the presence of 20 ng/ml IL-21 or mock supernatants. The cells were then stimulated for 15 min with 1 ng/ml IL-12. Protein extracts were assessed for phosphorylated STAT-4 (p-STAT-4), STAT-4, and STAT-1 by Western blot analysis. Results are representative of four independent experiments. (D) Thp cells were cultured as in C. RNA was harvested and assessed for STAT-4 expression in duplicateby RealTime PCR and shown relative to GAPDH. Results are representative of three independent experiments.

decrease in STAT-4 protein levels is likely due to a decrease in STAT-4 mRNA (Fig. 4 D). These findings suggest that IL-21 treatment dampens the responsiveness of developing Th1 cells to IL-12 through a reduction of STAT-4 expression.

*IL-21 Signaling Is Required for Limiting a Th1 Response In Vivo.* Given our observation that IL-21 treatment could inhibit Th1 cell function in vitro, we wished to determine if endogenously produced IL-21 plays a role in limiting Th1 cell function in vivo. To this end, we examined a classic Th1 cell–mediated inflammatory response, the delayed-type hypersensitivity (DTH) reaction in IL-21R–deficient mice. DTH responses to a specific antigen (TNP-KLH) were examined by injection of antigen or PBS to the hind footpads of previously immunized wild-type or IL-21R–deficient mice. Footpad swelling was determined 24 h later. Wild-type animals responded to antigenic challenge with robust swelling of the footpad (Fig. 5 A). Interestingly, IL-21R–deficient mice mounted a much stronger DTH response resulting in an average of twice the swelling (Fig. 5 A). The increased DTH response in IL-21R–deficient animals correlated with a marked increase in IFN-γ

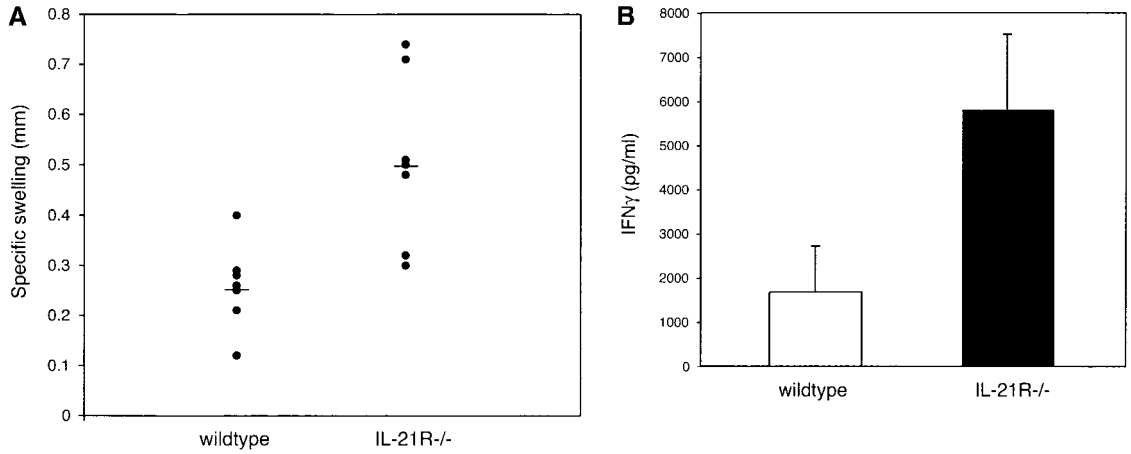


**Figure 5.** Enhanced DTH responses in IL-21R–deficient mice. (A) Specific footpad swelling of wild-type and IL-21R–deficient (IL-21R$^{-/-}$) mice was determined by subtracting nonspecific swelling in the PBS-injected footpad from the TNP-KLH-induced swelling. Each data point represents one mouse and horizontal lines indicate averages. Results are pooled of two independent experiments. (B) Purified CD4$^{+}$ T cells from the draining lymph nodes of immunized mice were stimulated in vitro with 250 μg/ml TNP-KLH and irradiated APCs. Supernatants were analyzed for IFN-γ levels by ELISA. Results shown are the average of two mice from each genotype performed in duplicate.

production from $CD4^+$ T cells purified from the draining lymph nodes and restimulated by antigen in vitro (Fig. 5 B). These results strongly support the notion that IL-21 is involved in limiting Th1 cell responses by suppressing the production of IFN-γ.

## Discussion

In this report, we demonstrate that the newly described cytokine, IL-21, is preferentially expressed in Th2 cells compared with Th1 cells. We have also shown that IL-21 influences Th cell differentiation by inhibiting the production of IFN-γ from developing Th1 cells, due to a decrease in the effectiveness of IL-12 signaling. The effect on IFN-γ expression is remarkably specific in that other characteristics of Th1 differentiation are intact (T-bet, IL-2, TNF-α, IL-12Rβ2). Lack of IL-21 signaling in vivo results in an increased DTH response, characterized by an increased inflammatory response and antigen-specific IFN-γ production by Th1 cells. Given the specific effect on IFN-γ production, IL-21 expression by previously activated Th2 cells could serve to bias away from a Th1 response and promote the expansion of Th2 cells by specifically suppressing IFN-γ.

Although Th2 cells express IL-21, the *IL-21* gene is not genetically linked to the Th2 cytokine gene cluster that includes *IL-4*, *IL-5*, and *IL-13*. Instead, *IL-21* is linked to *IL-2* and *IL-15* and, consistent with this, a sequence comparison of IL-21 with IL-4, IL-15, and IL-2 indicates that IL-21 shares most sequence identity with IL-2 (7). The Th2 locus is thought to be coordinately regulated by adjacent gene-specific promoters and enhancers as well as by an intergenic regulatory element, termed CNS, that has been shown genetically to be involved in the Th2-specific regulation of the locus (23). In addition, evidence suggests that the Th2-specific transcription factor GATA-3 directly regulates IL-4, IL-5, and IL-13 transcription and GATA-3 has also been linked with the chromatin remodeling that occurs in the Th2 locus during Th2 differentiation (24). IL-21 expression mirrors the other Th2 cytokines in that it is relatively low in activated Thp cells and high in Th2 effectors (Fig. 1). However, we do not observe a marked preferential expression of IL-21 under Th2 skewing conditions immediately after priming like that observed for IL-4 (IL-21 mRNA-Th1 = 0.0004, Th2 = 0.0009, IL-4 mRNA-Th1 = 0.0001, Th2 = 0.044; Fig. 1 B). One explanation for this observation is that the CNS element may be important for the early expression of Th2 cytokines and it is clearly not regulating IL-21 transcription. Another possibility is that IL-21 transcription may be more dependent on other Th2-specific transcription factors, such as c-maf, which are upregulated later than GATA-3 during Th2 differentiation (25). Additionally, we have observed that IL-21 can induce IL-21 expression in Th1 cells (unpublished data). This is in contrast to the inability of IL-4 to induce its own expression in Th1 cells, suggesting that IL-21 expression within Th cell subsets may be more plastic than IL-4 and subject to influences from neighboring cell populations. It should also be noted that a previous study on human IL-21 reported the detection of high levels of IL-21 mRNA from freshly isolated $CD4^+$ PBLs (7). In this study, the PBLs were not purified for naive Thps and presumably contained effector $CD4^+$ Th2 cells poised to express high levels of IL-21.

We demonstrate here that IL-21 inhibits STAT-4 activity by reducing STAT-4 expression. The expression of a number of STAT proteins, including STAT-4, has been shown to be downregulated by cytokine exposure primarily through proteasome mediated degradation (26–29). This does not appear to be the mechanism here as STAT-4 mRNA, as well as protein, is affected by IL-21 exposure (Fig. 4 D). Several examples of regulated STAT-4 mRNA expression have been reported. STAT-4 transcripts were found to be preferentially expressed in Th1 cells compared with Th2 cells (30). Moreover, the induction of STAT-4 message in maturing dendritic cells and activated macrophages is inhibited by exposure to the Th2 cytokines, IL-4, and IL-10 (31). Decreased STAT-4 expression may then in turn reinforce differentiation along the Th2 pathway. These observations suggest that IL-21 may be in part responsible for the reduced expression of STAT-4 in Th2 cells, however STAT-4 expression is equivalent between wild-type and IL-21R–deficient Th2 cells suggesting that other Th2 cell cytokines can mediate this effect as well (unpublished data).

Although we demonstrate that IL-21 compromises the ability to signal through STAT-4, it is possible that other STAT-4–independent pathways for IFN-γ production may also be affected by IL-21. First, the small amount of IFN-γ produced by STAT-4–deficient Th cells is also diminished by IL-21 exposure (unpublished data). In addition, our data suggest that T-bet expression is normal in IL-21–treated Th1 cells. Consistent with this observation and the recent report that T-bet targets chromatin remodeling at the IFN-γ locus, we have found that the IFN-γ locus is equally accessible to DNase 1 cleavage in both IL-21–treated and untreated Th1 cells (reference 32 and unpublished data). However, another potential IL-21 target is the GADD45 protein family. GADD45γ is preferentially expressed in Th1 cells and is able to directly activate p38 and JNK pathways, both of which have been suggested to be involved in IFN-γ production from Th1 cells (33, 34). Moreover, Th1 cells from GADD45γ-deficient mice are compromised in their ability to make IFN-γ, and like IL-21–treated Th1 cells, produce normal amounts of TNF-α (33). While preliminary studies do not suggest that IL-21 exposure results in gross changes in p38 and JNK activity or GADD45γ expression (unpublished data), more thorough analysis in the future will be required to determine the effect of IL-21 on these signaling pathways.

IL-21 has a unique immunosuppressive effect on IFN-γ production. Antiinflammatory cytokines, such as IL-10 and TGF-β, have been reported to have similar properties. TGF-β, like IL-21, has been reported to inhibit IFN-γ production from in vitro differentiating Th1 cells but not

Th1 effector cells (35). However, TGF-β treatment of Thp cells has an even more profound immunosuppressive effect on Th2 development compared with Th1 development, highlighting the specificity of IL-21 action on IFN-γ (35–37). IL-10 also strongly inhibits cytokine production by T cells, although most of these effects are thought to be indirect through inhibition of APC function (5). As our studies here were performed entirely with highly purified Thp cells in an APC free system, the ability of IL-21 to inhibit IFN-γ production must be a direct result of this cytokine on T cell function. Both IL-10 and TGF-β have also been associated with the development and/or function of regulatory $CD4^+$ T cells which appear to inhibit a number of inflammatory pathologies (38). It remains to be determined if IL-21 is expressed by or involved in the development of these regulatory cell populations.

We have demonstrated that IL-21 exhibits specific immunosuppressive properties on Th cells in both in vitro and in vivo settings. However, the biological outcome of IL-21 treatment will likely depend on the cellular context of the signal. For example, IL-21 has been shown to promote the expansion and lytic activity of human NK cells (7). Moreover, in contrast to what we observe in developing Th cells, IL-21 treatment of activated murine NK cells and $CD8^+$ T cells results in enhanced IFN-γ production (12). Interestingly, whether IL-21 enhances or inhibits murine NK cell function depends largely on the activation state of the NK cell (12). This supports our observation that IL-21 only inhibits IFN-γ production from Th1 cells during a discrete developmental window. Taken together, these observations suggest that the role IL-21 plays in an ongoing immune response will likely be complex. However, the highly specific modulation of Th1 cell function by this Th2 cytokine provides an attractive candidate for immunomodulatory therapies.

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## References

1. Abbas, A.K., K.M. Murphy, and A. Sher. 1996. Functional diversity of helper T lymphocytes. *Nature*. 383:787–793.
2. Glimcher, L.H., and K.M. Murphy. 2000. Lineage commitment in the immune system: the T helper lymphocyte grows up. *Genes Dev*. 14:1693–1711.
3. O'Garra, A. 1998. Cytokines induce the development of functionally heterogeneous T helper cell subsets. *Immunity*. 8:275–283.
4. Maggi, E., P. Parronchi, R. Manetti, C. Simonelli, M.P. Piccinni, F.S. Rugiu, M. De Carli, M. Ricci, and S. Romagnani. 1992. Reciprocal regulatory effects of IFN-γ and IL-4 on the in vitro development of human Th1 and Th2 clones. *J. Immunol*. 148:2142–2147.
5. Moore, K.W., R. de Waal Malefyt, R.L. Coffman, and A. O'Garra. 2001. Interleukin-10 and the interleukin-10 receptor. *Annu. Rev. Immunol*. 19:683–765.
6. Fitch, F.W., M.D. McKisic, D.W. Lancki, and T.F. Gajewski. 1993. Differential regulation of murine T lymphocyte subsets. *Annu. Rev. Immunol*. 11:29–48.
7. Parrish-Novak, J., S.R. Dillon, A. Nelson, A. Hammond, C. Sprecher, J.A. Gross, J. Johnston, K. Madden, W. Xu, J. West, et al. 2000. Interleukin 21 and its receptor are involved in NK cell expansion and regulation of lymphocyte function. *Nature*. 408:57–63.
8. Vosshenrich, C.A., and J.P. Di Santo. 2001. Cytokines: IL-21 joins the γ(c)-dependent network? *Curr. Biol*. 11:R175–R177.
9. Asao, H., C. Okuyama, S. Kumaki, N. Ishii, S. Tsuchiya, D. Foster, and K. Sugamura. 2001. Cutting edge: the common γ-chain is an indispensable subunit of the IL-21 receptor complex. *J. Immunol*. 167:1–5.
10. Ozaki, K., K. Kikly, D. Michalovich, P.R. Young, and W.J. Leonard. 2000. Cloning of a type I cytokine receptor most related to the IL-2 receptor β chain. *Proc. Natl. Acad. Sci. USA*. 97:11439–11444.
11. Kaplan, M.H., U. Schindler, S.T. Smiley, and M.J. Grusby. 1996. Stat6 is required for mediating responses to IL-4 and for development of Th2 cells. *Immunity*. 4:313–319.
12. Kasaian, M.T., M.J. Whitters, L.L. Carter, L.D. Lowe, J.M. Jussif, B. Deng, K.A. Johnson, J.S. Witek, M. Senices, R.F. Konz, et al. 2002. IL-21 limits NK cell responses and promotes antigen-specific T cell activation: a mediator of the transition from innate to adaptive immunity. *Immunity*. 16: 559–569.
13. Szabo, S.J., S.T. Kim, G.L. Costa, X. Zhang, C.G. Fathman, and L.H. Glimcher. 2000. A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell*. 100:655–669.
14. Bird, J.J., D.R. Brown, A.C. Mullen, N.H. Moskowitz, M.A. Mahowald, J.R. Sider, T.F. Gajewski, C.R. Wang, and S.L. Reiner. 1998. Helper T cell differentiation is controlled by the cell cycle. *Immunity*. 9:229–237.
15. Overbergh, L., D. Valckx, M. Waer, and C. Mathieu. 1999. Quantification of murine cytokine mRNAs using real time quantitative reverse transcriptase PCR. *Cytokine*. 11:305–312.
16. Szabo, S.J., B.M. Sullivan, C. Stemmann, A.R. Satoskar, B.P. Sleckman, and L.H. Glimcher. 2002. Distinct effects of T-bet in TH1 lineage commitment and IFN-γ production in CD4 and CD8 T cells. *Science*. 295:338–342.
17. Reiner, S.L., and R.M. Locksley. 1995. The regulation of immunity to Leishmania major. *Annu. Rev. Immunol*. 13: 151–177.
18. Takeda, K., T. Tanaka, W. Shi, M. Matsumoto, M. Minami, S. Kashiwamura, K. Nakanishi, N. Yoshida, T. Kishimoto, and S. Akira. 1996. Essential role of Stat6 in IL-4 signalling. *Nature*. 380:627–630.
19. Shimoda, K., J. van Deursen, M.Y. Sangster, S.R. Sarawar, R.T. Carson, R.A. Tripp, C. Chu, F.W. Quelle, T. Nosaka, D.A. Vignali, et al. 1996. Lack of IL-4-induced Th2 response and IgE class switching in mice with disrupted Stat6 gene. *Nature*. 380:630–633.
20. Wu, C., J. Ferrante, M.K. Gately, and J. Magram. 1997.

Characterization of IL-12 receptor β1 chain (IL-12Rβ1)-deficient mice: IL-12Rβ1 is an essential component of the functional mouse IL-12 receptor. *J. Immunol.* 159:1658–1665.
21. Szabo, S.J., A.S. Dighe, U. Gubler, and K.M. Murphy. 1997. Regulation of the interleukin (IL)-12R β2 subunit expression in developing T helper 1 (Th1) and Th2 cells. *J. Exp. Med.* 185:817–824.
22. Wurster, A.L., T. Tanaka, and M.J. Grusby. 2000. The biology of Stat4 and Stat6. *Oncogene.* 19:2577–2584.
23. Mohrs, M., C.M. Blankespoor, Z.E. Wang, G.G. Loots, V. Afzal, H. Hadeiba, K. Shinkai, E.M. Rubin, and R.M. Locksley. 2001. Deletion of a coordinate regulator of type 2 cytokine expression in mice. *Nat. Immunol.* 2:842–847.
24. Ouyang, W., M. Lohning, Z. Gao, M. Assenmacher, S. Ranganath, A. Radbruch, and K.M. Murphy. 2000. Stat6-independent GATA-3 autoactivation directs IL-4-independent Th2 development and commitment. *Immunity.* 12:27–37.
25. Ho, I.C., M.R. Hodge, J.W. Rooney, and L.H. Glimcher. 1996. The proto-oncogene c-maf is responsible for tissue-specific expression of interleukin-4. *Cell.* 85:973–983.
26. Kim, T.K., and T. Maniatis. 1996. Regulation of interferon-γ-activated STAT1 by the ubiquitin-proteasome pathway. *Science.* 273:1717–1719.
27. Rayanade, R.J., K. Patel, M. Ndubuisi, S. Sharma, S. Omura, J.D. Etlinger, R. Pine, and P.B. Sehgal. 1997. Proteasome- and p53-dependent masking of signal transducer and activator of transcription (STAT) factors. *J. Biol. Chem.* 272:4659–4662.
28. Wang, K.S., E. Zorn, and J. Ritz. 2001. Specific down-regulation of interleukin-12 signaling through induction of phospho-STAT4 protein degradation. *Blood.* 97:3860–3866.
29. Yu, C.L., and S.J. Burakoff. 1997. Involvement of proteasomes in regulating Jak-STAT pathways upon interleukin-2 stimulation. *J. Biol. Chem.* 272:14017–14020.
30. Chtanova, T., R.A. Kemp, A.P. Sutherland, F. Ronchese, and C.R. Mackay. 2001. Gene microarrays reveal extensive differential gene expression in both $CD4^+$ and $CD8^+$ type 1 and type 2 T cells. *J. Immunol.* 167:3057–3063.
31. Fukao, T., D.M. Frucht, G. Yap, M. Gadina, J.J. O'Shea, and S. Koyasu. 2001. Inducible expression of Stat4 in dendritic cells and macrophages and its critical role in innate and adaptive immune responses. *J. Immunol.* 166:4446–4455.
32. Mullen, A.C., F.A. High, A.S. Hutchins, H.W. Lee, A.V. Villarino, D.M. Livingston, A.L. Kung, N. Cereb, T.P. Yao, S.Y. Yang, and S.L. Reiner. 2001. Role of T-bet in commitment of TH1 cells before IL-12-dependent selection. *Science.* 292:1907–1910.
33. Lu, B., H. Yu, C. Chow, B. Li, W. Zheng, R.J. Davis, and R.A. Flavell. 2001. GADD45γ mediates the activation of the p38 and JNK MAP kinase pathways and cytokine production in effector TH1 cells. *Immunity.* 14:583–590.
34. Yang, J., H. Zhu, T.L. Murphy, W. Ouyang, and K.M. Murphy. 2001. IL-18-stimulated GADD45β required in cytokine-induced, but not TCR-induced, IFN-γ production. *Nat. Immunol.* 2:157–164.
35. Ludviksson, B.R., D. Seegers, A.S. Resnick, and W. Strober. 2000. The effect of TGF-β1 on immune responses of naive versus memory $CD4^+$ Th1/Th2 T cells. *Eur. J. Immunol.* 30: 2101–2111.
36. Heath, V.L., E.E. Murphy, C. Crain, M.G. Tomlinson, and A. O'Garra. 2000. TGF-β1 down-regulates Th2 development and results in decreased IL-4-induced STAT6 activation and GATA-3 expression. *Eur. J. Immunol.* 30:2639–2649.
37. Gorelik, L., P.E. Fields, and R.A. Flavell. 2000. Cutting edge: TGF-β inhibits Th type 2 development through inhibition of GATA-3 expression. *J. Immunol.* 165:4773–4777.
38. Read, S., and F. Powrie. 2001. $CD4^+$ regulatory T cells. *Curr. Opin. Immunol.* 13:644–649.