T-bet is rapidly induced by interferon-γ in lymphoid and myeloid cells

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Differentiation of naive CD4$^+$ T cells into IFN-γ-producing T helper 1 (T_H1) cells is pivotal for protective immune responses against intracellular pathogens. T-bet, a recently discovered member of the T-box transcription factor family, has been reported to play a critical role in this process, promoting IFN-γ production. Although terminal T_H1 differentiation occurs over days, we now show that challenge of mice with a prototypical T_H1-inducing stimulus, *Toxoplasma gondii* soluble extract, rapidly induced IFN-γ and T-bet; T-bet induction was substantially lower in IFN-γ-deficient mice. Naive T cells expressed little T-bet, but this transcription factor was induced markedly by the combination of IFN-γ and cognate antigen. Human myeloid antigen-presenting cells showed T-bet induction after IFN-γ stimulation alone, and this induction was antagonized by IL-4 and granulocyte/macrophage colony-stimulating factor. Although T-bet was induced rapidly and directly by IFN-γ, it was not induced by IFN-α, lipopolysaccharide, or IL-1, indicating that this action of IFN-γ was specific. Moreover, T-bet induction was dependent on Stat1 but not Stat4. These data argue for a model in which IFN-γ gene regulation involves an autocrine loop, whereby the cytokine regulates a transcription factor that promotes its own production. These findings substantially alter the current view of T-bet in IFN-γ regulation and promotion of cell-mediated immune responses.

T helper 1 | transcription factor | Stat

A critical process in developing immune responses is the differentiation of naive CD4$^+$ T cells into either T helper 1 (T_H1) or T_H2 cell subsets, which are defined by their characteristic cytokine production profiles. T_H1 cells secrete IFN-γ and tumor necrosis factor β (TNFβ) and are central to cellular immunity against intracellular pathogens. T_H2 cells, in contrast, produce IL-4, IL-5, IL-6, IL-9, and IL-13 and promote antihelminthic immunity and allergic inflammation (1, 2).

The differentiation of naive T_H cells into T_H1 and T_H2 cells is influenced greatly by the cytokines present during and after antigen presentation. The appearance of IFN-γ-producing cells is promoted by IL-12 signaling, which is mediated by signal transducer and activator of transcription 4 (Stat4) (3, 4). Stat4-deficient mice do not respond to IL-12 normally, having a marked impairment in T_H1 differentiation and a propensity for T_H2 differentiation (5, 6). Conversely, IL-4 signals by means of Stat6 to promote T_H2 differentiation. Stat6-deficient mice, in turn, have impaired IL-4 signaling and diminished T_H2 responses (7–9). Moreover, IFN-γ can suppress T_H2 differentiation (10).

In addition to Stats, other transcription factors are important for T helper differentiation. GATA-3 inhibits T_H1 while promoting T_H2 differentiation (11, 12). More recently, the T-box transcription factor, T-bet, has been shown to promote T_H1 development and IFN-γ production (13). T-bet is expressed preferentially in T_H1 and natural killer (NK) cells, and its expression correlates with expression of IFN-γ. Overexpression of T-bet in T_H2 cells confers a T_H1 cytokine production profile on these cells, with induction of IFN-γ and repression of IL-4 and IL-5 expression.

Because the expression of T-bet appears to be critical to T_H1 differentiation, we examined its regulation in response to a known T_H1-inducing stimulus, i.p. administration of soluble antigen from the pathogen *Toxoplasma gondii*. Within 6 h of stimulation, an increase in T-bet mRNA levels was detected. This correlated with an increase in IFN-γ mRNA levels and, strikingly, was impaired in IFN-$\gamma^{-/-}$ mice. We therefore investigated the role of IFN-γ in T-bet regulation and found that IFN-γ induces T-bet expression in monocytes, macrophages, and dendritic cells (DC). Moreover, in association with cognate antigen, IFN-γ, in the absence of IL-12, markedly enhances T-bet expression in T_H cells. This indicates a role for IFN-γ in evoking a T_H1 response through a previously unrecognized, positive feedback loop.

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Materials and Methods

***In Vivo* Stimulation with *T. gondii* Antigen.** C57BL/6 (DCT; National Cancer Institute, Frederick, MD) and age-matched C57BL/6/IFN-$\gamma^{-/-}$ (Taconic Farms) female mice were injected i.p. with 20 μg of soluble *T. gondii* tachyzoite antigen (STAg) by using an established protocol (14). Spleens were harvested 6 h after injection, along with spleens from littermate controls. The spleens then were homogenized mechanically, and total RNA was isolated by using RNA STAT-60, following the manufacturer's protocol (Tel-Test, Friendswood, TX) (14).

Cell Purification and Culture Conditions. Human monocytes were purified from the peripheral blood of normal donors by elutriation (Department of Transfusion Medicine, Clinical Center, National Institutes of Health, Bethesda, MD) and depletion of contaminating cells, using negative selection with magnetic beads (Miltenyi Biotec, Auburn, CA). The resulting monocytes were found to be >99% CD14$^+$ and CD45$^+$. DC were derived from purified monocytes cultured in granulocyte/macrophage colony-stimulating factor (GM-CSF) and IL-4 for 7 days (1,000 units/ml and 5 ng/ml, respectively; R & D Systems). Cells were stimulated by recombinant IFN-γ or IFN-α (both from Biological Resource Bank, National Cancer Institute, Bethesda) or lipopolysaccharide (LPS; Sigma catalog no. L3129) in complete medium [RPMI with 10% heat-inactivated FCS/2 mM L-glutamine (GIBCO)/antibiotic—antimycotic solution (Biofluids, Rockville, MD)]. Murine splenocytes were obtained from

Abbreviations: T_H, T helper; Stat, signal transducer and activator of transcription; DC, dendritic cells; STAg, soluble *T. gondii* tachyzoite antigen; LPS, lipopolysaccharide; APC, antigen-presenting cell; NK, natural killer; RT-PCR, reverse transcription–PCR; GM-CSF, granulocyte/macrophage colony-stimulating factor.

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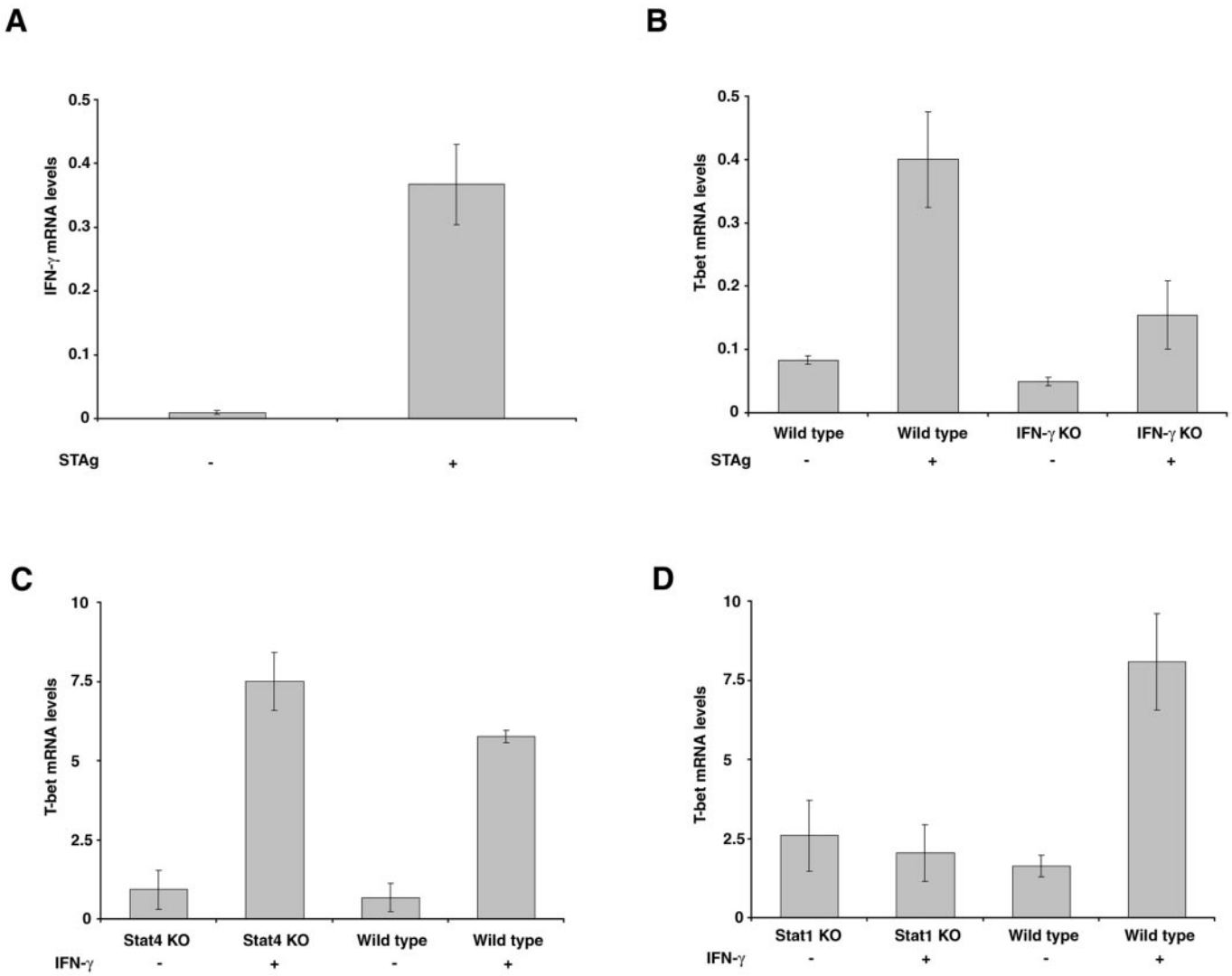

Fig. 1. IFN-γ signaling regulates T-bet expression. Murine splenocytes were collected from four wild-type or four IFN-$\gamma^{-/-}$ mice 6 h after *T. gondii* antigen challenge as well as from four untreated mice from each strain. IFN-γ (*A*) and T-bet (*B*) mRNA levels were measured by quantitative real-time reverse transcription–PCR (RT-PCR). (*C*) Splenocytes obtained from BALB/c/Stat4$^{+/+}$ (wild type) and BALB/c/Stat4$^{-/-}$ (Stat4 KO) mice were treated with IFN-γ for 3 h. T-bet mRNA levels were assayed by quantitative real-time RT-PCR. (*D*) Splenocytes obtained from 129/SvEv/Stat1$^{+/+}$ (wild type) and 129/SvEv/Stat1$^{-/-}$ (Stat1 KO) mice were treated with IFN-γ for 3 h *in vitro*. T-bet mRNA levels were assayed by quantitative real-time RT-PCR, using mRNA from Con-A-activated murine splenocytes for normalization. This level is arbitrarily designated as 1.0.

129/SvEv/Stat1$^{-/-}$, 129/SvEv/Stat1$^{+/+}$, BALB/c/Stat4$^{+/+}$ (Taconic Farms), and BALB/c/Stat4$^{-/-}$ (The Jackson Laboratory) mice and subsequently stimulated with recombinant murine IFN-γ (R & D Systems) in complete medium.

Analysis of RNA Expression. In real-time PCR experiments, cDNA was reverse-transcribed from 1 μg of total RNA by using a first-strand cDNA synthesis kit (Roche, Gipf-Oberfrick, Switzerland) and analyzed by using the ABI PRISM7700 Sequence Detection System (Applied Biosystems). Designer primer and probe sets specific to human T-bet (primers, 5′-GATGTTTGTGGACGTGGTCTTG-3′ and 5′-CTTTCCACACTGCACCCACTT-3′; probe, 5′-6FAM-CCAGCACCACTGGCGGTACCAG-TAMRA-3′; 6FAM is 6-carboxyfluorescein and TAMRA is *N*,*N*,*N*′,*N*′-tetramethyl-6-carboxyrhodamine), mouse T-bet (primers, 5′-GCCAGGGAACCGCTTATATG-3′ and 5′-GACGATCATCTGGGTCACATTGT-3′; probe, 5′-6FAM-ACCCAGACTCCCCCAACACCGG-TAMRA-3′) were used along with commercially available human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA and murine 18S rRNA housekeeping controls (Applied Biosystems) to determine T-bet mRNA expression. As indicated, cDNA derived from phytohemagglutinin-activated human peripheral blood mononuclear cells (24 h, 1 μg/ml; Sigma), the human NK3.3 cell line, and Con A-stimulated (48 h, 1 μg/ml) murine splenocytes was used for generating a standard curve and normalizing data. These standards were arbitrarily designated as 1.0. For Northern analysis, 10 μg of total RNA was separated on 1% agarose glyoxylate gel, transferred to nylon membranes, and hybridized with radiolabeled mouse and human T-bet and GAPDH probes. Blots were washed and exposed to film for autoradiography.

***In Vitro* Antigen Stimulation of Naive T Cells.** CD4$^+$ T cells were isolated from lymph nodes of B10.A, Rag2-deficient, 5C.C7 TCR transgenic mice (Taconic Farms) that recognize a pigeon cytochrome peptide and I-E^k, by depleting contaminating cells by using FITC-anti-CD8, anti-I-A^k, anti-B220, anti-FcγRII/III, anti-NK1.1, and anti-heat-shock antigen and anti-FITC mi-

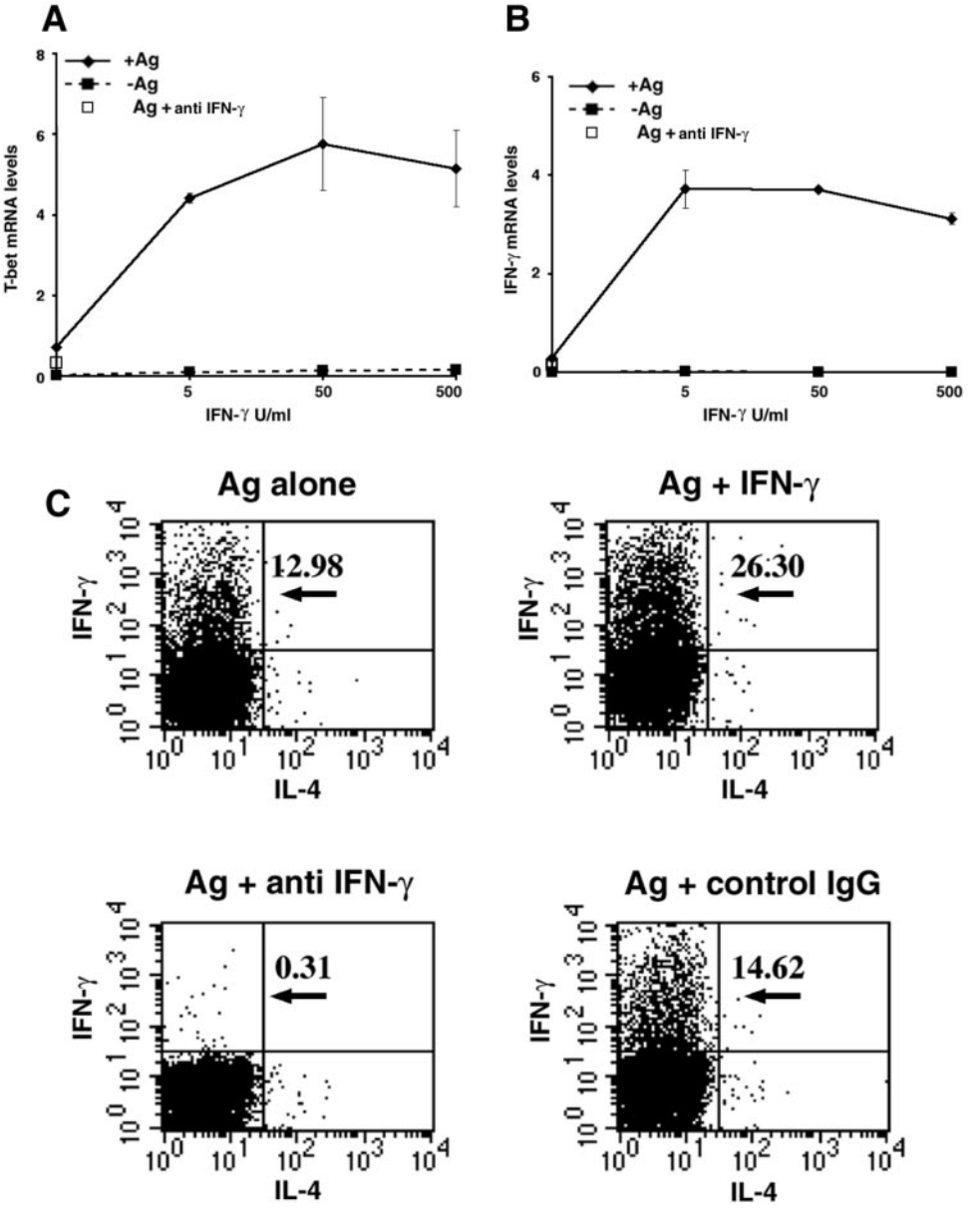

Fig. 2. IFN-γ in conjunction with cognate antigen (Ag) induces T-bet and T_H1 differentiation in naive $CD4^+$ T cells. Naive $CD4^+$ T cells were isolated from lymph nodes of 5C.C7 T cell receptor transgenic/Rag2-deficient mice, incubated with APCs and anti-IL-12 (10 μg/ml) in the presence (solid lines) or absence (dashed lines) of peptide antigen (100 μM) and various amounts of exogenous IFN-γ as indicated. Neutralizing anti-IFN-γ antibody (10 μg/ml) was added to selected cultures stimulated with peptide without exogenous IFN-γ (□). At 24 h, T-bet mRNA (*A*) and IFN-γ mRNA (*B*) levels were determined by quantitative RT-PCR. (*C*) In similar experiments, exogenous IFN-γ (5 units/ml) and anti-IFN-γ antibody blockade were analyzed for their effects on IFN-γ and IL-4 production, as detected by staining fixed, permeabilized cells for cytosolic cytokine content, at 72 h. Numbers in the upper right quadrant of each histogram indicate the percentage of IFN-γ-positive cells.

crobeads. $CD4^+$ T cells then were negatively selected by using an autoMACS magnetic bead column (Miltenyi Biotec). The purity of $CD4^+$ cells in this cell population was more than 99.5%, and no $FITC^+$ cells were detected by fluorescence-activated cell sorting (FACS) analysis. More than 95% were $CD44^{low}/CD62L^{high}$ naive $CD4^+$ T cells, and no $CD44^{high}/CD62L^{low}$ memory $CD4^+$ T cells were detected by FACS analysis. Six million naive $CD4^+$ T cells were cocultured with 100 μM pigeon cytochrome peptide (Laboratory of Molecular Structure–Peptide Synthesis Laboratory, National Institute of Allergy and Infectious Diseases) in the presence of 1.5×10^6 P13.9 cells in 1.5 ml per well in a 24-well plate for 24 h (RNA analysis) or 72 h (intracellular cytokine staining). The P13.9 cell line is a supertransfected derivative of the DAP.3 fibroblast-derived transfectant DCEK Hi7 that expresses high constitutive levels of $I\text{-}E^k$, CD80, and intercellular adhesion molecule 1 (15). These cells were treated with 50 μg/ml mitomycin C (Calbiochem) before use as antigen-presenting cells (APCs). In selected cultures, recombinant IFN-γ (5–500 units; R & D Systems), anti-IFN-γ (10 μg/ml; Harlen Biosciences, Madison, WI), or anti-IL-12 (10 μg/ml; Harlen Biosciences) was added.

Intracellular Cytokine Staining. Primed $CD4^+$ T cells were restimulated with plate-bound anti-CD3 and anti-CD28 (3 μg/ml each) for 6 h, and 2 μM monensin (PharMingen) was added for the last 2 h of culture. Cells were harvested, washed, fixed with

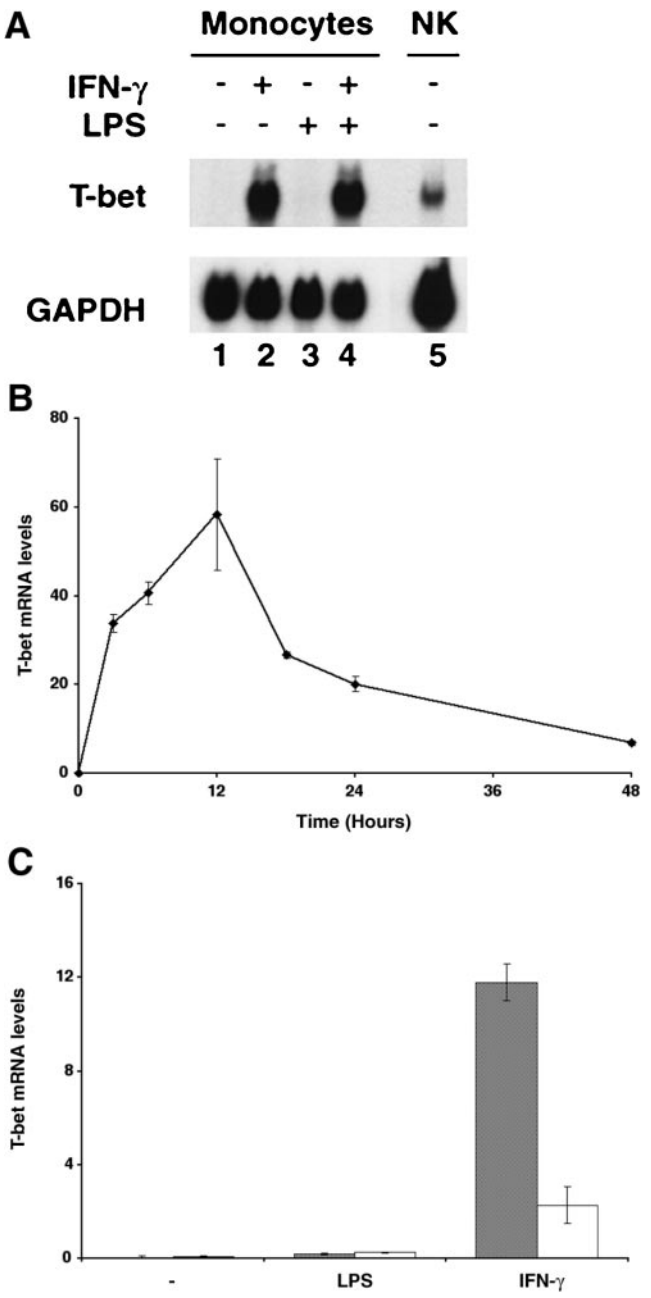

Fig. 3. T-bet is induced rapidly in monocytes and DC by IFN-γ. Purified human monocytes were treated as indicated with IFN-γ and/or LPS, and T-bet mRNA levels were measured by Northern blotting (*A Upper*) and by real-time PCR (*B*). The experiment shown in *A* represents 3 h of stimulation; a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe was used to confirm equal loading (*A Lower*). (*C*) Cultured human myeloid DC were either removed (shaded) or maintained (unshaded) in IL-4- and GM-CSF-containing medium before treatment with IFN-γ or LPS as indicated. T-bet levels were assayed by quantitative RT-PCR.

4% paraformaldehyde, and permeabilized with 0.5% Triton X-100 in PBS containing 10 mM Hepes and 0.1% BSA. Cells were stained simultaneously with FITC-anti-CD4, phycoerythrin-conjugated anti-IL-4, and APC-anti-IFN-γ and then analyzed by flow cytometry.

Monoclonal Antibodies. The antibodies used for T cell isolation and flow cytometry analysis were FITC-conjugated anti-CD4 (RM4–5), anti-CD8 (53–6.7), anti-I-A^k (11–5.2), anti-B220 (RA3–6B2), anti-FcγRII/III (2.4G2), anti-NK1.1 (PK136), anti-heat-shock antigen (M1/69), phycoerythrin-conjugated anti-IL-4 (11B11), APC-conjugated anti-IFN-γ (XMG1.2), rat IgG1 (A110–1), and rat IgG2a (R35–95) obtained from PharMingen.

Results

T-bet Is Regulated by IFN-γ. Given its critical role in cell-mediated immunity, we examined the regulation of T-bet expression in response to a prototypical T_H1-inducing pathogen, *T. gondii* (14). Expression of IFN-γ and T-bet mRNA in splenocytes was measured after injection of STAg. Rapid induction of both IFN-γ and T-bet messages was observed (Fig. 1 *A* and *B*). Six hours after injection, a 30-fold increase in IFN-γ mRNA was detected as measured by real-time PCR (Fig. 1*A*). Splenic T-bet mRNA levels also increased 4-fold during this period (Fig. 1*B*), but the induction of T-bet was significantly lower in IFN-$\gamma^{-/-}$ mice, suggesting that IFN-γ plays a role in regulating T-bet levels.

To analyze further the role of IFN-γ in regulating T-bet expression, we used *in vitro* stimulation of splenocytes obtained from mice with targeted deletions of *Stat1* and *Stat4*, genes whose protein products are required for signal transduction for IFN-γ and IL-12, respectively. As shown in Fig. 1*C,* T-bet was constitutively expressed in splenocytes and was up-regulated 3- to 4-fold within 3 h by IFN-γ. The induction of T-bet in Stat4-deficient splenocytes was comparable to that of wild-type control splenocytes, whereas T-bet induction was abrogated in Stat1-deficient mice, supporting the conclusion that IFN-γ induction of T-bet expression in splenocytes is independent of IL-12.

IFN-γ and TCR Engagement Synergistically Induces T-bet in Naive $CD4^+$ T Cells. These findings led us to examine the role of IFN-γ in regulating T-bet expression in purified cell populations. Purified naive $CD4^+$ T cells were obtained from $Rag2^{-/-}$ mice that expressed a transgenic TCR specific for a pigeon cytochrome *c* peptide (5C.C7 $Rag2^{-/-}$ mice). These cells were stimulated in the presence of P13.9 APCs and anti-IL-12 antibody with or without cytochrome *c* peptide (100 μM) and IFN-γ concentrations varying from 0 to 500 units/ml. T-bet and IFN-γ mRNA levels were determined 24 h later. Unstimulated naive $CD4^+$ T cells expressed very low levels of T-bet mRNA. When stimulated with cytochrome *c* peptide alone, a small but significant increase in T-bet was observed (Fig. 2*A*). The addition of anti-IFN-γ to the peptide-stimulated cells reduced T-bet expression 3-fold. Adding IFN-γ to the peptide-stimulated cells caused a striking increase in T-bet mRNA. By contrast, adding IFN-γ in the absence of peptide caused only a minimal increase in T-bet levels. Thus, the induction of T-bet in $CD4^+$ T cells stimulated with their cognate antigen is strikingly dependent on the presence of IFN-γ. This IFN-γ effect is IL-12-independent, because anti-IL-12 was present in the culture medium.

The IFN-γ mRNA response pattern at 24 h was very similar (Fig. 2*B*). There was very modest induction of IFN-γ mRNA in response to the addition of cytochrome *c* peptide only, which was strikingly up-regulated by the addition of as little as 5 units/ml IFN-γ. IFN-γ, by itself, induced no detectable IFN-γ mRNA.

T_H1 differentiation was assessed after 3 days of priming by enumerating cells that produced IFN-γ in response to stimulation with plate-bound anti-CD3 and anti-CD28 (Fig. 2*C*). Priming with cytochrome *c* peptide in the presence of anti-IL-12 without added IFN-γ resulted in 13% of the cells becoming IFN-γ producers; this rose to 26% with the addition of IFN-γ. However, adding anti-IFN-γ during the priming culture reduced the frequency of IFN-γ-producing cells to less than 1%. T-bet mRNA expression at 3 days (as analyzed by semiquantitative

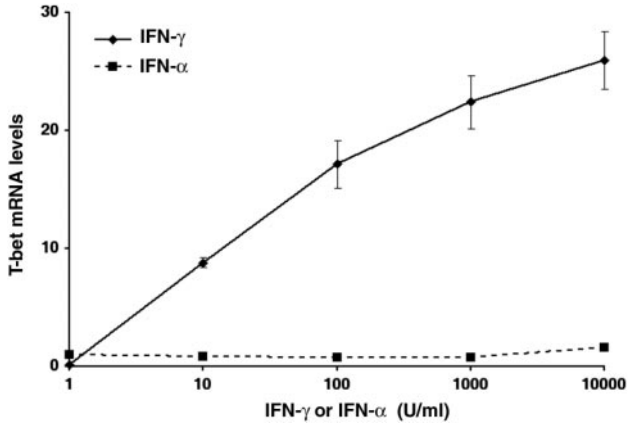

Fig. 4. T-bet mRNA is induced specifically by IFN-γ but not IFN-α. Human monocytes were stimulated for 3 h with various concentrations of IFN-γ and IFN-α as indicated. T-bet mRNA levels were measured by real-time RT-PCR.

PCR) paralleled the frequency of IFN-γ-producing cells (data not shown).

Thus, in the absence of IL-12, naive T cell expression of T-bet in response to TCR stimulation, through antigen and APCs, is strikingly dependent on IFN-γ. Similarly, induction of IFN-γ mRNA is also IFN-γ-dependent in these cells.

IFN-γ Induces T-bet Expression in Macrophages and DC. We, along with others, have shown that DC and macrophages can be induced to express molecules expressed in T_H1 cells, including the IL-12R and Stat4, and that the expression of these molecules correlates with the ability of these nonlymphoid cells to produce IFN-γ. We have proposed that this may be one mechanism by which components of the innate immune system might promote T_H1 differentiation (16–19). This directed us to investigate whether T-bet is expressed in myeloid cell populations as well.

In initial experiments with a human monocyte cell line, THP-1, T-bet was not detected in resting conditions. IFN-γ stimulation of THP-1 cells induced T-bet levels 21-fold at 24 h (11% of T-bet mRNA levels present in phytohemagglutinin-activated human peripheral blood mononuclear cells). Similarly, primary human monocytes expressed undetectable levels of T-bet, but T-bet was induced to very high levels in IFN-γ-activated monocytes (Fig. 3*A*). T-bet mRNA was detectable 30 min after stimulation of human monocytes with IFN-γ and increased markedly to a peak at 12 h (Fig. 3*B*), thereafter declining at 24 and 48 h. Remarkably, the levels of T-bet in IFN-γ-stimulated monocytes were much greater than those of NK cells, one of the cell types first described to express T-bet (13). The induction was specific, because other monocyte activators (LPS, IL-1, and TNF-α) had no effect on T-bet levels (Fig. 3*A* and data not shown).

The critical role of DC in regulating immune responses is now well recognized (20). We have shown that human DC generated by culturing monocytes in IL-4 and GM-CSF do not express Stat4 at rest, but Stat4 is induced to high levels after activation with the combination of LPS and IFN-γ (16). We therefore analyzed the regulation of T-bet expression under these circumstances. As shown in Fig. 3*C*, IL-4- and GM-CSF-generated DC expressed little, if any, T-bet. When these cells were removed from IL-4 and GM-CSF and then stimulated with IFN-γ, very high levels of T-bet mRNA were induced at 24 h. However, this induction was attenuated by ≈75% when the DC were maintained in culture conditions that included IL-4 and GM-CSF.

T-bet Up-Regulation Is IFN-γ- but Not Type I IFN-Specific. Type I and type II IFN both activate Stat1. Because type I IFNs can promote T_H1 differentiation in human cells (21), we next determined whether Stat1 activation was sufficient for T-bet induction. Measurement of T-bet transcription in human monocytes at 3 h (Fig. 4) and at 24 h (not shown) in response to various concentrations of IFN-α and IFN-γ revealed that induction of T-bet was specific to IFN-γ. Although there was a >60-fold induction of T-bet mRNA in human monocytes at the lowest level of IFN-γ tested (10 units/ml), no T-bet induction was measured in response to IFN-α, even at the highest dose tested (10,000 units/ml). This rapid induction of T-bet at low IFN-γ levels emphasized the role of IFN-γ in biologically relevant conditions. Moreover, this suggested that activation of Stat1 is necessary, but not sufficient, for T-bet induction by IFN-γ.

Discussion

T-bet has been identified as a key transcription factor that promotes T_H1 differentiation and IFN-γ production. A role of Stat4 upstream of T-bet induction was reported in initial studies (22); others showed that T-bet can be induced in a Stat4-independent fashion (23). Our findings are consistent with the latter. We now show a major role for IFN-γ, independent of IL-12, in the induction of T-bet in antigen-stimulated $CD4^+$ T cells. The data presented here support a model that includes autoregulation of IFN-γ production by means of T-bet. Specifically, our findings argue that IFN-γ controls the expression of a key transcription factor that regulates its own production in an autocrine feedback loop. IL-12 can enhance IFN-γ production and, thus, indirectly increase T-bet expression. However, these results do not exclude a direct role for IL-12 in T-bet induction. In the absence of IL-12, the T_H1 priming of $CD4^+$ T cells is completely IFN-γ-dependent.

It is important to note, however, that although IFN-γ clearly regulates T-bet, its action appears not to be exclusive. T-bet is not completely absent in IFN-γ- and Stat1-deficient mice, implying the existence of other regulators of T-bet expression.

Furthermore, our data clearly demonstrate that T-bet expression is induced very rapidly *in vitro* and *in vivo*. In this manner, T-bet is central to an important autocrine feedback loop in which T-bet and IFN-γ both up-regulate each other, an interplay that substantially revises previous models (13, 22). For example, our findings can explain previous data showing that little T-bet is produced in cells from Stat4-deficient mice (22). Presumably, this finding resulted from $Stat4^{-/-}$ mice having reduced IFN-γ production. In fact, our studies demonstrate that Stat4-deficient splenocytes can up-regulate T-bet to wild-type levels upon the addition of exogenous IFN-γ (Fig. 1*C*). Stat1-deficient splenocytes, in comparison, cannot (Fig. 1*D*). Our findings confirm and extend those of Mullen *et al.* (23), who demonstrated that expression of T-bet by using retroviral transduction resulted in the up-regulation of endogenous T-bet. Our data would suggest that the autoregulation of T-bet in these experiments could be mediated through a positive feedback loop involving IFN-γ. Indeed, this would appear to place IFN-γ and IL-4 in a comparable position regarding T_H1 and T_H2 cells, respectively. In both cases, the principal product of the differentiated cell determines the direction of polarization of naive cells.

Finally, our data expand the range of T-bet-producing cells to include nonlymphoid cells. Although T-bet initially was reported to be present in T and NK cells, we find that T-bet is present in a variety of other cells, including monocytes, macrophage cell lines, and DC. Myeloid T-bet expression may provide a functional basis for IFN-γ production by myeloid cells, a concept that has been controversial but recently gaining acceptance (19). We have proposed that this production of IFN-γ by DC and macrophages could have important immunoregulatory functions and could be a mechanism by which DC can regulate T_H1 differentiation. DC express considerable levels of T-bet and can be induced to express Stat4. Moreover, T-bet and Stat4 both can be

down-regulated by cytokines. The function and regulation of these transcription factors in APCs is an exciting area of investigation. Whether other roles exist for T-bet in innate immune responses, beyond the regulation of IFN-γ, remains an intriguing possibility.

Given the importance of T-bet in controlling IFN-γ production, it is important to understand in detail the mechanisms underlying T-bet expression and activation. We propose to amend the current model for T-bet function to include the rapid induction (within 30 min) of T-bet expression in myeloid and lymphoid cells by IFN-γ, a process that occurs in a Stat4-independent and Stat1-dependent manner. T-bet induction by IFN-γ introduces a positive feedback loop that could be active both early in infection and later during T cell differentiation.

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