Role of T-bet in Commitment of T_H1 Cells Before IL-12–Dependent Selection

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How cytokines control differentiation of helper T (T_H) cells is controversial. We show that T-bet, without apparent assistance from interleukin 12 (IL-12)/STAT4, specifies T_H1 effector fate by targeting chromatin remodeling to individual interferon-γ (IFN-γ) alleles and by inducing IL-12 receptor β2 expression. Subsequently, it appears that IL-12/STAT4 serves two essential functions in the development of T_H1 cells: as growth signal, inducing survival and cell division; and as trans-activator, prolonging IFN-γ synthesis through a genetic interaction with the coactivator, CREB-binding protein. These results suggest that a cytokine does not simply induce T_H fate choice but instead may act as an essential secondary stimulus that mediates selective survival of a lineage.

Helper T (T_H) cells differentiate into at least two classes of effector cells, which mobilize different arms of the immune system (*1*). T_H1 cells express IFN-γ and mediate cellular immunity. T_H2 cells express IL-4, IL-5, and IL-13 and mediate nonphagocytic immunity. Cytokines control the outcome of T_H immunity, with IL-12, acting via signal transducer and activator of transcription 4 (STAT4), to promote T_H1 development, whereas IL-4 promotes T_H2 development via the actions of STAT6 (*2*). An unresolved question in T_H development is whether cytokines instruct cells to adopt fates or act as growth signals to select cells with predetermined fates (*3*, *4*). In addition to the critical role of STAT proteins in T_H development, transcription factors that act as putative master regulators of effector differentiation have also recently been described (*5*, *6*). When ectopically expressed under nonpermissive conditions, T-bet (*5*) and Gata-3 (*7*) are sufficient to induce IFN-γ and IL-4 synthesis, respectively. However, it remains unresolved whether STAT proteins

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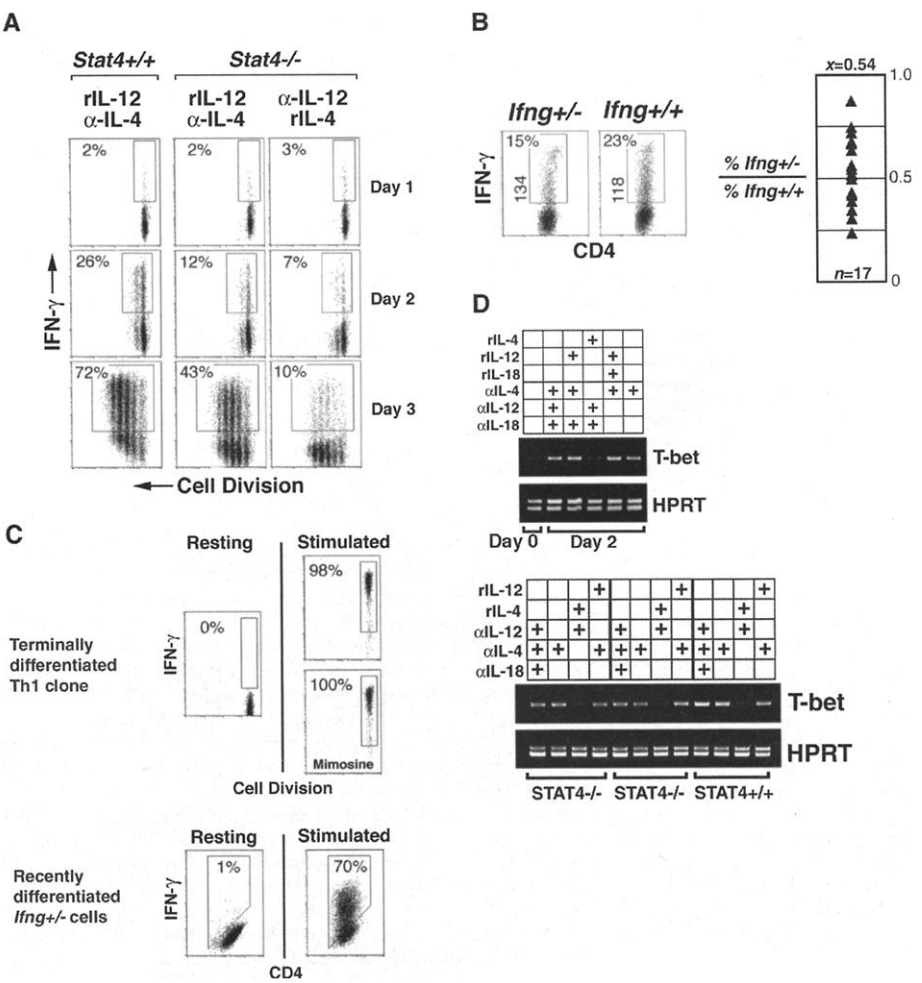

Fig. 1. Nonessential roles for STAT4 in T_H1 differentiation and T-bet induction. (**A**) CFSE-labeled, *Stat4*$^{+/+}$ and *Stat4*$^{-/-}$ cells were stimulated (*14*) for indicated times in the presence of antibody against IL-18 and other listed additions, before analysis of cell division (*x* axis) and IFN-γ expression (*y* axis). Throughout this report, only CD4$^+$ flow cytometry events are displayed. Rectangular gates indicate specific IFN-γ staining compared to control mAb. Percentages are always oriented horizontally; geometric mean fluorescence intensity (MFI) values are all oriented vertically. (**B**) Cells from *Ifng*$^{+/-}$ and *Ifng*$^{+/+}$ littermates were stimulated for 2 days without rIL-12, before analysis of CD4 (*x* axis) and IFN-γ (*y* axis) expression (left panels). Ratios of IFN-γ expression in 17 experiments are depicted (right). (**C**) T_H1 clone, PGL2 (*17*) (upper panels), was CFSE-labeled, rested (left), or restimulated (right) in the absence or presence of mimosine (400 μM) (to impose G_1 arrest) for 6 hours before analysis of cell division (*x* axis) and IFN-γ expression (*y* axis). Naïve *Ifng*$^{+/-}$ cells (lower panels) were stimulated for 5 days in antibody against IL-12, before rest or restimulation in antibody against IL-12 for 4 hours and analysis of CD4 (*x* axis) and IFN-γ (*y* axis) expression. Cells from *Ifng*$^{+/-}$ mice were used to avoid potentially unstable contributions from two alleles. (**D**) Lymph node cells from *Stat4*$^{+/+}$ (upper panel), and groups of two *Stat4*$^{-/-}$, one *Stat4*$^{+/+}$ mice (lower panel), were stimulated in the indicated conditions for 2 days, unless specified, before reverse-transcription PCR (RT-PCR) of T-bet and hypoxanthine-guanine phosphoribosyltransferase (HPRT) expression (*17*). For standardization, HPRT reactions contained a competitive template (upper band) (*17*).

are upstream of these master regulator proteins (*4*, *7*–*9*).

Previous studies have shown that expression of IFN-γ or IL-4 by $T_{H}1$ cells can occur in the absence of STAT4 or STAT6, respectively (*7*, *9*–*11*). However, it is uncertain whether these cells can be considered bona fide $T_{H}1$ and $T_{H}2$ effectors, because the absence of STAT activation results in defective $T_{H}1$ and $T_{H}2$ responses (*12*, *13*). We, therefore, tested the relative roles of STAT4 and T-bet in the initial steps of $T_{H}1$ differentiation. Cells from wild-type (*Stat4*$^{+/+}$) and STAT4-deficient (*Stat4*$^{-/-}$) mice (*12*) were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) and stimulated with combinations of mitogen and different polarizing cytokines (*14*). In all groups tested, induction of IFN-γ within the initial cell divisions was fundamentally stochastic, with only a fraction of each cell generation capable of expressing cytokine (Fig. 1A). Induction of IFN-γ was clearly STAT4-independent, consistent with prior in vitro (*10*) and in vivo (*15*) findings, and, as expected (*16*), emergence of IFN-γ–expressing cells was markedly limited by IL-4 signaling. Combinations of neutralizing antibodies and cytokines further confirmed the specificity of these pathways (*17*).

We also examined allelic contributions (*18*–*20*) to the stochastic pattern of gene induction. If cytokine induction was occurring at both alleles, there should be equal frequency of IFN-γ expression in cells from wild-type and IFN-γ haplo–insufficient mice (*18*). Instead, cells with only one functional allele (*Ifng*$^{+/-}$) consistently (n = 17) displayed approximately half the frequency of IFN-γ expression (mean 0.54) as cells with two functional alleles (*Ifng*$^{+/+}$) (Fig. 1B). Furthermore, the level of IFN-γ expression per cell with either one or two alleles was roughly equivalent, suggesting that induction of IFN-γ is monoallelic. The specificity of this approach was established by similar analyses of two other cytokines that exhibit monoallelic expression (*17*). Thus, induction of IFN-γ from $T_{H}1$ cells is not dependent on IL-12/STAT4 and is a stochastic process, reflected by monoallelic expression.

We next examined whether IL-12–independent $T_{H}1$ differentiation is heritable. Quiescent $T_{H}1$ clones that had been stimulated with mitogen uniformly expressed IFN-γ without entering the cell cycle (Fig. 1C). This is consistent with the stable remodeling of IFN-γ alleles during $T_{H}1$ differentiation (*21*) allowing the rapid recall of cytokine synthesis. We, therefore, examined whether this could occur in the absence of IL-12 signaling. Five days after initial stimulus in the presence of antibody against IL-12, cells had ceased synthesizing IFN-γ (Fig. 1C). Within 4 hours of restimulation, most cells could reiterate IFN-γ expression (Fig. 1C) without entering the cell cycle (*22*), unlike the slow, stochastic pattern of the naïve progenitor (Fig. 1A). These results suggest that IFN-γ expression by $T_{H}1$ cells is IL-12–independent and that early progeny acquire an epigenetic memory (*23*) of allelic induction (*19*, *20*).

We next examined whether T-bet activation requires IL-12/STAT4 or is regulated independently of this signal. T-bet, also known as T-box 21 (Tbx21) (*24*), was recently described as a trans-activator specific to IFN-γ–expressing lineages that is sufficient to induce IFN-γ even under $T_{H}2$-polarizing conditions (*5*). When mRNA levels from cells stimulated in various cytokine environments were tested, T-bet induction was not found to require IL-12/STAT4 or IL-18 (Fig. 1D). Induction of T-bet was, however, suppressed by IL-4 (Fig. 1D) acting via STAT6 (*17*). Thus, T-bet expression and $T_{H}1$ differentiation are coordinately induced without requiring IL-12/STAT4 and are coordinately suppressed by IL-4/STAT6.

In light of the IL-12–independent regulation of T-bet, we tested whether T-bet could mediate IL-12–independent $T_{H}1$ differentiation. We used bicistronic retroviral vectors containing a green fluorescent protein (GFP) marker (*14*) to reconstitute *Stat4*$^{-/-}$ cells that were cultured under nonpermissive condi-

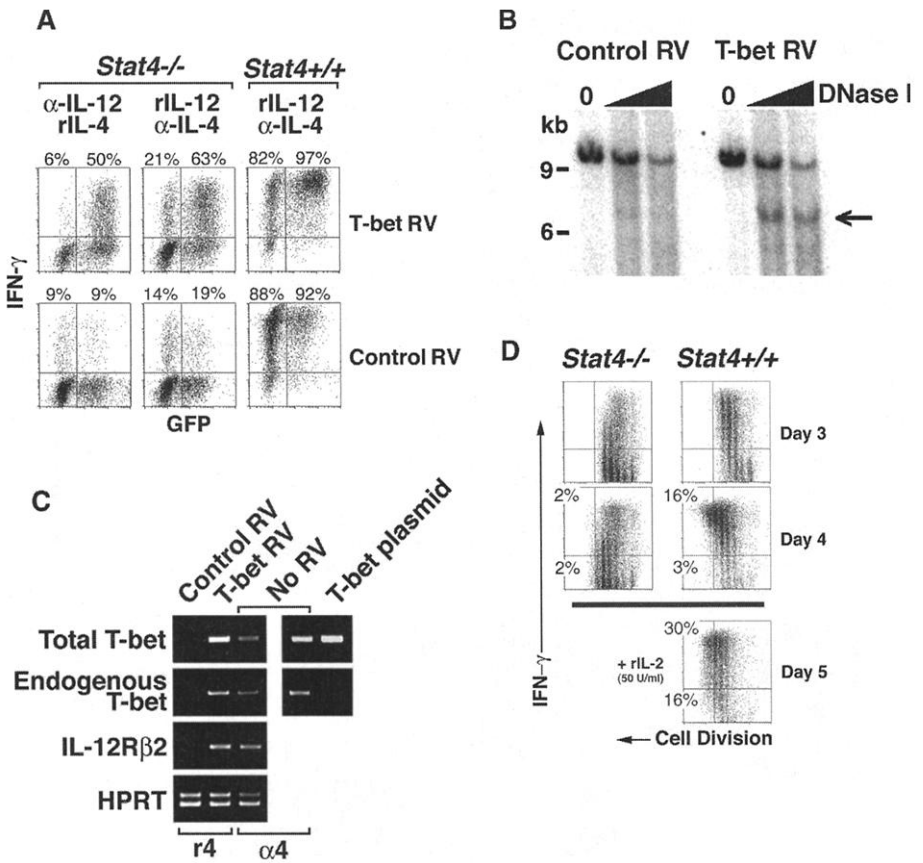

Fig. 2. $T_{H}1$ programming and IL-12 signaling downstream of T-bet. (**A**) *Stat4*$^{+/+}$ and *Stat4*$^{-/-}$ cells were stimulated as indicated for 24 hours before infection (*14*) with T-bet retrovirus (RV) or control RV, 3 days additional culture with indicated cytokines, and analysis of GFP (*x* axis) and IFN-γ expression (*y* axis). Percent IFN-γ^{+} among GFP^{-} and GFP^{+} cells is indicated above each column. (**B**) *Stat4*$^{-/-}$ cells were stimulated with rIL-4 and infected with RV as in (A). Cells were sorted for GFP expression 2 days after infection, and DNase I hypersensitivity of the IFN-γ locus of was determined (*14*). Canonical HS site I is indicated by arrow. (**C**) *Stat4*$^{-/-}$ cells were stimulated under $T_{H}2$ conditions [rIL-4 ("r4") and antibody against IL-12] for 24 hours before RV infection, additional culture for 4 days in indicated cytokines, and RT-PCR analysis of total (cellular plus RV) T-bet, endogenous (cellular) T-bet, and IL-12Rβ2 expression (*17*). Primers for endogenous T-bet were designed to prevent amplification of RV T-bet. Sample cultured without IL-4 ("α4", "No RV"), was used to standardize sensitivity (left panel) and specificity (right panel). T-bet RV plasmid was not reverse-transcribed. (**D**) B10.D2 *Stat4*$^{+/+}$ and BALB/c *Stat4*$^{-/-}$ cells were mixed, CFSE-labeled, and stimulated in $T_{H}1$ (rIL-12 and antibody against IL-4) conditions before analysis of cell division (*x* axis) and IFN-γ expression (*y* axis) at indicated times. Ly9.1 staining was used to separate the *Stat4*$^{-/-}$ (left) from *Stat4*$^{+/+}$ (right) cells. The percentage of cells after five divisions, a threshold to achieve tissue-homing capacity (*28*, *29*), among IFN-γ^{+} or IFN-γ^{-} cells is indicated left of each row. Identical results were obtained when B10.D2 *Stat4*$^{-/-}$ and BALB/c *Stat4*$^{+/+}$ cells were mixed (*22*).

tions for endogenous T-bet induction using recombinant IL-4 (rIL-4). Ectopic T-bet, but not sham virus, fully corrected the IFN-γ defect (Fig. 2A), and transduced cells maintained heritable IFN-γ expression (*22*), suggesting that T-bet is responsible for STAT4-independent T_H1 differentiation. Therefore, we examined accessibility of the IFN-γ locus by assessing DNase I hypersensitivity (HS) in transduced *Stat4*$^{-/-}$ cells. The canonical HS site I of T_H1 cells (*21*) was induced specifically by T-bet retrovirus (Fig. 2B), suggesting that chromatin remodeling is targeted to the IFN-γ locus by T-bet. Finally, we also found that ectopic T-bet specifically induced robust transcription of both endogenous (cellular) T-bet and IL-12 receptor β2 (IL-12Rβ2) (Fig. 2C). Thus, consistent with its ability to induce other T_H1 attributes (*5*), T-bet can activate its own expression, target remodeling of the IFN-γ locus, and induce expression of IL-12Rβ2.

If T-bet induces IL-12Rβ2, then IL-12 might simply promote selective survival or proliferation of committed T_H1 cells (Fig. 2A). Distinct populations of *Stat4*$^{+/+}$ and *Stat4*$^{-/-}$ cells that had differentiated together in T_H1 conditions (*14*) were separately analyzed on the basis of their Ly9 allotype (Fig. 2D). Only *Stat4*$^{+/+}$ cells expressing IFN-γ proliferated and survived beyond the fifth cell division. IFN-γ–negative *Stat4*$^{+/+}$ cells, like *Stat4*$^{-/-}$ cells, proliferated little beyond the fifth cell division. IFN-γ–negative *Stat4*$^{+/+}$ cells specifically lacked IL-12 responsiveness, because provision of an additional growth factor, IL-2, restored their proliferation. Thus, the coordinated programming of IFN-γ expression and IL-12 responsiveness appeared to be downstream effects of T-bet expression. Likewise, IL-12–associated increase in T-bet mRNA (*5*, *9*) during stochastic T_H1 commitment might simply reflect a survival advantage for cells already expressing T-bet (selection) rather than a direct effect on the T-bet gene itself (instruction). Consistent with this hypothesis, we found that ectopic expression of IL-12Rβ2 during T_H1-promoting conditions did not increase T-bet mRNA levels (*17*).

In addition to growth and survival effects, STAT4 also induces higher levels of IFN-γ in individual cells, reflected by an increased fluorescence intensity of IFN-γ staining (Figs. 1 and 2). We found that the effect of STAT4 could be temporally separated from the initial act of T_H1 differentiation. *Ifng*$^{+/-}$ cells that had differentiated in the absence of IL-12 exhibited robust enhancement of IFN-γ expression from the single functional allele when restimulated in the presence of IL-12 (Fig. 3A). Thus, although T_H1 cells could differentiate without STAT4, IL-12 appeared to mediate a potent secondary effect on IFN-γ expression, which was also reversible in the absence of IL-12 (*17*). Additionally, histone deacetylase inhibitors, such as sodium butyrate (Fig. 3B) or trichostatin A (*22*), could completely mimic the IL-12/STAT4 effect, suggesting that secondary enhancement of IFN-γ expression in T_H1 cells may involve changes in histone acetylation.

A candidate mediator of trans-activation by STAT4 is CREB-binding protein (CBP), a coactivator with intrinsic histone acetyltransferase activity, known to associate with some other STAT proteins (*25*). Cells stimulated under T_H1 conditions, from wild-type (*Cbp*$^{+/+}$) and CBP haplo–insufficient (*Cbp*$^{+/-}$) mice (*26*) had comparable frequency and intensity of IFN-γ staining when IL-12 was still present (Fig. 3C). However, a striking defect became apparent when cells were restimulated without IL-12. CBP deficiency caused rapid destabilization of the IL-12 effect, which normally persisted more than 2 days in isolation from IL-12 signaling (Fig. 3C). We further characterized the interaction by CFSE-labeling T_H1 cells and restimulating them in various cytokine environments (Fig. 3D). Initially, each group expressed high levels of IFN-γ. Between 48 and 72 hours, however, synthesis of IFN-γ decreased in a time-dependent manner, with a clear hierarchy of cytokine influence. Thus, absence of IL-12 led to the shortest burst of IFN-γ synthesis, and IL-12 significantly prolonged this duration, which was increased further by the addition of IL-18, a signal known to interact with IL-12 (*27*). STAT4 appears, therefore, to require genetic cooper-

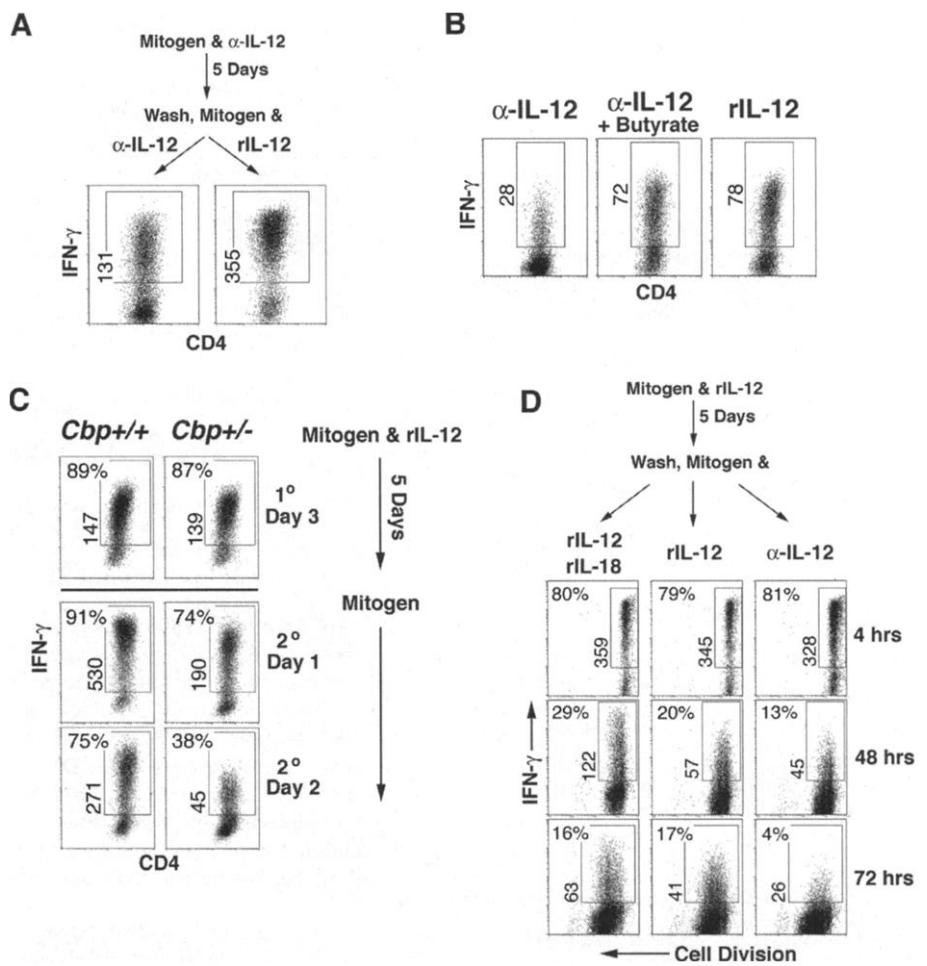

Fig. 3. Enhancement of IFN-γ expression in T_H1 cells by IL-12/STAT4. (**A**) *Ifng*$^{+/-}$ cells were stimulated for 5 days in the presence of antibody against IL-12, before restimulation in the presence of antibody against IL-12 or rIL-12 for 4 hours and analysis of CD4 (*x* axis) and IFN-γ (*y* axis) expression. (**B**) *Ifng*$^{+/-}$ cells were stimulated for 3 days in antibody against IL-12, with (middle) or without (left) sodium butyrate (1 mM), or in rIL-12 (right) before analysis of CD4 (*x* axis) and IFN-γ (*y* axis) expression. (**C**) *Cbp*$^{+/+}$ and *Cbp*$^{+/-}$ cells were stimulated in T_H1 conditions for 5 days, washed extensively, and restimulated with mitogen alone for an additional 2 days. Analysis of CD4 (*x* axis) and IFN-γ (*y* axis) expression was performed at the times indicated during the stimulation (1°) and restimulation (2°). (**D**) C57BL/6 T_H cells were stimulated in the presence of rIL-12 for 5 days. Cells were then washed, CFSE-labeled, and restimulated as indicated. Analysis of cell division (*x* axis) and IFN-γ expression (*y* axis) was performed at the indicated times, with brefeldin A (2 μg/ml) present during the last 2 hours of each time point.

ation from a cofactor with acetylating activity to sustain IFN-γ synthesis in T_H1 cells. The precise biochemical nature of the interactions and the actual targets of acetylation are being investigated (*22*).

Our results prompt reexamination of how the identity of the T_H1 lineage is specified. Upon stimulation, naïve cells activate T-bet, which coordinates a program of autoinduction, chromatin remodeling of IFN-γ alleles, and IL-12Rβ2 expression. STAT4 is not essential in T-bet induction and is not required to aid T-bet in inducing the T_H1 identity. This appears to place T-bet upstream of the STAT4 pathway, not by acting on the *Stat4* gene itself, but by specifying that a cell stably expressing T-bet (and capable of reiterating IFN-γ) has the ability to activate STAT4 (*17*). STAT4 nevertheless confers benefits that are essential to cell-mediated immunity, perhaps by ensuring that some T_H1 progeny will undergo sufficient cell divisions to emigrate from lymph nodes (*28*, *29*) and, through genetic interactions with CBP and IL-18, arrive at tissue maximally armed.

Although Gata-3 can autoinduce itself and remodel the IL-4 locus without assistance from STAT6 (*7*), neither selection nor enhancement of IL-4 gene expression have yet been implicated as critical roles for STAT6 (*4*, *7*–*9*). In addition to their positive effects on T_H2 and T_H1 development, IL-4 (Fig. 1) and IL-12 (*7*, *30*), acting via STAT proteins, potently suppress induction of T-bet and Gata-3, thereby limiting differentiation of T_H1 and T_H2 subsets, respectively. It is, therefore, likely that parallel pathways of instruction, in which cytokines repress or induce activators of specific lineages, can coexist with mechanisms of selection.

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14. Cell stimulation, CFSE labeling, intracellular cytokine staining, and flow cytometry were performed as previously described (*23*). Stimulation used CD8-depleted splenocytes from naïve mice (2×10^6 cells/ml), with monoclonal antibody (mAb) against CD3 (0.1 μg/ml, all Pharmingen unless indicated), mAb against CD28 (0.5 μg/ml), human recombinant IL-2 (rIL-2, 10 U/ml, LifeSciences), and, if indicated, rIL-4 (5 U/ml, Roche), rIL-12 (2.5 ng/ml), rIL-18 (50 ng/ml, R&D Systems); antibodies against IL-4 (11B11, 10 μg/ml), IL-12 (1 μg/ml), and IL-18 (2 ng/ml, R&D Systems). Phorbol 12-myristate 13-acetate (PMA) (50 ng/ml) and ionomycin (500 ng/ml) were used for restimulation of less than 6 hours. PMA (1 ng/ml) and ionomycin (500 ng/ml) were used for restimulation of greater than 12 hours. BALB/c *Stat4*$^{-/-}$ (*12*), C57BL/6 *Ifng*$^{+/-}$ (*17*), and wild-type mice were obtained from the Jackson Laboratories. C57BL/6 *Cbp*$^{+/-}$ mice were generated as described (*26*). All animal work was performed in accordance with University of Pennsylvania guidelines.

For retroviral transduction, polymerase chain reaction (PCR) was used to add a consensus Kozak sequence upstream of the T-bet start ATG, and the cDNA was cloned into vector MigRI upstream of an internal ribosomal entry sequence followed by GFP cDNA. Transfection of a packaging cell line was performed as described (*17*). CD8-depleted splenocytes were stimulated in the presence of mAb against CD3 (0.4 μg/ml), mAb against CD28 (0.5 μg/ml), rIL-2 (20 U/ml), and additional cytokines as specified. After 24 hours, cells were harvested, resuspended in viral supernatant containing polybrene (8 μg/ml), and centrifuged at 6000*g* for 90 min at 25°C. After centrifugation, cells were resuspended in media containing rIL-2 (40 U/ml) and their initial cytokine conditions.

For DNase I hypersensitivity analysis, *Stat4*$^{-/-}$ cells were stimulated in rIL-4 and infected with T-bet or control retrovirus. GFP-positive cells were sorted using a MoFlo cytometer (Cytomation) 2 days after infection. DNase I digestions were performed on isolated nuclei, as described (*17*). Briefly, nuclei were incubated for 15 min at 37°C with DNase I (0, 0.25, or 0.5 μg/ml) at a density of 4×10^7 nuclei/ml. Analysis of the IFN-γ locus was performed as described (*21*). Briefly, genomic DNA was digested overnight with Bam HI and resolved through 0.8% agarose before transfer to nylon membranes. Blots were probed with a 410-bp fragment of IFN-γ exon 4 generated by PCR using the following primers: sense GCGCCAAGCATTCAATGAGCTCATCCGAG and antisense GAGTTCAGTCAGCCGCTTGGCTGTC. All experiments in this report were performed at least twice.
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A p53 Amino-Terminal Nuclear Export Signal Inhibited by DNA Damage–Induced Phosphorylation

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The p53 protein is present in low amounts in normally growing cells and is activated in response to physiological insults. MDM2 regulates p53 either through inhibiting p53's transactivating function in the nucleus or by targeting p53 degradation in the cytoplasm. We identified a previously unknown nuclear export signal (NES) in the amino terminus of p53, spanning residues 11 to 27 and containing two serine residues phosphorylated after DNA damage, which was required for p53 nuclear export in colloboration with the carboxyl-terminal NES. Serine-15–phosphorylated p53 induced by ultraviolet irradiation was not exported. Thus, DNA damage–induced phosphorylation may achieve optimal p53 activation by inhibiting both MDM2 binding to, and the nuclear export of, p53.

The gene encoding p53 mediates a major tumor suppression pathway that is frequently altered in human cancers (*1*). p53 is inhibited during normal cell growth by MDM2, a proto-oncogene discovered by its genomic amplification on a murine double minute chromosome, through either ubiquitin-dependent p53 degradation in the cytoplasm (*2*) or repression of p53's transcriptional activity in the nucleus (*3*, *4*). p53 is activated after DNA damage through p53 phosphorylation (*5*, *6*), or in response to oncogenic insults by the activation of ARF, a tumor suppressor encoded by the alternative reading frame of the INK4a locus that is frequently altered in human cancers (*7*). Blocking p53 nuclear export

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