

Sequential Polarization and Imprinting of Type 1 T Helper Lymphocytes by Interferon- γ and Interleukin-12

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SUMMARY

Differentiation of naive T lymphocytes into type I T helper (Th1) cells requires interferon- γ and interleukin-12. It is puzzling that interferon- γ induces the Th1 transcription factor T-bet, whereas interleukin-12 mediates Th1 cell lineage differentiation. We use mathematical modeling to analyze the expression kinetics of T-bet, interferon- γ , and the IL-12 receptor β 2 chain (IL-12R β 2) during Th1 cell differentiation, in the presence or absence of interleukin-12 or interferon- γ signaling. We show that interferon- γ induced initial T-bet expression, whereas IL-12R β 2 was repressed by T cell receptor (TCR) signaling. The termination of TCR signaling permitted upregulation of IL-12R β 2 by T-bet and interleukin-12 signaling that maintained T-bet expression. This late expression of T-bet, accompanied by the upregulation of the transcription factors Runx3 and Hlx, was required to imprint the Th cell for interferon- γ re-expression. Thus initial polarization and subsequent imprinting of Th1 cells are mediated by interlinked, sequentially acting positive feedback loops of TCR-interferon- γ -Stat1-T-bet and interleukin-12-Stat4-T-bet signaling.

INTRODUCTION

T lymphocytes integrate multiple signals to make decisions on proliferation and differentiation. At the molecular level, this has been intensely studied for the differentiation of naive T helper (Th) cells, in which the external stimuli converge on the induction of a small number of transcription factors (Murphy and Reiner, 2002). The decision to differentiate along the Th1, Th2, or Th17 cell pathways is based on the cytokine environment experienced during antigenic stimulation. In response to infections with intracellular pathogens, Th1 cell differentiation is induced by interleukin-12 (IL-12) and Th1 effector cells then promote a cell-mediated immune response through production of interferon- γ (IFN- γ). Differentiated Th1 cells react to re-encounter of the pathogen with rapid IFN- γ production independent of IL-12 stimulation (Zhu and Paul, 2008).

The transcription factor T-bet (Tbx21) has been recognized as the “master” regulator of Th1 cell differentiation (Szabo et al., 2000). When expressed ectopically in cells stimulated with interleukin-4 in the absence of IL-12, T-bet induces Th1 cell differentiation, including a high degree of IFN- γ expression and silencing of Th2-specific genes (Szabo et al., 2000). T-bet-deficient mice are unable to clear infections that require a type I response, and their Th cells show defective IFN- γ production when primed with antigen and IL-12 in vitro (Szabo et al., 2002). Several functions have been attributed to T-bet at the molecular level. It induces a transcriptionally favorable chromatin structure at the *Ifng* locus (Chang and Aune, 2005; Hatton et al., 2006; Tong et al., 2005), in cooperation with other transcription factors, such as Hlx and Runx3 (Djuretic et al., 2007; Mullen et al., 2002). Moreover, T-bet inhibits GATA-3, the transcription factor driving Th2 differentiation (Hwang et al., 2005), and renders Th cells responsive to IL-12 by, directly or indirectly, inducing the β 2 chain of the IL-12 receptor (IL-12R β 2) (Afkarian et al., 2002; Mullen et al., 2001).

The expression of T-bet is controlled by IFN- γ signaling in synergy with T cell receptor (TCR) stimuli (Afkarian et al., 2002; Lighvani et al., 2001). Because T-bet in turn can activate IFN- γ expression, a self-reinforcing feedback loop between T-bet and IFN- γ may be established. Consequently, IFN- γ could play a dual role as effector cytokine and autocrine Th1 cell differentiation signal. Indeed, inhibition of IFN- γ signaling strongly impairs IL-12-dependent Th1 differentiation (Macatonia et al., 1993; Seder et al., 1993). However, despite being a potent activator of T-bet, IFN- γ cannot induce Th1 cell differentiation in the absence of IL-12 (Macatonia et al., 1993; Seder et al., 1993).

Several mechanisms of action have been described for the primary Th1 cell-inducing signal, IL-12. IL-12 stimulation, mediated by Stat4, enhances IFN- γ production in naive Th cells (Afkarian et al., 2002; Hsieh et al., 1993). There is also evidence that Stat4 induces epigenetic changes at the *Ifng* locus (Chang and Aune, 2005; Zhang and Boothby, 2006). Moreover, IL-12 has been suggested to selectively promote survival and proliferation of differentiating Th1 cells (Mullen et al., 2001). An important factor controlling the action of IL-12 is the activation-dependent expression of the IL-12 receptor β 2 subunit (Szabo et al., 1997). IFN- γ , acting at least in part via induction of T-bet expression, and IL-12 itself, acting through an unknown mechanism, have been implicated in the regulation of IL-12R β 2 (Rogge et al., 1997; Szabo et al., 1997).

Different models have been proposed to explain how cytokine signals and transcription factors function together in inducing and stabilizing the Th1 cell phenotype. In one model, Stat4, activated by IL-12, controls imprinting of the *Ifng* gene, whereas the main function of T-bet is the downregulation of GATA-3, which otherwise would inhibit Th1 cell differentiation by repression of Stat4 and IL-12R β 2 (Usui et al., 2006). However, T-bet has been shown to be required for Th1 cell differentiation even when induction of GATA-3 is prevented by blocking IL-4 signaling (Szabo et al., 2002; Zhang and Boothby, 2006). Because T-bet also directly activates IFN- γ transcription (Chang and Aune, 2005; Hatton et al., 2006), a model has been proposed in which T-bet is the central factor regulating transcriptional competence of the *Ifng* locus (Murphy and Reiner, 2002). In that model, IL-12 signaling induces IFN- γ expression in naive cells, which then, in an autocrine feedback loop, acts to stabilize T-bet expression (Afkarian et al., 2002; Lighvani et al., 2001). However, this action of IL-12 upstream of IFN- γ cannot explain why Th1 cell differentiation can be induced by IL-12, but not by IFN- γ (Macatonia et al., 1993; Seder et al., 1993). Therefore, the link between IL-12 and T-bet remains enigmatic.

The complex interactions between IFN- γ , T-bet, the IL-12R, and Stat4, on the one hand, and GATA-3, on the other, point to the existence of an intricate gene network governing Th1 cell differentiation. In this work, we closely combine experimental measurements and theoretical approaches to dissect the functioning of the Th1 cell differentiation network. We simultaneously monitored the expression kinetics of critical genes, including *Tbx21*, *Ifng*, and *Il12rb2*, during Th1 cell differentiation of primary murine Th cells in vitro. In parallel, we developed a mathematical model of the underlying regulatory network from the kinetic data and tested its central predictions experimentally. We uncovered, in Th cells, a previously unknown pathway of T-bet induction that was driven by IL-12 stimulation and was independent of IFN- γ . Moreover, we show that IL-12 signaling was inhibited by TCR stimulation through repression of IL-12R β 2. Given that antigen signaling synergized with IFN- γ in induction of T-bet expression, but inhibited IL-12-signaling, T-bet was expressed in two consecutive waves during Th1 cell differentiation: a rapid, IFN- γ -driven phase during the acute effector response was followed by an IL-12-driven phase after termination of antigen stimulation. We find that cytokine memory for IFN- γ was controlled by T-bet expression in the late, IL-12-driven phase. Therefore, our results provide a mechanism for the long-known unique role of IL-12 in Th1 cell differentiation.

RESULTS

T-Bet Is Expressed in Two Distinct Waves Driven by IFN- γ and IL-12

To distinguish the roles that IL-12 and IFN- γ play during Th1 priming, we analyzed their effects on the expression kinetics of T-bet over a full time course (6 days) of Th1 polarization in vitro. Naive murine CD4 $^+$ T cells were stimulated for 48 hr with antibodies to CD3 and CD28, mimicking TCR stimulation by cognate antigen. To induce Th1 cell polarization, we added IL-12 and blocking antibodies to IL-4 to the culture. T-bet mRNA showed biphasic expression kinetics with two maxima at 24 hr and 120 hr after the onset of stimulation (Figure 1A, solid

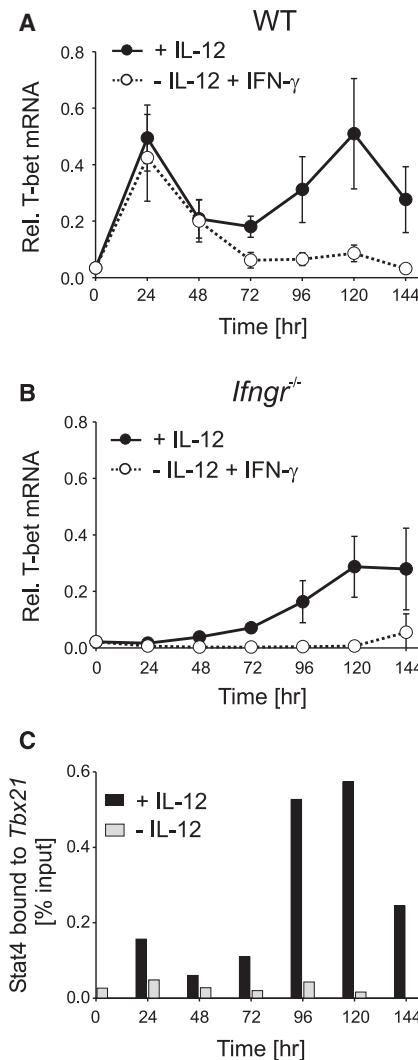
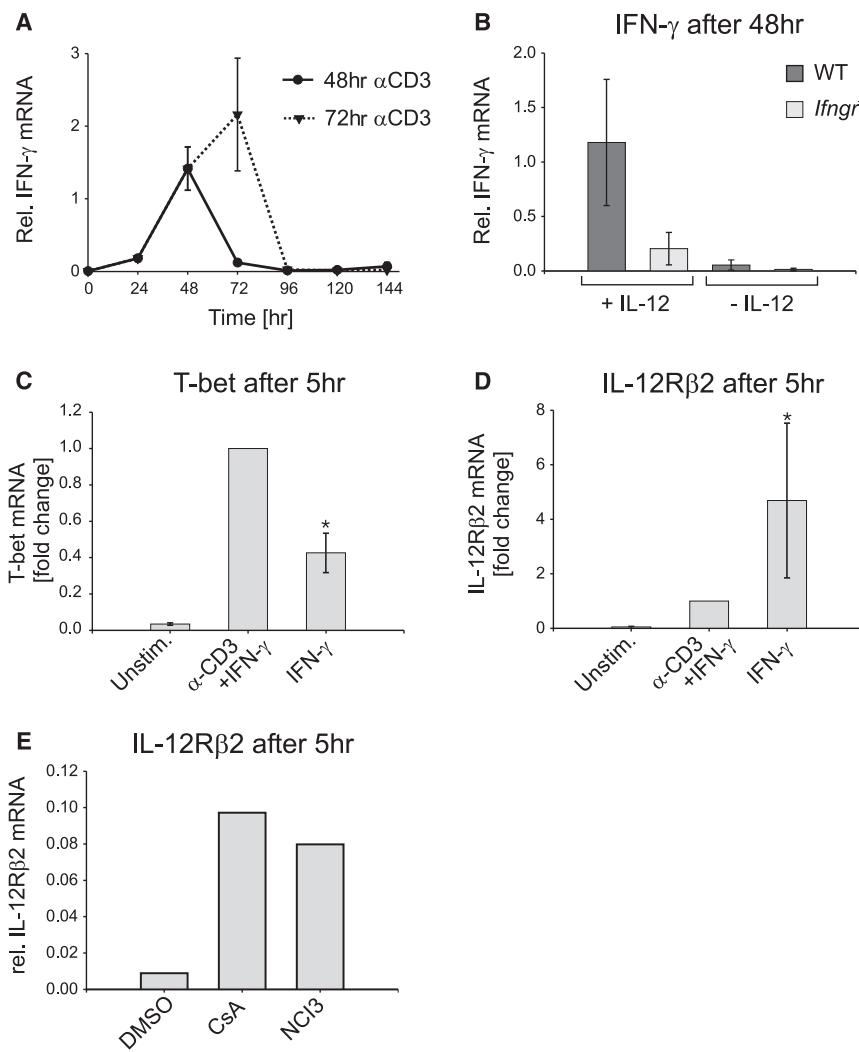


Figure 1. T-Bet Expression in the First 48 hr of Primary Activation Requires IFN- γ Signaling, Whereas Later Expression Is Directly Induced by IL-12 and Stat4

(A and B) Naive Th cells from C57BL/6 (A) or *Ifngr*^{-/-} mice (B) were stimulated with plate-bound CD3-specific antibodies for 48 hr, and antibodies to CD28 and IL-4 were included in the medium. The cells were activated in the presence of IL-12 (solid lines) or IFN- γ and blocking antibodies to IL-12 (dotted lines). T-bet mRNA was quantified over 6 days with quantitative RT-PCR. Mean and SD of five independent experiments are shown.

(C) Cells were treated as in (A) and chromatin was isolated for ChIP assay and precipitated with Stat4-specific antibodies. Quantitative PCR was performed with primers specific for the T-bet enhancer element. Bound Stat4 is expressed as percentage of the input used for the ChIP assay. The results are representative of two independent experiments.

line). To define the role of IFN- γ in T-bet regulation, we interrupted IFN- γ signaling by using cells lacking the IFN- γ receptor 1 chain (*Ifngr*^{-/-}) (Figure 1B, solid line). This perturbation eliminated the first peak of T-bet expression, corroborating the previous observation that T-bet expression at early time points (24–48 hr) is IFN- γ dependent (Afkarian et al., 2002; Lighvani et al., 2001). However, the second T-bet peak was still present, albeit the expression was reduced. In the absence of IL-12,



this second T-bet peak disappeared (Figure 1B, dotted line). These data show that the early expression of T-bet during Th1 cell priming (<48 hr) is driven by IFN- γ , whereas in the late phase (>72 hr), T-bet expression directly depends on IL-12. Thus, we examined T-bet expression in the absence of IL-12 in wild-type (WT) cells, adding recombinant IFN- γ to avoid potential indirect effects caused by reduced IFN- γ production when IL-12 is missing. The first T-bet peak remained unchanged but the second peak was strongly reduced to a low basal expression (Figure 1A, dotted line). Therefore, we concluded that IL-12 induces T-bet expression independently of IFN- γ . Given that Stat4 is the main transcription factor activated by IL-12, we examined whether Stat4 binds to the *Tbx21* locus. Using chromatin immunoprecipitation, we detected Stat4 binding to the conserved T-bet enhancer element 13 kilobases upstream of the transcriptional start site (Yang et al., 2007). Stat4 binding required the presence of IL-12 and strong binding occurred exclusively in the late phase of primary activation (Figure 1C).

Taken together, our observations show that T-bet is expressed in two distinct waves during Th1 priming. For the first

Figure 2. Effects of Antigenic Stimulation: Repression of IL-12R β 2, Induction of T-Bet and Activation of IFN- γ Expression in Synergy with IL-12 and IFN- γ Signaling

(A) Naive Th cells from C57BL/6 mice were cultivated under Th1 cell-inducing conditions on plate-bound antibodies to CD3 (α CD3) for 48 hr (solid lines) or for 72 hr (dotted lines). IFN- γ mRNA was quantified with RT-PCR. Mean and SD of three independent experiments are shown.

(B) IFN- γ mRNA expression was quantified after 48 hr of stimulation with IL-12 ("+IL-12") or with IFN- γ and blocking antibodies to IL-12 ("−IL-12"). Naive Th cells from C57BL/6 (dark bars) or $Ifngr^{-/-}$ mice (light bars) were used. Mean and SD of five experiments are shown.

(C and D) Naive Th cells from Balb/c mice were stimulated for 5 hr with IFN- γ in the presence or absence of CD3+CD28-specific antibodies or left unstimulated as indicated. T-bet (C) and IL-12R β 2 (D) mRNA was quantified with RT-PCR and the fold change in expression relative to stimulation with CD3+CD28-specific antibodies and IFN- γ was calculated. Mean and SD of four to six experiments are shown. As represented by the asterisk, the fold change was highly significant in a paired t test, $p < 0.01$.

(E) Naive Th cells from Balb/c mice were stimulated for 5 hr with IFN- γ and CD3+CD28-specific antibodies. Calcineurin inhibitors CsA (50 nM) or NCI3 (10 μ M) were added as indicated and compared to control cells cultured with DMSO. The results shown are representative of five (CsA) and two (NCI3) independent experiments.

T-bet wave, IFN- γ signaling is required but IL-12 is dispensable, whereas the second wave is driven predominantly by an IL-12-dependent and IFN- γ -independent T-bet induction mechanism and coincides with binding of Stat4 to the T-bet enhancer.

Antigen Controls the Switch from IFN- γ - to IL-12-Dependent T-Bet Expression

Having identified IFN- γ as an early (<48 hr) and IL-12 as a late (>72 hr) inducer of T-bet expression, we investigated which mechanisms restrict the activity of these signals to distinct phases. We reasoned that the antigen signal may be involved in the temporal control of T-bet expression because it is terminated after 48 hr of stimulation.

In the initial phase, IFN- γ secreted by the stimulated Th cells can activate T-bet expression in an autocrine fashion (Afkarian et al., 2002; Lighvani et al., 2001). During primary Th cell activation, IFN- γ mRNA and intracellular protein peaked after 48 hr and subsided completely after 72–96 hr (Figure 1A, solid line and Figure S1 available online). Prolonging TCR stimulation by an additional 24 hr extended the period of IFN- γ expression by the same time interval, demonstrating its strict dependence on antigen signaling (Figure 1A, dashed line). Moreover, IFN- γ expression was activated in a synergistic fashion by IL-12- and

IFN- γ signaling (Figure 2B). In particular, the much reduced IFN- γ mRNA expression measured in *Ifngr*-deficient cells is likely to be due to the lack of T-bet induction (see Figure 1B), which is required for IFN- γ production (Szabo et al., 2002). We conclude that high IFN- γ expression in naive Th cells requires the joint action of IL-12 and TCR signaling as well as T-bet and is therefore limited to the early phase of primary activation when all three factors are present simultaneously.

However, this temporal restriction of IFN- γ transcription cannot fully explain why T-bet mRNA concentration also declined between 48 and 72 hr. Even the addition of recombinant IFN- γ to the culture did not prolong T-bet expression (Figure 1A, dotted line). T-bet expression could be limited in time by the TCR stimulus, as TCR signaling synergizes with IFN- γ in inducing T-bet expression (Lighvani et al., 2001). To assess the role of TCR-dependent signals, we analyzed T-bet expression after 5 hr of stimulation because prolonged exposure to IFN- γ in the absence of TCR signals induced cell death. Stimulation of naive Th cells with IFN- γ in the presence of the CD3+CD28 stimulus resulted in ~2- to 3-fold higher T-bet mRNA expression than stimulation with IFN- γ alone (Figure 2C). Thus, the termination of antigen signaling appears to limit the initial, IFN- γ -driven wave of T-bet expression.

The IL-12-dependent mode of T-bet expression was not observed in the early phase of activation (Figure 1B). This might be due to the limited availability of the IL-12R β 2 chain, resulting in reduced IL-12 signaling in the first days of primary stimulation (Afkarian et al., 2002; Szabo et al., 1997). We observed a basal induction of IL-12R β 2 when stimulating naive Th cells with CD3+CD28-specific antibodies and IFN- γ , a known inducer of IL-12R β 2 (Szabo et al., 1997) (Figure 2D). However, an IFN- γ stimulus without CD3+CD28 stimulation resulted in ~4-fold-higher IL-12R β 2 mRNA expression, suggesting the repression of the *Il12rb2* gene by TCR signaling. CD3+CD28 stimulation with simultaneous application of either of the two calcineurin inhibitors, Cyclosporine A (CsA) or NCI3, resulted in a strong increase of IL-12R β 2 mRNA expression (Figure 2E). This observation suggests that the TCR-dependent IL-12R β 2 repression is mediated by the calcineurin-NFAT pathway.

Taken together, these findings support a model in which antigen-induced signaling coordinates the two distinct waves of T-bet expression. Antigen signaling promotes T-bet induction synergistically with IFN- γ and, at the same time, inhibits IL-12 signaling through repression of IL-12R β 2. The termination of antigen signaling allows the upregulation of IL-12R β 2 expression and thus the delayed induction of T-bet by IL-12.

The Core Network Driving Th1 Cell Polarization

Our data uncover several hitherto unrecognized molecular processes involved in T-bet regulation. Together with previously identified mechanisms, they appear to form a complex regulatory network that underlies Th1 cell differentiation. To enable a rigorous statistical analysis on which regulatory mechanisms must be invoked to account for the experimental observations, we developed mathematical models for the expression kinetics of T-bet, IFN- γ , and IL-12R β 2 and tested them against kinetic data.

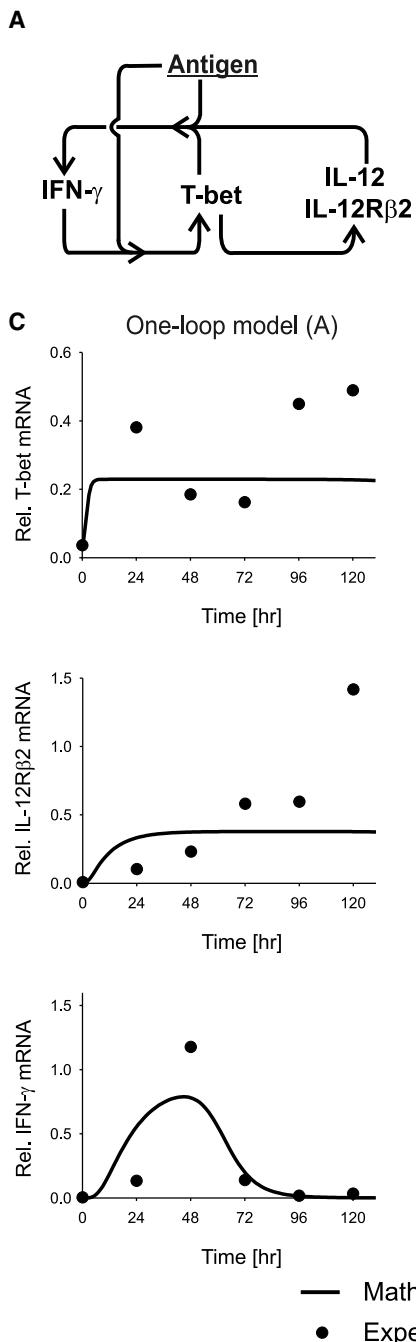
In a model based on the existing literature (one-loop model, Figure 3A), T-bet indirectly promotes its own expression through induction of IFN- γ , such that T-bet and IFN- γ form a positive

feedback loop (Afkarian et al., 2002; Lighvani et al., 2001). In addition, IFN- γ production is augmented by IL-12 (Hsieh et al., 1993) and IL-12R β 2 expression is stabilized by T-bet (Afkarian et al., 2002). Attempting to simultaneously fit this model to the kinetics of T-bet, IFN- γ , and IL-12R β 2 expression, we found that it cannot explain the observed kinetics (Figure 3C).

Therefore, we constructed a second model (two-loop model, Figure 3B) by including the regulatory interactions described here, namely IL-12-dependent T-bet expression and repression of IL-12R β 2 by TCR signaling. In this model, T-bet can promote its own expression through another, IL-12-dependent positive feedback loop by augmenting the expression of the IL-12 receptor and thereby enhancing IL-12-dependent T-bet expression. To test whether this model accounts for the observed behavior, we set translation and degradation rate constants to reasonable values and simultaneously fitted the 11 remaining model parameters to the mRNA kinetics of T-bet, IL-12R β 2, and IFN- γ in WT and *Ifngr*^{-/-} cells, in the presence and absence of IL-12 (parameters in Table 1). The two-loop model accounts for the observed expression kinetics of all three genes simultaneously under standard Th1 conditions (Figure 3D) and the various perturbed conditions (below and Figure S8). We also tested two additional models, each including only one of the new features, either IL-12-dependent T-bet expression or TCR-dependent IL-12R β 2 repression. We found that the two-loop model, which includes both mechanisms, explained the measured data significantly better ($p < 0.01$) than any of the alternative models (Supplemental Data). Therefore, IFN- γ induced T-bet expression, IL-12-induced T-bet expression, and the dual role of antigen signaling in augmenting the former and inhibiting the latter T-bet induction pathway are necessary for accounting for the expression kinetics of T-bet, IFN- γ , and IL-12R β 2 during Th1 cell differentiation.

Experimental Validation of the Two-Loop Model

To investigate the role of the feedback loops controlling T-bet expression, we blocked IFN- γ - or IL-12-signaling (Figures 4A–4C, blue and red lines) in experiment and simulation and compared the resulting changes in the expression kinetics with unperturbed conditions (black lines). In the absence of IFN- γ signaling, the first wave of T-bet expression was completely abolished, whereas the second wave was induced with a delay of ~24 hr (Figure 4A, compare blue and black lines). In the absence of IL-12, the first T-bet wave was left intact, but the second wave was strongly diminished (Figure 4A, compare red and black lines). Expression of IL-12R β 2 was upregulated synchronously with the second, IL-12-dependent wave of T-bet expression under all three conditions (compare Figures 4A and 4B), supporting the existence of the proposed T-bet-IL-12R β 2 feedback loop. IL-12R β 2 expression was strongly diminished in the absence of IL-12 signaling (Figure 4B, red lines), whereas induction was delayed by ~24 hr in *Ifngr*-deficient cells (Figure 4B, compare black and blue lines). Therefore, IFN- γ seemed to accelerate activation of the T-bet-IL-12R feedback loop. In summary, the two-loop model explains well the experimentally observed independent regulation of the two feedback loops as well as acceleration of the IL-12-dependent loop by IFN- γ . Having corroborated the model with mRNA kinetics, we tested whether key features of the model were also observed on the protein level. We measured T-bet expression by



flow cytometry in the early phase and in the late phase in WT and *Ifngr*^{-/-} cells in the presence and absence of IL-12 and found good agreement between experimental measurements and the protein expression pattern assumed by the model (Figure S2).

In the two-loop model, the antigen stimulus directs the two waves of T-bet expression through activating the IFN- γ -T-bet loop, while inhibiting the IL-12-T-bet feedback. Therefore, we tested whether the model could predict the response to a perturbation of TCR stimulation. In simulations, inhibition of TCR signaling after 24 hr of stimulation resulted in accelerated induc-

Figure 3. The Two-Loop Model, Not the One-Loop Model, Can Explain Expression Kinetics of T-Bet, IL-12R β 2, and IFN- γ during Th1 Cell Priming

(A) Network structure of the literature-based one-loop model.

(B) Schematic representation of the two-loop model, integrating the regulatory interactions described in Figures 1 and 2: IL-12-dependent T-bet expression and antigen-dependent repression of IL-12R β 2.

(C and D) Circles represent the expression of T-bet, IL-12R β 2, and IFN- γ mRNA, measured over 5 days of Th1 cell differentiation with naive Th cells, isolated from C57BL/6 mice. A representative example of five independent experiments is shown. Lines represent the best fit of the one-loop (C) or the two-loop (D) model.

tion of IL-12R β 2 and advanced onset of the second wave of T-bet expression (Figures 4D and 4E, left). Using CsA to block activation of the calcineurin-NFAT pathway, we found that experimental inhibition of antigen-dependent signals after 24 hr of TCR stimulation resulted in the predicted effect (Figures 4D and 4E, right).

It has been shown previously that the induction of IFN- γ and IL-12R β 2 by T-bet could be indirect and mediated by T-bet-dependent repression of GATA-3, which otherwise would repress Stat4 (Usui et al., 2006). In the presence of T-bet, elevated Stat4 would enhance IL-12 signaling, induce IFN- γ , and increase IL-12R β 2 chain expression. We measured Stat4 and GATA-3 in the course of T cell activation and found, in general, no correlation with T-bet concentrations (Figure S3). In particular, we find no difference in Stat4 and GATA-3 mRNA expression when comparing WT and *Ifngr*-deficient cells in the first 48 hr of stimulation, a phase when T-bet and IFN- γ expression are vastly different (Figures 4A and 4C). Similarly, at 72–120 hr after the onset of stimulation, comparison of cells cultured

in the presence and absence of IL-12 showed that, although the expression of T-bet and IL-12R β 2 differed greatly, there was no significant difference in the expression of Stat4 and GATA-3 ($p > 0.05$). When comparing *Ifngr*-deficient cells with WT cells 96 hr after the onset of stimulation in the presence of IL-12, a small but significant decrease in Stat4 mRNA was observed; such a decrease could reflect GATA-3 mediated inhibition of Stat4 expression. Thus, there may be a small additional effect on Th1 cell polarization through the repression of GATA-3. We conclude that regulation of IFN- γ and IL-12R β 2 by T-bet is predominantly via the mechanisms of the two-loop model and without

Table 1. Parameter Values of the Two-Loop Model

Activation Rate Constants	Parameter Values	90% Confidence Intervals
α_1	0.044 h ⁻¹	[0.032 0.054]
α_2	0.42 h ⁻¹	[0.29 1.28]
α_3	0.00051 h ⁻¹	[0.00044 0.00060]
α_4	0.0028 h ⁻¹	[0.0024 0.0035]
α_5	3.7 h ⁻¹	[1.6 9.6]
Half-Saturation Constants		
K_1	0.46	[0.14 2.88]
K_2	2.1	[0.6 7.2]
K_4	0.013	[0.0073 0.020]
K_5	0.029	[2.3 × 10 ⁻⁸ 36]
K_6	66	[18 155]
K_7	0.014	[0.0061 0.025]
mRNA Degradation		
γ_{Tbet}	1 h ⁻¹	n.a.
γ_{Rec}	1 h ⁻¹	n.a.
γ_{IFN}	1 h ⁻¹	n.a.
Translation Rate		
β	100 h ⁻¹	n.a.
Protein Degradation		
δ_{Tbet}	0.1 h ⁻¹	n.a.
δ_{Rec}	0.1 h ⁻¹	n.a.
δ_{IFN}	1 h ⁻¹	n.a.

a substantial effect via repression of GATA-3 and Stat4 transcription, under the conditions employed here.

Late, Not Early, T-Bet Is Predictive of IFN- γ Re-expression

Why is IL-12 a more potent Th1 cell-polarizing signal than IFN- γ (Macatonia et al., 1993; Seder et al., 1993; Wenner et al., 1996), although we find that T-bet is induced by both cytokines? Given that IFN- γ controls T-bet expression in the early phase of priming and IL-12 in the late phase, we hypothesized that the time of T-bet expression is a critical determinant for Th1 cell differentiation. We investigated how T-bet expression at different time points affected the polarization efficiency, measured as the frequency of IFN- γ -producing cells during the recall response after 6 days of differentiation.

To test whether T-bet expression during the first 24 hr influenced polarization, we compared cells that had expressed different amounts of T-bet in this early phase (Figure 5A, black and red lines). Through addition of recombinant IFN- γ , T-bet induction kinetics were accelerated, but no increase in the polarization efficiency was observed (Figure 5B, compare black to red). Next, we inhibited T-bet expression in the first 48 hr by using blocking antibodies to interrupt IFN- γ signaling (Figure 5A, blue line). This perturbation resulted in a moderate decrease of the number of IFN- γ -producing cells in the recall response by ~20% (Figure 5B, blue). Then, we analyzed *Ifngr*-deficient cells in which T-bet expression was abolished in the first 48 hr and expression was reduced in the late phase of priming (Figure 1B).

The polarization efficiency in these cells was reduced by ~50% compared to WT cells (Figure 5C). Finally, we investigated the role of the second wave of T-bet expression, which we have shown to be IL-12 dependent. When cells were primed with IFN- γ in the absence of IL-12, T-bet expression in the first 48 hr was left intact, whereas expression in the late phase was strongly reduced (Figure 1A). Under these conditions, IFN- γ cytokine memory was reduced by ~80% (Figure 5C). No polarization occurred when *Ifngr*-deficient cells were primed in the absence of IL-12 because T-bet was not expressed under these conditions (Figures 1B and 5C). These data suggest that the second, IL-12-dependent wave of T-bet expression controls IFN- γ expression in the recall response, whereas the first, IFN- γ dependent wave has a minor impact. Given that in addition to controlling the first wave of T-bet expression, IFN- γ can accelerate onset of the second wave (Figure 4A), *Ifngr*-deficient cells also exhibit reduced polarization efficiency.

To test the hypothesis that the expression of T-bet in the late phase controls the polarization efficiency, we assessed the correlation between T-bet expression and the frequency of IFN- γ -producing cells in the recall response, by using the data from five experiments under Th1 cell-inducing and perturbation conditions. We found that cytokine memory for IFN- γ was uncorrelated with T-bet expression after 24 hr (Figure 6A), but highly correlated with its expression after 96 hr (Figure 6B). By calculating this correlation for each time point during Th1 cell priming, we found that late (72–120 hr), but not early (0–48 hr) T-bet expression was predictive of IFN- γ cytokine memory (Figure 6C). The correlation was maximal between 72 and 120 hr but decreased at 144 hr, right before the recall response was induced. Taken together, these findings are consistent with the second, IL-12-driven wave of T-bet expression determining the polarization efficiency.

Previous work has shown that T-bet does not act independently in Th1 cell differentiation but cooperates with the transcription factors Runx3, Hlx, and Stat4 (Djuretic et al., 2007; Mullen et al., 2002; Thieu et al., 2008). Measuring the mRNA kinetics of Hlx and Runx3 during primary Th1 cell differentiation, we found that both factors were expressed at very low amounts during the first two days of priming and were upregulated between 72 and 120 hr of stimulation (Figure 6D). Similar kinetics were observed for activity of Stat4 as measured by its phosphorylation status by flow cytometry: although no phosphorylation was observable after 24 hr, the amount gradually increased between 48 and 72 hr, resulting in phosphorylation of Stat4 in nearly all cells after 96 hr (Figure 6E). Therefore, the entire concert of Th1 cell-inducing transcription factors T-bet, Runx3, Hlx, and active Stat4 becomes available only during the second, IL-12-dependent phase of T-bet induction.

DISCUSSION

In this work, we provide evidence that differentiation of activated naive Th cells into Th1 cells occurs in two steps. In the early Th1 cell-polarizing phase (0–48 hr), TCR signaling induces T-bet expression synergistically with IFN- γ , but represses activation of the IL-12-Stat4 pathway. In the late imprinting phase (>48 hr), after termination of antigenic stimulation, this repression is released, and enhanced IL-12-Stat4 signaling occurs and maintains T-bet

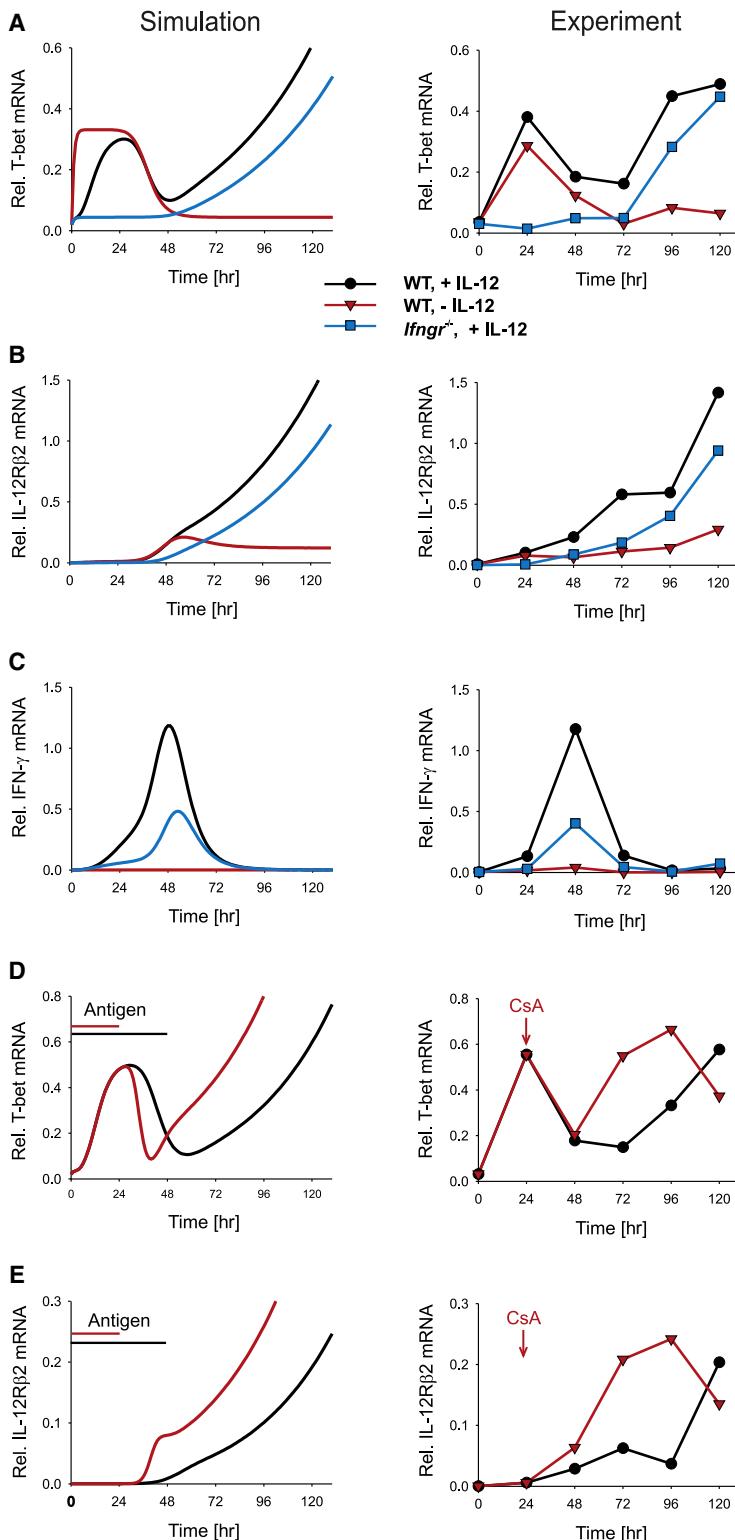


Figure 4. The Two-Loop Model Can Simulate and Predict Expression Kinetics under Various Experimental Conditions

(A-C) The left column shows the expression kinetics of T-bet (A), IL-12R β 2 (B), and IFN- γ mRNA (C) simulated on the basis of the two-loop model (Figure 3B) under Th1 cell-inducing conditions (black) and in the absence of IFN- γ (blue) or IL-12 signaling (red). All parameters were simultaneously fitted to experimental data. As shown in the right column, naive Th cells, isolated from C57BL/6 (black and red) or *Ifng^{-/-}* mice (blue), were stimulated for 5 days with IL-12 (black and blue) or IFN- γ , and blocking antibodies to IL-12 (red). T-bet (A), IL-12R β 2 (B), and IFN- γ mRNA kinetics (C) were quantified. The experiment was performed three times; one representative example is shown.

(D and E) As shown in the left column, expression kinetics of T-bet (D) and IL-12R β 2 mRNA (E) are compared in silico in cells stimulated with antigen for 24 hr (red) or 48 hr (black) under Th1 cell-inducing conditions. As shown in the right column, naive Th cells, isolated from C57BL/6 mice, were cultured under Th1 cell-inducing conditions, and cyclosporine A (CsA) was added after 24 hr of stimulation as indicated (red). T-bet (D) and IL-12R β 2 mRNA (E) were quantified over 5 days of culture. One representative example out of three independent experiments is shown.

expression. Through a mathematical model we could show that these regulatory interactions between T-bet, IFN- γ , IL-12R β 2, and the antigen stimulus can explain the major features of the expression kinetics during primary activation, such as two-peaked T-bet expression with a single IFN- γ peak and delayed induction of IL-12R β 2. Therefore, we propose that the model described here constitutes the core of the gene network controlling Th1 cell differentiation.

T-bet imprints the Th1 cell phenotype in that histones are modified and chromatin is remodeled in regulatory regions on the *Ifng* gene (Chang and Aune, 2005; Hatton et al., 2006; Shnyreva et al., 2004). Here, we show a quantitative dose dependence of the frequency of IFN- γ -producing cells in a recall stimulation on the expression of T-bet in the late phase of primary activation (>48 hr). Importantly, this T-bet dose dependence of Th1 cell priming held true for different stimulation protocols (e.g., with or without IFN- γ signaling), strongly suggesting that T-bet expression in a specific time window, rather than other factors, is the decisive event. The underlying mechanism might involve epigenetic regulation rather than direct transcriptional activation, given that the observed dose dependence is maximal between 72 and 120 hr and decreased after 144 hr when the recall response is induced.

A temporal constraint on Th1 cell priming has been shown previously in that memory expression of the *Ifng* gene requires entry into the S phase of the first cell cycle (Bird et al., 1998; Richter et al., 1999).

However, because this occurs at ~20 hr of stimulation, whereas the T-bet effect is strongest at ~96 hr, S phase entry seems to be only one of a number of necessary events that allow or mediate T-bet action. In agreement with previous reports, we show that Runx3 and Hlx, which are induced by and cooperate with T-bet, were specifically expressed in the late phase of

expression through a positive feedback loop of IL-12R and T-bet. The expression of T-bet in the late phase, and not in the early phase, correlates strongly with the frequency of IFN- γ -producing cells in a later recall response, and thus this late IL-12-dependent T-bet induction governs the imprinting of the Th1 cell for IFN- γ re-

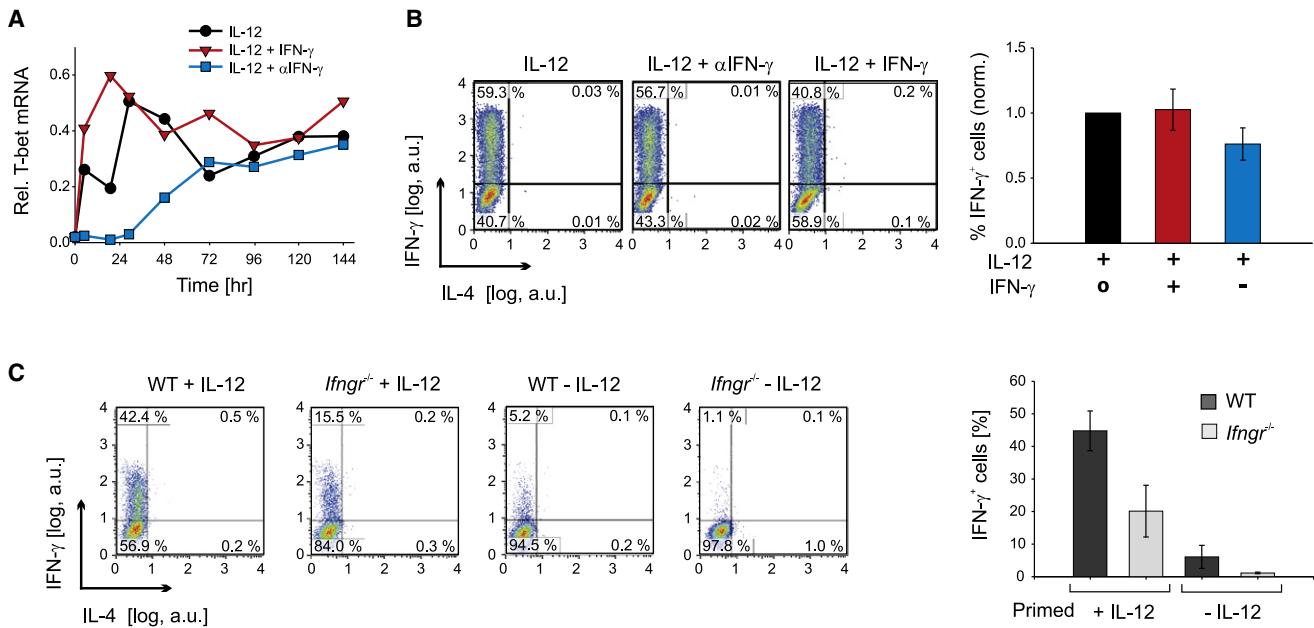


Figure 5. T-Bet Expression in the Late Phase of Primary Activation Controls Cytokine Memory for IFN- γ

(A) Naive Th cells ($CD4^+ CD62L^{hi} CD44^{lo}$) purified by flow cytometry, isolated from Balb/c mice, were stimulated for 6 days with IL-12 (black), with IL-12 and IFN- γ (red), or with IL-12 and blocking antibodies to IFN- γ (blue). T-bet mRNA kinetics were quantified by RT-PCR. One representative example out of three independent experiments is shown.

(B) Cells cultured as in (A) were restimulated with PMA and ionomycin after 6 days of culture and stained intracellularly for IFN- γ and IL-4. As a positive control, Th2 cells were stained with the same protocol (Figure S4 online). As shown in the right column, mean and SD of the frequency of IFN- γ -producing cells under all three conditions was calculated from three independent experiments, normalized to conventional Th1 cell-inducing conditions (IL-12).

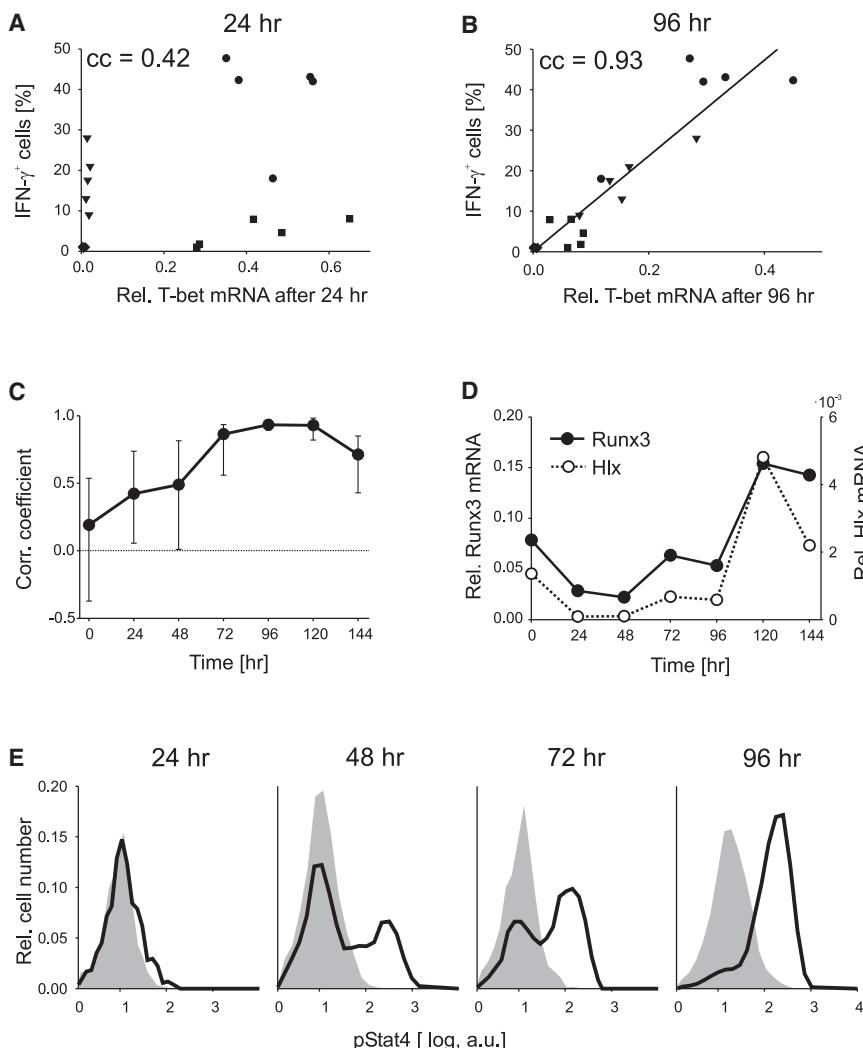
(C) Naive Th cells, isolated from C57BL/6 or Ifngr^{-/-} mice, were stimulated with IL-12 ("+IL-12") or with IFN- γ and blocking antibodies to IL-12 ("−IL-12"). After 6 days, cells were restimulated with PMA and ionomycin and stained intracellularly for IFN- γ and IL-4. As shown on the right, mean and SD of the frequency of IFN- γ -producing cells was calculated from four independent experiments.

primary activation (Djuretic et al., 2007; Mullen et al., 2002). Moreover, Stat4 activation was restricted to the late phase and also cooperates with T-bet on the induction of multiple Th1 cell-specific genes (Thieu et al., 2008). In addition, it has been reported recently that Stat5-dependent induction of chromatin accessibility, occurring between 24 and 48 hr of primary activation, is required for binding of T-bet to the *Ifng* locus (Shi et al., 2008). We propose that T-bet acts in a concert with other transcription factors to imprint the *Ifng* gene, restricting this ability to the late imprinting phase of Th1 cell differentiation.

We show here that in the late phase of primary Th cell activation, IL-12, acting via Stat4, directly controlled T-bet expression. IL-12 signaling was restricted to the late phase of priming because the IL-12 receptor $\beta 2$ subunit was repressed by TCR signaling earlier. We show that Stat4 became phosphorylated as soon as IL-12R $\beta 2$ expression started to increase, at time points when Stat4 mRNA expression remained at a constant low amount (48–72 hr). Therefore, availability of IL-12R $\beta 2$, not Stat4, limits IL-12 signaling in the early phase of priming. As IL-12 maintains T-bet expression, which in turn induces expression of IL-12R $\beta 2$ (Afkarian et al., 2002; Mullen et al., 2001), IL-12 enhances indirectly expression of its own receptor and T-bet indirectly promotes its own expression by inducing IL-12R $\beta 2$. Through this positive feedback loop, IL-12 maintains sustained T-bet expression in the late phase of primary Th cell activation, which is required for establishing IFN- γ cytokine memory. This

mechanism links IL-12, the major Th1 cell-inducing cytokine, and T-bet, the Th1 cell "master" transcription factor.

Until now, expression of T-bet has been considered to be regulated by IFN- γ and Stat1 (Afkarian et al., 2002; Lighvani et al., 2001). We find that IFN- γ controlled T-bet expression only in the first two days of activation, a period when also previous measurements were conducted. In the later phase of primary Th cell activation, however, T-bet expression was controlled by IL-12, and this T-bet expression was required for Th1 cell imprinting. Therefore, IFN- γ cannot drive Th1 cell differentiation in the absence of IL-12 (Macatonia et al., 1993; Seder et al., 1993). Yet IFN- γ signaling promotes IL-12-driven Th1 cell differentiation in vitro (Macatonia et al., 1993; Seder et al., 1993), possibly by making cells responsive to IL-12 through inducing expression of the IL-12R $\beta 2$ chain (Hu-Li et al., 1997; Wenner et al., 1996). We show here that, in the presence of IL-12, expression of IL-12R $\beta 2$ was induced through a positive feedback loop with T-bet. If both genes initially are expressed at low amounts, mutual induction is inefficient and thereby proceeds with slow kinetics. We find that this slow induction of IL-12R $\beta 2$ and T-bet was accelerated significantly, if T-bet expression was induced by IFN- γ in the early polarization phase. Already within hours of activation, low IFN- γ expression has been observed (Grogan et al., 2001) and might then activate the T-bet-IFN- γ feedback loop, which in turn will promote IL-12R $\beta 2$ expression. Taken

Sequential Th1 Cell Induction by IFN- γ and IL-12

together, these findings show that IFN- γ and IL-12 seem to play distinct roles in the regulation of T-bet and IL-12R β 2: expression is started up by the transient action of IFN- γ and is later maintained by IL-12.

We find that IFN- γ and IL-12 act strictly sequentially during primary activation of naive Th cells. In functional terms, initial polarization accompanied by IFN- γ expression is dissociated from the IL-12-driven Th1 cell lineage decision. Initially, antigen presentation elicits IFN- γ production by the T cell. In turn, IFN- γ , in synergy with CD40 stimulation, enhances IL-12 production by the antigen-presenting cell (APC) (Trinchieri, 2003). The synergistic activation of IFN- γ production by APC-derived IL-12 together with antigen and (IFN- γ -induced) T-bet will close a positive feedback loop between APC and T cell. During interaction with the APC, the T cell responds only weakly to IL-12 because TCR-dependent signals suppress IL-12R β 2 expression through a calcineurin-dependent mechanism, as we show here. Consequently, Th1 cell differentiation, requiring strong IL-12 signaling, occurs only after termination of TCR stimulation and will then be responsive to the amount of IL-12 produced in the course of the APC-T cell interaction.

Figure 6. Late, Not Early, T-Bet Expression Correlates with IFN- γ Recall Expression under Various Culture Conditions

(A and B) Naive Th cells isolated from C57BL/6 (circles, squares) or *Ifng^r/r* mice (triangles, diamonds) were stimulated with IL-12 (circles, triangles) or with IFN- γ and blocking antibodies to IL-12 (squares, diamonds). After 6 days of primary activation, cells were restimulated with PMA and ionomycin and the frequency of IFN- γ producing cells was determined by intracellular cytokine staining. For five independent experiments, T-bet mRNA expression after 24 hr (A) or 96 hr (B) of stimulation was plotted against the frequency of IFN- γ -producing cells in the recall response. The correlation coefficient (cc) is indicated and linear regression is shown for (B).

(C) The correlation coefficient as indicated in (A) and (B) was determined for each time point. The error bars indicate a 95% confidence interval, as estimated by bootstrap analysis.

(D and E) Naive Th cells from C57BL/6 mice were cultivated under Th1 cell-inducing conditions for 6 days. As shown in (D), Hlx and Runx3 mRNA expression was quantified by RT-PCR. As shown in (E), at indicated time points during stimulation, phosphorylated Stat4 was measured by intracellular staining and flow cytometry (solid lines). To assess background staining, we used cells stimulated in the absence of IL-12 (shaded area). One representative example out of three (Runx3) or two (Hlx and pStat4) independent experiments is shown.

Thus, the final lineage decision can be made on the basis of the signals produced throughout the acute response. Interestingly, IL-12 is also involved in termination of the immune response by inducing expression of the anti-inflammatory cytokine IL-10 in Th1 cells (Chang et al., 2007; O'Garra and Vieira, 2007). IL-12 production itself is inhibited by IL-10, which thereby acts as a negative feedback regulator (O'Garra and Vieira, 2007). T cells might be particularly competent for IL-10 production during the APC-T cell interaction because IL-10 expression seems to require concomitant stimulation of Notch by Notch ligands expressed on the APC surface (Rutz et al., 2008). Inhibition of IL-12 signaling during TCR stimulation could be important to delay IL-10 production by Th1 cells (Assenmacher et al., 1998), thereby preventing instant negative feedback regulation of T cell activation.

In summary, we present a new model of Th1 cell priming supported by kinetic experiments and mathematical modeling. Th1 cell differentiation is induced in a two-step process controlled by two sequentially acting positive feedback loops. In the early phase, when the TCR is stimulated, the Th1 cell "master" transcription factor T-bet is induced by a positive feedback loop mediated by IFN- γ . During that phase, expression of the IL-12 receptor β 2 subunit is repressed by TCR signaling. Therefore, IL-12R β 2 is induced by T-bet only after

termination of the antigen stimulus. This allows activation of Stat4 by IL-12, which is required to maintain T-bet expression in the late phase of activation, when it is required to drive Th1 cell differentiation.

EXPERIMENTAL PROCEDURES

Mice, Antibodies, and Chemical Reagents

Balb/c, C57BL/6, and *Ifngr*^{-/-} mice on a C57/Bl6 background were bred under pathogen-free conditions. All animals were handled in strict accordance with good animal practice as defined by the German animal welfare bodies, and all animal work was approved by the appropriate committee. Antibodies to IFN- γ (AN18.17.24), IL-12 (C17.8), IL-4 (11B11), CD3 (145-2C11), CD28 (37.51), CD4 (GK1.5), CD62L (MEL14), and CD44 (IM7) were prepared from hybridoma supernatants. Cyclosporine A (CsA) was obtained from AWD, and phorbol mysterate acetate (PMA), ionomycin and brefeldin A were supplied by Sigma-Aldrich. The calcineurin inhibitor NCI3 was a kind gift of M. Sieber (Sieber et al., 2007).

T Cell Culture

Naive CD4 $^+$ T cells were purified and cultivated as described previously (Niesner et al., 2008). If not stated otherwise, CD4 $^+$ CD62L hi splenocytes were purified by magnetic cell sorting (98%–99% purity). Naive cells (2×10^6 cells/ml) were stimulated with plate-bound CD3-specific antibodies (10 μ g/ml) for 48 hr. Soluble CD28-specific antibodies (1 μ g/ml), IL-2 (10 ng/ml), and blocking antibodies to IL-4 (10 μ g/ml) were included in the medium. To induce Th1 cell differentiation, we added IL-12 (5 ng/ml) to the culture. Where indicated, IFN- γ (10 ng/ml), cyclosporine A (50 nM) or blocking antibodies to IFN- γ (10 μ g/ml) or IL-12 (10 μ g/ml) were used.

mRNA Quantification

mRNA was quantified by RT-PCR as described before (Chang et al., 2007). In brief, we used Nucleospin RNA II (Macherey-Nagel) for RNA isolation, TaqMan Reverse Transcription Reagents (Applied Biosystem) for cDNA synthesis, and LightCycler FastStart DNA Master SYBR Green I with a LightCycler 2.0 Instrument (Roche) for the PCR reaction. Primer sequences and PCR conditions are provided in Table S5 online.

Flow Cytometry

Cytokine staining was performed as described previously (Chang et al., 2007). For intracellular T-bet staining, we used the Foxp3 staining buffer set (eBioscience) according to the manufacturer's instructions together with an Alexa Fluor 647-coupled T-bet antibody (eBioscience, 4B10). We estimated the mean relative T-bet expression by subtracting the mean background (isotype control staining) from the mean T-bet signal. For pStat4 staining, we used an Alexa Fluor 647-conjugated pStat4 antibody (BD); for fixation and permeabilization, Phosflow Lyse/Perm buffer and Perm Buffer III (BD) were used according to the manufacturer's instructions.

Chromatin Immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed as described previously (Chang et al., 2007) with the following modifications: Prior to formaldehyde fixation, DNA and proteins were fixed with dithiobis (succinimidyl propionate) (2 mM final concentration) for 30 min at room temperature and fixation was stopped by addition of glycine (125 mM final concentration) for 10 min. Moreover, the preclear step was omitted and dry milk in PBS (final concentration of 3.5%) was added to the lysate before loading of the column. A polyclonal Stat4-specific antibody (Santa Cruz, C-20) was used for precipitation. Primer sequences can be found in Table S5 online.

Mathematical Modeling

All mathematical models considered in this report describe production and degradation of protein and mRNA of T-bet, IFN- γ , and IL-12R β 2 (Rec) by differential equations. As external IL-12 concentrations were saturating, IL-12-dependent signaling was controlled by the expression of IL-12R β 2.

Box 1. Two-Loop Model

$$\begin{aligned} \frac{d Tbet_{mRNA}}{dt} &= \alpha_1 + \alpha_2 \cdot \frac{Ag(t)}{K_1 + Ag(t)} \cdot \frac{IFN_{Prot}}{K_2 + IFN_{Prot}} \\ &\quad + \alpha_3 \cdot Rec_{Prot} - \gamma_{Tbet} \cdot Tbet_{mRNA} \\ \frac{d Tbet_{Prot}}{dt} &= \beta \cdot Tbet_{mRNA} - \delta_{Tbet} \cdot Tbet_{Prot} \\ \frac{d Rec_{mRNA}}{dt} &= \alpha_4 \cdot Tbet_{Prot} \cdot \frac{K_4}{K_4 + Ag(t)} - \gamma_{Rec} \cdot Rec_{mRNA} \\ \frac{d Rec_{Prot}}{dt} &= \beta \cdot Rec_{mRNA} - \delta_{Rec} \cdot Rec_{Prot} \\ \frac{d IFN_{mRNA}}{dt} &= \alpha_5 \cdot \frac{Tbet_{Prot}}{K_5 + Tbet_{Prot}} \cdot \frac{Rec_{Prot}}{K_6 + Rec_{Prot}} \cdot \frac{Ag(t)}{K_7 + Ag(t)} \\ &\quad - \gamma_{IFN} \cdot IFN_{mRNA} \\ \frac{d IFN_{Prot}}{dt} &= \beta \cdot IFN_{mRNA} - \delta_{IFN} \cdot IFN_{Prot} \end{aligned}$$

The two-loop model is deposited at www.ebi.ac.uk/biomodels-main/ (ID: MODEL9284712021).

Using a simulated-annealing algorithm (Kirkpatrick et al., 1983) implemented in Matlab (MathWorks), we simultaneously fitted activation rate constants and half-saturation constants to mRNA kinetics of all three genes in WT and *Ifngr*-deficient cells, in the presence and absence of IL-12 (Table 1). Confidence intervals were estimated through a bootstrapping approach, in which new data sets were generated from the experimental data by addition of noise, estimated from repeated measurements. From the distribution of parameter values determined by fitting each data set, the 5th and 95th percentile was calculated to estimate the 90% confidence interval. The two-loop model was compared to three alternative models through a statistical analysis (Timmer et al., 2004): the one-loop model lacking TCR-dependent repression of IL-12R β 2 and IL-12-dependent T-bet expression and two other models, each lacking one of these features. A detailed description of the model comparison can be found in the Supplemental Data.

Statistical Analysis

Statistical analysis was performed with Matlab (Mathworks). We applied a paired t test to the logarithm of the expression to assess the significance of the measured relative increase of T-bet and IL-12R β 2 in the absence of CD3-stimulation or after addition of CsA. Log-normal distribution was verified by the Lilliefors test. The 95% confidence intervals for correlation coefficients were estimated from a distribution of correlation coefficients of 100 data sets obtained by a bootstrapping strategy with replacement. The interval was bias corrected with the activated percentile method (Efron and Tibshirani, 1993).

SUPPLEMENTAL DATA

Supplemental Data include eight figures, mathematical models, and five tables and can be found with this article online at [http://www.cell.com/immunity/supplemental/S1074-7613\(09\)00183-6](http://www.cell.com/immunity/supplemental/S1074-7613(09)00183-6).

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