IL-21 Inhibits IFN-γ Production in Developing Th1 Cells through the Repression of Eomesodermin Expression

Akira Suto, Andrea L. Wurster, Steven L. Reiner and Michael J. Grusby

J Immunol 2006; 177:3721-3727; doi: 10.4049/jimmunol.177.6.3721
http://www.jimmunol.org/content/177/6/3721

Why The JI?

- Rapid Reviews! 30 days* from submission to initial decision
- No Triage! Every submission reviewed by practicing scientists
- Speedy Publication! 4 weeks from acceptance to publication

References  This article cites 25 articles, 10 of which you can access for free at:
http://www.jimmunol.org/content/177/6/3721.full#ref-list-1

Subscription  Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions  Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts  Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
IL-21 Inhibits IFN-γ Production in Developing Th1 Cells through the Repression of Eomesodermin Expression

Akira Suto,* Andrea L. Wurster,²* Steven L. Reiner,† and Michael J. Grusby³*‡

Exposure of naive Th cell precursors (Thp) to IL-21 inhibits IFN-γ production from developing Th1 cells. The inhibition of IFN-γ seen in IL-21-treated Thp cells is specific as the expression of other Th1 cytokines is unaffected. Recently, it has been reported that Eomesodermin (Eomes), a member of the T-box gene family, is expressed in developing CD8⁺ T cells and plays an important role in regulating IFN-γ production and cytolytic effector function. In this study, we show that Eomes mRNA and protein are also expressed in developing Th1 cells, and exposure of naive Thp cells to IL-21 results in a decrease in Eomes expression. Moreover, the repression of Eomes expression by IL-21 is not due to an indirect effect of IL-21 on the expression of IFN-γ or STAT4 and is independent of STAT1 and T-bet expression. Finally, we show that ectopic expression of Eomes prevents the inhibition of IFN-γ production from IL-21-treated Thp cells. Taken together, these results demonstrate that Eomes plays a role in regulating IFN-γ production in CD4⁺ T cells and IL-21 inhibits IFN-γ production in developing Th1 cells through the repression of Eomes expression. The Journal of Immunology, 2006, 177: 3721–3727.

activated CD4⁺ T cells differentiate into at least two functionally distinct subsets, Th1 and Th2 cells, as defined by their patterns of cytokine production (1, 2). Th1 cells produce IFN-γ and lymphotoxin and are responsible for delayed-type hypersensitivity reactions and promoting control of intracellular pathogens (1, 2). Th2 cells produce IL-4, IL-5, and IL-13 and provide a helper function for Ab production, particularly IgE (1, 2). The cytokine expression profile of each Th cell subset is regulated by specific transcription factors. Th1 lineage commitment and IFN-γ production are controlled by T-bet (3), while in Th2 cells specific cytokine production is mediated by c-Maf and GATA3 (3–7).

Like Th1 cells, CD8⁺ T cells produce IFN-γ when activated by intracellular pathogens. CD8⁺ effector T cell development is regulated by two T-box transcription factors, T-bet and Eomesodermin (Eomes)⁴ (8, 9). Eomes was initially reported as a transcription factor that initiates mesodermal cell fate in most vertebrates (10), and Eomes⁻/⁻ mice die early in embryogenesis. Recently, it has been reported that Eomes, a paralogue of T-bet, is significantly induced in effector CD8⁺ T cells and plays an important role in IFN-γ production and cytolytic effector mechanisms (9). Moreover, ectopic expression of Eomes in Th2 cells is sufficient to induce IFN-γ in the absence of T-bet (9).

IL-21 is a four-helix-bundle type 1 cytokine with significant homology to IL-2, IL-15, and IL-4 and mediates its effects through a ligand-specific IL-21R associated with the common cytokine receptor γ-chain (11–13). IL-21 has pleiotropic effects on the proliferation, differentiation, and effector function of T cells, B cells, NK cells, and dendritic cells. IL-21 is produced by activated CD4⁺ T cells, and predominantly by Th2 cells (14). Exposure to IL-21 during the priming of highly purified naive Th precursor (Thp) cells results in a decrease in the production of IFN-γ upon secondary stimulation. Interestingly, the production of other Th1 cell cytokines is intact following exposure of developing Th1 cells to IL-21 and the suppression of IFN-γ is specific. Although IL-21 inhibits IL-12 signaling through the repression of STAT4 mRNA and protein, the mechanism by which IFN-γ production in developing Th1 cells is repressed by IL-21 is not completely understood.

In this study, we found that IL-21 represses Eomes expression in CD4⁺ T cells. Repression of Eomes expression by IL-21 correlates with an inhibition of IFN-γ expression and is independent of any effect of IL-21 on IFN-γ, T-bet, STAT1, or STAT4 expression. Moreover, ectopic expression of Eomes completely protects developing Th1 cells from the inhibition of IFN-γ production seen in IL-21-treated cells. These results indicate that IL-21 inhibits IFN-γ production in developing Th1 cells through the repression of Eomes expression.

Materials and Methods

Mice

Mice 4–8 wk of age were used in all experiments. BALB/c and C57BL/6 mice were purchased from Taconic Farms. IFN-γ⁻/⁻ mice on a C57BL/6 background and STAT4⁻/⁻ mice on BALB/c background were purchased from The Jackson Laboratory. STAT1⁻/⁻, T-bet⁻/⁻, and IFN-γ⁻/⁻ T-bet⁻/⁻ mice were obtained from L. Glimcher (Harvard School of Public Health, Boston, MA). All mice were housed in microisolator cages under specific pathogen-free conditions at the Harvard School of Public Health, and all animal studies were performed according to institutional and National Institutes of Health guidelines for animal use and care.

Reagents

Abs to CD3, CD28, and IL-4 used in Th cell differentiation cultures were obtained from BD Pharmingen. rIL-2 was provided by Chiron. Murine
rIL-21 was obtained from R&D Systems. Cytochrome C (CHX) and actinomycin D were obtained from Sigma-Aldrich.

Flow cytometric analysis
Cells were stained and analyzed on a FACScalibur (BD Biosciences) using CellQuest software. The following Abs were purchased from BD Pharmingen: anti-CD4 PerCP (H129.19), anti-CD8 FITC (53-6.7), and anti-CD62L PE (MEL-14). Before staining, Fc receptors were blocked with anti-CD16/32 Ab (2.4G2; BD Pharmingen). Negative controls consisted of isotype-matched, directly conjugated, nonspecific Abs (BD Pharmingen).

Cell culture
Lymphocytes were cultured in RPMI 1640 supplemented with 10% heat-inactivated FCS, 50 μM 2-ME, 2 mM l-glutamine, and antibiotics (complete RPMI 1640). Naive cells were purified from lymph nodes of mice by cell sorting using anti-CD4 or anti-CD8 and anti-CD62L (BD Pharmingen) to 96–98% purity. Naive cells were plated onto 1 cell sorting using anti-CD4 or anti-CD8 and anti-CD62L (BD Pharmingen) to 96–98% purity. Naive cells were plated onto 1×10^6 cells/ml in the presence of 2 μg/ml anti-CD28 and 100 U/ml IL-2 (neutral conditions), 1 ng/ml IL-12 and 10 μg/ml anti-IL-4 (Th1 conditions), or 10 ng/ml IL-4 and 10 μg/ml anti-IFN-γ (Th2 conditions) in the presence or absence of IL-21 (100 ng/ml). Cells were expanded in 100 U/ml IL-2 3 days after initiation of culture. After 5 days in culture, the cells were restimulated with anti-CD3/CD28 (second stimulation).

Western blot analysis
Whole cell extracts were prepared by lysing cells in 20 mM Tris, 0.5% Nonidet P-40, 250 mM NaCl, 5 mM EDTA, 50 mM NaF, 50 mM β-glycerophosphate, 0.5 mM Na3VO4, 1 mM DTT, and 0.1 mM PMSF, and clearing the lysates by centrifugation. Protein extracts were separated on 7.5% polyacrylamide gel and transferred to a poly(vinylidene difluoride) membrane. The immunoblots were blocked in 1% milk in PBST (PBS and 0.1% Tween 20) and incubated with the indicated Ab overnight at 4°C. Ab specific for Eomes (ab31717) was obtained from Abcam. Ab specific for heat shock protein 90 (H-114) was obtained from Santa Cruz Biotechnology. The blots were washed with PBST and incubated with anti-rabbit HRP-conjugated Ab (Zymed Laboratories). Detection was performed using ECL substrate (Amersham Biosciences).

Retrovirus transduction
Retrovirus transduction of primary CD4^+ T cells was performed, as described previously (3). The retroviral vectors for GFP (control-RV) and Eomes GFP RV (Eomes-RV) have previously been described (9). For real-time PCR and ELISA analysis, GFP-positive cells were sorted on day 4 and restimulated on day 7 with anti-CD3 for 24 h. For intracellular cytokine staining, cells were washed on day 4, maintained under neutral conditions, and subjected to intracellular cytokine staining.

Intracellular staining for IFN-γ
Cells were stimulated with PMA/ionomycin for 5 h. Monensin (2 μM; Sigma-Aldrich) was added for the final 4 h to prevent cytokine release. After surface staining, cells were fixed with IC FIX (BioSource International), permeabilized with IC PERM (BioSource International), and stained with anti-IFN-γ allophycocyanin (XMG1.2; BD Pharmingen).

Real-time PCR analysis
Total cellular RNA was extracted with TRizol solution (Invitrogen Life Technologies). Reverse transcription was conducted using an iScript cDNA synthesis kit (Bio-Rad). Primers and TaqMan probes for T-bet, GATA-3, IFN-γ, hypoxanthine phosphoribosyltransferase, and β-actin have previously been described (14). PCR primers and a fluorigenic probe for Eomes were forward, 5'-CTCTCACCCTCTCAGAGACA CAGTT-3'; reverse, 5'-TGATCTTCTAGCTGGTATACC-3'; probe, 5'-FAM-TGCTGTTAGCGCCTACAAAACA-3'-TAMRA.

CHX treatment
Naive Tn cells stimulated with anti-CD3/CD28 and IL-2 for 48 h were harvested, washed, and recultured for an additional 18 h in medium alone. The rested cells were then treated with or without CHX (0.5 mM) for 1 h, washed, and restimulated with anti-CD3/CD28 and IL-2 in the presence or absence of IL-21 (200 ng/ml) with or without CHX (0.5 mM). After 5 h, total RNA was prepared and analyzed by real-time PCR for detection of Eomes mRNA.

Decay of Eomes mRNA
Naive CD4^+ T cells were cultured with anti-CD3/CD28 and IL-2 for 72 h, after which actinomycin D (10 μg/ml) was added to the cultures. Cells were then incubated with or without IL-21 (200 ng/ml) at 37°C for 0, 1, 3, and 5 h. Total RNA was isolated and Eomes mRNA expression was determined by real-time PCR. No significant loss of cell viability was observed by trypan blue exclusion after the incubation.

Data analysis
Data are presented as mean ± SD. Statistical analysis of the results was performed using the unpaired t test. Values of p < 0.05 were considered significant.

Results
IL-21 inhibits IFN-γ production in CD4^+ T cells independent of T-bet
We have reported previously that exposure of highly purified naive Tn cells to IL-21 inhibits IFN-γ production from these cells as they differentiate into Th1 cells. T-bet was an attractive potential target for this IL-21-mediated effect because T-bet is specifically expressed in differentiating Th1 cells, and this transcription factor is critical for high level IFN-γ expression. Although T-bet^-/- cells cultured under either neutral or Th1 skewing conditions produce less IFN-γ upon restimulation as compared with wild-type (WT) cells, exposure of T-bet^-/- cells to IL-21 at the initiation of culture results in a further reduction in their capacity to produce IFN-γ (Fig. 1). Moreover, T-bet protein levels are not affected by IL-21 treatment of naive Tn cells cultured under Th1 skewing conditions (14). Taken together, these results suggest that IL-21 inhibits IFN-γ production in developing Th1 cells independent of T-bet.

IL-21 represses Eomes expression in CD4^+ T cells upon primary stimulation
To identify genes that are regulated by IL-21 and that may control IFN-γ expression, we performed DNA microarray analysis. Naive Tn cells stimulated with anti-CD3/28 and IL-21 were compared with naive Tn cells stimulated with anti-CD3/28 alone for 24 h. From this analysis, we found that Eomes was down-regulated ~2-fold by IL-21 treatment (data not shown). Eomes was first reported to initiate mesodermal cell fate in most vertebrates (10), and Eomes^-/- mice die early in embryogenesis. Eomes and T-bet belong to the same subfamily of T-box factors (15) and are significantly induced in effector CD8^+ T cells. In addition, Eomes plays an important role in IFN-γ production and

![FIGURE 1. IL-21 represses IFN-γ production in T-bet^-/- CD4^+ T cells. Tn cells from WT and T-bet^-/- mice were cultured under neutral or Th1 conditions for 6 days in the presence or absence of IL-21, at which time the cells were washed and stimulated with 1 μg/ml anti-CD3. Culture supernatants were collected 24 h after anti-CD3 stimulation, and the amounts of IFN-γ in the supernatants were measured by ELISA.](http://www.jimmunol.org/)
cytolytic effector mechanisms in CD8+ T cells (9). Therefore, we focused on the potential role of Eomes in IL-21-mediated inhibition of IFN-γ expression in CD4+ T cells.

First, we measured the kinetics of Eomes mRNA production in CD4+ T cells upon primary stimulation in the presence or absence of IL-21 by real-time PCR (Fig. 2). Naive CD4+ T cells were stimulated with anti-CD3/CD28 and IL-2 for 0, 6, 24, 48, and 72 h in the presence or absence of IL-21. Eomes mRNA was barely detectable in naive CD4+ T cells. We found that the expression levels of Eomes mRNA increased over time upon primary stimulation until 72 h. By contrast, the inclusion of IL-21 remarkably repressed Eomes expression at each time point examined. The increase in Eomes mRNA expression required TCR signaling because we could not detect the Eomes mRNA expression in naive CD4+ T cells stimulated with IL-2 alone for 24 h (data not shown). Taken together, these results indicate that IL-21 represses Eomes mRNA expression in TCR-stimulated CD4+ T cells.

IL-21 represses Eomes expression in developing Th1 cells

To determine whether Eomes protein expression is also repressed by IL-21, we performed Western blot analysis. Naive Thp cells were cultured under neutral conditions in the presence or absence of IL-21 for 72 h (primary stimulation). The cells were maintained with IL-2 for 2 days and restimulated with anti-CD3/CD28 for

FIGURE 2. Kinetics of Eomes mRNA production in CD4+ T cells upon primary stimulation. Naive Thp cells were stimulated with anti-CD3/CD28 and IL-2 in the presence or absence of IL-21. RNA was purified at indicated time points during stimulation. Eomes expression was assessed by real-time PCR. Values show the relative expression levels normalized to β-actin.

FIGURE 3. IL-21 represses Eomes expression in developing Th1 cells. A, Naive Thp cells were stimulated in neutral condition in the presence or absence of IL-21. Cells were lysed after 72 h of primary stimulation or 24 h of secondary stimulation. As positive control, naive CD8+ T cells were stimulated with anti-CD3/CD28 and IL-2 for 72 h. The lysates from 0.5 million cells each were subjected to Western blot analysis for Eomes and then for heat shock protein 90. B, Naive Thp cells from lymph nodes of C57BL/6 mice were cultured under neutral, Th1, and Th2 conditions for 72 h. Eomes, T-bet, and GATA-3 expression was assessed by real-time PCR. Values show the relative expression levels normalized to β-actin. Data are representative of three experiments. C, Naive Thp T cells from lymph nodes of C57BL/6 mice were cultured under neutral, Th1, and Th2 conditions for 5 days and restimulated with anti-CD3. RNA was purified 5 h after restimulation, and Eomes, T-bet, and GATA-3 expression was assessed by real-time PCR. Values show the relative expression levels normalized to GAPDH. Data are mean ± SD from three independent experiments.
24 h on day 5 (secondary stimulation). As a positive control, CD8\(^+\) T cells were stimulated with anti-CD3/CD28 and IL-2 for 72 h (Fig. 3A). Eomes protein in either naive CD4\(^+\) or naive CD8\(^+\) T cells could not be detected by Western blot (data not shown). We found that Eomes protein is up-regulated in primary stimulated CD4\(^+\) T cells, although the level is lower than that seen in primary stimulated CD8\(^+\) T cells. Strikingly, IL-21 represses Eomes protein expression in CD4\(^+\) T cells upon primary stimulation. Moreover, exposure of naïve Thp cells to IL-21 results in a dramatic decrease in the expression of Eomes protein upon secondary stimulation. The expression level of T-bet protein was not decreased by IL-21 treatment, as previously reported (14) (data not shown).

We next examined whether IL-21 affects Eomes, T-bet, and GATA3 mRNA expression in developing Th1 and Th2 cells. Naïve Thp cells were cultured under neutral, Th1, or Th2 skewing conditions in the presence or absence of IL-21, and mRNA expression was assessed by real-time PCR upon primary stimulation (Fig. 3B) or secondary stimulation (Fig. 3C). We found that Eomes is expressed in Th1 cells, but at barely detectable levels in Th2 cells following either primary or secondary stimulation. Exposure of naïve Thp cells to IL-21 results in a significant decrease in the expression of Eomes mRNA in either neutral or Th1 cell skewing conditions upon primary and secondary stimulation. This was a direct effect through the IL-21R complex, because IL-21 did not repress Eomes expression in IL-21R\(^{-/-}\) cells (data not shown). IL-21 did not repress T-bet mRNA expression in developing Th1 cells in either neutral or Th1 condition, as previously reported (Fig. 3, B and C) (14). Finally, IL-21 did not affect GATA-3 mRNA levels in either Th1 or Th2 conditions (Fig. 3. B and C). These results indicate that IL-21 specifically represses Eomes expression in developing Th1 cells.

Ectopic expression of Eomes rescues IFN-\(\gamma\) production from IL-21-treated Thp cells

To determine whether maintenance of Eomes expression can rescue IFN-\(\gamma\) production from IL-21-treated cells, we used retroviral transduction to ectopically express Eomes in developing Th1 cells. Naïve Thp cells from C57BL/6 mice were cultured under neutral conditions in the presence or absence of IL-21 for 24 h before infection with a retrovirus that expresses either GFP alone (control-RV) or coexpresses Eomes and GFP (Eomes RV). Cells were washed and maintained under neutral conditions for an additional 4 days, at which time IFN-\(\gamma\) production was assessed by intracellular cytokine staining after restimulation with PMA/ionomycin (Fig. 4A). We found that IL-21 does not impair the development of IFN-\(\gamma\)-producing cells when the cells were transduced with Eomes-RV (PBS, 93% IFN-\(\gamma\)\(^+\) vs IL-21, 87% IFN-\(\gamma\)\(^+\)). In addition, the mean fluorescence intensity of IL-21-treated cells is comparable to PBS-treated cells in Eomes-RV infected cells (PBS 281 vs IL-21 356). In contrast, addition of IL-21 resulted in a marked reduction in the number of IFN-\(\gamma\)-producing cells in control GFP-infected cells (PBS, 39% IFN-\(\gamma\)\(^+\) vs IL-21, 19% IFN-\(\gamma\)\(^+\)) as expected from our previous results (Fig. 1) (14). Consistent with the results from intracellular cytokine staining, IL-21 inhibits IFN-\(\gamma\)-production in control GFP-infected cells (PBS, 44.7 ± 10.4 ng/ml vs IL-21, 8.3 ± 2.0 ng/ml; n = 3 in each group), but not in Eomes GFP-infected cells, as measured by ELISA (PBS, 189.1 ± 56.3 ng/ml vs IL-21, 221.9 ± 48.5 ng/ml; n = 3 in each group) (Fig. 4B). Thus, ectopic expression of Eomes prevents a decrease in IFN-\(\gamma\)-production of IL-21-treated cells. Taken together, these data suggest that IL-21 inhibits IFN-\(\gamma\) production from Th1 cells through the repression of Eomes expression.

**FIGURE 4.** Ectopic expression of Eomes rescues IFN-\(\gamma\) production from IL-21-treated cells. Naïve Thp cells from C57BL/6 mice were cultured under neutral conditions in the presence or absence of IL-21 (200 ng/ml). On day 1 of culture, cells were infected with a retrovirus that either expresses GFP alone (Control RV) or coexpresses Eomes and GFP (Eomes RV). Infected cells were maintained under neutral conditions in the presence or absence of IL-21 (PBS vs IL-21). A, Cells were washed on day 4 and maintained under neutral conditions, and IFN-\(\gamma\) production was assessed by intracellular cytokine staining 5 h after restimulation with PMA/ionomycin on day 7. IFN-\(\gamma\) staining among GFP-positive cells is shown. Numbers indicate mean fluorescence intensity and percentage of GFP-positive cells expressing IFN-\(\gamma\). The results are representative of five independent experiments. B, GFP-positive cells were purified by flow cytometry on day 4, maintained under neutral conditions, and restimulated with anti-CD3 for 24 h at 0.5 × 10^6 cells/ml on day 7. The amount of IFN-\(\gamma\) in the supernatants was measured by ELISA. Data are mean ± SD from three independent experiments.

IL-21 represses Eomes expression independent of IFN-\(\gamma\) and T-bet

IFN-\(\gamma\) receptor signaling is required for the induction of T-bet expression following TCR occupancy (16, 17). Because IL-21 inhibits IFN-\(\gamma\) expression in developing Th1 cells, it was possible that IL-21 might repress Eomes expression indirectly through the inhibition of IFN-\(\gamma\) production. To determine whether IL-21 can repress Eomes expression independent of IFN-\(\gamma\), naïve Thp cells from IFN-\(\gamma\)\(^{-/-}\) mice were cultured in the presence or absence of IL-21 and Eomes expression was assessed by real-time PCR upon restimulation (Fig. 5A). We found that Eomes mRNA is expressed in IFN-\(\gamma\)\(^{-/-}\) Th1 cells and repressed by IL-21 treatment. Because T cells from IFN-\(\gamma\)\(^{-/-}\) mice are not quite the same as WT cells in that they are already biased to Th2 cytokine production, we performed a similar experiment in which we used Abs to neutralize IFN-\(\gamma\) in Th1 culture conditions (Fig. 5B). Similar to that seen with IFN-\(\gamma\)\(^{-/-}\) cells, we found that IL-21 represses Eomes mRNA in the presence of anti-IFN-\(\gamma\) in Th1 conditions. These results indicate that IL-21 represses Eomes mRNA independent of IFN-\(\gamma\) inhibition. It is likely, however, that IFN-\(\gamma\) signaling does, directly
or indirectly, contribute to Eomes expression because the expression level of Eomes mRNA is lower in IFN-γ−/− cells compared with WT cells. In contrast to Eomes expression, IL-21 does not repress T-bet mRNA in WT cells and T-bet expression is nearly absent in IFN-γ−/− Th1 cells, as previously reported (Fig. 5A) (14).

We next examined whether IL-21 can repress Eomes expression in the absence of both T-bet and IFN-γ. Naive Thp cells from T-bet−/−, IFN-γ−/− T-bet−/−, and WT mice were cultured in the presence or absence of IL-21 and Eomes expression was assessed by real-time PCR following secondary stimulation (Fig. 5C). We found that IL-21 can still repress Eomes expression in the absence of T-bet and IFN-γ, although the expression level of Eomes is lower in T-bet−/− and IFN-γ−/− T-bet−/− cells compared with WT cells. Taken together, these results demonstrate that IL-21 can repress Eomes expression independent of any effect it may have on IFN-γ and T-bet expression.

**Eomes mRNA expression is independent of STAT1 and STAT4 signaling**

T-bet is induced by IFN-γ and STAT1 signaling during T cell activation (17). Because IFN-γ signaling contributes to Eomes mRNA expression, we next examined whether STAT1 is essential for the Eomes expression (Fig. 6A). Naive Thp cells from STAT1−/− and WT mice were cultured under neutral conditions. Three days after activation, Eomes was expressed in both WT and STAT1−/− CD4+ T cells and is repressed by IL-21 treatment. Thus, STAT1 is not required for Eomes mRNA expression.

Because IL-21 inhibits STAT4 signaling by the reduction of STAT4 expression (14), we next examined whether IL-21 can repress Eomes expression in the absence of STAT4. Naive Thp cells from STAT4−/− and WT mice were cultured in the presence or absence of IL-21, and Eomes expression upon secondary stimulation was assessed by real-time PCR (Fig. 6B). We found that IL-21 can repress Eomes in the absence of STAT4, although the expression level of Eomes mRNA is lower in STAT4−/− mice compared with WT mice. Taken together, IL-21 can repress Eomes expression independent of STAT1 and STAT4.

**FIGURE 5.** IL-21 represses Eomes expression in the absence of T-bet and IFN-γ. A, Naive Thp cells from C57BL/6 (WT) mice and IFN-γ−/− mice on C57BL/6 background were cultured under neutral conditions in the presence or absence of IL-21 for 5 days, and then washed and restimulated with anti-CD3. RNA was isolated after 5 h of secondary stimulation. B, Naive Thp cells from WT mice were cultured under Th1 conditions with and without anti-IFN-γ (10 μg/ml) in the presence or absence of IL-21. RNA was isolated after 72 h of primary stimulation. C, Naive Thp cells from T-bet−/−, IFN-γ−/− T-bet−/−, or BALB/c (WT) mice were cultured under neutral conditions in the presence or absence of IL-21 for 5 days, and then washed and restimulated with anti-CD3. RNA was purified 5 h after secondary stimulation. Eomes (A–C) and T-bet (A) expression were assessed by real-time PCR. Values show the relative expression levels normalized to GAPDH. Representative data from three independent experiments are shown.

**FIGURE 6.** STAT1 and STAT4 are not required for Eomes expression. A, Naive Thp cells from C57BL/6 (WT) mice and STAT1−/− mice on C57BL/6 background were cultured under neutral conditions in the presence or absence of IL-21. RNA was prepared after 60 h of primary stimulation. Eomes mRNA expression was assessed by real-time PCR. Values show the relative expression levels normalized to β-actin. Representative data from two independent experiments are shown. B, Naive Thp cells from BALB/c (WT) mice and STAT4−/− mice on BALB/c background were cultured under neutral conditions in the presence or absence of IL-21 for 5 days and then washed and restimulated with anti-CD3. RNA was purified 5 h after secondary stimulation. Values show the relative expression levels normalized to GAPDH. Representative data from two independent experiments are shown.
De novo protein synthesis is not required for the repression of Eomes mRNA by IL-21 treatment

To determine whether the inhibitory effects of IL-21 on TCR-induced expression of Eomes mRNA are direct or indirect, we used CHX to inhibit de novo protein synthesis (Fig. 7). Naïve Thp cells stimulated with anti-CD3/CD28 and IL-2 for 48 h were harvested, washed, and recultured for an additional 18 h in medium alone. These rested cells were then treated with or without CHX (0.5 mM) for 1 h, washed, and restimulated with anti-CD3/CD28 and IL-2 in the presence or absence of IL-21 (200 ng/ml) with or without CHX (0.5 mM). Weak induction of Eomes was observed in the absence of IL-21 (200 ng/ml). After 5 h, total RNA was prepared and analyzed by real-time PCR for detection of Eomes mRNA. Values show the relative expression levels normalized to hypoxanthine phosphoribosyltransferase. Bars show the mean ± SD from triplicate culture wells. Statistical analyses were performed using Student’s t test. Asterisk indicates significantly different from the mean value; p < 0.05.

IL-21 does not accelerate the decay of Eomes mRNA

We next examined whether IL-21 can accelerate the decay of Eomes mRNA. After naïve CD4$^+$ T cells were stimulated with anti-CD3/CD28 and IL-2 for 72 h, de novo transcription was inhibited by the addition of actinomycin D (10 μg/ml). Cells were then incubated with or without IL-21 (200 ng/ml) for 1, 3, or 5 h, and the remaining Eomes mRNA was measured by real-time PCR (Fig. 8). We found that IL-21 did not accelerate the decay of Eomes mRNA. Taken together with the results of CHX experiment, IL-21 directly represses TCR-triggered Eomes mRNA up-regulation in CD4$^+$ T cells, probably by affecting transcription initiation.

**Discussion**

Previously, we have shown that addition of IL-21 to in vitro cultures of naïve Thp cells results in cells that produce reduced amounts of IFN-γ upon secondary stimulation. In contrast, addition of IL-21 upon secondary stimulation does not affect IFN-γ production by differentiated Th1 cells. The effect of IL-21 on IFN-γ expression is specific, and other characteristics of Th1 cell differentiation (IL-2 and TNF-α production and IL-12Rβ2 expression) are intact (14). T-bet was an attractive potential target for IL-21 because T-bet is specifically expressed in developing Th1 cells (3) and acts on the IFN-γ locus independently of STAT4 (18). However, the expression level of T-bet is not affected by IL-21 (14) (Fig. 3). Moreover, we found that IL-21-treated T-bet$^{−/−}$ Th cells also leads to inhibition of IFN-γ expression (Fig. 1). Because of these findings, T-bet is most likely not a target of IL-21.

We have also reported that IL-21 inhibits STAT4 activity by reducing STAT4 mRNA and protein levels (14). STAT4 plays an important role in amplifying the amount of IFN-γ production in Th1 cells, although the IL-12/STAT4 pathway does not appear to be as important as STAT1 and T-bet signaling in initiating IFN-γ production (19–21). Recently, it has been reported that GATA-3 expression inhibits Th1 cell development through the inhibition of STAT4 and IL-12Rβ2 expression (22). Therefore, it was possible that IL-21 may repress STAT4 expression through the up-regulation of GATA-3. However, IL-21 treatment of naïve Thp cells does not significantly alter either GATA-3 mRNA expression levels or IL-12Rβ2 expression levels (14) by real-time PCR analysis (Fig. 3). These results suggest that GATA-3 is also not likely a target of IL-21. Moreover, we have reported that the small amount of IFN-γ production produced by STAT4$^{−/−}$ Th cells is also diminished by IL-21 treatment (14). Thus, the pathway by which IL-21 inhibits IFN-γ production must also be independent of STAT4.

In this study, we demonstrate that Eomes plays a role in regulating IFN-γ production in CD4$^+$ T cells and IL-21 inhibits IFN-γ expression in developing Th1 cells through a repression of Eomes expression independent of its effects on IFN-γ, T-bet, GATA-3, STAT1, or STAT4 expression. The regulation of Eomes expression has not been extensively studied. Activin and BMP4, two TGF-β family cytokines, have been reported to up-regulate Eomes expression in Xenopus (10) and IL-4 has been shown to repress Eomes expression (23). Our data suggest that IFN-γ itself leads to an up-regulation in Eomes expression as IFN-γ$^{−/−}$ cells express less Eomes compared with WT cells. In addition, Eomes expression is remarkably repressed in Tfh cells cultured in Th2 skewing conditions and is most likely due to the action of IL-4 and/or IL-21 produced by developing Th2 cells. Taken together, these observations suggest that Eomes is up-regulated by IFN-γ and down-regulated by IL-4 and IL-21 in CD4$^+$ T cells.

Finally, we find that IL-21 represses Eomes mRNA expression most likely by affecting transcriptional initiation, because IL-21 can repress Eomes mRNA independent of de novo protein synthesis and does not accelerate the decay of Eomes mRNA. IL-21 activates STAT1, STAT3, and, to a lesser degree, STAT5A and STAT5B. IL-2 also activates these STATs (24, 25). Because...
STAT1 is not required for Eomes mRNA expression (Fig. 6), it is possible that the balance of STAT3, STAT5A, and/or STAT5B activation may contribute to the repression of Eomes mRNA by IL-21.

Acknowledgments
We thank Dr. L. Glimcher for T-bet−/− and IFN-γ−/− T-bet−/− mice, and Dr. M. Nakajima and Dr. A. Becskei for valuable discussion.

Disclosures
The authors have no financial conflict of interest.

References