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Pro- and Antiinflammatory Cytokine Signaling: Reciprocal Antagonism Regulates Interferon-gamma Production by Human Natural Killer Cells

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Summary

Activated monocytes produce proinflammatory cytokines (monokines) such as interleukin (IL)-12, IL-15, and IL-18 for induction of interferon-γ (IFN-γ) by natural killer (NK) cells. NK cells provide the antiinflammatory cytokine transforming growth factor (TGF)-β, an autocrine/negative regulator of IFN-γ. The ability of one signaling pathway to prevail over the other is likely important in controlling IFN-γ for the purposes of infection and autoimmunity, but the molecular mechanism(s) of how this counterregulation occurs is unknown. Here we show that in isolated human NK cells, proinflammatory monokines antagonize antiinflammatory TGF-β signaling by downregulating the expression of the TGF-β type II receptor, and its signaling intermediates SMAD2 and SMAD3. In contrast, TGF-β utilizes SMAD2, SMAD3, and SMAD4 to suppress IFN-γ and T-BET, a positive regulator of IFN-γ. Indeed, activated NK cells from *Smad3*$^{-/-}$ mice produce more IFN-γ in vivo than NK cells from wild-type mice. Collectively, our data suggest that pro- and antiinflammatory cytokine signaling reciprocally antagonize each other in an effort to prevail in the regulation of NK cell IFN-γ production.

Introduction

Interferon-γ (IFN-γ), a cytokine essential for both innate and adaptive immune responses, is produced principally by $CD4^+$ and $CD8^+$ T cells, as well as natural killer (NK) cells. It is critical for successful clearance of intracellular pathogens and also in host defense against malignant transformation (Dunn et al., 2004; Jouanguy et al., 1999). Overproduction of IFN-γ can lead to autoimmune disorders, such as Crohn's disease, and contributes to septic shock, while IFN-γ deficiency can result in a higher susceptibility to infection or malignancy (Bouma and Strober, 2003; Dunn et al., 2002; Schroder et al., 2004; Shankaran et al., 2001; Trinchieri, 1995). IFN-γ production should therefore be subject to intense positive and negative regulation in cells of the immune system.

Monocyte-derived proinflammatory cytokines (monokines), such as IL-12, IL-15, and IL-18—especially in combination—positively regulate IFN-γ production (Cooper et al., 2001; Fehniger et al., 1999). IL-12 is a particularly potent inducer of IFN-γ synthesis in T cells and NK cells. Its high-affinity receptor is a heterodimer comprised of two subunits, the β1 and the β2 chains (Chua et al., 1994; Presky et al., 1996). IL-12 activates the Janus kinases Tyk2 and Jak2, leading to phosphorylation and activation of signal transducer and activator of transcription (STAT)-4 and other STATs as well (Watford et al., 2004). In addition, IL-12 also activates p38 mitogen-activated protein kinase (MAPK) in NK cells and T cells (Visconti et al., 2000; Zhang and Kaplan, 2000). Importantly, all of these signaling molecules or pathways activated by IL-12 play a role in positively regulating IFN-γ production (Visconti et al., 2000; Watford et al., 2004; Zhang and Kaplan, 2000). IL-15 receptors are comprised of IL-15Rα, IL-2/15Rβ, and γ_c and mainly signal through Janus kinases (Jak) 1 and 3 and STAT-3 and -5 (McInnes and Gracie, 2004; Ohteki, 2002). IL-18 is a member of the IL-1 family and its receptor is composed of two subunits: the ligand binding subunit, IL-1Rrp (IL-1 receptor related protein), and the signal transducing subunit, AcPL (accessory protein-like) (Akira, 2000; Born et al., 1998). IL-18 signaling is suggested to be mediated by MyD88-NFκB, MAPK, and STAT-3 (Nakanishi et al., 2001). IL-18 alone is a poor inducer of IFN-γ production by naive T cells and NK cells, due to their weak IL-18R constitutive expression (Sareneva et al., 2000; Yoshimoto et al., 1998). However, the combination of IL-12 and IL-18 results in remarkably synergistic IFN-γ production, due in part to induction of the IL-18R on the cell surface by IL-12 and subsequent reciprocal upregulation of their receptors (Akira, 2000). The synergistic stimulation of IFN-γ production by IL-12 and IL-18 also results from the orchestrated action of transcription factors NFκB, STAT-4, and AP-1, whose binding sites are located in the *IFNG* gene (Akira, 2000; Nakanishi et al., 2001).

Interestingly, it has been shown that all of the above proinflammatory cytokines activate transcription of T-bet, a member of the T-box transcription factor family (Strengell et al., 2002; Townsend et al., 2004). Glimcher and colleagues have shown that IFN-γ production in $CD4^+$ T cells, NK cells, and effector $CD8^+$ T cells is controlled by T-bet (Sullivan et al., 2003; Szabo et al., 2002). Furthermore, T-bet is a master regulator of lineage commitment of T_H1 $CD4^+$ T cells capable of IFN-γ production (Mullen et al., 2001a, 2001b; Szabo et al., 2000, 2002). T-bet is able to induce itself and two target genes, *Hlx1*

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and *IL-12Rβ2*, and to remodel the chromatin structure at the *Ifng* locus in T cells (Afkarian et al., 2002; Avni et al., 2002; Mullen et al., 2001a, 2002). T-bet is also required in dendritic cells for the optimal production of IFN-γ and optimal activation of antigen-specific T_H1 cells (Lugo-Villarino et al., 2003).

In contrast to proinflammatory cytokines, TGF-β is an immunosuppressive antiinflammatory cytokine implicated as a negative regulator of IFN-γ production by T and NK cells (Dennler et al., 2002; Letterio and Roberts, 1998). TGF-β is produced by most blood mononuclear cells, especially NK cells (Horwitz et al., 1997; Letterio and Roberts, 1998). In most cases, TGF-β signaling is accomplished via its mediators, SMAD2, SMAD3, and SMAD4. In the presence of active TGF-β, its type II receptor (TGF-βRII) transphosphorylates the type I receptor (TGF-βRI) to phosphorylate receptor-regulated SMADs (R-SMADs), i.e., SMAD2 and SMAD3, which in turn form heteromeric complexes with a common-partner SMAD (Co-SMAD), i.e., SMAD4 (Shi and Massague, 2003; Wrana et al., 1994). Subsequently, the heterodimers, i.e., SMAD2/SMAD4 and SMAD3/SMAD4, translocate into the nucleus and bind to the promoter regions of TGF-β-responsive genes (Hata et al., 1997). The N-terminal regions (MH1 domain) in SMAD3 and SMAD4 are able to bind to DNA directly at the core sequences of GTCT or AGAC, whereas SMAD2 does not bind directly to DNA. Promoter bound SMADs can recruit both transcriptional coactivators and transcriptional corepressors (Derynck et al., 1998; Liu et al., 2001; Prunier et al., 2003; Takeda et al., 2004; Wotton and Massague, 2001), and the balance between the two in the promoter regions most likely determines whether SMADs activate or repress the transcription of target genes. TGF-β can also signal through SMAD-independent pathways such as p38 MAPK (Bhowmick et al., 2001; Yu et al., 2002). While it is known that TGF-β suppresses the induction of IFN-γ expression (Bellone et al., 1995; Bright and Sriram, 1998; Espevik et al., 1987; Pardoux et al., 1999; Sudarshan et al., 1999) and that this is accomplished at least in part via inhibition of T-bet (Gorelik et al., 2002; Lin et al., 2005; Neurath et al., 2002), it is unknown if this critical negative regulation is dependent or independent of the SMAD transcription factor pathway or if this suppression can occur in a T-bet-independent fashion.

The interaction of different signaling pathways is important to provide an integrated response to the cell's total signal input. This principle would likely apply to the monocyte-NK cell innate effector loop after activation by gram-negative organisms or obligate intracellular pathogens where both pro- and antiinflammatory monokines are released to modulate NK IFN-γ production (Reed, 1999). In this study, we investigated the interaction between pro- and antiinflammatory cytokine signaling pathways, focusing on their physiological role in regulating IFN-γ production by human NK cells. We demonstrate that proinflammatory monokines IL-12, IL-15, IL-18, or their combinations are able to antagonize the TGF-β signaling pathway in human NK cells by downregulating TGF-βRII, SMAD2, and SMAD3. On the other hand, we found that TGF-β signaling represses monokine-induced IFN-γ gene expression and T-BET expression via SMAD-dependent signaling.

Results

Proinflammatory Cytokines Antagonize Antiinflammatory Cytokine TGF-β Signaling

Monocyte-derived proinflammatory cytokines such as IL-12, IL-15, IL-18, and their combinations are strong stimulators of IFN-γ production in NK cells through their activation of transcription factors including STATs, AP-1, T-BET, and NFκB (Strengell et al., 2002; Townsend et al., 2004). NK cells constitutively produce TGF-$β_1$ and can secrete nanogram per milliliter amounts of the latent form and significant amounts of the active form (Bellone et al., 1995; Gray et al., 1998; Horwitz et al., 1999). We therefore asked if the induction of IFN-γ gene expression by these monokines might also simultaneously benefit from an antagonism of the antiinflammatory cytokine TGF-β signaling that inhibits IFN-γ gene expression.

Our microarray data indicated that the costimulation of human NK-92 cells by IL-12 and IL-18 decreased the mRNA level of *TGF-βRII* over time (data not shown), a result we confirmed by real-time RT-PCR and Western blot analysis (Figure 1A, top, and see Figure S1, top, in the Supplemental Data available with this article online). Similar results were obtained in primary human NK cells when they were purified to ≥99% and costimulated with IL-12 and IL-15 or IL-18 (Figure 1A, middle). We also treated NK-92 cells with IL-12, IL-15, and IL-18 alone and in combination and noted by real-time RT-PCR that each of these treatments significantly suppressed *TGF-βRII* mRNA expression (Figure 1A, bottom).

We next assessed the TGF-βRII signaling molecule SMAD2, an R-SMAD. Results from our microarray and semiquantitative RT-PCR analysis showed that costimulation of NK-92 cells with IL-12 and IL-18 downregulated *SMAD2* transcript (data not shown). Western blotting demonstrated that this downregulation also occurred over time at the protein level (Figure 1B, top). The inhibition of SMAD2 by the costimulation of IL-12 and IL-18 also occurred in primary NK cells (Figure 1B, middle). Interestingly, the treatment of NK-92 cells with IL-12, IL-15, and IL-18 alone and their combinations revealed that only the most potent stimulus of IFN-γ among these proinflammatory cytokine treatments, IL-12 plus IL-18 (Fehniger et al., 1999), and to a much lesser degree, IL-15 plus IL-18, downregulated SMAD2 expression (Figure 1B, bottom, and Figure S1, bottom).

Similar experiments were performed for SMAD3, but results were distinct from SMAD2 and are summarized in Figure 1C. A Western blot showed inhibition of SMAD3 protein expression over time following monokine costimulation in NK-92 (Figure 1C, top), and this decrease was confirmed in primary NK cells (Figure 1C, middle). Further assessment showed that IL-12 or IL-15 alone, and the costimulation of IL-12 and IL-15 or IL-18 but not IL-18 alone, inhibited the protein expression of SMAD3 in NK-92. Monokine costimulation that normally shows synergy in the induction of IFN-γ gene expression (Cooper et al., 2001; Fehniger et al., 1999) also showed synergy of SMAD3 inhibition in comparison to individual cytokines (Figure 1C, bottom). The lack of SMAD3 inhibition by IL-18 alone is likely due to the fact that the surface density expression of the IL-18R in NK-92 cells is weak in the absence of IL-12 stimulation

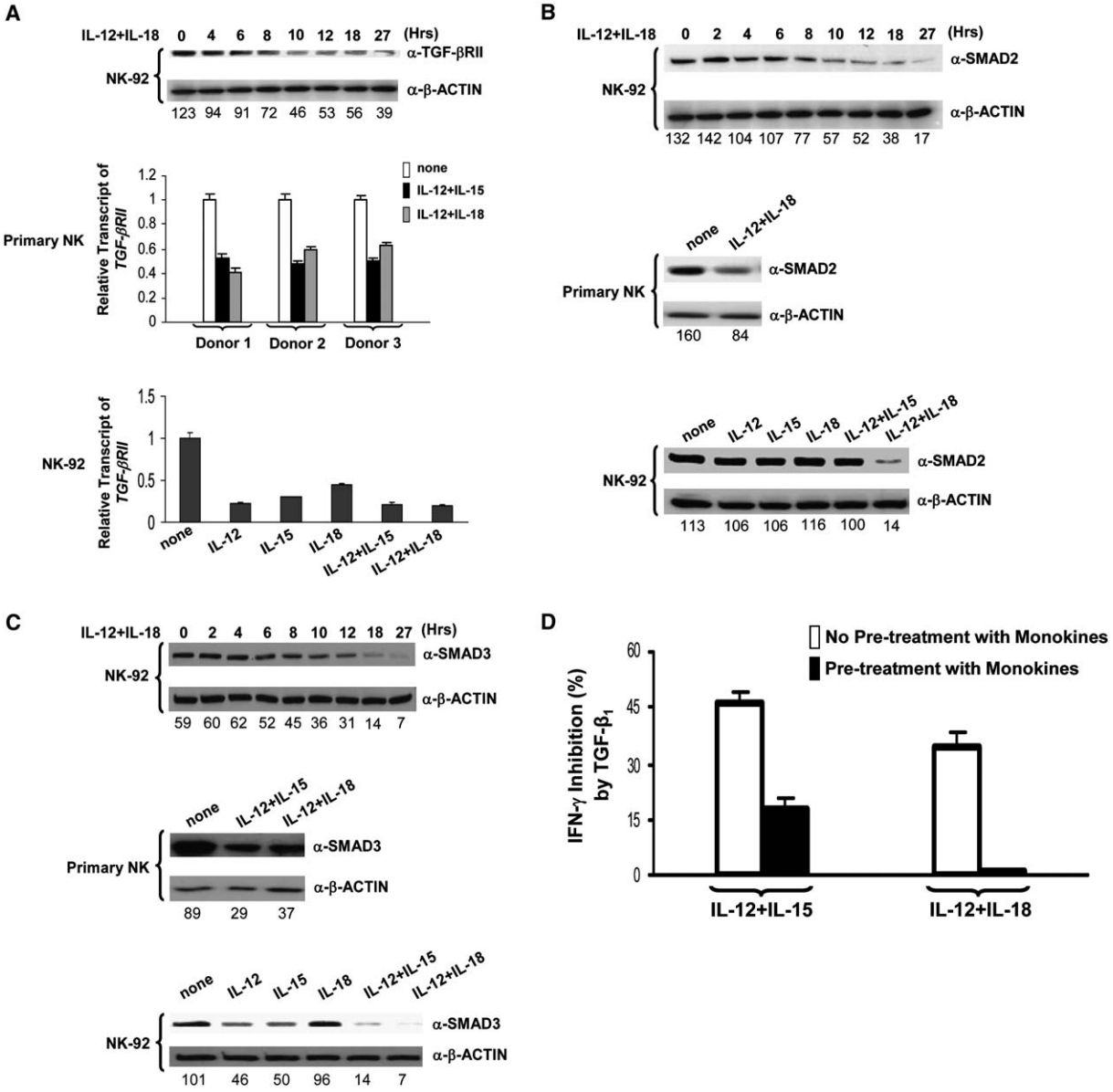

Figure 1. Proinflammatory Cytokines Antagonize Antiinflammatory Cytokine TGF-β Signaling

(A) Top: Protein quantification of TGF-βRII in NK-92 cells by Western blotting. Numbers beneath each lane represent quantification of TGF-βRII by densitometry, normalized for equivalent loading as determined by β-actin. Middle: Transcript quantification of *TGF-βRII* in primary NK cells by real-time RT-PCR relative to the level of the untreated samples that were normalized to 1 ($p < 0.01$, $n = 7$). Results from three representative donors are shown. Bottom: Transcript quantification of *TGF-βRII* as noted above except that NK-92 cells were treated as noted on x axis for 24 hr. Each condition showed a significantly lower *TGF-βRII* transcript level compared to the untreated sample that was normalized to 1 ($p < 0.01$, $n = 3$).
(B) Protein quantification of SMAD2 by Western blot. Cells and culture conditions are indicated in the figure and Experimental Procedures.
(C) Protein quantification of SMAD3 by Western blot. Cells and culture conditions are indicated in the figure and Experimental Procedures.
(D) Monokine activation reduces NK cell sensitivity to TGF-β_1 signaling. NK-92 cells were pretreated with or without monokines for 20 hr, and then restimulated with the same monokines in the presence or absence of TGF-β_1 (10 ng/ml) for 6 hr. The inhibition of IFN-γ secretion reflects the NK cell's sensitivity to TGF-β_1 and was less in the NK cells that received pretreatment with monokines ($p < 0.01$, $n = 3$). Error bars in (A) and (D) indicate SD for triplicates in one representative experiment.

(Sareneva et al., 2000). Consistent with this, we found that IL-18 inhibited *TGF-βRII* expression to a lesser degree than other proinflammatory cytokines or their combinations (Figure 1A, bottom). Microarray and semiquantitative RT-PCR indicated that the SMAD3 inhibition also occurred at the mRNA level (data not shown).

Interestingly, our microarray and/or Western data showed that the proinflammatory cytokines we used did not antagonize SMAD4 or TGF-βRI (data not shown). To prove that the downregulation of TGF-β signaling components by monokines is physiologically relevant, we demonstrated that NK-92 cells pretreated with the combination of IL-12 and IL-15 or IL-18 showed less sensitivity to inhibition of IFN-γ production by TGF-β_1 (Figure 1D). We also found that IL-2 showed effects equal to those of IL-15 in downregulating TGF-β signaling (Figure S2).

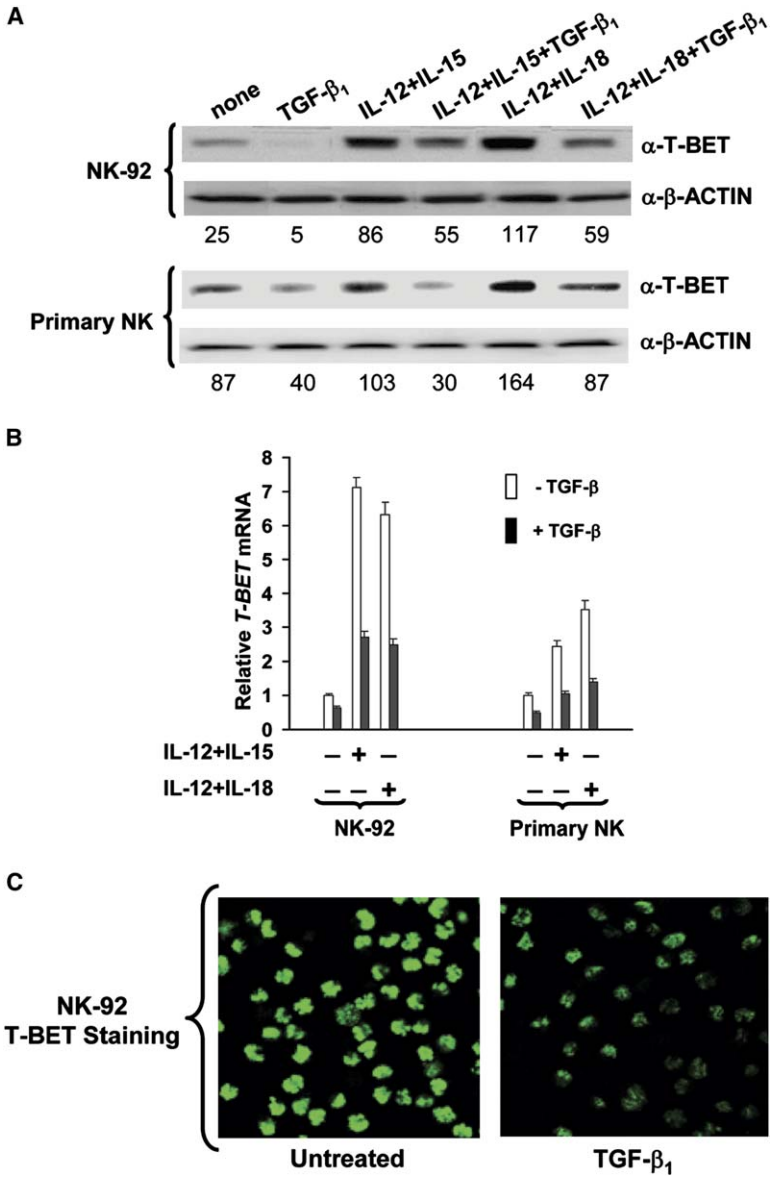

Figure 2. Regulation of Human NK Cell T-BET Expression by Proinflammatory Monokines and Antiinflammatory TGF-β_1

(A) Protein quantification of T-BET expression via Western blotting. Numbers below each lane indicate quantification of T-BET protein under each condition by densitometry, normalized for equivalent loading as determined by β-actin.

(B) Quantification of *T-BET* mRNA by real-time RT-PCR after 6 hr of culture ($p < 0.01$, $n = 3$). Error bars indicate SD for triplicates in one representative experiment.

(C) NK-92 cells were treated with or without TGF-β_1 for 24 hr and then stained by immunocytochemistry with an anti-T-BET antibody. These data are representative of at least three experiments.

The Antiinflammatory Cytokine TGF-β Antagonizes the Induction of T-BET by Proinflammatory Cytokines in Human NK Cells

T-bet has been shown to be a master positive regulator of IFN-γ production in murine $CD4^+$ T cells, NK cells, and $CD8^+$ T cells (Sullivan et al., 2003; Szabo et al., 2002), but the role of its human homolog, T-BET, in the regulation of IFN-γ in NK cells has not been reported. We first determined that T-BET is a positive regulator of human NK cell IFN-γ production as well. Dominant-negative *T-BET* was overexpressed in the human NK cell line NK-92, which led to significantly decreased IFN-γ production after costimulation by IL-12 and IL-15 or IL-18 (Figure S3). Further, we found that costimulation with IL-12 and IL-15 or IL-18 consistently induced T-BET protein and transcript in NK-92 and in primary human NK cells (Figures 2A and 2B), consistent with previous data from the murine system (Gorelik et al., 2002; Neurath et al., 2002; Szabo et al., 2002; Townsend et al., 2004). In each instance, the T-BET protein levels directly correlated with the levels of IFN-γ secretion (data not shown).

TGF-β_1 has previously been shown to downregulate T-bet in murine T cells (Gorelik et al., 2002; Lin et al., 2005; Neurath et al., 2002). We found that during the above same set of experiments, the addition of TGF-β_1 significantly reduced T-BET protein expression in both resting and activated NK-92 cells and primary human NK cells compared to vehicle-treated controls (Figure 2A). This TGF-β-mediated downregulation of T-BET was further demonstrated by immunochemical staining of individual NK-92 cells (Figure 2C) and of primary NK cells (data not shown). Among the nine normal donors that we examined, the inhibition of T-BET protein by TGF-β_1 was significant but variable: in resting NK cells, it was seen in seven of nine donors, and in activated NK cells in six of nine donors, a result that may in part be due to a *T-BET* polymorphism (Akahoshi et al., 2005). To test whether or not the inhibition of T-BET

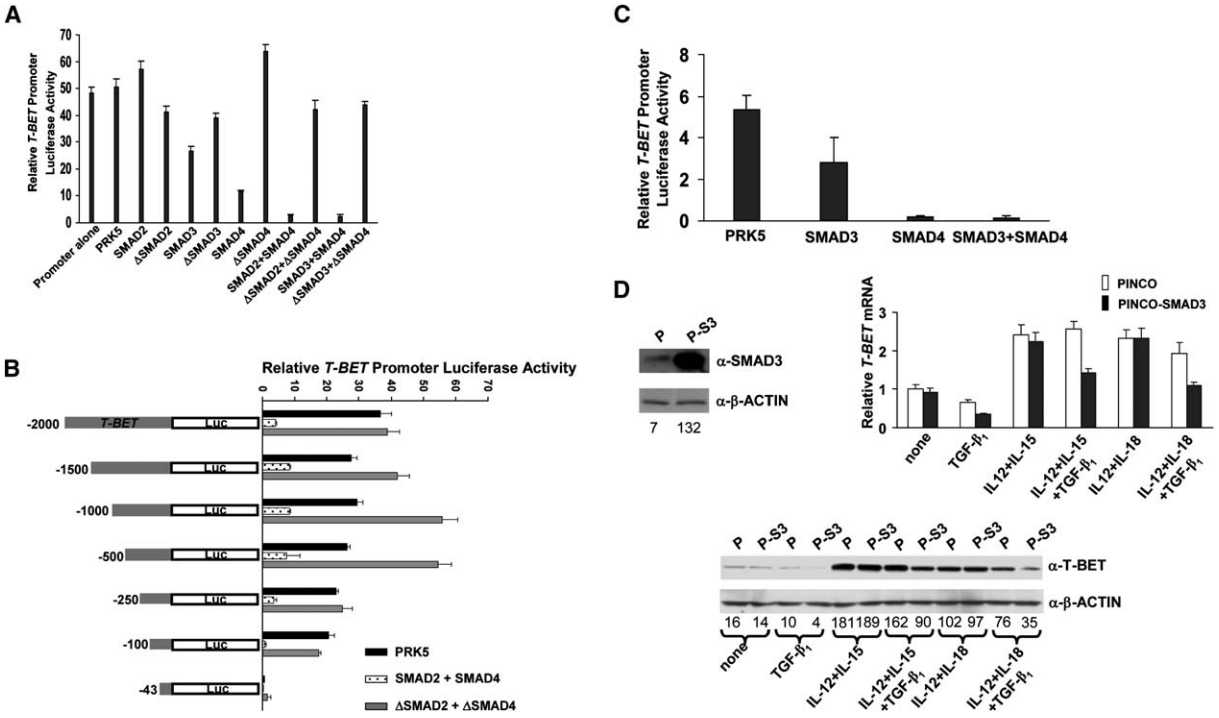

Figure 3. Antagonism of T-BET by TGF-β Is Mediated by SMAD Proteins

(A) 293T cells were transfected with the 2 kb *T-BET-Luc* reporter construct together with various SMAD expression plasmids and their deletion mutants (Δ) or the empty vector (PRK5) as indicated. Transfection of each SMAD expression plasmid and their combinations except SMAD2 alone resulted in significantly lower expression of luciferase compared to the PRK5 control plasmid transfection ($p < 0.01$, $n = 3$). This effect was lost upon transfection with each paired Δ plasmid.

(B) The 2 kb *T-BET-Luc* promoter construct or its deletions were transfected into 293T cells together with SMAD2 and SMAD4, or ΔSMAD2 and ΔSMAD4, or the empty vector (PRK5), as indicated. Experiments shown here are representative of two performed with similar results.

(C) The −100 bp *T-BET-Luc* promoter construct was transfected into the DERL-7 NK cell line by electroporation together with SMAD3, SMAD4, and their combination, or the empty vector (PRK5) as indicated. Transfection of SMAD3, SMAD4, and their combination resulted in significantly lower expression of luciferase compared to the PRK5 control plasmid transfection ($p < 0.01$, $n = 3$).

(D) NK-92 cells were transduced with PINCO-SMAD3 (P-S3) or PINCO (P) only and enriched to 99% purity by FACS (data not shown). Top left: Verification of SMAD3 overexpression by Western blot. Top right: Quantification of *T-BET* transcript by real-time RT-PCR. Overexpression of SMAD3 resulted in significant inhibition of *T-BET* transcript in both resting and monokine-activated NK-92 cells in the presence of TGF-β_1 (10 ng/ml) ($p < 0.05$, $n = 4$) but not in the absence of TGF-β_1. Bottom: Identical results were obtained for T-BET at the protein level determined by Western blotting. Numbers below each lane indicate quantification of T-BET protein under each condition by densitometry, normalized for equivalent loading as determined by β-actin. Experiments shown here are representative of three performed with similar results. Error bars in (A)–(D) indicate SD for triplicates in one representative experiment.

expression by TGF-β_1 also occurred at the mRNA level, we assayed the *T-BET* transcript after TGF-β_1 treatment by real-time RT-PCR. TGF-β_1 inhibited *T-BET* transcript at baseline and after costimulation by IL-12 and IL-15 or IL-18 in both NK-92 and primary NK cells with the same frequency as seen in our protein assay (Figure 2B). We also assessed the levels of EOMES mRNA, a T-BET-related transcription factor implicated in IFN-γ production by murine $CD8^+$ T cells (Pearce et al., 2003), but did not observe any change upon TGF-β_1 treatment (data not shown).

Antagonism of T-BET by TGF-β Is Mediated by SMAD Proteins

We next investigated the mechanism by which TGF-β_1 inhibits T-BET expression. Since TGF-β signaling can be mediated by both SMAD-dependent and -independent pathways (Bhowmick et al., 2001; Yu et al., 2002), we first tested whether SMAD transcription factors repress T-BET gene expression. To this end, we cloned a 2 kb promoter of human *T-BET* into the pGL3 luciferase reporter vector. The cloned promoter is 2 kb upstream of the potential transcription start site determined by the published mRNA sequence of *T-BET* in NCBI GenBank (accession no. NM_013351) and is referred to as the −2 kb promoter. To ensure that this transcription start site was correct, we used the computer program FirstEF (Davuluri et al., 2001) to query the genomic sequence around the *T-BET* region and found the transcription initiation region identified by the program to be consistent with the published mRNA sequences in GenBank. As shown in Figure 3A, the luciferase reporter assays in 293T cells indicated that transfection with SMAD2 alone, which lacks a DNA binding domain, did not inhibit *T-BET* promoter activity. Further, TGF-β_1 treatment of these SMAD2-transfected cells had no effect, likely due to the very weak intrinsic TGF-β signaling in 293T cells (data not shown and Y. Chen, personal communication). However, transfection of SMAD3 and SMAD4 significantly suppressed *T-BET* promoter activity by 47% and 77%, respectively, in comparison to the empty vector PRK5 (Figure 3A). Cotransfection of SMAD2

and SMAD4 or SMAD3 and SMAD4 resulted in synergistic suppression of *T-BET* promoter activity by 94% and 96%, respectively. In contrast, none of the SMAD deletion constructs significantly inhibited constitutive *T-BET* promoter activity when transfected into 293T cells.

To find the minimum *T-BET* promoter region responsible for inhibition by SMAD proteins, we performed a series of deletions of the 2.0 kb *T-BET* promoter to make −1.5 kb, −1.0 kb, −500 bp, −250 bp, −100 bp, and −43 bp promoter reporter constructs. Luciferase assays indicated that the relative activity of −2.0 kb, −1.5 kb, −1.0 kb, −500 bp, −250 bp, −100 bp, and −43 bp promoters was 37, 28, 29, 26, 23, 21, and 0.8, respectively (Figure 3B). Therefore, the basal activity of the −100 bp *T-BET* promoter was not significantly different from the larger promoters with the exception of the −2.0 kb promoter. Moreover, cotransfection of SMAD2 and SMAD4 dramatically repressed the promoter activity of all six constructs ranging from −2.0 kb to −100 bp, while the activity of the −43 bp *T-BET* promoter was too low to judge the inhibition (Figure 3B). These data suggested that the *T-BET* promoter region responsible for the inhibition by SMAD proteins lies within the −100 bp proximal promoter region, and the region from −43 to −100 may be important for the basal promoter activity.

The luciferase assay was repeated in a human $CD3^{-}CD56^{+}$ NK cell line called DERL-7 (Di Noto et al., 2001), confirming that SMAD3 and SMAD4 alone and in combination were able to repress the activity of the −100 bp *T-BET* promoter (Figure 3C).

To provide further evidence that antagonism of T-BET by antiinflammatory TGF-β signaling is mediated by SMAD proteins, we investigated whether or not overexpression of SMAD proteins in human NK cells was able to suppress T-BET expression. Since we showed in Figure 3A that the overexpression of SMAD3 or SMAD4 repressed T-BET promoter activity in 293T cells, we tried to overexpress SMAD3 and SMAD4 in the human NK-92 cell line by using the GFP-expressing EBV/retroviral PINCO hybrid vector (Becknell et al., 2005; Grignani et al., 1998; Trotta et al., 2005). Immunoblotting indicated substantial overexpression of SMAD3 in the GFP(+) fraction of cells sorted by FACS (Figure 3D, top left); however, the overexpression of SMAD4 was unsuccessful (data not shown). In the presence of TGF-β_1, both resting and monokine-activated NK-92 cells overexpressing SMAD3 had significantly less T-BET transcript and protein than did NK-92 cells overexpressing the vector-GFP control (Figure 3D, top right and bottom). Although overexpression of SMAD3 was also able to dampen T-BET promoter activity in 293T and DERL-7 cells in the absence of TGF-β_1 (Figures 3A and 3C), the overexpression of SMAD3 in NK-92 cells did not suppress T-BET in the absence of TGF-β_1 (Figure 3D). This suggests that for human NK cells, TGF-β_1-mediated suppression of T-BET likely requires more than SMAD3 (e.g., SMAD3 phosphorylation by TGF-βRI and dimerization with SMAD4). This notion is consistent with the observed synergy of T-BET promoter suppression in 293T cells by combination of SMAD3 and SMAD4 (Figure 3A).

To complement these gain-of-function studies on T-BET expression, we performed loss-of-function studies with NK cells derived from $Smad3^{-/-}$ mice ($Smad2^{-/-}$ and $Smad4^{-/-}$ mice are embryonic lethal) (Yang et al., 1999). We analyzed six pairs of $Smad3^{-/-}$ and wt mice and found that NK cells from only three of six $Smad3^{-/-}$ mice showed an increase of T-bet expression over wt mice (data not shown), which may be explained by redundant functions from intact Smad2 and Smad4. These results are consistent with our findings in NK-92 (Figure 3D) and primary human NK cells (n = 6, data not shown) in that overexpression of SMAD3 alone, in the absence of TGF-β_1, did not consistently inhibit expression of T-BET expression after monokine activation.

SMAD Proteins Negatively Regulate IFN-γ Gene Expression in Human and Murine NK Cells

The above results suggest that TGF-β_1-mediated activation of SMAD transcription factors could inhibit IFN-γ production indirectly by targeting *T-BET*, a master positive regulator of *IFNG*. We also tested whether TGF-β_1-mediated activation of SMAD transcription factors could target *IFNG* in a direct manner that was independent of T-BET. We investigated the effect of SMAD expression plasmids and their deletion mutants on the activity of an *IFNG* promoter reporter construct in 293T cells due to their extremely low T-BET expression (data not shown). The construct ranges from −3600 to +70 and also includes 680 bp of intron 1 downstream of luciferase (Figure 4A, top). As can be seen at the bottom of Figure 4A, SMAD3 alone and SMAD4 alone significantly inhibit the activity of the *IFNG* promoter in 293T cells. SMAD2 alone again did not show inhibition, likely due to the lack of a DNA binding domain, yet the cotransfection of SMAD2 and SMAD4 demonstrated synergy in suppressing the activity of the *IFNG* promoter. The SMAD deletion mutants reversed the suppression. To determine which region of the *IFNG* promoter is most responsible for the inhibition of activity by SMAD proteins, we performed a promoter mapping analysis. The results suggested that the most active inhibitory site is likely within the −204 proximal promoter region (Figure 4B). Collectively, these results suggested that TGF-β signaling could target *IFNG* gene expression in a SMAD-dependent fashion that is independent of T-BET.

Interestingly, SMAD proteins also inhibit the transactivation of the *IFNG* promoter by T-BET. As shown in Figure 4C, the introduction of T-BET induced an 8- to 10-fold increase in the promoter activity of *IFNG*, yet overexpression of SMAD3 alone or SMAD4 alone but not SMAD2 was able to significantly inhibit this induction. Additionally, SMAD2 and SMAD4 or SMAD3 and SMAD4 produced synergistic inhibition, similar to the inhibition of basal *T-BET* and *IFNG* promoter activity (Figures 3A and 4A).

The luciferase assay was repeated in the DERL-7 human NK cell line, confirming that SMAD3 and SMAD4 alone and in combination were able to repress the activity of the −204 bp *IFNG* promoter (Figure 4D).

To demonstrate that the regulation of both *T-BET* and *IFNG* genes by SMAD-mediated TGF-β signaling is physiologically important in modulating IFN-γ levels in human NK cells, we investigated whether overexpressed SMAD3 in primary human NK cells and in the NK-92 cell line was able to inhibit IFN-γ production. Primary NK

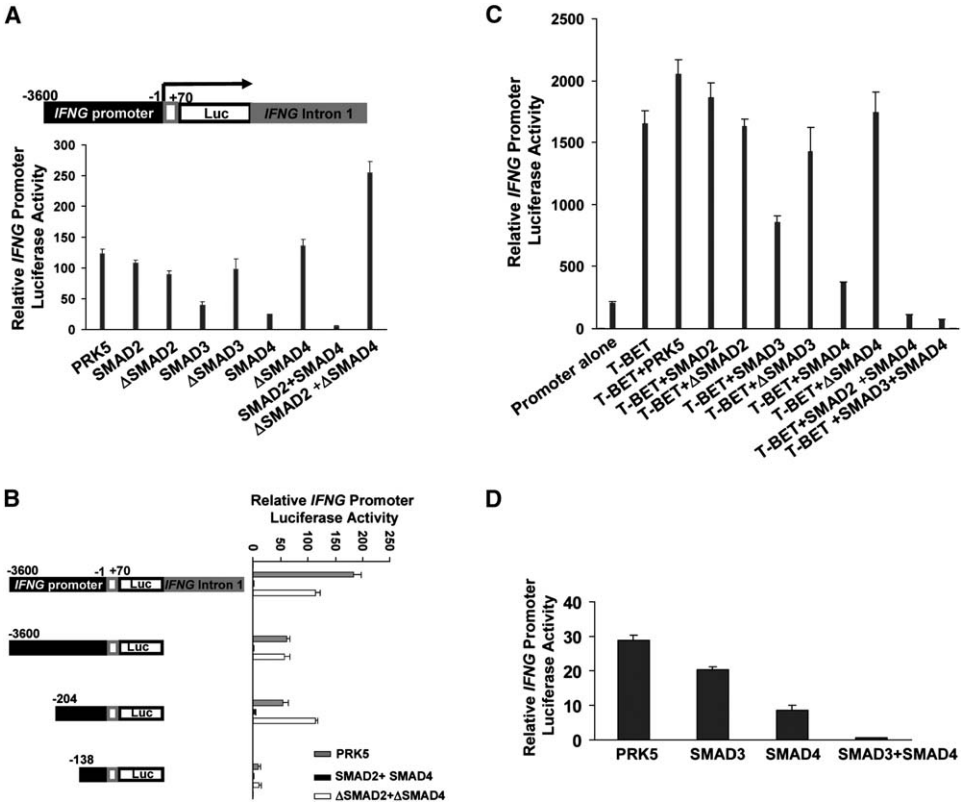

Figure 4. SMAD Proteins Inhibit *IFNG* Promoter Activity in the −204 bp Proximal Region in Both the 293T Cell Line and the DERL-7 NK Cell Line and Inhibit Transactivation by T-BET

(A) 293T cells were transfected with the 3.6 kb *IFNG-Luc* reporter construct with intron 1 and either various SMAD expression plasmids, the respective deletion mutants (Δ), or the empty vector (PRK5), as indicated. Transfection of each SMAD expression plasmid and their combinations except SMAD2 alone resulted in significantly lower expression of luciferase compared to the PRK5 control plasmid transfection ($p < 0.01$, $n = 3$), which was lost upon transfection with each paired Δ plasmid.

(B) Full-length and portions of the 3.6 kb *T-BET-Luc* promoter were transfected into 293T cells together with the empty vector (PRK5), SMAD2 and SMAD4, or ΔSMAD2 and ΔSMAD4, as indicated. The y axis indicates relative *IFNG* promoter luciferase activity. Experiments shown here are representative of three performed with similar results.

(C) The 3.6 kb *T-BET-Luc* promoter construct with intron 1 was transfected into 293T cells alone or with various plasmids as indicated. Transfection of each SMAD expression plasmid and their combination except SMAD2 alone resulted in significantly lower expression of luciferase compared to the *T-BET* plus PRK5 control plasmid transfection ($p < 0.01$, $n = 3$), which was lost upon transfection with each paired Δ plasmid.

(D) The −204 bp *IFNG-Luc* promoter construct was transfected into the DERL-7 NK cell line by electroporation together with SMAD3, SMAD4, and their combination or the PRK5 control plasmid as indicated. Transfection of SMAD3, SMAD4, and their combination resulted in significantly lower expression of luciferase compared to PRK5 ($p < 0.01$, $n = 3$). Error bars in (A)–(D) indicate SD for triplicates in one representative experiment.

cells were first infected by PINCO-SMAD3 retrovirus or PINCO alone, enriched for purity by FACS (Figure 5A), and confirmed to overexpress SMAD3 (Figure 5B). We showed that PINCO-SMAD3-infected cells produce less IFN-γ when stimulated by IL-12 and IL-18 in the absence or presence of TGF-β_1 compared to controls (Figure 5C). The same results were obtained with NK-92 cells, where we were able to assess both IFN-γ protein and transcript (Figures 5D and 5E). In contrast to the results from assessing T-BET expression in NK-92 cells overexpressing SMAD3 (Figure 3D), monokine-induced IFN-γ expression was repeatedly inhibited in the absence of TGF-β_1, consistent with the notion put forth earlier that IFN-γ may be suppressed via SMADs in a direct manner that is independent of T-BET. The enhanced inhibition in the presence of TGF-β_1 suggests that additional TGF-β1-activated signaling components are required for SMAD3 to achieve optimal inhibition. Indeed, we found that levels of overexpressed SMAD3 increased in the nucleus and decreased in the cytoplasm in a time course that was dependent upon treatment with TGF-β_1 (data not shown).

Finally, we undertook a loss-of-function approach to address the effects of SMAD proteins on IFN-γ gene expression, utilizing NK cells from *Smad3*$^{-/-}$ mice. We analyzed IFN-γ production by using NK cells obtained from seven pairs of *Smad3*$^{-/-}$ and wt mice, and we found that in all instances, monokine-costimulated *Smad3*$^{-/-}$ NK cells showed a significantly greater increase of IFN-γ protein production compared to wt NK cells, in both the absence and presence of TGF-β_1 (Figure 5F). Similar results were quantified at the level of the IFN-γ transcript (data not shown). Likewise, *Smad3*$^{-/-}$ NK cells show less sensitivity to TGFβ_1-mediated inhibition of IFN-γ gene expression when compared to wt NK cells (Figure 5G). These mouse loss-of-function data are consistent with the gain-of-function data in monokine-activated primary human NK cells and NK-92 (Figures 5C–5E) in that overexpression of SMAD3 alone, in both the absence and presence of TGF-β_1, inhibits expression of IFN-γ.

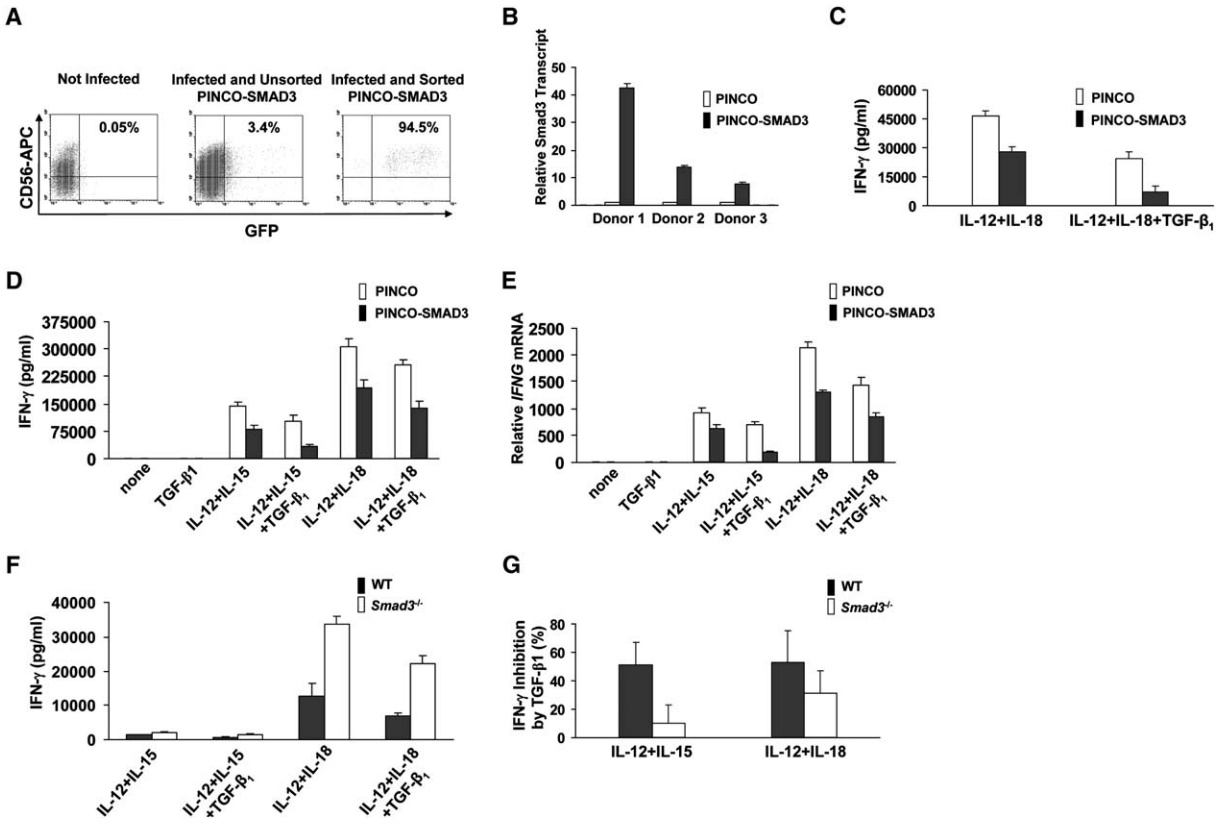

Figure 5. Suppression of IFN-γ in Human NK Cells Overexpressing SMAD3 and Confirmation in *Smad3*$^{-/-}$ Mice

(A) Primary NK cells were retrovirally infected with the PINCO vector or PINCO-SMAD3 and enriched by FACS.

(B) SMAD3 was quantified by real-time RT-PCR in each donor.

(C) The NK cells were costimulated with IL-12 and IL-18 in the presence or absence of TGF-β_1 (10 ng/ml) and assayed for IFN-γ by ELISA. Results are representative of at least three donors and show that overexpression of SMAD3 results in significant inhibition of IFN-γ ($p < 0.05$).

(D and E) NK-92 cells were infected as above, enriched by FACS, and costimulated with IL-12 and IL-15 or IL-18 in the presence or absence of TGF-β_1 (10 ng/ml) for 6 hr and assayed for IFN-γ secretion (D) by ELISA or *IFN*-γ transcript (E) by real-time RT-PCR. All experiments from (D) and (E) use nonstarved cells and show a statistically significant difference between the control and SMAD3-infected cells ($p < 0.01$, $n = 3$).

(F) NK cells were isolated from spleens of *Smad3*$^{-/-}$ and wild-type (wt) mice, costimulated for 24 hr in vitro with rmIL-12 and rmIL-15 or rmIL-18, and assayed for IFN-γ secretion by ELISA. NK cells from *Smad3*$^{-/-}$ mice produce significantly more IFN-γ than NK cells from wt mice ($p < 0.05$, $n = 7$ pairs).

(G) *Smad3*$^{-/-}$ NK cells are significantly less sensitive to TGF-β_1-mediated IFN-γ inhibition compared to wt NK cells ($p < 0.05$, $n = 7$). Error bars in (B)–(F) indicate SD for triplicates in one representative experiment and in (G) represent SE for three independent experiments.

Direct Association of SMAD Proteins on the Human IFNG Promoter in NK Cells

The gain-of-function and loss-of-function studies above suggested that SMAD3 might directly regulate IFN-γ expression, and our earlier work (Figure 4B) suggested that a direct binding site could be located in the −138 to −204 region of the *IFNG* promoter. Examination of the sequence revealed three potential SMAD binding sites (GTCT) in this proximal region, each with a high degree of conservation between human and mouse (Figure 6A). Two of the three binding sites are adjacent to each other. We performed EMSAs and found that a single band formed when using a digoxigenin-labeled oligonucleotide containing the two adjacent SMAD binding sites or the third site with nuclear extract of NK-92 cells overexpressing SMAD3 (Figure 6B and data not shown). The 50X or 125X unlabeled identical oligonucleotide successfully competed with the labeled sequence for SMAD binding, and a mutated oligonucleotide resulted in a substantial decrease of SMAD binding. The addition of anti-SMAD3 antibody to the binding reaction produced a gel supershift. We next performed ChIP assays of both endogenous and overexpressed SMAD3 in NK-92 cells and found that SMAD3 was enriched on the *IFNG* promoter in both cases (Figure 6C and data not shown). Finally, we found that inhibition of IFN-γ by TGF-β_1 or by overexpressed SMAD3 did not require de novo protein synthesis (Figure S4). These data support our hypothesis that SMAD proteins (at least SMAD3) directly bind to and act at the SMAD binding sites located in the −138 to −204 *IFNG* promoter, consistent with our luciferase assay results.

NK Cells from *Smad3*$^{-/-}$ Mice Produce More IFN-γ in the Generalized Shwartzman Reaction than NK Cells from Wild-Type Mice

We previously showed that proinflammatory cytokine IL-15 stimulates the generalized Shwartzman reaction and increases IFN-γ production by NK cells in this process (Fehniger et al., 2000). However, to our knowledge,

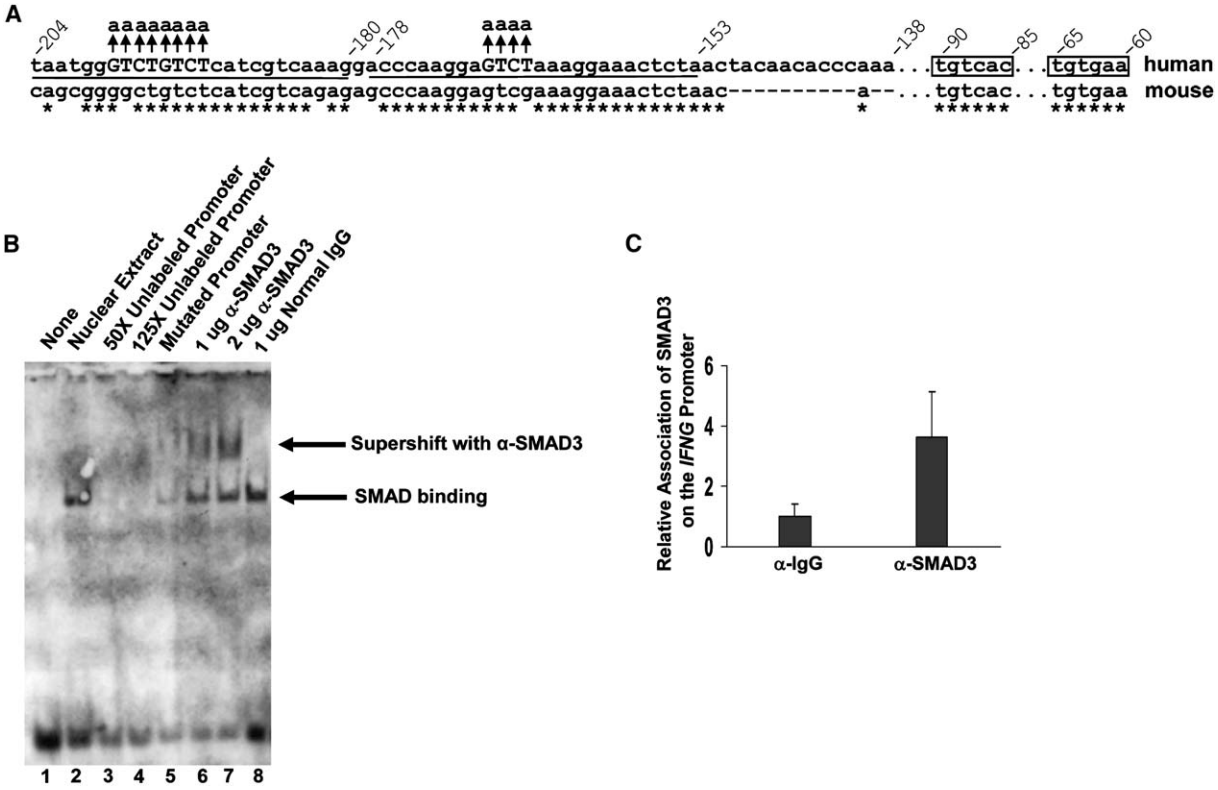

Figure 6. Direct Association of SMAD3 on the −204 *IFNG* Promoter

(A) Partial sequences of the proximal human (top) and mouse (bottom) *IFNG* promoters are shown. Three potential SMAD binding sites (GTCT) are shown as upper case. The two oligos containing SMAD binding sites and their mutants (arrows) used for electrophoretic mobility shift assays (EMSAs) are underlined. Two characterized T-BET binding sites (Cho et al., 2003) are boxed. The numbers indicate the position of nucleotides (transcription start site is referred to as +1) and the stars indicate the sequence homology between mouse and human.

(B) The labeled oligo containing two adjacent SMAD binding sites (lanes 1–4 and 6–8) or its mutant (lane 5) was incubated with (lanes 2–8) or without (lane 1) nuclear extract of NK-92 cells overexpressing SMAD3 in the presence (lanes 3 and 4) or absence (lanes 1, 2, and 5–8) of excess unlabeled competitor oligo. SMAD3 antibody (lanes 6 and 7) or normal IgG antibody (lane 8) was also included in the supershift experiment. The DNA bound SMAD complex and DNA bound SMAD3-α-SMAD3 supershift complex are marked.

(C) Chromatin immunoprecipitation (ChIP) of endogenous SMAD3 in NK cells showed a significant association of SMAD3 on the proximal −204 *IFNG* promoter in comparison to normal IgG ($p < 0.05$, $n = 3$). The amount of the *IFNG* promoter DNA recovered from ChIP was detected by real-time PCR. An equal amount (10 μg) of SMAD3 antibody and normal IgG antibody were used in the experiment. Error bars indicate SE for three independent experiments.

the role of TGF-β signaling, especially the Smad protein cascade, in this lethal in vivo process is unknown. Above, we demonstrated that SMAD3 is an important transcription factor to mediate the antagonism between pro- and antiinflammatory cytokine signaling and to suppress IFN-γ production, a cytokine that is central to the lethal generalized Shwartzman reaction (Heremans et al., 1989). We therefore analyzed the role of Smad3 in the generalized Shwartzman reaction. We found that in vivo, $CD3^{-}NK1.1^{+}$ NK cells from *Smad3*$^{-/-}$ mice produce higher IFN-γ than NK cells from wt mice by 2-fold (Figure 7, $p < 0.01$, $n = 3$). No IFN-γ was detectable by flow cytometry in NK cells from PBS-treated mice or in $CD3^{+}NK1.1^{-}$ T cells from LPS-treated mice (data not shown), suggesting that the regulation of IFN-γ produced by NK cells is critical in the generalized Shwartzman reaction.

Discussion

The opposing actions of pro- and antiinflammatory cytokines are very important for the maintenance of immune system homeostasis, as cytokine imbalance is implicated in the genesis or exacerbation of disease (Antonelli, 1999; Bouma and Strober, 2003; Dunn et al., 2002; Schroder et al., 2004; Shankaran et al., 2001). Proinflammatory cytokines are primarily responsible for initiating an effective defense against pathogens that require a type 1 immune response for clearance (Hill and Sarvetnick, 2002; Yoshimura et al., 2003). However, overproduction of these mediators can be harmful and is implicated in the pathogenesis of inflammatory bowel disease and septic shock (Bouma and Strober, 2003; Dinarello, 1997, 2000; Schroder et al., 2004). Insufficient proinflammatory cytokines can result in immunodeficiency associated with increased risk of infectious disease and cancer (Bouma and Strober, 2003; Shankaran et al., 2001). In contrast, antiinflammatory or type 2 cytokines are critical for elimination of some parasitic infections and for temporizing the proinflammatory process (Hill and Sarvetnick, 2002; Yoshimura et al., 2003). Excessive production of antiinflammatory cytokines may also result in the inappropriate suppression of the immune response (Bone, 1996).

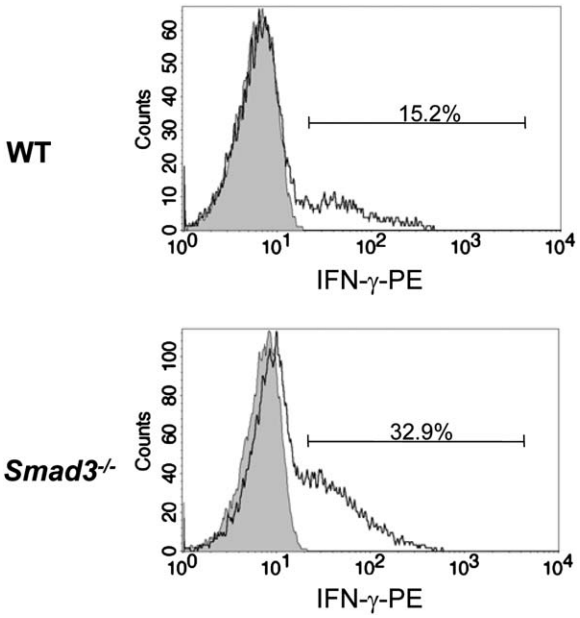

Figure 7. NK Cells from *Smad3*$^{-/-}$ Mice Produce More IFN-γ in the Generalized Shwartzman Reaction than NK Cells from Wild-Type Mice

Splenocytes processed from LPS-primed and -challenged mice were treated with GolgiPlus (2 μl/ml) and LPS (10 μg/ml) for 4–6 hr, followed by surface staining with CD3-FITC and NK1.1-APC antibodies and subsequent intracellular staining with rat anti-mouse IFN-γ-PE or rat IgG1 Isotype-PE antibodies. The NK cell population gated on CD3^{-}NK1.1^{+} was analyzed for IFN-γ production for both IFN-γ-PE staining (solid line, open) and IgG1 isotype control (shaded region). NK cells from *Smad3*$^{-/-}$ mice produced higher IFN-γ than NK cells from wt mice by 2-fold ($p < 0.01$, $n = 3$). Results shown represent one of three pairs of littermate mice.

One example of coordinate regulation between pro- and antiinflammatory cytokines is the monocyte/macrophage-NK cell innate effector loop. In this loop, host invasion by either obligate intracellular pathogens or gram-negative bacteria activates monocyte/macrophages to produce proinflammatory cytokines such as IL-15, -12, and -18, which quickly activate NK cells. The monokine-stimulated NK cells in turn promptly produce IFN-γ (Fehniger et al., 1999; Tripp et al., 1993), the prototypic macrophage-activating cytokine required to effectively contain the pathogen. IFN-γ production by NK cells is a key amplification step within this signaling circuit as it further induces monocyte/macrophage production of IL-15, IL-12, and IL-18 (Hayes et al., 1998; Ma et al., 1996; Trinchieri, 2003). Several aspects of this proinflammatory innate effector loop are poorly understood. It is unknown, for example, if monokine production of IL-10 (Tripp et al., 1993) or monokine-induced NK cell apoptosis (Ross and Caligiuri, 1997) are the only mechanisms preventing uncontrolled amplification of this positive feedback loop resulting in massive cytokine release. It is also unknown if "resting" NK cells actively suppress IFN-γ production prior to its rapid induction via proinflammatory cytokine stimulation.

TGF-β is an excellent antiinflammatory cytokine candidate to mediate suppression of NK cell IFN-γ production (Bellone et al., 1995; Bright and Sriram, 1998; Espevik et al., 1987; Pardoux et al., 1999; Sudarshan et al., 1999). TGF-β_1 is produced by every leukocyte lineage, including NK cells, macrophages, and dendritic cells (Horwitz et al., 1997; Letterio and Roberts, 1998). Furthermore, NK cells appear to be the only lymphocyte population that constitutively produces TGF-β_1, with the ability to secrete nanogram per milliliter amounts of the latent form and significant amounts of the active form (Bellone et al., 1995; Gray et al., 1998; Horwitz et al., 1999). Consistent with this notion, we found that resting human NK cells show constitutive expression of TGF-βRII, SMAD2, SMAD3, and SMAD4. Collectively, we hypothesize that resting NK cells have a dynamic TGF-β-TGF-βR autocrine loop constitutively active in the absence of proinflammatory immune stimulation. Validation of this signaling loop is currently underway.

In this study we investigate the hypothesis that, after activation by proinflammatory monokines, the TGF-β signaling pathway in human NK cells is disabled. We show that IL-12, IL-15, and IL-18 individually and/or in combination are able to antagonize several components of the TGF-β signaling pathway in NK cells at the mRNA and protein levels, including its receptor TGF-βRII and R-SMADs, SMAD2 and SMAD3. While costimulation with IL-12 and either IL-15 or IL-18 shows synergistic inhibition of SMAD3, only the combination of IL-18 and IL-12 or IL-15 results in downregulation of SMAD2. The antagonism of TGF-β signaling by these proinflammatory monokines reveals, to our knowledge, a new mechanism by which IFN-γ production may be maximized during the early innate immune response to infection or malignant transformation. Taken together, our results suggest that proinflammatory monokines stimulate robust IFN-γ production in NK cells by downregulating multiple components of the inhibitory TGF-β signaling pathway, in addition to upregulating transcriptional activators of IFN-γ production such as STAT and T-BET proteins.

It had previously been shown that IFN-γ itself is able to antagonize TGF-β signaling in human monocytes (Ulloa et al., 1999), but through a mechanism that is distinct from what is described here for human NK cells responding to proinflammatory monokines. In the U937 monocytic leukemia cell line, IFN-γ was shown to antagonize TGF-β signaling by a Jak1/STAT-1-mediated induction of SMAD7, an antagonistic SMAD, which in turn prevents the interaction of TGF-βRI with SMAD3 (Ulloa et al., 1999). In a wound model and compared to wt mice, IFN-$\gamma^{-/-}$ mice demonstrated enhanced production of TGF-β1 and phosphorylated SMAD2 and decreased levels of SMAD7 (Ishida et al., 2004). It is not surprising that the mechanism of IFN-γ-induced suppression of the TGF-β signaling pathway is distinct from the pathway we describe here for monokine-induced suppression in NK cells. The human NK cell line NK-92 is only weakly responsive to IFN-γ (H.A. Young, personal communication) and IFN-γ-stimulated NK-92 cells do not increase STAT-1 phosphorylation (data not shown). Indeed, stimulation of NK-92 cells with IL-12, IL-15, or IL-18 and their combinations does not upregulate SMAD7 protein expression (data not shown).

TGF-β exerts a profound influence on dampening NK cell effector function and is likely the best candidate cytokine for returning and maintaining activated NK cells

to their resting state, at least in part by inhibiting IFN-γ production. Although the phenomenon of TGF-β-mediated suppression of IFN-γ production has been widely reported (Bellone et al., 1995; Bright and Sriram, 1998; Espevik et al., 1987; Pardoux et al., 1999; Sudarshan et al., 1999), the mechanisms by which this occurs are not fully elucidated. One mechanism that is clear in murine T cells is the downregulation of T-bet (Gorelik et al., 2002; Lin et al., 2005; Neurath et al., 2002), something we confirm here in human NK cells.

It has also been postulated that TGF-β interferes with early signaling events activated by proinflammatory cytokines, i.e., TGF-β might antagonize the activation of JAKs or STATs. However, there are conflicting reports on this possibility. Two independent groups showed that in human T cells TGF-β_1 inhibits IL-12-induced phosphorylation of JAK2, TYK2, and STAT4 (Bright and Sriram, 1998; Pardoux et al., 1999). In contrast, Sudarshan et al. reported that, in primary T cells and the NK3.3 cell line, TGF-β_1 had no effect on IL-12-induced phosphorylation of JAK2, TYK2, or STAT4, although it did inhibit IL-12-induced IFN-γ production (Sudarshan et al., 1999). There are also conflicting reports for the inhibitory effects of TGF-β on IL-2-induced activation of JAK and STAT proteins (Bright et al., 1997; Han et al., 1997; Sudarshan et al., 1999). These results suggested to us that TGF-β may inhibit NK cell IFN-γ production by alternative mechanisms.

In the current study, we provide new data demonstrating that TGF-β downregulates T-BET, the positive regulator of IFN-γ, via SMAD2, SMAD3, and SMAD4. We also show that these same SMAD proteins can decrease T-BET-mediated transactivation of the *IFNG* promoter to less than basal level (Figure 4C), suggesting that the suppression of IFN-γ expression by the SMAD proteins is able to override the activation by T-BET likely in part via negative regulation of IFN-γ that is independent of SMADs' direct effect on T-BET.

It is unclear how these SMAD transcription factors are able to inhibit T-BET transactivation of IFN-γ. There may be a convergence of these factors at a common location within the *IFNG* promoter. In support of this, we found that there is an enriched region for SMAD and T-BET binding sites at the proximal promoter of *IFNG* from −58 to −217. In this region, there are at least four GTCT/AGAC SMAD binding sites and two previously characterized T-BET binding sites (Cho et al., 2003), suggesting that these transcription factors may interfere with each other at this region. We were able to show the direct binding of the SMAD3 protein to specific sites within the human *IFNG* proximal promoter as well as SMAD-mediated suppression of the *IFNG* promoter activity by luciferase assays in both 293T cells and the DERL-7 NK cell line. Our preliminary data also indicate the direct binding of T-BET to the human *IFNG* proximal promoter (data not shown). By coimmunoprecipitation, we could not detect a significant interaction between SMAD3 and T-BET, suggesting that they bind to this proximal promoter region independently.

We found that negative regulation of IFN-γ by TGF-β can be independent of SMAD-mediated T-BET inhibition. We noted that, in the absence of TGF-β_1, overexpression of SMAD3 in NK-92 cells led to a significant decrease of IFN-γ production after monokine activation

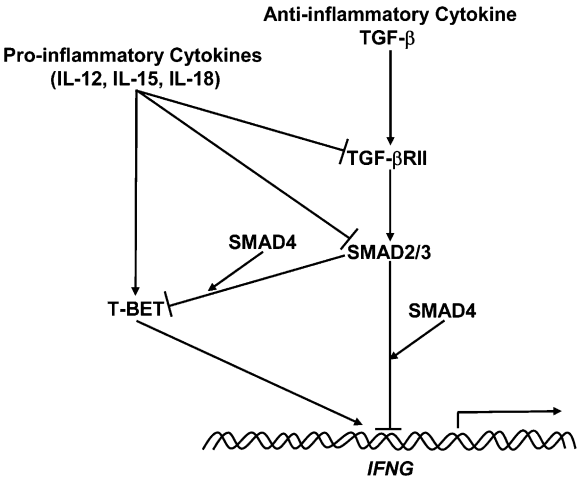

Figure 8. A Model of Reciprocal Antagonism between Proinflammatory and Antiinflammatory Cytokine Signaling Pathways in Regulating IFN-γ Production

The model is suggested by our results presented here and by previously published studies (Gorelik et al., 2002; Neurath et al., 2002; Szabo et al., 2002; Townsend et al., 2004). Proinflammatory cytokines, such as IL-12, IL-15, and IL-18 alone and especially in combination, induce the expression of T-BET, a positive, direct regulator of IFN-γ gene expression. They also inhibit the expression of TGF-β type II receptor (TGF-βRII), SMAD2, and SMAD3. TGF-β signaling inhibits IFN-γ gene expression via SMAD proteins in a manner that is both T-BET dependent and independent.

without a concomitant T-BET decrease (see Figures 3D, 5D, and 5E and data not shown). Additionally, we showed that monokine-stimulated *Smad3*$^{-/-}$ NK cells consistently express more IFN-γ than NK cells from wt mice (Figure 5F), while expression of T-bet under identical conditions was highly variable and showed no significant difference (data not shown). Finally, monokine-stimulated NK-92 cells expressing a dominant-negative T-BET also exhibited significant inhibition of IFN-γ by TGF-β_1 (data not shown). It will be interesting to see if TGF-β_1 is able to downregulate IFN-γ production in T-bet$^{-/-}$ mice. These results suggest that TGF-β may inhibit other transcription factors as well, such as AP1, NF-κB, and STATs. However, our lab and other groups have found that IFN-γ production cannot be completely suppressed by TGF-β, suggesting that cooperation with other cytokine pathways is likely.

Our reproduction of the generalized Shwartzman reaction provides in vivo evidence that the TGF-β signaling mediator Smad3 can suppress IFN-γ production. We found that NK cells of *Smad3*$^{-/-}$ mice produce 2-fold higher IFN-γ than wt mice. This finding may prove useful in either explaining excessive immune activation involving IFN-γ in illnesses such as inflammatory bowel disease or in partially blocking the effects of excess TGF-β in vivo.

Our data also provide the molecular basis for the earlier observations that blocking endogenous TGF-β in fresh human endometrial cell cultures increases the production of IFN-γ by uterine NK cells (Eriksson et al., 2004), and blockade of TGF-β signaling in mice results in the accumulation of a large pool of NK cells secreting copious IFN-γ responsible for T_H1 differentiation and

protection from leishmania infection (Laouar et al., 2005). Thus, we hypothesize that the "resting state" for human NK cells likely consists of active antiinflammatory cytokine signaling that results in suppression of proinflammatory immune activation.

In summary, we provide new mechanistic data demonstrating reciprocal antagonism between pro- and antiinflammatory cytokine signaling pathways operating in human NK cells (Figure 8). We show that monokines IL-12, IL-15, and IL-18 act alone and/or in combination to antagonize TGF-β signaling via downregulation of TGF-βRII and its signaling mediators, SMAD2 and SMAD3. We show that TGF-β utilizes SMAD2, SMAD3, and SMAD4 to antagonize IFN-γ gene expression via suppression of T-BET and also via a direct, T-BET-independent negative regulatory effect on the *IFNG* promoter. Given the role that IFN-γ has in promoting immune editing of tumors (Dunn et al., 2002; Shankaran et al., 2001) and TGF-β's role in promoting late-stage oncogenesis via immune suppression (Bellone et al., 1995; Castriconi et al., 2003; Lee et al., 2004), developing a targeted strategy to modulate SMAD gene expression may add a useful and novel component to the treatment of malignant disease.

Experimental Procedures

Cell Culture and Isolation of Primary NK Cells

The human IL-2-dependent NK cell line NK-92, a generous gift of Dr. Hans G. Klingemann (Rush University Medical Center, Chicago, IL) (Gong et al., 1994), and DERL-7, a kind gift of Drs. Rosella Di Noto (Università Federico II, Naples, Italy) and Francesco Salvatore (The CEINGE Bank of Human and Animal Continuous Cell Lines, Napoli, Italy) (Di Noto et al., 2001), were maintained in culture in RPMI-1640 medium (Invitrogen, Carlbad, CA) containing Glutamax, supplemented with 20% heat-inactivated FBS (Invitrogen), and 150 IU/ml rhIL-2 (Hoffman-LaRoche Inc., Nutley, NJ) at 37°C. The amphotropic-packaging cell line Phoenix (gift from Dr. Garry P. Nolan, Stanford University, CA) was maintained in culture in Dulbecco modified Eagle medium (DMEM) (Invitrogen)/10% FBS medium and grown for 16 to 18 hr to 80% confluence prior to transfection by calcium phosphate-DNA precipitation (Promega, Madison, WI). Human primary NK cells were isolated from peripheral blood leukopacks of healthy donors (American Red Cross, Columbus, OH) by incubation for 30 min with RosetteSep cocktail (StemCell Technologies Inc., Vancouver, Canada), followed by Ficoll-Hypaque density gradient centrifugation, and by plastic adherence overnight to remove the monocyte population. Enriched NK cells were ~80% $CD56^+$, as determined by fluorescence activated cell sorter (FACS) analysis with an anti-CD56 phycoerythrin (PE)-conjugated monoclonal antibody (Immunotech, Marseille Cedex 9, France). The enriched NK cells were directly used for retroviral infection and infected cells were then purified by FACS (see below). For all other experiments, enriched NK cells were further enriched to ≥99% $CD56^+CD3^-$ by cell sorting or by using CD56 MicroBeads and MACS LS Separation Columns (Miltenyi Biotec, Auburn, CA). NK cells from splenocytes of *Smad3*$^{-/-}$ and wild-type (wt) C57BL/6 or A/J strain mice (8 to 15 weeks old) were enriched to 70%–80% by DX5-PE/NK1.1-PE antibodies and PE Microbeads by LS Separation Columns and were further purified to ≥99% DX5/$NK1.1^+CD3^-$ by cell sorting.

Plasmid Construction

The 2.0 kb (−1 to −2000) sequence of the *T-BET* gene promoter was generated by PCR from human genomic DNA, and the promoter sequences of 1.5 kb (−1 to −1500), 1.0 kb (−1 to −1000), 500 bp (−1 to −500), 250 bp (−1 to −250), and 100 bp (−1 to −100) were amplified from a plasmid containing the 2.0 kb fragment. The upstream primers for the application of these sequences were: T-BET-2KbXho, 5′-ACTCGAGAGCCAAGCACTGAGCAAAAATGT-3′; T-BET-1.5KbXho, 5′-ACTCGAGCAGAAATGTAGAGCTGGGGC-3′; T-BET-1.0KbXho, 5′-ACTCGAGATCTCCGAGGCAGCCCTTCA-3′; T-BET-500bpXho, 5′-ACTCGAGGGAGAAAGAGGGCAACCCGA-3′; T-BET-250bpXho 5′-ACTCGAGCTTCAAAGCTGGGCTGAATT-3′; T-BET-100bpXho, 5′-ACTCGAGGGCGGGGTGGGGCGAGGCGG-3′. The downstream primer was T-BETPrtHindIII 5′-TAAAGCTT CTGT CACTAGAGTCGCAGCGCTTT-3′. The amplified promoter fragments were cloned into the PCR2.1 vector (Invitrogen) and the sequences were confirmed by DNA sequencing. The sequenced fragments then were digested from the PCR2.1 vector by *Xho*I and *Hin*dIII and cloned into the same restriction sites of the pGL3 luciferase basic reporter vector (Promega) to generate *T-BET-LUC* reporter constructs. The −43 bp *T-BET* promoter construct was made by ligating the *Xho*I-*Hin*dIII pGL3 fragment with two annealed oligos: -43T-BETXho1Up, 5′-TCGAAGCTTCATAAAGCCACAGCAAAGCGCTGC GACTCTAGTGACAG-3′ and -43T-BETHindDN, 5′-AGCTCTGTCAC TAGAGTCGCAGCGCTTTGCTGTGGCTTTATGAAGCT-3′. A series of *IFNG* promoter constructs with different lengths were kind gifts from Dr. Howard A. Young and Dr. Jay Bream (National Cancer Institute, National Institutes of Health, Frederick, MD) or Dr. Rivkah Gonsky (Cedars-Sinai, Los Angeles, CA).

Dominant-negative T-BET (dnT-BET) is a chimeric fusion of the human T-BET N terminus (amino acids 1–371, accession number NP_037483) with the *Drosophila* Engrailed repression domain (amino acids 2–298, accession number XP_080792). The T-BET portion was obtained by RT-PCR from human peripheral blood mononuclear cell cDNA. The Engrailed portion was obtained by RT-PCR from *Drosophila* embryo cDNA (Clontech, Palo Alto, CA). The chimeric cDNA was cloned in a quaternary ligation reaction consisting of *Bgl*II-*Sph*I T-BET, *Sph*I-*Bsr*BI T-BET, *Sfo*I-*Xho*I Engrailed, and *Bgl*II-*Xho*I pCMV-HA (Clontech). This cDNA was sequenced bidirectionally and subcloned to the *Bam*HI-*Eco*RI sites of PINCO retroviral vector (Becknell et al., 2005; Grignani et al., 1998; Trotta et al., 2005), after blunting the *Xho*I and *Eco*RI sites with Klenow fragment. A C-terminal MYC epitope was subsequently added to permit detection of the dnT-BET protein. Alternatively, as a negative control, the Engrailed repression domain was amplified along with a start codon and a C-terminal MYC tag and ligated into pCR2.1 (Invitrogen). After sequencing, this cDNA was subcloned to the *Bam*HI-*Eco*RI sites of PINCO retroviral vector. The PINCO retroviral vector was kindly provided by Dr. Martin Sattler (Dana-Farber Cancer Institute, Harvard University, Boston, MA).

SMAD2, SMAD3, and SMAD4 expression plasmids on the PRK5 vector, their deletion mutants, and the empty vector PRK5 for cloning SMAD genes have been previously described (Zhang et al., 1996, 1998) and were kind gifts from Dr. Rik Derynck (University of California at San Francisco, San Francisco, CA). We reamplified SMAD3 from the PRK5 backbone by PCR and the amplified fragment with a *Bam*H1 restriction site before the start codon and an *Eco*R1 restriction site after the stop codon was cloned into the PCR2.1 vector (Invitrogen). The sequenced SMAD3 from PCR2.1 was subcloned into *Bam*HI-*Eco*RI sites of PINCO to create PINCO-SMAD3.

Luciferase Reporter Assays

293T cells were seeded into 24-well plates at a concentration of 2.5×10^5 per well and grown overnight. The *T-BET-Luc* or the *IFNG-Luc* construct (1 μg) and various SMAD expression plasmids or the empty vector PRK5 (1 μg each) were cotransfected into 293T cells by Lipofectamine 2000 with Plus Reagent (Invitrogen) according to the manufacturer's protocol. The pGL3 basic reporter vector was used as a control for basal-promoter activity. A renilla-luciferase vector (5 ng), pRL-TK (Promega), was cotransfected to serve as an internal control for transfection efficiency. Cells were harvested after 48 hr of transfection and assessed for luciferase activity by the dual luciferase reporter assay system (Promega). To transfect the DERL-7 NK cell line, the promoter and/or expression constructs (5 μg each) and pRL-TK (50 ng) were electroporated into 4.0×10^6 cells by Cell Line Nucleofector Kit V and an Amaxa electroporator (Amaxa, Inc, Gaithersburg, MD) according to the manufacturer's protocol. Cells were harvested after 20 hr of transfection and assessed for luciferase activity by the dual luciferase reporter assay system. Total amounts of transfected DNA were adjusted to be the same using the empty vector in all assays of each experiment. All assays were done in triplicate and all values were normalized for transfection efficiency

against renilla-luciferase expression directed from the cotransfected pRL-TK plasmid. The activity of the pGL3 basic reporter vector alone (always between 1/10 and 1/5 of experimental values) was subtracted from that of the vector with a promoter, and the mean ± SD of the triplicate values of the difference is shown in the figures.

NK Cell Stimulation and IFN-γ Immunoassay by ELISA

IL-2-dependent NK-92 cells, starved for ~24 hr unless otherwise indicated, or purified human primary NK cells were treated with or without varied cytokines, IL-12, IL-15, IL-18, and TGF-β_1 (R & D Systems, Minneapolis, MN), for 24 hr or the indicated time. Cells were harvested to extract RNA for synthesizing cDNA or to extract proteins for Western blotting. Cell-free supernatants were collected for detecting IFN-γ protein by enzyme-linked immunosorbent assay (ELISA) with commercially available mAb pairs (Endogen Inc., Woburn, MA) according to the manufacturer's protocol. Murine NK cell stimulation and ELISA were performed similarly except that murine monkines and antibodies (R & D Systems) were used. Results are shown as a mean of triplicate wells ± SD. Recombinant human IL-12 (rhIL-12) and murine IL-12 (rmIL-12) were kindly provided by the Genetics Institute Inc. (Cambridge, MA), rhIL-18 and rmIL-18 by BASF Bioresearch Corporation (Worcester, MA), and rhIL-15 and rmIL-15 by Amgen (Thousand Oaks, CA). Unless specified, the concentration used for rhIL-12 was 10 ng/ml, rhIL-15 100 ng/ml, rhIL-18 100 ng/ml, rmIL-12 20 ng/ml, rmIL-15 20 ng/ml, rmIL-18 10 ng/ml, and TGF-β_1 20 ng/ml.

Retroviral Infection of the NK-92 Cell Line and Primary Human NK Cells

For infection, the NK-92 cell line and enriched primary human NK cells were pretreated with 900 IU/ml IL-2 for 1 or 2 days and infected in the presence of 900 IU/ml IL-2. Retroviral infections were performed by utilizing a hybrid EBV-retroviral vector following previously published protocols (Becknell et al., 2005; Grignani et al., 1998; Trotta et al., 2005). In brief, infectious supernatants from PINCO and PINCO-SMAD3 transiently transfected Phoenix cells were collected 48 hr after transfection and used for three cycles of infections. Upon infection, NK-92 cells were sorted (FACS Vantage; BD Biosciences, San Jose, CA) for green fluorescent protein (GFP) expression, and primary NK cells were sorted for GFP and CD56 expression upon staining with an anti-CD56 allophycocyanin (APC)-conjugated monoclonal Ab (Immunotech). GFP^+CD56^+ primary NK cells were used immediately after sorting. Overexpression of SMAD3 was confirmed in NK-92 cells by Western blotting and in primary NK cells by real-time RT-PCR (see below).

Cell Lysis and Immunoblotting

Cells were harvested, washed once with ice-cold PBS, and lysed (10^8 cells/ml lysis buffer) in hypertonic buffer (1% NP-40, 10 mM HEPES [pH 7.5], 0.5 M NaCl, 10% glycerol supplemented with protease and phosphatase inhibitors [1 mM phenylmethylsulfonylfluoride (PMSF), 1 mM Na_3VO_4, 50 mM NaF, 10 mM β-glycerol-phosphate, 1 mM EDTA, and protease inhibitor cocktail tablet (Roche Applied Science, Indianapolis, IN)]). Alternatively, for direct lysis, 3×10^5 cells were washed once with ice-cold PBS and directly lysed in 30 μl of Laemmli buffer. Lysates were electrophoretically separated on 4%–15% gradient SDS-PAGE gels (Bio-Rad Lab, Hercules, CA) and were transferred to nitrocellulose membranes. The membranes were blocked with 5% milk in TBS-0.1% Tween 20. The primary antibody was usually diluted 1:1000 with 5% milk in TBS-0.1% Tween 20 and was added to react with the membrane overnight. The membrane was then washed three times in TBS-0.1% Tween 20. The HRP bound secondary antibody (Amersham Corp., Arlington Heights, IL) was diluted 1:5000 with 5% milk in TBS-0.1% Tween 20 and was added to the membrane to stand for 1 hr. The membrane was again washed four times in TBS-0.1% Tween 20 and an enhanced chemiluminescence (ECL; Amersham Corp.) was added for 1 min. The blot was then exposed to film for various lengths of time. The β-actin Western was included as a control to demonstrate equal loading.

Antibodies used were: mouse monoclonal anti-T-BET (Santa Cruz Biotechnology, Santa Cruz, CA); rabbit polyclonal anti-SMAD2 (Cell Signaling Technology, Beverly, MA); rabbit polyclonal anti-human TGF-β type II receptor (Upstate Biotechnology, Lake Placid, NY); rabbit polyclonal anti-SMAD3 (Zymed Laboratory Inc, South San Francisco, CA); mouse Myc-tag monoclonal antibody (Cell Signaling Technology), and goat polyclonal anti-β-actin (Santa Cruz Biotechnology).

Immunocytochemistry of T-BET

NK-92 cells or primary human NK cells were treated with or without TGF-β_1 for 24 hr and fixed with 4% paraformaldehyde for 20 min. Slides were prepared by depositing 3×10^5 cells onto glass slides with the use of a Cytospin 3 centrifuge (Thermo Shandon, Pittsburgh, PA). Cells were permeabilized for 5 min at room temperature with 0.2% Triton X-100, and then blocked with blocking buffer (10% normal goat serum in PBS) for 1 hr at room temperature before incubating overnight at 4ºC with anti-mouse T-BET (Santa Cruz Biotechnology) diluted 1:8 in blocking buffer. After washing two times in PBS, the slides were stained with AlexFlour 488-goat-anti-mouse IgG (Molecular Probes, Eugene, OR) for 1.5 hr, washed, and mounted with Vectshield Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA). In a parallel control experiment, it was observed that omission of the primary antibody eliminated staining. Fluorescence, corresponding to presence of anti-T-BET, was detected by means of a Zeiss LSM 510 microscope equipped with Sapphire multiphoton and Argon/HeNe lasers.

Real-Time RT-PCR

Total RNA from NK-92 cells or primary NK cells was isolated with RNeasy kit (Qiagene, Valencia, CA). cDNA was synthesized from 1–3 μg total RNA with random hexamers (Invitrogen). Real-time RT-PCR reactions for the human IFN-γ transcript were performed as a multiplex reaction with the primer/probe set specific for the IFN-γ cDNA (forward primer, 5′-GAAAAGCTGACTAATTATTCGGTAACT G-3′; reverse primer, 5′-GTTCAGCCATCACTTGGATGAG-3′; probe, 5′FAM-CTTGAATGTCCAACGCAAAGCAATACATGA-3′TAMRA) and an internal control (18S rRNA, PE Applied Biosystems, Foster City, CA). The reactions for the human *TGF-βRII* transcript (forward primer, 5′-TGTCTGTGGATGACCTGGCTAA-3′; reverse primer, 5′-TTCTAGGACTTCTGGAGCCATGT-3′; probe, 5′FAM-AGTGGGCAG GTGGGAACTGCAAGA-3′TAMRA), *SMAD2* transcript (forward primer, 5′-CGAAATGCCACGGTAGAAAT-3′; reverse primer, 5′-GGCACTCAGCAAAAACTTCC-3′; probe, 5′FAM-AGGAAGAGGAGT GCGCTTAT-3′TAMRA), and *SMAD3* transcript (forward primer, 5′-CCGAATCCGATGTCCCC-3′; reverse primer, 5′-AAGGCCGGCTCG CAGT-3′; probe, 5′FAM-CACATAATAACTTGGACCTGCAGCCAGT TACC-3′TAMRA) were performed similarly. Alternatively, reactions for the human *T-BET* transcript (forward primer, 5′-CAACAATGT GACCCAGATGAT-3′; reverse primer, 5′-AATCTCGGCATTCTGGT AGG-3′; probe, 5′FAM-CCGGCTGCATATCGTTGAGGTGAAC-3′TA MRA) were performed separately from the 18S control. cDNA from phytohemagglutinin (PHA)-activated human lymphocytes served as positive controls and water without a cDNA template was used as a negative control. Conditions for real-time RT-PCR were: stage 1, 50ºC 2 min; stage 2, 95ºC 10 min; stage 3, 95ºC 15 s, 60ºC 1 min with 40 cycles. Real-time RT-PCR reactions were performed with an ABI prism 7700 sequence detector (Taqman; PE Applied Biosystems), and data were analyzed with the Sequence Detector version 1.6 software to establish the PCR cycle at which the fluorescence exceeded a set threshold, C_T, for each sample. Data were analyzed according to the comparative CT method, as previously described (Fehniger et al., 1999), using the internal control (18S) transcript levels to normalize differences in sample loading and preparation. Results represent the n-fold difference of transcript levels between different samples and are expressed as the mean ± SD of triplicate reaction wells.

Electrophoretic Mobility Shift Assays

Electrophoretic mobility shift assays (EMSAs) were performed with the Dig Gel Shift Kit, 2nd Generation (Roche Applied Science) according to the manufacturer's protocol. The digoxigenin-labeled oligos, the unlabeled competitor oligos, and the mutant oligos corresponded to two different putative SMAD3 binding sites within the proximal *IFNG* promoter (−138 to −204). Where indicated, a 50-fold or a 125-fold molar excess of unlabeled competitor oligo DNA was used in competition experiments. For antibody supershift, rabbit anti-SMAD3 antibody (Zymed Laboratory Inc.) or normal rabbit IgG antibody (as a control, Upstate Biotechnology) was added to

the reaction mixture. DNA-protein complexes were separated in a 5% polyacrylamide gel in 1× TBE buffer and were exposed to film.

Chromatin Immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed with the ChIP Assay Kit (Upstate Biotechnology) according to the manufacturer's protocol. An equal amount (10 μg) of rabbit polyclonal anti-SMAD2/3 (Santa Cruz Biotechnology) and normal rabbit anti-IgG (Upstate Biotechnology) was used to precipitate a cross-linked DNA/protein complex. The *IFNG* primer set and the probe used to detect the amount of precipitated DNA from the −138 to −204 region were described previously (Messi et al., 2003).

IFN-γ Production in the Generalized Shwartzman Reaction

Sex-matched littermates of *Smad3*$^{-/-}$ and wt C57BL/6 mice (18 to 20 weeks old) were subcutaneously injected with 40 μg lipopolysaccharide (LPS, Sigma, St. Louis, MO) per mouse for priming and after 24 hr were i.v. challenged with 400 μg LPS. After one of the paired littermates died (usually within 20 hr), another littermate was sacrificed to ensure equal time periods of the LPS treatment. Single-cell-suspension splenocytes processed from spleens were cultured in the presence of GolgiPlus (BD Biosciences, 2 μl/ml) and LPS (10 μg/ml) for 4–6 hr to allow IFN-γ protein to accumulate in the golgi, harvested, and stained with CD3-FITC and NK1.1-APC (BD Biosciences) by surface staining and with rat anti-mouse IFN-γ-PE and rat IgG1 Isotype-PE (BD Biosciences) by intracellular staining. The IFN-γ production by CD3^{-}NK1.1^{+} NK cells was determined by flow cytometry.

Mice

Smad3$^{-/-}$ and wt C57BL/6 or A/J mice were used for comparing IFN-γ or T-BET expression after monokine stimulation in vitro or after induction of the generalized Shwartzman reaction in vivo. All animal work was approved by The Ohio State University Animal Care and Use Committee, and mice were treated in accordance with the institutional guidelines for animal care.

Microarray Analysis

NK-92 cells were cultured in the medium without IL-2 for 24 hr. The starved cells were costimulated with IL-2 (150 U/ml), IL-12 (10 ng/ml), and IL-18 (20 ng/ml) for 0 hr, 1 hr, 2 hr, 4 hr, 6 hr, 10 hr, and 12 hr, respectively. The cells were harvested and total RNA was isolated with TriZOL Reagent (Invitrogen) and the RNeasy kit (Qiagene). Preparation of cRNA, hybridization, and scanning of the human genes U95A arrays were performed according to the manufacturer's protocol (Affymetrix, Santa Clara, CA). The chips of the Human Genome U95Av2 Array were used and they represent approximately 10,000 full-length genes.

Statistics

Data were compared by Student's two-tailed t test. p less than 0.05 was considered statistically significant.

Supplemental Data

Supplemental Data include four figures and can be found with this article online at http://www.immunity.com/cgi/content/full/24/5/575/DC1/.

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