

Transforming growth factor- β controls T helper type 1 cell development through regulation of natural killer cell interferon- γ

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Interferon- γ and interleukin 12 produced by the innate arm of the immune system are important regulators of T helper type 1 (T_H1) cell development, but signals that negatively regulate their expression remain controversial. Here we show that transforming growth factor- β (TGF- β) controlled T_H1 differentiation through the regulation of interferon- γ produced by natural killer (NK) cells. Blockade of TGF- β signaling in NK cells caused the accumulation of a large pool of NK cells secreting copious interferon- γ , responsible for T_H1 differentiation and protection from leishmania infection. In contrast, blockade of TGF- β signaling in dendritic cells did not affect dendritic cell homeostasis or interleukin 12 production, thus indicating a previously undescribed demarcation of the function of TGF- β in NK cells versus dendritic cells.

Interferon- γ (IFN- γ) secreted by the innate immune cells (called 'innate IFN- γ ') during the early phase of infection is a determining factor for efficient responses to pathogens. Direct evidence of the importance of IFN- γ has come from defects in IFN- γ or IFN- γ receptor expression, which cause profound deficiency in responses to intracellular pathogens^{1,2}. The ability of natural killer (NK) cells to rapidly and efficiently produce IFN- γ is recognized as an important innate mechanism of resistance to many pathogens³. Once stimulated by dendritic cell (DC)-derived interleukin 12 (IL-12), NK cells produce IFN- γ , which in turn activates a variety of antimicrobial responses designed to limit pathogen growth⁴⁻⁶. This pathway is important for resistance to many bacterial and parasitic pathogens, including *Listeria monocytogenes*⁷, *Trypanosoma gondii*⁸ and *Leishmania major*⁹. IFN- γ produced by the innate immune system is essential for the development of subsequent adaptive immune responses. Specifically, NK cell production of IFN- γ is a decisive factor in T helper type 1 (T_H1) development that is essential for the long-term control of many of these pathogens¹⁰⁻¹².

NK cells are endowed with two features to ensure a quick and efficient innate defense. The first involves the expression of multiple activation receptors by individual NK cells, thereby allowing an individual NK cell to respond to multiple activating receptor ligands¹³. The second is related to their constitutive expression of cytokine receptors that permits many NK cells to be stimulated by proinflammatory cytokines produced early in the course of an immune response¹⁴. NK cells are quiescent cells that undergo low proliferation, with only 1-3% of splenic NK cells dividing in naive mice¹⁵. After pathogen challenge, they rapidly proliferate in an initial nonspecific

phase resembling the 'bystander' proliferation seen in T cells. This nonspecific phase is followed by a specific phase of proliferation of NK cells expressing the activation receptor that recognize infected cells in a way somewhat analogous to T cell clonal expansion. Finally, NK cell function is terminated by a resolution phase during which NK cell numbers contract and rapidly return to steady state in a way similar to the resolution of an adaptive immune response¹⁶.

TGF- β is arguably one of the most potent immunosuppressive cytokines. It is produced by almost all cells, including leukocytes, in which its expression controls differentiation and proliferation of innate and adaptive immune cells. Precise understanding of the mechanisms and outcomes of the effects of TGF- β on NK cell activity, however, is lacking¹⁷⁻¹⁹. Conceivably, one mechanism would be that TGF- β exerts direct effects on NK cells to suppress the production of IFN- γ necessary for T_H1 development. Alternatively, an indirect effect through inhibition of IL-12 production by DCs may act to shut off NK cell function. To address this, we used our initial finding that NK cells and DCs share the expression of CD11c to design a transgenic mouse model that expresses a dominant negative form of TGF- β receptor II under control of the CD11c promoter (CD11c-dnTGF β RII) in both NK cell and DC compartments. Whereas blockade of TGF- β signaling in DCs did not affect DC homeostasis or DC production of IL-12, blockade of TGF- β signaling in NK cells caused the generation of large numbers of NK cells capable of producing large amounts of IFN- γ responsible for T_H1 development and protection from *L. major* infection. Our results suggest a previously undescribed demarcation of the function of TGF- β in NK cells versus DCs.

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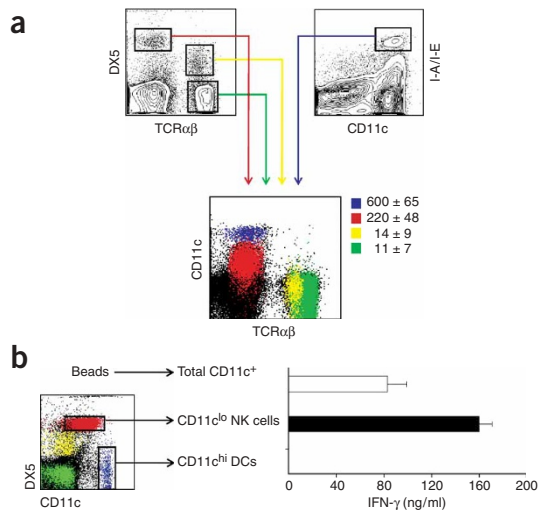


Figure 1 Phenotypic and functional characterization of NK cell and DC subsets. **(a)** Phenotypic characterization of NK cells and DCs. Spleen cells from 8-week-old C57BL/6 mice were analyzed by four-color flow cytometry staining. A combination of four antibodies was used to compare four cell subsets of interest: DCs, NK cells, NKT cells and T cells. Top left, distribution of DX5 versus TCRαβ; gates delineate NK cells (DX5^{hi}TCRαβ⁻), NKT cells (DX5^{lo}TCRαβ⁺) and T cells (DX5⁻TCRαβ⁺). Top right, distribution of CD11c versus major histocompatibility complex class II molecules (I-A and I-E; I-A/I-E); gate delineates the DC (CD11c⁺I-A/I-E⁺) subset. Bottom: when overlaid, gated cell subsets have distinct colors (DCs, blue; NK cells, red; NKT cells, yellow; T cells, green) that allow direct comparison of their localization in the total distribution of CD11c versus TCRαβ. Numbers indicate values of the mean fluorescence intensity of CD11c expression ± s.e.m. on the cell populations. Data were similar regardless of mouse age. Results are from six independent experiments with $n = 3$ mice for each experiment. **(b)** Production of IFN-γ in NK cells versus DCs. Spleen cells were first stained with four antibodies as described in **a**; CD11c^{hi} DCs and CD11c^{lo} NK cells were then sorted by flow cytometry based on their differential expression of CD11c and DX5. Total CD11c-expressing cells were isolated by enrichment with anti-CD11c magnetic beads (control). Cells were stimulated with IL-12 and IL-18; data represent IFN-γ after 24 h and are representative of three independent experiments with $n = 4$ mice for each experiment.

RESULTS

NK cells are the main source of innate IFN-γ

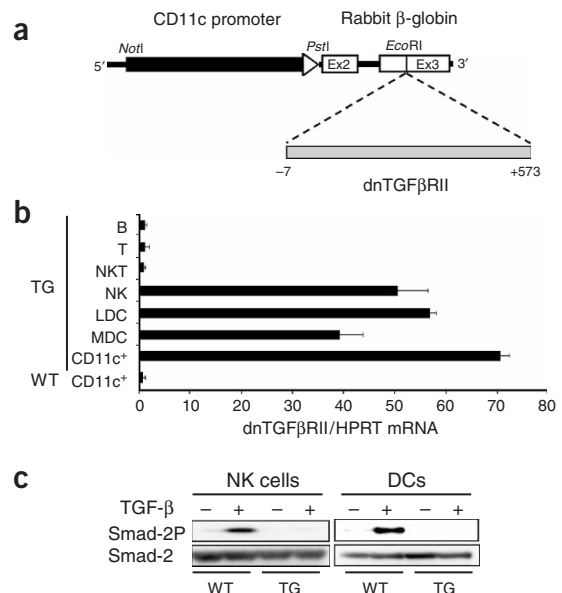
To address the effect of TGF-β on the production of innate IFN-γ, we first revisited the idea that both NK cells and DCs produce IFN-γ²⁰. NK cells and DCs were initially characterized as two distinct components of the innate immune response, with one defined by expression of DX5 and NK1.1 markers and the other characterized by high expression of major histocompatibility complex class II and CD11c markers. We investigated the expression of myeloid markers on NK cells and found that both NK cells and DCs shared expression of CD11c (**Fig. 1**). We analyzed samples using four-color flow cytometry staining to compare the compartment localization of various cell subsets within the total distribution of CD11c versus TCRαβ (**Fig. 1a**) or CD11c versus DX5 (**Fig. 1b**). DCs, used as positive control for CD11c expression, had substantial surface amounts of CD11c; neither NKT cells nor T cells expressed the CD11c marker. NK cells had intermediate expression of CD11c compared with that of DCs (mean

fluorescence of CD11c expression relative to each cell population, **Fig. 1a**). CD11c^{lo} DX5^{hi}TCRαβ⁻ cells showed the typical profile of NK cells, as assessed by expression of the NK1.1, IL2Rβ and NK receptors (data not shown).

The fact that CD11c was expressed on both NK cells and DCs suggested that contamination by CD11c^{lo} NK cells might account for IFN-γ production in CD11c-expressing DC populations. To test this possibility, we assessed the production of IFN-γ in response to IL-12 and IL-18, both stimuli known to elicit substantial production of IFN-γ²¹. We first isolated CD11c-expressing cells by enrichment with beads coated with antibody to CD11c (anti-CD11c), a method commonly used for DC preparation (**Fig. 1b**). In this case, considerable IFN-γ was produced by this cell preparation, which contained all CD11c-expressing cells, including CD11c^{hi} DCs and CD11c^{lo} NK cells. Next we sorted CD11c^{lo} NK cells and CD11c^{hi} DCs by flow cytometry based on their differential expression of CD11c and DX5 (**Fig. 1b**) and compared their ability to produce IFN-γ. In these conditions, only NK

Figure 2 Generation and characterization of CD11c-dnTGFβRII transgenic mice.

(a) CD11c-dnTGFβRII transgene construct. dnTGFβRII, gray box; rabbit β-globin gene exon 3 (Ex3), white box; plasmid CD11c promoter vector, black box. **(b)** Expression of the dnTGFβRII transgene. Sorted cells were isolated from pooled spleens ($n = 5$) from wild-type (WT) or transgenic (TG) mice. Total RNA was extracted and transgene expression was analyzed by real-time PCR. Cell types: myeloid DCs (MDC; CD11c^{hi}I-A/I-E⁺CD8α⁻); lymphoid DCs (LDC; CD11c^{hi}I-A/I-E⁺CD8α⁺); NK cells (NK; CD11c^{lo}DX5^{hi}TCRαβ⁻); NKT cells (NKT; CD11c⁻DX5^{lo}TCRαβ⁺); T cells (T; TCRαβ⁺); and B cells (B; CD19⁺). Data represent values normalized to HPRT mRNA and are representative of three independent experiments. **(c)** Function of the dnTGFβRII transgene. NK cells and DCs were sorted from pooled spleens ($n = 8$) as described in **Figure 1b**. NK cells were stimulated for 30 min with IL-12 and IL-18 and DCs were stimulated for 30 min with lipopolysaccharide in the absence (-) or presence (+) of TGF-β. Total cell lysates were analyzed by immunoblot with anti-phosphorylated Smad2 (Smad-2P). Blots were stripped and were subsequently reprobated with anti-Smad2 (control for protein loading). Data are representative of two independent experiments.



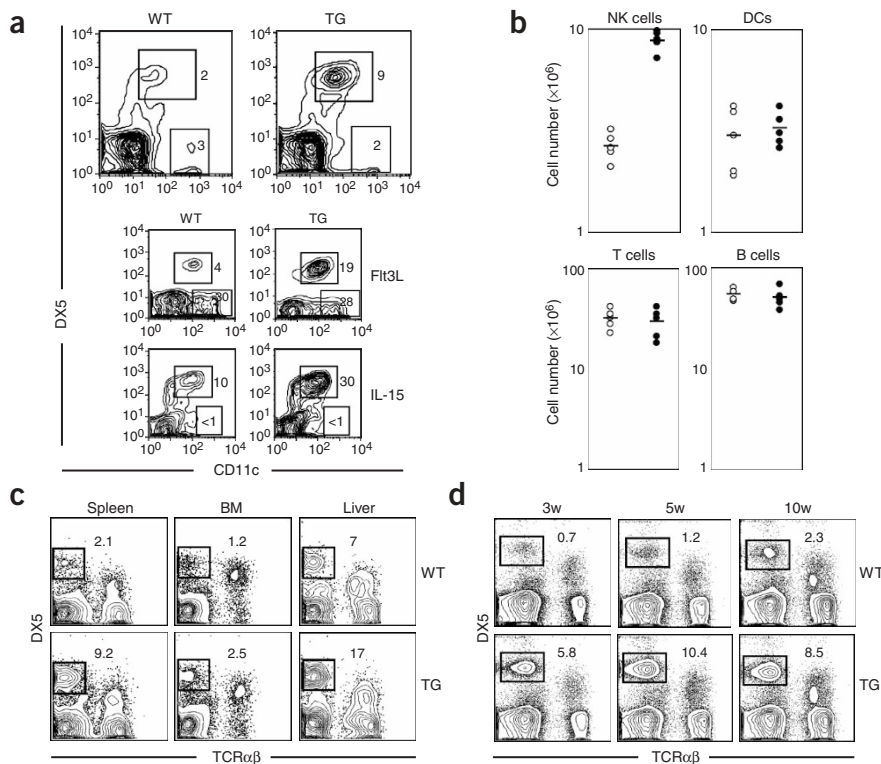


Figure 3 CD11c-dnTGFβRII mice are characterized by a large NK cell pool size. (a) NK cell frequency. Spleens were isolated and cells were stained with anti-CD11c, anti-DX5, anti-I-A/I-E and anti-TCRαβ. Numbers beside and in boxed areas (gates) show the frequency of NK cells (CD11c^{lo}DX5^{hi}) and DCs (CD11c^{hi}DX5^{lo}) from untreated mice (top; $n = 5$) and FIt3L-treated mice (middle; $n = 3$). Spleen cells were cultured with IL-15 (bottom; $n = 3$) and NK cell and DC frequency were assessed after 7 d of culture. (b) NK cell, DC, T cell and B cell subset numbers in spleen cell samples isolated from individual ($n = 5$) transgenic mice (filled circles) and control mice (open circles). (c) Distribution of NK (DX5^{hi}TCRαβ⁺) cells in spleen, bone marrow (BM) and liver. (d) Distribution of NK (DX5^{hi}TCRαβ⁺) cells in mice 3, 5 and 10 weeks of age (3w, 5w and 10w, respectively). Data in c and d are representative of three independent experiments with $n = 2$ mice for each experiment. Numbers beside boxed areas indicate the percentage of NK cells.

cells were able to produce IFN-γ (Fig. 1b). These results indicated that contaminating NK cells accounted for IFN-γ production in DC populations stimulated with IL-12 and IL-18. Consistent with our data, NK cells but not DCs have been reported to be the source of IFN-γ in response to infection²² and in NK cell-contaminated cultures of macrophages stimulated with IL-12 and IL-18 (ref. 23).

To better characterize the response of CD11c^{lo} NK cells versus CD11c^{hi} DCs, we used a cytokine detection array specific for 20 different cytokines and chemokines (Supplementary Fig. 1 online). We sorted NK cells and DCs and stimulated them with IL-12 and IL-18 as described above. After 24 h, we collected supernatants and incubated them with the array membrane. IFN-γ was present in supernatants of stimulated NK cells, whereas there were no secreted cytokines in supernatants of stimulated DCs. Thus, NK cells are the main source of IFN-γ and IFN-γ is the main product of NK cells.

Generation of CD11c-dnTGFβRII-transgenic mice

To definitively address the function of TGF-β in the regulation of NK cell versus DC, we used CD11c-dnTGFβRII to target both NK cell and DC compartments (Fig. 2a). This dnTGFβRII blocks TGF-β signaling when its expression is sufficiently high to interfere with the assembly

of a functional signaling complex consisting of TGF-β and type II and type I TGF-β receptors²⁴. To characterize transgene expression, we assessed dnTGFβRII mRNA in various cell populations by real-time RT-PCR (Fig. 2b). We found specific expression of dnTGFβRII in CD11c-expressing cells, including NK cell and DC subsets; we detected no expression in NKT cells, T cells or B cells isolated from CD11c-dnTGFβRII-transgenic mice. Finally, we tested whether the dnTGFβRII expression was sufficient to block TGF-β signaling. Binding of TGF-β to the functional TGF-β receptor leads to phosphorylation of the signaling molecule Smad2 on Ser465 and Ser467 residues²⁵. Whereas TGF-β was able to induce phosphorylation of Smad2 in DCs and NK cells from control nontransgenic mice, phosphorylation of Smad2 was undetectable in extracts of cells from transgenic mice (Fig. 2c). We conclude that dnTGFβRII expression was sufficient to block TGF-β signaling through the functional TGF-β receptor exclusively in DCs and NK cells.

TGF-β controls NK cell homeostasis

One outcome of the blockade of TGF-β signaling in CD11c-expressing cells was the selective dysregulation of NK cell homeostasis (Fig. 3). Mouse DCs and NK cells are both minor cell populations, representing about 3% of total cells in peripheral lymphoid organs of adult mice. In CD11c-dnTGFβRII mice, NK cell homeostasis was significantly disrupted, as indicated by the higher frequency (10% ± 3% versus 3% ± 1% in transgenic versus wild-type, respectively; $n = 5$; $P < 0.05$) and increased number

($9 \times 10^6 \pm 3 \times 10^6$ versus $2 \times 10^6 \pm 1 \times 10^6$ in transgenic versus wild-type, respectively; $n = 5$; $P < 0.05$) of spleen NK cells (Fig. 3a,b). Similarly, there was a higher frequency of NK cells in other organs of transgenic mice, including bone marrow and liver (Fig. 3c). In contrast, the size of the transgenic DC compartment was unaffected despite efficient blockade of TGF-β signaling in these cells (Fig. 3a-c). To further substantiate that finding, we compared the expansion of DC versus NK cell populations in response to the ligand for the cytokine receptor FIt3 (FIt3L; Fig. 3a). FIt3L is a potent inducer of DC population expansion and, to a lesser extent, it also promotes the expansion of NK cell populations²⁶. As expected, FIt3L induced considerable expansion of the DC compartment but with no difference in transgenic versus control (wild-type) mice ($37\% \pm 8\%$ versus $42\% \pm 11\%$, respectively; $n = 3$). In comparison, analysis of the NK cell response to FIt3L directly confirmed greater expansion of cell numbers in the absence of TGF-β signaling ($20\% \pm 4\%$ versus $5\% \pm 2\%$ in transgenic versus wild-type, respectively; $n = 3$). Similarly, when splenocytes were cultured in the presence of IL-15, a cytokine known to promote NK cell division²⁷, the frequency of NK cells was about fourfold higher in transgenic cultures (Fig. 3a). These data demonstrate a selective inhibitory function of TGF-β in the regulation of NK cell homeostasis.

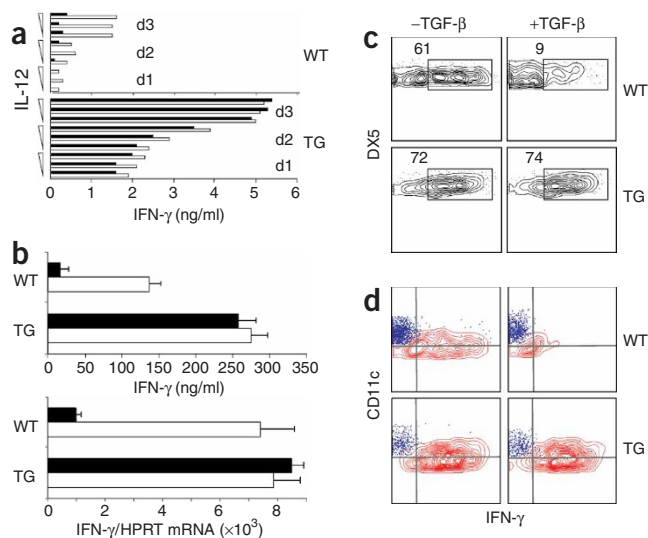


Figure 4 TGF- β is a potent suppressor of NK cell production of IFN- γ . (a,b) Sorted NK cells were stimulated with IL-12 alone (a) or with IL-12 plus IL-18 (b) in the absence (open bars) or presence (filled bars) of 5 ng/ml of TGF- β . (a) IFN- γ secreted 1, 2 and 3 d (d1, d2 and d3, respectively) after stimulation with 2, 10 and 20 ng/ml of IL-12 (wedges). (b) IFN- γ production 24 h after stimulation with IL-12 plus IL-18, assessed by enzyme-linked immunosorbent assay for secreted IFN- γ (top) and by real-time PCR for mRNA expression (bottom; relative to HPRT mRNA). Data are representative of two (a) and five (b) independent experiments, with $n = 10$ pooled spleens for each experiment. (c,d) IFN- γ expression of total splenocytes stimulated for 24 h with IL-12 and IL-18 in the absence (-) or presence (+) of 5 ng/ml of TGF- β . GolgiStop added during the last 6 h of culture permitted intracellular detection of IFN- γ expression. (c) Splenocytes were surface-stained with anti-CD11c, anti-DX5 and anti-I-A/I-E; numbers beside boxed areas indicate the frequency of IFN- γ -expressing cells among gated NK cells (CD11c^{lo}DX5^{hi}). Data are representative of four independent experiments, with $n = 2$ for each experiment. (d) IFN- γ expression compared directly in NK cell (CD11c^{lo}DX5^{hi}; red) versus DC (CD11c^{hi}I-A/I-E⁺; blue) subsets. The frequency of IFN- γ -expressing cells is less than 1% among DC subsets ($n = 5$).

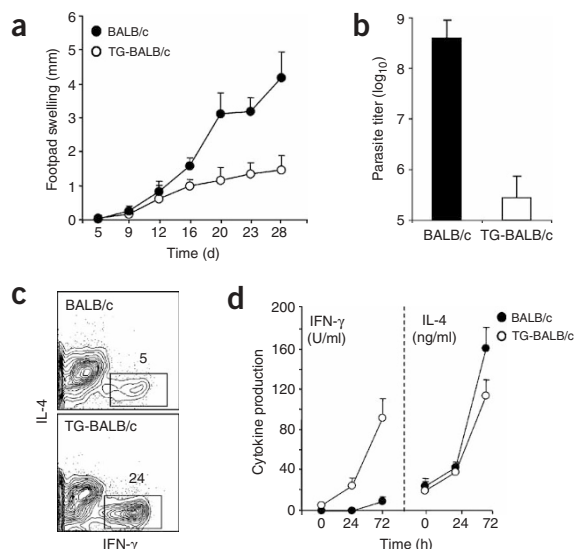
Studies of NK cell homeostasis have established that the steady-state numbers of NK cells increase rapidly during early life, from about 3×10^5 per spleen at days 1–2 after birth to about 2×10^6 cells at 3 weeks of age, finally achieving a plateau of 2×10^6 to 4×10^6 per spleen in 2- to 8-month-old mice²⁸. To address the kinetics of NK cell accumulation in CD11c-dnTGF β RII mice, we assessed NK cell frequency in mice at different ages. The frequency of NK cells was consistently higher in transgenic than in control mice (Fig. 3d). By 3 weeks of age, NK cells were about fourfold more abundant in transgenic mice and this threshold was sustained for the rest of adult life. Notably, the frequency of transgenic NK cells reached a plateau by 2 months of age, consistent with typical NK cell kinetics (Supplementary Fig. 2 online). Furthermore, assessment of NK cell turnover by *in vivo* incorporation of 5-bromodeoxyuridine demonstrated transgenic and control mice had a similar frequency of cycling NK cells (Supplementary Fig. 3 online). Similarly, analysis of the spectrum of expression of NK receptors showed no differences in transgenic versus control NK cells (Supplementary Fig. 4 online). Compared with the effects of TGF- β signaling blockade in T cells²⁹, these data are consistent with a unique outcome that leads to a higher

steady-state NK cell frequency with a normal proliferation and maturation profile.

TGF- β suppresses NK cell IFN- γ

NK cells respond to stimulation with a combination of IL-12 and IL-18 by producing abundant IFN- γ ³⁰. However, IL-12 alone cannot elicit IFN- γ production, despite constitutive expression of IL-12 receptor on NK cells³¹. We verified that NK cells had a low capacity to respond to IL-12 alone but that the addition of TGF- β completely abrogated IFN- γ production (Fig. 4a). In comparison, analysis of IFN- γ production relative to different doses of IL-12 showed four- to fivefold more IFN- γ in TGF- β -resistant NK cells (Fig. 4a). When we combined IL-12 with IL-18, overnight stimulation of NK cells was sufficient to induce efficient expression of IFN- γ mRNA and substantial IFN- γ protein (Fig. 4b). This rapid burst of IFN- γ production, however, was efficiently suppressed in control NK cells by the addition of TGF- β , as demonstrated at both the protein and mRNA level. In contrast, as expected, TGF- β continued to have no effect on transgenic NK cell production of IFN- γ (Fig. 4b). Flow cytometry of the frequency of IFN- γ -expressing NK cells further confirmed the potent

Figure 5 CD11c-dnTGF β RII mice develop efficient leishmania-specific T_H1 responses. (a) CD11c-dnTGF β RII mice show increased resistance to infection with *L. major*. BALB/c mice and transgenic BALB/c mice (TG-BALB/c) were infected in the rear footpads with 1×10^6 metacyclic *L. major* promastigotes and lesions were measured (time, horizontal axis). Data represent the mean \pm s.d. from four mice/group and are representative of two independent experiments. The difference between the groups is statistically significant at day 20 and beyond ($P < 0.05$). (b) Quantification of parasite burden 50 d after infection by limiting dilution analysis. Data represent the mean \pm s.d. of lesions from three mice/group. (c,d) CD11c-dnTGF β RII mice show efficient T_H1 differentiation. At 4 weeks after infection, draining lymph nodes were isolated and cells were stimulated with 50 μ g/ml of soluble leishmania antigen. For intracellular cytokine expression, cells were incubated for 24 h, with GolgiStop added during the last 6 h. (c) Frequency of IFN- γ -expressing cells among gated CD4⁺ cell subsets. (d) IFN- γ and IL-4 in cell culture supernatants collected after 24 and 72 h. Data in c,d are representative of two independent experiments, with $n = 3$ for each experiments.



ability of TGF- β to suppress NK cell production of IFN- γ (Fig. 4c). Whereas the addition of TGF- β efficiently suppressed the differentiation of NK cell into IFN- γ -expressing cells ($61\% \pm 7\%$ without TGF- β versus $9\% \pm 3\%$ with TGF- β ; $n = 8$; $P < 0.05$), as expected, it continued to have no effect on TGF- β -resistant NK cells ($72\% \pm 9\%$ in the absence of TGF- β versus $74\% \pm 5\%$ with TGF- β ; $n = 8$).

The stimulation of TGF- β -resistant NK cells consistently resulted in more IFN- γ (Fig. 4b) and a higher frequency of IFN- γ -expressing cells (Fig. 4c) than did stimulation of controls. Consequently, we considered the possibility that blockade of TGF- β signaling in DCs might demonstrate the potential of TGF- β -resistant DCs to produce IFN- γ . For this, we stimulated total spleen cells with IL-12 and IL-18 and compared IFN- γ -expressing cell frequency directly in NK cell versus DC subsets (Fig. 4d). DCs failed to differentiate into IFN- γ -expressing cells regardless of whether they were sensitive or resistant to TGF- β , consistent with our finding that DCs are not the main source of IFN- γ (Fig. 1b,c). The production of IL-12 by DCs, however, is indisputable, yet the effects of TGF- β remain controversial^{32–34}. Given the importance of IL-12 in the production of IFN- γ by NK cells, we next investigated whether TGF- β can affect DC production of IL-12. However, we found no substantial difference in DC production of IL-12 in the presence of TGF- β (Supplementary Fig. 5 online), consistent with the hypothesis that IL-10 is the main inhibitor of DC-derived IL-12 (ref. 35). IFN- γ and IL-12 are both important regulators of T_{H1} differentiation, and thus our data suggest that the function of TGF- β is to control T_{H1} responses through regulation of NK cell production of IFN- γ but not IL-12 production by DCs.

Enhanced T_{H1} response in CD11c-dnTGF β RII mice

Leishmania infection provides an excellent model for defining the function of TGF- β -mediated suppression of NK cells on T cell differentiation, because T_{H1} cells confer protection in the resistant mouse strain (C57BL/6), whereas T_{H2} cells are associated with disease progression in susceptible mice (BALB/c). We infected control and transgenic mice from BALB/c background with *L. major* promastigotes in the hind footpads and monitored lesion development by footpad swelling (Fig. 5). As expected, infection in control BALB/c mice resulted in lesion progression demonstrated by footpad swelling (Fig. 5a) and increased parasite burden (Fig. 5b). In contrast, CD11c-dnTGF β RII BALB/c mice showed increased resistance to *L. major* infection (Fig. 5a,b), indicating that susceptibility to *L. major* infection requires TGF- β signaling in innate effector cells. To address whether protection against *L. major* infection in CD11c-dnTGF β RII BALB/c mice was due to a leishmania-specific T_{H1} response, we isolated T lymphocytes from draining lymph nodes and restimulated them with soluble leishmania antigen. Whereas control BALB/c mice showed a strong T_{H2} response and lacked IFN- γ secretion, infection of transgenic BALB/c mice resulted in a potent antigen-specific T_{H1} response, as demonstrated by efficient differentiation into IFN- γ -expressing CD4⁺ T cells (Fig. 5c) and confirmed by substantial IFN- γ production by CD4⁺ T cells (Fig. 5d). Although not as notable as the difference in IFN- γ secretion, there was a considerable decrease in IL-4 production in transgenic BALB/c mice (Fig. 5d). Finally, to confirm that the development of T_{H1} responses in CD11c-dnTGF β RII BALB/c mice was not the result of a blockade of TGF- β signaling in T cells (for example, by induction of CD11c-dnTGF β RII transgene expression in activated T cells), we assessed transgene expression in draining lymph nodes CD4⁺ T cells from uninfected and infected mice (Supplementary Fig. 6 online). Our results confirmed that the dnTGF β RII transgene was not expressed in CD4⁺ T cells regardless

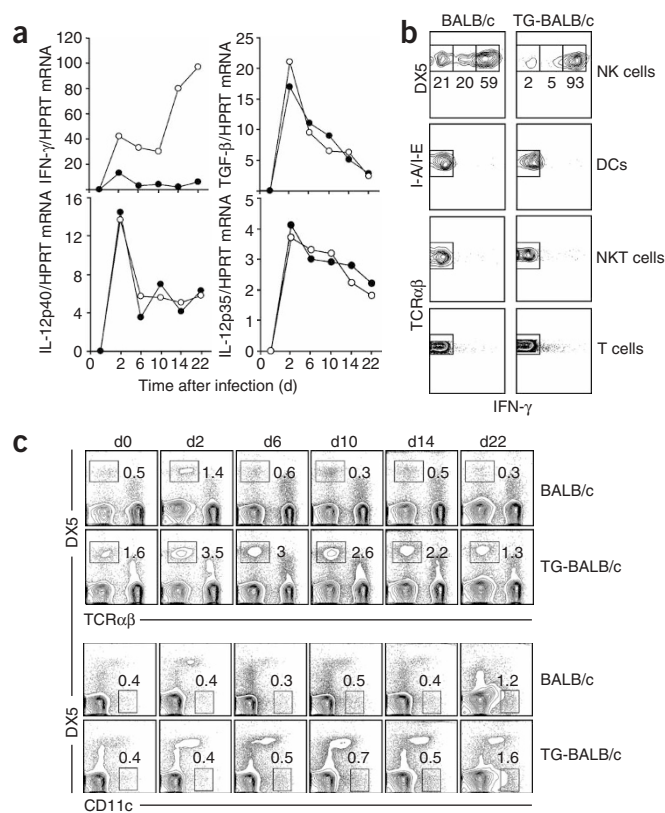


Figure 6 CD11c-dnTGF β RII mice develop efficient NK cell responses to *L. major* infection. (a) CD11c-dnTGF β RII mice produce more innate IFN- γ . Mice were infected in the rear footpads with 1×10^6 metacyclic *L. major* promastigotes and draining lymph nodes were isolated after infection (time, horizontal axes). RNA was prepared from total organs and expression of IFN- γ , TGF- β , IL-12p40 and IL-12p35 mRNA was assessed by real-time PCR. Data are normalized to HPRT mRNA from transgenic BALB/c mice (open circles) and control BALB/c mice (filled circles). (b) NK cell samples from CD11c-dnTGF β RII mice show a higher frequency of IFN- γ -expressing cells. At 2 d after infection, draining lymph nodes were isolated and total cells were stimulated for 24 h with IL-12 and IL-18. GolgiStop added during the last 6 h of culture permitted intracellular detection of IFN- γ expression. Data represent distribution of IFN- γ -expressing cells among NK cell (CD11c^{lo}DX5^{hi}), DC (CD11c^{hi}I-A/I-E⁺), NKT cell (DX5^{lo}TCR $\alpha\beta$ ⁺) and T cell (DX5^{lo}TCR $\alpha\beta$ ⁺) subsets. Numbers beneath boxed areas represent the percentage of NK cells with high (right gate), low (middle gate) or no (left gate) intracellular expression of IFN- γ . Data are representative of three mice per group. (c) CD11c-dnTGF β RII mice show higher NK cell frequency. As described in a, draining lymph nodes were isolated after infection (time (in days), above plots) and cells were stained with anti-CD11c, anti-DX5, anti-TCR $\alpha\beta$ and anti-I-A/I-E. Numbers beside boxed areas indicate the frequency of NK cell (DX5^{hi}TCR $\alpha\beta$ ⁺; top) and DC (CD11c^{hi}DX5⁺; bottom) subsets. Data are representative of two independent experiments with $n = 3$ mice per time point.

of whether they were activated (infected transgenic mice) or not (uninfected transgenic mice).

Enhanced NK cell responses in CD11c-dnTGF β RII mice

To obtain a comprehensive understanding of leishmania-specific T_{H1} response in CD11c-dnTGF β RII BALB/c mice, we used real-time RT-PCR to assess the kinetics of innate cytokine expression in the draining lymph nodes during the early phase of response to *L. major* infection (Fig. 6a). Both control and transgenic BALB/c mice responded to *L. major* infection with a rapid burst of IFN- γ , IL-12

and TGF- β mRNA that peaked by day 2 after infection. However, peak expression of IFN- γ mRNA was at least threefold higher in CD11c-dnTGF β R2 BALB/c mice (Fig. 6a). Flow cytometry showed that NK cells accounted for the rapid burst of IFN- γ expression on day 2 after infection (Fig. 6b). Notably, transgenic NK cells also had more expression of intracellular IFN- γ than did control cells ($93\% \pm 5\%$ versus $59\% \pm 3\%$ in transgenic versus wild-type NK cells, respectively; $n = 3$). After day 2, IFN- γ mRNA expression rapidly decreased in control BALB/c mice, consistent with a suppressive microenvironment highly rich in TGF- β expression (Fig. 6a). Despite having kinetics of TGF- β mRNA expression similar to those of wild-type mice, transgenic BALB/c mice with TGF- β -resistant NK cells continued to have high and sustained expression of IFN- γ during the early phase of response to *L. major* infection. This resulted in a second burst of IFN- γ mRNA by day 14, consistent with the development of an adaptive T_H1 response in CD11c-dnTGF β R2 BALB/c mice. In contrast, control BALB/c mice with baseline expression of IFN- γ mRNA during the innate response phase failed to mount a T_H1 response (Fig. 6a). Notably, transgenic and control BALB/c mice showed no difference in mRNA expression of IL-12 subunits p40 and p35 (Fig. 6a), indicating that early production of substantial IFN- γ was the decisive factor that led to 'successful' T_H1 responses in CD11c-dnTGF β R2 BALB/c mice.

To further extend that finding, we addressed the kinetics of NK cell accumulation in response to *L. major* infection. The frequency of NK cells correlates with the magnitude of T_H1 development³⁶. Our analysis showed that the rate of NK cell accumulation paralleled the kinetics of innate IFN- γ mRNA expression in draining lymph nodes, consistent with higher NK cell frequency and higher IFN- γ expression in CD11c-dnTGF β R2 BALB/c mice (Fig. 6b). The peak IFN- γ mRNA expression corresponded to maximum proliferation and/or accumulation of NK cells, whereas the rapid decrease in IFN- γ mRNA expression in control BALB/c mice was associated with a rapid decrease of the number of NK cells. Similarly, higher and sustained expression of IFN- γ mRNA was associated with higher and sustained TGF- β -resistant NK cell numbers in transgenic BALB/c mice. As for IL-12 mRNA expression in draining lymph nodes, the frequency of lymph node DCs was not different in control versus transgenic BALB/c mice (Fig. 6c). These results demonstrate that TGF- β signaling is responsible for the fall in NK cell numbers and/or activity characteristic of susceptible BALB/c mice.

DISCUSSION

Innate IFN- γ is emerging as a signal that controls T_H1 development. Our central finding here has been that TGF- β controls T_H1 differentiation through regulation of innate IFN- γ production. We have shown that NK cells are the main source of innate IFN- γ and that blockade of TGF- β signaling in NK cells caused increased NK cell numbers and more production of innate IFN- γ responsible for T_H1 differentiation and protection from *L. major* infection. A chief pursuit in the field of T cell differentiation is deciphering the factors behind the 'decision' to mount a T_H1 or T_H2 response; we have provided here direct evidence that TGF- β participates in this decision through regulation of NK cell-derived IFN- γ .

Innate IFN- γ production is induced mainly by IL-12. IL-12 participates in a positive feedback loop by promoting IFN- γ secretion by NK cells that in turn potently primes DCs for further IL-12 production^{5,6}. The hypothesis that TGF- β can inhibit innate effector cell functions has been suggested, but its effects on NK cells versus DCs has remained controversial^{17-19,32-34}. Our data support a model in which TGF- β signaling suppresses NK cell production of IFN- γ but does not affect DC production of IL-12. Blockade of TGF- β signaling caused the

generation of a high frequency of NK cells, whereas it had no effect on DC homeostasis in CD11c-dnTGF β R2 mice. Also, *L. major* infection resulted in high expression of innate IFN- γ mRNA but unchanged expression of innate IL-12 mRNA in CD11c-dnTGF β R2 mice. Furthermore, the addition of exogenous TGF- β did not affect IL-12 production in DC cultures, whereas it efficiently abrogated production of IFN- γ in NK cell cultures. Innate IFN- γ and IL-12 signals are important regulators for T_H1 development³⁷, and our data delineate a previously undescribed demarcation of the effects of TGF- β on NK cell production of IFN- γ versus DC production of IL-12.

The involvement of innate IFN- γ was initially established in a mouse model of cutaneous leishmaniasis using treatment with anti-IFN- γ versus IFN- γ in resistant versus susceptible strains, respectively^{10,38}. Whereas treatment with anti-IFN- γ abrogates the resistance to leishmaniasis in the C3H/HeN mouse strain, administration of IFN- γ offers protection in the susceptible BALB/c strain^{10,38}. NK cell depletion in resistant mice results in considerably decreased innate IFN- γ and an abrogated 'downstream' T_H1 response¹¹. The association of a high NK cell frequency with efficient T_H1 priming has been demonstrated via the actions of several adjuvants³⁶. A direct correlation between the frequency of NK cells and the frequency of IFN- γ -expressing T_H1 cells has been directly established. Given those findings, our data have demonstrated that blockade of TGF- β signaling in NK cells caused the generation of large numbers of NK cells capable of producing considerable IFN- γ , which led to downstream T_H1 polarization and protection from *L. major* infection in CD11c-dnTGF β R2 BALB/c mice.

Innate IFN- γ seems to have a dual function in T_H1 polarization, as it increases the production of IL-12 by DCs³⁹ and it acts synergy with TCR signals to induce expression of IFN- γ by T cells⁴⁰. The fact that large amounts of innate IFN- γ in CD11c-dnTGF β R2 BALB/c mice do not affect innate IL-12 expression supports the model that innate IFN- γ is directly involved in the control of T_H1 development. In line with that observation, the recruitment of a large number of IFN- γ -expressing NK cells correlates with induction of T_H1 cells but does not affect IL-12 expression by DCs³⁶. Given that IL-12 is required for NK cell activation^{5,6}, we established that the lack of NK cell function characteristic of susceptible BALB/c strain was not due to defects in IL-12 production. Evidence for this came from the similar kinetics of IL-12p40 and IL-12p35 mRNA expression in response to *L. major* infection in transgenic compared with control BALB/c mice. In support of those data, normal IL-12 expression has been reported in susceptible versus resistant mouse strains⁴¹, indicating that a lack of NK cell activation is more likely to be due to cytokines that inhibit IL-12 function. From our study, TGF- β emerges as a factor responsible for the lack of IL-12-induced NK cell responsiveness. Responsiveness to IL-12 is mediated through a specific, high-affinity receptor composed of IL-12R β 1 and IL-12R β 2 subunits that exist mainly on T cells and NK cells⁴²⁻⁴⁴. In T cells, TGF- β has been shown to suppress IL-12-induced production of IFN- γ but direct effects of TGF- β in regulating IL-12R have proven controversial. Studies of IL-12 responsiveness in BALB/c T cells have demonstrated the action of endogenous TGF- β in inhibiting the expression of IL-12R β 2 but not IL-12R β 1 (ref. 45). However, analysis of IL-12-mediated immune modulation specific to a peptide (Ac1-11) of myelin basic protein has indicated effects of TGF- β on both IL-12R β 1 and IL-12R β 2 (ref. 46). Similarly, the response of peripheral blood T cells from patients with tuberculosis stimulated in the presence of anti-TGF- β suggests effects on both IL-12R β 1 and IL-12R β 2 downregulation⁴⁷. In contrast, human alloactivated T cells show no effects on the expression of IL-12R β ⁴⁸. Viral transduction of exogenous IL-12R β 2 fails to restore T_H1 differentiation in the presence of TGF- β , indicating that downregulation of

IL-12R β 2 is not key in the TGF- β -mediated direct effect⁴⁹. In contrast to what is known about T cells, little is known regarding the effects of TGF- β in suppressing IL-12-induced IFN- γ in NK cells. Further investigation using this mouse model system may directly address this issue.

One principal advance in understanding T cell differentiation was the recognition that components of the innate immune system shape the adaptive immune response. We have demonstrated that TGF- β signaling is a checkpoint event that tightly controls T_H1 development through regulation of innate NK cell IFN- γ . Defining the mechanism involved in the selective development of T_H1 or T_H2 cells will have a direct fundamental influence on future vaccine development and the design of effective therapies for infectious diseases. Our results provide a rationale for targeting NK cells during vaccination to augment IFN- γ at the time of antigen challenge and thus subsequently promote cell-mediated immunity.

METHODS

CD11c-dnTGF β RII-transgenic mice. The human TGF- β type II receptor sequence between nucleotides -7 and +573, encoding the extracellular and transmembrane regions of the TGF- β type II receptor (dnTGF β RII), was a gift from L. Wakefield (National Cancer Institute, Bethesda, Maryland)⁵⁰. The dnTGF β RII fragment was cloned into the *Eco*RI site of rabbit β -globin gene exon 3 of plasmid CD11c promoter vector pDOI-5, a gift from T. Brocker (Ludwig-Maximilians University, Munich, Germany)⁵¹. The orientation and integrity of dnTGF β RII transgene was subsequently checked by DNA sequencing (the Keck sequencing facility, Yale University, New Haven, Connecticut). The plasmid vector containing the CD11c promoter, dnTGF β RII and polyadenylation sequence was excised by digestion with *Not*I, was purified and then was microinjected into (C57BL/6 \times C3H) F₁ fertilized eggs. Offspring transgenic for CD11c-dnTGF β RII were identified by PCR of genomic DNA with 5'-ACTTGAC TGACCGTTGTGTG-3' and 5'-ATGCTTCTCTCTTTCCTAC-3 primers and were backcrossed at least seven times onto C57BL/6 and BALB/c strains. Compared with mice transgenic for CD4-dnTGF β RII (ref. 29), the mice transgenic for CD11c-dnTGF β RII seemed healthy with no signs of autoimmune disease. Transgenic and control mice were maintained in specific pathogen-free conditions and were used between 3 and 10 weeks of age. All animal experiments were done with approved Institutional Animal Care and Use Committee protocols (Yale University, New Haven, Connecticut).

Parasites and infections. A clone of *L. major* (WHO MHOM/IL/80/Friedlin; a gift from D. Mosser, University of Maryland, College Park, Maryland) was used. Promastigotes were grown in Schneider's insect cell culture medium (Gibco) supplemented with 20% heat-inactivated FBS, 2 mM L-glutamine, 100 U/ml of penicillin G and 100 μ g/ml of streptomycin. Metacyclics were prepared from day-7 stationary-phase cultures by a density-gradient centrifugation method⁵². Mice were injected in the hind footpad with 1×10^6 metacyclic promastigotes. Lesion size was determined by measurement of the thickness of the footpad with a caliper and subtraction of the thickness of the uninfected contralateral footpad. Parasite burdens in footpads were determined by limiting serial dilution of single-cell suspensions made from individual excised lesions as described⁵³.

In vitro and in vivo cell population expansion. For induction of *in vivo* NK cell population expansion, mice were treated intraperitoneally for 9 consecutive days with 10 μ g Flt3L (Immunex) plus 1 μ g mouse serum albumin, as described⁵⁴. Spleens were collected and NK cell frequency was determined by flow cytometry. For induction of *in vitro* NK cell population expansion, total splenocytes were cultured in complete medium supplemented with 10 ng/ml of IL-15 (R&D Systems) as described²⁷. At 7 d after treatment, NK cell frequency was determined by flow cytometry.

Cell staining and sorting. Single-cell suspensions were treated with Fc receptor block (2-4G2) and were stained anti-CD11c (HL3), anti-CD49/Pan NK (DX5), anti-TCR $\alpha\beta$ (H57-597), anti-I-A/I-E (M5/114.15.2) and anti-CD4 (GK1.5) conjugated to fluorescein isothiocyanate, phycoerythrin, peridinin chlorophyll

protein or allophycocyanin (PharMingen). Cells were analyzed with a FACS-Calibur or were sorted with a FACSVantage (Becton Dickinson). Data were analyzed with FlowJo software (Tree Star). Where indicated (Fig. 1b), cells were sorted with anti-CD11c-conjugated MicroBeads according to the manufacturer's procedure (Miltenyi Biotec). Splenocytes were magnetically labeled with CD11c-MACS MicroBeads, and cells labeled with MicroBeads were retained on the MACS Column while the unlabeled cells passed through. The column was removed from the separator and the retained cells were eluted as the enriched, positively selected cell fraction. The enrichment of cells was confirmed by flow cytometry (more than 95% CD11c⁺).

Cytokine expression and/or production. Total splenocytes were stimulated for 24 h with IL-12 (10 ng/ml; Peprotech) and IL-18 (20 ng/ml; R&D) in the presence or absence of 5 ng/ml of TGF- β (R&D Systems), with GolgiStop (PharMingen) added during the last 6 h. Cells were surface-labeled (anti-DX5, anti-CD11c and anti-TCR $\alpha\beta$), were fixed and permeabilized, then were stained with anti-IFN- γ (XMG1.2) according to the manufacturer's procedure (PharMingen). Results are presented as the frequency of IFN- γ -expressing cells among gated DCs and NK cells.

At 4 weeks after infection with *L. major*, draining lymph nodes were isolated and total cells were stimulated for 24 h with 50 μ g/ml of soluble leishmania antigen, with GolgiStop (PharMingen) added during the last 6 h. Cells were surface-stained (anti-CD4 and anti-TCR $\alpha\beta$) and intracellular staining was done with IFN- γ (XMG1.2) and IL-4 (11B11; PharMingen). Results are presented as the frequency of IFN- γ - and IL-4-expressing CD4⁺ T cells.

For cytokine production, cells were sorted and were stimulated as described above. Cytokines released from DC, NK cell or CD4⁺ T cell cultures were measured by cytokine-specific sandwich enzyme-linked immunosorbent assay at various time points.

Real-time PCR. Total RNA was isolated with TRIzol reagent (Invitrogen) and contaminant DNA was removed by DNase (Ambion) according to the manufacturer's instructions. Total RNA (5 μ g) was reverse-transcribed with Superscript reverse transcriptase (Invitrogen). Expression of the dnTGF β RII transgene was determined by real-time PCR of RNA from various cell types, using 5'-CATCTTCTACTGCTACCGCGTTAA-3' and 5'-CACACCAGCCACCA CCTTC-3' at a final concentration of 500 nM and the internal TaqMan probe 5'-5-carboxyfluorescein (FAM)-CCGGGAGAAGTTGAGTCCATGTACGC-black hole quencher (BHQ)-1-3' at a final concentration of 200 nM. The gene encoding hypoxanthine guanine phosphoribosyl transferase (HPRT) was used as an internal reference and was analyzed using the primers 5'-CTGGTGAAAA GGACCTCTCG-3' and 5'-TGAAGTACTCATATAGTCAAGGGCA-3' at a final concentration of 200 nM and the TaqMan probe 5'-FAM-TGTTGGATACAGG CCAGACTTTGTTGGAT-BHQ-1-3' at a final concentration of 200 nM. The dnTGF β RII mRNA was normalized to HPRT mRNA.

Cytokine mRNA expression of total organs or sorted stimulated cells was assessed by real-time PCR. Expression of TGF- β and IL-12p40 mRNA was assessed with the following primers (500 nM) and probes (200 nM): TGF- β , 5'-CCCGAAGCGGACTACTATGC-3' and 5'-ATAGATGGCGTGTGTCGGT-3' and the internal TaqMan probe 5'-FAM-AGAGGTCACCCGCGTGCTAATGG TG-BHQ-1-3'; IL-12p40, 5'-CTCAGGATCGCTATACAAATTCCTC-3' and 5'-CCTCAGGCGGTGCCTATGT-3' and the internal TaqMan probe 5'-FAM-TGCAGCAAGTGGGCATGTGTTCC-BHQ-1-3'. Primers and probes used for IFN- γ mRNA expression have been described⁵⁵. Expression of IL-12p35 mRNA was determined using a TaqMan Gene Expression Assay kit (Applied Biosciences). Cytokine mRNA expression was normalized to HPRT mRNA.

Immunoblots. Sorted DCs (1×10^6 cells/ml) or sorted NK cells (1×10^6 cells/ml) were stimulated with lipopolysaccharide (10 ng/ml; Sigma) or with IL-12 (10 ng/ml; Peprotech) and IL-18 (20 ng/ml; R&D Systems), respectively. After 30 min of stimulation in the absence or presence of 5 ng/ml of TGF- β (R&D Systems), cells were lysed and total protein (20 μ g) was resolved by 10% SDS-PAGE and was transferred to Immobilon P membranes. Immunoblots used anti-Smad2 (PharMingen) and anti-phosphorylated Smad2 (Upstate Biotechnology). Bands were visualized with secondary horseradish peroxidase-conjugated antibodies and the ECL System (Amersham Pharmacia).

Statistics. Analyses were made with the nonparametric unpaired Mann-Whitney *U*-test. A *P* value less than 0.05 was considered statistically significant.

Note: Supplementary information is available on the Nature Immunology website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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