Immunity 25, 455–471, September 2006 ©2006 Elsevier Inc. DOI 10.1016/j.immuni.2006.07.011

Transforming Growth Factor-β Controls Development, Homeostasis, and Tolerance of T Cells by Regulatory T Cell-Dependent and -Independent Mechanisms

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Summary

The role of transforming growth factor-β (TGF-β) in inhibiting T cell functions has been studied with dominant-negative TGF-β receptor transgenic models; however, the full impact of TGF-β signaling on T cells and the mechanisms by which TGF-β signals remain poorly understood. Here we show that mice with T cell-specific deletion of TGF-β receptor II developed lethal inflammation associated with T cell activation and differentiation. In addition, TGF-β signaling positively regulated T cell development and homeostasis. Development of $CD8^+$ T cells and NKT cells, maintenance of peripheral Foxp3-expressing regulatory T cells, and survival of $CD4^+$ T cells all depended on TGF-β signaling. Both T helper 1 (Th1) differentiation and survival of activated $CD4^+$ T cells required T-bet, the TGF-β-regulated transcription factor, which controlled CD122 expression and IL-15 signaling in Th1 cells. This study reveals pleiotropic functions of TGF-β signaling in T cells that may ensure a diverse and self-tolerant T cell repertoire in vivo.

Introduction

The stochastic nature of T cell receptor (TCR) rearrangement results in a plethora of T cell clones that are subjected to the processes of positive and negative selection in the thymus. T cell clones with low-affinity TCRs to self antigens are positively selected, whereas T cells with high-affinity TCR interactions are mostly eliminated by negative selection to prevent the development of autoimmunity (Starr et al., 2003). A subset of self-reactive T cells avoid being destroyed in the thymus and further differentiate into mature T cells with regulatory activities; these include $CD4^+CD25^+$ regulatory T (Treg) cells and CD1d-dependent natural killer T (NKT) cells (Kronenberg and Rudensky, 2005). The Treg cell lineage, specified by the transcription factor Foxp3, essentially regulates peripheral T cell tolerance, as shown by the fact that mice and humans that lack these cells develop severe autoimmune diseases (Maloy and Powrie, 2001; Sakaguchi, 2004; Shevach, 2000). On the other hand, activation of NKT cells can prevent or exaggerate autoimmunity depending on the disease model (Kronenberg, 2005).

Transforming growth factor-β (TGF-β) family consists of potent regulatory molecules that play an essential role in T cell tolerance (Gorelik and Flavell, 2002). There are three TGF-β isoforms in mammals, TGF-β1, TGF-β2, and TGF-β3, which signal through two trans-membrane serine-threonine kinase receptors, TGF-β receptor I and II (TGF-βRI, TGF-βRII) (Massague, 1998). Among the three TGF-β isoforms, TGF-β1 is predominantly expressed in the immune system (Govinden and Bhoola, 2003). Deficiency of TGF-β1 results in an early lethal autoimmune phenotype in mice (Kulkarni et al., 1993; Shull et al., 1992), which is associated with circulating IgG antibodies to nuclear antigens and a progressive infiltration of leukocytes into multiple organs (Dang et al., 1995; Yaswen et al., 1996). T cells appear to be key mediators of the autoimmune disease in these mice, because depletion of $CD4^+$ or $CD8^+$ T cells alleviates the inflammatory phenotype (Kobayashi et al., 1999; Letterio et al., 1996).

Because TGF-β1 can modulate the activity of multiple cell types (Li et al., 2006), it was not clear from the early studies whether T cells are direct targets of TGF-β1. Several groups have used transgenic approaches to inhibit TGF-β signaling in T cells. Expression of a dominant-negative TGF-βRII from the *CD4* promoter (CD4-DNRII) leads to the generation of autoantibodies and the development of an inflammatory disease, but the mice survive to adulthood (Gorelik and Flavell, 2000). In another report, expression of DNRII from the *CD2* promoter results in a $CD8^+$ T cell lymphoproliferative disorder with little inflammation (Lucas et al., 2000). This difference could be due to the expression pattern and/or strength of the promoters used; alternatively, these transgenic mice may have only a partial loss of function of TGF-β signaling in T cells. Consistent with this, deletion of TGF-βRII in bone marrow cells results in a more severe inflammatory phenotype in mice (Leveen et al., 2002). However, because T cells are not the only cell type targeted in this model, it is possible that inhibition of TGF-β signaling in cell types other than T cells contributes to the stronger inflammatory phenotype. Thus, the ultimate role of TGF-β signaling in T cells remains to be established.

A recent study revealed that *Tgfb1*$^{-/-}$ mice fail to maintain peripheral Treg cells (Marie et al., 2005). Interestingly, the autoimmune phenotype developed in *Tgfb1*$^{-/-}$ mice resembles that of *Foxp3*$^{-/-}$ mice, which possess a specific defect of Treg cells (Fontenot et al., 2003). Therefore, it is an open question whether the T cell activation phenotype in *Tgfb1*$^{-/-}$ mice is due to loss of Treg cells and/or is a consequence of lack of Treg cell-independent control of T cell tolerance by TGF-β1. It has been well documented that TGF-β potently inhibits T cell proliferation and differentiation (Gorelik and Flavell, 2002). However, TGF-β induces the differentiation of Treg cells in vitro (Chen et al., 2003; Fantini et al., 2004; Wan and Flavell, 2005). Because Treg cells potently inhibit T cell proliferation and differentiation (Sakaguchi, 2004), it is not known whether the suppressive activity of TGF-β on T cells represents an autonomous pathway and/or is secondary to its induction of Treg cells.

The exact molecular mechanism responsible for the expansion and differentiation of T cells in *Tgfb1*$^{-/-}$

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mice or mice with inhibited TGF-β signaling in T cells is not known. $CD4^+$ T cells in CD4-DNRII mice differentiate into both IFN-γ-producing T helper 1 (Th1) cells and IL-4-producing Th2 cells in vivo (Gorelik and Flavell, 2000). In vitro, TGF-β inhibition of $CD4^+$ effector T cell differentiation has been attributed to its blockade of the expression of transcription factors T-bet in Th1 cells and GATA-3 in Th2 cells (Neurath et al., 2002; Gorelik et al., 2000, 2002; Heath et al., 2000). However, whether TGF-β exerts its in vivo effects by also modulating the function of these transcription factors remains unknown.

To determine a definitive function of TGF-β signaling in T cells in vivo, we generated mice with a T cell-specific deletion of TGF-βRII. $CD8^+$ T cell maturation and the development of NKT cells were inhibited in the thymus. In the periphery, Treg cells were reduced, and T cells were activated and differentiated notably into T-bet-expressing Th1 cells. T-bet determined both the IFN-γ expression and the survival of $CD4^+$ T cells, which was associated with the expression of CD122. Deletion of TGF-βRII in OT-II *Rag1*$^{-/-}$ T cells led to their partial activation and massive apoptosis in the periphery. These findings revealed both regulatory T cell-dependent and -independent mechanisms of T cell development, tolerance, and homeostasis specified by TGF-β signaling in T cells.

Results

Development of Lethal Autoimmunity in the Absence of TGF-β Signaling in T Cells

Previous studies with transgenic expression of dominant-negative TGF-βRII in T cells have shown varying phenotypes (Gorelik and Flavell, 2000; Lucas et al., 2000). We sought to determine a definitive function of TGF-β signaling in T cells by crossing a strain of floxed TGF-βRII mice (RII) with CD4-Cre transgenic mice (4cre) (Chytil et al., 2002; Lee et al., 2001). The *Cre* transgene in 4cre mice is expressed in $CD4^+CD8^+$ immature thymocytes (Lee et al., 2001). Consistent with this, $CD4^+CD8^+$ thymocytes in 4cre-RII/RII mice had no detectable surface TGF-βRII compared to that of control RII/RII mice (Figure 1A). Peripheral $CD4^+$ and $CD8^+$ T cells were also devoid of surface TGF-βRII (Figure 1A). Exogenous TGF-β induced Smad2 and Smad3 nuclear translocation in RII/RII $CD4^+$ T cells, which could not be detected in 4cre-RII/RII $CD4^+$ T cells (Figure 1B). TGF-β-induced Smad2 phosphorylation was also not detectable in 4cre-RII/RII $CD4^+$ T cells, whereas there was normal signaling in both RII/RII and heterozygous 4cre-RII/+ T cells (data not shown). These data demonstrate efficient deletion of TGF-βRII and abrogation of TGF-β signaling in 4cre-RII/RII T cells.

RII/RII and 4cre-RII/+ mice appeared healthy and had no signs of autoimmunity (Figure 1C and data not shown). In contrast, all 4cre-RII/RII mice developed a progressive wasting disease and succumbed to death before 5 weeks of age. Histological analysis revealed heavy infiltration of leukocytes into multiple tissues including stomach, lung, liver, pancreatic islets, and thyroid gland of 4cre-RII/RII mice but not those of 4cre-RII/+ mice (Figure 1C). Higher amounts of DNA autoantibody were also detected in the sera of 4cre-RII/RII mice (Figure 1D), suggesting that impaired TGF-β signaling in T cells results in the loss of B cell tolerance to self antigens. Similar leukocyte infiltration and development of nuclear antigen antibody is observed in aged CD4-DNRII transgenic mice (Gorelik and Flavell, 2000); however, CD4-DNRII mice survive to adulthood, and the inflammatory phenotype in CD4-DNRII mice is much less severe than that developed in 4cre-RII/RII mice. In fact, TGF-β treatment of CD4-DNRII $CD4^+$ T cells led to low but detectable nuclear translocation of Smad2 and Smad3 albeit reduced compared to that in control T cells (data not shown). Therefore, the previously reported dominant-negative TGF-βRII transgenic mice, which develop varying degrees of inflammation, most likely possess incomplete loss of function of TGF-β signaling in T cells. It is noteworthy that the wasting and inflammatory phenotype of 4cre-RII/RII mice was comparable in severity to that of *Tgfb1*$^{-/-}$ mice (Kulkarni et al., 1993; Shull et al., 1992), which highlights T cells as the key target of TGF-β1-mediated immune tolerance.

Inhibited Thymic $CD8^+$ T Cell Maturation and Compromised Peripheral T Cell Tolerance in 4cre-RII/RII Mice

To investigate the consequences of loss of TGF-β signaling in T cells, we first evaluated thymic T cell development in 4cre-RII/RII mice that were around 14 days old. At this age, 4cre-RII/RII mice appeared healthy and had comparable thymic cellularity to littermate controls, compared to older mice that had involuted thymus resulting from peripheral inflammation (data not shown). The CD4 and CD8 profile of 4cre-RII/RII thymocytes was not drastically different from that of control thymocytes, although a substantial reduction of $CD8^+$ T cells was observed (Figure 2A). Mouse thymic $CD8^+$ T cells are comprised of both the mature and the immature single-positive T cells (Paterson and Williams, 1987). To study the mature T cell compartment, we first gated on the mature TCR-β^{hi} T cells (see Figure S1A in the Supplemental Data available online). Among the TCR-β^{hi} T cells, a 2-fold increase of $CD4^+CD8^+$TCR-β^{hi} T cells was observed in 4cre-RII/RII mice (Figure 2B and Figure S1A). The number of $CD4^+$TCR-β^{hi} T cells was comparable, but the number of $CD8^+$TCR-β^{hi} T cells was reproducibly reduced by 50% in 4cre-RII/RII mice (Figure 2B and Figure S1A). These observations reveal a function for TGF-β signaling in the maturation of $CD8^+$TCR-β^{hi} T cells.

TGF-β potently suppresses T cell proliferation in vitro (Kehrl et al., 1986). To determine whether TGF-β affected T cell expansion in vivo, $CD4^+$ and $CD8^+$ T cell numbers were compared in the periphery. $CD4^+$ and $CD8^+$ T cell numbers in the spleens and lymph nodes were significantly higher in 4cre-RII/RII mice compared to littermate controls (Figure 2C). To determine whether the T cell expansion phenotype was associated with increased T cell proliferation, we pulse-labeled 4cre-RII/RII or the control RII/+ mice with BrdU for 16 hr. Foxp3-expressing Treg cells were excluded in the analysis by gating on $CD4^+$Foxp3$^-$ and $CD8^+$ T cells. During the 16 hr time window, we observed 5- to 6-fold increase of BrdU-positive T cells in the spleens of 4cre-RII/RII mice (Figure 2D). An increase in BrdU-positive T cells was also detected in the lymph nodes of 4cre-RII/RII mice (data not shown). These data demonstrate that similar to in vitro observations, in vivo blockade of TGF-β signaling

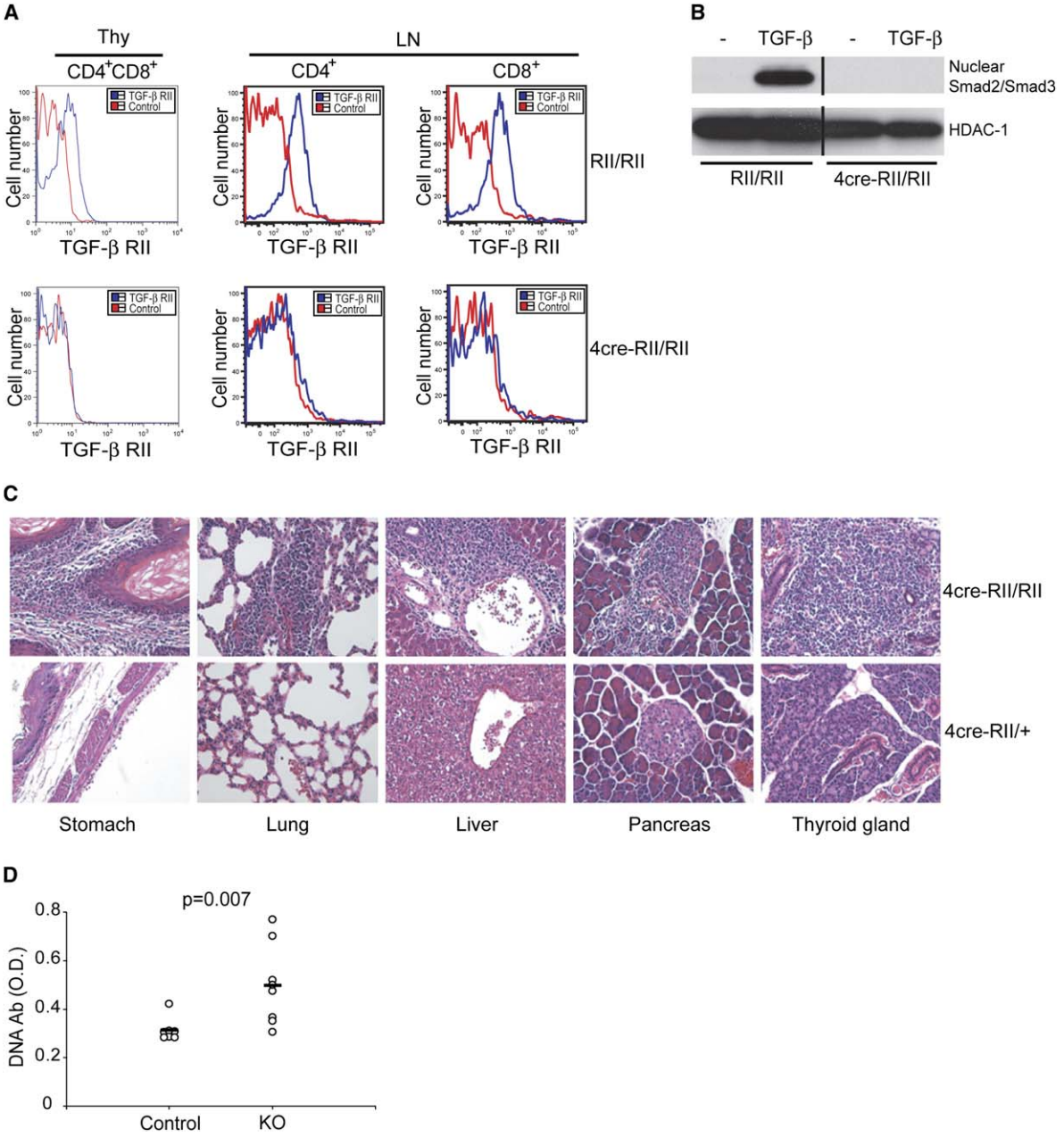

Figure 1. Abrogation of TGF-β Signaling in T Cells Led to Lethal Autoimmunity

(A) Surface staining of TGF-βRII (blue) in thymic (Thy) $CD4^+CD8^+$ and lymph node (LN) $CD4^+$ and $CD8^+$ T cells. The binding of an isotype control antibody is shown in red. These are representative results of two independent experiments.

(B) RII/RII or 4cre-RII/RII $CD4^+$ T cells were stimulated with anti-CD3 and anti-CD28 for 24 hr in the absence or presence of TGF-β. The amounts of Smad2 and Smad3 and HDAC-1 in the nuclear extracts were determined by immunoblotting.

(C) Hematoxylin and eosin staining of stomach, lung, liver, pancreas, and thyroid gland sections from the tissues of 4cre-RII/RII and 4cre-RII/+ mice (200×) at 21 days old. These are representative results of five independent experiments.

(D) Titers of dsDNA antibody in the sera of 4cre-RII/RII (KO), or littermate RII/RII and 4cre-RII/+ (control) mice aged between 21 and 26 days (n = 8). The p value between the two groups of dsDNA antibody titers is shown.

in T cells results in a lymphoproliferative phenotype in peripheral lymphoid organs.

Peripheral $CD4^+$ and $CD8^+$ T cells in 4cre-RII/RII mice had an activated phenotype as measured by CD44 and CD62L expression ($CD44^{hi}CD62L^{lo}$) (Figure 2E) and by the high expression of adhesion molecules CD18 and CD11a (Figure S1B). These T cells also expressed high amounts of CD43 (Figure S1C), a marker associated with effector T cells (Onami et al., 2002). To determine the cytokine production, as a measure of T cell differentiation, we stimulated lymph node T cells ex vivo with phorbol 12-myristate 13-acetate (PMA) and ionomycin for 4 hr and performed intracellular cytokine staining. In contrast to 4cre-RII/+ $CD4^+$ T cells, which had only a few cells capable of producing IFN-γ or IL-4, the majority of 4cre-RII/RII $CD4^+$ T cells produced high

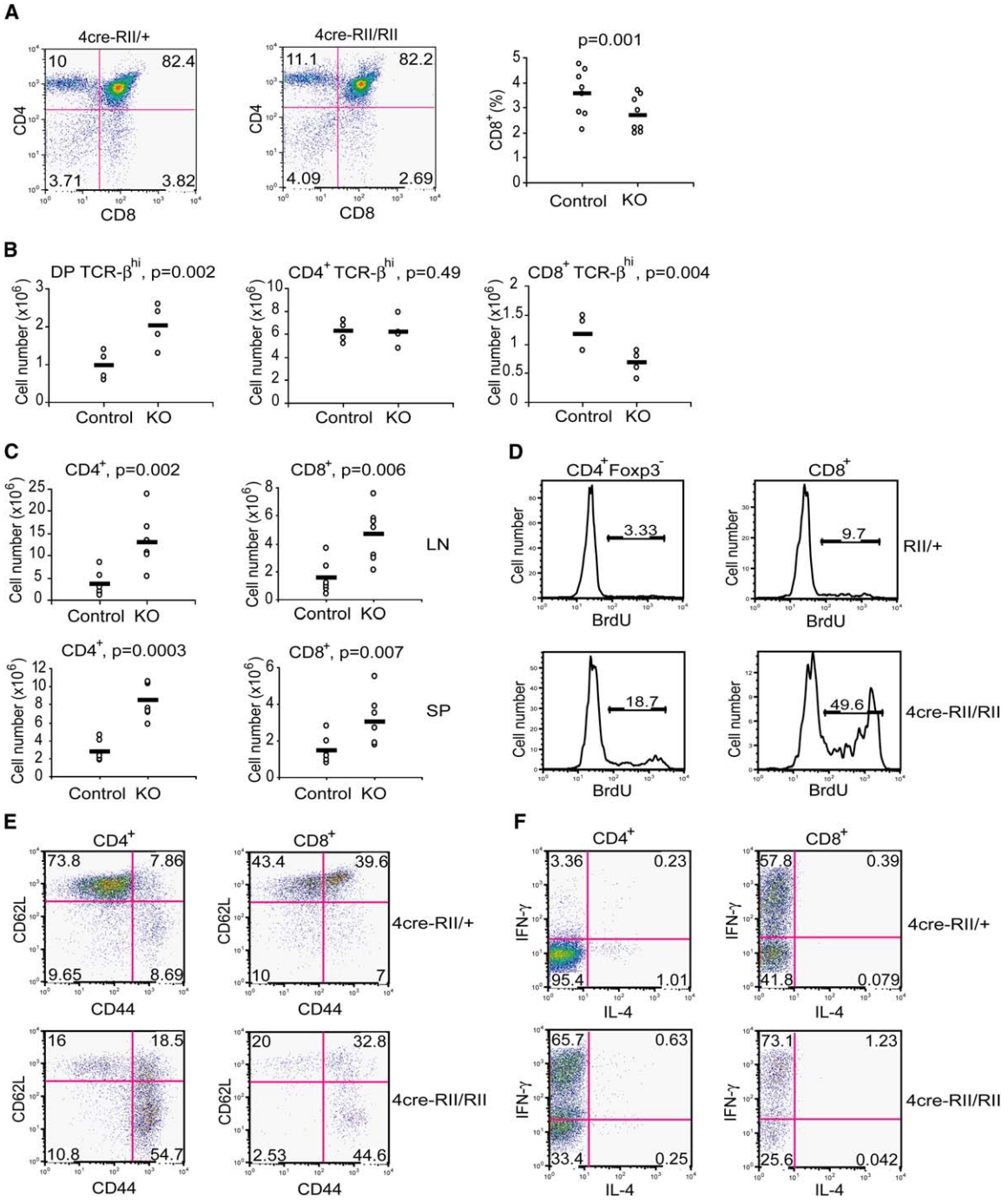

Figure 2. TGF-β Signaling in T Cells Was Required for the Maturation of Thymic CD8+ T Cells and the Inhibition of Peripheral T Cell Proliferation, Activation, and Differentiation

(A) The thymic CD4 and CD8 profile of 4cre-RII/+ and 4cre-RII/RII mice at 16 days old (left and middle). The percentage of thymic CD8+ T cells in 4cre-RII/RII (KO) or littermate RII/RII and 4cre-RII/+ (control) mice (n = 8) aged between 14 and 16 days (right).

(B) Number of thymic TCR-βhi T cells expressing CD4 and CD8 (DP), CD4, or CD8 in 4cre-RII/RII (KO) or littermate RII/RII and 4cre-RII/+ (control) mice (n = 4) aged between 14 and 16 days.

(C) Number of CD4+ or CD8+ T cells in the spleens (SP) and lymph nodes (LN) of 4cre-RII/RII (KO) or littermate RII/RII and 4cre-RII/+ (control) mice (n = 7) at 19–26 days of age.

(D) BrdU staining of splenic CD4+Foxp3− and CD8+ T cells in RII/+ and 4cre-RII/RII mice at 16 days old. These are representative results of two independent experiments.

(E) Expression of CD44 and CD62L of CD4+ and CD8+ T cells isolated from the lymph nodes of 4cre-RII/+ (top) and 4cre-RII/RII (bottom) mice at 16 days old. These are representative results of eight independent experiments.

(F) CD4+ and CD8+ T cells isolated from the lymph nodes of 4cre-RII/+ or 4cre-RII/RII mice were stimulated with PMA and ionomycin for 4 hr and analyzed for the expression of IFN-γ and IL-4 by intracellular cytokine staining. These are representative results of five independent experiments.

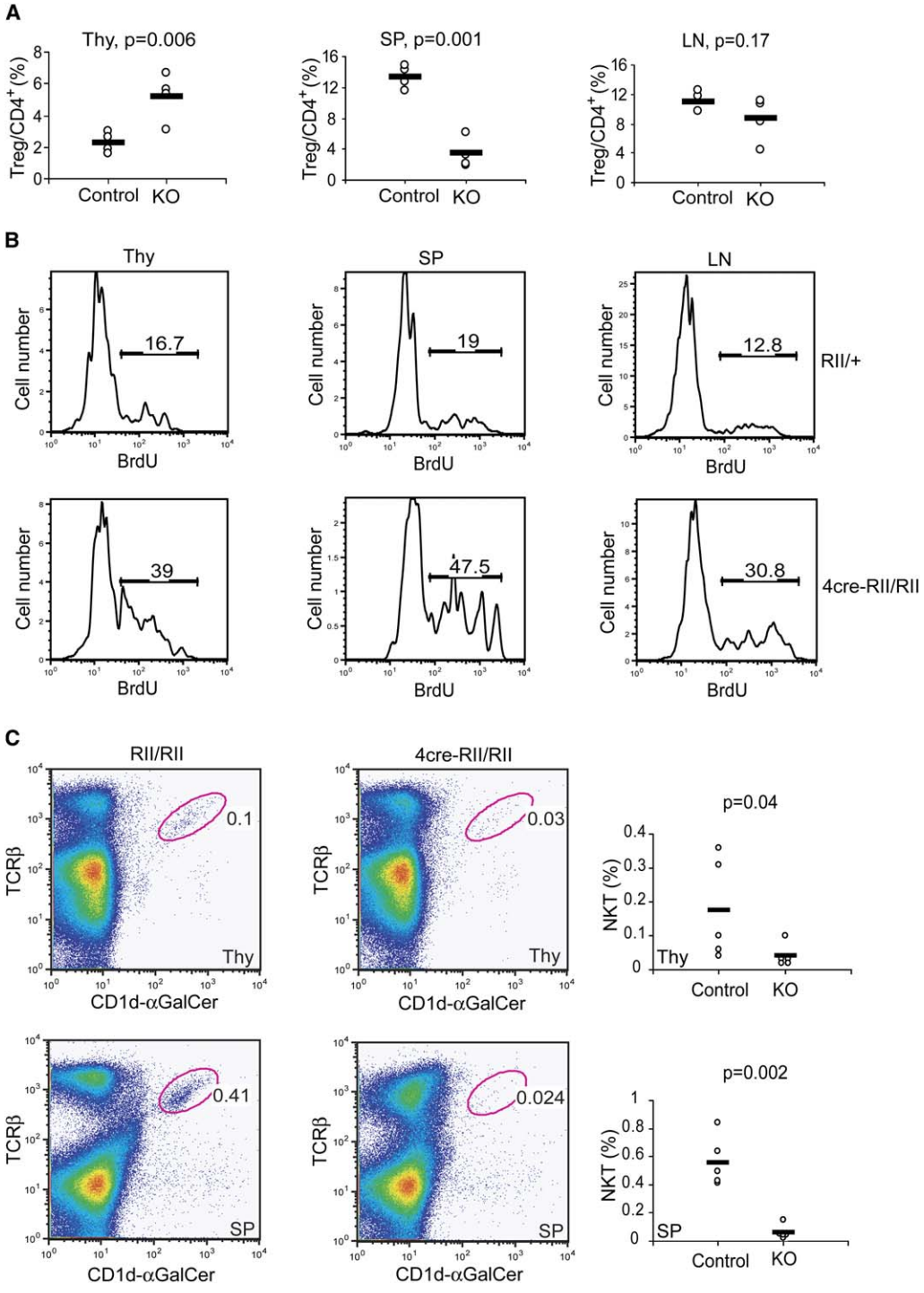

Figure 3. TGF-β Regulation of Treg Cell and NKT Cell Development and Homeostasis

(A) The percentage of $CD4^+Foxp3^+$ Treg cells among $CD4^+TCR\text{-}\beta^{hi}$ T cells in 4cre-RII/RII (KO), or littermate RII/RII and 4cre-RII/+ (control) mice (n = 4) is plotted. Thy, thymus; SP, spleen; LN, lymph node.

(B) BrdU staining of thymic, splenic, and lymph node $CD4^+Foxp3^+$ T cells in RII/+ and 4cre-RII/RII mice at 16 days old. These are representative results of two independent experiments.

(C) Thymic and splenic $TCR\text{-}\beta^{hi}CD1d\text{-}\alpha GalCer^+$ NKT cells in 4cre-RII/RII or RII/RII littermates at 16 days old (left and middle). The percentage of thymic and splenic NKT cells in 4cre-RII/RII (KO) or littermate RII/RII and 4cre-RII/+ (control) mice (n = 5) aged between 14 and 16 days (right).

amounts of IFN-γ but not IL-4 (Figure 2F). Increased IFN-γ-positive cells were also observed in 4cre-RII/RII $CD8^+$ T cells (Figure 2F). To investigate whether 4cre-RII/RII T cells produced increased IFN-γ in vivo, 4cre-RII/+ or 4cre-RII/RII mice were injected with Brefeldin A. 6 hr later, the expression of IFN-γ was determined from T cells isolated from the lymph nodes of these mice. A higher percentage of 4cre-RII/RII $CD4^+$ and $CD8^+$ T cells

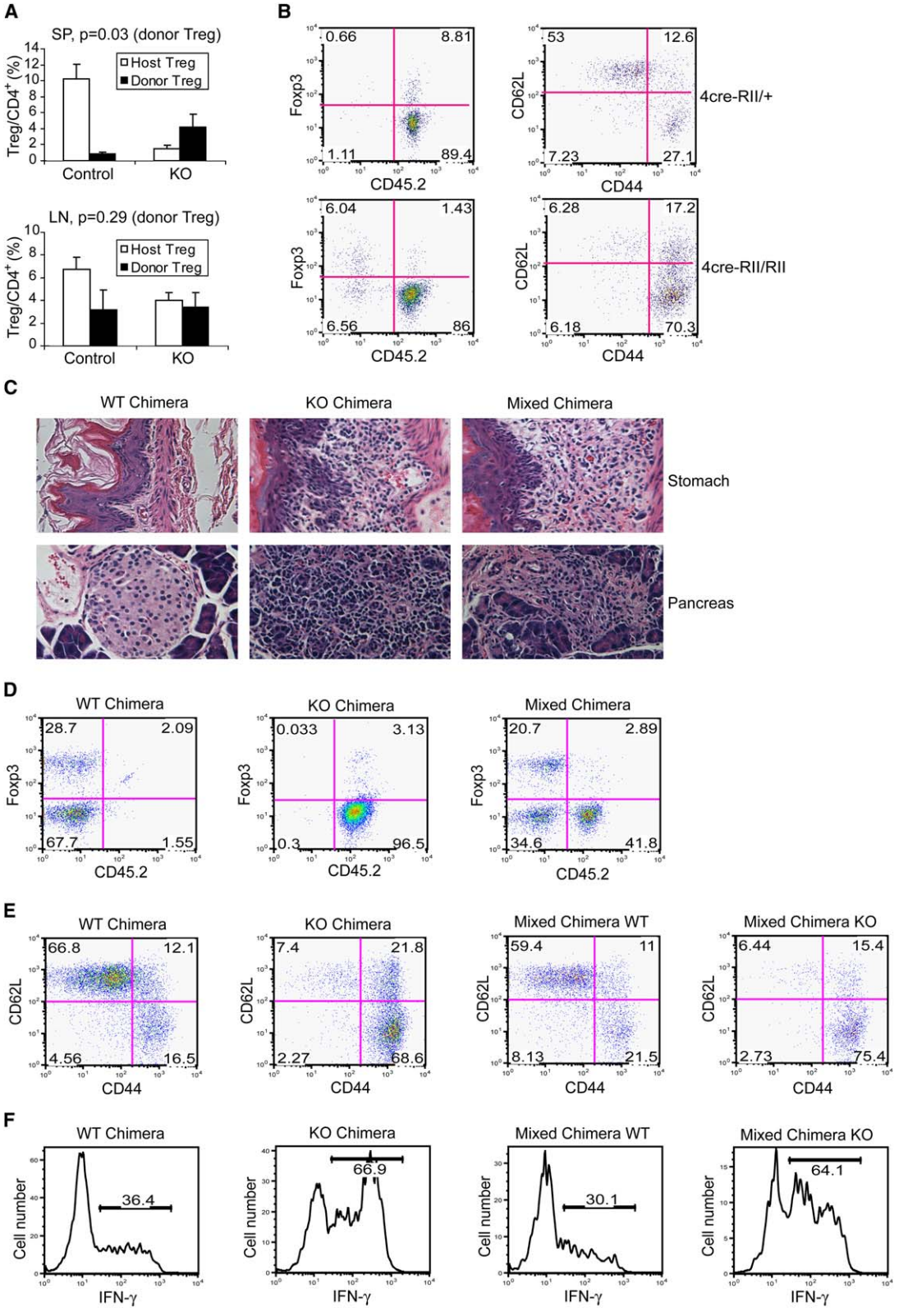

Figure 4. TGF-β Signaling in T Cells Autonomously Maintained Peripheral Treg Cells and Inhibited T Cell Activation and Differentiation

(A and B) Wild-type Treg cells were injected into neonatal mice.

(A) The percentage of donor and host $CD4^+Foxp3^+$ Treg cells among $CD4^+$ T cells in the spleens (SP, top) or the lymph nodes (LN, bottom) of 4cre-RII/RII (KO) or littermate RII/RII and 4cre-RII/+ (control) mice (n = 3) is plotted. The p values depict the difference of the donor Treg cells between the KO and control groups. These are representative results of two independent experiments. The error bars have been calculated with Excel STDEV, which gives the standard deviation.

produced IFN-γ during this time period (Figure S1D). Therefore, ablation of TGF-β signaling in T cells led to their activation and differentiation associated with the development of a lethal autoimmune syndrome.

Inhibited Thymic NK T Cell Development and Reduced Peripheral Treg Cells in 4cre-RII/RII Mice

Thymic T cell selection results in the generation of subsets of T cells that play important roles in regulating immunity and tolerance (Kronenberg and Rudensky, 2005). One type of such T cells is the Foxp3-expressing Treg cells that essentially control peripheral T cell tolerance (Sakaguchi, 2004). To investigate the role of TGF-β signaling in Treg cells, we first studied Treg cell development in 4cre-RII/RII mice. An increase in the percentage and number of $CD4^+Foxp3^+$ T cells was observed among $CD4^+TCR\text{-}\beta^{hi}$ T cells from thymus of 14-day-old 4cre-RII/RII mice (Figure 3A and data not shown). Consistent with this, a 2-fold higher percentage of 4cre-RII/RII thymic Treg cells incorporated BrdU during a 16 hr pulse-labeling period (Figure 3B). These observations reveal a previously unknown function for TGF-β in inhibiting the proliferation of thymic Treg cells.

Despite increased number of thymic Treg cells, the number of peripheral Treg cells was not markedly different between 14-day-old 4cre-RII/RII mice and the control mice (data not shown). However, when 4cre-RII/RII mice were aged to about 21 days, a decrease in the percentage of Treg cells among $CD4^+$ T cells was observed, which was more pronounced in the spleens than in the lymph nodes of these mice (Figure 3A). A previous study showed that Treg cells expressing a dominant-negative mutant of TGF-βRII failed to proliferate in a transfer model of colitis (Huber et al., 2004). To study whether decreased Treg cells in peripheral tissues was a consequence of failed proliferation, 4cre-RII/RII and RII/+ mice were pulse labeled with BrdU for 16 hr. Unexpectedly increased rather than decreased BrdU-positive Treg cells were detected in both the spleens and the lymph nodes of the 4cre-RII/RII mice (Figure 3B). These observations demonstrate that although TGF-β inhibits Treg cell proliferation, it is required for the maintenance of peripheral Treg cells. Because the cell number is a reflection of both the rate of proliferation and death, TGF-β signaling is likely essential for the survival of Treg cells in the periphery.

In addition to Treg cells, NKT cells are another major lineage of self-reactive T cells with an important function in immunity and tolerance. NKT cells express receptors that are also expressed on natural killer cells and TCRs that recognize glycolipids presented by the CD1d molecule (Bendelac et al., 1997). Most NKT cells express Vα14Jα18 TCRs and are referred to as Vα14 invariant (Vα14*i*) NKT cells (Kronenberg, 2005). Glycolipid α-glactosylceramide (αGalCer) presented by CD1d binds with high avidity on Vα14*i* NKT cells (Kronenberg, 2005). To study the function of TGF-β signaling in Vα14*i* NKT cell development, we used CD1d-αGalCer tetramer to identify these cells. 20%–50% thymic $Tetramer^+TCR\text{-}\beta^{hi}$ Vα14*i* NKT cells were present in 4cre-RII/RII mice compared to their littermate controls (Figure 3C, top). The spleens of 4cre-RII/RII mice were almost completely devoid of Vα14*i* NKT cells (Figure 3C, bottom). NKT cells were also not detectable in the peripheral blood and the livers of these mice (data not shown). These results suggest that TGF-β signaling is required for the development and homeostasis of Vα14*i* NKT cells.

TGF-β Autonomously Maintained Peripheral Treg Cells and Inhibited T Cell Activation and Differentiation

Mice devoid of the Treg cell lineage, i.e., $Foxp3^{-/-}$ mice (Fontenot et al., 2003), develop a similar autoimmune phenotype as 4cre-RII/RII mice; we therefore wanted to know whether the T cell activation phenotype in 4cre-RII/RII mice was a consequence of loss of peripheral Treg cells and/or was independent of Treg cells. To this end, we purified wild-type $CD4^+CD25^+$ T cells that were enriched for the Treg cell population from the lymph nodes and spleens of C57BL/6 mice that expressed the congenic marker CD45.1 and transferred them to 2-day-old 4cre-RII/RII or littermate control mice that expressed CD45.2. Consistent with a more dramatic reduction of Treg cells in the spleens than the lymph nodes of 4cre-RII/RII mice (Figure 3A), transferred wild-type Treg cells selectively repopulated the spleens of 4cre-RII/RII mice compared to the control group (Figure 4A). However, even in the presence of substantial numbers of splenic wild-type $CD4^+Foxp3^+$ Treg cells (Figure 4B, left), 4cre-RII/RII $CD4^+$ T cells exhibited an activated phenotype (Figure 4B, right). In addition, transfer of wild-type Treg cells did not alleviate the lethal inflammation in 4cre-RII/RII mice (data not shown). These observations suggest that depletion of peripheral Treg cells in 4cre-RII/RII mice is not the sole cause of the T cell activation and the autoimmune phenotype.

In addition to Treg cells, TGF-β also controls other subsets of regulatory T cell lineages, including NKT cells (Figure 3C). To study whether TGF-β regulation of T cell activation and differentiation is dependent on other non-Treg regulatory T cell subsets, we generated mixed bone marrow chimeric mice. Bone marrow cells from $CD45.2^+$ 4cre-RII/RII (KO) mice and $CD45.1^+$ wild-type

(B) Expression of Foxp3, CD45.2 (left), and CD44, CD62L (right) in splenic $CD4^+$ T cells of 4cre-RII/+ and 4cre-RII/RII mice. $CD45.2^+$ or $CD45.2^-$ T cells represent host or donor T cells, respectively.

(C–F) Bone marrow chimeras were generated and analyzed.

(C) Development of immunopathology in mice reconstituted with wild-type (wt), 4cre-RII/RII (KO), or 1:1 mixed wt and KO bone marrow cells. Hematoxylin and eosin staining of stomach and pancreas sections is shown (200×). These are representative results of three independent experiments.

(D) Expression of Foxp3 and CD45.2 in splenic $CD4^+$ T cells of wt, KO, or mixed bone marrow chimeras. A representative plot of six mice from each group is shown.

(E) Expression of CD44 and CD62L in $CD4^+$ T cells isolated from the lymph nodes of wt, KO chimeras, or of the wt or KO populations of the mixed bone marrow chimera. These are representative results of three independent experiments.

(F) Expression of IFN-γ in $CD4^+$ T cells isolated from the lymph nodes of wt, KO chimeras, or of the wt or KO populations of the mixed bone marrow chimera. These are representative results of three independent experiments.

(wt) mice were transferred either separately or in combination into sublethally irradiated *Rag1*$^{-/-}$ recipients. All chimeric mice reconstituted with KO bone marrow cells developed severe wasting disease with infiltration of leukocytes into multiple organs including stomach and pancreas (Figure 4C) and succumbed to death within 5 weeks. In contrast, none of the recipients of wt bone marrow cells developed disease (Figure 4C). Notably, four out of seven mice of the mixed chimeras also developed severe inflammation in the same time frame (Figure 4C). As expected, CD4$^+$Foxp3$^+$ Treg cells were greatly reduced in KO chimeras compared to those in wt chimeras (Figure 4D). Interestingly, the KO population from the mixed chimera also had lower Treg cell numbers than the wt population of the same mouse (Figure 4D), demonstrating a cell-intrinsic role for TGF-β signaling in the maintenance of Treg cells. In comparison to the wt population, a marked increase in the number of activated CD44^{hi}CD62L^{lo} CD4$^+$ T cells and CD8$^+$ T cells (data not shown) were present in the KO population of the mixed chimera, which was accompanied by the production of higher amounts of IFN-γ (Figures 4E and 4F). Together, these observations reveal a cell-autonomous function for TGF-β signaling in the maintenance of Treg cells and in inhibiting CD4$^+$ T cell activation and Th1 cell differentiation, which is essential for the prevention of autoimmune diseases.

An Essential Role for T-bet in Th1 Cell Differentiation and Survival of CD4$^+$ T Cells in 4cre-RII/RII Mice

Effector T cell differentiation is orchestrated by key transcription factors, including T-bet for Th1 cells (Szabo et al., 2000). Consistent with a dominant Th1 cell phenotype (Figure 2F), peripheral but not thymic 4cre-RII/RII CD4$^+$ T cells expressed high amounts of T-bet (Figure 5A). To determine the role of T-bet in these T cells, we generated 4cre-RII/RII mice deficient in T-bet (encoded by *Tbx21*). Deficiency of T-bet did not rescue the inflammatory phenotype or the Treg cell deficiency of 4cre-RII/RII mice (Figures S2 and S3A). In fact, 4cre-RII/RII *Tbx21*$^{-/-}$ mice developed an anemic phenotype resulting in earlier lethality than 4cre-RII/RII mice (data not shown). In addition, CD4$^+$ T cell activation was not altered in the absence of T-bet (Figure 5B), demonstrating that TGF-β inhibits T cell activation independent of regulating the expression of T-bet. Compared to 4cre-RII/RII CD4$^+$ T cells, 4cre-RII/RII *Tbx21*$^{-/-}$ CD4$^+$ T cells had greatly reduced IFN-γ-producing Th1 cells and increased IL-4-producing Th2 cells (Figure 5C). This is consistent with a role of T-bet in promoting Th1 while suppressing Th2 cell differentiation (Finotto et al., 2002). It is therefore possible that Th2 effector T cells and/or activated CD8$^+$ T cells may be responsible for the immunopathology in 4cre-RII/RII *Tbx21*$^{-/-}$ mice.

Intriguingly, deficiency of T-bet in 4cre-RII/RII mice also led to a reduced ratio of splenic CD4$^+$ T cells to CD8$^+$ T cells, which was not observed in *Tbx21*$^{-/-}$ mice (Figure 5D). A similar reversed CD4$^+$/CD8$^+$ T cell ratio was also observed in the lymph nodes, peripheral blood, and livers of these mice (Figure S3B), suggesting that diminished splenic CD4$^+$ T cells was not due to altered traffic of T cells to other tissues. In contrast to the expanded CD4$^+$ T cell compartment in 4cre-RII/RII mice, but consistent with the change in the CD4$^+$/CD8$^+$ ratio, 4cre-RII/RII *Tbx21*$^{-/-}$ spleens had reduced number of CD4$^+$ T cells (Figure 5E). This was a specific defect for CD4$^+$ T cells; CD8$^+$ T cell number increased in both 4cre-RII/RII and 4cre-RII/RII *Tbx21*$^{-/-}$ mice (Figure 5E). These observations suggest that T-bet is required not only for the Th1 cell differentiation but also for the survival of CD4$^+$ T cells in 4cre-RII/RII mice.

Il2rb, a Direct Target Gene of T-bet, Enabled IL-15 Signaling and Th1 Cell Survival

In CD8$^+$ T cells, T-bet and its paralogue eomesodermin regulate the expression of CD122 (Intlekofer et al., 2005), the receptor β chain for interleukin 15 (IL-15) and IL-2. T-bet also regulates the expression of CD122 in Th1 cells differentiated in vitro (L. Glimcher and G.M. Lord, personal communication). The IL-15-CD122 pathway is important in the homeostasis of effector and memory CD8$^+$ T cells (Judge et al., 2002; Ku et al., 2000), but its function in CD4$^+$ T cells remains largely undefined in vivo. To investigate whether the defective survival of CD4$^+$ T cells in 4cre-RII/RII *Tbx21*$^{-/-}$ mice was associated with compromised CD122 expression, we compared surface CD122 expression in these T cells by FACS analysis (Figure 6A). Although there were only a small percentage of CD4$^+$ T cells expressing CD122 in wild-type or *Tbx21*$^{-/-}$ mice, almost all splenic CD4$^+$ T cells expressed high amounts of CD122 in 4cre-RII/RII mice (Figure 6A, left). CD122 expression was markedly reduced in CD4$^+$ T cells from 4cre-RII/RII *Tbx21*$^{-/-}$ mice (Figure 6A, left). In contrast, the expression of CD122 on CD8$^+$ T cells in 4cre-RII/RII *Tbx21*$^{-/-}$ mice was not changed and thus was not solely dependent on T-bet (Figure 6A, right). These observations are consistent with the specific reduction of CD4$^+$ but not CD8$^+$ T cell number in 4cre-RII/RII *Tbx21*$^{-/-}$ mice (Figures 5D and 5E).

The increased CD122 expression in 4cre-RII/RII CD4$^+$ T cells rendered them responsive to IL-15 and IL-2, because both IL-2 and IL-15 stimulated the proliferation of 4cre-RII/RII CD4$^+$ T cells in vitro as revealed by CFSE dilution (Figure 6B, left), whereas naive wild-type CD4$^+$ T cells were not responsive to these cytokines (data not shown). More viable cells were recovered from the wells that were treated with IL-15 than IL-2 or antigen-presenting cell (APC) alone (Figure 6B, middle), suggesting that IL-15 treatment also promoted the survival of 4cre-RII/RII CD4$^+$ T cells. Consistent with reduced CD122 expression on 4cre-RII/RII *Tbx21*$^{-/-}$ CD4$^+$ T cells, IL-15 had minimal effect on these cells (Figure 6B, right). To investigate the function of IL-15 on survival of these cells in vivo, we labeled 4cre-RII/RII CD4$^+$ T cells with CFSE and transferred them into sublethally irradiated wild-type or *Il15*$^{-/-}$ mice. 4cre-RII/RII CD4$^+$ T cells proliferated extensively in both wild-type and *Il15*$^{-/-}$ hosts as revealed by CFSE dilution (Figure 6C, left). However, reduced numbers of 4cre-RII/RII CD4$^+$ T cells were recovered from *Il15*$^{-/-}$ mice compared to wild-type mice (Figure 6C, right). These observations suggest an important role of the IL-15-CD122 pathway in regulating the survival of 4cre-RII/RII CD4$^+$ T cells in vivo.

To further explore the molecular mechanisms by which TGF-β can modulate CD122 expression via T-bet in 4cre-RII/RII CD4$^+$ T cells, we first studied T-bet

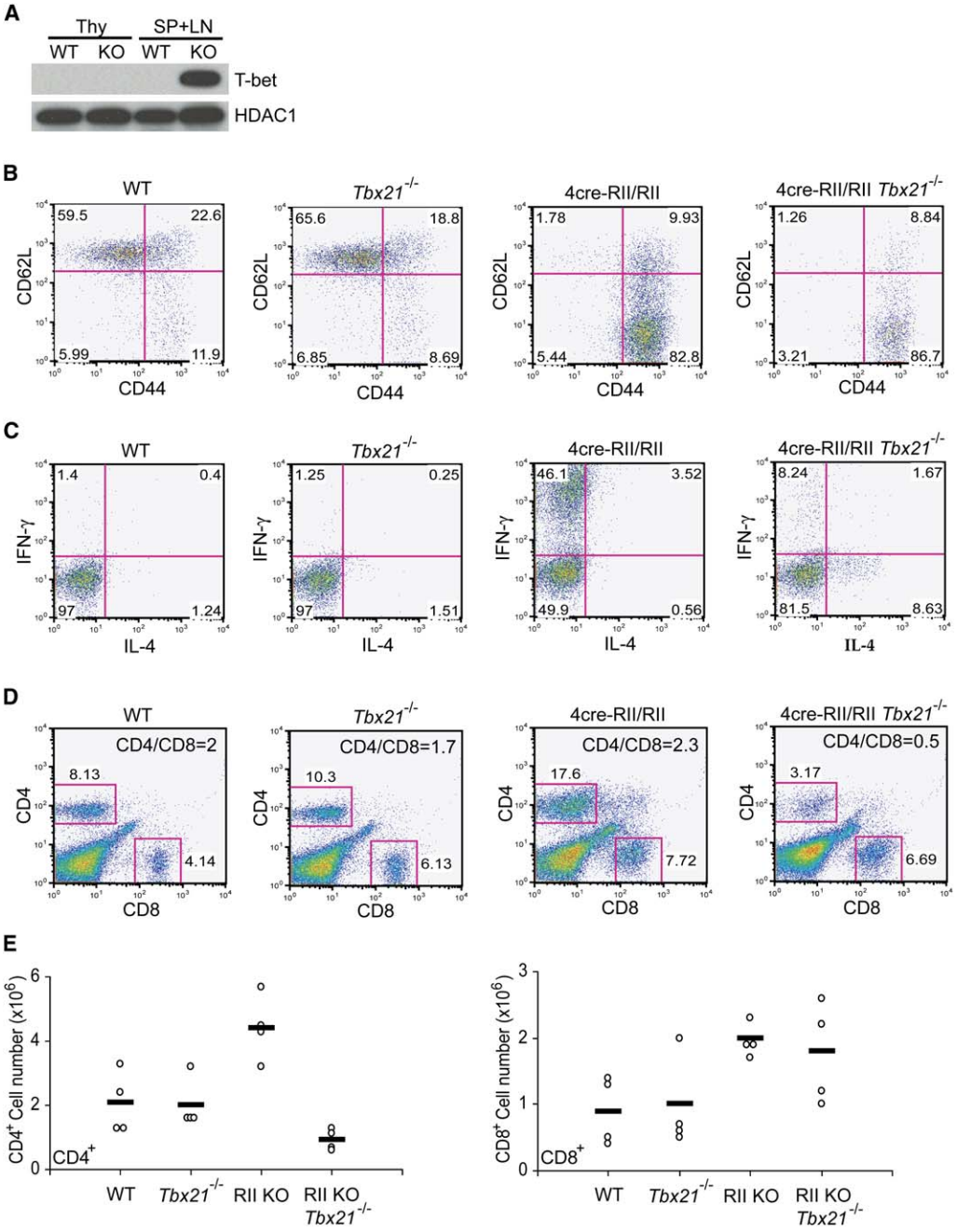

Figure 5. Requirement of T-bet for Th1 Cell Differentiation and CD4+ T Cell Survival in 4cre-RII/RII Mice

(A) Expression of T-bet in CD4+ T cells from RII/RII (wt) and 4cre-RII/RII (KO) mice at 14 days old. The amounts of T-bet and HDAC-1 were determined by immunoblotting from thymic (Thy) and peripheral (SP + LN) CD4+ T cell extracts.

(B) Expression of CD44 and CD62L in splenic CD4+ T cells from RII/RII (wt), *Tbx21*$^{-/-}$, 4cre-RII/RII, and 4cre-RII/RII *Tbx21*$^{-/-}$ mice at 16 days old. These are representative results of five independent experiments.

(C) Expression of IFN-γ and IL-4 in CD4+ T cells isolated from the lymph nodes of RII/RII (wt), *Tbx21*$^{-/-}$, 4cre-RII/RII, and 4cre-RII/RII *Tbx21*$^{-/-}$ mice. These are representative results of two independent experiments.

(D) The percentage of CD4+ and CD8+ splenic T cells in RII/RII (wt), *Tbx21*$^{-/-}$, 4cre-RII/RII, and 4cre-RII/RII *Tbx21*$^{-/-}$ mice. The ratios of CD4+ T cells to CD8+ T cells are shown. These are representative results of five independent experiments.

(E) The number of CD4+ and CD8+ splenic T cells in RII/RII (wt), *Tbx21*$^{-/-}$, 4cre-RII/RII, and 4cre-RII/RII *Tbx21*$^{-/-}$ mice at 16 days old (n = 4).

regulation of CD122 expression in in vitro differentiated wild-type CD4+ T cells. High expression of CD122 was found in Th1 but not in Th2 cells differentiated from wild-type naive CD4+ T cells isolated from C57BL/6 mice (Figure 6D, left). Inclusion of TGF-β1 in Th1 cell culture inhibited CD122 expression almost entirely (Figure 6D, right). This is consistent with high CD122 expression in 4cre-RII/RII CD4+ T cells (Figure 6A), which we showed to have differentiated into Th1 cells (Figure 2F), and with our previous finding that TGF-β1 inhibits T-bet expression and Th1 cell differentiation (Gorelik et al., 2002). To study whether increased CD122

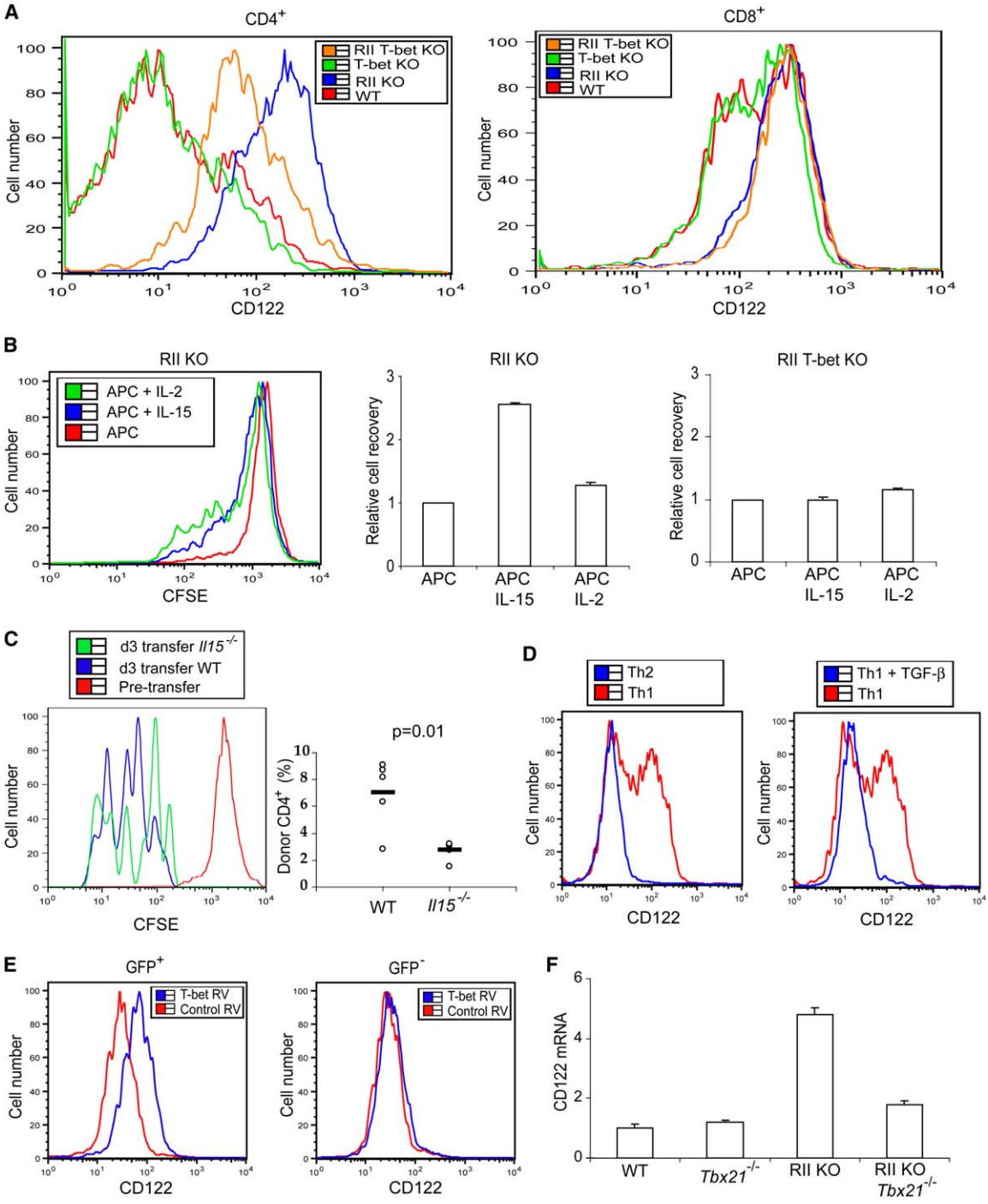

Figure 6. T-bet Regulation of CD122 Expression and IL-15 Responsiveness in Th1 Cells

(A) Expression of CD122 in splenic CD4$^+$ (left) and CD8$^+$ (right) T cells of RII/RII (wt), *Tbx21*$^{-/-}$ (T-bet KO), 4cre-RII/RII (RII KO), and 4cre-RII/RII *Tbx21*$^{-/-}$ (RII T-bet KO) mice. This is a representative plot of four mice from each group.

(B) 4cre-RII/RII CD4$^+$ T cells (RII KO) were CFSE labeled and cultured with antigen-presenting cells (APCs) in the absence or presence of IL-2 or IL-15 for 3 days. The amounts of CFSE (left) and the relative recovery of viable cells (middle) in these cultures are shown. 4cre-RII/RII *Tbx21*$^{-/-}$ CD4$^+$ T cells (RII T-bet KO) were cultured with APCs in the absence or presence of IL-2 or IL-15 for 3 days. The relative recovery of viable cells (right) is shown. These are representative results of two independent experiments. The error bars have been calculated with Excel STDEV, which gives the standard deviation.

(C) 4cre-RII/RII CD4$^+$ T cells were CFSE labeled and transferred into sublethally irradiated wild-type (wt) or *Il15*$^{-/-}$ mice. The representative CFSE profile of the transferred CD4$^+$ T cells in the blood at day 3 is shown (left). The percentage of the transferred CD4$^+$ T cells in the wt or *Il15*$^{-/-}$ recipients is depicted (n = 5, right).

(D) Naive CD4$^+$ T cells isolated from C57BL/6 mice were cultured under Th1 or Th2 conditions for 4 days (left) or Th1 condition in the absence or presence of TGF-β (right), and CD122 expression was compared between different conditions. These are representative results of two independent experiments.

expression in Th1 cells conferred their growth or survival advantage in response to IL-15 or IL-2, day 4 Th1 or Th2 cells were purified and recultured in the absence or presence of these cytokines. Increased viable Th1 cells but not Th2 cells were recovered when IL-15 was added to the cultures, and to a less extent when IL-2 was added (Figure S4A). These observations reveal that the IL-15-CD122 pathway selectively regulates the survival of Th1 cells.

To determine whether expression of T-bet was sufficient to induce CD122 expression under nonpermissive conditions, we transduced Th2 cells with a control or T-bet-expressing retrovirus and cultured these cells for another 3 days. Cells transduced with the T-bet retrovirus expressed higher amounts of CD122 than cells transduced with the control virus (Figure 6E), suggesting that T-bet may directly control CD122 expression. High amount of CD122 mRNA was found in 4cre-RII/RII $CD4^+$ T cells, which was substantially decreased in 4cre-RII/RII *Tbx21*$^{-/-}$ $CD4^+$ T cells (Figure 6F). Thus, increased CD122 surface expression in 4cre-RII/RII $CD4^+$ T cells was at least partly mediated by T-bet-dependent CD122 transcription. Several putative T-box transcription factor binding sites are present in the promoter region of the gene encoding CD122 (*Il2rb*) (Intlekofer et al., 2005). Eomesodermin is associated with the *Il2rb* promoter in $CD8^+$ T cells (Intlekofer et al., 2005). T-bet binds to *Il2rb* promoter in Th1 cells differentiated in vitro (L. Glimcher and G.M. Lord, personal communication). To investigate whether T-bet directly binds to the *Il2rb* promoter in 4cre-RII/RII $CD4^+$ T cells, we performed chromatin immunoprecipitation with a T-bet-specific antibody. Genomic fragments containing *Il2rb* promoter were highly enriched in 4cre-RII/RII $CD4^+$ T cells but not in the control T cells with the T-bet antibody (Figure S4B). In contrast, the promoter of the *Il4* gene, which is not a T-bet target, was only marginally enriched (Figure S4B). The augmented association of T-bet with *Il2rb* promoter in 4cre-RII/RII CD4 T cells supports *Il2rb* as a T-bet target gene in these cells.

Treg Cell-Independent TGF-β Regulation of OT-II T Cell Activation and Survival

Because TGF-β signaling affected thymic T cell development (Figures 2 and 3), it was possible that the peripheral T cell repertoire was altered in 4cre-RII/RII mice. In addition, in a Treg cell-centric view of peripheral T cell tolerance, the autonomous TGF-β regulation of T cell activation and differentiation (Figure 4) might simply reflect the requirement of TGF-β signaling for the suppressive function of Treg cells, as suggested by recent studies (Green et al., 2003; Chen et al., 2005; Fahlen et al., 2005; von Boehmer, 2005). In addition, previously reported studies of TGF-β on T cells in vitro were complicated by the fact that TGF-β induces Foxp3 expression and the differentiation of Treg cells in in vitro cultures (Chen et al., 2003; Fantini et al., 2004; Wan and Flavell, 2005). Therefore, it was not known whether TGF-β could regulate T cells independent of Treg cells. To address these questions, we crossed 4cre-RII/RII mice with OT-II ($CD4^+$ TCR specific for OVA peptide) transgenic mice onto *Rag1*$^{-/-}$ background, in order to exclude the T cell repertoire as a variable. 4cre-RII/RII OT-II T cells had no detectable surface expression of TGF-βRII (Figure S5A), revealing efficient deletion of TGF-βRII in these cells. Abrogation of TGF-βRII expression did not markedly affect thymic OT-II T cell development as determined by the expression of CD4, CD8, and TCR-β (Figure S5B and data not shown). Both RII/RII OT-II and 4cre-RII/RII OT-II T cells failed to differentiate into Foxp3-expressing Treg cells (Figure S5C), which rendered these mice an ideal model for the study of Treg cell-independent TGF-β regulation of T cell function.

In contrast to RII/RII OT-II T cells, most of which had a naive $CD44^{lo}CD62L^{hi}$ phenotype, a substantial proportion of splenic 4cre-RII/RII OT-II T cells exhibited an activated $CD44^{hi}CD62L^{lo}$ phenotype (Figure 7A). To determine whether 4cre-RII/RII OT-II T cells differentiated into effector Th1 or Th2 cells, we stimulated splenic OT-II T cells with PMA and ionomycin for 4 hr and performed intracellular cytokine staining with IFN-γ and IL-4 antibodies. The majority of 4cre-RII/RII OT-II T cells did not differentiate into either Th1 or Th2 cells in comparison to the dominant Th1 cell phenotype of polyclonal $CD4^+$ T cells from 4cre-RII/RII mice (Figure 7B). Lack of effector T cell differentiation of 4cre-RII/RII OT-II T cells was likely due to the absence of cognate antigens in vivo, because stimulation of these T cells with OVA peptide led to their differentiation into Th1 cells in vitro (Figure S5D). The majority of 4cre-RII/RII OT-II T cells also did not upregulate CD122 compared to RII/RII OT-II T cells (Figure 7C), which was consistent with the finding that CD122 was selectively expressed in Th1 cells. Therefore, abrogation of TGF-β signaling in OT-II T cells led to their partial activation but not differentiation into Th1 or Th2 cells.

Because we observed reduced number of $CD4^+$ T cells in 4cre-RII/RII *Tbx21*$^{-/-}$ mice associated with lack of Th1 differentiation and CD122 expression (Figures 5 and 6A), we investigated whether loss of TGF-β signaling in OT-II cells would affect their survival. Compared to RII/RII OT-II mice, the number of OT-II T cells was greatly reduced in the spleens and lymph nodes of 4cre-RII/RII OT-II mice (Figure 7D) and in the blood by 80%–90% (Figure 7D). We observed a 3- to 4-fold increase of annexinV-positive T cells in 4cre-RII/RII OT-II T cells compared to RII/RII OT-II T cells (Figure 7E), suggesting that the diminished number of T cells was due to increased apoptosis. These observations revealed an essential function for TGF-β signaling in promoting survival of peripheral OT-II T cells.

Discussion

Because of the diverse effects of TGF-β on multiple lineages of hemopoietic cells (Li et al., 2006), it has been

(E) Naive $CD4^+$ T cells were cultured under nonpolarizing conditions for 24 hr, transduced with T-bet or control retrovirus (RV), and recultured under Th2 conditions for 3 days. The expression of CD122 in GFP^+ (cells infected with retrovirus) and GFP^- populations is plotted.
(F) The relative expression of CD122 mRNA in $CD4^+$ T cells of RII/RII (wt), *Tbx21*$^{-/-}$, 4cre-RII/RII, or 4cre-RII/RII *Tbx21*$^{-/-}$ mice was determined by quantitative RT-PCR. These are representative results of two independent experiments. The error bars have been calculated with Excel STDEV, which gives the standard deviation.

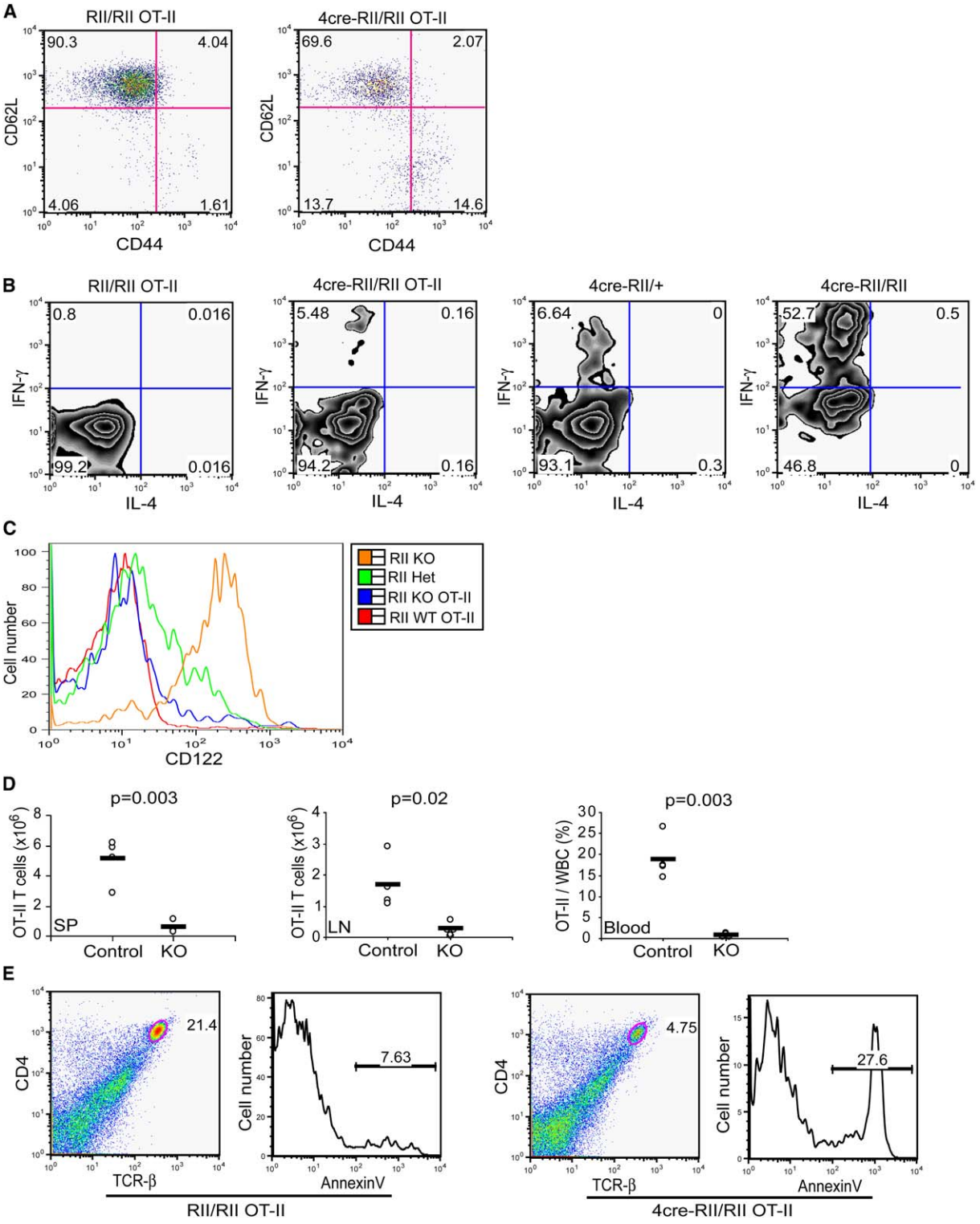

Figure 7. TGF-β Regulation of OT-II T Cell Activation, Differentiation, and Survival

(A) Expression of CD44 and CD62L in splenic RII/RII OT-II and 4cre-RII/RII OT-II T cells. These are representative results of five independent experiments.

(B) Splenic RII/RII OT-II, 4cre-RII/RII OT-II, 4cre-RII/+ CD4$^+$, and 4cre-RII/RII CD4$^+$ T cells were stimulated with PMA and ionomycin for 4 hr and analyzed for the expression of IFN-γ and IL-4 by intracellular cytokine staining.

(C) Expression of CD122 in splenic RII/RII OT-II (RII WT OT-II), 4cre-RII/RII OT-II (RII KO OT-II), 4cre-RII/+ CD4$^+$ (RII Het), and 4cre-RII/RII CD4$^+$ (RII KO) T cells.

(D) Number of OT-II T cells in the spleens (SP) and the lymph nodes (LN) of 4cre-RII/RII OT-II (KO), or littermate RII/RII OT-II and 4cre-RII/+ OT-II (control) mice (n = 4). The percentage of blood OT-II T cells among white blood cells (WBC) is also plotted.

very difficult to assess the role of TGF-β signaling on T cells with *Tgfb1*$^{-/-}$ mice. TGF-β has pleiotropic effects on T cells in vitro. In addition to the well-documented suppressive activity, studies have revealed positive functions for TGF-β on T cell differentiation (Halverson et al., 1997; Lingnau et al., 1998; Veldhoen et al., 2006), expansion, and/or survival (Cerwenka et al., 1994, 1996). Although the dominant-negative models (discussed in the Introduction) have helped to identify TGF-β as one of the most important molecules involved in T cell tolerance in vivo, the underlying mechanisms are not well understood. We have used the cre-loxp system to delete TGF-βRII in T cells and have explored its role in thymic T cell development and peripheral T cell activity. We found that TGF-β signaling promoted CD8$^+$ T cell maturation and NKT cell development, while inhibiting Treg cell proliferation in the thymus. The TGF-β signaling in T cells also maintained peripheral Treg cells, as well as inhibiting T cell proliferation, activation, and differentiation in a cell-autonomous manner, lack of which led to severe autoimmunity and early lethality in mice. Transcription factor T-bet regulated both Th1 differentiation and the survival of CD4$^+$ T cells in the absence of TGF-β signaling, which was associated with the induction of CD122 expression. Inhibition of TGF-β signaling in OT-II T cells resulted in their partial activation and marked apoptosis without differentiation into Th1 or Th2 cells. Our findings reveal previously undescribed potent and pleiotropic roles of TGF-β in T cells in vivo, which have important implications for our understanding of TGF-β regulation of T cell development, tolerance, and homeostasis.

Inhibition of TGF-β signaling caused reduced number of thymic mature CD8$^+$ T cells and impaired NKT cell development. A recent study with the transfer of TGF-βRII-deficient bone marrow cells concluded no thymocyte developmental defects in the absence of TGF-β signaling, although it reveals increased proliferation of CD8$^+$ thymocytes (Leveen et al., 2005). Importantly, in that report CD8$^+$ T cells were not separated into mature and immature single-positive T cells (ISPs). Since ISPs cycle more actively than mature T cells (Paterson and Williams, 1987), a defect in CD8$^+$ T cell maturation would lead to increased ISPs and the hyperproliferation phenotype of CD8$^+$ thymocytes. The mechanisms by which TGF-β regulates NKT cell development and CD8$^+$ T cell maturation remain to be defined. Signaling pathways originated from TCR and cytokines such as IL-7 critically regulate T cell-positive selection and lineage commitment (Aliahmad and Kaye, 2006). The potential crosstalk of TGF-β and these pathways in T cell development is currently under investigation.

Recent studies have highlighted a pivotal role for Treg cell-mediated immune suppression in peripheral T cell tolerance (Sakaguchi, 2004). However, signaling pathways that regulate Treg cell development and homeostasis remain largely undefined (Fontenot and Rudensky, 2005). Here we show that TGF-β signaling in T cells inhibited proliferation and expansion of Treg cells in the thymus, while it autonomously maintained Treg cells in peripheral tissues. Expansion of thymic Treg cells was not reported in *Tgfb1*$^{-/-}$ mice (Marie et al., 2005). It is possible that TGF-β2 and TGF-β3 may compensate for TGF-β1 in inhibiting the expansion of thymic Treg cells, as indicated by the fact that the activity of all three TGF-βs is blocked in our model of 4cre-RII/RII conditional knockout mice. Consistent with our findings, it was reported that Treg cells are reduced in the periphery of *Tgfb1*$^{-/-}$ mice (Marie et al., 2005). We further showed that reduced peripheral Treg cells in 4cre-RII/RII mice was not due to impaired proliferation of these cells; in fact, they underwent hyperproliferation. These observations suggested that the TGF-β pathway might be essential for the survival of peripheral Treg cells, which is supported by recent in vitro studies (Fu et al., 2004; Zheng et al., 2002). Alternatively, TGF-β may be required for the expression of Foxp3 and the prevention of Treg cells to differentiate into effector T cells, or the generation of Treg cells from naive T cells in the periphery (Kretschmer et al., 2005). Under either circumstance, the requirement of TGF-β signaling for the maintenance of Treg cells defines an important mechanism for TGF-β-induced T cell tolerance.

In addition to the maintenance of Treg cells, TGF-β autonomously inhibited T cell activation and differentiation. These findings revealed a cell-intrinsic requirement for TGF-β signaling in both Treg cells and naive T cells for maintaining T cell tolerance. In the absence of TGF-β signaling, CD4$^+$ T cells upregulated T-bet, differentiated into Th1 cells, and hyperproliferated. Here we demonstrate that T-bet regulated CD122 expression in Th1 cells and enabled these cells to respond to IL-15 and IL-2. These findings define a new role for IL-15 signaling in Th1 cells that is regulated by TGF-β. It is noteworthy that there is T-bet-independent CD122 expression in 4cre-RII/RII CD8$^+$ T cells and to a less extent in CD4$^+$ T cells. A recent study showed that both T-bet and its paralogue eomesodermin regulate CD122 expression in CD8$^+$ T cells (Intlekofer et al., 2005). Therefore, it is possible that in addition to T-bet, eomesodermin is capable of inducing CD122 expression in 4cre-RII/RII T cells, a hypothesis that is currently under investigation.

The nature of the antigens that drive the expansion and differentiation of effector T cells in 4cre-RII/RII mice remains to be fully characterized. Intriguingly, 4cre-RII/RII OT-II T cells on the *Rag1*$^{-/-}$ background were partially activated but not differentiated into Th1 or Th2 cells, which demonstrates that effector T cell differentiation is not a default pathway in the absence of TGF-β signaling. Stimulation of 4cre-RII/RII OT-II T cells with OVA peptides led to their differentiation to Th1 cells in vitro, suggesting that defective differentiation of 4cre-RII/RII OT-II T cells in vivo may be due to the absence of the cognate antigen stimulation.

Strikingly, TGF-βRII-deficient OT-II T cells underwent extensive apoptosis and were markedly depleted in peripheral tissues. Similar depletion of CD4$^+$ T cells was also found in 4cre-RII/RII *Tbx21*$^{-/-}$ mice in which CD4$^+$ T cells failed to differentiate into Th1 cells. In both cases, CD122 expression was diminished compared to that of 4cre-RII/RII CD4$^+$ T cells. Based on these observations, we hypothesize that lack of TGF-β signaling results in

(E) Splenic T cells from RII/RII OT-II or 4cre-RII/RII OT-II mice were gated by CD4 and TCR-β staining and analyzed for the presence of apoptotic cells by annexinV staining. These are representative results of two independent experiments.

apoptosis of CD4$^+$ T cells unless they differentiate into Th1 cells, which express T-bet and can be rescued from apoptosis by IL-15 signaling via CD122 upregulation. The effects of TGF-β signaling on CD4$^+$ T cell survival and differentiation may ensure a diverse and self-tolerant T cell repertoire in vivo, the lack of which results in the accumulation of effector CD4$^+$ Th1 cells in 4cre-RII/RII mice. We have shown that TGF-β inhibits CD8$^+$ T cell activation and differentiation. It remains to be determined whether TGF-β also regulates CD8$^+$ T cell survival. Interestingly, in 4cre-RII/RII mice, CD8$^+$ T cells were more actively cycling than CD4$^+$ T cells, yet CD8$^+$ T cells expanded similarly to CD4$^+$ T cells, suggesting that CD8$^+$ T cells underwent higher rates of apoptosis than CD4$^+$ T cells in the absence of TGF-β signaling. Collectively, these observations suggest that loss of peripheral T cell tolerance in 4cre-RII/RII mice is likely a combined effect of TGF-β regulation of T cell survival and effector T cell differentiation.

The mechanisms involved in TGF-β regulation of T cell survival remain to be determined. Previous studies with *Tgfb1*$^{-/-}$ mice showed increased thymic and peripheral T cell apoptosis associated with T cell activation (Chen et al., 2001). It is possible that increased T cell apoptosis in the absence of TGF-β signaling is a consequence of activation without effector T cell differentiation. However, we did not observe upregulation of death receptors Fas or TRAIL in 4cre-RII/RII OT-II T cells (unpublished observations), which are involved in activation-induced cell death. It is therefore possible that TGF-β may promote naive T cell survival independent of T cell activation. However, we conclude that TGF-β regulation of T cell activation and survival in 4cre-RII/RII mice is most likely Treg cell independent, since Treg cells were not present in OT-II *Rag1*$^{-/-}$ mice, and in a similar vein, deficiency of Foxp3 did not perturb OT-II T cell homeostasis (Fontenot et al., 2005).

In conclusion, in this report we have defined a multifaceted role for TGF-β signaling in T cell development, tolerance, and homeostasis and have revealed novel mechanisms by which TGF-β controls CD4$^+$ T cell differentiation and survival. These findings will advance our knowledge on the function of TGF-β in the immune system and can be exploited for the immunotherapy of autoimmune diseases and cancer.

Experimental Procedures

Mice

Floxed TGF-βRII mice, CD4-Cre transgenic mice, *Tbx21*$^{-/-}$ mice, and OT-II transgenic mice were previously reported (Barnden et al., 1998; Chytil et al., 2002; Lee et al., 2001; Szabo et al., 2002). C57BL/6 CD45.1 congenic mice and *Il15*$^{-/-}$ mice were obtained from Taconic Farms. Mice used in the transfer experiments have been backcrossed to C57BL/6 background for 5 to 6 generations. All mice were maintained under specific pathogen-free conditions. All animal experimentation was conducted in accordance with institutional guidelines.

Histopathology

Tissues from sacrificed animals were fixed in Streck Tissue Fixative (Streck Laboratories) and embedded in paraffin. 5 μm sections were stained with hematoxylin and eosin.

ELISA

The amounts of dsDNA antibody in mouse sera were determined with an ELISA kit from Alpha Diagnostic International. Sera from eight 4cre-RII/RII and eight littermate control mice aged from day 21 to 26 were assayed individually with 1:100 dilution in 1% BSA PBS.

Immunoblotting

To analyze Smad2 and Smad3 and T-bet expression, nuclear extracts were prepared as previously described (Li et al., 2001). 5 μg protein extract was separated on 4%–12% Bis-Tris NuPAGE Gels (Invitrogen) and transferred to PVDF membrane (Millipore). Membranes were probed with Smad2 and Smad3 antibody (clone 18, BD Transduction Laboratories) and T-bet antibody (clone 4B10, Santa Cruz Biotechnology). Subsequently, the membranes were striped with Restore Western Blot Stripping Buffer (Pierce) and reprobed with HDAC-1 antibody (Sigma).

Quantitative RT-PCR

CD4$^+$ T cells were purified with anti-CD4 microbeads (Miltenyi Biotec). RNA was isolated with RNeasy Mini Kit (Qiagen) and reverse transcribed into cDNA with proSTAR First-Strand RT-PCR Kit (Stratagene) according to the manufacturer's instructions. Presynthesized Taqman Gene Expression Assay (Applied Biosystems) was used to amplify mouse CD122 mRNA (*Il2rb*; Mm00434264_m1). Hypoxanthine phosphoribosyltransferase (*Hprt*) was used as an internal reference and measured with the primers 5′-CTGGTGAAAAG GACCTCTCG-3′ and 5′-TGAAGTACTCATTATAGTCAAGGGCA-3′ at a final concentration of 200 nM and with the TaqMan probe 5′-FAM-TGTTGGATACAGGCCAGACTTTGTTGGAT-BHQ-1-3′ at a final concentration of 200 nM. Relative expression of *Il2rb* normalized to *Hprt* was presented.

Chromatin Immunoprecipitation

CD4$^+$ T cells were fixed for 10 min at room temperature with 10% formaldehyde in 0.1 M NaCl, 1 mM EDTA, 0.5 mM EGTA, and 50 mM HEPES. After incubation, glycine was added to a final concentration of 0.125 M to "quench" the formaldehyde for 5 min. Cells were pelleted, washed once with ice-cold PBS, and lysed on ice for 10 min with lysis buffer A (10 mM Tris [pH 8], 10 mM EDTA, 0.5 mM EGTA, 0.25% Triton, 0.5% NP40, and the protease inhibitors [PIs] from Roche). Lysates were pelleted and washed once in lysis buffer B (10 mM Tris [pH 8], 1 mM EDTA, 0.5 mM EGTA, 0.2 M NaCl, and PIs) before being resuspended in the sonication buffer (10 mM Tris [pH 8], 1 mM EDTA, 0.5 mM EGTA, and PIs). Lysates were sonicated to reduce DNA length to between 500 and 1000 base pairs. The soluble fraction was reconstituted with 0.5% sarcosyl and 5% glycerol, and aliquoted. 20 μg of the chromatin was precleared with protein A and protein G agarose beads in RIPA.IP buffer (1% Triton, 0.1% DOC, 140 mM NaCl, and PIs) for 1 hr, and was then incubated with 5 μg of T-bet antibody (clone 4B10, Santa Cruz Biotechnology). Immune complexes were precipitated with protein A and protein G agarose beads preblocked in RIPA.IP buffer with 20 μg/ml salmon sperm DNA and 20 μg/ml BSA. Immunoprecipitates were washed seven times in the RIPA wash buffer (1% Triton, 0.1% DOC, 500 mM NaCl, 0.1% SDS, and PIs), and eluted in 100 μl of TE with 0.5% SDS and 200 μg/ml proteinase K. Precipitated DNA was further purified with phenol/chloroform extranction and ethanol precipitation and was analyzed by PCR. The primers used in the analysis of binding included: *Il2rb*, 5′-GTGCTCATGCGTGAG CAGAAG-3′ and 5′-GCCCACTTCCTGTATAGAGGATG-3′; *Il4*, 5′-AGAAGGTCTGCCTCCATCATCC-3′ and 5′-TTATCAGCGTAGGGTT GCCACTGG-3′.

T Cell Culture and Retroviral Transduction

Splenic and lymph node CD4$^+$ T cells were enriched by positive selection with anti-CD4 microbeads (Miltenyi Biotec). Naive T cells were further purified from C57BL/6 mice with a cell sorter (Becton Dickinson) by gating on the CD4$^+$CD25$^-$CD62L^{hi}CD44^{lo} population. For Th1 culture, 3 × 10^5 CD4$^+$ T cells were stimulated with 3 × 10^6 irradiated antigen-presenting cells (APCs) in the presence of 100 u/ml IL-2, 35 ng/ml IL-12, 5 μg/ml CD3, 2 μg/ml CD28, and 10 μg/ml IL4 antibodies (11B11 clone) for 4 days. Th1 cells were also differentiated in the presence of TGF-β1 for 4 days (2 ng/ml). For Th2 culture, CD4$^+$ T cells were stimulated with APCs in the presence of 100 u/ml IL-2, 1000 u/ml IL-4, 5 μg/ml CD3, 2 μg/ml CD28, and 10 μg/ml IFN-γ antibodies (XMG clone) for 4 days. Th1 or Th2 cells were Ficoll

purified and cultured in the absence or presence of IL-15 (20 ng/ml) or IL-2 (100 U/ml) for 3 days. For retroviral transduction experiments, $CD4^+$ T cells were stimulated with APCs in the presence of 100 u/ml IL-2, 5 μg/ml CD3, and 2 μg/ml CD28 antibodies. At 24 hr after stimulation, T cells were resuspended in T-bet or control retroviral supernatant and subjected to spin infection. Subsequently, cells were cultured in fresh medium under Th2 conditions for 3 days. For the analysis of 4cre-RII/RII or 4cre-RII/RII *Tbx21*$^{-/-}$ T cells, microbead-purified $CD4^+$ T cells were labeled with CFSE and cultured with APCs in the absence or presence of IL-15 or IL-2 for 3 days. For the differentiation of OT-II T cells in vitro, RII/RII OT-II or 4cre-RII/RII OT-II T cells were purified by positive selection with anti-CD4 microbeads, and splenic dendritic cells were purified from C57BL/6 mice with anti-CD11c microbeads (Miltenyi Biotec). 1×10^5 OT-II T cells were cocultured with 2×10^5 dendritic cells in the presence of 0.05 μM OVA peptides (ISQAVHAAHAEINEAGR) for 3 days.

BrdU Labeling

Day 16 4cre-RII/RII or littermate control mice were given 50 mg/kg BrdU by i.p. injection. 16 hr later, thymic, splenic, and lymph node T cells were prepared and analyzed.

Flow Cytometry

Cells from spleens, lymph nodes, or thymus were depleted of erythrocytes by hypotonic lysis. Cells were incubated with specific antibodies for 30 min on ice in the presence of 2.4G2 mAb to block FcγR binding. All samples were analyzed with FACSCalibur (Becton Dickinson) and FloJo (Tree Star) software. All antibodies were obtained from BD Biosciences PharMingen with the exception of biotinylated mouse TGF-βRII antibody (R&D Systems), Foxp3 antibody (e-Biosciences), and BrdU antibody (Becton Dickinson). PE-labeled Cd1d-aGalCer was kindly provided by Dr. M. Kronenberg (La Jolla Institute for Allergy and Immunology). For intracellular cytokine staining, single-cell suspensions of spleens or lymph nodes were stimulated with 50 ng/ml phorbol 12-myristate 13-acetate (PMA, Sigma) and 1 μM ionomycin (Sigma) for 4 hr; GolgiStop (BD Biosciences PharMingen) were added for the final 2 hr of culture. After stimulation, cells were first stained with CD4, CD8, TCR-β, and CD45.2 antibodies, then fixed and permeabilized with a Cytofix/Cytoperm kit (BD Biosciences PharMingen), and finally stained with IFN-γ and IL-4 antibodies. Mice were also injected i.p. with 0.25 mg Brefeldin A (Sigma). 6 hr later, peripheral T cells were harvested and stained with IFN-γ and IL-4 antibodies. Intracellular Foxp3 staining was carried out with a kit from e-Biosciences. After Foxp3 staining, some of the cells were also stained with BrdU antibody. To analyze cell apoptosis, cells were stained with FITC-labeled annexin V (BD Biosciences PharMingen) according to the manufacturer's instructions.

Adoptive Transfer of T Cells

Splenic and lymph node $CD45.1^+CD4^+CD25^+$ T cells were purified with microbeads. 3×10^5 T cells were injected i.p. to 2-day-old $CD45.2^+$ 4cre-RII/RII or littermate control mice. These mice were monitored for the development of disease and analyzed at day 21 of age. Splenic and lymph node 4cre-RII/RII $CD4^+$ T cells were purified with microbeads and labeled with CFSE. 2×10^6 T cells were injected i.v. to C57BL/6 or *Il15*$^{-/-}$ mice that were irradiated at 700 rad 1 day before the transfer. 3 days later, these mice were bled and analyzed for the proliferation and the recovery of the transferred T cells.

Generation of Bone Marrow Chimeras

Bone marrow cells isolated from 6-week-old $CD45.1^+$ C57BL/6 (wt) mice or 3-week-old $CD45.2^+$ 4cre-RII/RII (KO) mice were depleted of erythrocytes by hypotonic lysis and T cells and antigen-presenting cells by complement-mediated cell lysis. 2×10^6 wt, KO, or 1:1 mixed wt and KO bone-marrow cells were injected i.v. to 6- to 8-week-old sublethally irradiated (600 rad) *Rag1*$^{-/-}$ mice. Reconstituted mice were maintained on autoclaved Sulfatrim-containing water for 2 weeks and on regular water afterwards.

Statistical Analyses

Student's t test was used to calculate statistical significance for difference in a particular measurement between groups. p value of ≤ 0.05 was considered statistically significant.

Supplemental Data

Six Supplemental Figures can be found with this article online at http://www.immunity.com/cgi/content/full/25/3/455/DC1/.

Acknowledgments

We are indebted to H. Moses (Vanderbilt University Medical Center) for floxed TGF-βRII mice and L. Glimcher (Harvard School of Public Health) for *Tbx21*$^{-/-}$ mice. We thank M. Kronenberg (La Jolla Institute for Allergy and Immunology) for CD1d tetramer and Wyeth Laboratories for IL-12. We are also grateful to C. Spilianakis for the help with the ChIP experiment and M. Kriegl for critical commentary on the manuscript. R.A.F. is an investigator of the Howard Hughes Medical Institute. This work is supported by a postdoctoral fellowship grant from the American Cancer Society (M.O.L.), an NIH KO1 award (M.O.L.), and a postdoctoral fellowship grant from Cancer Research Institute (S.S.), with additional support from American Diabetes Association (R.A.F.) and NIH grant RO1DK51665 (R.A.F.). The authors declare that they have no competing financial interests.

Received: March 29, 2006
Revised: June 22, 2006
Accepted: July 12, 2006
Published online: September 14, 2006

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