Transforming Growth Factor-β Signaling Curbs Thymic Negative Selection Promoting Regulatory T Cell Development

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SUMMARY

Thymus-derived naturally occurring regulatory T (nTreg) cells are necessary for immunological self-tolerance. nTreg cell development is instructed by the T cell receptor and can be induced by agonist antigens that trigger T cell-negative selection. How T cell deletion is regulated so that nTreg cells are generated is unclear. Here we showed that transforming growth factor-β (TGF-β) signaling protected nTreg cells and antigen-stimulated conventional T cells from apoptosis. Enhanced apoptosis of TGF-β receptor-deficient nTreg cells was associated with high expression of proapoptotic proteins Bim, Bax, and Bak and low expression of the antiapoptotic protein Bcl-2. Ablation of Bim in mice corrected the Treg cell development and homeostasis defects. Our results suggest that nTreg cell commitment is independent of TGF-β signaling. Instead, TGF-β promotes nTreg cell survival by antagonizing T cell negative selection. These findings reveal a critical function for TGF-β in control of autoreactive T cell fates with important implications for understanding T cell self-tolerance mechanisms.

INTRODUCTION

The stochastic process by which T cell antigen receptors (TCRs) are generated produces T cells bearing TCRs with high affinity for self-antigens. Both cell-intrinsic and cell-extrinsic mechanisms have evolved to control pathogenic autoreactive T cells. T cells encountering high-affinity self-antigens in the thymus can be eliminated through apoptosis (negative selection), which is mediated in part by the proapoptotic molecule Bim (Bouillet et al., 2002; Hogquist et al., 2005; Mathis and Benoist, 2004; Palmer, 2003). In addition, regulatory T (Treg) cells expressing the transcription factor Foxp3 are required to keep in check the autoreactive T cells that evade negative selection (Feuerer et al., 2009; Josefowicz and Rudensky, 2009; Sakaguchi et al., 2008; Shevach, 2009).

Thymic differentiation of naturally occurring CD4^{+}Foxp3^{+} Treg (nTreg) cells is regulated by TCR affinity. Studies with TCR transgenic mouse models reveal that engagement of agonist

self-peptides induces not only T cell negative selection but also nTreg cell differentiation (Apostolou et al., 2002; Jordan et al., 2001; Kawahata et al., 2002; Walker et al., 2003). The mechanisms by which nTreg cells are protected from clonal deletion are unclear. nTreg cells or their precursors might be inherently more resistant to negative selection than conventional T cells (van Santen et al., 2004). The magnitude of clonal deletion may also be regulated so that large numbers of nTreg cells are produced to suppress autoreactive T cells. How TCR signaling is integrated to the differentiation program of nTreg cells, which culminates in the stable expression of Foxp3, also remains incompletely understood. However, additional signals from costimulatory receptors such as CD28 and cytokines including the common γ-chain cytokines appear essential for the lineage commitment of nTreg cells (Burchill et al., 2007; Fontenot et al., 2005; Malek et al., 2002; Salomon et al., 2000; Tai et al., 2005; Vang et al., 2008).

Transforming growth factor-β (TGF-β) is a regulatory cytokine with pleiotropic functions in control of T cell responses (Li and Flavell, 2008). TGF-β1-deficient mice or mice with T cell-specific deletion of TGF-β receptors develop early fatal multifocal inflammatory diseases, highlighting a pivotal role for TGF-β in T cell tolerance. How TGF-β regulates T cell tolerance and its interactions with other self-tolerance pathways including T cell-negative selection and Treg cell-mediated suppression have yet to be clarified. Activation of naive T cells in the presence of TGF-β induces Foxp3 expression and the differentiation of induced Treg (iTreg) cells (Chen et al., 2003; Kretschmer et al., 2005; Zheng et al., 2004). In contrast to the thymic origin of nTreg cells, iTreg cells are differentiated in the periphery, and they may control immune tolerance to innocuous environmental antigens such as those derived from commensal flora (Curotto de Lafaille and Lafaille, 2009). TGF-β-induced iTreg cell differentiation is in part mediated by the recruitment of its downstream transcription factor Smad3 to a *Foxp3* enhancer element and the consequent induction of *Foxp3* gene expression (Tone et al., 2008).

The function of and mechanism by which TGF-β controls nTreg cell differentiation and homeostasis remain ill-defined. Studies with mice with T cell-specific deletion of the TGF-β type II receptor (*Tgfbr2*) gene showed that TGF-β signaling is dispensable for the development of nTreg cells in 12- to 16-day-old mice (Li et al., 2006; Marie et al., 2006). A recent report, however, revealed an earlier requirement for TGF-β signaling in nTreg cell development. Conditional deletion of the TGF-β type I receptor (*Tgfbr1*) gene in T cells blocks thymic nTreg cell differentiation in 3- to 5-day-old mice but triggers nTreg cell expansion in

mice older than 1 week (Liu et al., 2008). It was postulated that TGF-β signaling was required for the induction of *Foxp3* gene expression and nTreg cell lineage commitment in neonatal mice similar to iTreg cells (Liu et al., 2008). The later expansion, a phenomenon also observed in mice deficient in TGF-βRII, was explained by the enhanced nTreg cell proliferation in response to increasing amounts of the cytokine interleukin-2 (IL-2) (Li et al., 2006; Liu et al., 2008). Despite uncompromised thymic production of nTreg cells in 12- to 16-day-old TGF-β receptor-deficient mice, Treg cells are reduced in the peripheral lymphoid organs of these mice, concomitant with the induction of rampant inflammatory diseases (Li et al., 2006; Liu et al., 2008; Marie et al., 2006). The mechanisms by which TGF-β maintains peripheral Treg cells remain to be determined.

In this study, with a T cell-specific TGF-βRII-deficient mouse model, we found that TGF-β signaling protected thymocytes from negative selection. In addition, TGF-β signaling inhibited nTreg cell apoptosis that was associated with imbalanced expression of anti- and proapoptotic Bcl-2 family proteins. Genetic ablation of the proapoptotic molecule Bim rescued nTreg cell death and restored the number of thymic nTreg cells in TGF-βRII-deficient mice. Bim deficiency also corrected the Treg cell homeostasis defects, attenuated T cell activation and differentiation, and prolonged the lifespan of TGF-βRII-deficient mice. These observations revealed a crucial function for TGF-β in inhibiting T cell-negative selection and nTreg cell apoptosis. This function was discrete from TGF-β induction of Foxp3 expression and iTreg cell differentiation. These findings also showed that T cell TGF-β signaling was essential for the survival of peripheral Treg cells and for the inhibition of autoreactive T cells. Collectively, our results demonstrate that TGF-β hinders deletional tolerance but promotes immune suppression to control T cell autoreactivity.

RESULTS

Enhanced Anti-CD3-Induced T Cell Apoptosis in the Absence of TGF-β Signaling

Among the numerous properties of TGF-β in the immune system is its ability to control T cell tolerance (Li and Flavell, 2008). We sought to investigate how T cell responses to high-affinity self-antigens are modulated by TGF-β signaling transduced by TGF-βRI and TGF-βRII receptors. To determine whether TGF-β receptor expression is regulated during T cell development, we examined mRNA expression in immature CD4$^+$CD8$^+$ and mature TCR-β^{hi}CD4$^+$ and TCR-β^{hi}CD8$^+$ thymocytes. mRNA encoding the ligand-binding receptor TGF-βRII, but not TGF-βRI, showed approximately 5-fold higher expression in mature T cells than in immature T cells (Figure 1A and data not shown), which was associated with the enhanced TGF-βRII protein expression (Figure 1B; Figure S1A available online). These observations suggested that TGF-βRII-dependent signaling might regulate T cell selection.

Thymocytes bearing high-affinity TCRs for self-antigens undergo clonal deletion or negative selection, which provides an important mechanism for the prevention of autoimmunity (Hogquist et al., 2005; Mathis and Benoist, 2004; Palmer, 2003). To determine whether TGF-βRII is required for clonal deletion, we used a T cell-specific TGF-βRII-deficient (*Tgfbr2$^{-/-}$*)

mouse model generated by crossing a strain of floxed *Tgfbr2* mice with the CD4-Cre transgene (Li et al., 2006). With these mice, we and others have shown that TGF-βRII-dependent signaling is essential for the maintenance of T cell tolerance (Li et al., 2006; Marie et al., 2006), but the underlying mechanisms remain elusive. Neonatal 4-day-old wild-type (*Tgfbr2$^{+/+}$*) and *Tgfbr2$^{-/-}$* mice were injected with either PBS or CD3 antibody to model high-affinity TCR ligation. 24 hr later, thymi from these mice were collected, and the immature and mature T cells were enumerated. As expected, T cell numbers from *Tgfbr2$^{-/-}$* and *Tgfbr2$^{+/+}$* mice in the PBS control group were comparable with the exception of a 50% reduction of TCR-β^{hi}CD8$^+$ T cells in *Tgfbr2$^{-/-}$* mice as previously reported (Figure 1C; Li et al., 2006). Surprisingly, thymocytes, notably TCR-β^{hi}CD4$^+$ and TCR-β^{hi}CD8$^+$ mature T cell subsets, were more profoundly depleted in *Tgfbr2$^{-/-}$* mice administrated with CD3 antibody (Figure 1C). Enhanced T cell deletion was associated with a 3-fold increase in the size of apoptotic areas in tissue sections from the thymi of *Tgfbr2$^{-/-}$* mice detected by TUNEL staining (Figure 1D). Therefore, intact TGF-β signaling appeared to be required to protect T cells from anti-CD3-induced T cell apoptosis.

Most peripheral T cells from 4-day-old *Tgfbr2$^{-/-}$* mice manifested a naive CD44^{lo}CD62L^{hi} phenotype similar to T cells from *Tgfbr2$^{+/+}$* mice (data not shown). However, CD3 antibody might activate these T cells and trigger the release of proinflammatory cytokines and stress hormone that could obscure TCR-induced deletion of thymocytes (Brewer et al., 2002). To avoid the potential complication of peripheral T cells, we isolated thymocytes from *Tgfbr2$^{-/-}$* and *Tgfbr2$^{+/+}$* mice and cultured them with CD3 and CD28 antibodies for 24 hr. Subsequently, apoptotic cells in culture were assessed with annexin V staining. Compared to T cells from *Tgfbr2$^{+/+}$* mice, increased apoptosis was observed in TCR-β^{hi}CD4$^+$ and TCR-β^{hi}CD8$^+$ T cells from *Tgfbr2$^{-/-}$* mice (Figures S1B and S1C). These observations supported a direct role for TGF-β signaling in inhibiting anti-CD3-induced T cell apoptosis.

Exaggerated T Cell Negative Selection in the Absence of TGF-β Signaling

To determine a definitive function for TGF-β in control of antigen-induced T cell negative selection, we used a TCR transgenic mouse model. OT-II (CD4$^+$ TCR specific for an ovalbumin peptide) transgenic mice were crossed to the RIP-mOVA transgene driving expression of a membrane-bound form of ovalbumin (mOVA) under the control of a rat insulin promoter (RIP) (Kurts et al., 1996). In addition to mOVA expression in the pancreatic β cells, mOVA is expressed in the medullary thymic epithelial cells, leading to a pronounced thymic deletion of OT-II T cells (Anderson et al., 2005; Gallegos and Bevan, 2004). OT-II mice were further crossed onto *Rag1$^{-/-}$* background to prevent the rearrangement of endogenous TCR that may alter T cell antigen specificity.

In line with our previous observations (Li et al., 2006), TGF-βRII deficiency did not affect OT-II T cell positive selection in the absence of mOVA expression (Figures 2A and 2B). However, thymic deletion of TCR-β^{hi} OT-II T cells was markedly enhanced in 5-week-old *Tgfbr2$^{-/-}$* mice on RIP-mOVA background (Figure 2A), which was associated with a profound reduction of
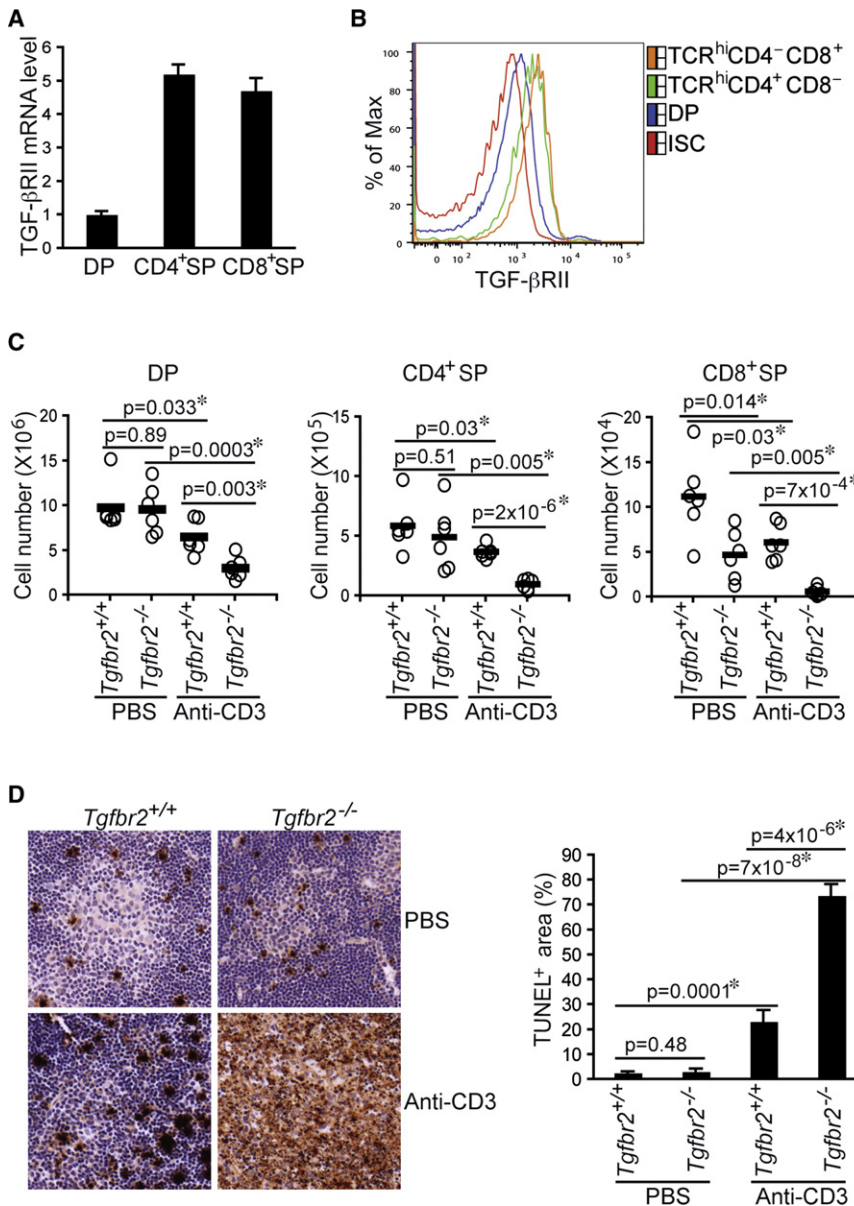
Figure 1. Enhanced Anti-CD3-Induced T Cell Apoptosis in TGF-βRII-Deficient Mice

(A and B) TGF-βRII expression in CD4⁺CD8⁺ double-positive (DP), CD4⁺CD8⁻ single-positive (CD4⁺SP), and CD4⁻CD8⁺ (CD8⁺SP) thymocytes was determined by quantitative PCR (A) and flow cytometric analysis (B). ISC stands for isotype control.

(C and D) Four-day-old wild-type ($Tgfbr2^{+/+}$) and TGF-βRII-deficient ($Tgfbr2^{-/-}$) mice were intraperitoneally injected with PBS or 20 μg/mouse of anti-CD3. Cell numbers of DP, CD4⁺SP, and CD8⁺SP thymocytes were determined 24 hr after the injection (C, n = 6). Thymocyte apoptosis was examined by TUNEL staining 48 hr after the injection (D, left, original magnification, 20×). TUNEL staining-positive area of four sections per group was quantified by MetaMorph software (D, right). Data are representative of two (A) and three (B–D) independent experiments. The p values between the groups are shown. Asterisk depicts significant difference.

TGF-β Control of Peripheral T Cell Tolerance to a Neo-self Antigen

Mice with T cell-specific deletion of TGF-β receptors develop fatal systemic inflammatory diseases on a polyclonal T cell background, which is associated with widespread T cell activation (Li et al., 2006; Liu et al., 2008; Marie et al., 2006). However, studies with TCR-transgenic mouse models reveal that most TGF-β receptor-deficient T cells specific for foreign antigens exhibit a naive T cell phenotype (Li et al., 2006; Marie et al., 2006). We wished to investigate whether cognate antigen stimulation was required for T cell hyperactivation in the absence of TGF-β signaling. To this end, we first determined whether TGF-βRII deficiency would affect T cell tolerance in OT-II RIP-mOVA mice. Consistent with previous reports (Anderson et al., 2005; Gallegos and Bevan, 2004), wild-type OT-II RIP-mOVA mice were tolerized to the oval-

mature CD69⁻CD62L⁺ OT-II T cells (Figure 2B). $Tgfbr2^{-/-}$ OT-II RIP-mOVA mice started to develop diabetes at 6 weeks of age (see below). It was possible that T cell negative selection may be affected by diabetes-induced stress in week-old mice. To minimize this effect, we determined T cell deletion in 8-day-old mice. Diminished TCR-β^hi and mature CD69⁻CD62L⁺ OT-II T cells were similarly observed in $Tgfbr2^{-/-}$ RIP-mOVA mice (Figure S2A). To directly assess T cell survival potential, thymic TCR-β^hi OT-II T cells were isolated from $Tgfbr2^{-/-}$ and $Tgfbr2^{+/+}$ RIP-mOVA mice by FACS sorting and cultured in medium for 12 hr. Compared to $Tgfbr2^{+/+}$ OT-II T cells, approximately 50% viable $Tgfbr2^{-/-}$ OT-II T cells were recovered (Figure 2C). Taken together, these findings revealed an unexpected function for TGF-β signaling in protecting T cells from antigen-induced negative selection.

bumin antigen and remained diabetes free for at least 8 months (Figure 3A and data not shown). In striking contrast, 6-week-old $Tgfbr2^{-/-}$ OT-II RIP-mOVA mice started to develop diabetes, and all mice became diabetic by 10 weeks of age (Figure 3A). Histological analysis of tissue sections from TGF-βRII-deficient, but not wild-type, OT-II RIP-mOVA mice revealed an aggressive leukocyte infiltrate in the islets of the pancreas (Figure 3B). In line with these observations, TGF-βRII-deficient but not wild-type OT-II T cells isolated from the pancreatic draining lymph nodes displayed an activated CD44^hiCD62L^lo phenotype (Figure 3C). However, most $Tgfbr2^{-/-}$ OT-II T cells from the nonpancreatic draining lymph nodes of RIP-mOVA mice, which presumably had not been exposed to mOVA antigen, exhibited a CD44^loCD62L^hi naive T cell phenotype (Figure 3C). These observations demonstrated that despite enhanced T cell-negative selection in the
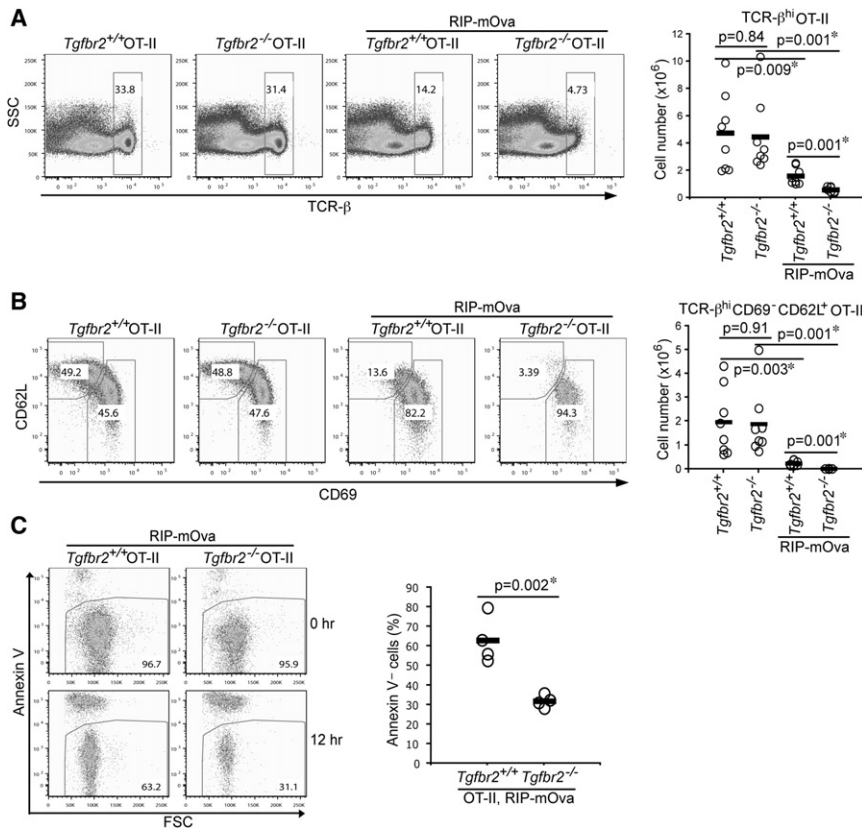
Figure 2. Exaggerated T Cell Negative Selection in the Absence of TGF-β Signaling

(A) Flow cytometric analysis of TCR-β expression in thymic OT-II T cells from 5-week-old $Tgfbr2^{+/+}$ and $Tgfbr2^{-/-}$ OT-II mice in the absence or presence of RIP-mOva transgene (left). The numbers of thymic TCR-β^{hi} OT-II T cells from eight groups of mice are shown (right).

(B) Flow cytometric analysis of CD69 and CD62L expression in thymic TCR-β^{hi} OT-II T cells from 5-week-old $Tgfbr2^{+/+}$ and $Tgfbr2^{-/-}$ OT-II mice in the absence or presence of RIP-mOva transgene (left). The numbers of thymic TCR-β^{hi}CD69$^-$ CD62L$^+$ OT-II T cells from eight groups of mice are shown (right).

(C) Survival of thymic OT-II T cells from $Tgfbr2^{+/+}$ and $Tgfbr2^{-/-}$ mice. Thymic TCR-β^{hi} OT-II T cells were purified by FACS sorting and cultured for 12 hr. T cell viability before and after the cell culture was determined by annexin V staining (left). Percentages of viable T cells at 12 hr from four pairs of mice are presented (right). The p values between the groups are shown. Asterisk depicts significant difference.

absence of T cell TGF-β signaling, an intact TGF-β pathway was essential for the inhibition of antigen-induced T cell activation and for the maintenance of peripheral T cell tolerance.

Increased Thymic nTreg Cell Death in the Absence of TGF-β Signaling

In addition to T cell negative selection, thymic generation of nTreg cells is essential for the establishment of immunological self-tolerance (Feuerer et al., 2009; Josefowicz and Rudensky, 2009; Sakaguchi et al., 2008; Shevach, 2009). Using TCR transgenic mice that express the cognate antigens in the thymus, it has been shown that nTreg cells can be generated in response to TCR agonist ligand stimulation (Apostolou et al., 2002; Jordan et al., 2001; Kawahata et al., 2002; Walker et al., 2003). To investigate whether TGF-β signaling regulates nTreg cell differentiation, we determined the frequency and number of thymic nTreg cells in OT-II mice. In the absence of mOVA, nTreg cells were barely detectable in either $Tgfbr2^{+/+}$ or $Tgfbr2^{-/-}$ OT-II mice (Figure 4A; Figure S2B). On the RIP-mOVA background, 1%–5% TCR-β^{hi} OT-II T cells differentiated into Foxp3$^+$ nTreg cells in $Tgfbr2^{+/+}$ and $Tgfbr2^{-/-}$ mice (Figure 4A; Figure S2B). However, as a consequence of enhanced mOVA antigen-induced T cell deletion (Figure 2), the numbers of both Foxp3$^+$ nTreg cells and Foxp3$^-$ mature T cells from $Tgfbr2^{-/-}$ mice were reduced to approximately 25% of those from $Tgfbr2^{+/+}$ mice (Figure 4A and data not shown). A recent study showed that nTreg cell development is compromised in neonatal mice with a T cell-specific inactivation of TGF-βRI, which was postulated to be caused by defective

Foxp3 induction (Liu et al., 2008). Because we observed similar frequencies of nTreg cells in $Tgfbr2^{-/-}$ and $Tgfbr2^{+/+}$ OT-II RIP-mOVA mice (Figure 4A; Figure S2B), we hypothesized that nTreg cell survival, rather than its lineage commitment, was dependent on TGF-β signaling.

We tested this hypothesis by using $Tgfbr2^{+/+}$ and $Tgfbr2^{-/-}$ mice on a polyclonal T cell background. Consistent with the previous report (Liu et al., 2008), the number of thymic nTreg cells in 3- to 5-day-old $Tgfbr2^{-/-}$ mice was about 25% of that in $Tgfbr2^{+/+}$ mice (Figure 4B). The numbers of TCR-β^{hi}CD4$^+$ Foxp3$^-$ conventional T cells were comparable between $Tgfbr2^{-/-}$ and $Tgfbr2^{+/+}$ mice (data not shown), most of which were probably low-affinity TCR T cells that had undergone positive selection. A higher proportion of $Tgfbr2^{-/-}$ nTreg cells expressed the cell proliferation marker Ki-67 than $Tgfbr2^{+/+}$ nTreg cells (Figure 4C), suggesting that reduction of $Tgfbr2^{-/-}$ nTreg cells was not caused by defective cell division. To directly assess T cell survival potential, we purified thymic nTreg cells and conventional TCR-β^{hi}CD4$^+$ T cells based on the expression of Foxp3 marked by a red-fluorescent protein (Wan and Flavell, 2005). Whereas $Tgfbr2^{+/+}$ and $Tgfbr2^{-/-}$ conventional T cells and $Tgfbr2^{+/+}$ nTreg cells had a comparable survival rate, 5-fold more $Tgfbr2^{-/-}$ nTreg cells underwent cell death after a 12 hr culture (Figure 4D). Reduced numbers of viable $Tgfbr2^{-/-}$ nTreg cells were observed as early as 6 hr after in vitro culture (Figure S3A). These observations revealed that concomitant with TGF-β inhibition of T cell-negative selection, TGF-β signaling promoted survival of thymic nTreg cells.

Bim Regulation of nTreg Cell Apoptosis in TGF-βRII-Deficient Mice

We next sought to investigate the mechanisms underlying the exaggerated cell death of $Tgfbr2^{-/-}$ nTreg cells. Deletion of
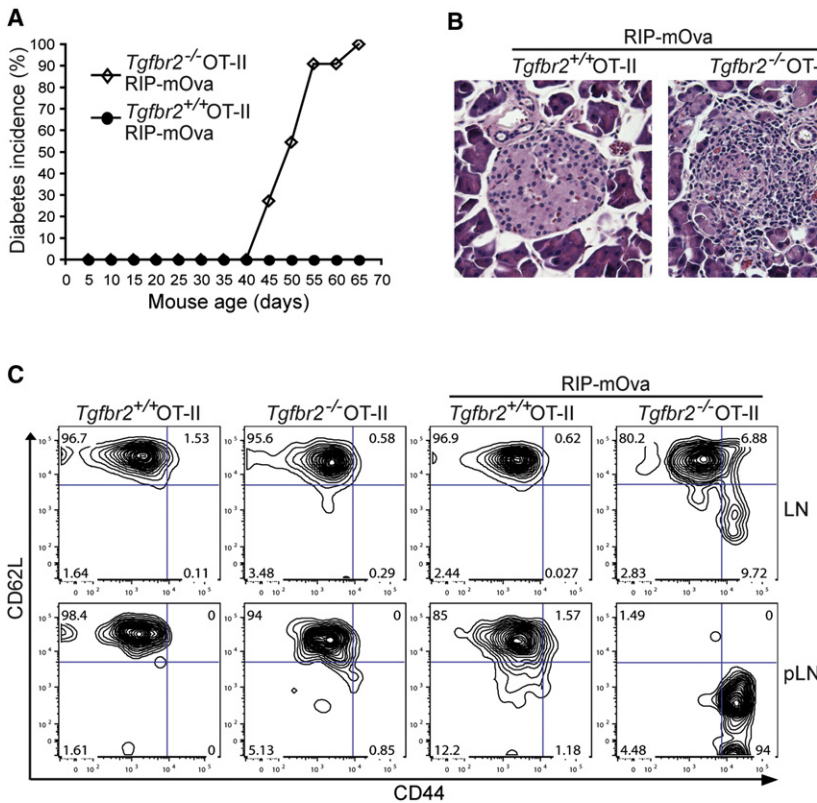
Figure 3. Diabetes Development and T Cell Activation in TGF-βRII-Deficient OT-II RIP-mOva Mice

(A) The incidence of diabetes in *Tgfbr2*^{+/+} and *Tgfbr2*^{−/−} OT-II RIP-mOva mice (n = 11).

(B) Hematoxylin and eosin staining of the pancreas of 8-week-old *Tgfbr2*^{+/+} and *Tgfbr2*^{−/−} OT-II RIP-mOva mice (original magnification, 20×). These are representative results of four mice per group analyzed.

(C) Flow cytometric analysis of CD44 and CD62L expression in OT-II T cells from nonpancreatic control lymph nodes (LN) and pancreatic lymph nodes (pLN) of 5-week-old *Tgfbr2*^{+/+} and *Tgfbr2*^{−/−} OT-II mice in the absence or presence of RIP-mOva transgene. Data are representative of three independent experiments.

autoreactive T cells occurs through apoptosis (Smith et al., 1989), which is mediated by the cysteine proteases, caspases (Lakhani et al., 2006). Approximately 10-fold more *Tgfbr2*^{−/−} nTreg cells exhibited high caspase activity than *Tgfbr2*^{+/+} nTreg cells isolated from 3- to 5-day-old mice (Figure 5A), supporting an apoptotic mechanism of cell death. As previously reported (Li et al., 2006; Marie et al., 2006), the frequency of thymic nTreg cells in 16-day-old *Tgfbr2*^{−/−} mice was no less than that of the control mice (Figure S3B). Importantly, elevated caspase activation was also detected in nTreg cells from 16-day-old *Tgfbr2*^{−/−} mice (Figure S3C). Recovery of thymic nTreg cells in these mice was probably due to the long-lasting enhanced nTreg cell proliferation (Figure 4C; Figure S3D). These findings demonstrated that TGF-β signaling was essential for inhibiting caspase activation and nTreg cell apoptosis in neonatal as well as week-old mice.

Signals from the common γ-chain cytokines, including IL-2, IL-15, and IL-7, are important regulators of T cell survival and nTreg cell differentiation (Burchill et al., 2007; Fontenot et al., 2005; Malek et al., 2002; Vang et al., 2008). To investigate whether compromised survival of *Tgfbr2*^{−/−} nTreg cells was due to defective signaling of IL-2, IL-15, or IL-7, we determined thymic expression of these cytokines and the cytokine receptors. With the exception of CD127 (IL-7 receptor α chain), the amounts of CD25 (IL-2 receptor α chain), CD122 (the shared IL-2 and IL-15 β chain), and CD132 (the common γ chain) were all elevated in *Tgfbr2*^{−/−} nTreg cells (Figure S4A), whereas the amounts of IL-2, IL-7, and IL-15 mRNA were comparable in the thymi of *Tgfbr2*^{+/+} and *Tgfbr2*^{−/−} mice (Figure S4B). To explore a potential role for the reduced CD127 expression in *Tgfbr2*^{−/−}

nTreg cells, we crossed *Tgfbr2*^{−/−} mice with a strain of IL-7R transgenic mice (Park et al., 2004). However, restoration of CD127 expression did not correct the reduced frequency of *Tgfbr2*^{−/−} nTreg cells in 3- to 5-day-old mice (Figures S4C and S4D). These observations suggested that nTreg cell apoptosis in *Tgfbr2*^{−/−} mice was unlikely caused by the compromised signaling of common γ-chain cytokines.

A prominent pathway of caspase activation and induction of apoptosis is through permeabilization of the outer mitochondrial membrane, which is regulated by proteins of the Bcl-2 family including both the prosurvival protein Bcl-2 and the proapoptotic proteins such as Bim, Bak, and Bax (Bouillet et al., 2002; Rathmell et al., 2002; Youle and Strasser, 2008). Associated with the enhanced caspase activation in *Tgfbr2*^{−/−} nTreg cells, Bcl-2 expression was downregulated (Figure 5B), whereas the expression of Bim, Bak, and Bax proteins was upregulated (Figure 5C). Previous studies have established Bim as a key regulator of T cell apoptosis during negative selection (Bouillet et al., 2002). Indeed, Bim deficiency corrected the enhanced OT-II T cell deletion in *Tgfbr2*^{−/−} RIP-mOVA mice (Figures 2A and 2B; Figures S2C and S2D). To determine whether the elevated Bim expression was causative of the enhanced apoptosis of *Tgfbr2*^{−/−} nTreg cells, we crossed *Tgfbr2*^{−/−} mice to the Bim-deficient (*Bcl2l11*^{−/−}) background. The frequency and number of thymic *Tgfbr2*^{−/−} nTreg cells from 3- to 5-day-old mice were corrected by 80% in the absence of Bim (Figure 5D), which was associated with decreased caspase activation (Figure 5E). It is noteworthy that Bim deficiency itself caused a general increase of thymic nTreg cells (Figure 5D; Figure S5A), which was likely a consequence of reduced T cell negative selection (Bouillet et al., 2002). CD28 costimulation is essential for nTreg cell development, which is possibly through CD28 regulation of *Foxp3* gene expression (Salomon et al., 2000; Tai et al., 2005). Importantly, Bim deletion did not rescue the thymic nTreg cell defects in CD28-deficient mice (Figure S5B). These observations suggested that TGF-β signaling
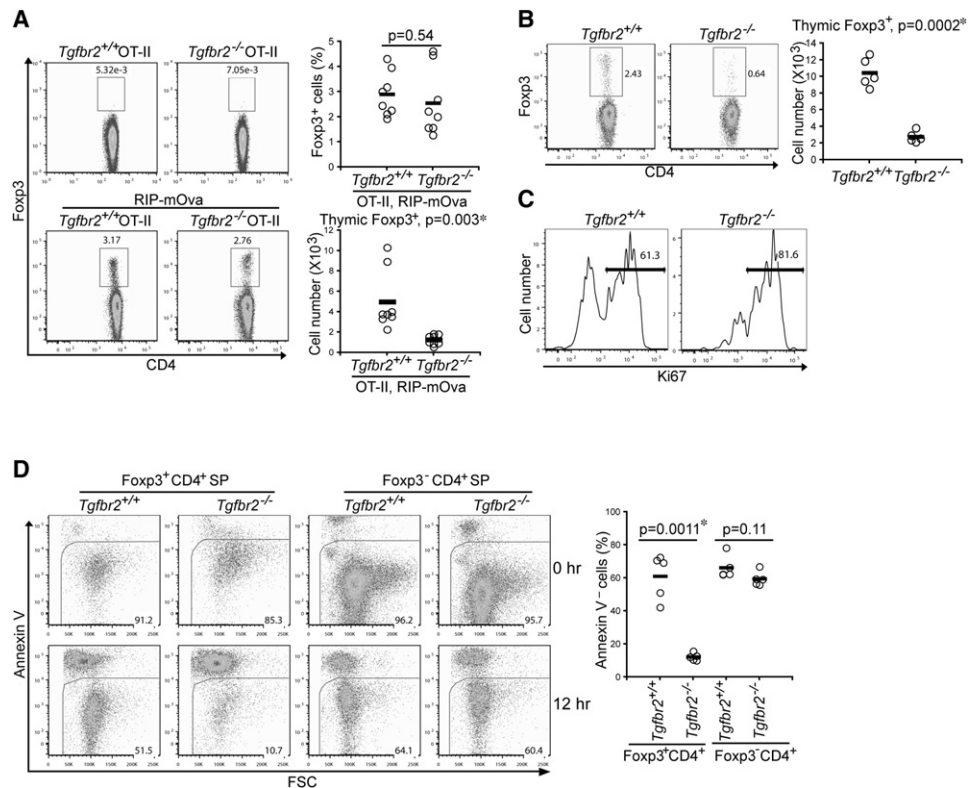
Figure 4. TGF-β Control of Thymic nTreg Cell Survival

(A) Thymic nTreg cells from 5-week-old $Tgfbr2^{+/+}$ and $Tgfbr2^{-/-}$ OT-II mice in the absence or presence of RIP-mOva transgene. Foxp3 expression in TCR-β^{hi} OT-II T cells (left) and the percentages (right top) and numbers (right bottom) of nTreg cells from $Tgfbr2^{+/+}$ and $Tgfbr2^{-/-}$ OT-II RIP-mOva mice are shown (n = 8).

(B) Thymic nTreg cells in 3- to 5-day-old $Tgfbr2^{+/+}$ and $Tgfbr2^{-/-}$ mice. Foxp3 expression in TCR-β^{hi}CD4$^+$ T cells (left) and nTreg cell numbers from $Tgfbr2^{+/+}$ and $Tgfbr2^{-/-}$ mice (right) are shown (n = 5).

(C) Flow cytometric analysis of Ki67 expression in thymic nTreg cells from 3- to 5-day-old $Tgfbr2^{+/+}$ and $Tgfbr2^{-/-}$ mice. These are representative results of four mice per group analyzed.

(D) Thymic Foxp3$^+$CD4$^+$ single-positive (SP) nTreg cells and Foxp3$^-$CD4$^+$ SP conventional T cells were purified by FACS sorting from 14- to 16-day-old $Tgfbr2^{+/+}$ and $Tgfbr2^{-/-}$ mice and cultured for 12 hr. T cell viability before and after the cell culture was determined by annexin V staining (left). Percentages of viable T cells at 12 hr from five pairs of mice are presented (right). The p values between the groups are shown. Asterisk depicts significant difference.

played a specific role in protecting nTreg cells from Bim-dependent apoptosis and was unlikely involved in the induction of Foxp3 expression in nTreg cells.

Bim Control of Treg Cell Homeostasis and T Cell Activation in TGF-βRII-Deficient Mice

Treg cells fail to be maintained in the peripheral lymphoid organs of TGF-β receptor-deficient mice (Li et al., 2006; Liu et al., 2008; Marie et al., 2006), but the underlying mechanisms have yet to be characterized. To determine whether Bim-dependent apoptosis accounted for this defect, we examined peripheral Treg cells in mice deficient in both TGF-βRII and Bim ($Tgfbr2^{-/-}Bcl2l11^{-/-}$). Whereas the frequency of splenic and lymph node Treg cells from $Tgfbr2^{-/-}$ mice was lower than that from $Tgfbr2^{+/+}$ mice, an almost complete rescue of the proportion of Treg cells was observed in $Tgfbr2^{-/-}Bcl2l11^{-/-}$ mice (Figures 6A and 6B; Figure S5C). Reduced Treg cells in $Tgfbr2^{-/-}$ mice were associated with an approximate 2-fold increase of Treg cell apoptosis, which was corrected by 60% in the absence of Bim (Figure 6C). Notably, Bim deletion did not rescue the peripheral Treg cell defects in CD28-deficient mice (Figure S5B). These observations

revealed a specific function for TGF-β signaling in protecting peripheral Treg cells from mitochondrion-dependent apoptosis.

To determine the effects of Bim deficiency on the anergic phenotype of Treg cells and Treg cell-suppressive function, we used CFSE-based cell proliferation assays. Whereas wild-type or $Bcl2l11^{-/-}$ Treg cells were refractory to anti-CD3-induced proliferation, $Tgfbr2^{-/-}$ and $Tgfbr2^{-/-}Bcl2l11^{-/-}$ Treg cells underwent substantial cell division (Figure S5D). Compared to wild-type Treg cells, $Tgfbr2^{-/-}$ Treg cells had enhanced suppressive activity on a per cell basis, whereas $Bcl2l11^{-/-}$ Treg cells were less suppressive (Figure 6D). Importantly, $Tgfbr2^{-/-}Bcl2l11^{-/-}$ Treg cells had comparably suppressive activity to wild-type Treg cells (Figure 6D). To investigate the impact of Bim deficiency on peripheral T cell tolerance, we examined the lifespan of $Tgfbr2^{-/-}Bcl2l11^{-/-}$ mice. Whereas 100% $Tgfbr2^{-/-}$ mice died by 5 weeks of age, all $Tgfbr2^{-/-}Bcl2l11^{-/-}$ mice survived during the same period (Figure 7A). Compared to CD4$^+$ T cells from $Tgfbr2^{-/-}$ mice, a smaller proportion of CD4$^+$ T cells from $Tgfbr2^{-/-}Bcl2l11^{-/-}$ mice exhibited an activated CD44^{hi}CD62L^{lo} phenotype (Figure 7B), and fewer CD4$^+$ T cells produced the effector cytokine IFN-γ
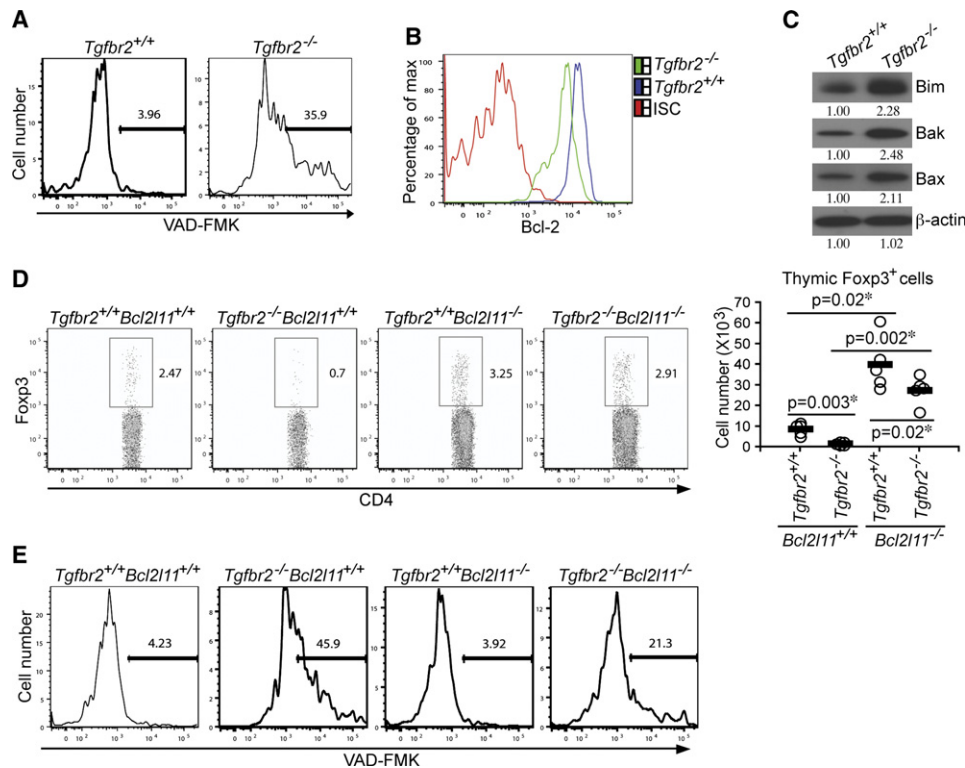
Figure 5. TGF-β Signaling Regulates Thymic nTreg Cell Development via the Inhibition of Bim-Dependent Apoptosis

(A) Flow cytometric analysis of caspase activation in 4-day-old thymic nTreg cells from $Tgfbr2^{+/+}$ and $Tgfbr2^{-/-}$ mice. These are representative results of four mice per group analyzed.

(B) Flow cytometric analysis of Bcl-2 expression in thymic nTreg cells from $Tgfbr2^{+/+}$ and $Tgfbr2^{-/-}$ mice. ISC stands for the isotype control antibody.

(C) The expression of Bim, Bak, and Bax in thymic nTreg cells from $Tgfbr2^{+/+}$ and $Tgfbr2^{-/-}$ mice was determined by immunoblotting. β-actin was used as a protein-loading control. Band densities were quantified by ImageJ. The relative protein amounts are shown. Data are representative of three independent experiments.

(D) Restoration of thymic nTreg cell development in $Tgfbr2^{-/-}$ mice by Bim deletion. Foxp3 expression in thymic TCR-β^{hi}CD4$^+$ T cells from 4-day-old $Tgfbr2^{+/+}$ $Bcl2l11^{+/+}$, $Tgfbr2^{-/-}Bcl2l11^{+/+}$, $Tgfbr2^{+/+}Bcl2l11^{-/-}$, and $Tgfbr2^{-/-}Bcl2l11^{-/-}$ mice (left) and nTreg cell numbers from five groups of 3- to 5-day-old mice are presented (right). The p values between the groups are shown. Asterisk depicts significant difference.

(E) Flow cytometric analysis of caspase activation in thymic nTreg cells from $Tgfbr2^{+/+}Bcl2l11^{+/+}$, $Tgfbr2^{-/-}Bcl2l11^{+/+}$, $Tgfbr2^{+/+}Bcl2l11^{-/-}$, and $Tgfbr2^{-/-}Bcl2l11^{-/-}$ mice. A representative of three independent experiments is shown.

(Figure 7C). Nevertheless, $Tgfbr2^{-/-}Bcl2l11^{-/-}$ mice eventually succumbed to a lethal multifocal inflammatory disorder similar to that of $Tgfbr2^{-/-}$ mice (Figure 7A; Figure S6). These observations revealed that Bim rescue of peripheral Treg cells was associated with a partial correction of the T cell activation and lethal autoimmune phenotype in $Tgfbr2^{-/-}$ mice.

DISCUSSION

Although TGF-β has been established as a pivotal regulator of T cell tolerance, the underlying mechanisms remain elusive. In this report, we used a T cell-specific TGF-βRII-deficient mouse strain to study the function of TGF-β signaling in control of T cell-negative selection and nTreg cell development, two established pathways of T cell self-tolerance. By using anti-CD3-induced and antigen-triggered T cell deletion models, we found that thymic-negative selection was repressed by TGF-β signaling in T cells. TGF-β inhibition of T cell deletion promoted thymic Treg cell survival, revealing a mechanism for TGF-β in regulation of nTreg cell development. Enhanced apoptosis of TGF-βRII-

deficient nTreg cells was associated with high expression of the proapoptotic Bcl-2 family protein Bim, lack of which partially rescued the nTreg cell death phenotype. In addition, we found that TGF-βRII-deficient Treg cells in peripheral tissues underwent exaggerated Bim-dependent apoptosis; Bim deficiency restored peripheral Treg cells, inhibited T cell activation, and extended the lifespan of T cell-specific TGF-βRII-deficient mice. These observations uncover previously undefined functions for TGF-β signaling in control of deletional tolerance and Treg cell-mediated immune suppression in vivo.

Autoreactive T cells can be readily found in the peripheral lymphoid organs of healthy individuals (Danke et al., 2004). In animal models of T cell-negative selection, a large fraction of T cells also escape clonal deletion (Bouneaud et al., 2000). Lack of complete deletion of autoreactive T cells may be a consequence of limited expression and/or presentation of self-antigens in the thymus. Here we showed that negative selection was also actively suppressed by TGF-β signaling in T cells. Although the significance of this regulatory pathway remains to be fully elucidated, we found that by inhibiting T cell clonal
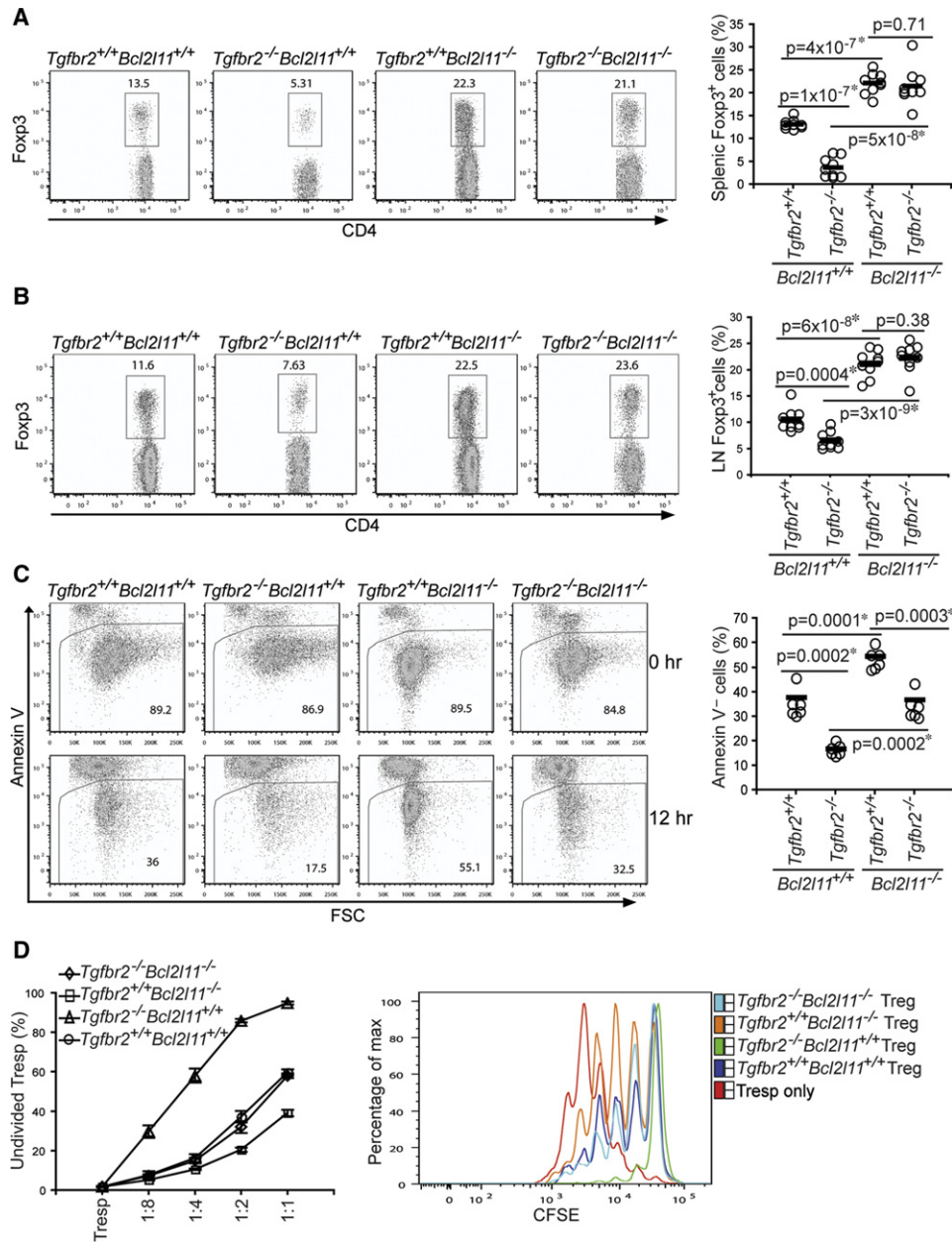
Figure 6. Bim Ablation Restores Peripheral Treg Cells in TGF-βRII-Deficient Mice

(A and B) Flow cytometric analysis of Foxp3 expression in splenic (A) and lymph node (LN) (B) CD4⁺ T cells from 16-day-old $Tgfbr2^{+/+}Bcl2l11^{+/+}$, $Tgfbr2^{-/-}Bcl2l11^{+/+}$, $Tgfbr2^{+/+}Bcl2l11^{-/-}$, and $Tgfbr2^{-/-}Bcl2l11^{-/-}$ mice (left). Treg cell percentages from eight groups of 14- to 16-day-old mice are shown (right).

(C) Splenic and lymph node Treg cells were purified from 16-day-old $Tgfbr2^{+/+}Bcl2l11^{+/+}$, $Tgfbr2^{-/-}Bcl2l11^{+/+}$, $Tgfbr2^{+/+}Bcl2l11^{-/-}$, and $Tgfbr2^{-/-}Bcl2l11^{-/-}$ mice by FACS sorting, and cultured for 12 hr. T cell viability before and after the cell culture was determined by annexin V staining (left). Percentages of viable T cells at 12 hr from six groups of 14- to 16-day-old mice are presented (right). The p values between the groups are shown. Asterisk depicts significant difference.

(D) Suppressive function of $Tgfbr2^{+/+}Bcl2l11^{+/+}$, $Tgfbr2^{-/-}Bcl2l11^{+/+}$, $Tgfbr2^{+/+}Bcl2l11^{-/-}$, and $Tgfbr2^{-/-}Bcl2l11^{-/-}$ Treg cells. Splenic and lymph node Foxp3⁺CD4⁺ Treg cells were isolated from $Tgfbr2^{+/+}Bcl2l11^{+/+}$, $Tgfbr2^{-/-}Bcl2l11^{+/+}$, $Tgfbr2^{+/+}Bcl2l11^{-/-}$, and $Tgfbr2^{-/-}Bcl2l11^{-/-}$ mice. CD44^{lo}CD4⁺ T cells from $Tgfbr2^{+/+}Bcl2l11^{+/+}$ mice were labeled with CFSE and used as responding T (Tresp) cells. Cell division was assessed by CFSE dilution. The percentages of undivided Tresp cells (y axis) and the ratios of cell numbers between Treg and Tresp cells (x axis) were plotted (left). A representative of CFSE dilution of Tresp cells cultured in the absence or presence of 1:2 ratio of Treg to Tresp cells was shown (right). These are representative of three independent experiments.

deletion, TGF-β enhanced nTreg cell production. nTreg cells exhibit an "antigen-experienced" phenotype, suggesting that their differentiation process is induced or accompanied by exposure to high-affinity self-antigens (Josefowicz and Rudensky, 2009; Sakaguchi et al., 2008). Recent studies of transgenic mice expressing TCRs derived from Treg cells demonstrate that nTreg cell differentiation is indeed instructed by TCR specificity (Bautista et al., 2009; DiPaolo and Shevach, 2009; Leung
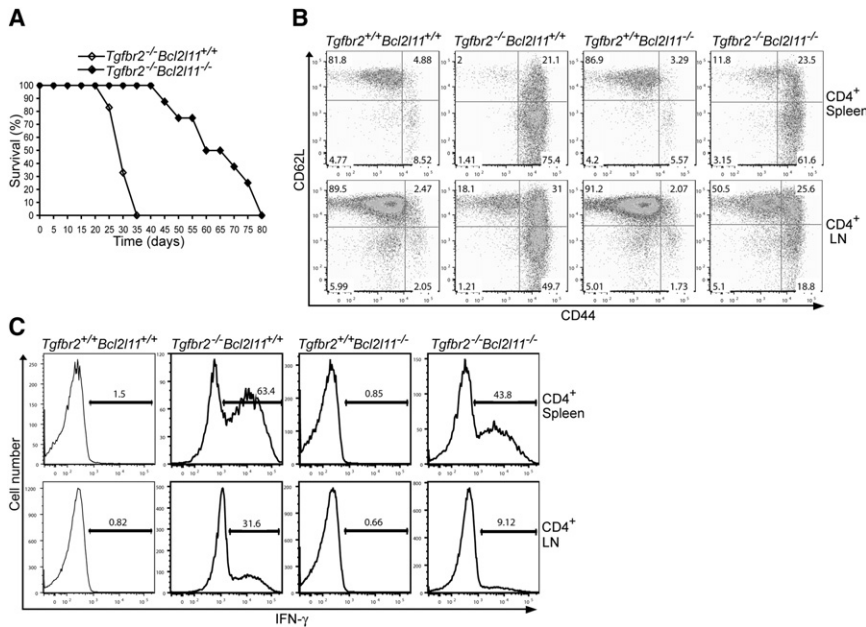
Figure 7. Bim Ablation Partially Restores T Cell Tolerance in TGF-βRII-Deficient Mice

(A) Survival of $Tgfbr2^{-/-}Bcl2l11^{+/+}$ (n = 18) and $Tgfbr2^{-/-}Bcl2l11^{-/-}$ (n = 8) mice.

(B) Flow cytometric analysis of CD44 and CD62L expression in splenic and lymph node (LN) CD4+ T cells from 14-day-old $Tgfbr2^{+/+}Bcl2l11^{+/+}$, $Tgfbr2^{-/-}Bcl2l11^{+/+}$, $Tgfbr2^{+/+}Bcl2l11^{-/-}$, and $Tgfbr2^{-/-}Bcl2l11^{-/-}$ mice. These are representative results of four mice per group analyzed.

(C) Splenic and LN CD4+ T cells from 14-day-old $Tgfbr2^{+/+}Bcl2l11^{+/+}$, $Tgfbr2^{-/-}Bcl2l11^{+/+}$, $Tgfbr2^{+/+}Bcl2l11^{-/-}$, and $Tgfbr2^{-/-}Bcl2l11^{-/-}$ mice were stimulated with PMA and ionomycin for 4 hr and analyzed for the expression of IFN-γ by intracellular staining. A representative of three independent experiments is shown.

et al., 2009). Intriguingly, the proportion of Treg cell-TCR transgenic T cells that mature into the Treg cell lineage is inversely correlated with the precursor frequency (Bautista et al., 2009; Leung et al., 2009), which could be explained by intraclonal competition for limited Treg cell-selecting high-affinity self MHC-peptide complexes. In one Treg cell-TCR transgenic mouse strain, profound T cell deletion was observed (DiPaolo and Shevach, 2009), revealing that at least some endogenous nTreg cells were differentiated in response to high-affinity self-antigens. Consistent with these and previous studies (Apostolou et al., 2002; Jordan et al., 2001; Kawahata et al., 2002; Walker et al., 2003), coexpression of mOVA in OT-II transgenic mice triggered nTreg cell differentiation, concomitant with the induction of T cell-negative selection. Blockade of TGF-β signaling in OT-II T cells resulted in heightened deletion of nTreg cells as well as conventional T cells in response to mOVA antigen. These observations are in line with the reports that Foxp3 deficiency does not affect the efficiency or sensitivity of T cell-negative selection (Chen et al., 2005; Hsieh et al., 2006). Collectively, our findings suggest that nTreg cells are not inherently more resistant to apoptosis than conventional T cells, but the magnitude of T cell clonal deletion can be regulated to ensure proper production of nTreg cells.

The Treg cell-specific transcription factor Foxp3 can be induced in both developing thymocytes and peripheral T cells in response to appropriate combinations of TCR, costimulatory molecule, and cytokine stimulation (Josefowicz and Rudensky, 2009). Activation of peripheral naive CD4+ T cells in the presence of TGF-β induces Smad-dependent Foxp3 expression and iTreg cell differentiation. Defective nTreg cell development had also been observed in neonatal mice with a T cell-specific inactivation of TGF-βRI, which was postulated to be caused by failed Foxp3 induction (Liu et al., 2008). Similar neonatal nTreg cell defects were present in TGF-βRII-deficient mice. However, we found that compromised differentiation of TGF-βRII-deficient nTreg cells was associated with high expression of the proapoptotic molecule Bim, enhanced caspase activation, and increased nTreg cell apoptosis. Importantly, genetic ablation of Bim restored nTreg cells in TGF-βRII-deficient mice. It is noteworthy that Bim deficiency did not correct the nTreg cell defects in mice lacking the costimulatory molecule CD28, the signals from which probably directly control Foxp3 gene transcription. These findings suggest that in contrast to iTreg cells, TGF-β signaling is not essential for the induction of Foxp3 expression in nTreg cells, but is required to inhibit Bim-dependent nTreg cell apoptosis.

The cellular and molecular mechanisms by which TGF-β regulates Bim expression and nTreg cell apoptosis are open for future studies. There are three types of TGF-β molecules in mammals, with TGF-β1 being the major protein expressed in the immune system (Li and Flavell, 2008). A previous report showed that T cells from TGF-β1-deficient mice are more susceptible to anti-CD3-induced T cell death, which was attributed to an intracellular function of TGF-β1 located in the mitochondrion (Chen et al., 2001). Because we found that TGF-βRII-deficient T cells were also hypersensitive to anti-CD3-induced apoptosis, TGF-β1 protection of T cell deletion was more likely mediated by the extracellular cytokine activity of TGF-β1. Whether TGF-β1 is involved in the inhibition of Bim expression and nTreg cell apoptosis has yet to be determined. In addition, multiple cell types produce TGF-β1 in vivo. By using a conditionally deficient TGF-β1 mouse model, we have identified T cells as an essential source of TGF-β1 for control of T cell tolerance and effector T cell differentiation (Li et al., 2007). The functions of T cell or other cell type-produced TGF-β1 in control of nTreg cell survival will be an interesting topic for future study.

TGF-β engagement of the receptor complex initiates diverse signaling pathways that enable TGF-β to exert its pleiotropic functions (Li and Flavell, 2008). The signaling pathways involved in TGF-β inhibition of Bim expression in nTreg cells are unknown. Two recent reports using a transgenic mouse strain expressing a dominant-negative mutant of TGF-βRII (DNRII) in the T cell compartment revealed that TGF-β signaling promotes apoptosis of pathogen-specific CD8+ effector T cells (Sanjabi et al., 2009;

Tinoco et al., 2009). In one study, decreased apoptosis of DNRII CD8$^+$ T cells is associated with reduced Bim expression (Tinoco et al., 2009). It is currently unknown how TGF-β opposingly regulates Bim expression in nTreg cells and effector CD8$^+$ T cells. We showed previously that the DNRII model, in contrast to the TGF-βRII conditional knockout mouse model used in this study, affords only a partial blockade of TGF-β signaling in T cells (Li et al., 2006), which might affect TGF-β regulation of Bim expression and T cell survival. It is also possible that TGF-β control of Bim expression is context dependent, with the ultimate effect being determined by the developmental stages of T cells and other environmental cues received by the T cells. It is noteworthy that Bim ablation did not completely rescue caspase activation in TGF-βRII-deficient mice. Other Bcl-2 family proteins including Bax, Bak, and Bcl-2 were dysregulated in TGF-βRII-deficient nTreg cells, raising the possibility that they might control Bim-independent caspase activation. How TGF-β regulates these and other alternative apoptosis pathways warrants further investigation.

Thymic nTreg cells from week-old TGF-βRII-deficient mice were equally susceptible to apoptosis as nTreg cells from neonatal mice. But thymic nTreg cell numbers were restored, which could be attributed to the enhanced cell proliferation. Nevertheless, the peripheral Treg cell numbers were reduced in TGF-βRII-deficient mice, revealing that intact TGF-β signaling was required for the maintenance of Treg cells. In this study, we found that compromised Treg cell maintenance was caused by Bim-dependent Treg cell apoptosis. Interestingly, Bim deficiency not only restored peripheral Treg cells, but also attenuated T cell activation and prolonged the lifespan of TGF-βRII-deficient mice. These findings suggest that TGF-β inhibition of peripheral Treg cell apoptosis contributes to the maintenance of T cell self-tolerance. A recent study showed that Bim-deficient T cells are refractory to TCR-induced calcium responses (Ludwinski et al., 2009), raising the possibility that T cell-intrinsic defects might also contribute to the attenuated T cell activation and differentiation phenotype in $Tgfbr2^{-/-}$ $Bcl2l11^{-/-}$ mice. However, Bim ablation could not completely prevent the lethal inflammation developed in TGF-βRII-deficient mice. These observations are in line with the findings that Treg cells are incapable of suppressing intestinal inflammation induced by naive CD4$^+$ T cells resistant to TGF-β signaling (Fahlen et al., 2005) and that transfer of wild-type Treg cells to neonatal $Tgfbr2^{-/-}$ mice fails to correct the systemic autoimmune disease (Li et al., 2006). Therefore, in addition to the maintenance of peripheral Treg cells, TGF-β signaling is indispensable for inhibiting autoreactive T cell activation and effector T cell differentiation.

In conclusion, in this report we have uncovered critical functions for TGF-β signaling in control of T cell negative selection, Treg cell development and homeostasis, and peripheral tolerance of autoreactive T cells. These findings refine and extend our understanding of T cell self-tolerance mechanisms and may be exploited for the immunotherapy of autoimmune diseases in the future.

EXPERIMENTAL PROCEDURES

Mice
Mice containing floxed $Tgfbr2$, $CD4$-Cre, OT-II, $Rag1^-$, RIP-$mOva$, $Bcl2l11^-$, $Il7rTg$, $CD28^-$, and $Foxp3$-RFP alleles were previously described (Bouillet

et al., 2002; Kurts et al., 1996; Li et al., 2006; Park et al., 2004; Wan and Flavell, 2005). Foxp3-RFP mice were provided by R. Flavell (Yale). RIP-mOva and IL-7R transgenic mice were provided by W. Heath (The Walter and Eliza Hall Institute of Medical Research) and A. Singer (National Cancer Institute), respectively. $Bcl2l11^{-/-}$, $CD28^{-/-}$, and $Rag1^{-/-}$ mice were purchased from the Jackson Laboratory. Mice with two floxed $Tgfbr2$ alleles were used as controls ($Tgfbr2^{+/+}$). T cell-specific TGF-βRII-deficient mice ($Tgfbr2^{-/-}$) were generated by crossing $Tgfbr2$-floxed mice with the CD4-Cre transgene. $Tgfbr2^{-/-}$ and $CD28^{-/-}$ mice were crossed with $Bcl2l11^{-/-}$ mice to produce mice devoid of both genes ($Tgfbr2^{-/-}Bcl2l11^{-/-}$ and $CD28^{-/-}Bcl2l11^{-/-}$). $Tgfbr2^{-/-}$ and $Tgfbr2^{-/-}Bcl2l11^{-/-}$ mice were crossed with Foxp3-RFP mice to mark Treg cells by red-fluorescent protein expression. OT-II RIP-mOva mice on a Rag1-deficient background were crossed with $Tgfbr2^{-/-}$ mice to generate $Tgfbr2^{-/-}$ OT-II RIP-mOva mice. Urine glucose concentrations in these mice were monitored by using Diastyx sticks (Bayer) on a weekly basis. Animals that had values of >250 mg/dl on two consecutive occasions were counted as diabetic. All mice were maintained under specific pathogen-free conditions, and animal experimentation was conducted in accordance with institutional guidelines.

Flow Cytometry
Fluorescent-dye-labeled antibodies against cell surface markers CD4, CD8, TCR-β, CD69, CD62L, CD44, CTLA4, GITR, ICOS, CD27, CD25, CD122, CD132, and CD127 were purchased from eBiosciences. Biotinylated mouse TGF-βRII antibody was obtained from R&D Systems. Thymic, splenic, and lymph node cells were depleted of erythrocytes by hypotonic lysis. Cells were incubated with specific antibodies for 15 min on ice in the presence of 2.4G2 mAb to block FcgR binding. All samples were acquired and analyzed with LSR II flow cytometer (Becton Dickinson) and FlowJo software (Tree Star). Intracellular Foxp3, CTLA4, Ki67, and Bcl-2 stainings were carried out with kits from eBiosciences and BD Biosciences. For intracellular cytokine staining, spleen and lymph node cells were stimulated with 50 ng/ml phorbol 12-myristate 13-acetate (PMA, Sigma), 1 μM ionomycin (Sigma) and GolgiStop (BD Biosciences) for 4 hr. After stimulation, cells were stained with cell surface marker antibodies, fixed and permeabilized with a Cytofix/Cytoperm kit (BD Biosciences), and stained with an IFN-γ antibody.

Immunoblotting
Thymic Foxp3$^+$ Treg cells marked by red-fluorescent protein expression were purified by FACS sorting. Protein extracts were prepared, separated on 15% SDS PAGE gels, transferred to PVDF membrane (Millipore), and probed with antibodies against Bim (Stressgen), Bax (Upstate), Bak (Upstate), and β-actin (Sigma).

Quantitative PCR
CD4$^+$CD8$^+$ double-positive (DP), TCR-β^{hi}CD4$^+$ single-positive (SP), and TCR-β^{hi}CD8$^+$ SP thymocytes were isolated by FACS sorting and used for RNA extraction. TGF-βRII mRNA amounts were determined by quantitative PCR (qPCR) with the primer set: 5′-atctggaaaacgtggagtcg-3′ and 5′-tccttca cttctcccacagc-3′. To determine the expression of common γ chain cytokines, thymus was lyzed by Trizol for RNA preparation. The amount of IL-2, IL-7, and IL-15 was measured by qPCR with the following primer sets: 5′-cccactt caagctccacttc-3′ and 5′-ttcaattctgtggcctgctt-3′ (IL-2), 5′-atccttgttctgctgc ctgt-3′ and 5′-tggttcattattcgggcaat-3′ (IL-7), 5′-cattttgggctgtgtcagtg-3′ and 5′-gcaattccaggagaaagcag-3′ (IL-15). The expression of TGF-βRII, IL-2, IL-7, and IL-15 was normalized to the β-actin amounts detected by qPCR with the primer set: 5′-ttgctgacaggatgcagaag-3′ and 5′-acatctgctggaaggtggac-3′.

Apoptosis Assays
Foxp3$^+$ Treg cells, conventional T cells, and OT-II T cells were purified by FACS sorting and cultured in T cell medium (RPMI1640 medium supplemented with 0.1 mM glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 55 μM 2-mercaptoethanol, 10 U/ml penicillin, 10 μg/ml streptomycin, and nonessential amino acids) for 6 and 12 hr. Cell apoptosis was determined by staining with FITC-labeled annexin V (BD Biosciences). To assess anti-CD3-induced thymocyte apoptosis in vitro, total thymocytes were stimulated with plate-bound anti-CD3 and anti-CD28 for 24 hr and stained with fluorescent-dye-labeled anti-TCR-β,

anti-CD4, anti-CD8, and annexin V. The annexin V$^+$ apoptotic cells in thymic subpopulations were determined by flow cytometry.

For active caspase measurements, thymocytes were cultured in T cell medium in the presence of 10 μM FITC-VAD-FMK (Promega) for 20 min, washed twice with cold PBS, and stained with antibodies against cell surface markers before subjected to flow cytometric analysis.

The TUNEL protocol with tyramide signal amplification was developed at the Molecular Cytology Core Facility at Memorial Sloan-Kettering Cancer Center. Four-micron tissue sections were treated with 20 μg/ml Proteinase K (Sigma) for 15 min at 37°C and fixed with 4% paraformaldehyde for 10 min at room temperature. Blocking of endogenous peroxidases was performed for 10 min in 1% H_2O_2 at room temperature. TdT (terminyl deoxynucleodityl transferase, Roche)-mediated biotin-dUTP end nick labeling reaction was performed at 4°C overnight. Tyramide-biotin (Perkin Elmer) was used to amplify the signal. Biotin was detected with a Vectastain ABC Kit (Vector Laboratories). Peroxidase activity was determined by incubating the slides in 0.2 mg/ml DAB in PBS with 50 μl of 30% H_2O_2 for 5 min at room temperature. Slides were counterstained with Harris Hematoxylin (Fisher Scientific), dehydrated, and mounted with Permount (Fisher Scientific). TUNEL-positive areas from four sections were quantified with the MetaMorph software.

Treg Cell Suppression Assay
Splenic and lymph node CD4$^+$Foxp3$^+$ regulatory T (Treg) cells were isolated from 16-day-old $Tgfbr2^{+/+}Bcl2l11^{+/+}$, $Tgfbr2^{-/-}Bcl2l11^{+/+}$, $Tgfbr2^{+/+}Bcl2l11^{-/-}$, and $Tgfbr2^{-/-}Bcl2l11^{-/-}$ mice by FACS sorting. CD44^{lo}CD4$^+$RFP$^-$ cells sorted from $Tgfbr2^{+/+}Bcl2l11^{+/+}$ mice were labeled with 4 μM of carboxyfluorescein diacetate succinimidyl ester (CFSE, Sigma) at 37°C for 10 min and used as responder T (Tresp) cells. 5×10^4 Tresp cells were cultured in 96-well plates with 10^5 irradiated splenocytes and 2 μg/ml CD3 antibody in the presence of different numbers of Treg cells for 72 hr.

Histopathology
Tissues from sacrificed animals were fixed in Safefix II (Protocol) and embedded in paraffin. 5 μm sections were stained with hematoxylin and eosin.

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

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and can be found with this article online at doi:10.1016/j.immuni.2010.04.012.

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