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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-$\beta^{hi}CD4^+$ and TCR-$\beta^{hi}CD8^+$ 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-$\beta^{hi}CD8^+$ T cells in *Tgfbr2*$^{-/-}$ mice as previously reported (Figure 1C; Li et al., 2006). Surprisingly, thymocytes, notably TCR-$\beta^{hi}CD4^+$ and TCR-$\beta^{hi}CD8^+$ 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-$\beta^{hi}CD4^+$ and TCR-$\beta^{hi}CD8^+$ 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