TGF-β signaling to T cells inhibits autoimmunity during lymphopenia-driven proliferation

Nu Zhang & Michael J Bevan

T cell-specific deletion of the receptor for transforming growth factor-β (TGF-β) mediated by Cre recombinase expressed early in T cell development leads to early-onset lethal autoimmune disease that cannot be controlled by regulatory T cells. However, when we deleted that receptor through the use of Cre driven by a promoter that is active much later in T cell development, adult mice in which most peripheral CD4+ or CD8+ T cells lacked the receptor for TGF-β showed no signs of autoimmunity. Because of their enhanced responses to weak stimulation of the T cell antigen receptor, when transferred into lymphopenic recipients, naive TGF-β-unresponsive T cells underwent much more proliferation and differentiation into effector cells and induced lymphoproliferative disease. We propose that TGF-β signaling controls the self-reactivity of peripheral T cells but that in the absence of TGF-β signals, an added trigger such as lymphopenia is needed to drive overt autoimmune disease.

Transforming growth factor-β (TGF-β) is a pleiotropic cytokine that regulates diverse biological processes during fetal development and in adults by binding to the type I (TGF-βRI) and type II (TGF-βRII) receptors for TGF-β found on the surface of most cells1–3. After binding TGF-β, TGF-βRI phosphorylates the signal transducers Smad2 and Smad3 to induce the formation of a complex with Smad4 and translocation of the complex to the nucleus. In addition, several Smad-independent pathways, such as activation of the kinases p38 and Jnk dependent on the ubiquitin ligase TRAF6 and kinase TAK1, are induced by TGF-β signaling4,5. During tumorigenesis, signaling via TGF-β may initially suppress tumor growth through the regulation of cell-cycle progression, differentiation and apoptosis. However, paradoxically, at later stages, it promotes tumors both by enhancing metastasis and by its immunosuppressive effect on antitumor immunity2,6,7. In line with the antiproliferative and immunosuppressive functions of TGF-β, mice deficient in TGF-β1 die before or shortly after birth, with an autoimmune phenotype that includes massive expansion of the T cell population and organ infiltration5,9. Depleting TGF-β-deficient mice of T cells alleviates the disease10,11, but because TGF-β has regulatory effects on many cell types, whether or not it has a direct effect on T cells in preventing the autoimmune phenotype has remained unclear. Therefore, to elucidate the role of direct signaling from TGF-β to T cells, researchers have studied mice that express a dominant-negative transgene encoding TGF-βRII expressed selectively in T cells14,15 or have engineered Cre recombinase-mediated deletion of loxP-flanked alleles encoding TGF-βRII specifically in the T cell lineage (Tgfbr2fl/f CD4-Cre)16,17.

In both of the models of T cell–specific unresponsiveness to TGF-β noted above, mice develop autoimmune pathology. In particular, mice with CD4-Cre–mediated deletion of loxP-flanked Tgfbr2 die very rapidly (by 3–5 weeks of age) with multiorgan autoimmune lesions, similar to what is observed in mice that completely lack TGF-β16,17. The severity and rapidity of disease onset in this model of T cell–specific deletion of TGF-βRII resembles that in mice that are deficient in the transcription factor Foxp3 and therefore lack regulatory T cells (Treg cells)18. Because signaling by TGF-β is required for the proper development, maintenance and function of Treg cells16,17,19–21, the question has arisen of to what extent the autoimmunity in Tgfbr2fl/f CD4-Cre mice is due to the failure of Treg cell function and how much is due to the intrinsic inability of TGF-β-unresponsive T cells to control their self-reactivity. The latter has been shown to be relevant because neither transfer of wild-type Treg cells into the mice with conditional deletion of TGF-βRII nor the generation of mixed–bone marrow chimeras reconstituted with bone marrow from wild-type mice and mice with conditional deletion of TGF-βRII prevents the onset of autoimmunity in the Tgfbr2fl/f CD4-Cre model16,17.

During their differentiation, T cells are positively selected for low-affinity reactivity to self antigens expressed in the thymus. T cells with too high of an affinity for self are deleted by negative selection, yet the population of peripheral CD4+ or CD8+ T cells retains an affinity for self antigen22. For a fraction of T cells, their self-reactivity is of sufficient potency to cause clinical signs of autoimmunity under various ‘provocations’. Tonic ‘tickling’ of the T cell antigen receptor (TCR) by engagement with self ligands in the periphery is needed to maintain the viability of naive T cells. Under normal, lymphoreplete conditions, this weak TCR signaling does not drive T cell division or overt activation. However, in lymphopenic conditions, combined signals from the TCR and the homeostatic γ-chain cytokines interleukin 7 (IL-7) and IL-15 drive the slow homeostatic proliferation of most T cells23. When transferred into lymphopenic hosts with long-term immunodeficiency, such as mice deficient in recombination-activating gene 1 (Rag1−/−) mice, a fraction of T cells undergo rapid
IL-7- and IL-15-independent proliferation that is dependent on signals from commensal microbiota24,25. Finally, lymphopenia has been associated with autoimmunity26–28.

We have studied a different model of T cell–specific conditional deletion of TGF-βRII by Cre driven by the distal promoter of the gene encoding the kinase Lck (dLck-Cre) in which deletion occurred slowly in the periphery. This was in contrast to the CD4-Cre–mediated deletion, in which all thymus emigrants lacked the receptor. Unexpectedly, in the dLck deletion model, we observed that adult mice in which most peripheral CD4+ T cells and CD8+ T cells lacked the TGF-β receptor did not develop signs of autoimmunity. We hypothesize that CD4-Cre–mediated early and efficient deletion of TGF-βRII coupled with neonatal lymphopenia were together responsible for the rapid, severe autoimmunity. In many in vivo and in vitro experiments, we found that TGF-β-unresponsive T cells differed from control, TGF-β-responsive T cells in their enhanced responses to weak TCR stimuli. We propose that constitutive signaling by TGF-β controls the self-reactivity of peripheral T cells, but that even in the absence of TGF-β responsiveness, an added trigger, such as lymphopenia, is needed to drive overt autoimmune disease.

RESULTS
Deletion of TGF-βRII via dLck-Cre in adult mice
To study the function of TGF-β signaling in mature T cells, we bred mice bearing loxP-flanked alleles encoding TGF-βRII (Tgfbr2flo/flo) with mice expressing Cre recombinase under the control of the distal Lck promoter that is only turned on after positive selection of thymocytes (dLck-Cre)29,30 to generate Tgfbr2flo/flo dLck-Cre mice (called ‘TGF-βRII-deficient mice’ here) and Tgfbr2flo/flo dLck-Cre and Tgfbr2flo/flo dLck-Cre mice (collectively called ‘control mice’ here). In adult mice, dLck-Cre–mediated deletion of TGF-βRII on peripheral CD4+ T cells and CD8+ T cells was almost complete, as shown by their surface staining of TGF-βRII, which was indistinguishable from that of cells stained with isotype-matched control antibody (Fig. 1a). The expression of CD103 (integrin αEβ7) on mature CD8+ T cells has been linked to TGF-β signaling31, and the lack of CD103 expression on TGF-βRII-deficient CD8+ T cells (Fig. 1a) supported the conclusion that dLck-Cre efficiently mediated the deletion of TGF-βRII on mature T cells in adult mice. CD4+ T cells and CD8+ T cells in adult TGF-βRII-deficient mice were skewed toward an effector or memory phenotype, defined by expression of CD44 and CD62L. (Fig. 1b), and this correlated with higher expression of the T helper type 1 cell–associated transcription factor T-bet (Fig. 1c). We did not observe more T-bet in T cells with a naive phenotype (CD44hi CD62L+) in TGF-βRII-deficient mice (Fig. 1c). However, we observed no lymphoproliferation or signs of autoimmune diseases in TGF-βRII-deficient mice, all of which remained healthy for up to a year. In contrast, Tgfbr2flo/flo CD4-Cre mice bred and maintained in the same animal facility showed considerable lymphocytic infiltration in many organs early in life (Supplementary Fig. 1) and were moribund by 3–5 weeks of age, as has been reported before for CD4-Cre–mediated deletion of TGF-βRII16,17. In contrast to the abundant lymphocytic infiltrates observed in 3-week-old Tgfbr2flo/flo CD4-Cre mice, the histology of Tgfbr2flo/flo dLck-Cre mice was normal even at 18 weeks of age (Supplementary Fig. 1).

Figure 1 Characterization of T cells in adult TGF-βRII-deficient mice. (a) Surface staining for TGF-βRII and CD103 on gated splenic CD4+ or CD8+ T cells from control (Ctrl) and TGF-βRII-deficient (KO) mice 7–9 weeks of age. Isotype, staining with isotype-matched control antibody. (b) Staining of CD62L and CD44 in splenic CD4+ or CD8+ T cells. Numbers in quadrants indicate percent cells in each throughout. (c) Intracellular staining for T-bet in total and naive CD44hi CD62Lhi CD4+ and CD8+ T cells. Data are representative of three experiments.

Inefficient deletion of TGF-βRII in neonatal mice
How could the strikingly different outcomes of the use of dLck-Cre versus CD4-Cre to delete TGF-βRII on T cells be explained? There are many factors that may have contributed to the difference. Given that lymphopenia has been linked to autoimmunity26–28, we turned our attention to the importance of lymphopenia during the neonatal period to explain the different phenotypes of these two models of T cell–specific unresponsiveness to TGF-β. At neonatal day 4, the expression of TGF-βRII on peripheral T cells was indistinguishable in TGF-βRII-deficient (Tgfbr2flo/flo dLck-Cre) mice and their TGF-βRII–sufficient (Tgfbr2flo/flo dLck-Cre and Tgfbr2flo/flo) littermates (Supplementary Fig. 2a). Even at 21 d of age, in TGF-βRII-deficient mice, a considerable proportion of thymic and peripheral T cells retained their expression of TGF-βRII. In contrast, all T cells that emerged from the thymus of mice expressing CD4-Cre had deletion of TGF-βRII (Supplementary Fig. 2b). Therefore, during the neonatal lymphopenic period in newborn mice, CD4-Cre efficiently deleted Tgfbr2 and led to autoimmunity, whereas dLck-Cre was effective later and mediated efficient deletion only in adult mice, with no resulting disease.

TGF-β inhibits lymphopenia-induced proliferation
To investigate whether lymphopenia could contribute to the autoimmune phenotype of mice with TGF-β-unresponsive T cells, we turned to mixed-cell transfer. We mixed together equal numbers of allelically marked naïve T cells isolated from control (Tgfbr2flo/flo dLck-Cre) mice and TGF-βRII-deficient mice and transferred the cells into Rag1-/- recipients (Fig. 2a,b). We labeled the cells with the cytosolic dye CFSE before transfer to monitor their proliferation. A fraction of the control T cells completely diluted their CFSE within a week (Fig. 2c), which corresponded to the fast proliferation dependent on complexes of self and/or environmental peptide and major histocompatibility complex reported in such recipient mice24,25. In addition, a subset of control T cells underwent a few rounds of slow IL-7- or IL-15-dependent homeostatic proliferation (Fig. 2c). In contrast to the activity of the control T cells, slow homeostatic proliferation was almost undetectable in the transferred TGF-βRII-deficient T cells, most of which underwent fast proliferation at both day 5.5 and day 7 (Fig. 2c). The enhanced proliferation of the TGF-βRII-deficient T cells resulted in a yield of CD4+ T cells and CD8+ T cells that was three- to fivefold greater (day 5.5) and fivefold greater (day 7) than...
Figure 2  Greatly enhanced lymphopenia-induced proliferation of TGF-βRII-deficient T cells in Rag1−/− mice. (a) Experimental design: naïve T cells purified from control (CD45.1+) mice and TGF-βRII-deficient (CD45.2+) mice were mixed at a ratio of 1:1 and labeled with CFSE, then 1 × 10⁶ cells were transferred into sex-matched Rag1−/− recipient mice; 5.5 and 7 d later, recipient mice were killed. (b) Staining of CD62L and CD44 in purified naïve CD4+ or CD8+ T cells. (c) CFSE profiles of donor CD4+ or CD8+ T cells in the spleen. (d) Quantification of TGF-βRII-deficient splenic CD4+ or CD8+ T cells, presented relative to control cell numbers (KO/ctrl). (e) Frequency of KLRG1+, granzyme B–positive (GzB+) and IFN-γ+ donor T cells in the spleen. Each symbol (d,e) represents an individual recipient mouse; small horizontal lines indicate the mean (and s.e.m.). *P < 0.05 and **P < 0.01 (Student’s t-test). Data are representative of two independent experiments.

that of control T cells (Fig. 2d). Furthermore, TGF-βRII-deficient CD4+ T cells and CD8+ T cells had enhanced effector phenotypes and functions, such as expression of the receptor KLRG1 and production of granulocyte B and interferon-γ (IFN-γ; Fig. 2e).

To study the long-term effects of the enhanced proliferation of TGF-βRII-deficient T cells, we isolated naïve T cells from control and TGF-βRII-deficient mice, and transferred the cells separately into Rag1−/− recipients. Starting at 4 weeks after transfer, Rag1−/− mice that had received TGF-βRII-deficient T cells showed more weight loss than did the control group (Fig. 3a). In line with that, both CD4+ and CD8+ TGF-βRII-deficient T cells accumulated much more than control cells did in all organs examined (five- to seven-fold more accumulation of TGF-βRII-deficient CD4+ T cells and nine- to seventeen-fold more accumulation of TGF-βRII-deficient CD8+ T cells; Fig. 3b). The considerably greater lymphocytic infiltration in the liver and small intestines of Rag1−/− mice that received TGF-βRII-deficient T cells than in those of mice that received control cells further confirmed that finding (Supplementary Fig. 3). Therefore, we concluded that signaling from TGF-β directly to naïve T cells inhibited their rapid proliferation, the acquisition of effector functions and autoimmunity in Rag1−/− recipient mice.

Microbiota-independent rapid proliferation

Because the rapid proliferation of T cells transferred into chronically immunodeficient recipients is much lower in germ-free mice, commensal flora are considered a prerequisite for this rapid T cell proliferation24–32. Both inflammatory signals and antigens from commensal bacteria are required for autoimmunity in these lymphopenic settings. To delineate the underlying mechanisms of the enhanced rapid proliferation of TGF-β-unresponsive T cells, we did experiments similar to those reported above (Fig. 2), except that we pre-treated the recipient Rag1−/− mice with antibiotics for 7 d before T cell transfer to diminish the commensal bacteria and basal inflammation (Supplementary Fig. 4a). As expected, the rapid proliferation of T cells from control mice was much lower in recipients treated with antibiotics (Fig. 2c and Supplementary Fig. 4b). However, the rapid proliferation of TGF-βRII-deficient T cells was similar with or without antibiotic treatment of the Rag1−/− recipients. As a result, the population expansion of TGF-βRII-deficient T cells relative to that of control T cells was greater in the antibiotic-treated hosts (Fig. 2d and Supplementary Fig. 4c).

In additional experiments, we transferred naïve T cells from control and TGF-βRII-deficient mice together into sublethally irradiated wild-type hosts (Fig. 4a). In contrast to hosts with chronic lymphopenia, these hosts with acute lymphopenia did not support the rapid proliferation of naïve T cells from control mice24,32 (Fig. 4b). However, a substantial proportion of naïve T cells derived from TGF-βRII-deficient mice underwent rapid proliferation in sublethally irradiated wild-type C57BL/6 (B6) mice (Fig. 4b). The enhanced proliferation of TGF-βRII-deficient T cells resulted in a much greater yield of both CD4+ T cells and CD8+ T cells than that of control T cells in all organs examined (Fig. 4c). Furthermore, TGF-βRII-deficient T cells had a greatly enhanced effector function and phenotype, including expression of IFN-γ, granzyme B and KLRG1 protein (Fig. 4d), which was consistent with the greater frequency TGF-βRII-deficient T cells that expressed T-bet and Eomes (Fig. 4d). We obtained similar results when we transferred purified naïve OT-I T cells (which have transgenic expression of a TCR restricted to major histocompatibility complex class I) into Rag1−/− mice or sublethally irradiated wild-type B6 mice (Supplementary Fig. 5). Consistent with published work24, wild-type OT-I T cells did not undergo fast proliferation in either the Rag1−/− or sublethally irradiated B6 recipients. In contrast, a considerable proportion of TGF-βRII-deficient OT-I T cells completely diluted their CFSE staining (Supplementary Fig. 5b,c). These data suggested that in contrast to control T cells, TGF-βRII-deficient T cells may not have required stimulation from bacterial flora or foreign antigens to undergo rapid proliferation in immunodeficient hosts. That interpretation was supported by our finding that the proliferation of TGF-βRII-deficient OT-I T cells in a lymphopenic host in response to Listeria monocytogenes or vesicular stomatitis virus expressing chicken ovalbumin was the same as that of control OT-I T cells transferred together with the TGF-βRII-deficient OT-I T cells (data not shown). We concluded that weak TCR stimuli from self peptides may have been the driving force for the rapid proliferation of TGF-β-unresponsive T cells in the lymphopenic environment.

TGF-β inhibits responses to weak TCR stimuli in vitro

To study the effect of TGF-β signaling on T cell proliferation in response to weak TCR stimuli, we stimulated OT-I CD8+ T cells with a panel of altered peptide ligands derived from the cognate SIINFEKL peptide of ovalbumin that have been characterized for their potency33.
We isolated naive CD8+ T cells from control and TGF-βRII-deficient OT-I mice, then mixed the cells together and labeled them with CFSE (Fig. 5a). We depleted splenocyte populations of T cells and natural killer (NK) cells, then pulsed the splenocytes with the various altered peptide ligands and used these cells as antigen-presenting cells (APCs) in the presence of added TGF-β1. We added IL-2 1 d later and monitored the cultures for another 4 d. We found that high-affinity peptide ligands such as N4 (the cognate ligand) and the variant Y3 induced the cultures for another 4 d. We found that high-affinity peptide ligands and used these cells as antigen-presenting cells (APCs) in the presence of added TGF-β1. We added IL-2 1 d later and monitored the cultures for another 4 d. We found that high-affinity peptide ligands such as N4 (the cognate ligand) and the variant Y3 induced only slightly enhanced population expansion of TGF-βRII-deficient OT-I cells during the 5-day culture (less than threefold; Fig. 5b). Notably, as the peptide potency decreased, the population expansion of TGF-βRII-deficient OT-I cells increased considerably, such that by day 4, TGF-βRII-deficient OT-I T cells outnumbered control OT-I T cells by 100-fold in cultures stimulated with T4, the ligand of lowest affinity (Fig. 5b,c). We detected no population expansion in the absence of peptide (Fig. 5b,c). The regulatory activity of TGF-β is modulated by the presence of inflammatory cytokines in other cell types4. Indeed, treatment with the proinflammatory cytokines IFN-α and IL-12 resulted in a much lower proliferative advantage of TGF-βRII-deficient OT-1 cells over control OT-1 cells, whereas other proinflammatory cytokines, such as IFN-γ, IL-6, TNF, IL-1β and IL-18, had a minimal effect or no effect (Fig. 5c and data not shown).

To rule out the possibility that the observed effects were unrelated to the TGF-β unresponsiveness of the TGF-βRII-deficient T cells, we added neutralizing antibody to TGF-β to the control cultures stimulated with T4; this resulted in the proliferation of control OT-1 cells to the same extent as that of TGF-βRII-deficient cells (Fig. 5c). Therefore, TGF-β signaling considerably inhibited the proliferative response of CD8+ T cells to weak TCR stimuli, whereas it had minimal effects on strong stimulation via the TCR.

To address the possibility that TGF-βRII-deficient T cells with a naive phenotype were already programmed to act differently than
control T cells acted, we sought to determine if the effect of TGF-β could be reproduced in wild-type naive cells. We purified wild-type naive OT-I cells, labeled them with CFSE and mixed them with peptide-pulsed APCs at a ratio of 1:1 in the presence of TGF-β1 or neutralizing antibody to TGF-β. We added IL-2 the next day and monitored the cultures for another 4 d. After strong stimulation of the TCR (N4 at a concentration of 5 ng/ml), the presence or absence of TGF-β signaling had a minimal effect on the proliferation of wild-type OT-I cells (Fig. 6). However, as the intensity of the TCR stimulation decreased, the inhibitory effects of TGF-β on wild-type OT-I cells increased considerably. For example, after stimulation with N4 at a concentration of 0.2 ng/ml (strong agonist peptide) or T4 at a concentration of 5 ng/ml (weak agonist peptide), inhibition of TGF-β signaling resulted in a sevenfold greater yield of OT-I cells during the 5-day culture (Fig. 6a). For stimulation with T4 at a concentration of 1 ng/ml, the yield of OT-I cells was 55-fold greater after inhibition of TGF-β signaling. Thus, we found that in the presence of neutralizing antibody to TGF-β, wild-type OT-I T cells acted like TGF-RII-deficient cells (Figs. 5 and 6). We concluded that TGF-β signaling specifically inhibited the response to weak TCR stimulation while largely sparing the response to strong TCR stimulation.

Another possible explanation for the greater proliferation of TGF-βRII-deficient T cells in a lymphopenic environment was that TGF-βRII-deficient T cells had an enhanced response to common γ-chain cytokines, such as IL-7, IL-15 or IL-2. However, IL-2, IL-7 or IL-15 alone did not induce the proliferation of TGF-βRII-deficient OT-I T cells in vitro (Fig. 5 and Supplementary Fig. 6). Furthermore, when we used sublethally irradiated Il15−/− mice as recipient mice, we obtained results similar to those obtained with sublethally irradiated wild-type hosts (Fig. 4), and in a lymphoreplete environment, TGF-βRII-deficient T cells did not respond better than wild-type cells to complexes of superagonist IL-2 and antibody to IL-2 (data not shown). Therefore, weak stimulation of the TCR was needed to induce hyperproliferative responses by TGF-βRII-deficient T cells.

CD4+ T cell help for TGF-βRII-deficient T cells
To elucidate the contributions of CD4+ T cells versus CD8+ T cells in eliciting autoimmunity in lymphopenic recipients of TGF-β-unresponsive T cells, we isolated naive CD4+ T cells and CD8+ T cells from control and TGF-βRII-deficient mice and transferred those cells separately into Rag1−/− recipients (Fig. 7a). There were tenfold

Figure 5  In vitro hyperproliferative response of TGF-βRII-deficient CD8+ T cells to weak stimulation. (a) Staining of CD62L and CD44 in the starting populations of control and TGF-βRII-deficient OT-I cells for the experiments below. (b) Quantification of OT-I T cells after culture of a mixture of equal numbers of CFSE-labeled naive control (CD45.1+CD45.2+) and TGF-βRII-deficient (CD45.1+) OT-I cells together with an equal number of B6 (CD45.2+) APCs (splenocyte populations that had been depleted of T cells and NK cells and incubated for 1 h at 37 °C with no peptide (No pep) or the ovalbumin peptide variants N4, Y3, Q4 or T4 (1 ng/ml each) plus LPS (1 µg/ml), then extensively washed); OT-I T cells and APCs were incubated together for 1 d in the presence of human TGF-β1 (2.5 ng/ml) without inflammatory cytokines, followed by the addition of IL-2 (50 U/ml).

(c) Flow cytometry of T4-stimulated OT-I T cells after 4 d of in vitro culture as in b, but with the addition of no cytokines (−), IFN-α (1000 U/ml), IFN-γ (20 ng/ml) or IL-12 (20 ng/ml) during the incubation with TGF-β1 (left), and flow cytometry of OT-I T cells without peptide stimulation (No pep), cultured with IL-2 and TGF-β1 (top right), or of T4-stimulated OT-I T cells cultured with IL-2 and neutralizing antibody to TGF-β (α-TGF-β, 10 µg/ml; bottom right). Numbers adjacent to outlined areas indicate percent CD45.1+CD45.2+ (TGF-βRII-deficient) cells (left area) or CD45.1+CD45.2+ (control) cells (right area). Data are representative of three independent experiments.

Figure 6  In vitro hyperproliferative response of wild-type CD8+ T cells to weak stimulation. (a) Quantification of live OT-I T cells in cultures of 1 × 10^6 CFSE-labeled naive wild-type (CD45.1+) OT-I cells and 1 × 10^5 B6 (CD45.2+) APCs (splenocyte populations depleted of T cells and NK cells and incubated for 1 h at 37 °C with various concentrations (above plots) of ovalbumin peptide variants plus LPS (1 µg/ml) and washed extensively), incubated in the presence of neutralizing antibody to TGF-β (10 µg/ml) or human TGF-β1 (2.5 ng/ml), with the addition of IL-2 (50 U/ml) 1 d after the start of coculture, assessed by flow cytometry with the addition of a specific number of beads (for quantification by comparison of beads and live OT-I T cells). (b) CFSE profiles of OT-I T cells after 4 d in vitro culture as in a. Data are representative of two independent experiments.
Figure 7 TGF-RII-deficient CD4+ T cells induce autoimmune disease in Rag1−/− mice, whereas TGF-RII-deficient CD8+ T cells do so only in the presence of CD4+ T cells. (a) Experimental design. 1 × 10⁶ naive CD4+ or CD8+ T cells from control or TGF-RII-deficient mice were transferred into Rag1−/− recipient mice, followed by analysis 5–7 weeks later. (b) Quantification of donor CD4+ T cells and CD8+ T cells in the spleen and lamina propria, calculated from total cellularity and percent of each population. (c,d) Body weight of Rag1−/− mice that received CD4+ T cells alone (c) or CD8+ T cells with or without CD4+ T cells (d). Each line represents an individual recipient mouse; small horizontal lines indicate the mean (and s.e.m.). Data are representative of two experiments.

more TGF-RII-deficient CD4+ T cells than control CD4+ T cells, whereas TGF-RII-deficient CD8+ T cells and control CD8+ T cells underwent similar population expansion (Fig. 7b). In line with that, TGF-RII-deficient CD4+ T cells rapidly induced weight loss in Rag1−/− recipients, whereas TGF-RII-deficient CD8+ T cells did not (Fig. 7c,d). However, even CD4+ T cells from control mice promoted the population expansion of TGF-RII-deficient CD8+ T cells in Rag1−/− recipients (Supplementary Fig. 7), and as a consequence, the transfer of control CD4+ T cells together with TGF-RII-deficient CD8+ T cells precipitated the development of autoimmunity (Fig. 7d). In summary, TGF-RII-deficient CD4+ T cells alone and TGF-RII-deficient CD8+ T cells with help from CD4+ T cells induced autoimmunity under lymphopenic conditions.

DISCUSSION
We have shown here that CD4+ T cells and CD8+ T cells that were unresponsive to TGF-β signals because they lacked TGF-RII remained quiescent in the lymphoid periphery of conventionally housed, immununized mice. Thus, adult mice in which most peripheral T cells lacked TGF-RII nonetheless seemed healthy, showing no signs of lymphoproliferation, weight loss or autoimmune disease for the duration of the analysis (18 weeks). That result, obtained by the T cell–specific deletion of Tgfbr2 by Cre expressed under the control of the distal Lck promoter was unexpected because it differed sharply from the outcome obtained by deletion of Tgfbr2 by Cre driven by the Cd4 promoter. Other studies have reported16,17, and we have confirmed here, that such Tgfbr2−/− CD4-Cre mice show signs of disease shortly after birth and die by ~3–5 weeks of age with massive lymphoproliferation and multiorgan lymphocytic infiltrates. We sought to explain the strikingly different outcomes of using dLck-Cre versus CD4-Cre to delete Tgfbr2. There are many differences between the two Cre deletion models. For example, CD4-Cre is turned on at the early stage of thymopoiesis (for example, CD4-Cre is turned on at the early stage of thymopoiesis whereas TGF-RII-deficient CD8+ T cells with or without CD4+ T cells (d). Each line represents an individual recipient mouse; small horizontal lines indicate the mean (and s.e.m.). Data are representative of two experiments.

Given that lymphopenia has been linked to autoimmunity26–28, we focused on the importance of the neonatal lymphopenic period to explain the different phenotypes of the two models of T cell–specific deletion of TGF-RII. This was sparked by our observation that deletion of TGF-RII was rather inefficient in the periphery and thymus of newborn and young Tgfbr2−/− dLck-Cre mice. In contrast, all T cells that emerged from the thymus in mice expressing CD4-Cre had deleted TGF-RII, and this included the cells that emerged during the neonatal period. Although our study has not ruled out the possibility of a contribution from other sources (such as differences in Treg cells, the presence or absence of NKT cells, or an altered TCR repertoire) for the different phenotypes of the two models, our data have shown that naive CD4+ T cells and CD8+ T cells were regulated by TGF-β signals in a lymphopenic environment. In particular, we have shown that after transfer, TGF-β-unresponsive T cells with a naive phenotype showed greater proliferation and acquisition of effector phenotype and more rapid disease induction in lymphopenic recipients than did TGF-β-responsive T cells.

Because the proliferation of naive T cells in a lymphopenic environment is partly dependent on the weak TCR stimulation derived from ligands composed of self major histocompatibility complex molecules plus self peptides37, we made a model of this by comparing the responses of control and TGF-RII-deficient CD8+ T cells to potent and weak TCR stimuli in vitro. In mixed cultures of control and TGF-RII-deficient T cells, we again observed the considerable proliferative advantage of the TGF-RII-deficient T cells in response to weak stimulation of the TCR. Published work has shown that the weakest ligand used, T4, is about 1.4% as potent as the cognate SIINFEKL peptide in activating OT-I cells37 and is reported to be close to the ‘threshold’ affinity for thymocyte positive selection versus negative selection36. Notably, neither differences in TCR repertoire nor
the action of Treg cells can explain that in vitro result obtained with purified OT-I CD8+ T cells as responder cells. To rule out the possibility that defects in TGF-βRII-deficient T cells other than acute unresponsiveness to TGF-β were related to the phenotype, we cultured wild-type OT-I cells with TGF-β or in the presence of blocking antibody to TGF-β. Indeed, in the absence of TGF-β signaling, wild-type OT-I cells acted like TGF-βRII-deficient cells.

Together our results allow us to hypothesize that under non-inflammatory conditions, but with enhanced exposure to self antigen, as in a lymphopenic environment, direct signaling from TGF-β to T cells is necessary for the control of T cell activation and proliferation. Our results and those of other studies have confirmed the dominant and persistent role of TGF-β in maintaining T cell homeostasis. The dampening effect on T cell activation and resultant autoimmunity is especially important when other factors, such as enhanced exposure to self antigen and greater access to homeostatic cytokines, affect peripheral T cells.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

N.Z. and M.I.R. designed experiments and wrote the paper, and N.Z. did the experiments.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Mice. Tgfbr2f/f mice were provided by S. Karlsson. Mice with transgenic expression of Cre from the distal Lck promoter were provided by P.J. Fink and were originally from N. Killeen (University of California, San Francisco). C57BL/6 mice (000664) and Rag1−/− mice (002216) from The Jackson Laboratory were housed in specific pathogen–free conditions in the animal facilities at the University of Washington. All recipient mice were used at 6–12 weeks of age. All experiments were done in accordance with the University of Washington Institutional Animal Care and Use Committee guidelines.

Adoptive transfer and antibiotic treatment. Total naive T cells, CD4+ T cells, CD8+ T cells and OT-I T cells were isolated from the spleen and lymph nodes with a Pan T cell isolation kit, a CD4 isolation kit or a CD8 isolation kit according to the manufacturer’s instructions (Miltenyi) but with the addition of biotin-conjugated antibody to CD44 (anti-CD44; IM7; BD) during the incubation with biotin-conjugated antibody ‘cocktail’. The appropriate number of T cells was transferred intravenously into the appropriate recipient mice. Some recipient mice had been treated with water containing antibiotics (100 U/ml polymixin B, 1 mg/ml neomycin and 1 mg/ml ampicillin) for 7 d before T cell transfer.

In vitro proliferation. Naive OT-I T cells were purified with a CD8 isolation kit (Miltenyi) with supplemental antibody to CD44 and were labeled with 5 µM CFSE (Invitrogen). Splenocyte populations depleted of T cells and NK cells were purified by a procedure similar to that used for OT-I T cells with an antibody mixture containing biotin–anti-TCRβ (H57-597), biotin–anti-TCRγδ (eBioGL3) and biotin–anti-NK1.1 (pk136; all from eBioscience) and were used as APCs. Those APCs were pulsed for 1 h at 37 °C with 0.2–5 ng/ml peptide (SIINFEKL and all variant peptides from Genemed Synthesis) and 1 µg/ml LPS (Sigma), followed by extensive washing. OT-I T cells and APCs were mixed and cultured in the presence of 2.5 ng/ml human TGF-β1 (R&D systems), with the addition of 1,000 U/ml IFN-α (PBL InterferonSource), 20 ng/ml IFN-γ and 20 ng/ml IL-12 (Peprotech) where appropriate. Then, 50 U/ml IL-2 (eBioscience) was added 1 d later. Where appropriate, 10 µg/ml neutralizing antibody to TGF-β (1D11; R&D Systems) was added in the absence exogenous human TGF-β1. For quantification of T cells during in vitro culture, 20 µl Flow Cytometry Absolute Count Standard beads (Bang’s Lab) were added to each sample before flow cytometry.

Antibodies and flow cytometry. Single-cell suspensions were prepared from the spleen, small intestine and liver after perfusion of the animal at the indicated time-points post cell transfer. Cells were typically stained with anti-CD8 (53-6.7), anti-CD4 (RM4-5), anti-CD44 (IM7; BD) during the incubation with biotin-conjugated antibody ‘cocktail’. The appropriate number of T cells was transferred intravenously into the appropriate recipient mice. Some recipient mice had been treated with water containing antibiotics (100 U/ml polymixin B, 1 mg/ml neomycin and 1 mg/ml ampicillin) for 7 d before T cell transfer.

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