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Control of CD8αα intestinal intraepithelial lymphocyte development by TGF-β

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Abstract

The molecular mechanisms directing the development of TCRαβ$^+$CD8αα$^+$ intestinal intraepithelial lymphocytes (IEL) are not thoroughly understood. Here we show that transforming growth factor-β (TGF-β) controls the development of TCRαβ$^+$CD8αα$^+$ IEL. Mice with either a TGF-β1 null mutation or a T cell-specific deletion of the TGF-β receptor I lacked TCRαβ$^+$CD8αα$^+$ IEL, whereas transgenic mice that over-expressed TGF-β1 had an increased population of TCRαβ$^+$CD8αα$^+$ IEL. Defective development of the TCRαβ$^+$CD8αα$^+$ IEL thymic precursors (CD4$^-$CD8$^-$TCRαβ$^+$CD5$^+$) was observed in the absence of TGF-β. In addition, we showed that TGF-β signaling induced CD8α expression in TCRαβ$^+$CD8αα$^+$ IEL thymic precursors and induced and maintained CD8α expression in peripheral populations of T cells. These data demonstrate a previously unrecognized role for TGF-β in the development of TCRαβ$^+$CD8αα$^+$ IEL and the expression of CD8 in T cells.

Keywords

TGF-β; CD8αα; CD4; IEL; Th-POK; Runx3

Intestinal intraepithelial lymphocytes (IEL) reside within the single cell epithelial layer of the intestine1, and are instrumental in safeguarding the integrity of mucosal immune responses. IEL contain two phenotypically distinct populations: conventional IEL,

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expressing the T cell receptor (TCR)αβ and either the co-receptor CD4 or heterodimers of CD8αβ, and unconventional IEL, which are either TCRαβ$^+$ or TCRγδ$^+$ and bear unique CD8αα homodimers.

TCRαβ$^+$CD8αα$^+$ IEL remain a somewhat elusive cell type, with their function and development still poorly understood. TCRαβ$^+$CD8αα$^+$ IEL exhibit an "activated but resting" phenotype and have been suggested to have a regulatory role within the gut1. They express high levels of CTLA-4, PD-1, Lag3 and transforming growth factor-β (TGF-β) and inhibitory NK receptors2-4, molecules associated with immunoregulation. TCRαβ$^+$CD8αα$^+$ IEL prevent colitis when co-transferred with CD4$^+$CD45RB^{high} T cells into immuno-compromised mice5. Lymphocytic choriomeningitis virus (LCMV) infection of transgenic mice which express an LCMV-reactive TCR plus a cognate antigen trans-gene reinforced the regulatory potential of TCRαβ$^+$CD8αα$^+$ IEL; upon infection, TCRαβ$^+$CD8αα$^+$ IEL showed signs of activation, however, unlike conventional IEL they did not become cytotoxic nor secrete pro-inflammatory cytokines3. The developmental pathway followed by TCRαβ$^+$CD8αα$^+$ IEL has been debated6; once thought to be of extra-thymic origin, TCRαβ$^+$CD8αα$^+$ IEL are now generally considered to arise in the thymus7, 8, where the TCRαβ$^+$CD8αα$^+$ IEL precursors develop under the condition of a high affinity self-antigen-agonist4, 9. "Forbidden" TCRs have been identified in the TCRαβ$^+$CD8αα$^+$ IEL population10. However, the molecular pathways and regulatory factors controlling the development of TCRαβ$^+$CD8αα$^+$ IEL remain largely undetermined. Interleukin 15 (IL-15) was suggested to be required for their development, but IL-15 is not needed in the thymus, instead it maintains TCRαβ$^+$CD8αα$^+$ IEL in the gut11.

The thymic "agonist selection" process utilized for the differentiation of TCRαβ$^+$CD8αα$^+$ IEL is shared with Foxp3$^+$ regulatory T cells (Tregs)12 and CD1-d restricted NK T cells (iNKT)13. Since both Foxp3$^+$ Tregs14 and iNKT15 require TGF-β and TCR ligation to develop and differentiate, we questioned whether TGF-β was also required for the development of TCRαβ$^+$CD8αα$^+$ IEL. We show here that TGF-β controls TCRαβ$^+$CD8αα$^+$ IEL development. Mice with either a null mutation in TGF-β1 (*Tgfb1*$^{-/-}$) or a T cell-specific deletion of TGF-β receptor I (TβRI) lacked TCRαβ$^+$CD8αα$^+$ IEL. In contrast, transgenic mice that over-expressed TGF-β1 had an increased population of TCRαβ$^+$CD8αα$^+$ IEL. Mechanistically, we show that TGF-β prevents apoptosis by regulating the expression of Bcl-2 and Bim and induces CD8α expression in TCRαβ$^+$CD8αα$^+$ IEL thymic precursors. TGF-β regulation of CD8α expression was not limited to the IEL, but was rather a more general phenomenon, as TGF-β induced the re-expression of repressed CD8α in lineage committed peripheral CD4$^+$ T cells. CD8α expression was associated with suppression of Th-POK up-regulation and prevention of Runx3 down-regulation in CD4$^+$ T cells. Thus we demonstrate a previously unrecognized role for TGF-β-signaling in the development of TCRαβ$^+$CD8αα$^+$ IEL and in the expression of CD8α in TCRαβ$^+$ cells.

Results

TGF-β deficiency reduces TCRαβ$^+$CD8αα$^+$ IEL

During the perinatal period lymphocytes accumulate in the gut, with a fully mature complement of IEL being present by three weeks of age16, 17. We examined the IEL

populations in *Tgfb1*$^{-/-}$ mice during this period of epithelial colonization by lymphocytes. TCRαβ^{+}CD8αα^{+} IEL were significantly reduced in *Tgfb1*$^{-/-}$ mice, most notably after two to three weeks of age (Fig.1a-c). The decrease in TCRαβ^{+}CD8αα^{+} IEL was accompanied by an increase in TCRαβ^{+}CD8αβ^{+} IEL. The decrease in TCRαβ^{+}CD8αα^{+} IEL was significant both in terms of frequency within TCRαβ^{+} lymphocytes of the gut (Fig.1b), or total cell numbers (Fig.1c) despite the overall increase in TCRαβ^{+} T cells that accompanies the systemic inflammation seen in *Tgfb1*$^{-/-}$ mice18. In contrast, there was no reduction in the frequency or total numbers of TCRγδ^{+}CD8αα^{+} IEL in *Tgfb1*$^{-/-}$ mice (Fig.1d-f). These data suggest TGF-β plays a role in the development of TCRαβ^{+}CD8αα^{+} IEL.

We next determined whether the requirement for TGF-β in the generation of TCRαβ^{+}CD8αα^{+} IEL was T cell autonomous by using mice in which TβRI was specifically deleted in T cells (*Tgfbr1*$^{f/f}$CD4-cre^{+})14. Examination of IEL in these mice revealed that they also lacked TCRαβ^{+}CD8αα^{+} IELs (Fig.1g). *Tgfbr1*$^{f/f}$CD4-cre^{+} mice had a reduction in the frequency and total number of TCRαβ^{+}CD8αα^{+} IEL (Fig.1h, i), but not in TCRγδ^{+}CD8αα^{+} IEL (Fig.1j-l). We also examined the IEL compartment in mixed bone marrow chimeras generated using CD45.1^{+} wild-type mixed with CD45.2^{+}*Tgfbr1*$^{f/f}$CD4-cre^{+} or CD45.2^{+} *Tgfbr1*$^{f/+}$CD4-cre^{+} donor cells injected into recombination-activating gene-1-deficient (*Rag1*$^{-/-}$) hosts. IEL populations derived from *Tgfbr1*$^{f/f}$CD4-cre^{+} bone marrow had a significantly lower proportion of TCRαβ^{+}CD8αα^{+} IEL compared to both CD45.1^{+} and *Tgfbr1*$^{f/+}$CD4-cre^{+} controls (Supplementary Fig.1). Thus, deletion of TGF-β signaling resulted in defective development of TCRαβ^{+}CD8αα^{+} IEL.

Canonical TGF-β signaling requires members of the Smad protein family19. *Smad3*$^{-/-}$ mice showed a significant reduction in the frequency of TCRαβ^{+}CD8αα^{+} IEL compared to littermate controls (Fig.2), suggesting Smad3 plays a role downstream of TGF-β in TCRαβ^{+}CD8αα^{+} IEL generation.

Over-expression of TGF-β1 increases TCRαβ^{+}CD8αα^{+} IEL

We next examined the IEL populations in mice that over-expressed active TGF-β1 specifically in T cells (Supplementary Fig. 2). In β1^{glo+}CD4-cre^{+} mice expression of an active TGF-β1, which is unable to associate with the latency associated protein (LAP), is driven by an ubiquitous promoter and marked by loss of eGFP expression20. These mice showed no signs of overt immune dysregulation (data not shown). However, TCRαβ^{+}CD8αα^{+} IELs were significantly enhanced in β1^{glo+}CD4-cre^{+} mice compared to non-transgenic littermate controls (Fig.3), further suggesting that TGF-β is required for the development of this population.

TGF-β deficiency reduces TCRαβ^{+}CD8αα^{+} IEL precursors

TCRαβ^{+}CD8αα^{+} IEL precursors are found within a population of CD4^{-}CD8^{-} double negative (DN) thymocytes which have a functionally rearranged TCR, thus have undergone positive selection, and are positive for CD57 (Fig. 4a). We investigated whether lack of TGF-β signaling affected their generation and/or phenotype. *Tgfb1*$^{-/-}$ mice (Fig.4b) and *Tgfbr1*$^{f/f}$CD4-cre^{+} mice (Supplementary Fig.3) had a significant reduction in the frequency of DN TCRαβ^{+}CD5^{+} thymocytes, but not in the frequency of total DN thymocytes (Fig.4b

and Supplementary Fig. 3). Increased cell death was observed in the DN TCRαβ$^+$CD5$^+$ thymocyte compartment in *Tgfb1*$^{-/-}$ mice compared to wild-type controls (Fig.4c). TGF-β-signaling is known to inhibit T cell apoptosis associated with increased mitochondria permeability21 and with imbalances in the expression of pro- and anti-apoptotic Bcl-2 family members22. DN TCRαβ$^+$CD5$^+$ thymocytes from *Tgfb1*$^{-/-}$ mice had reduced expression of the pro-survival protein Bcl-2 (Fig.4d) and increased expression of the pro-apoptotic protein Bim (Fig.4e). This was not the case for the total DN population, which, in *Tgfb1*$^{-/-}$ mice, exhibited enhanced expression of both Bim and Bcl-2 compared to wild-type controls, and, as previously described21, showed no increase in apoptosis (Supplementary Fig.4). Thus, TGF-β1 is required for the protection of DN TCRαβ$^+$CD5$^+$ thymocytes from cell death by safeguarding the balanced expression of Bcl-2 family members.

We used Ki-67 staining to look for a proliferation defect in TGF-β1-deficient DN TCRαβ$^+$CD5$^+$ thymocytes. *Tgfb1*$^{-/-}$ DN TCRαβ$^+$CD5$^+$ thymocytes exhibited no proliferative defect, rather there were more dividing (Ki-67$^+$) cells (Fig.4f). This was in contrast to the total DN thymocyte population, which had similar frequencies of Ki-67$^+$ cells in *Tgfb1*$^{-/-}$ and wild-type thymi (Supplementary Fig.4). These data suggest that TGF-β protects DN TCRαβ$^+$CD5$^+$ from death and exclude decreased precursor expansion as the primary cause for the lack of TCRαβ$^+$CD8αα$^+$ IEL in TGF-β-deficient mice.

Phenotypic alterations of thymic precursors in TGF-β-deficient mice

We further examined if TGF-β1-deficient DN TCRαβ$^+$CD5$^+$ thymocytes exhibited an altered phenotype that could influence their function, survival and/or migration to the gut. The few remaining DN TCRαβ$^+$CD5$^+$ thymocytes in *Tgfb1*$^{-/-}$ mice exhibited lower expression levels of IL-7 receptor α (CD127) (Fig.4g). IL-7 has both pro-survival effects for thymocytes23 and is implicated in CD8 lineage choice24, however, it remains unclear whether CD127 down-regulation has a causative role or just accompanies the TGF-β-mediated decrease in DN TCRαβ$^+$CD5$^+$ thymocytes. IL-2 causes down-regulation of CD127 expression25 and TGF-β-deficient mice have increased IL-2 levels14. However, heightened amounts of IL-2 cannot account for lack of TCRαβ$^+$CD8αα$^+$ IEL in TGF-β-deficient mice, as *IL-2*$^{-/-}$ × *Tgfbr1*$^{f/f}$Lck-cre$^+$ (IL-2 and TβRI double knockout) mice do not have TCRαβ$^+$CD8αα$^+$ IELs, and *IL-2*$^{-/-}$ mice do not have enhanced frequencies of these cells (Supplementary Fig.5). IL-15 is also involved in TCRαβ$^+$CD8αα$^+$ IEL generation11, however we saw no alteration in IL-15R expression that could account for decreased TCRαβ$^+$CD8αα$^+$ IEL in TGF-β-deficient mice (Supplementary Fig.5).

We next examined expression of adhesion molecules important for gut homing. Integrin α4β7 is important for trafficking of recent thymic emigrants (RTE) to the gut epithelium26 and is thought to direct homing of TCRαβ$^+$CD8αα$^+$ IEL precursors to the gut mucosa6, 27. Wild-type DN TCRαβ$^+$CD5$^+$ thymocytes expressed α4β7 (Fig.4h). The frequency of α4β7$^+$ cells was increased in *Tgfb1*$^{-/-}$ mice (Fig.4h), ruling out aberrant α4β7$^+$-mediated gut homing of precursors. CD103 expression, which is dependent on TGF-β28, was reduced on DN TCRαβ$^+$CD5$^+$ thymocytes from *Tgfb1*$^{--/-}$ mice (Fig.4i). However, CD103 is not required for trafficking of RTEs to the gut26. These data suggest that defective generation of

TCRαβ$^+$CD8αα$^+$ IEL in TGF-β-deficient mice could not be attributed to phenotypic changes affecting DN TCRαβ$^+$CD5$^+$ thymocyte gut homing and/or IL-7 responsiveness.

TGF-β induces CD8α expression in DN TCRαβ$^+$CD5$^+$ thymocytes

Although total DN thymocytes in TGF-β-deficient mice displayed normal survival, other thymocyte populations, such as single positive CD4$^+$ and CD8$^+$ and double positive thymocytes showed enhanced cell death21 and changes in the reciprocal expression of Bcl-2 and Bim similar to DN TCRαβ$^+$CD5$^+$ thymocytes (Supplementary Fig.4 and data not shown). To further elucidate the mechanisms by which TGF-β controls TCRαβ$^+$CD8αα$^+$ IEL development, we determined whether TGF-β1 could induce CD8α expression in the DN TCRαβ$^+$CD5$^+$ thymocytes. DN TCRαβ$^+$CD5$^+$ thymocytes were sorted from wild-type mice and cultured with TCR-simulation and with or without TGF-β1. TGF-β1 induced CD8α mRNA in DN TCRαβ$^+$CD5$^+$ thymocytes (Fig.5a) without affecting CD8β expression (Fig. 5b).

To determine what transcription factors were involved in the TGF-β mediated induction of CD8α expression in DN TCRαβ$^+$CD5$^+$ thymocytes, we examined the expression of Runx3 and Th-POK in DN TCRαβ$^+$CD5$^+$ thymocytes and TCRαβ$^+$CD8αα$^+$ IEL. Runx3 and Th-POK are transcription factors important in CD4 and CD8 lineage commitment; Runx3 inhibits CD4 and promotes CD8 T cell differentiation, and Th-POK inhibits CD8 and promotes CD4 T cell differentiation29. Using fluorescent-protein reporter mice30, 31 to examine Runx3 and Th-POK expression, we saw that a similar proportion of DN TCRαβ$^+$CD5$^+$ thymocytes and TCRαβ$^+$CD8αα$^+$ IEL were Runx3 positive, but no TCRαβ$^+$CD8αα$^+$ IEL and few TCRαβ$^+$CD5$^+$ thymocytes were Th-POK positive (Supplementary Fig.6), suggesting that the TCRαβ$^+$CD8αα$^+$ IEL program is set in the thymus. In addition, in DN TCRαβ$^+$CD5$^+$ thymocytes cultured under TCR-stimulation conditions, TGF-β1 did not change the proportion of Runx3$^+$ DN TCRαβ$^+$CD5$^+$ thymocytes (data not shown), nor the amount of Runx3 expressed per cell (MFI) (Supplementary Fig.6). However, TGF-β1 treatment resulted in a lower proportion of Th-POK-positive TCRαβ$^+$CD5$^+$ thymocytes compared to control cultures (Supplementary Fig.6). These data suggest that TGF-β alters the balance of these lineage commitment transcription factors in DN TCRαβ$^+$CD5$^+$ thymocytes to favor CD8α expression. Confirming a direct effect of TGF-β on Th-POK transcription, TGF-β1 reduced Th-POK mRNA amounts in sorted DN TCRαβ$^+$CD5$^+$ thymocytes cultured under TCR-stimulating conditions (Fig.5c). In addition, DN TCRαβ$^+$CD5$^+$ thymocytes from *Tgfb1*$^{-/-}$ mice had increased expression of Th-POK mRNA compared to littermate controls (Supplementary Fig.6). These data suggest that TGF-β favors CD8 expression by reducing Th-POK in the presence of maintained Runx3 expression.

TGF-β maintains CD8 expression on CD8$^+$ T cells

The demonstration that TGF-β could induce CD8α mRNA in DN TCRαβ$^+$CD5$^+$ thymocytes prompted us to ask whether TGF-β could also affect the levels of CD8α expression in other TCRαβ$^+$ T cell populations. In total TCRαβ$^+$CD8$^+$ (CD8αα$^+$ and CD8αβ$^+$) IELs and TCRαβ$^+$CD8αβ$^+$ IEL CD8α expression on a per cell basis was lower in TGF-β-deficient mice compared to controls (Fig.1, Supplementary Fig.7 and data not shown), despite the

increased frequency of TCRαβ$^+$CD8αβ$^+$IEL in TGF-β-deficient mice (Fig.1). In addition, peripheral CD8$^+$ T cells in *Tgfb1*$^{-/-}$ (Fig.6a, b) and *Tgfbr1*$^{f/f}$CD4-cre$^+$ mice (Fig.6c, d) exhibited a lower expression of CD8α per cell. CD8β expression was also reduced on TCRβ$^+$CD8αβ$^+$ and peripheral CD8$^+$ T cells in TGF-β-deficient mice (Fig.6). Reduced CD8 expression was accompanied by reduced frequencies of CD8$^+$ T cells, as reported for TGF-β-deficient mice32, and was detected on live and naïve TCRαβ$^+$CD8$^+$ gated cells (Supplementary Fig.7), suggesting decreased CD8 expression was unlikely due to cell death or activation. Expression of CD8 was also specifically reduced on *Tgfbr1*$^{f/f}$CD4-cre$^+$ cells in mixed bone-marrow chimeras (Supplementary Fig.7), suggesting a T cell intrinsic effect. Notably, decreased co-receptor expression was not seen in CD4$^+$ T cells (Fig.6 and Supplementary Fig.7), indicating a specific role for TGF-β in maintaining CD8 expression. Consistent with these *in vivo* observations, addition of TGF-β to cultures of peripheral wild-type CD8$^+$ T cells increased the expression of CD8α on a per cell basis (MFI) (Supplementary Fig.8). These data indicate that TGF-β maintains CD8α expression not only in TCRαβ$^+$ IELs, but also in peripheral CD8$^+$ T cells.

TGF-β induces expression of CD8α in CD4$^+$ T cells

T cells expressing both CD4 and CD8 exist *in vivo*33, and adoptive transfer of CD4$^+$ T cells into *Rag1*$^{-/-}$ mice leads to differentiation of a CD4$^+$CD8αα$^+$ population specifically in the gut34. Thus, we examined whether TGF-β could induce CD8α expression on CD4$^+$ T cells. A small fraction of peripheral CD4$^+$ T cells, a population with no CD8α expression, became CD8α$^+$ when cultured with TGF-β1 *in vitro* (Fig.7a). The induction of CD8α on CD4$^+$ T cells occurred whether the starting population was sorted naïve T cells, CD4$^+$CD62L^{hi} cells (Supplementary Fig.9), or sorted CD4$^+$CD25$^-$ cells (Fig.7). TGF-β-dependent induction of CD8α on CD4$^+$ T was independent of CD28-costimulation, since CD8α was induced in the presence and absence of anti-CD28 (data not shown and Supplementary Fig.9). Of those cells that were induced to express CD8α, one-third were CD8β$^-$ (CD4$^+$CD8αα$^+$), while the remaining cells co-expressed CD8β.

To confirm this was indeed *de novo* induction of CD8α expression, we examined CD8α mRNA expression in TGF-β1-treated CD4$^+$ T cells before proliferation of the cultured cells. At 6 hours following TGF-β1-treatment, CD8α gene transcription had already significantly increased in CD4$^+$ T cells (Fig.7b), with an even greater enhancement of CD8α mRNA at 48 hours. CD8β mRNA expression was not increased at 6 hours and was decreased at later time points (Fig.7b). Thus, similar to DN TCRαβ$^+$CD5$^+$ thymocytes, TGF-β1 induces CD8α expression in CD4$^+$ T cells.

Mechanisms of CD8α expression by TGF-β

We next determined the mechanism(s) of TGF-β-mediated induction of CD8α on CD4$^+$ T cells. Induction of CD8α occurred in wild-type CD4$^+$ T cells cultured with TGF-β1, but no CD4$^+$CD8α$^+$ T cells emerged in *Smad3*$^{-/-}$ T cell cultures (Fig.7c). In addition, *Smad3*$^{-/-}$ CD4$^+$ T cells failed to show up-regulation of CD8α mRNA following stimulation in the presence of TGF-β1 (Fig.7b). Thus, Smad3 is required for TGF-β induction of CD8α in CD4$^+$ cells.

We also examined if, similar to DN TCRαβ$^+$CD5$^+$ thymocytes, an alteration in the expression of the lineage commitment transcription factors Th-POK and/or Runx3 could also be found in peripheral CD4$^+$ T cells during TGF-β-mediated CD8α induction. Using Runx3 reporter mice30 we showed that TCR activation up-regulated Runx3 in CD4$^+$ T cells (Fig.8a). Addition of TGF-β1 to these cultures considerably reduced Runx3 expression in the emerging CD4$^+$CD8$^-$ subset, but had no such effect on the converted CD4$^+$CD8α$^+$ population (Fig.8a). These data indicate that resistance to TGF-β-mediated Runx3 down-regulation was associated with CD8α induction in CD4$^+$ T cells, although whether this maintenance of Runx3 has a causative effect on CD8α expression remains to be elucidated.

Lack of Th-POK in peripheral CD4$^+$ T cells allows re-expression of CD835. Using Th-POK reporter mice31, we show that CD4$^+$ T cell activation caused increased expression of Th-POK, consistent with previous studies35. However, addition of TGF-β1 to cultures abrogated the activation-induced increase in Th-POK expression (Fig.8b), suggesting that TGF-β1 had an inhibitory effect on Th-POK expression. There was a slight, but reproducible, decrease in Th-POK levels in CD4$^+$CD8α$^+$ T cells compared to CD4$^+$CD8α$^-$ single positive cells in TGF-β1 treated cultures (Fig.8c). In addition, TGF-β1 treatment of CD4$^+$ T cells from *Zbtb7b*$^{+/-}$ (Th-POK heterozygous) mice31, which express half the amount of Th-POK, led to increased frequencies of CD4$^+$CD8α$^+$ cells compared to wild-type controls (Fig.8d). In contrast, TGF-β1 treatment of CD4$^+$ T cells from mice that constitutively express Th-POK in T cells36, showed reduced frequencies of CD4$^+$CD8α$^+$ T cells (Fig.8e). Collectively, our data indicate that even slight changes in the expression levels of the fate-determining factors Th-POK and Runx3 allow CD8α expression on CD4$^+$ T cells. In particular, they suggest that a key step in inducing CD8α in CD4$^+$ T cells by TGF-β, is the down-regulation of Th-POK.

Because transfer of activated CD4$^+$ T cells into *Rag1*$^{-/-}$ mice leads to the generation/conversion of CD4$^+$CD8αα$^+$ T cells in the intraepithelial space of the gut34, we determined whether this effect was TGF-β-dependent by injecting *in vitro* activated CD4$^+$ T cells from *Tgfbr1*$^{f/+}$CD4-cre$^+$ or *Tgfbr1*$^{f/f}$CD4-cre$^+$ mice into *Rag1*$^{-/-}$ mice. As reported, after 4 weeks a proportion of control CD4$^+$ T cells became CD4$^+$CD8α$^+$ in the intraepithelial space (Supplementary Fig.10)34. However, this was not the case when TβRI-deficient CD4$^+$ T cells were transferred. No CD4$^+$CD8α$^+$ T cells were found in the spleen in either group (data not shown). These data indicate that TGF-β can function *in vivo* to drive expression of CD8α on a CD4+ population.

Discussion

Here we demonstrate that TGF-β is an essential regulator in the control of TCRαβ$^+$CD8αα$^+$ IEL development. Deletion of TGF-β ligand or receptor abrogated TCRαβ$^+$CD8αα$^+$ IEL development. Deletion of the important TGF-β-signaling intermediate Smad3, also led to a decrease in TCRαβ$^+$CD8αα$^+$ IEL. Over-expression of TGF-β1 in T cells enhanced TCRαβ$^+$CD8αα$^+$ IEL development. The defective development of IELs in the absence of TGF-β-signaling was restricted to the TCRαβ$^+$CD8αα$^+$ subset, while neither TCRαβ$^+$CD8αβ$^+$ nor TCRγδ$^+$CD8αα$^+$ populations decreased. TGF-β was required for the

generation of thymic precursors (DN TCRαβ$^+$CD5$^+$) of TCRαβ$^+$CD8αα$^+$ IELs and protected these cells from death.

Consistent with an anti-apoptotic role for TGF-β in T cells21, TGF-β functions to enhance the levels of pro-survival Bcl-2 and reduce expression levels of pro-apoptotic Bim molecules in DN TCRαβ$^+$CD5$^+$ thymocytes. Although TGF-β protection from cell death seems important, it may not be the sole mechanism for lack of TCRαβ$^+$CD8αα$^+$ IEL in the absence of TGF-β, as other populations of thymocytes exhibited increased Bim and decreased Bcl-2 expression and enhanced cell death similar to DN TCRβ$^+$CD5$^+$ thymocytes (a definite causative effect of changes in Bcl-2 and Bim on cell death in most TGF-β-deficient thymocytes remains to be determined). Thus, protection from apoptosis may represent only part of the complex molecular events by which TGF-β controls TCRαβ$^+$CD8αα$^+$ IEL development. Indeed, TGF-β could induce CD8α in DN TCRαβ$^+$CD5$^+$ thymocytes. TGF-β also decreased the expression of Th-POK, which inhibits CD8 expression, in DN TCRαβ$^+$CD5$^+$ thymocytes, providing further mechanisms of TGF-β-mediated control of TCRαβ$^+$CD8αα$^+$ IEL development.

Our finding that TGF-β, in addition to inducing expression of CD8α in DN TCRβ$^+$CD5$^+$ thymocytes, maintains and/or increases CD8α expression on TCRαβ$^+$ cells which already express CD8α, provides an additional mechanism for TGF-β regulation of TCRαβ$^+$CD8αα$^+$ IEL development. In a similar way, TGF-β induces the expression of Foxp3 in CD4$^+$Foxp3$^-$ cells37, but also maintains its expression in Foxp3$^+$Tregs38. Decreased CD8α expression in peripheral CD8$^+$ T cells in TGF-β-deficient mice was not due to either cell death or activation, and was cell intrinsic, because peripheral CD8$^+$ T cells lacking TβRI expression in bone marrow chimeras also had lower CD8 expression levels.

Induction of CD8α, as shown in DN TCRβ$^+$CD5$^+$ thymocytes, was also seen in peripheral CD4$^+$ T cells, and therefore could be a general feature of TGF-β signaling. Reprogramming of peripheral CD4$^+$ T cells by TGF-β was particularly unexpected, as peripheral CD4$^+$ T cells have active mechanisms that repress CD8 expression. A previous study reported that CD4$^+$ T cells isolated from the gut, but not the spleen, expressed CD8αα following transfer into *Rag1*$^{-/-}$ recipients34. The gut is a TGF-β rich environment. We confirmed these experiments and further showed that transferred CD4$^+$ cells that cannot respond to TGF-β do not express CD8α in the gut. A small population of TCRαβ$^+$CD4$^+$CD8α$^+$ is found in the epithelial cell layer of the gut in the steady-state39, but the cellular origin and molecular regulation of their development remain unknown. Thus, our data raise the possibility that TCRαβ$^+$CD8αα$^+$ IEL consist of a subpopulation converted from mature CD4$^+$ or CD8αβ$^+$ T cells in the TGF-β-rich mucosal intra-epithelial space.

Further analysis of TGF-β-induced CD4$^+$CD8α$^+$ cells identified both CD4$^+$CD8αα$^+$ and CD4$^+$CD8αβ$^+$ populations. Although we see expression of both CD8α and CD8β on the surface of the CD4$^+$ T cell cultured in the presence of TGF-β, only CD8α mRNA was induced by TGF-β. CD4$^+$ T cells have been reported to constitutively express CD8β40, but CD8β can only reach the cell surface in the presence of CD8α41. Hence induction of CD8α only, and not CD8β, might be sufficient to induce the expression of CD8αβ heterodimers at the cell surface.

If $CD4^+$ T cells have transcriptional programs repressing CD8 expression, then induction of CD8α in $CD4^+$ T cells indicates TGF-β actively reverses a “repressor” and/or promotes an “enhancer” of the CD8α gene transcription program. Our observations showed that TGF-β affected the expression of two transcription factors important in lineage commitment; Th-POK and Runx3. Compared with $CD4^+$ cells cultured with TCR stimulation alone, TGF-β treatment caused a considerable decrease in Th-POK expression, which could explain CD8α expression in $CD4^+$ T cells. Reduction in Th-POK expression in $CD4^+$ T cells was shown to allow for the induction of CD8, and maintaining Th-POK at high levels is important in keeping the CD8 program repressed35. We confirmed this view by examining CD8α-induction in $CD4^+$ T cells from mice that express different amounts of Th-POK protein. Thus, a decrease in Th-POK expression following TGF-β treatment could release CD8 repression, allowing CD8α expression on $CD4^+$ T cells.

Alternatively, but nonexclusively, a complementary change of positive factors could collaboratively accomplish CD8α expression on $CD4^+$ cells. Runx3 is mainly associated with the repression of CD4 and CD4-fate genes, but is known to bind to CD8 enhancers, predominately $E8_I$42. We observed increased levels of Runx3 in $CD4^+CD8\alpha^+$ compared to $CD4^+CD8\alpha^-$ cells in TGF-β treated cultures. Thus, in contrast to Th-POK, the levels of Runx3 expression in the converted $CD4^+CD8\alpha^+$ population were higher than that in $CD4^+CD8\alpha^-$ cells. Therefore we suggest that TGF-β can alter the balance of these two transcription factors within the cell to create an environment favorable for the expression of CD8α in $CD4^+$ T cells. In addition, induction of CD8α on $CD4^+$ T cells was Smad3 dependent, and interestingly Runx proteins and the Smad proteins have been shown to interact to regulate the IgCα promoter43. Other members of the TGF-β-super-family, such as the BMP (bone morphogenic proteins), have long been known to activate Runx proteins44. Runx proteins have also been shown to be important downstream mediators in the TGF-β-induced expression of Foxp3 and CD10345, 46. Whether TGF-β-mediated down-regulation of Th-POK is downstream of Smad proteins remains to be elucidated, but our data clearly link TGF-β signaling to Th-POK down-regulation.

Further, we show that TGF-β-signaling affected the balance between Th-POK and Runx3 in DN $TCR\alpha\beta^+CD5^+$ thymocytes. Thus, despite the different transcription networks active in $TCR\alpha\beta^+CD8\alpha\alpha^+$ precursors and $CD4^+$ T cells, TGF-β has the overall ability to alter Th-POK and Runx3 expression in a direction that favors CD8α expression.

If TGF-β can induce CD8α expression, it remains unclear why TGF-β deficient mice only lack the $TCR\alpha\beta^+CD8\alpha\alpha^+$ but not the $TCR\alpha\beta^+CD8\alpha\beta^+$ or $TCR\gamma\delta^+CD8\alpha\alpha^+$ IELs. $TCR\gamma\delta^+$ and $TCR\alpha\beta^+$ T cells undergo distinct developmental programs, and indeed TGF-β could represent a signal specific for the $TCR\alpha\beta^+CD8\alpha\alpha^+$ cell fate. The CD8 enhancer $E8_I$ is required for CD8α gene expression in $CD8\alpha\alpha^+$ IEL47, so TGF-β signaling may affect $E8_I$ regulation. Regarding the functional effect of CD8α expression in $CD4^+$ T cells, phenotypic examination of cells from TGF-β treated cultures showed that $CD4^+CD8\alpha^-$ and $CD4^+CD8\alpha^+$ cells expressed similar levels of Foxp3, and no increased expression of proteins associated with CD8-fate were seen in $CD4^+CD8\alpha^+$ cells (data not shown). The presence of $CD4^+CD8^+$ T cells has been described in a number of normal and pathological

conditions33, including autoimmune disorders and cancer. It will be interesting to examine the mechanisms by which these cells arise.

In conclusion, our findings highlight an intriguing common developmental pathway of "self-reactive" T cell populations such that Foxp3$^+$ Treg cells, iNKT cells, and now TCRαβ$^+$CD8αα$^+$ IEL, all require TGF-β for their development.

Methods

Methods and any associated references are available in the online version of the paper at http://www.nature.,com/natureimmunology/.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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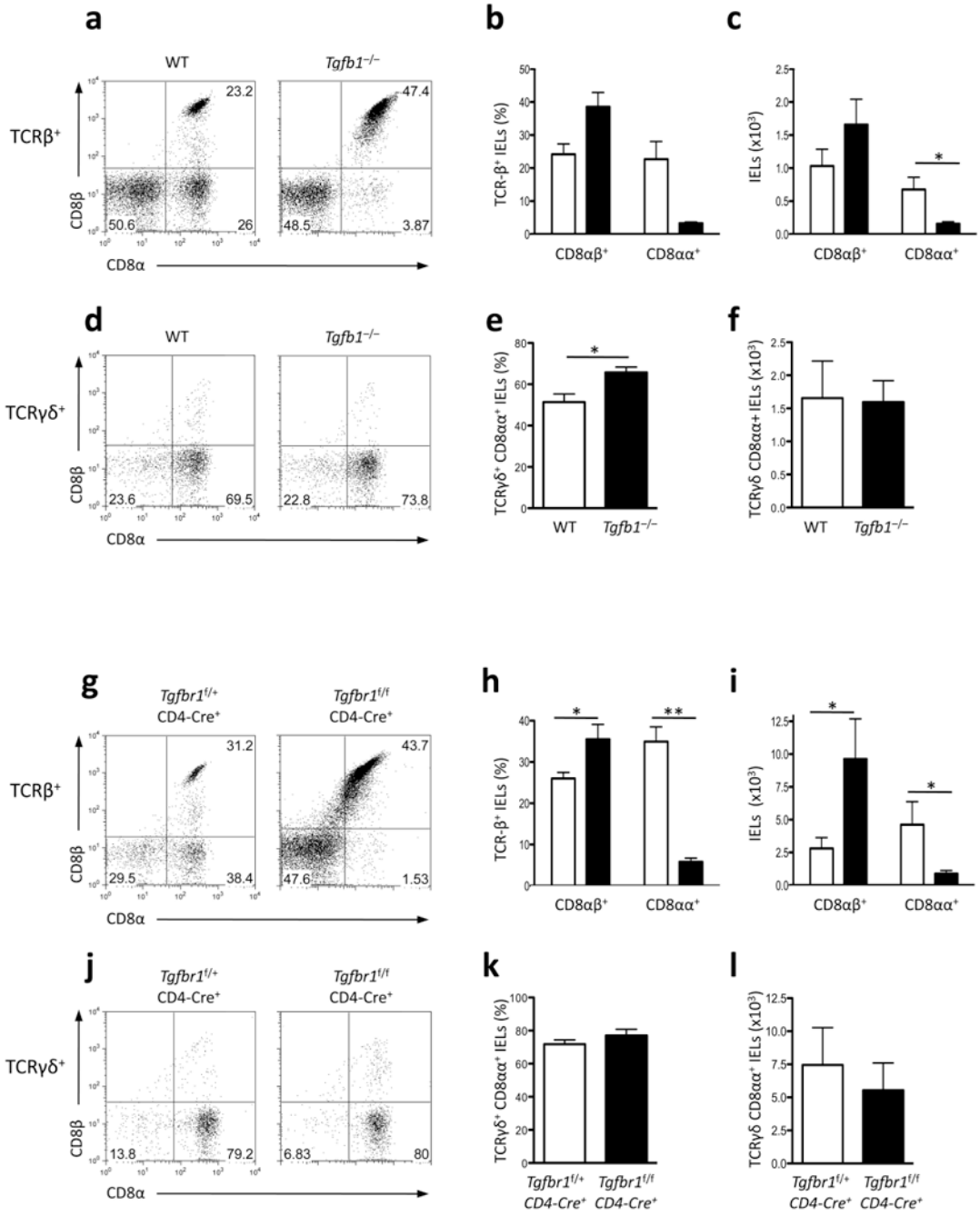

Figure 1. Reduction of TCRαβ$^+$CD8αα$^+$ IEL in mice which lack TGF-β-signaling

(**a, d**) Representative plots showing CD8α versus CD8β staining on TCRβ$^+$ (**a**) and TCRγδ$^+$ (**d**) cells in 2-3-week-old *Tgfb1*$^{-/-}$and age-matched littermate control wild-type (WT) mice. (**b**) frequency and (**c**) total number of CD8αβ$^+$ or CD8αα$^+$ TCRαβ$^+$ IEL in mice from **a** (mean ± SEM). (**e**) frequency and (**f**) total number of CD8αα$^+$ TCRαβ$^+$ IELs in mice from (**d**) (mean ± SEM). *n*=8 for *Tgfb1*$^{-/-}$ (black bars) and *n*=8 for wild-type controls (white bars). (**g, j**) Representative plots showing CD8α versus CD8β staining on TCRβ$^+$ (**g**) and TCRγδ$^+$ (**j**) cells in 3-4 week old *Tgfbr1*$^{f/f}$CD4-Cre$^+$ and age-matched littermate control

Tgfbr1$^{f/+}$CD4-cre^{+} mice. (**h**) frequency and (**i**) total number of CD8αβ^{+} or CD8αα^{+} TCRαβ^{+} IEL in mice from (**g**) (mean ± SEM). (**k**) frequency and (**l**) total number of CD8αα^{+} TCRαβ^{+} IELs in mice from (**j**) (mean ± SEM). *n*=18 for *Tgfbr1*$^{f/f}$CD4-cre^{+} (black bars) and *n*=19 for *Tgfbr1*$^{f/+}$ CD4-cre^{+} mice (white bars). *p<0.05, **p<0.005, **p<0.0001 (unpaired two-tailed student's *t*-test).

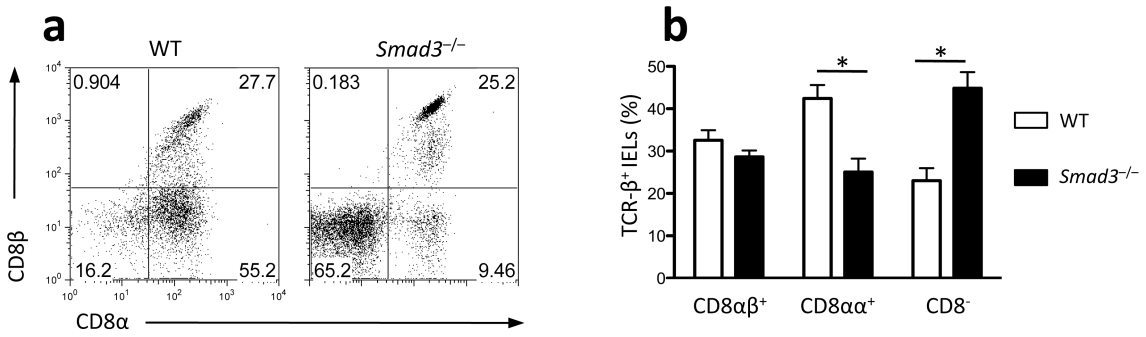

Figure 2. Smad3$^{-/-}$ mice have a reduction in TCRαβ^{+}CD8αα^{+} IEL

(**a**) Representative plots showing CD8α versus CD8β staining on TCRβ^{+} cells from wild-type (left) and *Smad3*$^{-/-}$ (right) mice. (**b**) Frequencies of TCRβ^{+}CD8αβ^{+}, TCRβ^{+}CD8αα^{+} and TCRβ^{+}CD8^{-} IELs in wild-type (n=14) and *Smad3*$^{-/-}$ (n=16) mice (mean ± SEM). *p<0.0007 (unpaired two-tailed student's *t*-test).

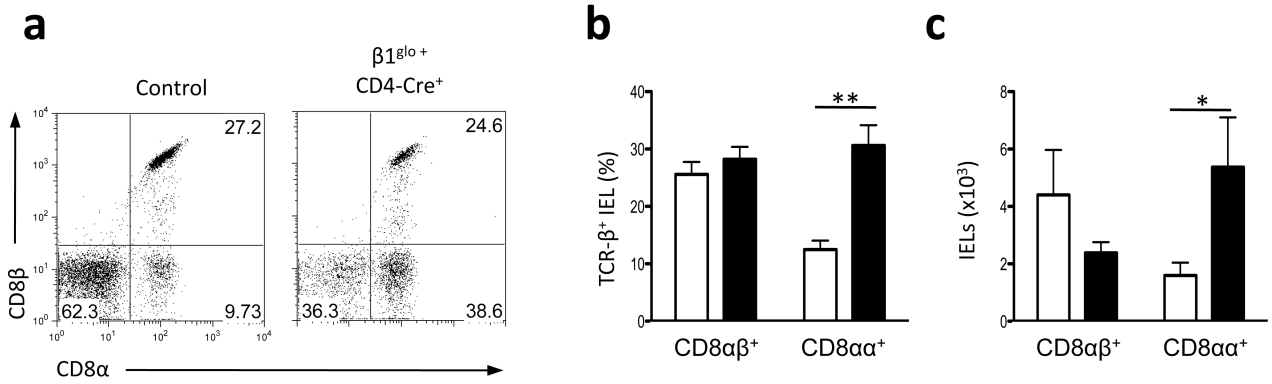

Figure 3. Over-expression of TGF-β from T cells leads to a greater population of TCRαβ$^+$CD8αα$^+$ IEL

(**a**) Representative plots showing CD8α versus CD8β staining on TCRβ$^+$ cells from β1^{glo+}CD4-cre$^+$ (8$^+$ weeks old) and age-matched littermate control mice (either β1^{glo-} CD4-cre$^+$, β1^{glo+} CD4-cre$^-$, or β1^{glo-} CD4-cre$^-$). (**b**) Frequency and (**c**) total number of CD8αβ$^+$ or CD8αα$^+$ TCRαβ$^+$ IEL in mice from (**a**) (mean ± SEM). *n*=7 for β1^{glo+}CD4- cre$^+$ (black bars) and *n*=8 for littermate controls (white bars) examined in at least 3 independent experiments. *p=0.0331, **p=0.0003 (unpaired two-tailed student's *t*-test).

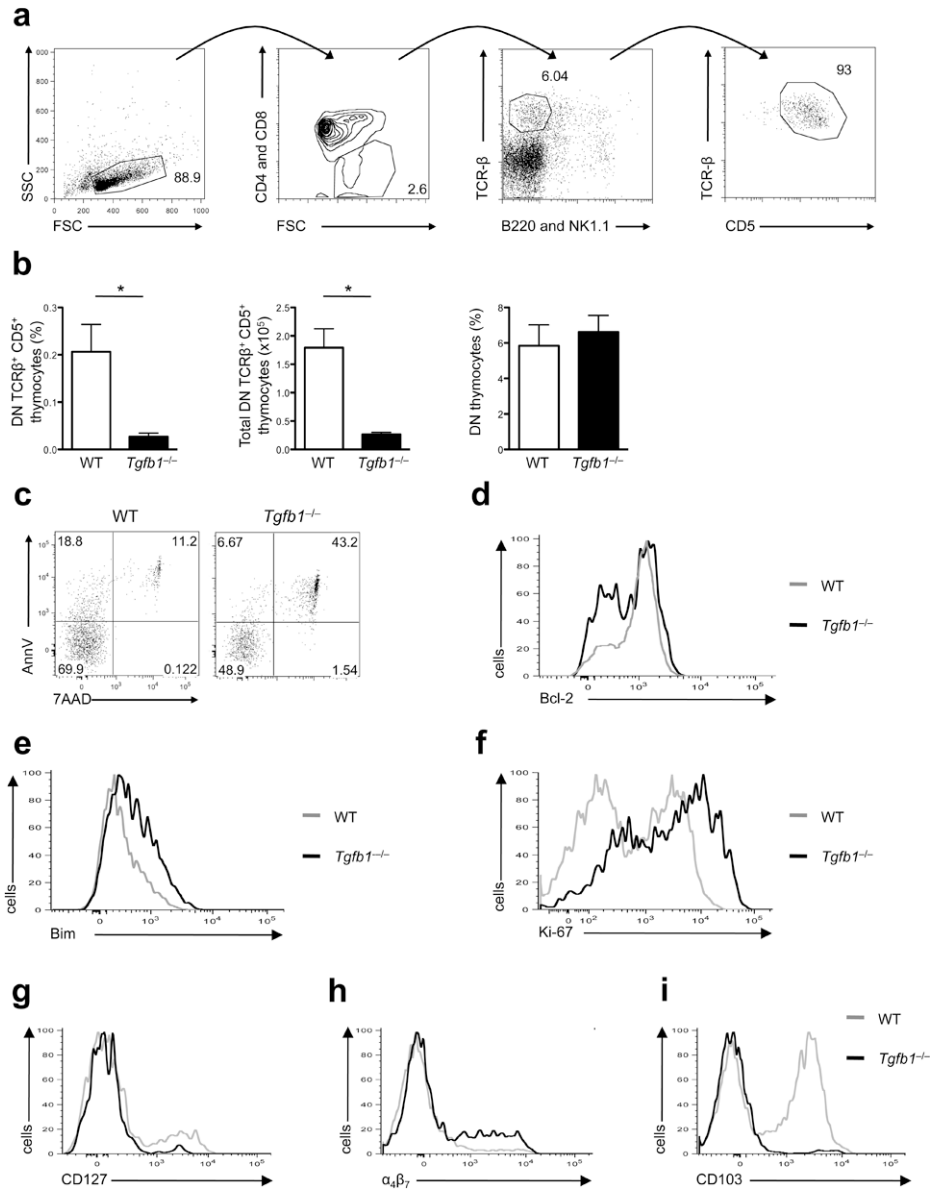

Figure 4. TGF-β-deficient mice have a reduced population of DN TCRαβ$^+$CD5$^+$ thymocytes

(**a**) Representative plots showing gating of DN TCRαβ$^+$CD5$^+$thymocytes. (**b**) Frequency (left) and total number (center) of DN TCRαβ$^+$CD5$^+$thymocytes and the frequency of all DN thymocytes (right) in WT littermate control (white bars) and *Tgfb1*$^{-/-}$ (black bars) mice. n=14 for *Tgfb1*$^{-/-}$ and n=12 for controls; *p<0.003. (**c**) Representative plots showing Annexin-V versus 7-AAD staining on DN TCRαβ$^+$CD5$^+$thymocytes from WT (left) and *Tgfb1*$^{-/-}$ (right) mice. (**d, e**) Histograms show Bcl-2 (**d**) and Bim (**e**) staining on DN TCRαβ$^+$CD5$^+$ thymocytes from WT (gray line) and *Tgfb1*$^{-/-}$ (black line) mice. (**f-i**) Representative plots showing (**f**) Ki-67, (**g**) CD127, (**h**) α4β7 and (**i**) CD103 staining on DN TCRαβ$^+$CD5$^+$ thymocytes from WT and *Tgfb1*$^{-/-}$ mice. Staining was performed on at least two mice per group and 2-5 times.

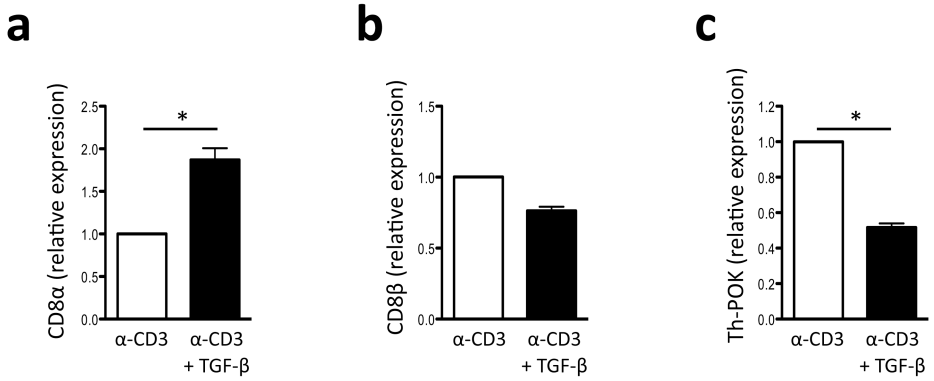

Figure 5. TGF-β induces CD8α expression on DN TCRαβ$^+$CD5$^+$ thymocytes

(**a, b, c**) Quantitative PCR analysis of (**a**) CD8α, (**b**) CD8β and (**c**) Th-POK expression in FACS sorted DN TCRαβ$^+$CD5$^+$ thymocytes from WT thymi stimulated overnight with anti-CD3 (1μg/ml) in the absence or presence of TGF-β1 (2ng/ml). Results are presented relative to control cultures without TGF-β1 treatment. Data are representative of 2 independent experiments. *p≤0.0244 (paired two-tailed student's *t*-test).

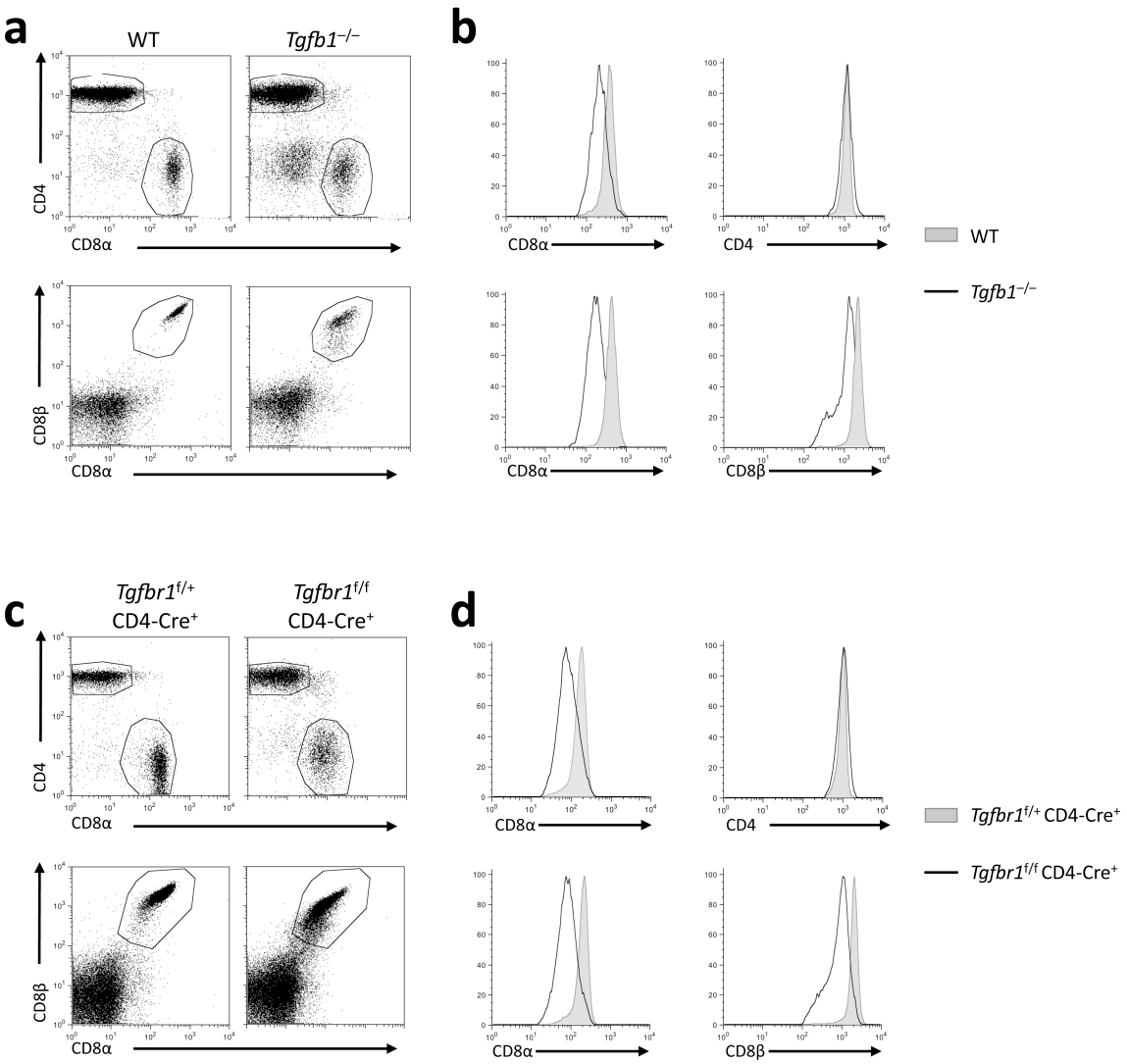

Figure 6. TGF-β is required to maintain expression of CD8 on peripheral T cells

(**a, c**) Representative plots showing CD4 versus CD8α (top panel) and CD8α versus CD8β (bottom panel) staining on TCRβ$^+$ splenocytes from (**a**) WT (left) and *Tgfb1*$^{-/-}$ (right) mice, and (**c**) *Tgfbr1*$^{f/+}$CD4-cre$^+$ and *Tgfbr1*$^{f/f}$CD4-cre$^+$ mice. (**b, d**) Representative histograms showing CD8α, CD8β and CD4 staining on (**b**) WT control (shaded) and *Tgfb1*$^{-/-}$ (black line) cells and (**d**) *Tgfbr1*$^{f/+}$CD4-cre$^+$ (shaded) and *Tgfbr1*$^{f/f}$CD4-cre$^+$ (black line) cells. Data represent staining on at least 2 mice per group examined in at least 3 independent experiments.

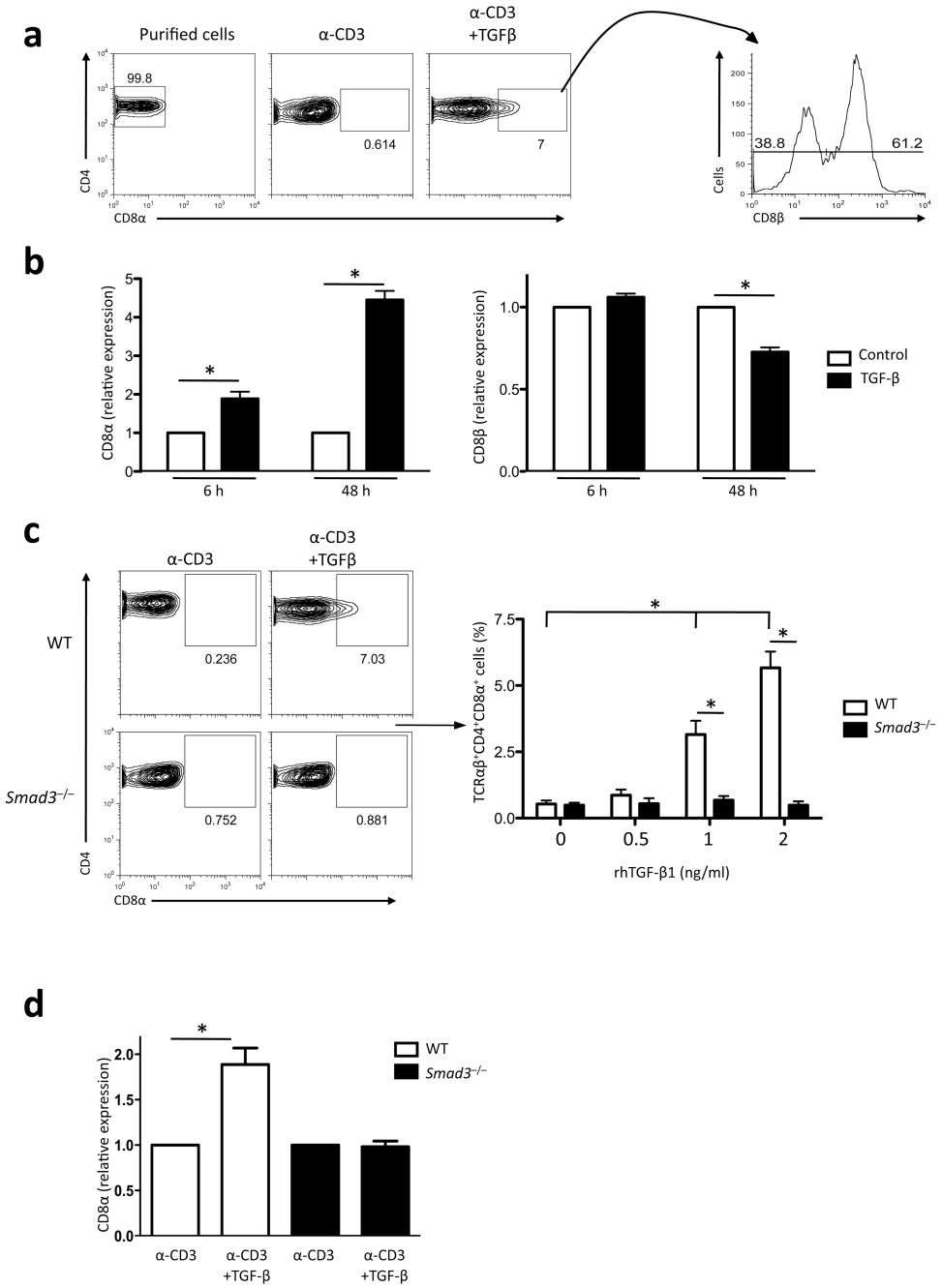

Figure 7. TGF-β induces expression of CD8α on peripheral CD4$^+$ T cells in a Smad3-dependent manner

(**a**) CD4$^+$CD25$^-$ cells were cultured with anti-CD3 in the absence or presence of TGF-β1 (2ng/ml) for 4 days. Representative plots showing CD4 versus CD8α staining on cultured cells, numbers reflect the frequency of CD8α$^+$CD4$^+$TCRβ$^+$ cells or the percent of CD4$^+$ cells in the starting population (purified cells). Representative histogram showing frequency of CD8β$^-$ (CD8αα$^+$) or CD8β$^+$ (CD8αβ$^+$) cells in the CD8α$^+$CD4$^+$TCRβ$^+$ population. Data shown are representative of 5 independent experiments. (**b**) Quantitative PCR analysis of

CD8α (left) and CD8β (right) expression in $CD4^+CD25^-$ cultures at 6 and 48 hours, stimulated with anti-CD3 (white bars) or anti-CD3 plus TGF-β1 (black bars). Results are presented relative to control cultures without TGF-β1 treatment. Data are representative of 4 independent experiments. *p<0.005 (paired two-tailed student's *t*-test). **c**, $CD4^+CD25^-$ cells from WT littermate control or *Smad3*$^{-/-}$ mice were cultured with anti-CD3 indicated concentrations of TGF-β1. Representative plots showing CD4 versus CD8α staining on cultured cells from WT (top) and *Smad3*$^{-/-}$ (bottom) cultures, Bar graph shows frequency of $CD8\alpha^+CD4^+TCR\beta^+$ cells following the different culture conditions (mean ± SEM). Data represent 3 independent experiments. p<0.004 (unpaired two-tailed student's *t*-test). (**d**) Quantitative PCR analysis of CD8α expression in WT (white bars) and *Smad3*$^{-/-}$ (black bars) $CD4^+CD25^-$ T cell cultures stimulated with anti-CD3 or anti-CD3 plus TGF-β1 for 6 hours. Results are presented relative control cultures without TGF-β1 treatment. Data are representative of 2 independent experiments. *p<0.005 (paired two-tailed student's *t*-test).

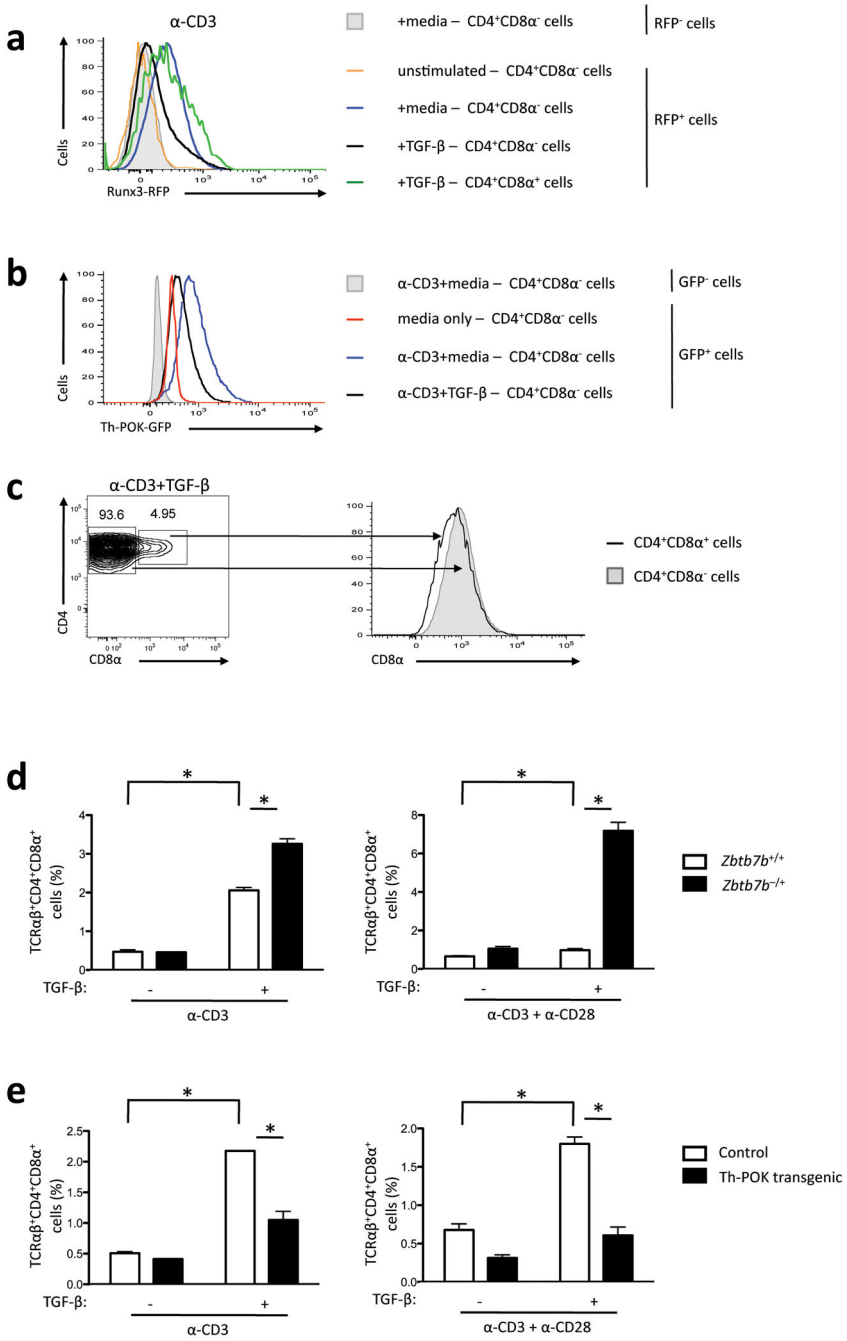

Figure 8. Expression of Th-POK and Runx3 in CD4$^+$CD8a$^-$ and CD4$^+$CD8a$^+$ cells

(**a-c**) CD4$^+$CD25$^-$ cells were cultured with anti-CD3 in the absence or presence of TGF-β1 (2ng/ml) for 4 days. (**a**) Representative histogram showing Runx3-RFP expression in Runx3-RFP$^-$ CD4$^+$CD8α$^-$ cells from anti-CD3 stimulated cultures (grey histogram), Runx3-RFP$^+$ CD4$^+$CD8α$^-$ cells from un-stimulated cultures (orange histogram), Runx3-RFP$^+$ CD4$^+$CD8α$^-$ cells from anti-CD3 plus media stimulated cultures (blue line), Runx3-RFP$^+$ CD4$^+$CD8α$^-$ from anti-CD3 plus TGF-β1 stimulated cultures (black line) and induced Runx3-RFP$^+$ CD4$^+$CD8α$^+$ from anti-CD3 plus TGF-β1 stimulated cultures (green line). (**b**)

Representative histogram showing Th-POK-GFP expression in GFP^- $CD4^+CD8\alpha^-$ cells from anti-CD3 stimulated cultures (shaded histogram), GFP^+ $CD4^+CD8\alpha^-$ cells from unstimulated cultures (red line), from anti-CD3 plus media stimulated cultures (blue line) or from anti-CD3 plus TGF-β1 stimulated cultures (black line). (**c**) Representative plot showing $CD4^+CD8\alpha^-$ and $CD4^+CD8\alpha^+$ populations generated in anti-CD3 plus TGF-β cultures. Representative histogram showing Th-POK-GFP expression in $CD4^+CD8\alpha^-$ (shaded) and $CD4^+CD8\alpha^+$ (black line) cells. Data shown (a-c) are representative of 3 independent experiments. (**d, e**) $CD4^+CD25^-$ cells from (**d**) *Zbtb7b*$^{+/+}$ (WT) and *Zbtb7b*$^{+/-}$ (heterozygous) mice and (**e**) Th-POK transgenic and littermate control mice were cultured with anti-CD3 (left) or anti-CD3 plus anti-28 (right) in the absence or presence of 1ng/ml TGF-β1 for 4 days. Bar graph shows frequency of $CD8\alpha^+CD4^+TCR\beta^+$ cells at day 4 (mean ± SEM). Data represent 3 independent experiments (*Zbtb7b*$^{+/-}$ mice) and 2 independent experiments (Th-POK-transgenic mice). $p<0.0202$ (unpaired two-tailed student's *t*-test).