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Item type	Article
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Citation	CD8+ Foxp3+ T cells share developmental and phenotypic features with classical CD4+ Foxp3+ regulatory T cells but lack potent suppressive activity. 2011, 41 (3):716-25 Eur. J. Immunol.
DOI	10.1002/eji.201040913
Journal	European journal of immunology
Downloaded	15-Sep-2016 21:56:59
Item License	http://creativecommons.org/licenses/by-nc-sa/4.0/
Link to item	http://hdl.handle.net/10033/346205



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(2011) European Journal of Immunology, 41 (3), pp. 716-725

CD8⁺ Foxp3⁺ T cells share developmental and phenotypic features with classical CD4⁺ Foxp3⁺ regulatory T cells but lack potent suppressive activity

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“Suppressor T cells” were historically defined within the CD8⁺ T-cell compartment and recent studies have highlighted several naturally occurring CD8⁺ Foxp3⁻ Treg populations. However, the relevance of CD8⁺ Foxp3⁺ T cells, which represent a minor population in both thymic and secondary lymphoid organs of nonmanipulated mice, remains unclear. We here demonstrate that *de novo* Foxp3 induction in peripheral CD8⁺ Foxp3⁻ T cells is counter-regulated by DC-mediated co-stimulation via CD80/CD86. CD8⁺ Foxp3⁺ T cells fail to develop in TCR-transgenic mice with Rag1^{-/-} background, similar to classical CD4⁺ Foxp3⁺ Tregs. Notably, both naturally occurring and induced CD8⁺ Foxp3⁺ T cells express bona fide Treg markers including CD25, GITR, CTLA4 and CD103, and show defective IFN- γ production upon restimulation when compared with their CD8⁺ Foxp3⁻ counterparts. However, utilizing DEREK transgenic mice for the isolation of Foxp3⁺ cells by eGFP reporter expression, we demonstrate that induced CD8⁺ Foxp3⁺ T cells similar to activated CD8⁺ Foxp3⁻ T cells only mildly suppress T-cell proliferation and IFN- γ production. We therefore categorize CD8⁺ Foxp3⁺ T cells as a tightly controlled population sharing certain developmental and phenotypic properties with classical CD4⁺ Foxp3⁺ Tregs, but lacking potent suppressive activity.

Keywords: CD8 · Foxp3 · TGF- β · Treg



Supporting Information available online

Introduction

Foxp3 is a master regulator of CD4⁺ Treg function [1–3] and its mutation or the depletion of Foxp3⁺ cells led to onset of

multiorgan autoimmunity [4–6]. The vast majority of Foxp3⁺ T cells are confined to TCR- $\alpha\beta$ ⁺ CD4⁺ T cells, and little is known about CD8⁺ T cells expressing Foxp3. Certain surface phenotypes such as CD28⁻ [7], CD122⁺ [8], CD8 $\alpha\alpha$ ⁺ [9, 10], latency-associated peptide (LAP)⁺ [11] and restriction to the nonclassical MHC I molecule Qa-1 [12] have been linked with immunosuppressive functions of CD8⁺ T cells. However, Foxp3 expression was either absent in these populations [8, 9, 13–15], incongruent

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with the defining surface phenotype [11] or was not investigated specifically on a protein level [16]. Additionally, the isolation of viable CD8⁺Foxp3⁺ populations was hampered by the nuclear localization of Foxp3 in conjunction with the occurrence of these cells at low numbers in nonmanipulated mice [2, 17], rendering the identity and relevance of mouse CD8⁺Foxp3⁺ T cells unclear.

Classical CD4⁺Foxp3⁺ Tregs develop either intrathymically (natural Tregs, nTregs) or in the periphery via conversion from Foxp3⁻ T cells (induced Tregs). Specialized dendritic cells (DC) can initiate the latter process by providing the key factors TGF- β and all-*trans*-retinoic acid (RA) [18, 19]. Although natural and in vitro induced CD4⁺Foxp3⁺ Tregs share key phenotypic and functional characteristics, they differ in the stability of Foxp3 expression, and different degrees of demethylation of an evolutionarily conserved region within the *foxp3* locus (TSDR; Treg-specific demethylated region) have been implicated in this observation [20]. To date, it is unclear if the same epigenetic mechanisms underlie the regulation of Foxp3 expression within CD8⁺ T cells and if DC are equally essential for Foxp3 induction.

Our study therefore aimed to systematically assess developmental, phenotypic and functional properties of CD8⁺Foxp3⁺ T cells in comparison to well-defined CD4⁺Foxp3⁺ Tregs.

Results

Foxp3 induction in CD8⁺Foxp3⁻ T cells requires TGF- β and TCR signals

Rag1^{-/-} mice crossed to TCR transgenic mice expressing MHC-class-II-restricted TCRs, which recognize nonself peptides, represent a widely used tool to study Foxp3 induction in CD4⁺ T cells as those mice are devoid of nTregs [21]. Conversely, we used Rag1^{-/-} \times OTI mice expressing a MHC-class-I-restricted OVA_{257–264}-specific TCR to study Foxp3 induction in CD8⁺ T cells, considering low numbers of CD8⁺Foxp3⁺ T cells in vivo and limited knowledge of their development. Activation of CD8⁺Foxp3⁻ T cells with OVA_{257–264} alone or in combination with RA failed to efficiently induce CD8⁺Foxp3⁺ T cells in both splenic and thymic cell suspensions, whereas stimulation in the presence of TGF- β induced Foxp3 in a substantial fraction of CD8⁺ T cells (Fig. 1A and B). Interestingly, CD8SP thymocytes up-regulated Foxp3 to a greater extent than CD8⁺ splenocytes, and RA could further amplify Foxp3 induction in both lymphoid compartments (Fig. 1A and B). This was also accompanied by a rise in absolute CD8⁺Foxp3⁺ cell numbers (Supporting Information Fig. 1A; data not shown). No gross differences were noted when comparing cells from spleens, subcutaneous and mesenteric lymph nodes (data not shown). The OVA_{257–264} peptide concentration influenced the efficiency of Foxp3 induction, being optimal between 0.01 and 0.1 μ g/mL and decreasing with higher or lower peptide concentrations (Supporting Information Fig. 2A and B). RA concentrations between 0.1 and 100 nM only in the presence of

peptide did not induce Foxp3. However, RA synergized with 2 ng/mL TGF- β to induce Foxp3 best at a concentration of 10 nM (Supporting Information Fig. 2C). TGF- β (0.2 ng/mL) displayed Foxp3-inducing activity although saturation required 100-fold higher concentrations (Supporting Information Fig. 2D).

As thymocytes include various stages of T-cell development that might give rise to CD8⁺Foxp3⁺ T cells during the culture period, we sorted DN, DP, CD4SP and CD8SP populations based on CD4 and CD8 expression and assessed their potential to up-regulate Foxp3. Only sorted CD8SP thymocytes significantly proliferated (Supporting Information Fig. 1B) and developed into CD8SP Foxp3⁺ T cells (Supporting Information Fig. 1A).

To further address the role of endogenous accessory cells for Foxp3 induction in this experimental system, we compared total spleen cell suspensions with purified CD8⁺ cells. Interestingly, splenic accessory cells were not only dispensable but also mildly inhibiting Foxp3 induction, as the percentage of Foxp3⁺ cells among CD8⁺ T cells increased slightly when purified T cells were used in the presence of RA (Fig. 1C). Similarly, sorted CD8SP thymocytes efficiently gave rise to CD8⁺Foxp3⁺ T cells (Supporting Information Fig. 1).

In summary, MHC-class-I-restricted peptide and TGF- β can mediate efficient de novo Foxp3 induction in CD8⁺Foxp3⁻ T cells in an accessory cell-independent manner.

DC inhibit Foxp3 induction in CD8⁺ T cells in part by co-stimulation via the CD80/CD86–CD28 axis

We next aimed to define the inhibitory mechanism of Foxp3 induction in total cell suspensions (Fig. 1C). It has been shown that co-stimulation via CD80/86 prevents CD4⁺Foxp3⁺ Treg induction in vitro, although this inhibition can be overcome by RA [22]. To explore if co-stimulation impairs Foxp3 induction in CD8⁺ T cells, the effects of agonistic α CD28 antibody were determined. We found a partial inhibition of Foxp3 induction both in the absence and presence of RA when co-stimulation was mimicked (Fig. 2A), which also correlated with a decrease in absolute numbers of CD8⁺Foxp3⁺ T cells (data not shown). Similar results were observed when using thymocytes (data not shown).

Given that splenic DC express high levels of CD80 and CD86 [22], we next hypothesized that the addition of DC inhibits Foxp3 induction in CD8⁺ T cells. Therefore, immature BM-derived DC, which express intermediate levels of CD80 and CD86, were titrated to in vitro cultures using CD8⁺ T cells from Rag1^{-/-} \times OTI mice. Interestingly, an increasing blockade of Foxp3 expression was obvious with decrease in the T/DC ratio (Fig. 2B). To address if this inhibition is mediated by co-stimulation via CD80 and CD86, we employed DC double deficient for both molecules. Indeed, a partial rescue of Foxp3 expression occurred, especially at higher T/DC ratios (Fig. 2B) closer resembling the physiological ratio between T cells and DC in lymphoid organs. Therefore, DC are not only dispensable but

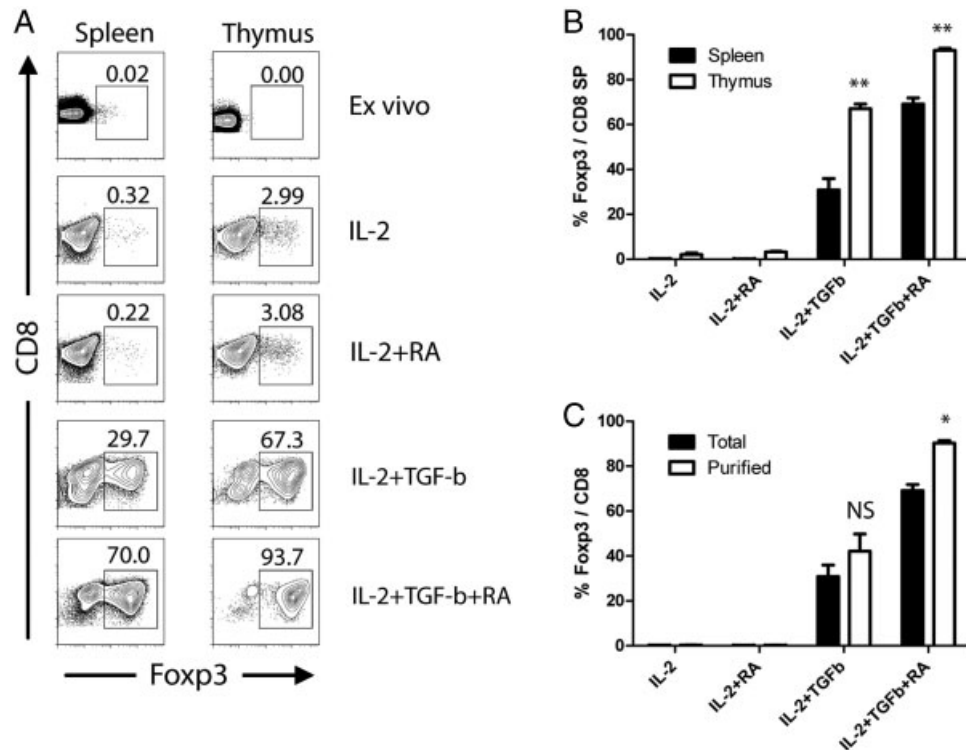


Figure 1. CD8⁺ T-cell activation in the presence of TGF- β induces Foxp3 expression in the absence of DC. (A and B) Total splenocytes and thymocytes of Rag1^{-/-} \times OTI mice were cultured in the presence of OVA_{257–264}, IL-2 and combinations of TGF- β and RA. Cells were analyzed for the expression of CD4, CD8 and Foxp3 on day 4 by flow cytometry. Untreated cells freshly isolated from the same genotype served as control (ex vivo). (A) Plots display percentages of Foxp3⁺ cells within CD8⁺ (spleen) and CD8⁺CD4⁻ (thymus) live gated cells. (B) Mean values+SD of triplicate measurements are shown. Data are representative of five independent experiments. (C) Splenocytes (total) or CD8⁺CD11c⁻ T cells (purified) FACS-sorted from Rag1^{-/-} \times OTI mice were plated at equal numbers, cultured as in (A and B) and analyzed for the frequency of Foxp3⁺ cells among CD8⁺ T cells 4 days later. Mean values+SD of triplicate measurements from one out of two independent experiments are shown (* p <0.05; ** p <0.005; NS: not significant, unpaired two-tailed Student's t -test).

actively inhibit Foxp3 induction in CD8⁺ T cells, in part by co-stimulation via CD80 and CD86.

Natural CD8⁺Foxp3⁺ T cells share developmental characteristics with classical CD4⁺Foxp3⁺ Tregs

Since CD4⁺Foxp3⁺ Tregs in nonmanipulated mice represent a polyclonal population developing both intra- and extrathymically [18], we next studied CD8⁺Foxp3⁺ T cells in untreated WT mice by flow cytometry. After exclusion of aggregates, we found that CD8⁺Foxp3⁺ T cells only constitute 0.1–0.4% of the CD8⁺ T-cell compartment in spleen (Fig. 3A), peripheral and mesenteric lymph nodes (data not shown), representing about 2% of the total Foxp3⁺ population. Interestingly, Foxp3⁺ cells were also identified among CD8SP thymocytes, and CD8⁺Foxp3⁺ cells were absent from both thymus and periphery of Rag1^{-/-} \times OTI mice (Fig. 3A). CD4⁺GFP⁺ nonfunctional Tregs are selected in the absence of functional Foxp3 in depletion of regulatory T cells (DEREG) \times scurfy (Sf) mice [3]. To assess if the selection of CD8⁺Foxp3⁺ T cells requires Foxp3, we analyzed GFP and Foxp3 expression among CD8⁺ splenocytes and CD8⁺CD4⁻ thymocytes from WT and DEREG \times Sf mice. Here, a CD8⁺Foxp3⁻GFP⁺

population could be detected at frequencies similar to that of CD8⁺Foxp3⁺ T cells in WT mice (Fig. 3B), demonstrating that the expression of functional Foxp3 protein is not essential for the generation of CD8⁺Foxp3⁺ T cells. Similarly, Foxp3-deficient DEREG \times Rag1^{-/-} \times OTI \times Sf CD8⁺ T cells up-regulated GFP upon culture with OVA_{257–264}, IL-2, TGF- β and RA, although with slightly reduced efficiency compared with Foxp3-sufficient cells (Supporting Information Fig. 3A and B), similar to our previous findings with CD4⁺ T cells [3].

Induced CD8⁺Foxp3⁺ T cells maintain complete TSDR methylation

Stable Foxp3 expression is epigenetically controlled by demethylation of the TSDR which is located within the *foxp3* gene locus [20]. Natural CD4⁺Foxp3⁺ Tregs contain a fully demethylated TSDR and were stable during in vitro culture, whereas in vitro induced CD4⁺Foxp3⁺ Tregs display a heavily methylated TSDR and loose Foxp3 expression upon in vitro culture in the absence of TGF- β [23]. To assess if similar mechanisms are operative in CD8⁺ T cells, we crossed Rag1^{-/-} \times OTI mice with bacterial artificial chromosome (BAC)-transgenic DEREG mice [6]

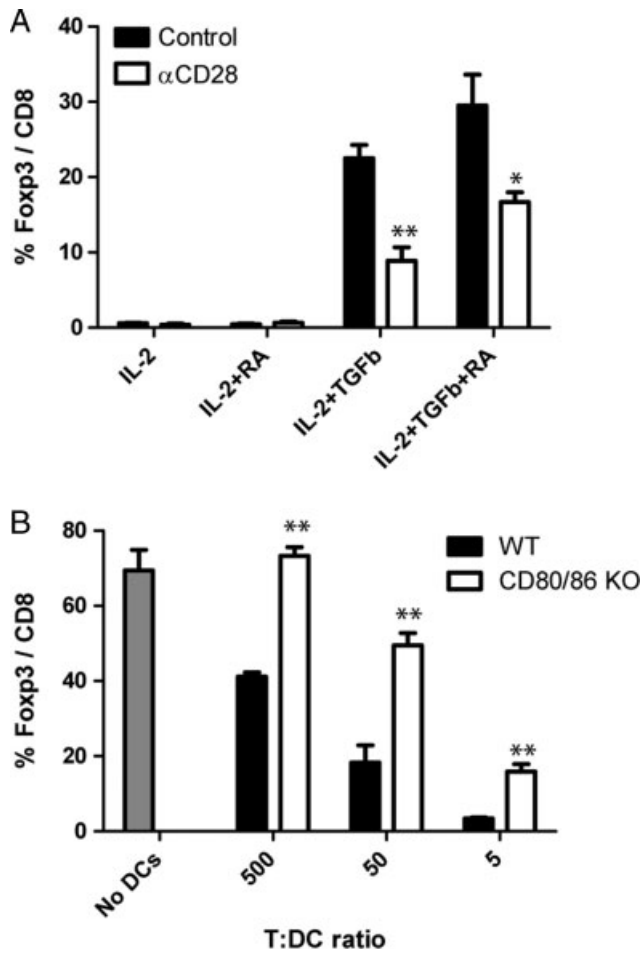


Figure 2. Co-stimulation via the CD80/CD86–CD28 axis inhibits Foxp3 induction in CD8⁺ T cells. (A) Splenocytes of Rag1^{-/-} × OTI mice were cultured in the presence of OVA_{257–264}, IL-2 and combinations of TGF- β and RA either in the absence (control) or presence of 2 μ g/mL agonistic α CD28 antibody (α CD28). Cells were analyzed for the expression of CD8 and Foxp3 on day 4 by flow cytometry. The frequency of Foxp3⁺ cells among CD8⁺ live gated cells is shown as mean+SD of triplicate measurements. Data are representative of three independent experiments. (B) BM-derived DC generated from WT or CD80/CD86 double-deficient mice (CD80/86 KO) were added at the indicated ratios to CD8⁺ Rag1^{-/-} × OTI T cells. Cultures were analyzed for the expression of CD8 and Foxp3 on day 4 by flow cytometry. Mean frequencies+SD of Foxp3⁺ cells among CD8⁺ T cells are shown as triplicate measurements and represent one out of two independent experiments. **p*<0.05, ***p*<0.005, unpaired two-tailed Student’s *t*-test.

allowing for selective isolation of induced Foxp3⁺ cells by eGFP reporter expression and assessed TSDR methylation within the *foxp3* gene locus (including BAC-encoded copies). All CpG motifs were completely methylated in freshly isolated CD8⁺Foxp3⁻ T cells (naïve) and no changes were observed upon T-cell activation (GFP⁻; Fig. 4A). Interestingly, induced CD8⁺Foxp3⁺ T cells maintained a fully methylated TSDR (GFP⁺; Fig. 4A), consistent with a rapid loss of Foxp3 expression upon in vitro stimulation in the absence of TGF- β (data not shown). Together, these data suggest that the stability of Foxp3 expression in both CD8⁺ T cells and CD4⁺ Tregs might underlie similar epigenetic mechanisms.

Natural and induced CD8⁺Foxp3⁺ T cells express classical Treg markers

As Foxp3 specifically defines mouse CD4⁺ Tregs [24], we next assessed if induced CD8⁺Foxp3⁺ T cells display expression of bona fide Treg markers. Therefore, induced CD8⁺GFP⁺, activated CD8⁺GFP⁻ and naïve CD8⁺Foxp3⁻ T cells were obtained from DEREK × Rag1^{-/-} × OTI mice. CD4⁺GFP⁺ nTregs sorted from DEREK mice served as the positive control. The expression of various markers was assessed by quantitative real-time PCR. As expected, CD8⁺GFP⁺ T cells and CD4⁺GFP⁺ nTregs expressed high levels of Foxp3, whereas only marginal Foxp3 expression was detected in CD8⁺GFP⁻ T cells, confirming that Foxp3 is not substantially induced by sole T-cell activation in mice (Fig. 4B). CD8⁺GFP⁺ T cells expressed CD25 and CTLA4 to equal or higher levels compared with nTregs; however, those markers were also induced in CD8⁺GFP⁻ T cells (Fig. 4B), consistent with their expression upon activation. Interestingly, CD73 was highly expressed by both nTregs and induced

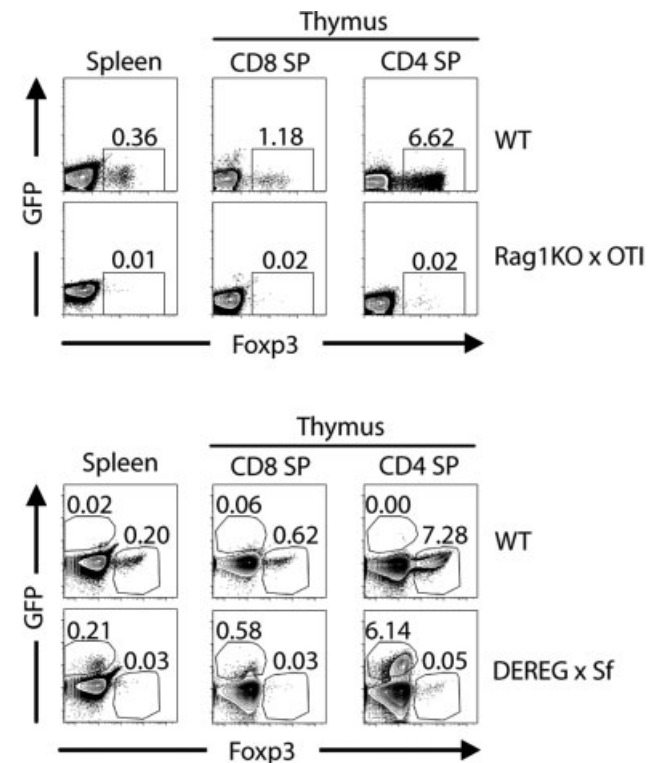
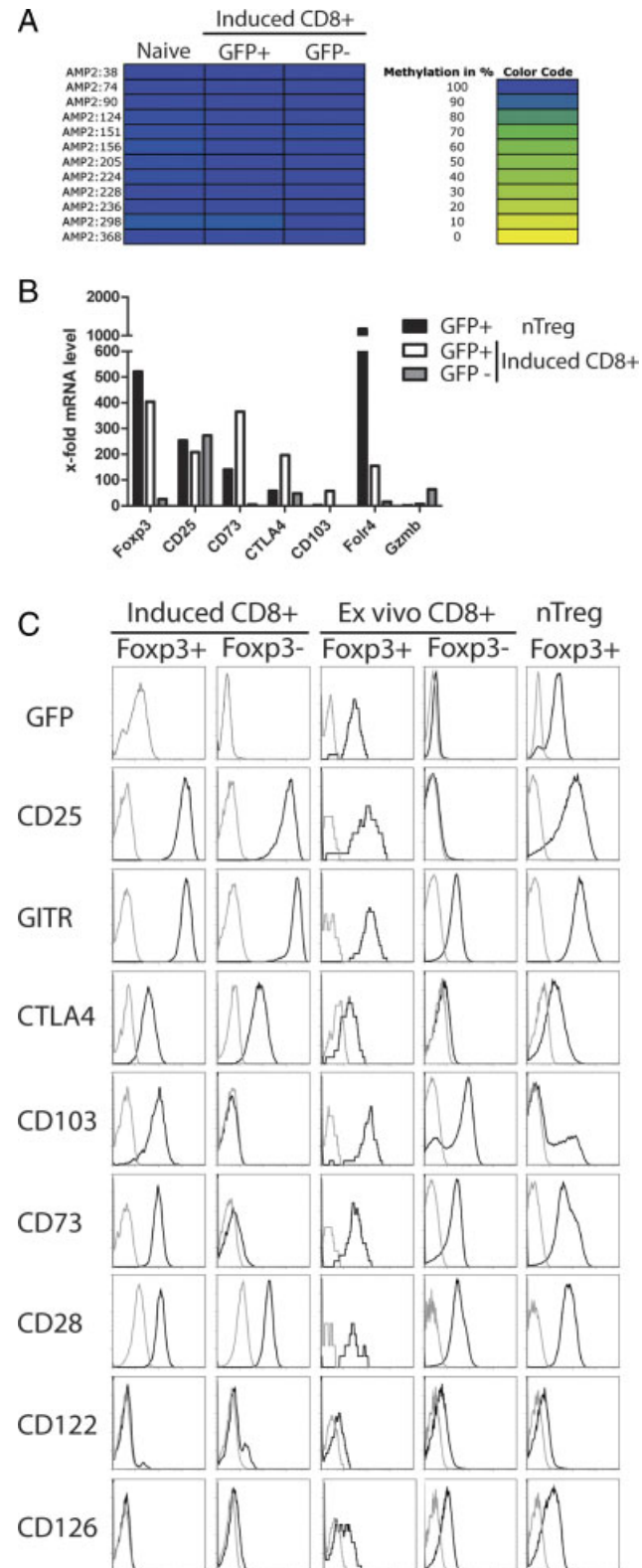


Figure 3. Naturally occurring CD8⁺Foxp3⁺ T cells share developmental features with classical CD4⁺Foxp3⁺ Tregs. (A) Splens and thymi of WT and Rag1^{-/-} × OTI mice were analyzed for CD8, CD4 and Foxp3 expression by flow cytometry. (A) Plots display percentages of Foxp3⁺ cells among live CD8⁺ (spleen), CD8⁺CD4⁻ (CD8SP, thymus) and CD8⁺CD4⁺ (CD4SP, thymus) cells. (B) Splens and thymi were prepared from 3-wk-old WT or DEREK × Sf mice. CD8, CD4 and Foxp3 expression was assessed by flow cytometry. Plots compare percentages of Foxp3⁺GFP⁻ and Foxp3⁺GFP⁺ cells among live CD8⁺ (spleen), CD8⁺CD4⁻ (CD8SP, thymus) and CD8⁺CD4⁺ (CD4SP, thymus) cells. Results are representative of three independent experiments.

CD8⁺GFP⁺ T cells, whereas activated T cells lacked CD73 mRNA. In contrast, the nTreg-associated marker folate receptor 4 (Folr4) showed low expression in both CD8⁺GFP⁺ and CD8⁺GFP⁻ T cells (Fig. 4B). CD103 was expressed at



low levels in CD8⁺GFP⁻-activated T cells, whereas induced CD8⁺GFP⁺ T cells and nTregs showed signals above untreated CD8⁺Foxp3⁻ T cells (Fig. 4B), the majority of which express CD103 protein (Fig. 4C). Notably, granzyme B mRNA was induced in CD8⁺GFP⁻-activated T cells but was low in CD8⁺GFP⁺ T cells and nTregs (Fig. 4B).

We next performed FACS analysis of CD8⁺ Rag1^{-/-} × OTI T cells similarly cultured in vitro. Additionally, DEREK and WT mice were used for ex vivo characterization of CD8⁺ T-cell populations. The expression of various markers of Foxp3⁺ and Foxp3⁻ cell populations was compared. CD4⁺Foxp3⁺ Tregs (nTregs) served as the positive control. As expected, the vast majority of induced CD8⁺Foxp3⁺ T cells and CD4⁺Foxp3⁺ nTregs co-expressed GFP due to the Foxp3 promoter-driven DEREK transgene, whereas GFP expression was absent in CD8⁺Foxp3⁻ T-cell populations (Fig. 4C). We found high expression of the classical Treg markers CD25, CTLA4 and GITR on both Foxp3⁺ and Foxp3⁻ in vitro activated CD8⁺ T cells, whereas their constitutive high expression ex vivo was selective for the Foxp3⁺ subset, similar to CD4⁺Foxp3⁺ Tregs (Fig. 4C). CD103 and CD73 were selectively expressed on the CD8⁺Foxp3⁺ subset in vitro, whereas significant yet lower expression was also detected on CD8⁺Foxp3⁻ populations ex vivo when compared with the CD8⁺Foxp3⁺ subset (Fig. 4C). Of note, the expression of CD25, CD103 and GITR was predominantly independent of functional Foxp3 as demonstrated using cells from DEREK × Rag1^{-/-} × OTI × Sf mice (Supporting Information Fig. 3C). CD122 expression and lack of CD28 expression were previously used to define naturally

◀ **Figure 4.** Natural and induced CD8⁺Foxp3⁺ T cells phenotypically resemble CD4⁺Foxp3⁺ Tregs. (A) CD8⁺ T cells were isolated from DEREK × Rag1^{-/-} × OTI mice and cultured in the presence of OVA_{257–264}, IL-2, TGF-β and RA. CD8⁺GFP⁺ (GFP⁺) cells were isolated by cell sorting on day 4. CD8⁺GFP⁻ activated T cells (GFP⁻) isolated from cultures stimulated with OVA_{257–264} and IL-2 only served as control, as did freshly isolated untreated CD8⁺Foxp3⁻ T cells (naïve). DNA was extracted and the TSDR methylation status was determined by bisulfite sequencing. The methylation status of individual CpG motifs is color-coded according to the degree of methylation at that site. The color code ranges from yellow (0% methylation) to blue (100% methylation) according to the color scale on the right. One representative of three independent sets of experiments is shown. (B) CD4⁺GFP⁺ natural Tregs were isolated from DEREK mice in addition to the GFP⁺ and GFP⁻ populations described in (A), and cDNA was prepared. Expression of several Treg-associated markers was assessed by real-time PCR. Results were normalized to the housekeeping gene Hprt and to untreated CD8⁺Foxp3⁻ T cells isolated from DEREK × Rag1^{-/-} × OTI mice. The graph displays x-fold mRNA levels as mean value from duplicate measurements. Results are representative of two independent sets of cDNA preparations. (C) In vitro cultures (induced CD8⁺) of CD8⁺ T cells isolated from DEREK × Rag1^{-/-} × OTI mice were established as described in (A) in the presence (Foxp3⁺) or absence (Foxp3⁻) of TGF-β/RA. Additionally, spleens and lymph nodes of WT mice (except GFP where DEREK mice were used) were prepared and single cell suspensions were obtained (ex vivo). Cells were analyzed by flow cytometry for the expression of CD4, CD8, Foxp3 and the indicated Treg-associated markers. Live CD8⁺Foxp3⁺ T cells (Foxp3⁺), CD8⁺Foxp3⁻ T cells (Foxp3⁻) and CD4⁺Foxp3⁺ natural Tregs (nTreg, Foxp3⁺) were gated. Gray lines indicate isotype control and solid black lines indicate specific staining. GFP signals of the DEREK transgene are overlaid with WT controls or are controlled internally. Results are representative of three independent experiments (C).

occurring CD8⁺ Treg populations [7, 8]. To assess on a protein level whether these populations relate to the here described Foxp3⁺ T-cell population, we compared CD122 and CD28 expression between CD8⁺Foxp3⁺-gated T-cell populations ex vivo and in vitro. CD122 was expressed at only marginal levels by both induced and natural CD8⁺Foxp3⁺ T cells (Fig. 4C), consistent with the finding that CD8⁺CD122⁺ Tregs lack Foxp3 expression [8]. In contrast, all T-cell populations were predominantly CD28⁺ (Fig. 4C). IL-6 was recently suggested to positively regulate the expansion of CD8⁺Foxp3⁺ T cells in vitro and in vivo [17]. We, therefore, compared IL-6R α (CD126) expression among the different subsets to judge their potential sensitivity towards IL-6. Interestingly, CD126 expression was absent from both induced CD8⁺Foxp3⁺ and CD8⁺Foxp3⁻ T-cell populations, whereas CD126 expression was noted on all T-cell populations ex vivo (Fig. 4C). Notably, naturally occurring CD8⁺Foxp3⁺ T cells expressed a CD8- $\alpha\beta$ heterodimer, TCR- $\alpha\beta$, CD3- ϵ (data not shown) and partially CD4 (Supporting Information Fig. 4); the latter consistent with previous reports [2, 25]. In summary, CD8⁺Foxp3⁺ T cells express classical CD4⁺Foxp3⁺ Treg markers in a pattern distinct from activated CD8⁺Foxp3⁻ T cells and previously described CD8⁺ Tregs.

CD8⁺Foxp3⁺ T cells display reduced IFN- γ production and only weakly suppress T-cell proliferation

Since Foxp3 is expressed by certain effector T-cell populations in humans [26] and IFN- γ is an important effector molecule of CD8⁺ T cells, we next asked whether CD8⁺Foxp3⁺ and CD8⁺Foxp3⁻ T-cell populations differ in IFN- γ expression. CD8⁺Foxp3⁺ and CD8⁺Foxp3⁻ T cells were generated from Rag1^{-/-} \times OTI mice. Additionally, WT splenocytes were obtained and all populations were restimulated with PMA/ionomycin. Importantly, the majority (75.8%) of activated CD8⁺Foxp3⁻ T cells produced IFN- γ , whereas almost no IFN- γ production (5.5%) was observed in induced CD8⁺Foxp3⁺ cells (Fig. 5A), consistent with a previous study [27]. Similarly, fewer CD8⁺Foxp3⁺ T cells produced IFN- γ in comparison to their Foxp3⁻ counterpart ex vivo (Fig. 5A). IFN- γ production by CD8⁺ T cells activated under Foxp3-inducing conditions could be partially restored when Foxp3 was mutated (Supporting Information Fig. 3D), yet Foxp3-independent mechanisms also seem to be involved in the repression of IFN- γ . Since suppressive function is a hallmark of Tregs, we finally tested induced CD8⁺Foxp3⁺ T cells in in vitro suppression assays. Suppressive activity was compared with activated CD8⁺Foxp3⁻ T cells, CD4⁺Foxp3⁺ nTregs and induced CD4⁺Foxp3⁺ Tregs, all isolated based on eGFP reporter expression. Interestingly, not only CD8⁺GFP⁺ T cells but also activated CD8⁺GFP⁻ T cells showed a mild suppressive effect on CD4⁺ (Fig. 5B) and CD8⁺ (Supporting Information Fig. 5) T-cell proliferation and on IFN- γ production by CD8⁺ T cells (Fig. 5C), which was however inferior to that of CD4⁺GFP⁺ natural and induced Tregs (Fig. 5B and C).

In conclusion, CD8⁺Foxp3⁺ T cells are actively restricted in pool size and not enriched in suppressive function, although they

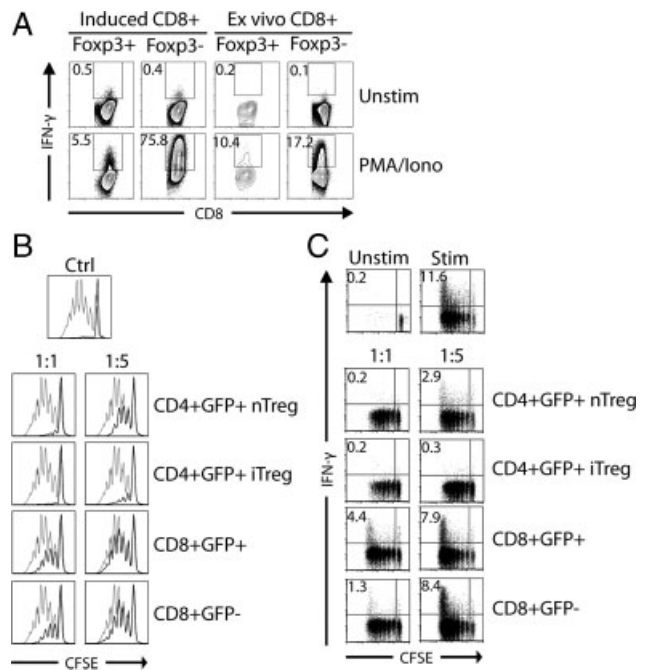


Figure 5. CD8⁺Foxp3⁺ T cells show defective IFN- γ production but only display mild suppressive activity. (A) In vitro cultures (induced CD8⁺) of CD8⁺ T cells isolated from Rag1^{-/-} \times OTI mice were established with OVA_{257–264} and IL-2 either in the presence (Foxp3⁺) or absence (Foxp3⁻) of TGF- β and RA. Additionally, spleens and lymph nodes of WT mice were prepared and single cell suspensions were obtained (ex vivo). Cells were restimulated with PMA/ionomycin or were left unstimulated, stained for CD8, Foxp3 and IFN- γ and analyzed by flow cytometry. Live CD8⁺Foxp3⁺ (Foxp3⁺) and CD8⁺Foxp3⁻ (Foxp3⁻) cells were gated and the frequency of IFN- γ ⁺ cells is displayed. (B) CFSE-labeled CD4⁺ T cells were co-cultured at the indicated ratios (Treg/responder cells) with CD4⁺GFP⁺ natural Tregs, CD4⁺GFP⁺ iTregs, induced CD8⁺GFP⁺ T cells and activated CD8⁺GFP⁻ T cells (all generated as described in the Materials and Methods Section) for 4 days in the presence of α CD3. CFSE dilution of CD4⁺CD45.1⁺ gated responder T cells is shown. Wells with responder T cells only in the presence (gray line) or absence (black line) of α CD3 served as controls (Ctrl). CFSE profiles of co-cultured responder T cells (black lines) are overlaid with α CD3-stimulated control (gray lines) to assess the suppressive activity of the indicated cell types. (C) CFSE-labeled CD8⁺CD45.1⁺ responder T cells were co-cultured at the indicated ratios (Treg/responder cells) with CD4⁺GFP⁺ natural Tregs, CD4⁺GFP⁺ iTregs, induced CD8⁺GFP⁺ T cells and activated CD8⁺GFP⁻ T cells for 4 days in the presence of α CD3. Wells were then restimulated with PMA/ionomycin. Wells with responder T cells only in the absence of α CD3 and restimulation (Unstim) and with responder T cells only in the presence of α CD3 and restimulation (Stim) served as controls. Plots are gated on live CD8⁺CD45.1⁺ responder T cells and display CFSE dilution versus IFN- γ expression. Frequencies within the upper left quadrants are indicated. (A–C) All results are representative for two to three independent experiments.

share certain developmental and phenotypic characteristics with CD4⁺Foxp3⁺ Tregs.

Discussion

Foxp3 can be efficiently induced in Rag1^{-/-} \times OTI CD8⁺Foxp3⁻ T cells without accessory cells in the presence of TGF- β , RA and OVA_{257–264} peptide (Fig. 1C). We observed that CD8SP

thymocytes up-regulated Foxp3 more efficiently than CD8⁺ T cells from peripheral sources which might relate to a T-cell intrinsic capability of immature T cells for Foxp3 induction as previously observed for CD4⁺ T cells [28, 29]. Although CD80/CD86 was reported to be essential for the generation of CD4⁺ Foxp3⁺ Tregs in vivo [30], DC actively repressed Foxp3 induction in part via CD80/CD86-mediated co-stimulation in vitro. This is in line with a previous report demonstrating lack of Foxp3 induction in CD8⁺ T cells upon polyclonal stimulation in the presence of 1 µg/mL αCD28 (similar concentration as used in our study) and TGF-β, although contrary effects were reported with higher agonist concentrations [31]. RA could overcome DC-mediated inhibition to some extent (data not shown), similar to previous findings with CD4⁺ Foxp3⁺ Tregs [22]. TCR ligand density and potency might, however, additionally influence Foxp3 induction [32]. Our results are in harmony with a study from Mucida et al. where CD8⁺ OTI cells were cultured with identical factors but in the presence of DC to induce Foxp3 [33]. Notably, the Foxp3 induction efficiency in this setting was about five times lower, probably due to the inhibitory effects of DC. Foxp3 induction was similarly suboptimal when a different TCR transgenic system and mLN-DC or polyclonal stimulation in the presence of BM-derived DC were used [17, 34]. Considering that MHC-class-I is broadly expressed, it is possible that CD8⁺ Foxp3⁺ T cells might preferentially develop in response to endogenous or foreign intracellular antigens presented by cell types incapable of co-stimulation, in specific compartments where TGF-β and RA are available. Indeed, ectopic antigen expression controlled by the villin promoter has recently been shown to result in expansion of intestinal CD8⁺ Foxp3⁺ T cells when crossed to TCR transgenic mice specific for the same antigen [34]. Additionally, CD8⁺ Foxp3⁺ T cells have been shown to expand during simian immunodeficiency virus infection at sites of viral replication [35] and accumulate in colorectal cancer tissue [36], which may be a result of direct antigen presentation by infected or transformed cells, respectively. On the other hand, CD8⁺ Foxp3⁺ T cells represent a highly size-restricted population in unmanipulated mice (Fig. 3A), consistent with previous observations [2, 17].

Interestingly, a CD8⁺ Foxp3⁺ population expands in MHC-class-II-deficient mice and shares phenotypic and functional features with CD4⁺ CD25⁺ Tregs [37], whereas the absence of CD8⁺ Foxp3⁺ T cells in MHC-class-I-deficient mice suggests MHCI restriction [25]. The presence of Foxp3⁺ cells among CD8SP thymocytes suggests at least a partial thymic origin of CD8⁺ Foxp3⁺ T cells, similar to CD4⁺ Foxp3⁺ Tregs [18], although re-immigration into the thymus after peripheral conversion cannot be formally excluded. Furthermore, the lack of CD8⁺ Foxp3⁺ T cells in Rag1^{-/-} × OTI mice underlines the requirement for certain defined TCR specificities in order to develop CD8⁺ Foxp3⁺ T cells.

A detailed phenotypic characterization of induced CD8⁺ Foxp3⁺ T cells revealed high expression of classical Treg markers including CD25, GITR and CTLA4, consistent with previous reports [17, 31] and likely reflecting T-cell activation, although

one study reported low CD25 expression on CD8⁺ Foxp3⁺ T cells [38]. Interestingly, the classical Treg markers CD73 and CD103 were selectively expressed by induced CD8⁺ Foxp3⁺ T cells, underlining that their expression is dependent on TGF-β, RA and/or Foxp3. In line with this, CD8⁺ T cells deficient in TGF-β signaling fail to up-regulate CD103 in a GVHD model [39], and Foxp3 has been shown to directly bind the CD103 promoter [40]. However, Foxp3-independent mechanisms can also activate CD103 [3], consistent with the only mildly reduced induction of CD103 expression in stimulated T cells from DEREK × Rag1^{-/-} × OTI × Sf mice (Supporting Information Fig. 3C).

CD8⁺ Foxp3⁺ T cells only displayed little suppressive capacity compared with CD4⁺ Foxp3⁺ Tregs, and CD8⁺ Foxp3⁻ T cells showed similarly low suppressive activity in vitro. Furthermore, adoptive transfer of induced CD8⁺ Foxp3⁺ T cells did not ameliorate disease in an OVA-based allergic airway inflammation model (data not shown). Previous studies have reported the suppressive capacity of TGF-β-induced CD8⁺ T cells [17, 31, 34, 38], which in principle does not contradict our data. First, several studies did not compare the strength of suppression to that of CD4⁺ Tregs [31, 34, 38], which depend on Foxp3 [3]. Second, suppressive CD8⁺ T cells were isolated either based on CD25 expression [17] (also broadly up-regulated on activated Foxp3⁻ T cells, at least in the absence of IL-6), or were tested without further separation for suppressive function [31, 38], thereby not allowing for discrimination between Foxp3⁺ and Foxp3⁻ subsets. Third, DC or agonistic αCD28 antibodies were used during in vitro differentiation in all these studies. Therefore, it cannot be formally excluded that the low suppressive function observed in our study is caused by the lack of signals provided by either DC or αCD28. However, this would underlie Foxp3-independent mechanisms, since CD8⁺ Foxp3⁺ T cells can be efficiently generated without co-stimulation (Fig. 1). Strikingly, co-stimulation even represses Foxp3 induction in CD8⁺ T cells (Fig. 2A and B) suggesting that CD80/CD86-αCD28 would rather modulate suppressive activity in a Foxp3⁻ subset. In sum, our results suggest that Foxp3 alone is not sufficient to confer strong suppressive activity to CD8⁺ T cells. Although transgenic mice with forced overexpression of Foxp3, but not WT mice, were described to harbor suppressive CD8⁺ T cells, Foxp3 was similarly considered as implicated but not sufficient to confer suppressive activity in a previous study [41].

Suppressive CD8⁺ Foxp3⁺ T cells and CD4⁺ Foxp3⁺ Tregs were recently described to expand in response to αCD3 treatment [27]. However, there is no direct evidence provided that CD8⁺ Foxp3⁺ T cells contribute significantly to suppression in vivo, and no suppression data of CD8⁺ Foxp3⁻ T cells (which we here show to have comparable suppressive activity) are available.

In summary, while we recently excluded an importance of Foxp3 expression in nonhematopoietic cells for the suppression of autoimmunity [24], we show here that Foxp3 can be expressed in a highly restricted subset of CD8⁺ T cells sharing phenotypic and developmental characteristics with CD4⁺ Foxp3⁺ Tregs. However,

induced CD8⁺Foxp3⁺ T cells are not enriched in suppressive activity on T-cell proliferation and IFN- γ production compared with Foxp3⁻ counterparts and show rather weak suppressive activity compared with CD4⁺Foxp3⁺ Tregs. Additionally, the Foxp3⁺ niche is predominantly populated by CD4⁺CD8⁻ Tregs under physiological conditions, including the intestine which is rich in Foxp3-inducing factors. Therefore, the physiological relevance of CD8⁺Foxp3⁺ T cells as suppressive population might have been previously overestimated. In fact, multiple mechanisms seem to prevent the generation/expansion of CD8⁺Foxp3⁺ T cells, including Dnmt1 [42]. The underlying mechanisms and physiological importance of this “natural imbalance” remain to be further explored. This study now provides an additional possible mechanism (co-stimulation by DC) and a rationale explanation (lack of strong suppressive activity). Future studies will have to define if certain pathological conditions can significantly alter the pool size and suppressive activity of CD8⁺Foxp3⁺ T cells.

Material and methods

Mice

Rag1^{-/-}, OTI, OTII, CD45.1, CD80KOxCD86KO and Sf mice were purchased from Jackson. DEREK mice were described previously [6]. All mice were bred at the Twincore (Hannover, Germany) or the Helmholtz Centre for Infection Research (Braunschweig, Germany). All animal experiments were performed under specific pathogen-free conditions and in accordance with institutional, state and federal guidelines.

Flow cytometry and cell sorting

The following antibodies and secondary reagents were purchased from eBioscience: α -CD4 (GK1.5), α -CD8- α (53-6.7), α -CD25 (PC61.5), α -CD45.1 (A20), α -CD73 (TY/11.8), α -CD103 (M290), α -CTLA4 (UC10-4B9), α -IFN- γ (XMG1.2), α -Foxp3 (FJK-16s), α -GITR (DTA-1), streptavidin and appropriate isotype controls. For intracellular cytokine staining, the IC fixation/permeabilization kit from eBioscience was used. Foxp3 staining was carried out using the Foxp3 fixation/permeabilization kit (eBioscience).

Cytometric analysis was performed using LSRII (BD) and FlowJo software (Treestar). Dead cells were excluded by propidium iodide or ethidium bromide monoazide staining, and cellular aggregates were excluded by SSC-W. For ex vivo analysis of CD8⁺Foxp3⁺ T cells, secondary lymphoid organs were digested with collagenase D and DNaseI (both Roche). Calcium/magnesium-free PBS containing 2 mM EDTA and 1% BSA were used during the entire staining procedure.

Cell sorting was carried out at the Cell Sorting Core Facility of the Hannover Medical School on FACSaria (BD), XDP or MoFlo (both Beckman Coulter) machines.

Real-time RT-PCR

cDNA was prepared using the μ MACS One-Step cDNA kit and a ThermoMACS magnetic separator (both from Miltenyi Biotec) according to the manufacturer's instructions. Validated intron-spanning primer sets were designed employing the Universal Probe Library Assay Design Centre (www.roche-applied-science.com). The following primer pairs were used: Foxp3 (5'-agaagctgggagctatgcag-3', 5'-gctacgatgcagcaagagc-3'); CD25 (Il2ra) (5'-ccaacacagtctatgaccaa-3', 5'-agattctcttggaaatcttcatgttc-3'); CD73 (5'-atgaacatctgggctacga-3', 5'-gtcctccacacggtatcaa-3'); CD103 (Itgae variant 2) (5'-cctggaccactacaaggaacc-3', 5'-ttgcagctctctctagtagg-3'); CTLA4 (5'-tcactgctgtttcttgagca-3', 5'-ggctgaaattgctttccatc-3'); Folr4 variant 2 (5'-gcctgccatcatcttga-3', 5'-tcattgatagaagacccttgacc-3'); GzmB (5'-gctgctcactgtgaaggaagt-3', 5'-tggggaatgattttaccat-3'); Hprt (5'-tcctctcagaccgctttt-3', 5'-cctgttcatcatcgctaac-3'). Quantitative real-time PCR was performed using the Mouse Universal Probe Library, the LightCycler480 Probes Master Kit and a LightCycler480 (all from Roche) according to the manufacturer's instructions. Integrated system software was used to obtain second derivative crossing point (C_p) values, and relative mRNA levels were calculated using the Hprt housekeeping gene.

Generation and isolation of CD8⁺Foxp3⁺ and CD8⁺Foxp3⁻ T cells

CD8⁺ T cells were obtained from secondary lymphoid organs of Rag1^{-/-} × OTI mice by negative magnetic isolation (Invitrogen) if not indicated otherwise. In some cases, total cell suspensions from spleens and thymi, or sorted CD8⁺CD11c⁻ splenocytes were used. To study the mechanisms of Foxp3 induction, 1 × 10⁴ CD8⁺ T cells were seeded in 96-well round bottom plates and cultured in RPMI medium (10% FCS supplemented) containing 200 U/mL rhIL-2 (Roche) and 0.01 μ g/mL OVA_{257–264} (Biosynthan). Some wells were additionally supplemented with 2 μ g/mL α -CD28 (37.51; eBioscience), 10 nM RA (Sigma), 2 ng/mL rhTGF- β 1 (Peprotech) or different combinations of the latter reagents. After 2 days, all wells were supplemented with 200 U/mL fresh rhIL-2, and Foxp3 expression was assessed by flow cytometry on day 4. Equal cell numbers and conditions were used when total cell suspensions were cultured, with the exception that 5 × 10⁴ total thymocytes were initially seeded. BM-derived DC were generated using GM-CSF (hybridoma supernatant) and added at indicated ratios to CD8⁺ T cells in some experiments.

For the generation of CD8⁺Foxp3⁺ T cells, 10 mL cultures were established in 10 cm dishes using 5 × 10⁶ CD8⁺ T cells negatively isolated cells from spleens and lymph nodes of DEREK × Rag1^{-/-} × OTI mice.

Cultures were supplemented with IL-2, OVA_{257–264}, TGF- β 1 and RA at the same concentrations as described above. Two days later, 200 U/mL IL-2 was supplemented and on day 3 10 mL of fresh medium was added if necessary. On day 4, CD8⁺GFP⁺ cells were isolated by FACS sorting. Activated CD8⁺Foxp3⁻ T cells were

generated identically except that TGF- β 1 and RA were excluded from the cultures and CD8⁺GFP⁻ cells were sorted on day 4.

TSDR methylation status

CD8⁺GFP⁺ T cells and CD8⁺GFP⁻-activated T cells were generated from male DERE \times Rag1^{-/-} \times OTI mice and sorted as described before. DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen) and bisulfite sequencing of the TSDR was performed as described previously [23].

In vitro restimulation

Cells were restimulated at a concentration of 1×10^7 /mL if not indicated otherwise with 100 ng/mL PMA and 1 μ g/mL ionomycin (both Sigma) for 6 h at 37°C. Brefeldin A (eBioscience) was added during the last 2 h, followed by intracellular cytokine staining and FACS analysis.

In vitro suppression assays

CD4⁺ T cells and CD8⁺ T cells were isolated from spleens and lymph nodes of CD45.1⁺ mice by negative selection (Invitrogen). CD25⁺ cells were subsequently depleted by α -CD25-PE and anti-PE microbeads (Miltenyi Biotec) according to the manufacturer's instructions. Responder T cells were then labeled with 5 μ M CFSE and seeded at 5×10^4 cells per 96-round bottom well together with 3×10^3 BM-derived DC in complete RPMI medium. CD4⁺GFP⁺ nTregs were sorted ex vivo from DERE \times OTI mice. CD8⁺GFP⁺ or CD8⁺GFP⁻ T cells were induced and sorted as detailed before. For the generation of induced CD4⁺GFP⁺ Tregs, CD4⁺ T cells were negatively selected from spleens and lymph nodes of DERE \times OTI mice followed by depletion of CD25⁺ cells. Cells were cultured in 96-well round-bottom plates at 5×10^4 T cells per well in the presence of 3×10^3 BM-DC (generated with FLT3L hybridoma supernatant), 0.06 μ g/mL OVA_{323–339} (Biosynthon), 200 U/mL IL-2, 2 ng/mL TGF- β and 10 nM RA. After 2 days, 200 U/mL IL-2 was supplemented and CD4⁺GFP⁺ cells were FACS-sorted on day 4. All populations were added at to responder T cells at indicated ratios (Treg/responder cells). Responder T cells were activated by the addition of 1 μ g/mL α -CD3 antibody. CFSE dilution of CD4⁺CD45.1⁺ responder T cells was assessed by flow cytometry on day 4. In case of CD8⁺ T cells, wells were restimulated on day 4 as described above and CD8⁺CD45.1⁺ cells were analyzed for CFSE dilution and IFN- γ production.

Statistical analysis

Unpaired two-tailed Student's *t*-test was performed (Microsoft Excel) to determine the statistical significance (**p*<0.05; ***p*<0.005).

Acknowledgements: We thank Stephanie Dippel, Martina Thiele, Christine Jaencke and Esther Ermeling for technical assistance. This work was supported by the SFB587, SFB738 and SFB900. Christian T. Mayer was supported by a stipend from the German National Academic Foundation. We would further like to thank the Cell Sorting Core Facility of the Hannover Medical School supported in part by the Braukmann-Wittenberg-Herz-Stiftung and Deutsche Forschungsgemeinschaft.

Conflict of interest: The authors declare no financial or commercial conflict of interest.

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Abbreviations: DREG: depletion of regulatory T cells · nTreg: natural Treg · RA: all-trans-retinoic acid · Sf: scurfy · TSDR: Treg-specific demethylated region

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Received: 6/8/2010
Revised: 25/11/2010
Accepted: 21/12/2010
Accepted article online: 4/1/2011