

Unique Phenotype of Human Tonsillar and In Vitro-Induced FOXP3⁺CD8⁺ T Cells¹

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Forkhead box p3 (FOXP3) is known to program the acquisition of suppressive capacities in CD4⁺ regulatory T cells (Treg), whereas its role in CD8⁺ T cells is unknown. The current study investigates whether FOXP3 also acts as a Treg master switch in peripheral blood and tonsillar CD8⁺ T cells. Single-cell analyses reveal the existence of a FOXP3⁺CD8⁺ population in human tonsils, whereas FOXP3⁺CD8⁺ T cells are rarely detected in peripheral blood. Tonsillar FOXP3⁺CD8⁺ T cells exhibit a Treg phenotype with high CTLA-4 and CD45RO and low CD127 and CD69 expression. Interestingly, the tonsillar FOXP3⁺CD8⁺ T cells are mostly CD25^{negative} and some cells also express the proinflammatory cytokines TNF- α , IFN- γ , or IL-17A. Particularly, IL-17A-expressing cells are present among FOXP3⁺CD8⁺ T cells. Even though FOXP3 expression is at the detection limit in peripheral blood CD8⁺ T cells ex vivo, it can be induced in vitro in naive CD8⁺ T cells by polyclonal stimulation. The induced FOXP3⁺CD8⁺ T cells are predominantly CD25^{high} and CD28^{high} and similar to tonsillar cells, they produce high levels of TNF- α , IFN- γ , and granzyme B. However, IL-4 expression is mutually exclusive and IL-17A expression is not detectable. These FOXP3⁺CD8⁺ T cells suppress the proliferation of CD4⁺ T cells in cocultures, while showing no direct cytotoxic activity. In conclusion, the current study characterizes FOXP3-expressing CD8⁺ T cells from human tonsils and shows that in vitro activation leads to FOXP3 expression in CD8⁺ T cells and gain of suppressive activity. *The Journal of Immunology*, 2009, 182: 2124–2130.

Regulatory T cells (Treg)³ are critical mediators of dominant immune tolerance to self- and foreign Ags. In contrast to CD4⁺ MHC class II-restricted Treg cells, little is known about CD8⁺ Treg cells, which are MHC class I restricted. Thus, the CD8⁺ Treg cells have access to Ag presentation by virtually all nucleated cells in the body. Several induced or naturally occurring CD8⁺ Treg cell subsets have been described (e.g., CD122⁺ (1), CD75s⁺ (2), CD103⁺ (3), Lag3⁺ (4), and CD28[−] (5)). CD8⁺CD28[−] T cells inhibit T cell activation indirectly by promoting a tolerogenic phenotype of APC (5). Furthermore, CD25⁺CD8⁺ Treg cells that express forkhead box p3 (FOXP3) and show a phenotype similar to their CD4⁺ counterparts have been identified in the thymus (6, 7).

FOXP3 is a transcriptional regulator, repressing proinflammatory cytokine expression while inducing the expression of immune regulatory cytokines and Treg markers (8). By interacting with other transcription factors and chromatin modifying enzymes,

FOXP3 induces epigenetic modifications that lead to the generation of Treg cells (9). Thus, FOXP3 acts as a master switch factor for Treg development similar to T-bet, GATA3, and retinoic acid receptor-related orphan receptor γ (ROR γ t; also known as RORC2) for the Th1, Th2, and Th17 cells, respectively. Initially, FOXP3 expression was detected only in CD25^{high}CD4⁺ T cells in mice and humans (10–13). However, its transgenic expression conferred suppressive activity to CD25[−]CD4⁺ T cells as well as to the CD8⁺ subset of T cells (13, 14). Whereas FOXP3⁺CD8⁺ T cells constitute a minuscule fraction of circulating CD8⁺ T cells in human blood, polyclonal activation in vitro induces expression of FOXP3 (15–19). Currently, it is not known how human FOXP3⁺CD8⁺ Treg cells develop in vivo and whether they possess suppressive activity.

The development of anti-inflammatory CD4⁺ Treg cells and the expression of their lineage-specific transcription factor FOXP3 has been shown to require TGF- β (20). Surprisingly, the development of the proinflammatory Th17 cells and the expression of their suggested lineage-specific transcription factor ROR γ t depend also on TGF- β (21–23). In contrast to Treg cells, Th17 cell development requires the cytokine IL-6 in addition to TGF- β , especially for the expression of the Th17 signature cytokine IL-17A (21). TGF- β is abundantly expressed in the MALT and plays a critical role in oral tolerance induction. As part of the MALT, palatine tonsils are located at the gateway of the respiratory and alimentary tracts where they are continually exposed to airborne and ingested Ags (24). Since the discrimination between harmless and harmful potential pathogens takes place in tonsils, they are a key site of effective immunity or tolerance. Whether CD8⁺ Treg cells are present in tonsils and participate in the regulation of their immune response is currently unknown.

In this study, we identify a FOXP3⁺CD8⁺ T cell population in human tonsils that is predominantly CD25[−] and includes some IFN- γ -, TNF- α -, and IL-17A-expressing cells. In vitro stimulation of naive CD8⁺ T cells induces FOXP3⁺CD8⁺ T cells exhibiting

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³ Abbreviations used in this paper: Treg, regulatory T; ROR γ t, retinoic acid receptor-related orphan receptor γ t; EF-1 α , elongation factor 1 α ; FOXP3, forkhead box p3; LDH, lactate dehydrogenase; Fwd, forward; rev, reverse; iTreg, inducible regulatory T cell; ECD, energy-coupled dye.

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a similar phenotype to the tonsillar subset. In addition, they acquire the capacity to suppress the proliferation of autologous CD4⁺ T cells.

Materials and Methods

Tissue samples, cell isolation, and purification of T cell subsets

Human PBMCs were isolated from heparinized whole blood from in-house donors or from buffy coats of healthy donors (obtained from Blutspende-Zentrum Zürich, Zurich, Switzerland) by centrifugation on a density cushion (Biocoll; Biochrom). Human palatine tonsils were obtained from the hospital of Davos and tonsillar cells were isolated directly after surgery. Only noninflamed, hypertrophic tonsils from children (average age of 13.2 years) not taking any immunosuppressive drugs were used for the analyses. Single-cell suspensions of human tonsils were prepared by mechanical disruption. Naive (CD45RO⁻) CD8⁺ T cells were isolated from PBMCs by negative selection using a pan T cell isolation kit, CD45RO and CD4 microbeads, and the AutoMACS magnetic separation system (Miltenyi Biotec). CD4⁺ T cells were isolated from PBMCs using a CD4 Positive Isolation Kit (Dyna) according to the manufacturer's instructions. Tonsil tissue was embedded in Tissue-Tek (Sakura Finetek), frozen in liquid nitrogen-cooled 2-methylbutane (Fluka), and stored at -80°C until cryosections were cut using a HM 500 OM microtome (Mikrotom). Tonsil studies have been reviewed and approved by the ethic committee of Graubünden.

Cell cultures

For differentiation cultures, the sorted T cell subsets were stimulated polyclonally with soluble anti-CD3 (OKT3; American Type Tissue Collection) and anti-CD28 (15E8; CLB) Abs (2.5 µg/ml of each) in serum-free AIM-V plus AlbuMAX (Life Technologies). Both Abs were produced in our laboratory. The cells were split at day 3 at a ratio of 1:2 with RPMI 1640, which was supplemented with heat-inactivated FCS (Amimed), antibiotics (penicillin, streptomycin, and kanamycin; Life Technologies), and MEM vitamin, L-glutamine, nonessential amino acids, sodium pyruvate (Life Technologies), designated as complete RPMI 1640. The naive T cells were cultured in the presence of neutralizing anti-IL-12 and IL-4 Abs (5 µg/ml; R&D Systems) without or with the addition of TGF-β (5 ng/ml; R&D Systems) or anti-TGF-β Ab (2.5 µg/ml; R&D Systems). Anti-IL-4 and IL-12 Abs were included in the cultures to inhibit the development of Th1 and Th2 cells and increase the efficiency of inducible regulatory T cell (iTreg) generation.

For coculture experiments, T cells were cultured as described above. At day 6, the cells were harvested, washed once, and cultured in complete RPMI supplemented with IL-2 (100 U/ml, Proleukin Prorepha Pharma). At day 10 of the culture, cells were cocultured with freshly isolated CD4⁺ T cells and irradiated CD4/CD8-depleted PBMCs as APCs from the same donor in the presence of anti-CD3 Ab (1 µg/ml) in complete RPMI. CD4⁺ T cells were labeled with CFSE (Molecular Probes) as previously described (25). Unlabeled "suppressor" cells were mixed with CFSE-labeled "responder" cells at different ratios. Throughout the assay, the number of T cells (sum of CD4⁺ responder and CD8⁺ suppressor T cells) was consistently 150,000 cells/well plus 300,000 APCs. Therefore, the ratio of 1:2 of T cells to APCs was equal in all wells at the start of coculture. For each condition, triplicates were analyzed at day 5 of coculture by flow cytometry. To determine the role of granzyme B (grzB)-mediated killing, the inhibitor Z-AAD-CMK (1.25 µM; BioVision) was included in the assay. To exclude dead cells, propidium iodide (Sigma-Aldrich) was added. Proliferation analysis is based on the percentage of undivided CFSE-positive cells.

For the analyses of the IFN-γ secretion, the cell-free supernatant was harvested at day 5 and the IFN-γ concentration was determined with a cytometric bead array (Bio-Rad) according to the manufacturer's protocol.

Cytotoxicity detection assay

To determine cytotoxicity, a lactate dehydrogenase (LDH)-based cytotoxicity detection system was used according to the manufacturer's protocol (Roche Applied Science). Briefly, the LDH activity in the culture supernatant was determined by a coupled enzymatic reaction which leads to the product formazan, which can then be measured with an ELISA reader. The measured absorbance values correlate directly with the amount of dead cells that had released LDH into the assay medium. The assay medium was RPMI 1640 supplemented with antibiotics and amino acids as described above but with only 1% FCS (which might be also a source of LDH). For the assay, 200,000 CD8⁺ T cells (at day 10 of differentiation or freshly isolated) were cultured with 200,000 CD4⁺ T cells from the same donor in the presence of anti-CD3 and anti-CD28 Abs (1 µg/ml) in a 96-well plate. All conditions were measured in triplicate. After 22 h of culture, the plate

was centrifuged, the supernatant was harvested, and the LDH activity was analyzed. CD4⁺ T cells in assay medium without CD8⁺ T cells were used as low control (spontaneous cell death) and CD4⁺ T cells in the presence of 1% Triton X-100 served as high control (maximal cell death). The cell death for each cell type was determined separately which allowed the calculation of the percent viable CD4⁺ T cells. CD4⁺ T cells without coculture of CD8⁺ T cells were defined as 100% viable cells.

RNA extraction, cDNA synthesis, and quantitative real-time RT-PCR

Total RNA was isolated using the RNeasy mini kit (Qiagen) and reverse transcription was performed with Revert Aid M-MuLV Reverse Transcriptase (Fermentas) using random hexamer primers according to the manufacturer's protocol.

Gene expression was analyzed by quantitative real-time PCR using iTaq SYBR Supermix with ROX (Bio-Rad) on a 7900HT Fast Real-Time PCR instrument (Applied Biosystems). The primer pairs were designed based on the sequences reported in GenBank using Primer Express software (Applied Biosystems). The primer sequences were as follows: elongation factor 1α (EF-1α) (fwd, CTGAACCATCCAGGCCAAAT; rev, GCCGTG TGGCAATCCAAT), FOXP3 (fwd, GAAACAGCACATTTCCAGAGTT C; rev, ATGGCCCCAGCGGATGAG), GATA3 (fwd, GCGG GCTCTATC ACAAATGA; rev, GCTCTCTGGGTGCAGACAGC), T-bet (fwd, GA TGCGCCAGGAAGTTTCAT; rev, GCACAATCATCTGGGTACATT), RORγt (fwd, CAGTCATGAGAACAATTTGAAGTG; rev, CAGGTG ATAACCCCGTAGTGGAT), and perforin (fwd, CACGAGACAGTA CAGCTTCA; rev, GGGAGTGTGTACCACATGGAAA). Primer pairs were evaluated for integrity by analysis of the amplification plot, dissociation curves, and efficiency of PCR amplification. PCR conditions were 3 min at 95°C, followed by 40 cycles of 15 s at 95°C and 60°C for 1 min. Relative quantification and calculation of the range of confidence are performed using the comparative ΔΔCT method as previously described (26). All amplifications were conducted in triplicate. The housekeeping gene *EF-1α* was used for normalization.

Immunofluorescence, flow cytometry, and Western blot

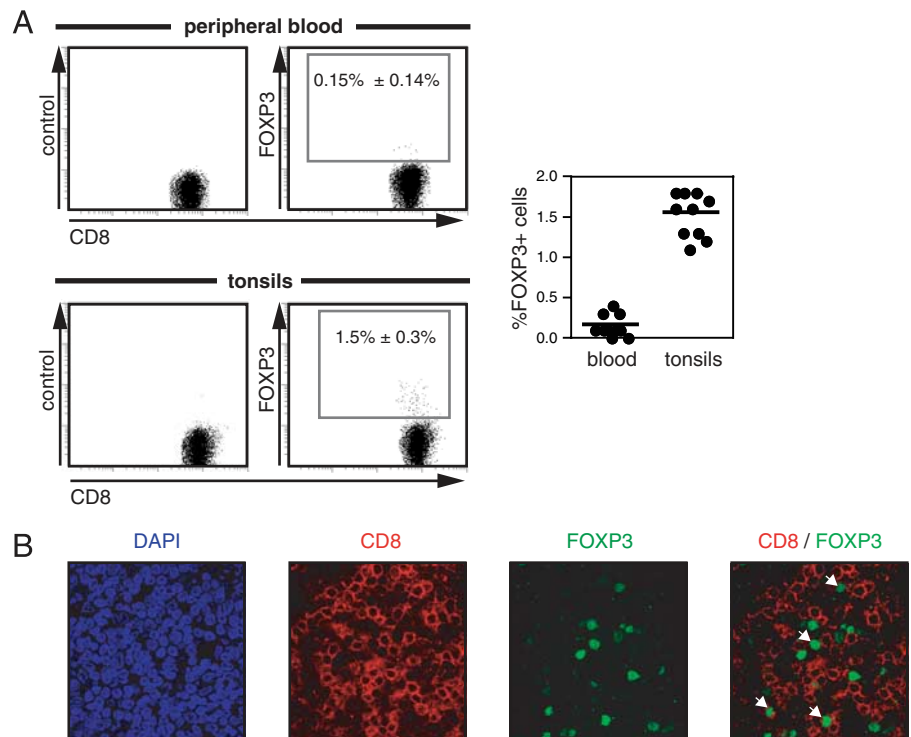
Cryosections were fixed in 4% paraformaldehyde for 10 min at room temperature. After washing with PBS, the slides were incubated for 30 min with PBS, 1% BSA, and 0.2% Triton X-100 containing 10% normal rat serum, followed by a 1-h incubation with the following Abs: anti-CD8 Alexa Fluor 647 (Biolegend), anti-FOXP3 Alexa Fluor 488 (eBioscience), or appropriate isotype controls. The tissue sections were then washed with PBS containing 0.05% Tween 20 and mounted with Vectashield (Vector Laboratories) containing 4',6-diamidino-2-phenylindole for nuclear staining. Microscopic analyses were performed on a confocal TCS SPE microscopy system (Leica Microsystems).

The following Abs were used for flow cytometry: anti-CD25 PC5, anti-CD8 energy-coupled dye (ECD)/PC5, anti-CD4 ECD/PC5, anti-CD3 ECD/FITC, anti-CD45RO PE, and anti-CD127 PE (all from Beckman Coulter), anti-CTLA-4 PE, anti-CD69 PE, anti-CD28 FITC, anti-IFN-γ FITC/PE, anti-IL-4 Alexa Fluor 488/PE, anti-TNF-α PE, anti-IL-10 PE, anti-IL-2 FITC (all from BD Biosciences), anti-IL-17A PE (eBioscience), anti-CD25 PE, anti-CD103 FITC/PE (DakoCytomation), and anti-grzB PE (Caltag Laboratories). Flow cytometric analyses were performed on an EPICS XL-MCL using the Expo32 software (Beckman Coulter).

FOXP3 staining was performed using the PE anti-human FOXP3 (PCH101) staining set (eBioscience) according to the manufacturer's instructions. To detect intracellular cytokines, cells were polyclonally stimulated with 2.5 µg/ml anti-CD3 and anti-CD28 Abs or with 25 ng/ml PMA and 1 µg/ml ionomycin (σ) for 6 or 4 h, respectively. Brefeldin A (10 µg/ml; Sigma-Aldrich) was added after 1 h of stimulation. Following surface staining, the cells were fixed and permeabilized using a BD Cytofix/Cytoperm kit (BD Bioscience) according to the manufacturer's instructions. For FOXP3 and cytokine costaining, the Alexa Fluor 488 or PE FOXP3 staining kit from Biolegend was used.

For SDS-PAGE and Western blot, 1 × 10⁶ cells were lysed in radioimmunoprecipitation assay buffer supplemented with DNase and incubated for 10 min at room temperature. Then DTT and NuPAGE sample buffer were added and the samples were loaded next to a protein-mass ladder (Fermentas) on a NuPAGE 4–12% bis-Tris gel (Invitrogen). The proteins were electroblotted onto a polyvinylidene difluoride membrane (Amersham Biosciences). Unspecific binding was blocked with 3% nonfat milk powder. The following Abs were used: goat anti-FOXP3 (Abcam), mouse anti-GAPDH (Ambion), anti-goat HRP-labeled, and sheep anti-mouse HRP-labeled Abs (Amersham Biosciences). For detection, the ECL Plus Western blotting detection system (Amersham Biosciences) was used. The bands were visualized with a LAS 1000 camera (Fuji).

FIGURE 1. FOXP3⁺CD8⁺ T cells are rare in human peripheral blood, but do exist in human tonsils. **A**, The expression of FOXP3 in CD8⁺ T cells from human blood or palatine tonsils was analyzed by flow cytometry. Representative dot plots for CD8 and FOXP3 or isotype control gated on CD8⁺CD3⁺ T cells of one donor are shown. The mean frequency ($\pm$ SD) of FOXP3⁺ cells is included in the dot plots. The graph shows the frequency of FOXP3⁺ cells among CD8⁺ T cells from blood and tonsils. Each symbol represents data from one donor ($n = 10$). **B**, Confocal microscopic analyses of human tonsil sections stained for FOXP3, CD8, and 4',6-diamidino-2-phenylindole (DAPI) (nuclear stain). The magnification is 630-fold. The white arrows indicate FOXP3⁺CD8⁺ T cells.



Statistical analyses

Significance was determined by the Wilcoxon signed rank test. Values of $p < 0.01$ (**) and $p < 0.05$ (*) were considered significant and are indicated in the figures.

Results

A FOXP3⁺CD8⁺ T cell population exists in human tonsils

To investigate whether human CD8⁺ T cells express the Treg-specific transcription factor FOXP3 and are potentially involved in immune tolerance, we analyzed FOXP3 expression of CD8⁺ T cells from human peripheral blood by flow cytometry. FOXP3⁺CD8⁺ T cells constituted a minuscule fraction among PBMCs, with an average of $0.15 \pm 0.14\%$ of CD8⁺ T cells expressing FOXP3 (Fig. 1A).

In contrast to peripheral blood, a FOXP3⁺CD8⁺ T cell population was detected in palatine tonsils (Fig. 1A). The existence of FOXP3-expressing CD8⁺ T cells in tonsils was confirmed by confocal microscopy analyses. These analyses showed a clear nuclear localization of FOXP3 (Fig. 1B).

Phenotype of FOXP3⁺CD8⁺ T cells from tonsils

The influence of FOXP3 on the phenotype of CD8⁺ T cells from human tonsils regarding cytokine and Treg marker expression was analyzed by flow cytometry. Among the FOXP3⁺CD8⁺ population cells expressing IFN- γ ($24.6 \pm 10.2\%$), IL-17A ($29.2 \pm 17.6\%$), or TNF- α ($36.3 \pm 14.8\%$) were most frequent (Fig. 2A), while only a small subset of FOXP3⁺CD8⁺ T cells expressed grzB, IL-2, or IL-10. The IL-4 expression pattern was remarkably different from the other cytokines by the fact that the IL-4 and FOXP3 expression was mutually exclusive. When the frequency of cytokine-producing cells between FOXP3⁺CD8⁺ and FOXP3⁻CD8⁺ T cells from tonsils was compared, a positive correlation was observed only for IL-17A. And this was also obvious when the FOXP3 expression among IL-17A⁺ and IL-17A⁻ T cells was compared (supplemental Fig. 1⁴).

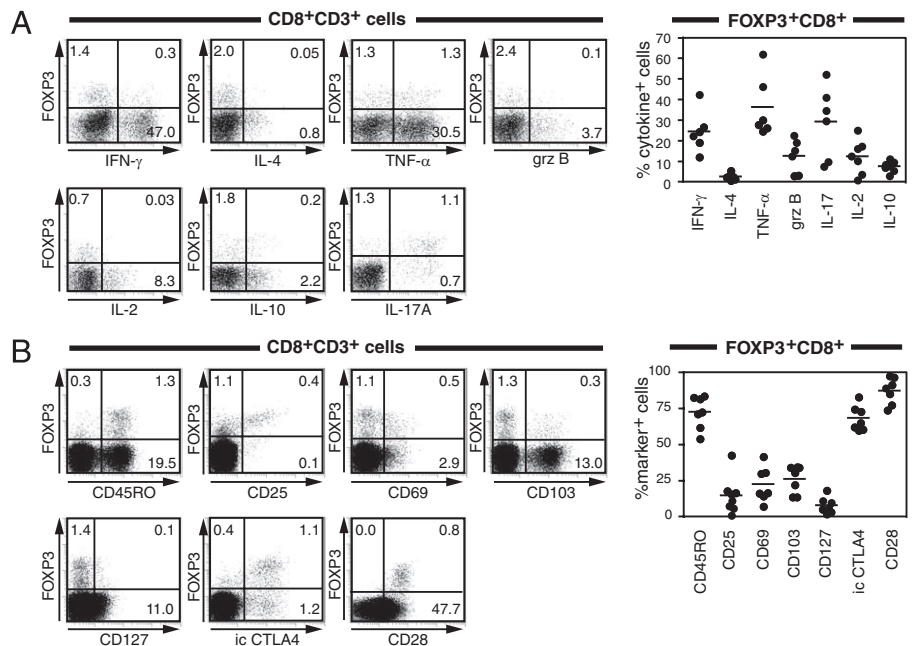
Analyses of activation and Treg marker expression showed that tonsillar FOXP3⁺CD8⁺ T cells belong predominantly to the CD45RO^{high} memory compartment ($72.8 \pm 11.3\%$), with a small fraction expressing the early activation marker CD69 ($22.7 \pm 12.0\%$; Fig. 2B). Furthermore, FOXP3⁺CD8⁺ T cells expressed high intracellular CTLA-4 ($68.5 \pm 8.7\%$) and low CD127 ($8.0 \pm 5.6\%$), which are characteristic for CD4⁺ Treg cells. The FOXP3⁺CD8⁺ population was predominantly CD25 negative, with only $14.9\% (\pm 12.7\%)$ expressing CD25. However, the less frequent CD25^{high}CD8⁺ T cell population expressed higher levels of FOXP3 than the CD25⁻CD8⁺ T cells. The expression of the integrin CD103, which is described to be a marker for effector/memory Treg cells in the murine system, was heterogeneous with $26.3 \pm 9.6\%$ of FOXP3⁺CD8⁺ T cells being positive for this marker. The majority of tonsillar FOXP3⁺CD8⁺ T cells expressed CD28 ($87.5 \pm 9.0\%$) and thus were distinct from the described CD8⁺CD28⁻ regulatory T cells.

FOXP3 expression induced in CD8⁺ T cells in vitro depends on TCR signal strength and TGF- β concentration

Since a FOXP3⁺CD8⁺ T population was detected only in tonsils (Fig. 2), we analyzed whether FOXP3 expression can be induced in peripheral blood CD8⁺ T cells. Naive CD8⁺ T cells isolated from peripheral blood, that were stimulated during 4 days with anti-CD3 and anti-CD28 Abs, expressed 126 (± 99) times more FOXP3 mRNA than ex vivo CD8⁺ T cells (Fig. 3A). Kinetic analysis revealed peak expression of FOXP3 mRNA after 4 days and FOXP3 protein after 6 days (supplemental Fig. 2). Addition of TGF- β , which is the main FOXP3-inducing factor in CD4⁺ T cells, had a weak, but consistent enhancing effect on FOXP3 mRNA expression (2.7 ± 1.4 -fold; Fig. 3, A and E), as well as on FOXP3 protein expression (2.9 ± 1.6 -fold; Fig. 3B). This increase of FOXP3 expression could be abolished by addition of anti-TGF- β Ab (supplemental Fig. 3A). There was no difference in CD103 or FOXP3 expression between cells treated with anti-TGF- β alone (data not shown) and those treated without any

⁴ The online version of this article contains supplemental material.

FIGURE 2. Phenotype of CD8⁺ T cells from tonsils. *A*, The tonsil cells were restimulated with PMA and ionomycin in the presence of brefeldin A and the cytokine expression was analyzed by intracellular staining and flow cytometry. The dot plots are gated for CD8⁺CD3⁺ T cells and the graph shows the frequency of cytokine⁺ cells among tonsillar FOXP3⁺CD8⁺ T cells ($n = 6$ or 7, respectively). *B*, Flow cytometric analyses of activation- and Treg marker by tonsillar FOXP3⁺CD8⁺ T cells. Representative dot plots of one donor (gated for CD8⁺CD3⁺ T cells) are shown and the graph depicts the frequency of marker⁺ cells among tonsillar FOXP3⁺CD8⁺ T cells ($n = 7$).



TGF- β . cells The differences in FOXP3 protein expression between cells cultured with or without TGF- β were also shown by Western blot analysis (Fig. 3C). To clarify the impact of TGF- β on FOXP3 induction in CD8⁺ T cells, the concentration of TGF- β in the cultures was titrated. TGF- β enhanced the frequency of FOXP3-expressing cells in a concentration-dependent manner and had also an enhancing effect on the FOXP3 expression level per cell, evidenced by the increased median fluorescence intensity of

the FOXP3⁺ cells (Fig. 3D). Furthermore, the TCR signal strength was shown to be of critical importance for the induction of FOXP3 expression, as increasing concentrations of anti-CD3 Ab enhanced the FOXP3 expression (Fig. 3E).

Phenotype of *in vitro*-differentiated CD8⁺ T cells

To investigate whether TGF- β treatment alters the phenotype of CD8⁺ T cells, the expression of the Treg markers CD103 and

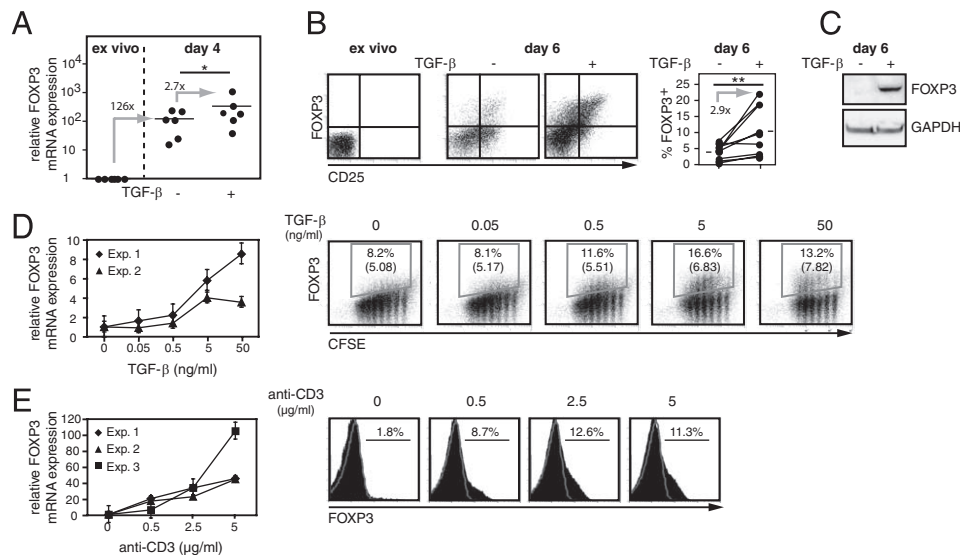


FIGURE 3. Induction of FOXP3 expression *in vitro* depends on TGF- β concentration and TCR signal strength. FOXP3 mRNA and protein expression were analyzed by quantitative real-time PCR (at day 4) and flow cytometry (at day 6), respectively. *A*, The graph shows the FOXP3 mRNA expression in naive CD8⁺ T cells *ex vivo* and at day 4 of the cultures with or without TGF- β . The expression is depicted as fold *ex vivo* expression, which was set as 1. *B*, FOXP3 and CD25 expression was analyzed by flow cytometry in naive CD8⁺ T cells *ex vivo* and at day 6 of the cultures in the presence or absence of TGF- β . One representative flow cytometric data set and the cumulated data (graph) are shown. *C*, Western blot analyses show the expression of FOXP3 in CD8⁺ T cells cultured with or without TGF- β at day 6 of culture. GAPDH was used as housekeeping gene. *D*, Analyses of the effect of the TGF- β concentration on FOXP3 mRNA and protein expression. The relative expression of FOXP3 mRNA is shown as fold expression without addition of TGF- β , which was set as 1. The graph shows real-time PCR results of two independent experiments (Exp.). The frequency of FOXP3⁺ cells and the median fluorescence intensity in brackets are shown in the dot plots ($n = 2$). *E*, Analyses of the effect of the TCR signal strength on FOXP3 mRNA and protein expression. The relative expression of FOXP3 mRNA is depicted as fold expression of cells cultured without addition of anti-CD3 Ab, which was set as 1. Flow cytometry data of one experiment are shown ($n = 3$). The gray line in the histograms represents the control staining. In all experiments, the FOXP3 mRNA data are normalized to the housekeeping gene EF-1 α . Statistical significance (**, $p < 0.01$ and *, $p < 0.05$) is indicated by asterisks.

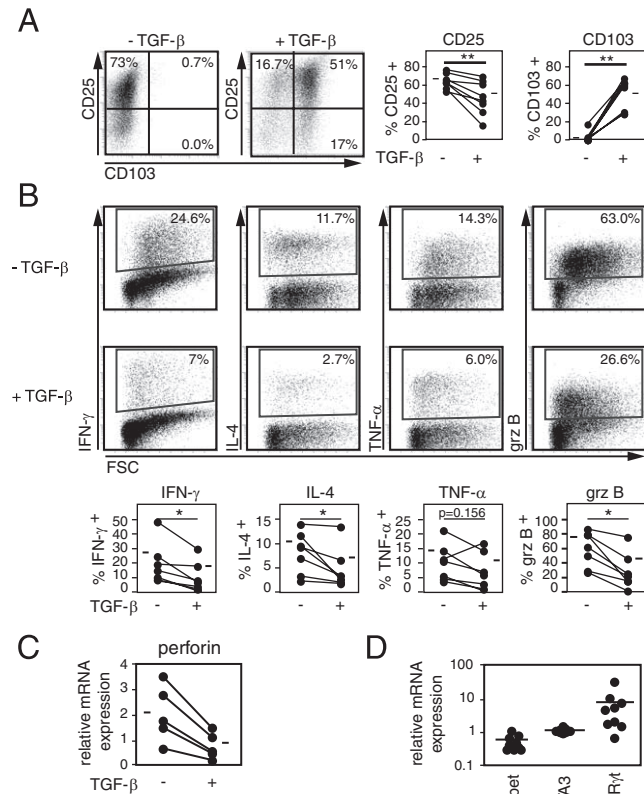


FIGURE 4. Phenotype of CD8⁺ T cells differentiated in vitro in the presence or absence of TGF-β. *A*, The expression of CD103 and CD25 was analyzed by flow cytometry at day 6 of culture. Flow cytometric data of one representative experiment and the graph summarizing the results of the independent experiments plus the mean are shown ($n = 9$). *B*, The cytokine expression was analyzed at day 6 of culture after restimulation with anti-CD3 and anti-CD28 Abs in the presence of brefeldin A by intracellular staining and flow cytometry. The graphs show the frequency of cytokine-expressing cells plus the mean ($n = 7$). *C*, The perforin mRNA expression was analyzed by real-time PCR at day 4 of culture. The graph shows relative expression as fold expression of cells ex vivo, which was set as 1. *D*, The expression of the lineage-specific transcription factors Tbet, GATA3, and RORγt was analyzed at day 4 of culture in the presence of TGF-β by real-time PCR. In each experiment, the expression without TGF-β is set as 1. In all experiments, the mRNA data are normalized to the housekeeping gene EF1α. Statistical significance (**, $p < 0.01$ and *, $p < 0.05$) is indicated by asterisks. FSC, forward scatter.

CD25, cytokines, and T cell lineage-specific transcription factors was analyzed in the presence or absence of TGF-β. Although TGF-β treatment highly enhanced the expression of CD103 from $2.6 \pm 5.7\%$ to $51.4 \pm 16.7\%$, it had only a minor but significant suppressive effect on the expression of CD25 (from $66.9 \pm 9.6\%$ to $50.8 \pm 20.9\%$; Fig. 4A). This effect of TGF-β was concentration dependent and could be reversed by the use of neutralizing anti-TGF-β Ab (supplemental Fig. 3).

Addition of TGF-β to the cultures slightly, but significantly ($p = 0.0156$), decreased the frequency of cells expressing IFN-γ, IL-4, and grzB, while the decrease in TNF-α expression did not reach significance (Fig. 4B). Furthermore, TGF-β treatment decreased perforin mRNA expression (Fig. 4C). Analyses of the expression of the Th1, Th2, and Th17 lineage-specific transcription factors Tbet, GATA3, and RORγt showed that RORγt mRNA expression (13.1 ± 10.7 -fold) was increased in cells differentiated in the presence of TGF-β, whereas Tbet and GATA3 mRNA ex-

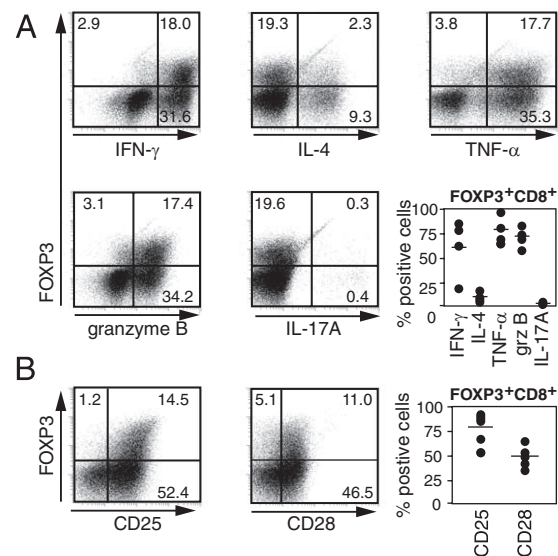


FIGURE 5. Phenotype of in vitro-generated FOXP3⁺CD8⁺ T cells. *A*, The coexpression of FOXP3 with cytokines in the presence of TGF-β was analyzed by flow cytometry at day 6 of culture after restimulation with ionomycin and PMA in the presence of brefeldin A. The graph shows the frequency of cytokine-producing cells among FOXP3⁺CD8⁺ T cells ($n = 4$). *B*, Flow cytometric analyses of one experiment and the frequency of CD25⁺ and CD28⁺ cells among FOXP3⁺CD8⁺ T cells (in the graph) are shown ($n = 6$).

pression was slightly decreased or remained unchanged, respectively (Fig. 4D).

Since TGF-β treatment enhanced FOXP3 expression, while decreasing the frequency of cytokine-expressing cells, we analyzed whether these TGF-β-mediated effects occur in the same cell. Flow cytometric analyses of FOXP3 and intracellular cytokine expression showed that the majority of the in vitro-generated FOXP3⁺CD8⁺ T cells expressed IFN-γ ($62.7 \pm 30.3\%$), TNF-α ($79.5 \pm 13.8\%$), or grzB ($73.0 \pm 11.2\%$). In contrast, FOXP3 and IL-4 expression was mutually exclusive, similar to the phenotype observed for tonsillar FOXP3⁺CD8⁺ T cells (Fig. 5A). IL-17A-expressing cells were almost absent in the cultures and showed no preferential expression of FOXP3 as observed for the IL-17A⁺CD8⁺ T cells from tonsils.

Our analyses had shown that TGF-β slightly decreased the frequency of CD25⁺ cells in the cultures (Fig. 4A); however, FOXP3⁺CD8⁺ T cells were predominantly CD25^{high} ($80.0 \pm 16.0\%$, Fig. 5B). Since down-regulation of CD28 was described to be another characteristic for CD8⁺ Treg cells, the coexpression of CD28 and FOXP3 was analyzed. Similar to the tonsillar FOXP3⁺CD8⁺ T cells, no specific down-regulation of CD28 expression on FOXP3⁺CD8⁺ T cells was observed at day 6 of the cultures in the presence of TGF-β (Fig. 5B).

In vitro-differentiated CD8⁺ T cells suppress the proliferation of CD4⁺ T cells

To analyze whether CD8⁺ T cells, induced to express FOXP3, gain suppressive activity, coculture experiments were performed. The induced FOXP3⁺CD8⁺ T cells (iTreg) were able to suppress the proliferation of autologous CD4⁺ responder cells, visualized by CFSE dilution and depicted as frequency of nondivided responder cells (Fig. 6A). Unstimulated and anti-CD3-stimulated CD4⁺ T cells in the absence of iTreg cells served as control for no and maximal proliferation, respectively. The degree of suppression was dependent on the ratio of iTreg to responder cells (Fig. 6A). In contrast, ex vivo CD8⁺ T cells showed a minor suppressive activity at a ratio of 1:1 only,

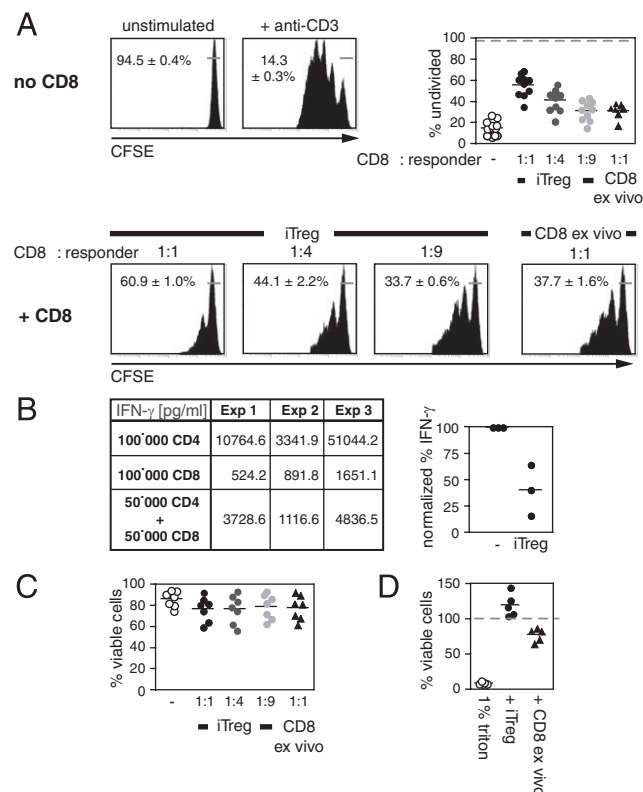


FIGURE 6. In vitro differentiated CD8⁺ T cells suppress the proliferation of CD4⁺ T cells in cocultures. **A**, Proliferation of CFSE-labeled CD4⁺ T cells (responder cells) was analyzed at day 5 of coculture with the induced FOXP3⁺CD8⁺ T cells (iTreg) or freshly isolated CD8⁺ T cells (ex vivo). Histograms were gated on propidium iodide negative CFSE⁺ cells. Results of one representative experiment and the mean ± SD of triplicates are shown. The graph shows the frequency of nondivided responder cells in the cocultures. Each symbol represents the mean of triplicates from independent experiments and the dashed line depicts the frequency of nondivided cells without stimulation ($n = 10$ or $n = 8$). **B**, The IFN- γ secretion of cultured T cells (per 100,000 cells) was determined by a cytometric bead array. The table shows the IFN- γ concentration in the supernatant determined in 3 individual experiments. The graph depicts the IFN- γ secretion normalized to CD4⁺ T cells that were cultured alone (defined as 100%). **C**, The frequency of viable responder cells in cocultures is shown. Each symbol represents the mean of triplicates from independent experiments ($n = 7$). **D**, The cytotoxic activity of CD8⁺ T cells was determined by a cytotoxicity detection kit that is based on the LDH activity released in the supernatant by damaged cells (for details see Material and Methods). CD8⁺ T cells and responder CD4⁺ T cells were cocultured at ratio 1:1 in the presence of anti-CD3 and anti-CD28 for 22h. The viability was calculated based on the ELISA reader values and untreated CD4⁺ T cells were defined as 100% viable cells (dashed line). Each symbol represents the mean of triplicates from independent experiments ($n = 5$).

which was comparable to the suppression observed with culture CD8⁺ T cells at a ratio of 1:9 (triangles in Fig. 6A).

In addition to the proliferation, the IFN- γ secretion of CD4⁺ T cells cultured with or without iTreg cells and of iTreg cells cultured alone was analyzed (Fig. 6B). These experiments showed that iTreg cells suppressed also the secretion of IFN- γ into the supernatant.

To determine the viability of the responder cells in the cocultures, the frequency of propidium iodide-negative CFSE-labeled cells was analyzed by flow cytometry (Fig. 6C). The percentage of viable responder cells slightly decreased when cocultured with iTreg cells or freshly isolated CD8⁺ T cells (ex vivo) from the same donor. When the viability without iTreg cells was defined as

100%, the viable cell frequency decreased to $88.7 \pm 8.1\%$ or $89.6 \pm 6.0\%$ by addition of the iTreg cells or CD8⁺ T cells ex vivo at a ratio 1:1, respectively (Fig. 6C). The inclusion of the grzB inhibitor Z-AAD-CMK in the coculture experiments showed no effect on the suppressive capacity of the CD8⁺ T cells (supplemental Fig. 4). To address killing of the responder CD4⁺ T cells by the CD8⁺ T cells more directly, the cytotoxic activity was determined with an assay that determines LDH activity released from the cytosol of damaged cells into the culture medium. These experiments revealed no cytotoxic effect of CD8⁺ T cells that were in vitro differentiated in the presence of TGF- β (Fig. 6D). In contrast, freshly isolated CD8⁺ T cells slightly decreased the viability of cocultured CD4⁺ T cells. All together, these data argue against cell death as the main mechanism of suppression.

Discussion

The current study discovers a FOXP3⁺CD8⁺ population in human palatine tonsils, which shows a unique phenotype regarding its cytokine profile. In contrast, only a negligible number of FOXP3⁺ cells were detected in human peripheral blood within the CD8⁺ T cell compartment. However, FOXP3⁺CD8⁺ T cells showing suppressive activity could be induced in vitro from naive CD8⁺ T cells by stimulation via the TCR.

The finding that FOXP3⁺CD8⁺ T cells are clearly detected in tonsils, but not in the circulation, suggests a preferential accumulation or local differentiation of FOXP3⁺CD8⁺ T cells in tonsil tissue. The main function of tonsils is to discriminate between potentially infectious pathogens and innocuous Ags, thus leading to the onset of the adaptive immunity or tolerance. It is well established that Treg cells are important players in the maintenance of tolerance to self- as well as to foreign Ags (24). Recently, the importance of the in vivo localization of Treg cells for their suppressive function has been demonstrated (27, 28). Distinct Treg subpopulations are responsible for the suppression of the initiation of immune reactions in lymphoid tissue and the suppression of effector cell function within inflamed tissue. These Treg subpopulations differ in their migration pattern; however, both subsets express high levels of the transcription factor FOXP3.

The tonsillar FOXP3⁺CD8⁺ T cells possess a typical Treg phenotype with high CTLA-4 expression and low CD127 and CD69 expression. However, they are distinct from the described thymic CD25^{high}CD8⁺ Treg cells (6) and the CD8⁺CD28⁻ Treg cells (reviewed in Ref. 29) in that they are predominantly CD25^{low} and CD28^{high}. Surprisingly, tonsillar FOXP3⁺CD8⁺ T cells express high levels of IFN- γ , TNF- α , and IL-17A and thus show a unique cytokine profile. In contrast, the expression of FOXP3 and IL-4 was mutually exclusive, consistent with our previous findings, demonstrating inhibition of Treg differentiation by IL-4 (30). Remarkably, the expression of TNF- α and especially IL-17A was even higher among FOXP3⁺CD8⁺ than FOXP3⁻CD8⁺ T cells. The coexpression of the signature Th17 cytokine IL-17A and the Treg-specific transcription factor FOXP3 within the same cell suggests an IL-17A expression plasticity between Treg and Th17 cells. This concept is supported by recent observations in mice showing the existence of cells coexpressing FOXP3 and the Th17 lineage-specific transcription factor ROR γ t (31–33). These studies showed, however, that FOXP3 inhibits the ROR γ t-induced IL-17A expression, which could be overcome by exogenous IL-6 (31). IL-6 converts natural Treg cells to Th17 cells (32, 34) and also induces coexpression of IL-17A and FOXP3 in a small subset of cells (35). IL-6, which is highly expressed in inflamed tissue, might also be expressed in tonsils and contribute to the development of IL-17⁺FOXP3⁺ T cells. This potential plasticity between Treg and Th17 cells might contribute to effective immune response

or to tolerance induction depending on the signals the cells are exposed to in the tissue.

Currently, it is not clear where and how FOXP3⁺CD8⁺ T cells develop in vivo or how they relate to IL-17A-expressing CD8⁺ T cells. However, it has been shown that TGF- β is required for the differentiation of CD4⁺ Treg as well as Th17 cells. In contrast, the current data demonstrate only an enhancing effect of TGF- β on FOXP3 expression, while stimulation via the TCR was sufficient to induce FOXP3 expression in vitro. The induced FOXP3⁺CD8⁺ T cells show a similar phenotype to the tonsillar FOXP3⁺CD8⁺ population with regard to expression of IFN- γ , IL-4, TNF- α , and grzB. However, FOXP3⁺CD8⁺ T cells generated in vitro in the presence of TGF- β express, in contrast to their counterparts from tonsils, only very low levels of IL-17A, even though ROR γ t expression was induced in CD8⁺ T cells by the TGF- β treatment. The differences in IL-17A expression between the tonsil and the in vitro-generated FOXP3⁺CD8⁺ T cells might be due to factors like IL-6 that are present within the tonsil tissue but not in our in vitro system.

Even though the in vitro-differentiated CD8⁺ T cells expressed proinflammatory cytokines, they gained suppressive activity and suppressed the proliferation of cocultured responder cells. The plasticity in IL-17A regulation and its epigenetic control requires further investigation and could be critical in inflammatory immune regulation. The current findings on the divide of inflammation and immune tolerance are important for future design and safety of immune-modulating therapies.

Disclosures

The authors have no financial conflict of interest.

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Supplementary Figure legend:

Supplementary Figure 1: Cytokine expression of tonsillar CD8⁺ T cells. Tonsil cells were re-stimulation with PMA and ionomycin in the presence of brefeldin A and the cytokine expression was analyzed by intracellular staining and flow cytometry. The frequency of cytokine⁻FOXP3⁺ and cytokine⁺FOXP3⁺ cells among CD8⁺CD3⁺ T cells is shown for each analyzed cytokine in a separate graph. B, The graph shows the frequency of FOXP3⁺ cells among IL-17A⁻ and IL-17A⁺ CD8⁺ T cells analyzed by flow cytometry (n=6). C, The graph shows the frequency of cytokine⁺ cells among FOXP3⁻ and FOXP3⁺ CD8⁺ T cells (n=6). Statistical significance (* p < 0.05) is indicated by asterisks. grz B: granzyme B

Supplementary Figure 2: Kinetics of FOXP3 expression in CD8⁺ T cells after stimulation *in vitro*. Naive CD8⁺ T cells were stimulated with anti-CD3 and anti-CD28 antibodies in the presence of TGF-β for the indicated time and the FOXP3 expression analyzed on mRNA and protein level. A, The relative expression of FOXP3 mRNA is depicted as fold expression *ex vivo*, which was set as 1 (n=8). B, Flow cytometry analyses for FOXP3 and CD25 of one representative experiment out of 4 are shown.

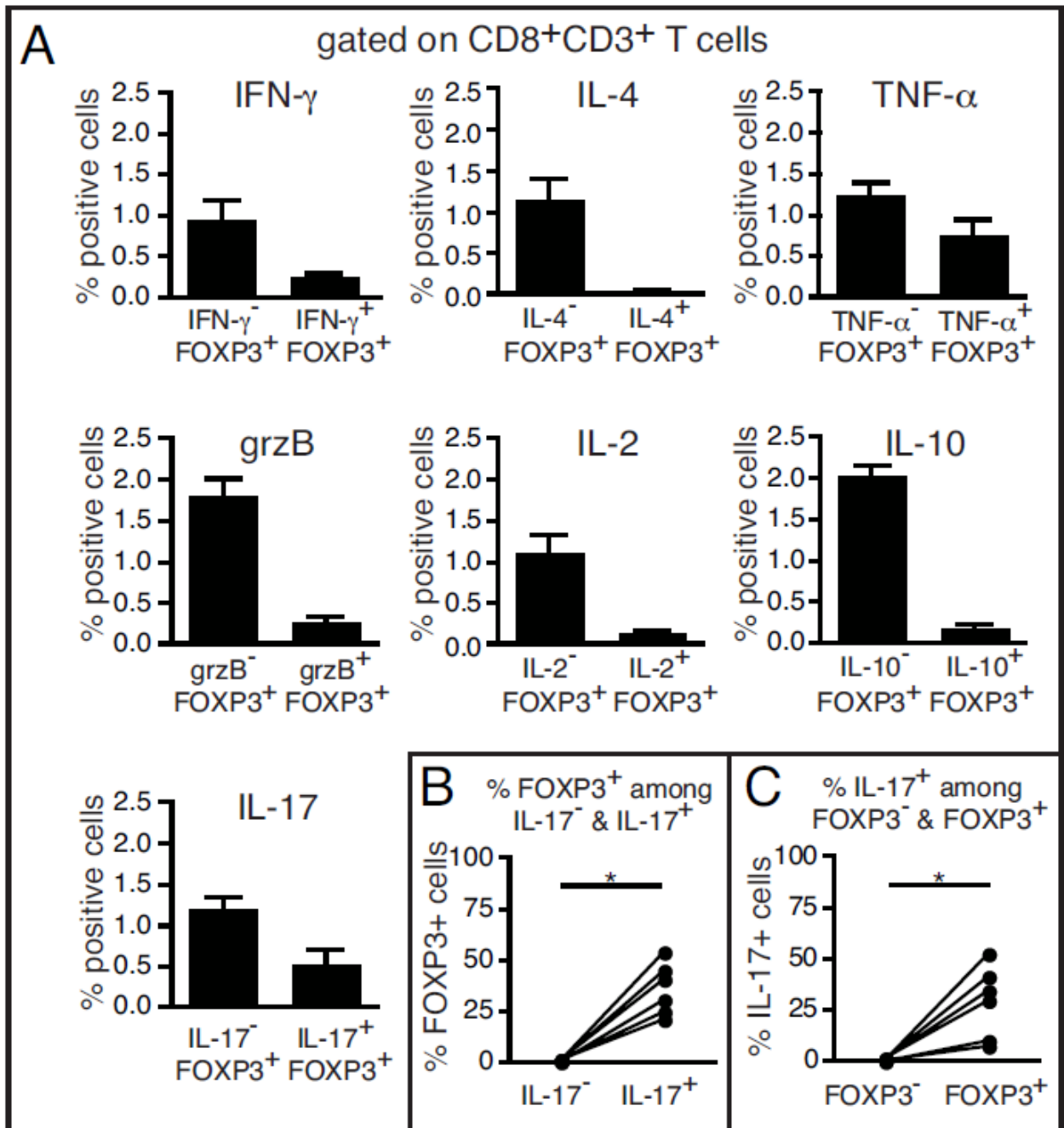
Supplementary Figure 3: Effect of TGF-β on the expression of CD103, CD25 and FOXP3. A, The expression of CD103 and FOXP3 was analyzed by flow cytometry at day 6 of culture in the absence of TGF-β, in the presence of 5 ng/ml TGF-β and in the presence of TGF-β plus additional anti-TGF-β antibody (2.5 μg/ml). Flow cytometric data of one representative experiment and the graph shows the results of the independent experiments plus the mean frequency are shown (n=4 or 6). B, The

26 CD103 and CD25 expression was analyzed by flow cytometry at day 6 of culture at
 27 different concentrations of exogenous TGF- β . The flow cytometric analysis of one
 28 representative experiment is shown and the graph shows the results of two
 29 independent experiments.

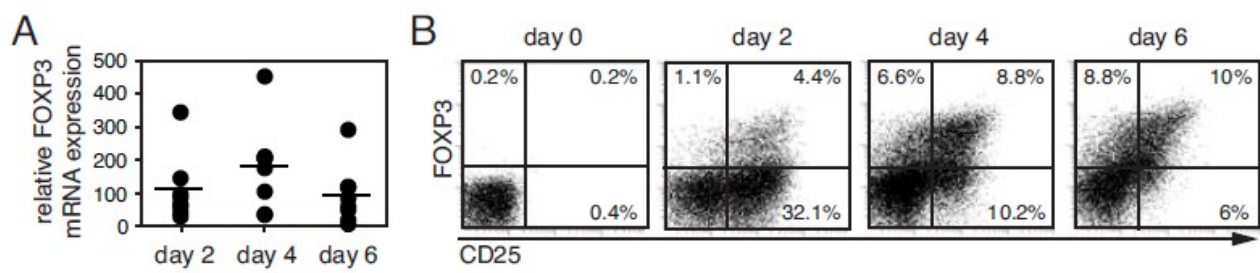
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31 **Supplementary Figure 4:** Inhibition on granzyme B activity has no impact on iTreg
 32 mediated suppression. Proliferation of CFSE-labelled CD4⁺ T cells (responder cells)
 33 was analyzed at day 5 of co-culture without or with induced FOXP3⁺CD8⁺ T cells
 34 (iTreg) in the presence or absence of the granzyme B inhibitor Z-AAD-CMK.
 35 Histograms were gated on propidium iodide negative CFSE⁺ cells. Results of one
 36 representative experiment and the mean $\pm$ SD of triplicates are shown (n=4). The
 37 percentage of non-divided responder cells is shown in the graph.

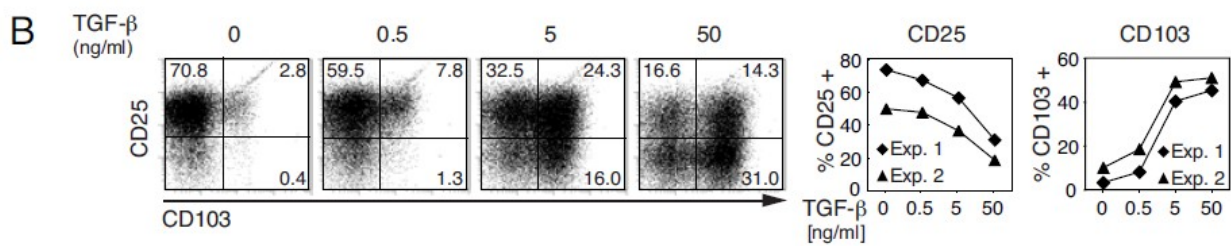
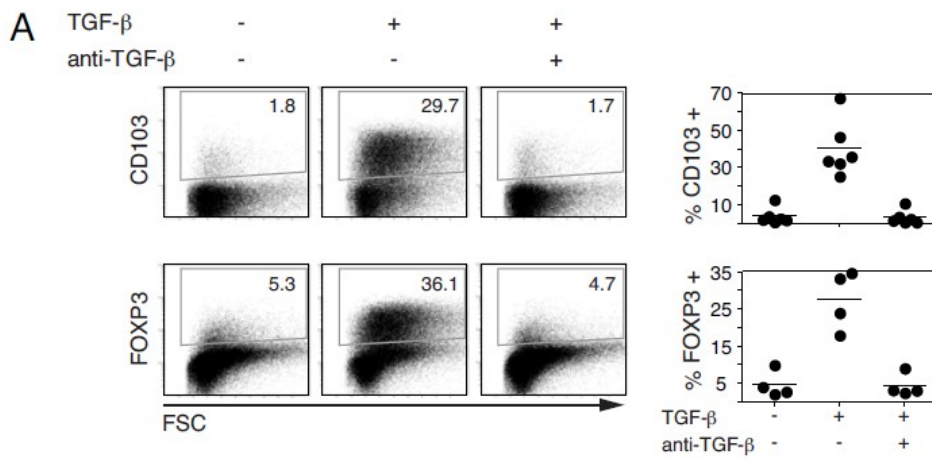
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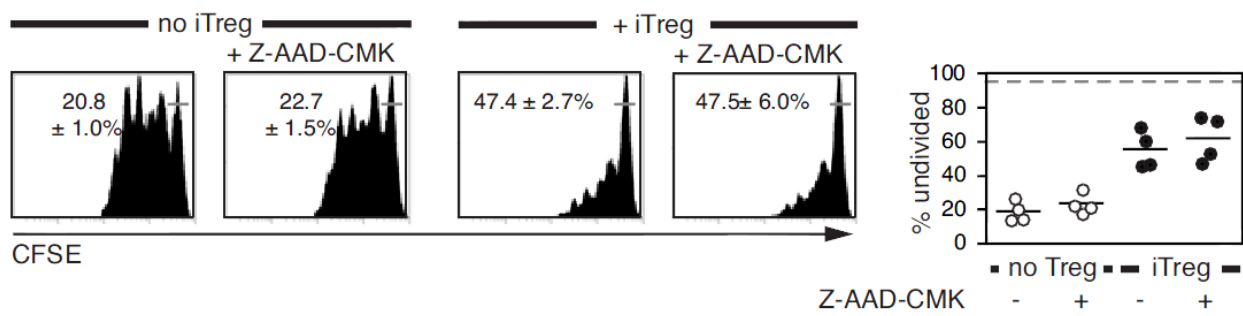
Suppl. Figure 1



Suppl. figure 2



Suppl. figure 3



Suppl. figure 4