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Dominant Th2 Differentiation of Human Regulatory T Cells upon Loss of FOXP3 Expression

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CD4+CD25+FOXP3+ regulatory T cells (Treg) are pivotal for peripheral self-tolerance. They prevent immune responses to auto- and alloantigens and are thus under close scrutiny as cellular therapeutics for autoimmune diseases and the prevention or treatment of alloresponses after organ or stem cell transplantation. We previously showed that human Treg with a memory cell phenotype, but not those with a naive phenotype, rapidly downregulate expression of the lineage-defining transcription factor FOXP3 upon in vitro expansion. We now compared the transcriptomes of stable FOXP3+ Treg and converted FOXP3− ex-Treg by applying a newly developed intranuclear staining protocol that permits the isolation of intact mRNA from fixed, permeabilized, and FACS-purified cell populations. Whole-genome microarray analysis revealed strong and selective upregulation of Th2 signature genes, including GATA-3, IL-4, IL-5, and IL-13, upon downregulation of FOXP3. Th2 differentiation of converted FOXP3− ex-Treg occurred even under nonpolarizing conditions and could not be prevented by IL-4 signaling blockade. Thus, our studies identify Th2 differentiation as the default developmental program of human Treg after downregulation of FOXP3. *The Journal of Immunology*, 2012, 188: 1275–1282.

Natural regulatory T cells (Treg) are indispensable for the maintenance of dominant self-tolerance and can suppress the activation, proliferation, and effector function of a wide range of immune cells, including CD4+ and CD8+ T cells, NK, NK T cells, B cells, and APC. They are thymus-derived and characterized by the expression of CD4, CD25, and the transcription factor FOXP3 (1). FOXP3 is pivotal for the development and function of Treg, and loss-of-function mutations of FOXP3 cause lethal autoimmune syndromes in mice and man (2, 3). During Treg development and in mature peripheral Treg, FOXP3 represses many inflammation-associated genes but also positively induces a gene expression profile that supports Treg function (4, 5). This Treg profile seems to depend on the strength and stability of FOXP3 expression, as Treg function is partially lost in genetically modified mice that express only low FOXP3 levels (6). The stability of FOXP3 expression is in parts regulated by epigenetic mechanisms, as shown by the differential DNA methylation pattern of the FOXP3 locus and differential histone modifications in Treg and conventional CD4+ T cells (7–11). Recent studies employing conditional Foxp3 knockout mice revealed that peripherally induced suppressor cell populations do not compensate for the lack of Treg (12, 13). Thus, thymus-derived Treg are crucial for the preservation of peripheral tolerance, and their adoptive transfer is a promising strategy for the treatment of inflammatory bowel disease (14), autoimmunity (9, 15), and the prevention of alloresponses after solid organ (16) or stem cell transplantation (17–19). For such applications, we previously described culture methods that permit the 100–1000-fold expansion of human Treg in vitro within 2 to 3 wk (20, 21). Yet, we and others (22) reported that Treg selected on the basis of a CD4+CD25(high) CD127(low/neg) phenotype were heterogeneous with respect to the frequency of FOXP3+ cells after in vitro expansion, and we confirmed the downregulation of FOXP3 in Treg clones. The loss of FOXP3 was almost exclusively confined to CD45RA− memory-type Treg, whereas CD45RA+ naive Treg homogeneously maintained FOXP3 expression even after 3 wk in culture (9, 20). Based on these findings, we suggested selecting CD45RA+ Treg for the generation of Treg products for clinical trials, whereas the fate of CD45RA− Treg after in vitro stimulation required further clarification. For this purpose, we now developed new methods that permit the isolation of intact mRNA from fixed, permeabilized, and FOXP3-stained, FACS-sorted cells to compare the differential gene expression profiles in converted versus stable Treg using whole-genome microarrays. We found that Treg rapidly and strongly upregulate Th2 genes upon loss of FOXP3 expression. These findings were confirmed on a protein level as converted Treg secrete high amounts of IL-4, IL-5, and IL-13, but hardly any Th1 or Th17 cytokines. Thus, using new technologies that permit the examination of human Treg with the same accuracy as in murine Foxp3-reporter models, we now demonstrate the dominant conversion of human Treg into Th2 cells upon in vitro stimulation. These findings are highly relevant for researchers planning adoptive cell therapies with in vitro-expanded Treg.
Materials and Methods

Isolation and cultivation of human Treg
PBMC were isolated from leukapheresis products of healthy volunteers (approved by the ethics committee and after their informed consent) by density gradient centrifugation. CD4+CD25highCD45RA− naïve and CD4+ CD25highCD45RA+ memory Treg were purified by FACS (BD FACSaria; BD Biosciences, Heidelberg, Germany) from MACS (Miltenyi Biotec, Bergisch Gladbach, Germany) pre-enriched CD25+ cells. Treg were cultured for 11 d on huFcγR+ murine feeder cells (L929) with anti-CD3 and anti-CD28 Abs in the presence of IL-2 (300 U/ml) and rested for an additional 3 to 4 d in medium with IL-2, as described before (20, 23). For inhibition of IL-4 signaling, anti-IL-4 (clone MP4-25D2, 1 μg/ml; eBioscience) and 20 μg/ml anti-human phospho-STAT6 Abs (Tyr641, #9361; Cell Signaling Technology, Frankfurt, Germany) or anti-human phospho-STAT6 Abs (Tyr317, #9361; Cell Signaling Technology) at 4°C overnight. Blots were washed and incubated with alkaline phosphatase-conjugated goat anti-rabbit Ab (Dako, Cytomation, Hamburg, Germany). Bands were visualized with ECL solution on Hyperfilm, scanned using a Molecular Dynamics personal densitometer SI, and quantified with ImageQuant 5.2 (all from GE Healthcare).

Surface staining
PBMC were isolated from leukapheresis products of healthy volunteers by density gradient centrifugation. CD4+CD25highCD45RA− naïve and CD4+ CD25highCD45RA+ memory Treg were purified by FACS (BD FACSaria; BD Biosciences, Heidelberg, Germany) from MACS (Miltenyi Biotec, Bergisch Gladbach, Germany) pre-enriched CD25+ cells. Treg were cultured for 11 d on huFcγR+ murine feeder cells (L929) with anti-CD3 and anti-CD28 Abs in the presence of IL-2 (300 U/ml) and rested for an additional 3 to 4 d in medium with IL-2, as described before (20, 23). For inhibition of IL-4 signaling, anti-IL-4 (clone MP4-25D2, 1 μg/ml; eBioscience, San Diego, CA), anti–IL-4R (clone 25463, 500 ng/ml; R&D Systems, Minneapolis, MN), or both Abs were added during expansion and resting.

Intracellular FOXP3 staining for subsequent RNA extraction (ethanol/trypotone method)
Surface staining of CD4 (FITC-conjugate; BD Biosciences) was carried out in PBS/2% FCS. For FOXP3 staining and subsequent RNA extraction, PBS with 2% tryptone (Roht, Karlsruhe, Germany) and 0.1% diethyl pyrocarbonate (DEPC; Roth) was used. The buffer was autoclaved, cooled to 4°C, and used for all washing and incubation steps throughout the procedure unless otherwise stated. Following surface staining, cells were washed once and blocked in ice-cold 70% ethanol (up to 7 × 10⁶ cells in 2 ml), and fixed for 15 min at −20°C, then washed twice and resuspended in 1 ml tryptone/DEPC buffer containing 20 μl rat serum (eBioscience) and 20 μl recombinant RNasin RNase inhibitor (20–40 U/μl; Promega, Madison, WI). After 5 min incubation at 4°C, 50 μl anti-human FOXP3 (allophycocyanin conjugate, clone PCH101; eBioscience) was added, and cells were incubated for another 25 min at 4°C in the dark. Cells were washed, resuspended at 1 × 10⁷ cells/ml, and FACS-sorted into tryptone/DEPC buffer. RNA was extracted immediately and its integrity checked with the RNA 6000 Nano Kit on an Agilent Bioanalyzer (Agilent Technologies, Boxböingen, Germany).

Transcription factor and intracellular cytokine staining
Cells were stimulated for 5 h with PMA (20 ng/ml)/ionomycin (1 μM) in the presence of GolgiStop (BD Biosciences) and stained using the FOXP3 staining buffer set (eBioscience) and the following anti-human Abs: FOXP3 (PE-, eFluor 450-, or allophycocyanin-conjugated; clone PCH101), IL-4 (allophycocyanin- or AF488-conjugated; clone 8D4-8), IL-5 (PE-conjugated; clone TRFK5), IL-13 (FITC-conjugated; clone PVM13-1), IL-17A (FITC- or allophycocyanin-conjugated; clone eBio64DE1C7) (all from eBioscience), IFN-γ (FITC-, PE-, or allophycocyanin-conjugated; clone B27; BD Biosciences), IL-10 (AF488- or allophycocyanin-conjugated, clone 10H4; BD Biosciences), anti-mouse/human GATA-3 (PE-conjugated; clone TWAJ), and anti-mouse/human T-bet (AF647-conjugated; clone eBio4B10), both from eBioscience. Data were acquired on a BD LSR II or FACSCalibur (BD Biosciences) and analyzed with FlowJo software (Tree Star, Ashland, OR).

RNA extraction
RNA was isolated from FACS-sorted cells using the RNeasy Micro Kit (Qiagen, Hilden, Germany) or TRIzol Reagent (Invitrogen, Darmstadt, Germany). When RNA was extracted from <1 × 10⁶ cells, glycen (Roche, Mannheim, Germany) was added during the TRIzol procedure.

Microarray analysis of gene expression
RNA preparations from FOXP3-sorted CD4+CD25highCD45RA− Treg from five donors were analyzed using Whole Human Genome Oligo Microarrays (Agilent Technologies). Labeling and hybridization were performed using the Agilent Gene Expression system according to the manufacturer’s instructions (Agilent Technologies). In brief, 50–200 ng high-quality RNA were amplified and cyanine 3-CTP labeled with the One Color Low Input Quick Amp Labeling Kit (Agilent Technologies). Six hundred nanograms labeled cRNA was fragmented and hybridized on the Whole Human Genome Expression Array G4851A (8 × 60K; Agilent Technologies). Images were scanned using a DNA microarray scanner, processed using Feature Extraction Software, and further analyzed using GeneSpring GX (all from Agilent Technologies). Fluorescence signals were normalized to the 75th percentile and baseline corrected to the median of all samples. Features were discarded that did not have a minimum raw expression value of 40 in at least 3 out of 10 samples. Different microarray probes covering the same gene were combined using the gene-level technology of the GeneSpring GX software (Agilent Technologies). Expression data for the donor-matched comparisons of variance (Supplemental Fig. 2) were percentile normalized as above, but not baseline transformed. Microarray data are available from the National Center for Biotechnology Information/Gene Expression Omnibus repository (accession number GSE26190; http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE26190).

Quantitative RT-PCR
RNA was transcribed into cDNA with Reverse Transcriptase (Promega) and analyzed on an Eppendorf Realplex4 S Cycler (Eppendorf International). mRNA expression levels were normalized to β2-microglobulin or 18S RNA. Primer sequences are listed in Supplemental Table I.

Western blot analysis
For preparation of whole-cell extracts, ~1 × 10⁶ cells were washed with PBS, pelleted, dissolved in 100 μl 2% SDS sample buffer with complete protease and phosphate inhibitors (Roche) per 5 × 10⁶ cells, heated at 95°C for 10 min, and vortexed for 1 min. For Western blots, equivalents of 0.5–0.75 × 10⁶ cells were separated by SDSPAGE and blotted on polyvinylidene difluoride membranes, followed by blocking (1 h at room temperature with TBS/5% milk powder/0.1% Tween 20) and probing with polyclonal rabbit anti-human STAT6 (9362; Cell Signaling Technology, Frankfurt, Germany) or anti-human phospho-STAT6 Abs (Tyre12, #9361; Cell Signaling Technology) at 4°C overnight. Blots were washed and incubated with alkaline phosphatase-conjugated goat anti-rabbit Ab (Dako, Cytomation, Hamburg, Germany). Bands were visualized with ECL solution on Hyperfilm, scanned using a Molecular Dynamics personal densitometer SI, and quantified with ImageQuant 5.2 (all from GE Healthcare).

Cytokine detection in culture supernatants
Supernatants were collected on day 11 of Treg cultures. Concentrations of IL-4, IL-5, IL-13, IL-10, IL-17, and IFN-γ were determined using Cytokine Bead Array Flex-sets (BD Biosciences) according to the manufacturer’s instructions.

Statistical analysis
Statistical significance was determined by paired or unpaired two-tailed Student t test, as indicated in the figure legends. A p value ≤0.05 was considered significant.

Results
Isolation of intact RNA from FACS-sorted cells after intracellular FOXP3 staining
There is increasing evidence for plasticity within the Treg lineage, which includes the loss of FOXP3 and the conversion of Treg into potentially proinflammatory Th cell subsets (24–27). The study of gene expression patterns in FOXP3-losing Treg could extend our understanding of this plasticity and reveal the underlying molecular mechanisms. However, paraformaldehyde (PFA) contained in commercially available FOXP3 staining kits induces conformational changes and covalent as well as noncovalent cross-links in nucleic acids and proteins and thus impedes the subsequent extraction of intact RNA. We therefore established an alternative FOXP3 staining method using 70% ethanol for fixation and permeabilization that does not induce such cross-links and thus allows the extraction of intact high-quality RNA. Staining for FOXP3 and subsequent flow cytometric sorting was carried out in tryptone- and RNase inhibitor-containing PBS. Both additives were essential for maintaining RNA integrity during the staining and sorting procedure. The frequencies of FOXP3+ cells among in vitro-expanded CD45RA− Treg detected with this protocol were comparable to those obtained with a commercial FOXP3 staining kit (Fig. 1A; divergence ranged from −10.6 to 5.7%; n = 4). FACS-separated populations routinely showed >95% purity upon reanalysis (96.9 ± 1.2% for FOXP3+ and 98.1 ± 0.9% for FOXP3− cells, respectively, n = 5; see also Supplemental Fig. 1). RNA extracted from sorted cells displayed little or no signs of
Likewise, key transcription factors for Th2 differentiation, namely kit (stained for FOXP3 using either a commercially available FOXP3 staining kit (left panel) or the ethanol-tryptone method (right panel). Plots are representative of n = 4 independent experiments. Numbers indicate percentages of cells. B, Electropherograms of RNA extracted from expanded human CD45RA− Treg. Cells were either stained for FOXP3 using the ethanol/tryptone method and separated by FACS (left panel, FOXP3+ population; representative of n = 14 independent RNA preparations) or remained unfixed before RNA extraction (right panel, representative of n = 5 independent preparations). RIN, RNA integrity number.

degradation (Fig. 1B), and mean RNA integrity numbers (28) were 7.9 (range: 7.3–9.0; n = 14) in fixed samples and 9.4 (range: 9.2–9.5; n = 5) in unfixed controls. RNA yield and quality depended on the time span between fixation and cell lysis for RNA extraction; best results were obtained by keeping preparation times short and samples cold and RNase-free. Thus, this PFA-free FOXP3 staining protocol (referred to as the ethanol/tryptone method) allowed the reliable extraction of largely intact RNA from intranuclearly stained and FACS-sorted cells.

In vitro-expanded CD45RA− Treg show a Th2 gene expression signature upon loss of FOXP3 expression

We previously showed that FOXP3 expression is primarily lost in CD45RA− memory Treg after in vitro stimulation (20, 22). To analyze the fate of FOXP3-losing memory Treg on the level of gene expression, FACS-sorted CD45RA−CD25hiCD45RA− Treg were in vitro expanded and subsequently sorted into FOXP3+ and FOXP3− populations applying the ethanol/tryptone method. RNA was extracted and analyzed using whole-genome expression microarrays. Combined results of five independent experiments are presented as heat maps for selected gene classes in Fig. 2A. Detailed mRNA expression levels of individual cell cultures can be found in Supplemental Fig. 2.

As expected, we observed significantly higher mRNA levels of Treg signature genes (e.g., FOXP3, CTLA4, and IL2RA) in the FOXP3+ population as compared with the corresponding FOXP3− population. Yet, surprisingly, a dramatic increase of Th2 cytokine mRNA (IL4, IL5, and IL13) was detected in FOXP3− cells, but no significant difference between the two subpopulations with respect to other Th cell lineage- and inflammation-associated cytokine genes (IFNG, IL6, IL9, IL10, IL12, IL17, IL21, IL23, and IL24). Likewise, key transcription factors for Th2 differentiation, namely GATA3 and GFI1, showed a significantly higher expression in FOXP3− cells, whereas the genes RORC and STAT1, responsible for Th17 and Th1 lineage commitment, respectively, were suppressed. In addition, FOXP3− cells highly expressed GPR44, also known as CRTH2, which encodes the Th2 cell-specific G protein-coupled receptor CD294 (29) (Fig. 2A). Thus, the FOXP3− population showed a gene signature dominated by Th2 lineage-associated genes including cytokines, transcription factors, signaling molecules, and cell-surface receptors.
Because STAT6 is critically involved in IL-4–induced Th2 development (30), we also asked whether the expression of typical STAT6 target genes (including *LTB*, *SOCS1*, and *IRF8*) would be altered during Treg conversion. Interestingly, their expression levels were not significantly different between FOXP3+ and FOXP3− populations, nor were the levels of MAF encoding a *trans-activator* of the IL-4 gene (31).

Microarray data were validated at the single gene level using quantitative RT-PCR (qRT-PCR) (Fig. 2B). As controls, RNA isolated from in vitro-expanded, FOXP3-stained, and sorted CD45RA+ Treg as well as from expanded bulk CD45RA− Treg without prior fixation were analyzed (Fig. 2B). As expected, FOXP3+ cells from cultures of CD45RA− and CD45RA+ Treg showed comparably high FOXP3 and low *GATA3* mRNA expression. When total CD45RA− unfixed cells were compared with ethanol/tryptone-fixed subpopulations, they mainly showed intermediate mRNA expression, confirming the reliability of the RNA preparation procedure (Fig. 2B). Taken together, the gene expression profile of FOXP3+ cells developing from initially FOXP3*CD45RA− Treg upon in vitro stimulation indicated Th2 differentiation.

**FOXP3− cells from CD45RA− Treg cultures predominantly produce Th2 cytokines**

To correlate the mRNA expression data with cytokine production, freshly isolated as well as in vitro-expanded CD45RA− and CD45RA+ Treg were stained for FOXP3 and Th cell lineage-defining cytokines (Fig. 3). In freshly isolated cells, the expression frequency for one or more Th cell lineage signature cytokines (IL-4, IL-10, IL-13, IFN-γ, and IL-17) never exceeded 5%. In contrast, after in vitro expansion, ~30% of the cells within CD45RA− Treg cultures started to produce one or more of the Th2 cytokines IL-4, IL-5, and IL-13 (Fig. 3A). Although we also detected IFN-γ+ and/or IL-17+ cells in these cultures (as previously described by us and others (22, 25, 32, 33)), their frequencies were significantly lower than those of the Th2 cytokine producers (Fig. 3A). Th2 cytokine production was largely confined to the FOXP3− subpopulation, whereas IFN-γ, IL-10, and IL-17 producers were almost equally distributed between FOXP3+ and FOXP3− cells (Fig. 3B). Confirming our previous findings, CD45RA+ Treg remained uniformly FOXP3+ and comprised hardly any cytokine-producing cells before and after in vitro expansion (20, 22 and data not shown).

**Th2 cytokine production in expanded CD45RA− Treg is associated with high GATA3 expression**

GATA3, the lineage-defining transcription factor for Th2 cells, was highly expressed in CD45RA− Treg that had downregulated FOXP3 upon in vitro stimulation (Fig. 2). To analyze whether GATA3 expression was restricted to Th2 cytokine producers, in vitro expanded CD45RA− Treg were simultaneously stained for cytokine production and the presence of GATA3, GATA-3 expression was significantly higher in IL-4–producing cells when compared with IL-17 and IFN-γ producers (Fig. 4).

**Inhibition of IL-4 signaling does not block Th2 differentiation in CD45RA− Treg**

Th2 differentiation from naive conventional T cells (Tconv) is mainly driven by IL-4 (30). Because CD45RA− Treg secreted IL-4 and other Th2 cytokines under the nonpolarizing culture conditions applied in this study, we next asked to which extent endogenous IL-4 supported the conversion of initially FOXP3+ Treg into cells of the Th2 lineage. To block IL-4 signaling, CD45RA− Treg were cultured in the presence of anti–IL-4 Abs, anti–IL-4R Abs, or both during the entire expansion period. The treatment effectively prevented phosphorylation of the transcription factor STAT6, a crucial step in IL-4 signaling and Th2 induction (Fig. 5A, 5B). However, despite profound inhibition of STAT6 activation, the frequencies of cytokine-secreting cells in CD45RA− Treg cultures were only marginally altered, with a slight but not statistically significant reduction of Th2 cytokine producers and a minor, statistically insignificant increase in IFN-γ– or IL-17–
secretion of IL-4, IL-5, and IL-13 (44–47). T-bet and GATA-3 reciprocally repress each other (48–50), and FOXP3 in Treg is supposed to prohibit expression of both of these lineage-defining transcription factors (51). Nevertheless, coexpression of Foxp3 and T-bet has been observed in activated murine Treg under type 1 inflammatory conditions, which permits their migration to inflammatory sites, but does not induce Th1 cytokine secretion (52). Our comparative analysis of human CD45RA+ memory Treg-derived FOXP3+ and FOXP3− subpopulations now revealed that typical marker genes of Treg, such as CTLA4, LAG3, IL2RA, LGALS3, and LRRC32, are downregulated upon loss of FOXP3 expression, whereas IL7R and CD40LG, usually not expressed by Treg, are upregulated. More strikingly, the cells losing FOXP3 upon in vitro stimulation under nonpolarizing conditions converted into Th2-like cells as they started to overexpress a number of Th2-specific genes, such as the signature cytokines IL4, IL5, and IL13, the transcription factors GATA3 and GF1I, and the surface receptor GPR44. In contrast, expression of Th1 or Th17 signature genes, such as TBX21 and RORC, was even lower in FOXP3− as compared with FOXP3+ cells isolated from the same cultures (Fig. 2). The conversion into Th2 cells upon loss of FOXP3 expression dramatically exceeded the previously described differentiation into Th1 or Th17 cells (22, 25, 32, 33). Both the high frequency of Th2 cytokine producers among FOXP3+ CD45RA+ Treg and the substantial concentrations of Th2 cytokines in CD45RA+ Treg culture supernatants corroborated these results. In murine studies, induction of cytokine secretion in former Treg has also been observed, either upon Foxp3 deletion (53) or upon expression of a nonfunctional Foxp3 protein (54). Whereas Rudensky and coworkers (53) found only Th1 cytokines in C57BL/6 animals, Chatila’s group (54) also observed a dramatic increase in IL-4 secretion after Foxp3 downregulation in BALB/c mice. Thus, a genetic predisposition may influence the conversion of Treg. Interestingly, however, artificial attenuation of Foxp3 expression has been described to induce predominantly a Th2 phenotype in Foxp3low-expressing cells even in C57BL/6 mice (6), and this conversion seems to be an intrinsic developmental program that occurs independently of IL4/STAT6 signaling, yet still requires GATA-3 expression (55). In Th2 differentiation of naive Tconv, IL-4 causes STAT6 phosphorylation by JAK1 and JAK3 kinases, leading to STAT6 dimerization and nuclear translocation that in turn supports IL4 and IL4R as well as GATA3 transcription (30). Blocking IL-4/STAT6 signaling in human Treg by the addition of anti-IL-4 Abs, anti–IL-4R Abs, or both during culture resulted in a dramatic reduction of STAT6 phosphorylation, as expected. Yet, the frequencies of IL-4+, IL-5−, and IL-13−producing cells and the amount of secreted Th2 cytokines were only marginally diminished, suggesting that autocrine IL-4/STAT6 signaling is not the main cause of Th2 conversion. To which extent IL-4–independent signaling cascades are involved in Th2 conversion of human Treg requires further clarification. For murine T cells, it has previously been shown that

Discussion

There is increasing evidence that Treg lineage commitment may not be irreversible, as downregulation of FOXP3 and consecutive expression of Th1 and Th2 cytokines by Treg has been observed by several groups (25, 27, 32, 34–36). We showed that, in particular, human CD45RA− memory-type Treg lose FOXP3 expression upon in vitro stimulation, whereas CD45RA− naive Treg maintain high FOXP3 levels even after extended culture periods. Importantly, both FACS-purified starting populations contained equivalent frequencies of FOXP3+ cells before in vitro stimulation (>93%), and both showed equally homogeneous demethylation at the Treg-specific demethylated region, a sensitive epigenetic mark for the identification of Treg and currently the most reliable marker for the exclusion of contaminations by induced or non-Treg (7, 10, 37). Furthermore, we ultimately proved loss of FOXP3 expression by the serial examination of human Treg clones in our previous studies (22). Because Treg instability may be detrimental for future adoptive cell therapies [or those already underway at some centers (17, 38)], we now examined the fate of Treg that lose FOXP3 upon in vitro stimulation. For this purpose, we developed a cell fixation and permeabilization protocol that allowed the isolation of intact mRNA from FOXP3-stained and FACS-sorted cells. In contrast to previously described methods (39), the isolated RNA was not degraded but of high quality and suited for sensitive downstream assays, such as qRT-PCR and microarray-based whole-genome expression analyses. The use of ethanol for fixation prevented the cross-linking of nucleic acids and proteins (40) that is caused by PFA contained in commercial staining kits. Furthermore, our method improves previously described RNA isolation protocols developed by Esser and colleagues (41) because it permits the simultaneous detection of fluorescently labeled surface markers. Because this technology is applicable to a wide variety of transcription factors and cell types, it may advance many areas in cellular biology.

Classical differentiation of naive CD4+ T cells into Th1 or Th2 cells is mainly induced by the local cytokine milieu as well as the type of Ag and APC. IFN-γ and IL-12 polarize toward Th1 differentiation via the transcription factors STAT4, STAT1, and T-bet (42, 43). Th2 cells are induced by IL-4 that induces GATA-3, usually in a STAT6-dependent fashion, and the hallmark of Th2 cells is their secretion of IL-4, IL-5, and IL-13 (44–47). T-bet and GATA-3 were stimulated for 5 h with PMA/ionomycin, then stained for GATA-3 and indicated cytokines. The histogram shows GATA-3 expression of IL-4+, IL-17+, or IFN-γ+ cells, respectively. Mean fluorescence intensity for GATA-3 was significantly different between IL-4+ and IFN-γ+ cells (p = 0.046; n = 6; two-tailed unpaired Student t test) as well as IL-4+ and IL-17+ cells (p = 0.009; n = 5; two-tailed unpaired Student t test).
IL-2 combined with CD28 costimulation can induce Th2 differentiation in an IL-4/STAT6-independent but STAT5-dependent manner (56, 57). Because our stimulation conditions provide all required components (CD3, CD28, and high-dose IL-2 stimulation), this pathway may be involved in Treg conversion. However, we reason instead that Th2 conversion of CD45RA\(^{2-}\)Treg represents a developmental program that is imprinted on the cells by repeated contact to (self-) Ag in vivo, as CD45RA+ naive Tregs do not convert although exposed to the same in vitro conditions. In support of this hypothesis, an increased GATA-3 expression in CD45RA\(^{2-}\)Treg (as compared with CD45RA\(^{+}\) Treg and Tconv) was detectable already before in vitro expansion (Supplemental Fig. 3). In mice, Th2 conversion of wild-type Tregs in vivo has been described (55) as well as conversion into IL-4–secreting follicular B Th cells (58), and it remains to be determined whether this is also a frequent event in humans, in whom diminished FOXP3 expression of Treg has been observed in patients with atopic diseases (59).

In summary, by performing, to our knowledge, the first comparative transcriptome analyses of human Treg and their converted

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**FIGURE 5.** Inhibition of IL-4 signaling does not block Th2 differentiation of CD45RA\(^{-}\) Treg during in vitro expansion. CD45RA\(^{-}\) Treg were expanded in the presence or absence of anti-IL-4, anti-IL-4R, or both Abs. A, Phosphorylated STAT6 (pSTAT6) in whole-cell extracts was analyzed by Western blot. Total STAT6 was analyzed as a loading control. Shown is one representative immunostaining (n = 3). B, Western blot band intensities were quantified with a densitometer. Phospho-STAT6 band intensities of unblocked cultures were set to 100%. Values represent means + SD (n = 3; two-tailed unpaired Student t test performed with absolute values). C and D, Cells were stimulated for 5 h with PMA/ionomycin, then stained for FOXP3 and indicated cytokines. Dot plots in C show one representative experiment. Bars in D represent mean percentages + SD of cytokine producers among expanded CD45RA\(^{-}\) Treg. For better comparison of n = 5–9 individual cultures, the numbers of cytokine-producing cells in unblocked cultures were scaled to 100%. Unblocked cultures contained on average 29.1, 15.5, and 26.4% IL-4, IL-5, and IL-13 producers, respectively, and 9.3, 9.9, and 7.1% IL-10, IFN-\(\gamma\), and IL-17 producers, respectively. E, Supernatants from treated or untreated CD45RA\(^{-}\) Treg cultures were analyzed for cytokine content on day 11 of in vitro expansion. Bars represent means + SD of n = 5–9 independent cultures. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 as compared with unblocked cultures; two-tailed unpaired Student t test.
progeny identified and separated on the basis of their FOXP3 expression level, we show that differentiation toward a Th2 phenotype represents the dominant pathway of human Treg upon loss of FOXP3 expression. These findings further elucidate the developmental plasticity of Treg in humans (60) and are thus of high relevance for current as well as future adoptive Treg therapies.

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