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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 allosponses after organ or stem cell transplantation. We previously showed that human Treg with a memory cell phenotype, but not those with a naïve 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.

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^+CD25^{high}CD45RA^− naive and CD4^+ CD25^{high}CD45RA 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 huFcR^− marine feeder cells (L929 cells) 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 MP4-2SD2, 1 μg/ml; eBioscience, San Diego, CA), anti–IL-4R (clone 254G6, 500 ng/ml; R&D Systems, Minneapolis, MN), or both Abs were added during expansion and resting.

Intracellular FOX3 staining for subsequent RNA extraction (ethanol/trypomine method)
Surface staining of CD4 (FITC conjugate; BD Biosciences) was carried out in PBS/2% FCS. For FOX3 staining and subsequent RNA extraction, PBS with 2% trypomine (Roth, 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 with ice-cold 70% ethanol (up to 7 × 10^7 cells in 2 ml) and fixed for 15 min at −20 °C, then washed twice and resuspended in 1 ml trypomine/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 FOX3 (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 × 10^6 cells/ml, and FACS-sorted into trypomine/DEPC buffer. RNA was extracted immediately and its integrity checked with the RNA 6000 Nano Kit on an Agilent Bioanalyzer (Agilent Technologies, Böblingen, 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 FOX3 staining buffer set (eBioscience) and the following anti-human Abs: FOX3 (PE-, eFlour 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 eBio64DEC17) (all from eBioscience), IFN-γ (FITC-, PE-, or allophycocyanin-conjugated; clone B27; BD Biosciences), IL-10 (AF488- or allophycocyanin-conjugated, clone 701; BD Biosciences), anti-IFN-γ (PE-conjugated; clone TR7), anti-IL-10 (AF647-conjugated; clone eBio4B10), and anti-mouse/human T-bet (AF647-conjugated; clone eBio4B10), both from eBioscience. Data were acquired on a BD LSR II or FACS caliber (BD Biosciences) and analyzed by 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 × 10^6 cells, glycogen (Roche, Mannheim, Germany) was added during the TRIzol procedure.

Microarray analysis of gene expression
RNA preparations from FOX3-sorted CD4^+CD25^{high}CD45RA^− 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 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 transformed 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, 1–5 × 10^6 cells were washed with PBS, pelleted, dissolved in 100 μl 2× SDS sample buffer with complete protease and phosphatase inhibitors (Roche) per 5 × 10^6 cells, heated at 95 °C for 10 min, and vortexed for 1 min. For Western blots, equivalents of 0.5–0.75 × 10^6 cells were separated by SDS-PAGE 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 (Ty#15, #9361; Cell Signaling Technology) at 4 °C overnight. Blots were washed and incubated with alkaline phosphatase-conjugated goat anti-rabbit Ab (Dako Cytometry, 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 FOX3 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 formalin did not induce such cross-links and thus allows the extraction of intact RNA. We therefore established an alternative formaldehyde that does not induce such cross-links and thus allows the extraction of intact high-quality RNA. Staining for FOX3 and subsequent flow cytometric sorting was carried out in trypomine- 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 memory Treg 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 divergence ranging from 96.9 ± 2.3% to 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.

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 CD4+CD25highCD45RA− 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. A. De-
Because STAT6 is critically involved in IL-4-induced Th2 development (30), we also asked whether the expression of typical STAT6 target genes (including IL1B, 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 GATA-3. 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-12R 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-
secretory cells (Fig. 5C, 5D). In line with these findings, cytokine concentrations in supernatants of blocked and unblocked cultures showed no significant differences, except for IL-4, because IL-4 neutralization by anti–IL-4 Abs expectedly reduced IL-4 levels, whereas blockade of IL-4R increased IL-4 concentrations, proving that IL-4 is consumed in such cultures (Fig. 5E). Overall, these data indicate that T2 differentiation of CD45RA<sup>−</sup> Treg does not require endogenous IL-4 but seems to represent the default developmental pathway upon loss of FOXP3.

**Discussion**

There is increasing evidence that Treg lineage commitment may not be irreversible, as downregulation of FOXP3 and consecutive expression of T1h and T2 cytokines by Treg has been observed by several groups (25, 27, 32, 34–36). We showed that, in particular, human CD45RA<sup>−</sup> memory-type Treg lose FOXP3 expression upon in vitro stimulation, whereas CD45RA<sup>−</sup> naive Treg maintain high FOXP3 levels even after extended culture periods. Importantly, both FACS-purified starting populations contained equivalent frequencies of FOXP3<sup>+</sup> 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 examine 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<sup>+</sup> 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 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<sup>−</sup> memory Treg-derived FOXP3<sup>+</sup> and FOXP3<sup>−</sup> 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 GFI1, and the surface receptor GPR44. In contrast, expression of Th1 or Th17 signature genes, such as TBX21 and RORC, was even lower in FOXP3<sup>−</sup> as compared with FOXP3<sup>+</sup> 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<sup>−</sup> CD45RA<sup>−</sup> Treg and the substantial concentrations of Th2 cytokines in CD45RA<sup>−</sup> 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 auto- or paracrine 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...
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\(^{-}\) 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\(^{-}\) 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...
progonym 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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