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GPA33: A Marker to Identify Stable Human Regulatory T Cells

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FOXP3-expressing regulatory T (Treg) cells safeguard immunological tolerance. Treg cells can be generated during thymic development (called thymic Treg [tTreg] cells) or derived from mature conventional CD4$^+$ T cells that underwent TGF-β–mediated conversion in the periphery (called peripheral Treg [pTreg] cells). Murine studies have shown that tTreg cells exhibit strong lineage fidelity, whereas pTreg cells can revert into conventional CD4$^+$ T cells. Their stronger lineage commitment makes tTreg cells the safest cells to use in adoptive cell therapy, increasingly used to treat autoimmune and inflammatory disorders. Markers to distinguish human tTreg cells from pTreg cells have, however, not been found. Based on combined proteomic and transcriptomic approaches, we report that the Ig superfamily protein GPA33 is expressed on a subset of human Treg cells. GPA33 is acquired late during tTreg cell development but is not expressed on TGF-β–induced Treg cells. GPA33 identifies Treg cells in human blood that lack the ability to produce effector cytokines (IL-2, IFN-γ, IL-17), regardless of differentiation stage. GPA33^{high} Treg cells universally express the transcription factor Helios that preferentially marks tTreg cells and can robustly and stably be expanded in vitro even without rapamycin. Expanded GPA33^{high} Treg cells are suppressive, unable to produce proinflammatory cytokines, and exhibit the epigenetic modifications of the FOXP3 gene enhancer CNS2, necessary for indelible expression of this critical transcription factor. Our findings thus suggest that GPA33 identifies human tTreg cells and provide a strategy to isolate such cells for safer and more efficacious adoptive cell therapy. ***The Journal of Immunology*****, 2020, 204: 3139–3148.**

Forkhead box protein 3-expressing CD4$^+$ regulatory T (Treg) cells inhibit undesirable immune responses through a variety of mechanisms and thereby limit immunopathology (1). Two lineages of Treg cells have been described, which differ in their developmental origin. So-called thymic Treg (tTreg) cells develop from immature thymocytes in response to recognition of self-antigen (2). Mature conventional CD4$^+$ T cells can, however, also differentiate into Treg cells, known as peripheral Treg (pTreg) cells, in response to recognition of Ags derived from commensal organisms or food (3–5). tTreg and pTreg cells are both needed for full protection from (auto)inflammatory disease. Still, major functional differences between these cell types have not been found, suggesting that their complementarity may stem from their distinct Ag specificities. One important difference between these Treg cell types has, however, been identified. Whereas tTreg cells are thought to be irreversibly committed to the Treg cell lineage, pTreg cells may have the capacity to revert into conventional T (Tconv) cells and can cause pathologic conditions upon adoptive transfer (6–8).

Adoptive cell therapy (ACT) with Treg cells is a powerful method to attenuate inflammation and promote acceptance of organ allografts in mice (9–14). Clinical trials are ongoing to mitigate graft-versus-host disease (15–22), autoimmune diseases like type 1 diabetes mellitus (23, 24), and to prevent rejection of organ grafts (25). Despite encouraging preliminary results, mouse studies suggest that greater efficacy must be possible (24), especially through the use of Treg cells with AgRs specific for peptides presented in the tissues that must be protected (26–34).

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Abbreviations used in this article: ACT, adoptive cell therapy; Ct, cycle threshold; DN, double negative; DP, double positive; eTreg, effector Treg; Fr. III, Fraction 3; HSD, honestly significant difference; iBAQ, intensity-based absolute protein quantification; IM, ionomycin; iTreg, TGF-β–induced Treg; MS, methylation-specific; mTconv, memory Tconv; nTconv, naive Tconv; nTreg, naive Treg; pTreg, peripheral Treg; qPCR, quantitative PCR; RTE, recent thymic emigrant; SP, single positive; Tconv, conventional T; Treg, regulatory T; TSDR, Treg cell–specific demethylated region; tTreg, thymic Treg.

There are several hurdles for ACT with Treg cells (35). First, pure populations of human Treg cells are difficult to obtain. $CD4^+$ Treg cells express CD25 and lack CD127 (36–38), but these markers do not allow rigorous separation between Treg and Tconv cells. To limit the presence of contaminating Tconv cells, $CD4^+CD25^+CD127^-$ cells are often expanded in vitro in the presence of the drug rapamycin. This drug inhibits Tconv cells, whereas Treg cells are relatively tolerant to it (39–42). Nonetheless, rapamycin does reduce the Treg cell proliferative capacity and may affect their function (43–45). Moreover, rapamycin induces conversion of Tconv into Treg cells, which, however, readily lose their Treg cell properties upon withdrawal of this drug (44). Finally, rapamycin presumably does not exclude pTreg cells from populations of Treg cells used for ACT. After adoptive transfer, unstable (rapamycin-induced or peripheral) Treg cells can lose expression of FOXP3, acquire the ability to produce proinflammatory cytokines (like IL-2, IFN-γ, and IL-17), and cause pathology (6–8, 46–49). This is a particular risk for next generation treatments using Treg cells carrying tissue-specific AgRs (26, 27, 29–35, 50). It is important, therefore, to develop methods to isolate pure Treg cell populations devoid of Tconv cells and unstable Treg cells.

Because of their superior lineage fidelity, tTreg cells are probably the cell type of choice for ACT (51–53). Reliable markers to identify human tTreg cells have, however, not been found. One candidate, neuropilin 1, is preferentially expressed on tTreg cells in mice, but is not present on human Treg cells in blood (49, 54, 55). Murine tTreg cells characteristically coexpress the transcription factors Foxp3 and Helios. Although FOXP3 can transiently be expressed by activated human Tconv cells, Treg cells express this transcription factor regardless of activation status (56, 57), and this is essential for maintenance of Treg cell identity and function (58, 59). Helios is also not a perfect marker (60), as $Helios^-$ Treg cells and recent thymic emigrant (RTE) Tconv can convert into $Helios^+$ Treg cells under certain conditions (61, 62). Nonetheless, tTreg cells reportedly never lose expression of Helios (63). Importantly, Helios helps maintain Treg cell stability (64–66) and the production of Tconv cell–specific effector cytokines in $FOXP3^+$ T cells is predominantly found in cells lacking expression of Helios, both in mice and humans (67–70). Because transcription factors cannot be used for isolation of viable cells, there is a great need for markers that identify viable stable human Treg cells. Through combined transcriptomic and proteomic analyses (71), we found that the transmembrane protein GPA33 (72) is expressed by a subset of human Treg cells that exhibit the hallmarks of tTreg cells. These cells can efficiently be expanded in vitro in the absence of rapamycin without loss of salient Treg cell properties, allowing their study as well as yielding a pure product for Treg cell–based ACT.

Materials and Methods

Abs

The following Abs were used: anti-CD1a (clone HI149), anti-CD226 (clone DX11), anti-CCR7 (clone 150503), anti-CD4 (clone RPA-T4), anti-CD8 (clone RPA-T8), anti-CD127 (clone HIL-7R-M21), anti-CD25 (clone 2A3), and anti-CD45RA (clone HI100) from BD Biosciences; anti-CD27 (clone 0323), anti-TIGIT (clone A15153G), anti-CD127 (clone A019D5), anti–IFN-γ (clone B27), and anti–IL-17A (clone BL168) from BioLegend; anti-CD31 (clone WM59), anti-CD3 (clone OKT3), anti–IL-2 (clone MQ1-17H12), anti-FOXP3 (236A/E7), and anti-Helios (clone 22F6) from eBioscience; anti-GPA33 (clone 402104) from R&D Systems; anti-GPA33 from the Olivia Newton-John Cancer Research Institute, Heidelberg, AU (73), labeled with Alexa Fluor 647 Succinimidyl Ester (Thermo Fisher Scientific); and an in house anti-GPA33 (SQ-GPA33—available upon request).

Sample preparation and cell sorting

Blood samples and Treg cell isolation. Human materials were obtained in accordance with the Declaration of Helsinki and the Dutch rules with respect to the use of human materials from volunteer donors. Umbilical cord blood was obtained from the Sanquin cord blood bank, according to the Eurocord guidelines, with informed consent from the mothers. Donors were full-term infants of healthy mothers after uncomplicated pregnancy. Buffy coats from healthy anonymized donors were obtained after their written informed consent, as approved by Sanquin internal ethical board. PBMCs were isolated up to 72 h after blood harvesting, using a Ficoll-Paque Plus (GE Healthcare) gradient. $CD4^+$ cells were extracted through magnetic sorting using CD4 microbeads (Miltenyi Biotec) according to the manufacturer's instructions. For long-term storage in liquid nitrogen, samples were frozen in FCS with 10% DMSO. Sorting of viable $CD4^+$ T cell subsets was done on a FACSAria III (BD Biosciences), based on surface expression of a live/dead marker, CD127, CD25, CD45RA, and/or GPA33.

Thymus samples. Anonymous human thymus tissue was obtained from surgical specimens of children up to 3 y of age undergoing open heart surgery at the Leiden University Medical Centre. Informed consent was gathered in accordance with the Declaration of Helsinki and was approved by the Medical Ethical Committee of the Leiden University Medical Centre. The tissue was disrupted mechanically and pressed through a 100-μm mesh filter to make single-cell suspensions.

Cell culture

T cells were cultured at 10^4 or 2×10^4 cells per well in 96-well U-bottom plates (Greiner Bio-One) in IMDM + 10% FCS + 1% L-glutamine + 1% penicillin/streptomycin (T cell medium) for up to 14 d with or without 100 nM rapamycin (Sigma-Aldrich) and 300 IU/ml IL-2 (proleukin; Novartis). On day 0, cells were stimulated with soluble anti-CD3 mAb (PeliCluster, 0.1 μg/ml) and anti-CD28 mAb (0.1 μg/ml; eBioscience). Fresh medium with IL-2 with or without rapamycin was added on day 4 and 11 of culture. On days 7 and 14, cells were counted, harvested, partly used for experiments, partly split to the original starting concentration, and restimulated as above. For iTreg cell cultures, 10^4 Tconv cells were cultured with anti-CD3/CD28 mAb and 10 ng/ml recombinant human TGF-β (Peprotech). On day 4, fresh T cell medium supplemented with TGF-β was added. Cells were harvested at day 7.

Flow cytometry

Surface staining of cells was done in PBS containing 0.5% FCS for 15 min at room temperature. To exclude dead cells from the analysis, Near IR (Life Technologies) was used as a live/dead marker. For intracellular staining of FOXP3, Helios, and cytokines, cells were fixed and permeabilized after surface staining with the FOXP3/transcription factor fixation/permeabilization buffers (eBioscience) following the manufacturer's instructions. Data were acquired on an LSR II Cytometer (BD Biosciences) and analyzed using the FlowJo software (version 10; Tree Star). To measure cytokine production, cells were washed with culture medium and stimulated with 20 ng/ml PMA (Sigma-Aldrich) and 1 μM ionomycin (IM; Sigma-Aldrich) in the presence of brefeldin A solution (eBioscience) for 4 h at 37°C.

Suppression assay

PBMCs were washed in PBS and labeled with 5 μM CellTrace Violet (Thermo Fisher Scientific/Molecular Probes). After 8 min, an equivalent volume of FCS was added, and cells were washed twice in IMDM/8% FCS. Labeled PBMCs were cocultured with expanded, and rested Treg cells and stimulated with anti-CD3 mAb (PeliCluster; 0.05 μg/ml). Proliferation was analyzed by flow cytometry after 4 d of coculture.

Treg cell–specific demethylated region assay

Sorted T cells were snap frozen in liquid nitrogen. Bisulfite conversion of DNA was performed using the EZ DNA Methylation-Direct Kit according to the manufacturer's protocol (Zymo Research). Cell pellets were taken up in PBS, and 1×10^4 cells were used for proteinase K digestion. Methylation-specific (MS) quantitative PCR (qPCR) was performed using iQ SYBR Green Supermix (Bio-Rad Diagnostics) (74). MS-qPCR was performed in 8 μl of reactions, containing 2 μl of the eluted bisulfite-treated DNA solution, 0.5 μM of MS or demethylation-specific primers for the *FOXP3* Treg cell–specific demethylated region (TSDR) and 4 μl SYBR Green reagent. MS-qPCR was conducted at 98°C for 10 min, 40 cycles of 98°C for 15 s and 60°C for 1 min, followed by melt curve

analysis. The methylation rate (in percentage) was calculated using the following formula: $100/(1 + 2^{Ct\ CG - Ct\ TG})$, where Ct CG is defined as cycle threshold (Ct) values using MS primers and Ct TG defined as Ct values using demethylation-specific primers.

Proteomics and mRNA sequencing data

Cellular proteomes and transcriptomes were measured as described (71). Absolute protein abundance was estimated using the intensity-based absolute protein quantification (iBAQ) (75) approach, with a few modifications described elsewhere (76). Briefly, peptide intensities were summed up and divided by the number of observable peptides per protein. The iBAQ values were then normalized based on the total sum of all protein intensities to be able to compare abundances across samples. The iBAQ values of three technical replicates were averaged based on the median. Raw files, MaxQuant output files, and normalized iBAQ values are deposited at the Proteomics Identification Database repository (PXD005477; http://proteomecentral.proteomexchange.org). RNA sequencing data can be accessed with accession number GSE90600 (https://www.ncbi.nlm.nih.gov/geo).

Data processing and statistical analysis

Statistical tests were performed using Prism 6.0 (GraphPad Software). Group differences were assessed using Row-matched or normal one-way ANOVA, followed by Tukey honestly significant difference (HSD) multiple comparison test. A $p < 0.05$ was considered statistically significant at a 95% confidence level. Data are presented as mean ± SD. Significance levels are as follows: $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$, unless otherwise indicated in the figure legends.

Results

CD45RA does not identify a pure FOXP3$^+$Helios$^+$ population

CD25$^+$CD127$^-$ Treg cells in human blood can be subdivided into CD45RA$^+$ naive and CD45RA$^-$ effector Treg (eTreg) cells (77). Because pTreg cells derive from Tconv cells that have been activated under tolerogenic conditions, one would expect that such cells lack expression of CD45RA, the prototypical marker of naive T cells. The CD45RA$^+$ Treg cell population might thus theoretically only contain tTreg cells (78, 79). However, in vitro–expanded cultures of CD45RA$^+$ Treg cells (gating strategy in Fig. 1A) still contained a sizable population of FOXP3$^+$Helios$^-$ single positive (SP) cells and a small FOXP3$^-$Helios$^-$ double negative (DN) population (Fig. 1B). Importantly, unlike FOXP3$^+$Helios$^+$ double positive (DP) Treg cells, a substantial proportion of these FOXP3$^+$Helios$^-$ SP cells produced IL-2, IL-17, and IFN-γ when stimulated with PMA + IM (Fig. 1C). The FOXP3$^+$Helios$^-$ SP cells could be activated Tconv that have upregulated FOXP3 (57) or genuine FOXP3$^+$Helios$^-$ SP Treg cells. To distinguish between these possibilities, we mixed Tconv cells from one donor with Treg cells from another donor (using an HLA-mismatch for identification) and examined their phenotype after coculture. Although some Tconv cells did acquire expression of FOXP3, its expression level was much lower than

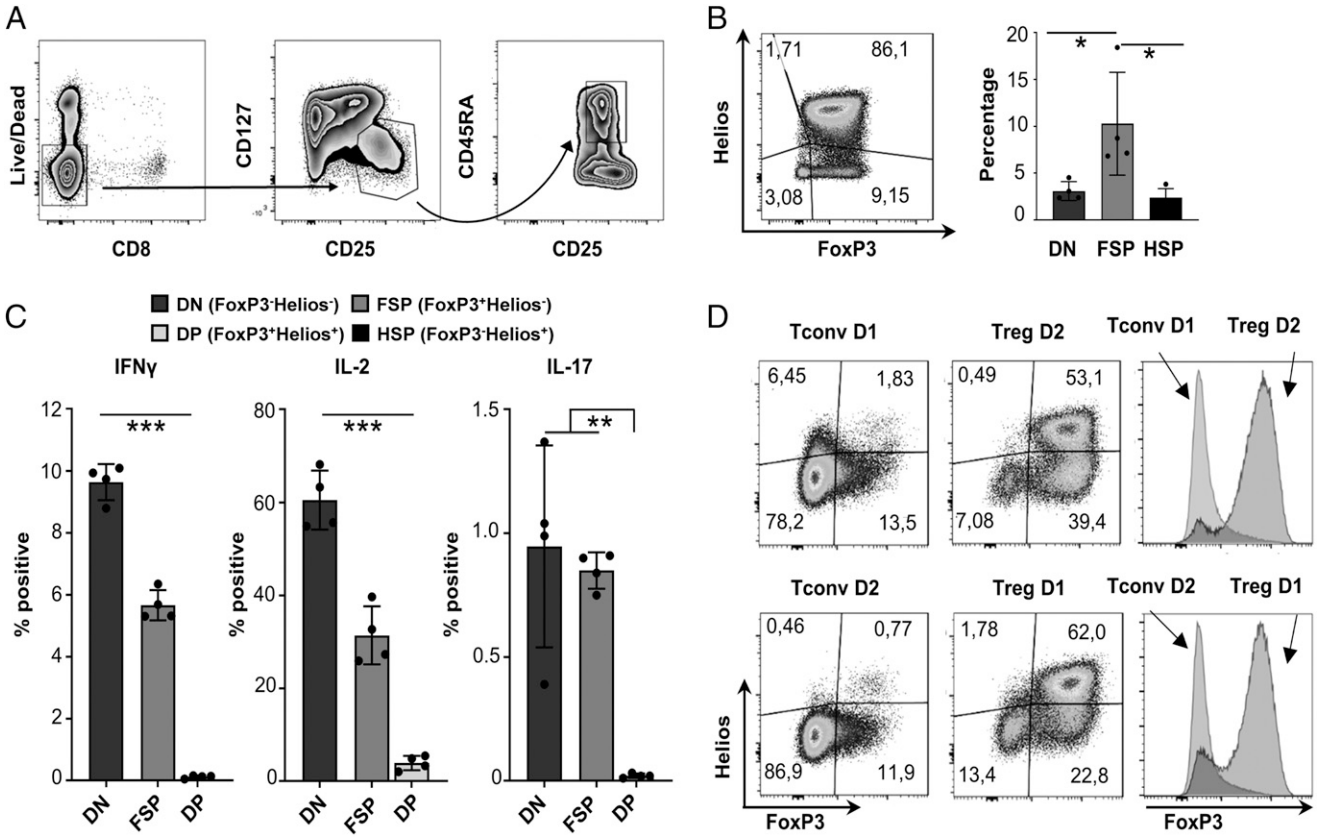

FIGURE 1. Selection of nTreg cells does not yield pure FOXP3$^+$Helios$^+$ Treg cells. (**A**) FACS-sorted CD4$^+$CD25^{int}CD127$^-$CD45RA$^+$ Treg cells were stimulated with anti-CD3, anti-CD28, and IL-2. (**B**) After 7 d, cells were analyzed by flow cytometry for FOXP3/Helios expression and for production of IL-2, IFN-γ, and IL-17 upon stimulation with PMA + IM. Representative FACS plots for FOXP3/Helios (left) and quantification (right) of FOXP3$^-$Helios$^-$ (DN), FOXP3$^+$Helios$^-$ (FSP) and FOXP3$^-$Helios$^+$ (HSP) populations. (**C**) Percentage of cytokine-producing cells in FOXP3/Helios populations (FOXP3$^+$Helios$^+$ = DP). (**D**) CD25$^-$CD127$^+$ Tconv cells from one donor (D1) were cultured with CD25$^+$CD127$^-$ Treg cells from another donor (D2) as in (A) in a 1:3 ratio (top) and vice versa (bottom). Donors were distinguishable through an HLA-mismatch (HLA-B*08). Shown are FOXP3/Helios FACS plots of Tconv and Treg cells of both donors, and a histogram of FOXP3 overlaid for Tconv and Treg cells that were cultured together. Data are representative of at least three independent experiments with four donors (B and C) or were generated in a single experiment with two donors (D). Mean values ± SD are shown. Statistical comparisons were performed by one-way ANOVA, followed by Tukey HSD. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. FSP, FOXP3 SP; HSP, Helios SP.

that found in the FOXP3$^+$Helios$^-$ SP cells derived from the Treg cell population (Fig. 1D). The CD45RA$^+$ Treg cell population thus includes Treg cells that do not (stably) express Helios, possibly representing peripherally induced eTreg cells that have upregulated expression of CD45RA, as has been shown for some conventional terminally differentiated effector cells (80).

GPA33: a surface marker highly expressed by naive Treg cells

To select live FOXP3$^+$Helios$^+$ DP Treg cells, better cell surface markers are necessary. Such markers might be more abundant in CD45RA$^+$ Treg cells, as this population is probably still more enriched for tTreg cells than the CD45RA$^-$ Treg cell population. We therefore searched a dataset that we recently generated (PXD005477), in which we compared whole cell proteomes of five CD4$^+$ T cell populations from human blood (71). These included naive Treg (nTreg) (CD4$^+$CD25^{int}CD45RA$^+$), eTreg (CD4$^+$CD25^{hi}CD45RA$^-$), naive Tconv (nTconv) (CD4$^+$CD25$^-$CD45RA$^+$), memory Tconv (mTconv) (CD4$^+$CD25$^-$CD45RA$^-$), and a population of CD4$^+$CD45RA$^-$ T cells with intermediate expression of CD25 containing a mixture of eTreg and mTconv cells similar to the previously described fraction 3 (Fr. III) (Fig. 2A) (71, 77). We found that a molecule called GPA33 is prominently enriched in nTreg cells, at both the mRNA and protein level (Fig. 2B). GPA33 is a transmembrane receptor of unknown function, belonging to the Ig-domain family, originally identified in human gastrointestinal tissue and tumors (72, 73). It is preferentially expressed in the nTreg cell population at levels that fall within the range of well-known T cell surface molecules such as CD27 and CD28 (Fig. 2C). Although the other CD4$^+$ T cell populations also express this receptor, their GPA33 protein levels are much lower than those found in the nTreg cell population (Fig. 2C), and a similar pattern was found at the mRNA level (Fig. 2D).

GPA33^{high} Treg cells are FOXP3$^+$Helios$^+$ and do not produce effector cytokines

Flow cytometry confirmed that GPA33 is highly expressed on most nTreg cells (Fig. 3A). Subsets of cells in the eTreg cell population as well as in Fr. III also expressed high levels of GPA33, whereas a lower level of expression was found in nTconv cells and some mTconv cells. In all Treg cell subsets, GPA33^{high} cells lacked CD127 and expressed FOXP3 and Helios (Fig. 3B). Selection for cells expressing GPA33 strongly enriched the FOXP3$^+$Helios$^+$ DP subpopulation from the CD4$^+$CD25$^+$ Treg cell population or from the CD4$^+$CD25^{int}CD45RA$^+$ nTreg cell population (Fig. 3C). In fact, the combination of just CD4, CD25, and GPA33 yielded a FOXP3$^+$Helios$^+$ DP Treg cell population of superior purity than obtained by the combination of CD4, CD25, and absence of CD127, which is commonly used to identify Treg cells (81). Nearly pure FOXP3$^+$Helios$^+$ DP could be obtained by selecting for high expression of GPA33 within the Treg cell gate (Fig. 3B, Supplemental Fig. 1). This enrichment was also greater than obtained by selection for CD4$^+$CD25$^+$CD127$^-$ Treg cells expressing TIGIT or lacking expression of CD226 (Supplemental Fig. 1), two markers described to improve the purity of the

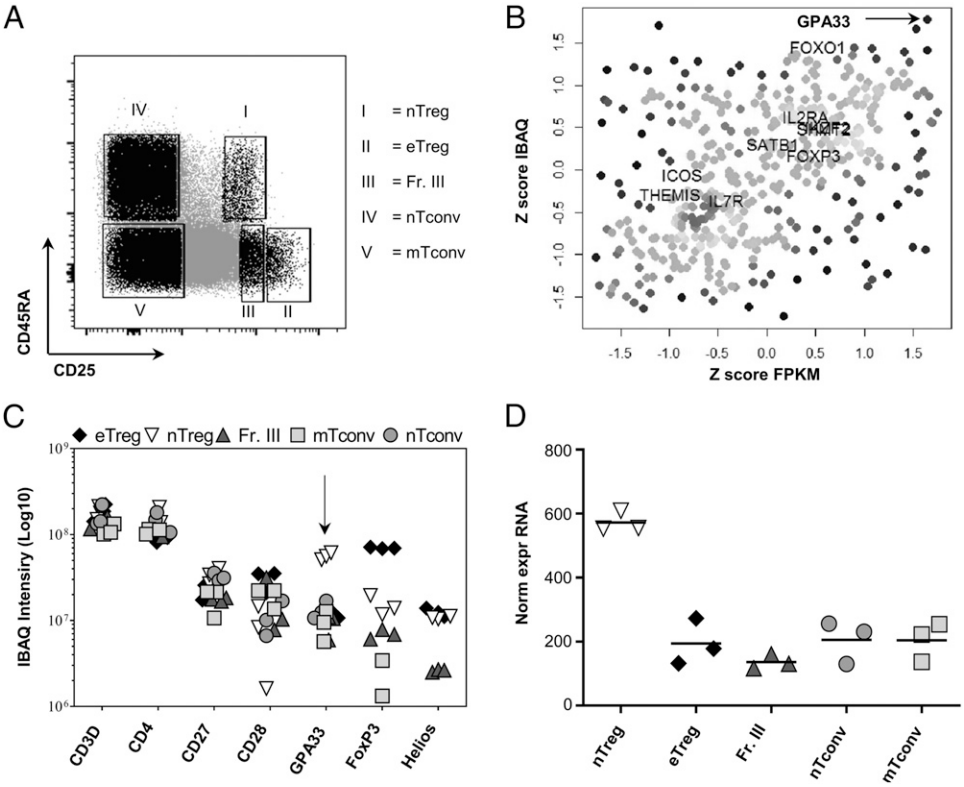

FIGURE 2. GPA33 expression is highest in nTreg cells at both protein and mRNA level. (**A**) CD4$^+$ T cells were sorted into CD25^{int}CD45RA$^+$ nTreg cells (I), CD25^{hi}CD45RA$^-$ eTreg cells (II), CD25^{int}CD45RA$^-$ Fr. III (III), CD25$^-$CD45RA$^+$ nTconv cells (IV), and CD25$^-$CD45RA$^-$ mTconv cells (V). (**B**) All five populations were analyzed by quantitative whole-cell shot gun mass spectrometry and mRNA sequencing. Relative abundance distribution of proteins (Z scores for $\log_{10}$ values of absolute iBAQ intensities) and mRNAs (Z scores for $\log_{10}$ values of fragments per kb of transcript per million mapped reads; colors match intensity quartiles) within the nTreg cell population. GPA33 (top right corner) is indicated with an arrow. (**C**) Protein abundance (expressed as IBAQ intensity) was normalized using the histone ruler, thus allowing direct comparison of abundance between different proteins. (**D**) GPA33 mRNA levels were determined by mRNA sequencing and are expressed as relative values. Data are from three independent experiments, each with technical triplicate samples.

FOXP3$^+$Helios$^+$ DP population (82, 83). Finally, absence of GPA33 identified CD4$^+$CD25$^+$CD127$^-$ T cells able to produce IL-2, IFN-γ, and IL-17, whereas Treg cells expressing the highest levels of GPA33 mostly lacked this ability (Fig. 3D, Supplemental Fig. 2). Expression of GPA33 even identified cells unable to produce IL-2, IFN-γ, and IL-17 in Fr. III, the FOXP3$^+$ population with the highest frequency of cells producing proinflammatory cytokines (71, 77). High expression of GPA33 thus marks CD4$^+$CD25$^+$CD127$^-$FOXP3$^+$Helios$^+$ Treg cells that are unable to produce Tconv effector cytokines. As such, selection for GPA33 may be useful for ACT. Treg cells from patients with type 1 diabetes do express GPA33, both immediately ex vivo and after 2 wk of expansion with a clinical grade protocol (Supplemental Fig. 3A, 3B). This shows that, in principle, GPA33 can be used to purify autologous Treg cells for treatment of such patients (23).

GPA33^{high} nTreg cells stably remain FOXP3$^+$Helios$^+$ in culture

For ACT, Treg cells must be expanded in vitro. Among the various subsets, the CD45RA$^+$ Treg cells possess the greatest capacity for in vitro expansion, far exceeding that of CD45RA$^-$ Treg cells (77, 84). We reasoned that selective culture of GPA33^{high} cells (all of which are FOXP3$^+$Helios$^+$) from the CD45RA$^+$ Treg cell population should eliminate the undesirable FOXP3$^+$Helios$^-$ and FOXP3$^-$Helios$^-$ cells that are present after expansion of the entire CD45RA$^+$ Treg cell population (Fig. 1B). To test this idea, nTreg

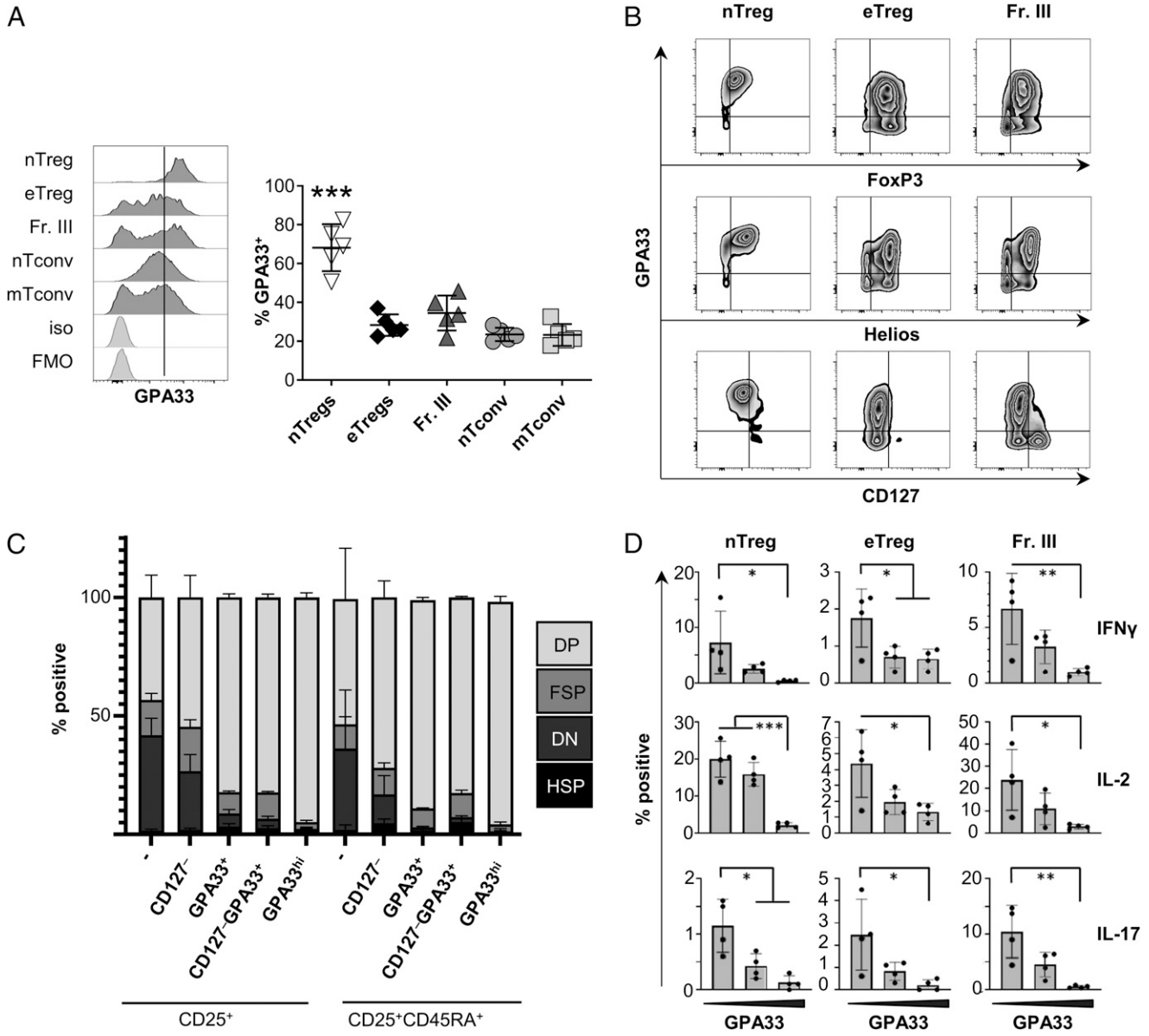

FIGURE 3. GPA33 is expressed on Helios$^+$ Treg cells that lack the capacity to produce effector cytokines. (**A**) Representative GPA33 histograms of the subsets defined in Fig. 2A (top, iso) and quantification (bottom) of the percentages of GPA33$^+$ cells in these same populations from human PBMCs. (**B**) FACS plots for GPA33 against FOXP3 (top), Helios (middle), and CD127 (bottom) on nTreg cells, eTreg cells, and Fr. III, as indicated at top of figure. (**C**) shows the cumulative results for FACS expression of FOXP3 and Helios from the CD4$^+$ fraction of three donors in the indicated gates. FOXP3$^+$Helios$^+$ (DP), FOXP3$^+$Helios$^-$ (FSP), FOXP3$^-$Helios$^-$ (DN), and FOXP3$^-$Helios$^+$ (HSP). (**D**) nTreg cells (left), eTreg cells (middle) and Fr. III cells (right) were sorted by FACS and then stimulated with PMA + IM for 4 h. Production of effector cytokines was measured by flow cytometry per GPA33 intensity (low to high, left to right on graphs). Gating strategy is shown in Supplemental Fig. 2. Data are representative of at least three independent experiments with three to five blood donors. Mean values ± SD are shown. Statistical comparisons were performed by one-way ANOVA, followed by Tukey HSD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. FMO, fluorescence minus one with no GPA33 Ab added; FSP, FOXP3 SP; HSP, Helios SP; iso, isotype control.

cells were isolated based on different expression levels of GPA33 (Fig. 4A, top) and expanded for 2 wk with anti-CD3/CD28 mAb and IL-2. Cells expressing the highest level of GPA33 remained almost exclusively FOXP3$^+$Helios$^+$ (Fig. 4A, bottom). Cells expressing lower levels of GPA33 yielded progressively more FOXP3$^+$Helios$^-$ SP and even DN cells (Fig. 4A, bottom). A similar pattern was observed after culture of Fr. III and eTreg cells that had been sorted based on different GPA33 expression levels (Supplemental Fig. 2). Furthermore, the ability to produce effector cytokines, such as IL-2 and IFN-γ, after expansion, correlated inversely with the GPA33 expression level of the original nTreg cell population used (Fig. 4C). The GPA33^{high} nTreg cell population therefore appears stably committed to the FOXP3$^+$Helios$^+$ Treg cell fate, at least during in vitro culture. Interestingly, GPA33 itself was also maintained during expansion of GPA33^{high} nTreg cells (Fig. 4D), GPA33^{high} eTreg cells, and Fr. III cells (Supplemental Fig. 2). GPA33 persisted on GPA33^{high} nTreg cell clones for as long as monitored (90 d; data not shown). In contrast, Tconv cells lost GPA33 during culture (Fig. 4D). Moreover, expression of GPA33 did not appear on TGF-β–induced Treg (iTreg) cells (Fig. 4D). GPA33^{high} nTreg cells stably remained FOXP3$^+$Helios$^+$ and were unable to produce proinflammatory cytokines, despite the fact that no rapamycin was added to the cultures. Importantly, these expanded GPA33^{high} nTreg cells suppressed proliferation of CD4$^+$ or CD8$^+$ Tconv cells as potently as unfractionated CD4$^+$CD25$^+$CD127$^-$ Treg cells that had been cultured in the presence of rapamycin (Fig. 5A), as is standard procedure for clinical grade Treg cell expansion (40). Similarly, their suppressive capacity was comparable to that of other Treg cell populations cultured without rapamycin but better than that of iTreg cells (Supplemental Fig. 4). GPA33^{high} nTreg cells also retained full CpG demethylation of the *FOXP3* CNS2 TSDR (Fig. 5B), which is a hallmark of stable Treg cells (24, 85), again without a requirement for rapamycin.

Stringent selection for a GPA33^{high} nTreg cell population from adult blood yields fewer cells than isolation of the unfractionated CD4$^+$CD25$^+$CD127$^-$ population. This disadvantage is, however, largely compensated by the much greater expansion that is possible in the absence of rapamycin (Fig. 5C). Selection for nTreg cells expressing high levels of GPA33 thus allows the isolation of Treg cells that can robustly be expanded in vitro and retain their salient properties without a need for rapamycin.

Treg cells acquire GPA33 during thymic development

GPA33^{high} nTreg cells fit the criteria for tTreg cells: stable coexpression of FOXP3 and Helios, inability to produce Tconv effector cytokines, and demethylation of *FOXP3* CNS2 TSDR. Furthermore, GPA33 is almost universally expressed on Treg cells from neonates, which have not yet been exposed to the commensal microorganisms and food Ags that induce differentiation of pTreg cells (Fig. 6A). To examine whether GPA33 identifies tTreg cells, we determined its expression on human thymocytes.

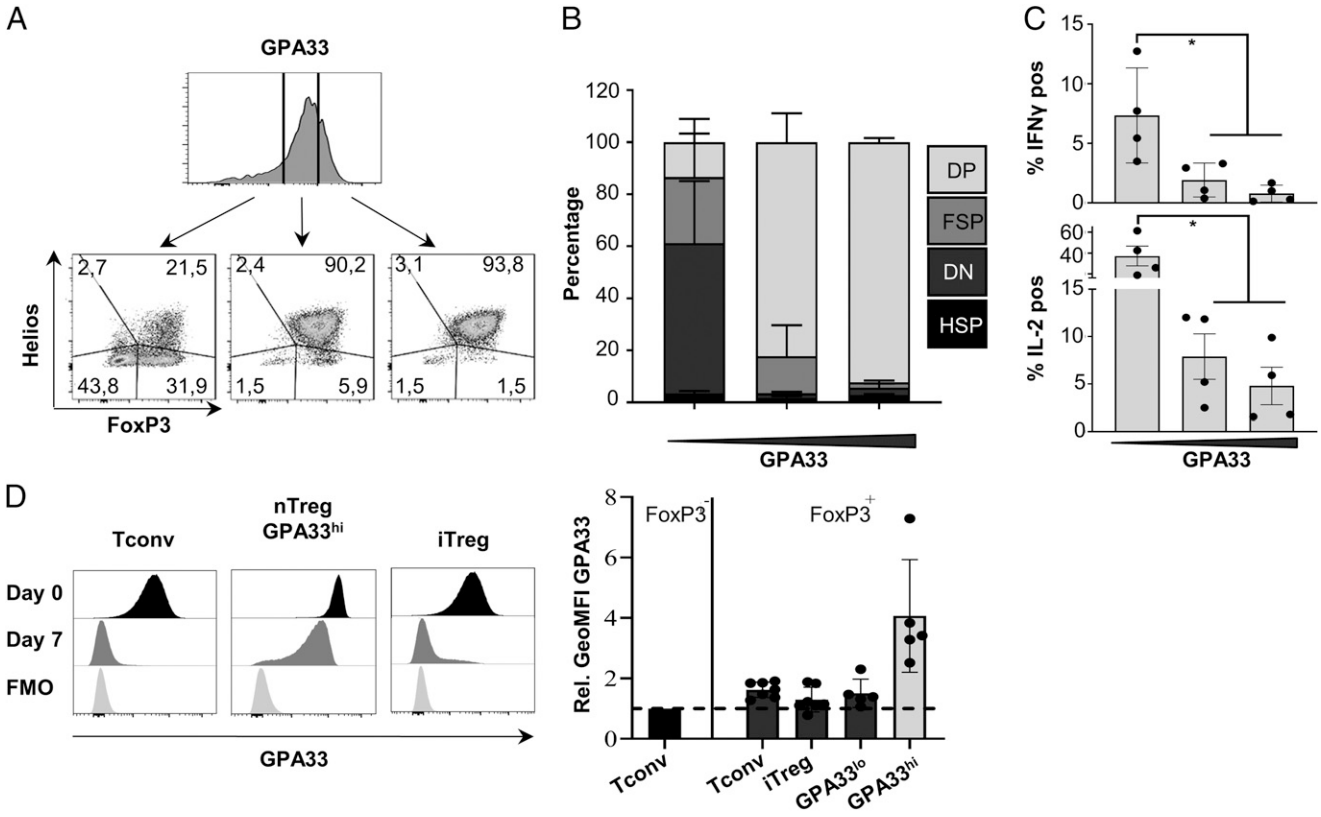

FIGURE 4. GPA33^{high} nTreg cells remain FOXP3$^+$Helios$^+$ DP after expansion without rapamycin. nTreg cells were sorted based on (from left to right) low, intermediate, or high expression of GPA33 and cultured for 2 wk with anti-CD3, anti-CD28, and IL-2 and then analyzed by FACS for expression of FOXP3 and Helios. FACS plots from a representative donor are shown in (**A**), and (**B**) shows the cumulative results for all donors. FOXP3$^+$Helios$^+$ (DP), FOXP3$^+$Helios$^-$ (FSP), FOXP3$^-$Helios$^-$ (DN), and FOXP3$^-$Helios$^+$ (HSP). (**C**) Production of effector cytokines after PMA + IM stimulation of cells cultured as described in (A). (**D**) GPA33 surface levels on Tconv cells and GPA33^{high}–sorted nTreg and iTreg cells of a representative donor after 1 wk of culture as in (A) (left) (note that the same day 0 GPA33 histogram is shown for Tconv and iTreg cells). Geometric mean fluorescence intensity (GeoMFI) for GPA33 of all donors is expressed relative to the Tconv FOXP3$^-$ cells within each population (right). Data are from three independent experiments with three to six blood donors and show mean ± SD. Statistical comparisons were performed by one-way ANOVA, followed by Tukey HSD. *$p < 0.05$. FSP, FOXP3 SP; HSP, Helios SP.

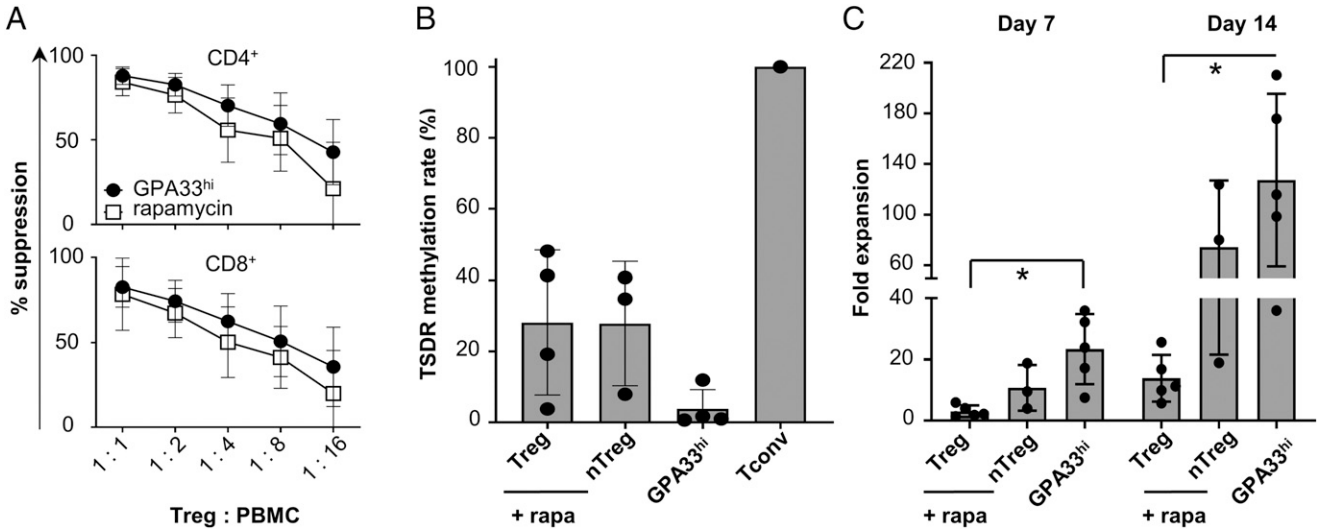

FIGURE 5. Expanded GPA33^{high} nTreg cells are suppressive and maintain a demethylated TSDR without rapamycin. (**A**) CD25$^+$CD127$^-$ Treg cells were cultured with 100 nM rapamycin (open squares), whereas GPA33^{high} nTreg cells were cultured without rapamycin (closed circles). After 7 d, suppressive capacity was tested using anti-CD3–stimulated PBMCs as responders in different ratios to Treg cells as indicated. Percent suppression is defined as 100 – (percentage of proliferation in test condition/percentage of proliferation of responders only condition) × 100% (**B**) TSDR methylation was assessed in CD25$^+$CD127$^-$ Treg cells, nTreg cells, GPA33^{high} nTreg cells, and Tconv cells after 14 d of culture with rapamycin (+rapa) or without rapamycin (as indicated in figure). (**C**) Expansion rates of CD25$^+$CD127$^-$ Treg cells and nTreg cells with rapamycin and GPA33^{high} nTreg cells without rapamycin after 7 and 14 d of culture. Note that the y-axis is interrupted for better visualization. Data are from at least two independently performed experiments with two to five blood donors and show mean ± SD. Statistical comparisons were performed by one-way ANOVA, followed by Tukey HSD. *$p < 0.05$.

CD3$^+$ thymocytes have been divided into four subpopulations based on expression of CD1a and CD27, from least mature (CD1a$^+$CD27$^-$, gate I) to most mature (CD1a$^-$CD27$^+$, gate IV) (86) (Fig. 6B). Stages I (CD1a$^+$CD27$^-$) and II (CD1a$^+$CD27$^+$) contain mainly late CD4$^+$CD8$^+$ DP thymocytes. At stages III (CD1a^{low}CD27$^+$) and IV (CD1a$^-$CD27$^+$), thymocytes have committed to the CD4 or CD8 lineage (86). FOXP3$^+$ Treg cells are found mostly in the more mature stages of development (86) (Fig. 6B). Although some Treg cells express GPA33 already at stage II, the percentage of GPA33$^+$ cells gradually increases with maturational stage (Fig. 6B). In the final steps of thymic development, Treg cells acquire CD31 and, just before exit from the thymus, the CD45RA isoform that also marks nTreg cells in blood (86). Importantly, nearly all cells at this late CD31$^+$CD45RA$^+$ stage express GPA33, whereas the CD45RA$^-$ cells are also mostly GPA33$^-$ (Fig. 6C). Therefore, GPA33 is upregulated during the final stages of tTreg cell development, just before egress into the peripheral blood. Indeed, GPA33 is universally expressed on CD31$^+$CD45RA$^+$ RTE Treg cells in blood (Fig. 6D).

Discussion

ACT with Treg cells can ameliorate autoimmune and pathologic conditions caused by alloreactivity after transplantation (17, 23). Many inflammatory disorders may be amenable to such treatments (35). However, the threshold to allow such therapies is high, given the existence of alternatives such as pharmacological immunosuppressants. ACT with Treg cells holds the potential for more effective mitigation of disease, with fewer side effects, than obtained with available drugs. Nonetheless, broad clinical implementation of ACT with Treg cells will only occur if this therapy is optimally safe. A key step in ensuring safety lies in removal of contaminating Tconv cells and unstable Treg cells (35). This is especially important for development of next generation therapies. The activity of Treg cells is controlled by their TCR (30–33, 87, 88). More potent regulation can be achieved with mono- or oligoclonal Treg cells expressing AgRs specific for the tissues that must be protected than with the currently used polyclonal Treg cell populations (28). Any Tconv cell carrying such a TCR, be it derived from contaminating Tconv cells or from unstable Treg cells, could provoke iatrogenic exacerbation of disease and should therefore be eliminated before infusion into patients.

Current protocols rely on rapamycin to limit the presence of Tconv cells in Treg cell populations cultured for ACT (39, 40). The use of this drug has several caveats. It does not preclude the presence of unstable Treg cells in expanded Treg cell populations and, in fact, induces generation of such cells (44). Furthermore, Treg cell–specific inactivation of mTORC1, the kinase complex that is inhibited by rapamycin, resulted in severe autoimmune disease in mice (43, 45). This suggests that rapamycin may even compromise functional properties of Treg cells. Ideally, therefore, Treg cell populations isolated for ACT should be sufficiently pure to obviate the need to use this drug.

Given their reported superior lineage fidelity (49, 51, 89), tTreg cells seem the most attractive cell type for ACT. A combined proteome/transcriptome database search (71) for markers that might allow their isolation led us to the Ig-domain protein GPA33 (72). A recent study found that FOXP3 binds to a region in the GPA33 gene, already suggesting that it might be expressed in Treg cells (90). We, in this study, showed that this protein is not expressed by all Treg cells but by a subset that has the characteristics of tTreg cells. The GPA33 protein is most closely related to CD2, and has relatives with important functions in T cells, such as CD276 (B7-H3) and VSIG4 [both inhibitors of T cell activation (91, 92)] and several cell adhesion molecules such as CEACAM, ICAM, and NCAM (3D-Protein Basic Local Alignment Search Tool, National Center for Biotechnology Information). GPA33 has been studied as a marker of intestinal epithelium and is highly expressed on some gastric and most colon carcinomas (73, 93). GPA33-deficient mice exhibit increased incidence of colitis and food intolerance (94), and variants at the GPA33 locus are associated with the rare autoimmune disorder eosinophilic granulomatosis with polyangiitis (P. Lyons, J. Peters, F. Alberici, J. Liley, R. Coulson, W. Astle, C. Baldini, F. Bonatti, M. Cid, H. Elding, et al., manuscript posted on bioRxiv). Although these

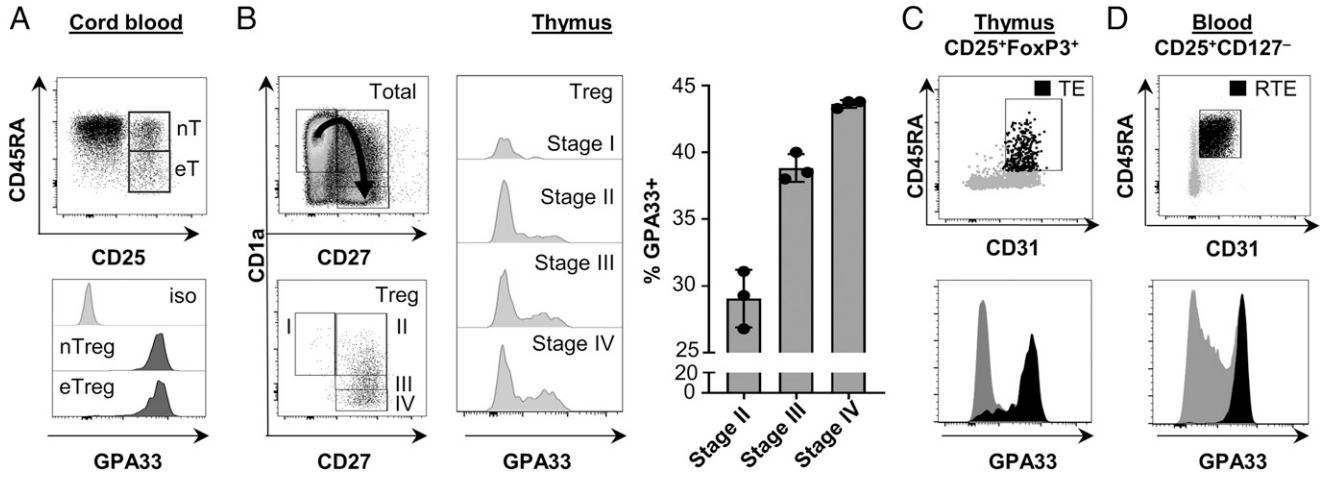

FIGURE 6. GPA33 expression on Treg cells appears late during thymic development. (**A**) Representative FACS profile for CD45RA versus CD25 (top) of $CD4^+$ T cells in human umbilical cord blood and GPA33 expression profile of the nTreg ($CD25^+CD127^-CD45RA^+$) and eTreg cell ($CD25^+CD127^-CD45RA^-$) populations (iso). (**B**) Representative FACS plot of maturation stages classified on the basis of CD1a and CD27 expression for the total human postnatal thymocyte population (top left) and the $CD4^+CD25^+FOXP3^+$ Treg cell population (bottom left). Maturational stages are indicated with roman numerals and range from stage I (most immature) to stage IV (most mature). Representative FACS plot for GPA33 per maturational stage in tTreg cells is shown (middle), and a graph of the percentage (mean ± SD) of $GPA33^+$ cells in stages II–IV of tTreg cells is shown for three donors (right). (**C**) Representative FACS plots of CD45RA and CD31 expression on $CD4^+CD25^+FOXP3^+$ thymocytes (top) indicating Treg cell–thymic emigrants (TE; $CD31^+CD45^+$) in black and $CD45RA^-$ tTreg cells in gray. The graph below depicts GPA33 expression of the populations shown in the top figure. (**D**) The same populations as in (C) are shown in blood $CD4^+CD25^+CD127^-$ Treg cells (RTE [$CD31^+CD45^+$] in black, $CD31^-$ cells in gray). Data are representative of at least three independent experiments. iso, isotype control.

results might reflect a role for GPA33 in epithelial barrier function (94), the expression of this molecule in Treg cells raises the possibility that a more direct role in T cell tolerance exists.

Although the function of GPA33 is not known, we found that it serves as a good marker to select pure populations of Treg cells. The purest $FOXP3^+Helios^+$ DP Treg cell populations are obtained by selecting $GPA33^{high}$ nTreg cells. Upon expansion without rapamycin, such cells stably retain expression of FOXP3 and Helios as well as full demethylation of the TSDR in the *FOXP3* gene. They furthermore abstain from producing effector cytokines and are fully suppressive. Although selection for $GPA33^{high}$ nTreg cells leads to relatively low yields, this disadvantage is largely offset by their superior expansion because of the absence of rapamycin. Moreover, the need for high cell numbers likely diminishes when Ag-specific Treg cells are used because of their greater potency (28). Nonetheless, when larger populations of Treg cells are required, selecting $GPA33^+$ cells from the entire $CD4^+CD25^+CD127^-$ or even from the $CD4^+CD25^+$ population combines high yield with increased purity of $FOXP3^+Helios^+$ DP Treg cells.

Our results suggest that GPA33 identifies Treg cells of thymic origin. It is expressed on almost all thymic $CD31^+CD45RA^+$ Treg cells, the population that is about to egress into the periphery (86), as well as on most peripheral nTreg cells, which should, theoretically, predominantly consist of tTreg cells. Moreover, GPA33 is universally expressed on Treg cells in cord blood, which are presumably all thymic-derived, as neonates have not been exposed in utero to the commensals and food Ags that drive differentiation of pTreg cells (95). Under no condition did we observe acquisition of GPA33 expression on Treg cells that lacked this marker initially and GPA33 does not appear on iTreg cells generated in vitro. It should be noted that some Tconv cells do express this receptor. GPA33 is, thus, not a unique Treg cell marker on its own, as is true for any other marker used to identify such cells, including FOXP3 (57) and Helios (61). However, Tconv cells lose expression of GPA33 after TCR-mediated activation, whereas this molecule remains stably expressed on Treg cells. This raises the possibility that GPA33 may be a lineage marker for tTreg cells. Consistent with that idea, GPA33 is found on a subset of cells in the $CD45RA^-$ Treg cell populations (eTreg cells and Fr. III), arguably mixtures of pTreg cells and activated tTreg cells (96, 97). The $GPA33^{high}$ cells in these fractions are universally $FOXP3^+Helios^+$ DP and lack ability to produce effector cytokines.

In conclusion, we have described GPA33 as a surface marker which, when used together with other Treg cell markers, allows isolation of pure and stable Treg cells, displaying the hallmarks of tTreg cells. On the basis of our findings, we consider it attractive to incorporate high expression of this marker as a selection criterion to generate clinical grade Treg cells for ACTs.

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Disclosures

A patent application for the use of GPA33 to select Treg cells for clinical use has been filed by D.A., J.B., and E.C. The other authors have no financial conflicts of interest.

References

1. Sakaguchi, S., T. Yamaguchi, T. Nomura, and M. Ono. 2008. Regulatory T cells and immune tolerance. *Cell* 133: 775–787.
2. Kieback, E., E. Hilgenberg, U. Stervbo, V. Lampropoulou, P. Shen, M. Bunse, Y. Jaimes, P. Boudinot, A. Radbruch, U. Klemm, et al. 2016. Thymus-derived regulatory T cells are positively selected on natural self-antigen through cognate interactions of high functional avidity. *Immunity* 44: 1114–1126.
3. Chen, W., W. Jin, N. Hardegen, K. J. Lei, L. Li, N. Marinos, G. McGrady, and S. M. Wahl. 2003. Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-β induction of transcription factor Foxp3. *J. Exp. Med.* 198: 1875–1886.
4. Hadis, U., B. Wahl, O. Schulz, M. Hardtke-Wolenski, A. Schippers, N. Wagner, W. Müller, T. Sparwasser, R. Förster, and O. Pabst. 2011. Intestinal tolerance

requires gut homing and expansion of FoxP3+ regulatory T cells in the lamina propria. *Immunity* 34: 237–246.
5. Lathrop, S. K., S. M. Bloom, S. M. Rao, K. Nutsch, C.-W. Lio, N. Santacruz, D. A. Peterson, T. S. Stappenbeck, and C.-S. Hsieh. 2011. Peripheral education of the immune system by colonic commensal microbiota. *Nature* 478: 250–254.
6. Komatsu, N., K. Okamoto, S. Sawa, T. Nakashima, M. Oh-hora, T. Kodama, S. Tanaka, J. A. Bluestone, and H. Takayanagi. 2014. Pathogenic conversion of Foxp3+ T cells into TH17 cells in autoimmune arthritis. *Nat. Med.* 20: 62–68.
7. Zhou, X., S. Bailey-bucktrout, L. T. Jeker, C. Penaranda, M. Ashby, M. Nakayama, W. Rosenthal, and A. Jeffrey. 2009. Instability of the transcription factor Foxp3 leads to the generation of pathogenic memory T cells in vivo. *Nat. Immunol.* 10: 1000–1007.
8. Duarte, J. H., S. Zelenay, M. L. Bergman, A. C. Martins, and J. Demengeot. 2009. Natural Treg cells spontaneously differentiate into pathogenic helper cells in lymphopenic conditions. *Eur. J. Immunol.* 39: 948–955.
9. Joffre, O., T. Santolaria, D. Calise, T. Al Saati, D. Hudrisier, P. Romagnoli, and J. P. M. van Meerwijk. 2008. Prevention of acute and chronic allograft rejection with CD4+CD25+Foxp3+ regulatory T lymphocytes. *Nat. Med.* 14: 88–92.
10. Golshayan, D., S. Jiang, J. Tsang, M. I. Garin, C. Mottet, and R. I. Lechler. 2007. In vitro-expanded donor alloantigen-specific CD4+CD25+ regulatory T cells promote experimental transplantation tolerance. *Blood* 109: 827–835.
11. Izquierdo, C., A. Z. Ortiz, M. Presa, S. Malo, A. Montoya, N. Garabatos, C. Mora, J. Verdaguer, and T. Stratmann. 2018. Treatment of T1D via optimized expansion of antigen-specific Tregs induced by IL-2/anti-IL-2 monoclonal antibody complexes and peptide/MHC tetramers. *Sci. Rep.* 8: 8106.
12. Hori, S., M. Haury, A. Coutinho, and J. Demengeot. 2002. Specificity requirements for selection and effector functions of CD25+4+ regulatory T cells in anti-myelin basic protein T cell receptor transgenic mice. *Proc. Natl. Acad. Sci. USA* 99: 8213–8218.
13. Taylor, P. A., C. J. Lees, and B. R. Blazar. 2002. The infusion of ex vivo activated and expanded CD4(+)CD25(+) immune regulatory cells inhibits graft-versus-host disease lethality. *Blood* 99: 3493–3499.
14. Edinger, M., P. Hoffmann, J. Ermann, K. Drago, C. G. Fathman, S. Strober, and R. S. Negrin. 2003. CD4+CD25+ regulatory T cells preserve graft-versus-tumor activity while inhibiting graft-versus-host disease after bone marrow transplantation. *Nat. Med.* 9: 1144–1150.
15. Brunstein, C. G., J. S. Miller, Q. Cao, D. H. McKenna, K. L. Hippen, J. Curtsinger, T. Defor, B. L. Levine, C. H. June, P. Rubinstein, et al. 2011. Infusion of ex vivo expanded T regulatory cells in adults transplanted with umbilical cord blood: safety profile and detection kinetics. *Blood* 117: 1061–1070.
16. Blazar, B. R., W. J. Murphy, and M. Abedi. 2012. Advances in graft-versus-host disease biology and therapy. *Nat. Rev. Immunol.* 12: 443–458.
17. Di Ianni, M., F. Falzetti, A. Carotti, A. Terenzi, F. Castellino, E. Bonifacio, B. Del Papa, T. Zei, R. I. Ostini, D. Cecchini, et al. 2011. Tregs prevent GVHD and promote immune reconstitution in HLA-haploidentical transplantation. *Blood* 117: 3921–3928.
18. Koenecke, C., C.-W. Lee, K. Thamm, L. Föhse, M. Schafferus, H.-W. Mittrücker, S. Floess, J. Huehn, A. Ganser, R. Förster, and I. Prinz. 2012. IFN-γ production by allogeneic Foxp3+ regulatory T cells is essential for preventing experimental graft-versus-host disease. *J. Immunol.* 189: 2890–2896.
19. Anasetti, C. 2015. Ex-vivo expanded donor regulatory T cells for prevention of acute graft-versus-host disease. H. Lee Moffitt Cancer Center and Research Institute, Tampa, FL. Available at: https://clinicaltrials.gov/ct2/show/NCT01795573.
20. Theil, A., S. Tuve, U. Oelschlägel, A. Maiwald, D. Döhler, D. Oßmann, A. Zenkel, C. Wilhelm, J. M. Middeke, N. Shayegi, et al. 2015. Adoptive transfer of allogeneic regulatory T cells into patients with chronic graft-versus-host disease. *Cytotherapy* 17: 473–486.
21. Brunstein, C. G., J. S. Miller, D. H. McKenna, K. L. Hippen, T. E. DeFor, D. Sumstad, J. Curtsinger, M. R. Verneris, M. L. MacMillan, B. L. Levine, et al. 2016. Umbilical cord blood-derived T regulatory cells to prevent GVHD: kinetics, toxicity profile, and clinical effect. *Blood* 127: 1044–1051.
22. Trzonkowski, P., M. Bieniaszewska, J. Juścińska, A. Dobyszuk, A. Krzystyniak, N. Marek, J. Myśliwska, and A. Hellmann. 2009. First-in-man clinical results of the treatment of patients with graft versus host disease with human ex vivo expanded CD4+CD25+CD127- T regulatory cells. *Clin. Immunol.* 133: 22–26.
23. Marek-Trzonkowska, N., M. Myśliwiec, A. Dobyszuk, M. Grabowska, I. Derkowska, J. Juścińska, R. Owczuk, A. Szadkowska, P. Witkowski, W. Młynarski, et al. 2014. Therapy of type 1 diabetes with CD4(+)CD25(high)CD127-regulatory T cells prolongs survival of pancreatic islets - results of one year follow-up. *Clin. Immunol.* 153: 23–30.
24. Bluestone, J. A., J. H. Buckner, M. Fitch, S. E. Gitelman, S. Gupta, M. K. Hellerstein, K. C. Herold, A. Lares, M. R. Lee, K. Li, et al. 2015. Type 1 diabetes immunotherapy using polyclonal regulatory T cells. *Sci. Transl. Med.* 7: 315ra189.
25. Todo, S., K. Yamashita, R. Goto, M. Zaitsu, A. Nagatsu, T. Oura, M. Watanabe, T. Aoyagi, T. Suzuki, T. Shimamura, et al. 2016. A pilot study of operational tolerance with a regulatory T-cell-based cell therapy in living donor liver transplantation. *Hepatology* 64: 632–643.
26. Putnam, A. L., N. Safinia, A. Medvec, M. Laszkowska, M. Wray, M. A. Mintz, E. Trotta, G. L. Szot, W. Liu, A. Lares, et al. 2013. Clinical grade manufacturing of human alloantigen-reactive regulatory T cells for use in transplantation. *Am. J. Transplant.* 13: 3010–3020.
27. Landwehr-Kenzel, S., F. Issa, S. H. Luu, M. Schmück, H. Lei, A. Zobel, A. Thiel, N. Babel, K. Wood, H. D. Volk, and P. Reinke. 2014. Novel GMP-compatible protocol employing an allogeneic B cell bank for clonal expansion of allospecific natural regulatory T cells. *Am. J. Transplant.* 14: 594–606.
28. Adair, P. R., Y. C. Kim, A. H. Zhang, J. Yoon, and D. W. Scott. 2017. Human tregs made antigen specific by gene modification: the power to treat autoimmunity and antidrug antibodies with precision. *Front. Immunol.* 8: 1117.
29. Jethwa, H., A. A. Adami, and J. Maher. 2014. Use of gene-modified regulatory T-cells to control autoimmune and alloimmune pathology: is now the right time? *Clin. Immunol.* 150: 51–63.
30. Tang, Q., K. J. Henriksen, M. Bi, E. B. Finger, G. Szot, J. Ye, E. L. Masteller, H. McDevitt, M. Bonyhadi, and J. A. Bluestone. 2004. In vitro-expanded antigen-specific regulatory T cells suppress autoimmune diabetes. *J. Exp. Med.* 199: 1455–1465.
31. Tarbell, K. V., L. Petit, X. Zuo, P. Toy, X. Luo, A. Mqadmi, H. Yang, M. Suthanthiran, S. Mojsov, and R. M. Steinman. 2007. Dendritic cell-expanded, islet-specific CD4+ CD25+ CD62L+ regulatory T cells restore normoglycemia in diabetic NOD mice. *J. Exp. Med.* 204: 191–201.
32. Fisson, S., F. Djelti, A. Trenado, F. Billiard, R. Liblau, D. Klatzmann, J. L. Cohen, and B. L. Salomon. 2006. Therapeutic potential of self-antigen-specific CD4+ CD25+ regulatory T cells selected in vitro from a polyclonal repertoire. *Eur. J. Immunol.* 36: 817–827.
33. Wright, G. P., C. A. Notley, S.-A. Xue, G. M. Bendle, A. Holler, T. N. Schumacher, M. R. Ehrenstein, and H. J. Stauss. 2009. Adoptive therapy with redirected primary regulatory T cells results in antigen-specific suppression of arthritis. *Proc. Natl. Acad. Sci. USA* 106: 19078–19083.
34. Bluestone, J. A., and Q. Tang. 2018. T_{reg} cells-the next frontier of cell therapy. *Science* 362: 154–155.
35. Trzonkowski, P., R. Bacchetta, M. Battaglia, D. Berglund, H. R. Bohnenkamp, A. ten Brinke, A. Bushell, N. Cools, E. K. Geissler, S. Gregori, et al. 2015. Hurdles in therapy with regulatory T cells. *Sci. Transl. Med.* 7: 304ps18.
36. Liu, W., A. L. Putnam, Z. Xu-Yu, G. L. Szot, M. R. Lee, S. Zhu, P. A. Gottlieb, P. Kapranov, T. R. Gingeras, B. Fazekas de St Groth, et al. 2006. CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells. *J. Exp. Med.* 203: 1701–1711.
37. Hartigan-O'Connor, D. J., C. Poon, E. Sinclair, and J. M. McCune. 2007. Human CD4+ regulatory T cells express lower levels of the IL-7 receptor alpha chain (CD127), allowing consistent identification and sorting of live cells. *J. Immunol. Methods* 319: 41–52.
38. Seddiki, N., B. Santner-Nanan, J. Martinson, J. Zaunders, S. Sasson, A. Landay, M. Solomon, W. Selby, S. I. Alexander, R. Nanan, et al. 2006. Expression of interleukin (IL)-2 and IL-7 receptors discriminates between human regulatory and activated T cells. *J. Exp. Med.* 203: 1693–1700.
39. Delgoffe, G. M., T. P. Kole, Y. Zheng, P. E. Zarek, K. L. Matthews, B. Xiao, P. F. Worley, S. C. Kozma, and J. D. Powell. 2009. The mTOR kinase differentially regulates effector and regulatory T cell lineage commitment. *Immunity* 30: 832–844.
40. Fraser, H., N. Safinia, N. Grageda, S. Thirkell, K. Lowe, L. J. Fry, C. Scottá, A. Hope, C. Fisher, R. Hilton, et al. 2018. A rapamycin-based GMP-compatible process for the isolation and expansion of regulatory T cells for clinical trials. *Mol. Ther. Methods Clin. Dev.* 8: 198–209.
41. Battaglia, M., A. Stabilini, and M. G. Roncarolo. 2005. Rapamycin selectively expands CD4+CD25+FoxP3+ regulatory T cells. *Blood* 105: 4743–4748.
42. Coenen, J. J. A., H. J. P. M. Koenen, E. van Rijssen, L. B. Hilbrands, and I. Joosten. 2006. Rapamycin, and not cyclosporin A, preserves the highly suppressive CD27+ subset of human CD4+CD25+ regulatory T cells. *Blood* 107: 1018–1023.
43. Zeng, H., K. Yang, C. Cloer, G. Neale, P. Vogel, and H. Chi. 2013. mTORC1 couples immune signals and metabolic programming to establish T(reg)-cell function. *Nature* 499: 485–490.
44. Valmori, D., V. Tosello, N. E. Souleimanian, E. Godefroy, L. Scotto, Y. Wang, and M. Ayyoub. 2006. Rapamycin-mediated enrichment of T cells with regulatory activity in stimulated CD4+ T cell cultures is not due to the selective expansion of naturally occurring regulatory T cells but to the induction of regulatory functions in conventional CD4+ T cells. *J. Immunol.* 177: 944–949.
45. Chapman, N. M., H. Zeng, T. M. Nguyen, Y. Wang, P. Vogel, Y. Dhungana, X. Liu, G. Neale, J. W. Locasale, and H. Chi. 2018. mTOR coordinates transcriptional programs and mitochondrial metabolism of activated T_{reg} subsets to protect tissue homeostasis. *Nat. Commun.* 9: 2095.
46. Tsuji, M., N. Komatsu, S. Kawamoto, K. Suzuki, O. Kanagawa, T. Honjo, S. Hori, and S. Fagarasan. 2009. Preferential generation of follicular B helper T cells from Foxp3+ T cells in gut Peyer's patches. *Science* 323: 1488–1492.
47. Sawant, D. V., and D. A. A. Vignali. 2014. Once a Treg, always a Treg? *Immunol. Rev.* 259: 173–191.
48. Bailey-Bucktrout, S. L., M. Martinez-Llordella, X. Zhou, B. Anthony, W. Rosenthal, H. Luche, H. J. Fehling, and J. A. Bluestone. 2013. Self-antigen-driven activation induces instability of regulatory T cells during an inflammatory autoimmune response. *Immunity* 39: 949–962.
49. Yadav, M., C. Louvet, D. Davini, J. M. Gardner, M. Martinez-Llordella, S. Bailey-Bucktrout, B. A. Anthony, F. M. Sverdrup, R. Head, D. J. Kuster, et al. 2012. Neuropilin-1 distinguishes natural and inducible regulatory T cells among regulatory T cell subsets in vivo. *J. Exp. Med.* 209: 1713–1722, S1–S19.
50. Safinia, N., C. Scotta, T. Vaikunthanathan, R. I. Lechler, and G. Lombardi. 2015. Regulatory T cells: serious contenders in the promise for immunological tolerance in transplantation. *Front. Immunol.* 6: 438.
51. Rubtsov, Y. P., R. E. Niec, S. Josefowicz, L. Li, J. Darce, D. Mathis, C. Benoist, and A. Y. Rudensky. 2010. Stability of the regulatory T cell lineage in vivo. *Science* 329: 1667–1671.

52. Fantini, M. C., C. Becker, G. Monteleone, F. Pallone, P. R. Galle, and M. F. Neurath. 2004. Cutting edge: TGF-beta induces a regulatory phenotype in CD4+CD25- T cells through Foxp3 induction and down-regulation of Smad7. *J. Immunol.* 172: 5149–5153.
53. Gagliani, N., M. C. Amezcua Vesely, A. Iseppon, L. Brockmann, H. Xu, N. W. Palm, M. R. de Zoete, P. Licona-Limón, R. S. Paiva, T. Ching, et al. 2015. Th17 cells transdifferentiate into regulatory T cells during resolution of inflammation. *Nature* 523: 221–225.
54. Weiss, J. M., A. M. Bilate, M. Gobert, Y. Ding, M. A. Curotto de Lafaille, C. N. Parkhurst, H. Xiong, J. Dolpady, A. B. Frey, M. G. Ruocco, et al. 2012. Neuropilin 1 is expressed on thymus-derived natural regulatory T cells, but not mucosa-generated induced Foxp3$^+$ T reg cells. *J. Exp. Med.* 209: 1723–1742, S1.
55. Milpied, P., A. Renand, J. Bruneau, D. A. Mendes-da-Cruz, S. Jacquelin, V. Asnafi, M. T. Rubio, E. MacIntyre, Y. Lepelletier, and O. Hermine. 2009. Neuropilin-1 is not a marker of human Foxp3+ Treg. *Eur. J. Immunol.* 39: 1466–1471.
56. Fontenot, J. D., J. P. Rasmussen, L. M. Williams, J. L. Dooley, A. G. Farr, and A. Y. Rudensky. 2005. Regulatory T cell lineage specification by the forkhead transcription factor foxp3. *Immunity* 22: 329–341.
57. Allan, S. E., S. Q. Crome, N. K. Crellin, L. Passerini, T. S. Steiner, R. Bacchetta, M. G. Roncarolo, and M. K. Levings. 2007. Activation-induced FOXP3 in human T effector cells does not suppress proliferation or cytokine production. *Int. Immunol.* 19: 345–354.
58. Fontenot, J. D., M. A. Gavin, and A. Y. Rudensky. 2003. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat. Immunol.* 4: 330–336.
59. Kim, J. M., J. P. Rasmussen, and A. Y. Rudensky. 2007. Regulatory T cells prevent catastrophic autoimmunity throughout the lifespan of mice. *Nat. Immunol.* 8: 191–197.
60. Thornton, A. M., P. E. Korty, Y. C. Kim, C. Martens, and E. M. Shevach. 2018. Helios expression defines a phenotypically distinct population of Treg cells. *J. Immunol.* 200(Suppl. 1): 116.9.
61. Akimova, T., U. H. Beier, L. Wang, M. H. Levine, and W. W. Hancock. 2011. Helios expression is a marker of T cell activation and proliferation. *PLoS One* 6: e24226.
62. Pratama, A., A. Schnell, D. Mathis, and C. Benoist. 2020. Developmental and cellular age direct conversion of CD4+ T cells into RORγ+ or Helios+ colon Treg cells. *J. Exp. Med.* 217: e20190428.
63. Thornton, A. M., G. Kilaru, P. Burr, S. Rieder, S. A. Muljo, and E. M. Shevach. 2016. Helios expression defines two distinct populations of Foxp3+ regulatory T cells. *J. Immunol.* 196(Suppl. 1): 125.6.
64. Chougnet, C., and D. Hildeman. 2016. Helios-controller of Treg stability and function. *Transl. Cancer Res.* 5(Suppl. 2): S338–S341.
65. Sebastian, M., M. Lopez-Ocasio, A. Metidji, S. A. Rieder, E. M. Shevach, and A. M. Thornton. 2016. Helios controls a limited subset of regulatory T cell functions. *J. Immunol.* 196: 144–155.
66. Nakagawa, H., J. M. Sido, E. E. Reyes, V. Kiers, H. Cantor, and H.-J. Kim. 2016. Instability of Helios-deficient Tregs is associated with conversion to a T-effector phenotype and enhanced antitumor immunity. *Proc. Natl. Acad. Sci. USA* 113: 6248–6253.
67. McClymont, S. A., A. L. Putnam, M. R. Lee, J. H. Esensten, W. Liu, M. A. Hulme, U. Hoffmüller, U. Baron, S. Olek, J. A. Bluestone, and T. M. Brusko. 2011. Plasticity of human regulatory T cells in healthy subjects and patients with type 1 diabetes. *J. Immunol.* 186: 3918–3926.
68. Baine, I., S. Basu, R. Ames, R. S. Sellers, and F. Macian. 2013. Helios induces epigenetic silencing of IL2 gene expression in regulatory T cells. *J. Immunol.* 190: 1008–1016.
69. Ayyoub, M., F. Deknuydt, I. Raimbaud, C. Dousset, L. Leveque, G. Bioley, and D. Valmori. 2009. Human memory FOXP3+ Tregs secrete IL-17 ex vivo and constitutively express the T(H)17 lineage-specific transcription factor RORgamma t. *Proc. Natl. Acad. Sci. USA* 106: 8635–8640.
70. Afzali, B., P. J. Mitchell, F. C. Edozie, G. A. Povoleri, S. E. Dowson, L. Demandt, G. Walter, J. B. Canavan, C. Scotta, B. Menon, et al. 2013. CD161 expression characterizes a subpopulation of human regulatory T cells that produces IL-17 in a STAT3-dependent manner. *Eur. J. Immunol.* 43: 2043–2054.
71. Cuadrado, E., M. van den Biggelaar, S. de Kivit, Y. Y. Chen, M. Slot, I. Doubal, A. Meijer, R. A. W. van Lier, J. Borst, and D. Amsen. 2018. Proteomic analyses of human regulatory T cells reveal adaptations in signaling pathways that protect cellular identity. *Immunity* 48: 1046–1059.e6.
72. Heath, J. K., S. J. White, C. N. Johnstone, B. Catimel, R. J. Simpson, R. L. Moritz, G.-F. Tu, H. Ji, R. H. Whitehead, L. C. Groenen, et al. 1997. The human A33 antigen is a transmembrane glycoprotein and a novel member of the immunoglobulin superfamily. *Proc. Natl. Acad. Sci. USA* 94: 469–474.
73. Herbertson, R. A., N. C. Tebbutt, F.-T. Lee, S. Gill, B. Chappell, T. Cavicchiolo, T. Saunder, G. J. O'Keefe, A. Poon, S. T. Lee, et al. 2014. Targeted chemoradiation in metastatic colorectal cancer: a phase I trial of 131I-huA33 with concurrent capecitabine. *J. Nucl. Med.* 55: 534–539.
74. Zhuo, C., Z. Li, Y. Xu, Y. Wang, Q. Li, J. Peng, H. Zheng, P. Wu, B. Li, and S. Cai. 2014. Higher FOXP3-TSDR demethylation rates in adjacent normal tissues in patients with colon cancer were associated with worse survival. *Mol. Cancer* 13: 153.
75. Schwanhäusser, B., D. Busse, N. Li, G. Dittmar, J. Schuchhardt, J. Wolf, W. Chen, and M. Selbach. 2011. Global quantification of mammalian gene expression control. [Published erratum appears in 2013 *Nature* 495: 126–127.] *Nature* 473: 337–342.
76. Wilhelm, M., J. Schlegl, H. Hahne, A. M. Gholami, M. Lieberenz, M. M. Savitski, E. Ziegler, L. Butzmann, S. Gessulat, H. Marx, et al. 2014. Mass-spectrometry-based draft of the human proteome. *Nature* 509: 582–587.
77. Miyara, M., Y. Yoshioka, A. Kitoh, T. Shima, K. Wing, A. Niwa, C. Parizot, C. Taflin, T. Heike, D. Valeyre, et al. 2009. Functional delineation and differentiation dynamics of human CD4+ T cells expressing the FoxP3 transcription factor. *Immunity* 30: 899–911.
78. Canavan, J. B., C. Scottà, A. Vossenkämper, R. Goldberg, M. J. Elder, I. Shoval, E. Marks, E. Stolarczyk, J. W. Lo, N. Powell, et al. 2016. Developing in vitro expanded CD45RA+ regulatory T cells as an adoptive cell therapy for Crohn's disease. *Gut* 65: 584–594.
79. Hoffmann, P., R. Eder, T. J. Boeld, K. Doser, B. Piseshka, R. Andreesen, and M. Edinger. 2006. Only the CD45RA+ subpopulation of CD4+CD25high T cells gives rise to homogeneous regulatory T-cell lines upon in vitro expansion. *Blood* 108: 4260–4267.
80. Tian, Y., M. Babor, J. Lane, V. Schulten, V. S. Patil, G. Seumois, S. L. Rosales, Z. Fu, G. Picarda, J. Burel, et al. 2017. Unique phenotypes and clonal expansions of human CD4 effector memory T cells re-expressing CD45RA. *Nat. Commun.* 8: 1473.
81. Yu, N., X. Li, W. Song, D. Li, D. Yu, X. Zeng, M. Li, X. Leng, and X. Li. 2012. CD4(+)CD25 (+)CD127 (low/-) T cells: a more specific Treg population in human peripheral blood. *Inflammation* 35: 1773–1780.
82. Fuhrman, C. A., W.-I. Yeh, H. R. Seay, P. Saikumar Lakshmi, G. Chopra, L. Zhang, D. J. Perry, S. A. McClymont, M. Yadav, M.-C. Lopez, et al. 2015. Divergent phenotypes of human regulatory T cells expressing the receptors TIGIT and CD226. *J. Immunol.* 195: 145–155.
83. Bin Dhuban, K., E. d'Hennezel, E. Nashi, A. Bar-Or, S. Rieder, E. M. Shevach, S. Nagata, and C. A. Piccirillo. 2015. Coexpression of TIGIT and FCRL3 identifies Helios+ human memory regulatory T cells. *J. Immunol.* 194: 3687–3696.
84. Putnam, A. L., T. M. Brusko, M. R. Lee, W. Liu, G. L. Szot, T. Ghosh, M. A. Atkinson, and J. A. Bluestone. 2009. Expansion of human regulatory T-cells from patients with type 1 diabetes. *Diabetes* 58: 652–662.
85. Floess, S., J. Freyer, C. Siewert, U. Baron, S. Olek, J. Polansky, K. Schlawe, H. D. Chang, T. Bopp, E. Schmitt, et al. 2007. Epigenetic control of the foxp3 locus in regulatory T cells. *PLoS Biol.* 5: e38.
86. Douaisi, M., R. S. Resop, M. Nagasawa, J. Craft, B. D. Jamieson, B. Blom, and C. H. Uittenbogaart. 2017. CD31, a valuable marker to identify early and late stages of T cell differentiation in the human thymus. *J. Immunol.* 198: 2310–2319.
87. Karim, M., G. Feng, K. J. Wood, and A. R. Bushell. 2005. CD25+CD4+ regulatory T cells generated by exposure to a model protein antigen prevent allograft rejection: antigen-specific reactivation in vivo is critical for bystander regulation. *Blood* 105: 4871–4877.
88. Levine, A. G., A. Arvey, W. Jin, and A. Y. Rudensky. 2014. Continuous requirement for the TCR in regulatory T cell function. *Nat. Immunol.* 15: 1070–1078.
89. Haribhai, D., J. B. Williams, S. Jia, D. Nickerson, E. G. Schmitt, B. Edwards, J. Ziegelbauer, M. Yassai, S. H. Li, L. M. Relland, et al. 2011. A requisite role for induced regulatory T cells in tolerance based on expanding antigen receptor diversity. *Immunity* 35: 109–122.
90. Sadlon, T. J., B. G. Wilkinson, S. Pederson, C. Y. Brown, S. Bresatz, T. Gargett, E. L. Melville, K. Peng, R. J. D'Andrea, G. G. Glonek, et al. 2010. Genome-wide identification of human FOXP3 target genes in natural regulatory T cells. *J. Immunol.* 185: 1071–1081.
91. Prasad, D. V. R., T. Nguyen, Z. Li, Y. Yang, J. Duong, Y. Wang, and C. Dong. 2004. Murine B7-H3 is a negative regulator of T cells. *J. Immunol.* 173: 2500–2506.
92. Vogt, L., N. Schmitz, M. O. Kurrer, M. Bauer, H. I. Hinton, S. Behnke, D. Gatto, P. Sebbel, R. R. Beerli, I. Sonderegger, et al. 2006. VSIG4, a B7 family-related protein, is a negative regulator of T cell activation. *J. Clin. Invest.* 116: 2817–2826.
93. Johnstone, C. N., S. J. White, N. C. Tebbutt, F. J. Clay, M. Ernst, W. H. Biggs, C. S. Viars, S. Czekay, K. C. Arden, and J. K. Heath. 2002. Analysis of the regulation of the A33 antigen gene reveals intestine-specific mechanisms of gene expression. *J. Biol. Chem.* 277: 34531–34539.
94. Williams, B. B., N. C. Tebbutt, M. Buchert, T. L. Putoczki, K. Doggett, S. Bao, C. N. Johnstone, F. Masson, F. Hollande, A. W. Burgess, et al. 2015. Glycoprotein A33 deficiency: a new mouse model of impaired intestinal epithelial barrier function and inflammatory disease. *Dis. Model. Mech.* 8: 805–815.
95. Hassan, J., and D. J. Reen. 2001. Human recent thymic emigrants--identification, expansion, and survival characteristics. *J. Immunol.* 167: 1970–1976.
96. Mohr, A., R. Malhotra, G. Mayer, G. Gorochov, and M. Miyara. 2018. Human FOXP3 + T regulatory cell heterogeneity. *Clin. Transl. Immunol.* 7: e1005.
97. Vukmanovic-stejic, M., Y. Zhang, J. E. Cook, J. M. Fletcher, A. Mcquaid, J. E. Masters, M. H. A. Rustin, L. S. Taams, P. C. L. Beverley, D. C. Macallan, and A. N. Akbar. 2006. Human CD4+ CD25hi Foxp3+ regulatory T cells are derived by rapid turnover of memory populations in vivo. *J. Clin. Invest.* 116: 2423–2433.