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Single-Cell Analysis of the Human T Regulatory Population Uncovers Functional Heterogeneity and Instability within FOXP3+ Cells

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Natural FOXP3+CD4+CD25High regulatory T cells are critical in immunological self-tolerance. Their characterization in humans is hindered by the failure to discriminate these cells from activated effector T cells in inflammation. To explore the relationship between FOXP3 expression and regulatory function at the clonal level, we used a single-cell cloning strategy of CD25-expressing CD4+ T cell subsets from healthy human donors. Our approach unveils a functional heterogeneity nested within CD4+CD25High FOXP3+ T cells, and typically not revealed by conventional bulk assays. Whereas most cells display the canonical regulatory T cell characteristics, a significant proportion of FOXP3+ T cells is compromised in its suppressive function, despite the maintenance of other phenotypic and functional regulatory T hallmark features. In addition, these nonsuppressive FOXP3+ T cells preferentially emerge from the CD45RO+ memory pool, and arise as a consequence of a rapid downregulation of FOXP3 expression upon T cell reactivation. Surprisingly, these dysfunctional Treg cells with unstable FOXP3 expression do not manifest overt plasticity in terms of inflammatory cytokine secretion. These results open a path to an extensive study of the functional heterogeneity of CD4+CD25HighFOXP3+ Treg cells and warrant caution in the sole use of FOXP3 as a clinical marker for monitoring of immune regulation in humans.

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The isolation of nTreg cells from human blood based on high CD25 expression (CD4+CD25HIg T cells) is the most common strategy to enrich for most, but not all, FOXP3+ nTreg cells and to assess their phenotype and function (8–10). However, CD25, along with other Treg cell-associated markers like CTLA-4, HLA-DR, and GITR, do not distinguish nTreg cells from activated T effector cells (11–13). Moreover, activated human Treg cells can transiently express FOXP3, and it remains controversial whether this expression leads to the acquisition of suppressive capacity (14–16). Hence, the isolation and functional characterization of human FOXP3+ nTreg cells, in particular in the context of inflammation, have been hindered by a lack of reliable phenotypic markers.

In addition, nTreg cells may not constitute a homogeneous population. Indeed, important biological differences between naive and memory human CD4+CD25High nTreg cells have been described (17, 18). Notably, the naive, CD45RA+ Treg cell subset has been reported to preferentially expand in vitro, whereas the memory, CD45RO+ Treg cell compartment displays greater suppressive activity directly ex vivo, although it is prone to cell death and expands poorly in vitro, and is often contaminated with non-Treg cells, including Th17 cells (17, 19). Thus, naive and memory FOXP3+ nTreg cells constitute distinct functional subsets within the human Treg cell pool.

In addition, recent studies in mice have shown that the Treg cell pool is prone to functional plasticity, enabling cells to adopt overlapping functional profiles or to switch to an inflammatory lineage. As such, functional induced Foxp3+ Treg cells can differentiate from murine T effector cells extrathympically from non-regulatory precursors under unique inflammatory settings in vitro and in vivo (20), a process also observed in human T cells (21). Conversely, nTreg cells can lose Foxp3 expression upon transfer into a lymphopenic host or in inflammatory settings (22, 23) (E. Yurchenko and C. Piccirillo, unpublished observations). Similarly, a subset of human CD4+CD25High T cells can lose FOXP3 expression and initiate inflammatory cytokine secretion upon

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Abbreviations used in this article: MFI, mean fluorescence intensity; nTreg, natural regulatory T; rhIL-2, recombinant human IL-2; T effector, T effector T; Treg, regulatory T.

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sequential TCR triggering in vitro, although the suppressive activity of these cells was not assessed (24). In contrast, FOXP3+ T cells can mediate suppressive activity while simultaneously expressing IFN-γ and IL-17 (25–27). Thus, it is largely unknown whether and how the plasticity in human nTREG cells relates with their functional stability.

Hence, CD4+CD25hiFOXP3+ T cells most likely represent a heterogeneous population that comprises genuine nTREG cells as well as a variety of T cell subsets with a spectrum of Ag experiences, phenotypes, and functional profiles. In this study, we assess the correlation between the magnitude and stability of FOXP3 expression and nTREG cell function at the single-cell level. We show that a significant proportion of FOXP3+ T cell clones is incapable of suppression, despite the maintenance of phenotypic TREG cell features, as a consequence of a rapid downregulation of FOXP3 expression upon TCR reactivation. These nonsuppressive FOXP3+ T cells are more prevalent in the CD45RO+ memory pool and do not manifest an overt proinflammatory phenotype. Thus, the dynamic modulation of FOXP3 expression, rather than its direct ex vivo measurement, is a more accurate indicator of the TREG cell functional status, and warrants caution in the sole use of FOXP3 as a marker of immunoregulation in humans.

Materials and Methods

Human donors

Blood was obtained from healthy volunteers after informed consent. This study was performed in agreement with the ethical review board of McGill University and the Research Institute of the McGill University Health Center.

Cell isolation

PBMC were isolated by Ficoll-Hypaque gradient centrifugation (Amersham Pharmacia, Piscataway, NJ) and resuspended in complete RPMI 1640, stained with FITC-conjugated anti-CD4 (clone RPA-T4) and PE-conjugated anti-CD25 (clone M-A251) Abs, according to manufacturer’s instructions, and CD25-expressing subsets were isolated using a FACS Aria II cell sorter (BD Biosciences, Mississauga, ON, Canada). The final CD4+ T cell subsets were gated according to their CD25 expression. As follows: CD4+CD25neg cells designated as TEff cells, CD4+ T cells expressing intermediate CD25 levels as CD25 low, and the top 1% CD25-expressing CD4+ T cells as CD4+CD25hi TREG cells (Fig. 1, Supplemental Fig. 1).

Single-cell cloning for the generation of CD4+ T cell lines

CD4+CD25neg, CD4+CD25low, and CD4+CD25hi (top 1%) T cell subsets were prepared, as described above, and isolated by single-cell FACS sorting. Individual T cells were activated in complete RPMI 1640 (10% FBS) with anti-CD3 (clone OKT3, 30 ng/ml), 200 U/ml recombinant human IL-2 (rIL-2; gift of the Surgery Branch, National Cancer Institute, National Institutes of Health), and irradiated allogeneic PBMC (5 × 10 5 cells) in 96-well U-bottom plates. Cultures were supplemented with fresh IL-2 medium every 3–4d, and were restimulated after 10–12 d. The expansion was stopped and the cells were harvested for analysis after a total of 21–24 d. The cloning efficiency reached 30–50%, 15–25%, and 5–15% for the CD25neg, CD25low, and CD25high subsets, respectively. In the experiments presented in this work, each donor was used no more than twice, and each cloning was done using freshly drawn blood.

Thymidine-based suppression and proliferation assays

Freshly sorted allogeneic T Eff cells (2 × 10 5 cells) were cocultured with individual TREG cell clones at 1:1 and 1:3 Treg/T Eff cell ratios in 96-well U-bottom plates in the presence of irradiated PBMC (8 × 10 5 cells) and anti-CD3 (30 ng/ml) for 96–120 h. [3H]Thymidine was added to the cultures for the last 24 h. Percentage of suppression was calculated, as previously shown (28). For anergy assays, individual T cell clones were stimulated as above in the presence or absence of exogenous rIL-2 (200 U/ml). Thymidine incorporation was measured, as previously shown (28).

CFSE-based suppression assays

CFSE-based assays for suppression of T Eff cell proliferation and cytokine secretion were performed, as previously described (29). Briefly, freshly sorted T Eff cells (7 × 10 3 cells) were labeled with CFSE (5 μM) and cocultured with individual Treg cells in 96-well U-bottom plates in the presence of irradiated PBMC (30 × 10 3 cells) and anti-CD3 (30 ng/ml) for 72 h. For cytokine suppression assays, PMA (25 ng/ml), ionomycin (1 μg/ml), and Golgi Stop (BD Biosciences) were added for the last 4 h of culture. Cells were then fixed and permeabilized using the Foxp3 staining buffer set (eBioscience), as per manufacturer’s instructions.

Multiparametric flow cytometry

Labeling of surface molecules was performed, as previously described (28). Briefly, individual CD4+ T cell clones (2 × 10 5 cells) were stained with PE-conjugated anti-CD25 (BC96 or m-A251) or anti-CD127 (eBioRDR5), as previously described. For FOXP3 staining, cells were fixed and stained intracellularly with allophycocyanin-conjugated anti-human FOXP3 (236A/E7), as per manufacturer’s instructions. For the detection of cytokines or FOXP3 postactivation, cells were activated in the presence of CFSE-labeled, irradiated PBMC, anti-CD3, and IL-2 for 24 h. For cytokerne detection, PMA (25 ng/ml), ionomycin (1 μg/ml), and Golgi Stop (BD Biosciences) were added for the last 4 h of incubation. Cytokine and FOXP3 detections were performed in separate assays. Cells were then treated with the eBioscience fixation and permeabilization kit, and stained intracellularly with PE-conjugated anti–IFN-γ (48.B3) and allophycocyanin-conjugated anti–IL-2 (Miq-17H12), or with PE-conjugated anti–IL-10 (JES3-9D7) and allophycocyanin-conjugated anti–IL-17 (eBio17B7) Abs. Samples were acquired on a FACS Calibur or FACS Canto II (BD Biosciences), and data were analyzed with the Flowjo software. CFSE+ cells were excluded from analysis in the cytokine-staining experiments. To pool results from different staining procedures, FOXP3 mean fluorescence intensity (MFI) values were normalized for each procedure and are expressed as a percentage of maximum.

Statistical analysis

Statistical analysis was performed using the Prism 5.0 software (GraphPad, San Diego, CA).

Results

Isolation of FOXP3+ TREG cell subsets from CD4+CD25hi T cell populations in PBMC

Previous studies have shown that CD4+ T cells expressing high levels of CD25 and low levels of CD127 constitute a population highly enriched for FOXP3+ nTREG cells (30–32). Indeed, the CD25hi nTREG cell population, representing the top 1–2% of CD4+ cells, can be isolated from PBMC, and comprises ≥95% FOXP3+CD127 low cells with high suppressive potency (>85% at a 1:4 Treg/target ratio) and high dependence to IL-2 for TCR-induced proliferation (>95% anergy) (Fig. 1). Inversely, CD4+CD25- T cells express high levels of CD127 (>85% CD127+), are unable of suppression (<10% at 1:1 ratio), and proliferate readily in the absence of IL-2 (<50% anergy). Whereas a high proportion of FOXP3+ cells is found within the CD4+CD25 low subset (~65%), CD4+CD25 low cells still possess suppressive function, albeit reduced compared with CD25 hi cells (62% at 1:1 ratio), suggesting that the remaining FOXP3+CD25 low cells are functional (Fig. 1B–E). However, this FOXP3+CD25 low subset is preferentially enriched for CD45RA expression compared with their CD25 hi counterparts (59 versus 30%, respectively), thus displaying an increased naive phenotype similar to FOXP3+CD25 neg T Eff cells (Fig. 1C). Hence, CD25 hi and CD25 low Treg cell subsets are selectively enriched for memory and naive FOXP3+ nTREG cells, respectively. Differential inclusion of FOXP3+CD25 low subset in Treg cell preparations may vary depending on the CD25 hi gating strategy used.

Single-cell analysis discriminates distinct phenotypic subsets comprised within the CD4+CD25 hi Treg cell population

To distinguish functional and phenotypic subsets comprised within the CD4+CD25 hi Treg cell population, we examined the Treg cell population at the single-cell level. To this end, three CD4+ T cell subsets expressing varying levels of CD25 (CD25 hi, CD25 low, CD25 low).
and CD25\textsuperscript{Neg}) were isolated by single-cell FACS sorting, and expanded in vitro to establish a pool of T cell clones for each subset (29) (Supplemental Fig. 1). We then assessed the level of FOXP3 expression for each cell line. The expansion conditions were carefully titrated to produce significant numbers of clones, yielding sufficient cell numbers for phenotypic and functional assessment, yet minimizing any potential biasing effects for each population. In addition, the testing procedures themselves were optimized to reduce their requirements in cell numbers. This ensured that the clones could be assessed even when very small (5 \times 10^4 cells), allowing for milder expansion conditions, and for the screening of a greater, and thus most representative, number of the obtained clones.

Over 80% of the CD25\textsuperscript{High}-derived clones express FOXP3, a proportion that decreases in the CD25\textsuperscript{Low} (45%) and more so in CD25\textsuperscript{Neg}-derived clones (8%), a trend also reflected by the population’s median (group median MFI of 39.7, 4.4, and 2.2% of maximum, respectively) (Fig. 2A, 2B). Hence, the in vitro expanded T cell clones preserve their ability to express FOXP3 to a similar extent as the parental bulk population from which they were expanded (Figs. 1B, 2B).

We also assessed the phenotype of the expanded T cell clones for other hallmark T\textsubscript{REG} cell markers, including high CD25 and low CD127 expression, as well as inflammatory cytokine production (IFN-\gamma). Whereas the majority of CD25\textsuperscript{High} clones maintain high levels of CD25 expression (group median MFI of 35.6), a small fraction (20–25%) presents low CD25 levels comparable to that of CD25\textsuperscript{Neg} clones (Fig. 2C). The clones expanded from the CD25\textsuperscript{Low} subset encompass a spectrum of CD25 expression levels, suggesting they comprise cells derived from both CD25\textsuperscript{Neg} and CD25\textsuperscript{High} subsets (Fig. 2C). CD127 expression in each subset also recapitulates the levels found in the respective parental bulk populations (Figs. 1B, 2D).

\section*{FIGURE 1. Isolation of FOXP3\textsuperscript{+} T\textsubscript{REG} cell subsets from CD4\textsuperscript{+}CD25\textsuperscript{High} T cell populations in PBMC.} A. PBMC were isolated from the blood of healthy donors, stained for CD4 and CD25, and analyzed by FACS. The gates show the different T cell populations studied. CD25\textsuperscript{High} cells are restricted to the top 1% of CD25-expressing CD4\textsuperscript{+} T cells. B. T cell subsets were FACS sorted according to the gates shown in A, and stained for FOXP3 and CD127. C. PBMC were stained for CD4, CD25, FOXP3, and CD45RA. CD45RA expression profiles of CD25\textsuperscript{High} and CD25\textsuperscript{Low} FOXP3\textsuperscript{+} T cells are overlaid, and the proportion of CD45RA\textsuperscript{+} cells is reported for each of the two subsets. D and E. Each subset was FACS sorted and tested for proliferation capacity in the absence of rhIL-2 (D) and suppressive potency against freshly sorted CD25\textsuperscript{Neg} T\textsubscript{Eff} cells at different ratios by [\textsuperscript{3}H]thymidine incorporation assays (E). Results in D and E are shown as mean of triplicates \pm SEM. Data from one representative experiment of at least three are shown.

\section*{FIGURE 2. Single-cell analysis allows for the discrimination of distinct phenotypic subsets comprised within the CD4\textsuperscript{+}CD25\textsuperscript{High} Tcell population.} A–D. Representative histograms (A) and all clones were grouped according to their subset origin, and examined for expression levels of FOXP3 (B), CD25 (C), and CD127 (D). E and F. Intracellular IFN-\gamma production was assayed in individual clones after 24 h of in vitro restimulation in the presence of irradiated PBMC feeders, anti-CD3, and rhIL-2. Feeder cells were initially labeled with CFSE and subsequently excluded from analysis. Representative clones (E) and all clones grouped according to their population of origin (F) are shown. Data show >300 clones, from three experiments carried on three different donors.
clones express little or no CD127 (group median MFI of 7.5), in contrast to CD25^Low and CD25^Neg clones, which maintain intermediate to high CD127 levels (group median MFI of 12.2 and 16.16, respectively) (Fig. 2D).

These results show that the CD25^High cell pool, albeit enriched in CD127^Low and FOXP3^+ T_{REG} cells, is also populated with cells that lack FOXP3 expression and display variable levels of CD127 (Fig. 2D, Supplemental Fig. 2A). These cells are reminiscent of the T_{EFF} cell phenotype, and could reflect an original contamination of the CD25^High pool with activated, CD25^+ effector-like T cells (22–24). This was confirmed by the examination of the relative ability of expanded clones from CD25^Neg, CD25^Low, and CD25^High subsets to produce the effector cytokine IFN-γ upon in vitro TCR restimulation. We find that the vast majority of CD25^Neg clones, and to a lesser extent CD25^Low clones, express robust IFN-γ levels in contrast to CD25^High clones (group medians of 47.3, 31.8, and 6.5% IFN-γ cells, respectively) (Fig. 2E, 2F). This capacity to produce IFN-γ is inversely correlated with FOXP3 expression (Supplemental Fig. 2B). The majority of CD25^High clones that lack FOXP3 also express levels of IFN-γ comparable to that of CD25^Neg clones. We also observe a population of CD25^High clones coexpressing FOXP3 and intermediate levels of IFN-γ, constituting ∼15% of the FOXP3^+ CD25^{High} clones. Importantly, these clones display a suppressive potency comparable to that observed in the FOXP3^+ IFN-γ^+ clones (Supplemental Fig. 2C).

Overall, CD4^+CD25^{High} clones are primarily populated with CD25^CD127^{Low}FOXP3^+ T cells, but also contain distinct subpopulations of effector-like FOXP3^+ T cells and IFN-γ^+ FOXP3^+ suppressive T cells. Thus, the top 1% of CD4^+CD25^{High} T_{REG} cells is phenotypically diverse and contains cells with a potential of T_{EFF} cell function.

A subset of CD25^{High} nT_{REG} cells expressing FOXP3 is devoid of regulatory function

A tight correlation between FOXP3 expression and human nT_{REG} cell function has been difficult to establish due to harsh procedures to detect intranuclear FOXP3, and by the discrepant results obtained from ectopic, retroviral expression systems (30, 33). After single-cell expansion, we directly examined the functional consequences of natural FOXP3 expression at the clonal level. To this end, individual clones derived from the CD25^{High}, CD25^{Low}, and CD25^{Neg} cell subsets were assessed for suppressive ability and proliferative potential. Most CD25^{High} clones, and a significant fraction of the CD25^{Low} clones, are capable of strong suppression...
Whereas a few suppressive clones are found within the CD25 Neg manifest functional features consistent with their respective bulk.

We identify a novel cell subset comprised within FOXP3+CD4+ that correlates negatively with the expression of FOXP3 (correlation obtained from CD25 High, CD25 Low, and CD25 Neg cell subsets).

Strikingly, levels of FOXP3 expression after restimulation correlate either proliferation or cytokine production of TEFF cells.

A subset of clones expressing FOXP3 that are unable to suppress expression in clones (\( R^2 = 0.38, p < 0.0001 \)) (Fig. 4B), we find that ∼30% of all the FOXP3+ clones are not suppressive. Interestingly, this subset is found in both the CD25 High and the CD25Low clone pool, with a higher relative prevalence in the latter compartment (23.7% of FOXP3+ clones in the CD25High group are nonsuppressive, versus 41.2% in the CD25Low group).

We also find that the capacity to proliferate in the absence of IL-2 correlates negatively with the expression of FOXP3 (correlation \( R^2 = -0.46, p < 0.0001 \)) (Fig. 3D). Nevertheless, 20% of all FOXP3+ clones can still significantly proliferate in the absence of IL-2. This subset of less anergic FOXP3+ T cells overlaps for the most part with the nonsuppressive FOXP3+ subset (Fig. 3A). Thus, we identify a novel cell subset comprised within FOXP3+CD4+CD25High cells of healthy subjects, in which the natural expression of FOXP3 is dissociated from Treg cell functional features.

**Nonsuppressive FOXP3+CD25High clones do not inhibit T_{eff} cytokine secretion**

T_{reg} cells can suppress various aspects of T_{eff} cell activation and function, including proliferation and cytokine production. As such, it has been shown that CD4+CD25High T_{reg} cells can rapidly and potently suppress IL-2 and IFN-\( \gamma \) production in murine T_{reg} cells (34, 35). To determine whether these mechanisms were affected in nonsuppressive FOXP3+ cells, we examined the correlation between cytokine secretion and inhibition of proliferation in T_{reg} cells in a CFSE-based suppression assay. We observe that IL-2 and IFN-\( \gamma \) can be strongly downregulated in the presence of CD25High clones (Fig. 4A, 4B). However, suppression of T_{reg}-derived IL-2 is seemingly not correlated with the level of FOXP3 expression in clones (\( R^2 = 8 \times 10^{-4}, p = 0.92 \)) (Fig. 4B), or to the level of suppression of proliferation (\( R^2 = 0.004, p = 0.64 \)) (Fig. 4C). In contrast, IFN-\( \gamma \) suppression by FOXP3+ clones is directly proportional to the FOXP3 expression level (\( R^2 = 0.28, p < 0.0001 \)), and to suppression of proliferation (\( R^2 = 0.42, p < 0.0001 \)) (Fig. 4B, 4C). Hence, CD4+CD25High T cells contain a subset of clones expressing FOXP3 that are unable to suppress either proliferation or cytokine production of T_{reg} cells.

**Unstable FOXP3 expression drives a loss of suppressive potency in FOXP3+ T_{reg} cells**

As FOXP3 levels can be modulated upon TCR signals, we hypothesized that full T cell activation could destabilize FOXP3 expression and adversely affect the phenotype or function among dysfunctional FOXP3+ T_{reg} cells. To this end, individual clones from each subset were restimulated via their TCR in the presence of rhIL-2, and levels of FOXP3 expression were measured 48 h postactivation. Whereas resting clones were largely FOXP3+ (>80% FOXP3+ cells) prior to reactivation, the majority of the T_{reg} cell clones display a significant drop in FOXP3 levels upon stimulation, thus ranging from 25 to 95% FOXP3+ cells (Fig. 5A, 5B).

Strikingly, levels of FOXP3 expression after restimulation correlate tightly with the magnitude of suppression of T_{reg} cell proliferation (Pearson test, \( r = 0.635, p < 0.0001 \)) (Fig. 5B). As such, clones that were FOXP3+ at harvest and demonstrate a suppressive function maintain the highest levels of FOXP3 after 48 h, whereas clones that were FOXP3+, but lack suppressive potency, have lost most FOXP3 expression post-T cell activation (Fig. 5B, 5D). This loss in FOXP3 observed immediately upon restimulation does not depend on initial FOXP3 expression levels, as shown by the lack of correlation between levels of FOXP3 expression or proportion of FOXP3+ cells measured at harvest (time 0) and after 48 h (Fig. 5C).

Thus, the expression of FOXP3 in human T_{reg} cells can be transiently lost upon stimulation. The ensuing levels of FOXP3, rather than the preactivation levels, govern the functionality of these cells.

**Nonsuppressive FOXP3+ cells preferentially arise from the CD45RO+ memory pool**

The naive and memory compartments within FOXP3+ T_{reg} cells constitute subsets endowed with different functional potentials (17, 24). We hypothesized that the functional clusters within FOXP3+ clones could be the reflection of Ag experience in individual T cells prior to expansion in vitro. To this end, we examined whether the nonregulatory FOXP3+ fraction nested within CD4+CD25High cells was found preferentially in the memory compartment. Whereas the pool of nonfunctional FOXP3+ clones is present in the CD25high, CD25highCD45RA− (naive), and that were FOXP3+.
CD25HighCD45RO+ (memory) T cell compartments (30, 38, and 60% of FOXP3+ clones, respectively) (Fig. 6A), the memory compartment yields a higher proportion of nonfunctional FOXP3+ clones than the naive CD45RA+ cells. Accordingly, FOXP3+ clones obtained from the CD45RO+ fraction are more susceptible to activation-induced FOXP3 loss than those obtained from CD45RA+ cells (28.9 versus 48.9% average loss in percentage of FOXP3+ cells for the CD45RA+ and CD45RO+ populations, respectively) (Fig. 6B). Thus, memory CD45RO+FOXP3+ TREG cells are more prone to a lack of regulatory function caused by a downregulation of FOXP3 expression upon restimulation.

To exclude the possibility that loss of FOXP3 expression is a consequence of in vitro expansion, we assessed the level of FOXP3 upon in vitro restimulation in freshly isolated total, CD45RA+ or CD45RA−CD4+CD25High TREG cells. We find that ~30% of CD45RA− cells and total CD25High TREG cells rapidly lose FOXP3 expression, in contrast to only 10% in CD45RA+ cells (Fig. 6C, 6D). Hence, the preferential activation-induced instability in FOXP3 expression found in CD45RO+ memory TREG cell clones is readily recapitulated in memory TREG cells directly ex vivo.

**Loss of FOXP3 does not result in acquisition of a T effector-like inflammatory phenotype**

In mice, Foxp3+ nTREG cells convert into bone fide T effector cells in vitro and in vivo upon loss of Foxp3 expression. To determine whether the nTREG cell clones that lose FOXP3 acquire a T effector-like phenotype, we examined the nTREG cell phenotype and ability to produce inflammatory cytokines of suppressive and non-suppressive FOXP3+ clones following in vitro restimulation. Both suppressive and non-suppressive FOXP3+ clones exhibit comparable levels of the classical TREG cell markers CD25 (mean MFI, 45.1 ± 2.4 versus 40.1 ± 2.1), FOXP3 (mean MFI, 53.7 ± 2.1 versus 48.1 ± 2.7), and CD127 (mean MFI, 9.7 ± 0.6 versus 12.5 ± 1) (Fig. 7A), suggesting that the phenotypic aspects of TREG cells remain intact in these cells. In addition, both functional and nonfunctional FOXP3+ clones show low levels of proinflammatory (IL-2, IL-17, and IFN-γ) and anti-inflammatory (IL-10) cytokines (mean percentage of positive cells <3%) (Fig. 7B). We also examined IL-2 and IFN-γ levels in freshly isolated naive and memory CD4+CD25High TREG cells, which we have shown to be differentially prone to downregulation of FOXP3 expression. We find that CD25High cells produce insignificant levels of cytokines when compared with CD25− T effector cells (maximum 3.7 versus 23.9% IL-2 and 1.37 versus 14.8% IFN-γ), irrespective of their ability to maintain FOXP3 (Fig. 7C, 7D). Overall, we show that the memory FOXP3+ TREG cell population is enriched for a subset of cells exhibiting activation-triggered FOXP3 instability, although this does not lead to loss of the TREG cell phenotype or the acquisition of inflammatory features.
Expression and FOXP3-driven phenotype and function in human population (36). The determination of the stability of FOXP3 expression has been hampered by the lack of specific markers for this population. Furthermore, we identify a significant functional heterogeneity within the single-cell level in human PBMC. The characterization of human CD4+FOXP3+CD25High nTREG cells represents a unique, molecular signature of lineage commitment in FOXP3+ nTREG cells (40, 41). We have not assessed the instability of FOXP3 expression by sequestering FOXO proteins away from the promoter of FOXP3 (37, 38). Interestingly, TREG cells present an altered activation of the Akt pathway upon TCR triggering (39). Hence, FOXP3-regulating pathways such as Akt/mTOR may be modified in nonfunctional FOXP3+ cells, leading to a differential regulation of FOXP3 expression upon T cell activation. In addition, FOXP3 expression is known to be regulated by chromatin remodelling, whereby a fully demethylated foxp3 locus represents a unique, molecular signature of lineage commitment in FOXP3+ nTREG cells (40, 41). We have not assessed whether foxp3 locus remodeling is involved in the FOXP3 downregulation we observe, but it has been shown that dynamic remodeling of chromatin by histone acetyl transferases and histone deacetylases is engaged upon T cell activation (42, 43). Hence, T cell activation signals could repress de novo active transcription at the remodelled foxp3 locus in unstable TREG cells.

Currently, the molecular pathways that enable and sustain FOXP3 expression in TREG cells are not entirely known. The expression and function of FOXP3 have been found to be under the control of the Akt/mTOR axis downstream of the TCR, whereby an overactivation of the Akt pathway can prevent de novo induction of FOXP3 expression by sequestering FOXO proteins away from the promoter of FOXP3 (37, 38). Interestingly, TREG cells present an altered activation of the Akt pathway upon TCR triggering (39). Hence, FOXP3-regulating pathways such as Akt/mTOR may be modified in nonfunctional FOXP3+ cells, leading to a differential regulation of FOXP3 expression upon T cell activation. In addition, FOXP3 expression is known to be regulated by chromatin remodelling, whereby a fully demethylated foxp3 locus represents a unique, molecular signature of lineage commitment in FOXP3+ nTREG cells.

Discussion

The characterization of human CD4+FOXP3+CD25High nTREG cells has been hampered by the lack of specific markers for this population (36). The determination of the stability of FOXP3 expression and FOXP3-driven phenotype and function in human FOXP3+ nTREG cells has to date been lacking. We addressed these questions by evaluating the nTREG cell phenotype and function at the single-cell level in human PBMC. In this study, we report the development of a highly standardized, short-term, single-cell cloning strategy to study the dynamics of FOXP3 expression in Treg and TREG cells in various phenotypic and functional microarrays. Our assays with cloned cells mimic the hallmark features of ex vivo TREG cells, and are sensitive enough to detect even moderate suppression defects. Using such an approach, we identify a significant functional heterogeneity within the human CD4+CD25High T cell pool. We find that 35–40% of FOXP3 clones obtained from CD4+CD25High T cells lack suppressive function, caused by a drastic downregulation of FOXP3 expression upon restimulation, an outcome that does not affect the canonical phenotype of nTREG cells. We further recapitulated these observations in freshly isolated populations of CD4+CD25High TREG cells, demonstrating that our observations are not merely the product of in vitro clonal expansion.

Interestingly, we show that CD45RO+FOX3+ memory TREG cells display a preferential capacity to lose suppressive activity as a result of FOXP3 downregulation following activation, a functional property also seen in CD45RO+ TREG cells directly ex vivo. Recently, important biological differences between naive and memory human CD4+CD25High nTREG cells have been described, although the division of labor between these subsets in vivo remains unclear (17). Notably, memory CD45RO+CD4+CD25High TREG cells represent 50–70% of FOXP3+ T cells and manifest a greater suppressive function ex vivo, but are also more prone to secrete IL-17 (17, 25). Paradoxically, our results show that memory TREG cells also comprise a greater proportion of less potent TREG cells. It is unclear how to reconcile these findings; however, the proportion of memory cells losing FOXP3 ex vivo occurs in a small fraction that could remain undetected in traditional assays. In addition, recent studies comparing the suppressive potency of CD45RA+ and CD45RO+ TREG cells in CFSE-based assays suggest that these two subsets may in fact not differ significantly in their functional potency (17). Taken together, these results show that Ag experience is a contributing factor to the instability of FOXP3 expression and human nTREG cell function.
Recent studies in mice show that nT<sub>reg</sub> cells can lose Foxp3 expression and suppressive capacity, and acquire an effector-like Th1/Th17 proinflammatory phenotype. In contrast, our data show that T<sub>reg</sub> cells that lack suppressive activity and downregulate FOXP3 expression after activation maintain a T<sub>reg</sub> cell phenotype and do not spontaneously produce inflammatory cytokines. This is surprising, considering that FOXP3<sup>-</sup> or immune dysregulation polyendocrinopathy and enteropathy X-linked T cells are hyperactive due to the absence of FOXP3 function. However, FOXP3 expression and the transcriptional repression of cytokine genes downstream of FOXP3 are mediated epigenetically and dynamically upon TCR re-engagement (44). Hence, our observation could be explained by a possible requirement for specific signals or cytokine cues in order for these cells to either maintain FOXP3 expression and suppressive function, or fully differentiate into an inflammatory/pathogenic lineage.

FOXP3 interacts with a diversity of other proteins and transcription factors to carry out its multifaceted function (45–47). Our findings suggest a functional dichotomy of the function of FOXP3, whereby acquisition of the nT<sub>reg</sub> cell phenotype and regulatory function correspond to two distinct pathways in FOXP3<sup>+</sup> T cells, suggesting they may require distinct molecular partners and signaling events (48–50). As such, AML/Runx1 has been shown to be necessary for the suppressive function of Foxp3 in mice (51), whereas NFAT and AP1 directly regulate cellular anergy and cytokine genes such as il-2 (46). These factors could be differentially regulated upon T cell activation, or come to be lost as a result of maturation. In vivo, it is known that the function of FOXP3<sup>+</sup> T<sub>reg</sub> cells can be modulated by the cytokine milieu at sites of inflammation. As such, dendritic cells secrete high levels of IL-6, which is known to interfere with TGF-β1-driven stabilization of FOXP3 expression and function (52). The inflammatory mediator TNF-α has also been shown to inhibit T<sub>reg</sub> cell function (53).

These cytokines are thought to contribute to the lack of function of tissue-infiltrating T<sub>reg</sub> cells in the murine models of multiple sclerosis and inflammatory bowel disease (54–56). Similar mechanisms could be at play in both the results we obtained in vitro and the pathophysiology of autoimmune diseases. Overall, our studies will refine our mechanistic view of FOXP3 function, and reveal the conditions and molecular mechanisms at play in the induction of the intrinsic T<sub>reg</sub> cell phenotype, or their regulatory function, downstream of FOXP3.

We examined whether nonsuppressive FOXP3<sup>+</sup> clones were also affected in other forms of regulation, such as the downregulation of cytokine secretion in their targets. We found the production of IFN-γ by T<sub>eff</sub> cells to be in direct correlation with their proliferation, suggesting that clones that are unable to suppress the proliferation of T<sub>reg</sub> cells do not act by shifting the cytokine pattern in T<sub>reg</sub> cells. Interestingly, our data also showed that, contrary to previous conclusions obtained in bulk studies, the suppression of IL-2 production by T<sub>reg</sub> cells in their targets is not correlated to the expression of FOXP3, suggesting that this particular T<sub>reg</sub> cell function requires additional environmental cues or intracellular factors, such as those discussed above. In addition, it is possible that clonal-level examination reveals subpopulations of T<sub>reg</sub> cells that diverge in their functional mechanism. Hence, the dissociation of the suppression of IL-2 and proliferation could be the reflection of the heterogeneity in effector mechanism of the T<sub>reg</sub> cell population at the single-cell level. Thus, some clones suppress proliferation and IL-2, whereas others suppress without affecting IL-2. The underlying mechanism and physiological relevance of such dichotomy are not clear at the moment.

We also detect other smaller subsets, which are nested in the CD4<sup>+</sup>CD25<sup>High</sup> T cell pool. First, ~10% of clones found within the CD25<sup>High</sup> group are FOXP3<sup>-</sup>, lose CD25 expression in the course of expansion, have the ability to express both IFN-γ and
CD127, and do not suppress, all characteristics reminiscent of Th1 cells. These cells could be the result of an initial contamination of the CD25^{hi} pool by recently activated TREG cells. Alternatively, these cells could be the result of loss of the TREG cell features upon in vitro culture, as was recently described in mice and in a fraction of human CD25^{hi} TREG cells (23, 24, 57). Second, a small fraction (<5%) of the CD25^{hi} lines consists of suppressive CD25^{-} FOXP3^{+} TREG cells and is reminiscent of the TREG 1-induced Foxp3^{+} TREG cells, which has been described to mediate their suppressive activity through the secretion of IL-10 and/or TGF-β (58, 59). Lastly, a subset of FOXP3^{+} clones secretes low levels of IFN-γ and is reminiscent of IFN-γ− or IL-17−producing FOXP3^{+} TREG cells, which retain their suppressive activity (25–27, 60). The origin and nature of these cells are still unclear, although cells bearing similar features have been described in cord blood, suggesting that a subset of genuine TREG cells can secrete IFN-γ.

In conclusion, we identify unique functional signatures within CD4^{+} TREG cell subsets, and show that FOXP3 expression is not an absolute marker of T cell regulation in human peripheral blood. Rather, the function of TREG cells depends on the dynamic nature of FOXP3 expression upon activation and on the antigenic history of TREG cell populations. Further characterization of the functional subsets within FOXP3^{+} cells will allow the identification of molecular events acting either upstream or downstream of FOXP3 expression and that synergize to determine the fate and function of FOXP3^{+} cells, thus offering new identification strategies to isolate and monitor functionally relevant TREG cell subsets. It remains to be elucidated how these observations translate in situ upon Ag-dependent, specific and/or inflammatory activation. Ultimately, these studies will allow the development of novel immunodiagnostic tools in various disease and therapeutic settings.

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Disclosures

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