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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 (TREG) 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+CD25High FOXP3+ TREG cells and warrant caution in the sole use of FOXP3 as a clinical marker for monitoring of immune regulation in humans. The Journal of Immunology, 2011, 186: 6788-6797.

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regulation of FOXP3 expression, rather than its intermediate CD25 levels as CD25 Low, and the top 1% CD25-expressing CD4+ T cells as CD4+CD25High TREG cells (Fig. 1, Supplemental Fig. 1). CD4+CD25Neg cells were designated as T EFF cells, CD4+ T cells expressing intermediate CD25 levels as CD4+CD25Low, and the top 1% CD25-expressing CD4+ T cells as CD4+CD25High TREG cells (Fig. 1, Supplemental Fig. 1).

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

CD4+CD25Neg, CD4+CD25Low, and CD4+CD25High (top 1%) T cell subsets were prepared, as described above, and isolated by single-cell FACS sorting. Individual T cell clones were activated 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 FACSria 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 were designated as Tnaive, cells CD4+ T cells expressing intermediate CD25 levels as CD4+CD25Low, and the top 1% CD25-expressing CD4+ T cells as CD4+CD25High TREG cells (Fig. 1).

Thymidine-based suppression and proliferation assays

Freshly sorted allogeneic Tnaive cells (2 × 10^5 cells) were cocultured with individual TREG cell clones in 1:1 and 1:3 TREG/Tnaive 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 18 h. Thymidine incorporation was measured, as previously shown (28).

CFSE-based suppression assays

CFSE-based assays for suppression of Tnaive cell proliferation and cytokine secretion were performed, as previously described (29). Briefly, freshly sorted Tnaive cells (7 × 10^3 cells) were labeled with CFSE (5 μM) and cocultured with individual TREG cell clones in 96-well U-bottom plates in the presence of irradiated PBMC (30 × 10^5 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 Fix/Perm staining buffer set (eBiosciences), 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 (eBioRD58), 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 cytokine 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 (MQ-17H12), or with PE-conjugated anti-IL-10 (JES3-9D7) and allophycocyanin-conjugated anti-IL-17 (eBio7B7) Abs. Samples were acquired on a FACS caliber or FACS Canto II (BD Biosciences), and data were analyzed with the Flow jo software. CFSE+ Tcell clones 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+CD25High 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+ TREG cells (30–32). Indeed, the CD25High TREG cell population, representing the top 1–2% of CD4+ T cells, can be isolated from PBMC, and comprises ≥95% FOXP3+CD127Low 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+CD25Low 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+CD25Low subset (~65%), CD4+CD25Low cells still possess suppressive function, albeit reduced compared with CD25High cells (62% at 1:1 ratio), suggesting that the remaining FOXP3+CD25Low cells are functional (Fig. 1B–E). However, this FOXP3+CD25Low subset is preferentially enriched for CD45RA expression compared with their CD25High counterparts (59 versus 30%, respectively), thus displaying an increased naive phenotype similar to FOXP3+CD25Low TREG cells (Fig. 1C). Hence, CD25High and CD25Low TREG cell subsets are selectively enriched for memory and naive FOXP3+ TREG cells, respectively. Differential inclusion of FOXP3+CD25Low subset in TREG cell preparations may vary depending on the CD25High gating strategy used.

Single-cell analysis discriminates distinct phenotypic subsets comprised within the CD4+CD25High TREG cell population

To distinguish functional and phenotypic subsets comprised within the CD4+CD25High 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 (CD25High, CD25Low, CD25Neg)
and CD25 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 High-derived clones express FOXP3, a proportion that decreases in the CD25 Low (45%) and more so in CD25 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 reg cell markers, including high CD25 and low CD127 expression, as well as inflammatory cytokine production (IFN-\(\gamma\)). Whereas the majority of CD25 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 Neg clones (Fig. 2C). The clones expanded from the CD25 Low subset encompass a spectrum of CD25 expression levels, suggesting they comprise cells derived from both CD25 Neg and CD25 High subsets (Fig. 2C). CD127 expression in each subset also recapitulates the levels found in the respective parental bulk populations (Figs. 1B, 2D).

FIGURE 1. Isolation of FOXP3+ T reg cell subsets from CD4+CD25\(^{\text{High}}\) 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\(^{\text{High}}\) cells are restricted to the top 1% of CD25-expressing CD4+ 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 High and CD25 Low FOXP3+ T cells are overlaid, and the proportion of CD45RA+ 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 Neg T eff cells at different ratios by [\(^{3}H\)]thyminde incorporation assays (E). Results in D and E are shown as mean of triplicates ± SEM. Data from one representative experiment of at least three are shown.

FIGURE 2. Single-cell analysis allows for the discrimination of distinct phenotypic subsets comprised within the CD4+CD25\(^{\text{High}}\) T reg cell population. A–D, Representative histograms (A) and all clones were grouped according to their subset origin, and examined for expression levels of FOXP3 (B) and CD25 (C). 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.

FIGURE 3. A subset of CD25 High nT REG cells expressing FOXP3 is devoid of regulatory function. After in vitro expansion, clones derived from CD25 Neg, CD25 Low, or CD25 High subsets were stained for FOXP3 and assayed for suppressive function and IL-2–dependent anergy. A, Postexpansion suppressive capacity at a 1:1 ratio against freshly sorted T EFF cells. B, Correlation of suppressive potency with FOXP3 expression at harvest (t0) in T cell clones. Correlation $R^2 = 0.38$, $p < 0.0001$. Linear regression with a slope of 0.8381 is shown with its 95% confidence interval (dotted lines). Results shown in A and B were pooled from 10 independent experiments carried out on six healthy donors, compiling 609 clones. Dotted quadrants represent cutoff for strong suppression, which was set at 40% based on assay sensitivity. Quadrant statistics are shown on the graph. Quadrant statistic values for each group of clones are shown on the side. Reproducibility of these observations across clonings and donors is illustrated in Supplemental Fig. 3B.
tightly with the magnitude of suppression of TEFF cell proliferation. We observe that CD4+CD25High clones (Fig. 4A),4 nonsuppressive FOXP3+ clones can still significantly proliferate in the absence of IL-2 = 0.0001) (Fig. 5A). Hence, clones that were FOXP3+, but lack suppressive potency, have lost most FOX3 expression post-T cell activation (Fig. 5B, 5D). This loss in FOX3 observed immediately upon restimulation does not depend on initial FOX3 expression levels, as shown by the lack of correlation between levels of FOX3 expression or proportion of FOX3+ cells measured at harvest (time 0) and after 48 h (Fig. 5C).

As FOX3 levels can be modulated upon TCR signals, we hypothesized that full T cell activation could destabilize FOX3 expression and adversely affect the phenotype or function among dysfunctional FOX3+ Treg cells. To this end, individual clones from each subset were restimulated via their TCR in the presence of rhIL-2, and levels of FOX3 expression were measured 48 h postactivation. Whereas resting clones were largely FOX3+ (>80% FOX3+ cells) prior to reactivation, the majority of the Treg cells display a significant drop in FOX3 levels upon stimulation. The ensuing levels of FOX3, rather than the preactivation levels, govern the functionality of these cells. Non-suppressive FOX3+ cells preferentially arise from the CD45RO+ memory pool. The naive and memory compartments within FOXP3+ Treg cells constitute subsets endowed with different functional potentials (17, 24). We hypothesized that the functional clusters within FOX3+ 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 FOX3+ fraction nested within CD4+CD25High cells was found preferentially in the memory compartment. Whereas the pool of non-functional FOX3+ clones are present in the CD25Heroch, CD25HerCD45RA− (naive), and

Nonsuppressive FOX3+CD25Her clones do not inhibit T effector cell functional features

Unstable FOX3 expression drives a loss of suppressive potency in FOX3+ Treg cells

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The naive and memory compartments within FOXP3+ Treg cells constitute subsets endowed with different functional potentials (17, 24). We hypothesized that the functional clusters within FOX3+ clones could be the reflection of Ag experience in individual T cells prior to expansion in vitro. To this end, we examined whether the non-regulatory FOX3+ fraction nested within CD4+CD25High cells was found preferentially in the memory compartment. Whereas the pool of non-functional FOX3+ clones are present in the CD25Heroch, CD25HerCD45RA− (naive), and
CD25<sup>High</sup>CD45RO<sup>+</sup> (memory) T cell compartments (30, 38, and 60% of FOXP3<sup>+</sup> clones, respectively) (Fig. 6A), the memory compartment yields a higher proportion of nonfunctional FOXP3<sup>+</sup> clones than the naive CD45RA<sup>+</sup> cells. Accordingly, FOXP3<sup>+</sup> clones obtained from the CD45RO<sup>+</sup> fraction are more susceptible to activation-induced FOXP3 loss than those obtained from CD45RA<sup>+</sup> cells (28.9 versus 48.9% average loss in percentage of FOXP3<sup>+</sup> cells for the CD45RA<sup>+</sup> and CD45RO<sup>+</sup> populations, respectively) (Fig. 6B). Thus, memory CD45RO<sup>+</sup>FOXP3<sup>+</sup> 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 expression in freshly isolated total, CD45RA<sup>+</sup> or CD45RA<sup>−</sup>CD4<sup>+</sup>CD25<sup>High</sup> TREG cells. We find that ~30% of CD45RA<sup>−</sup> cells and total CD25<sup>High</sup> TREG cells rapidly lose FOXP3 expression, in contrast to only 10% in CD45RA<sup>+</sup> cells (Fig. 6C, 6D). Hence, the preferential activation-induced instability in FOXP3 expression found in CD45RO<sup>+</sup> 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<sub>eff</sub>-like inflammatory phenotype

In mice, Foxp3<sup>−/−</sup> nTREG cells convert into bone fide T<sub>eff</sub> cells in vitro and in vivo upon loss of Foxp3 expression. To determine whether the nTREG cell clones that lose FOXP3 acquire a T<sub>eff</sub>-like phenotype, we examined the nTREG cell phenotype and ability to produce inflammatory cytokines of suppressive and non-suppressive FOXP3<sup>+</sup> clones following in vitro restimulation. Both suppressive and non-suppressive FOXP3<sup>+</sup> clones exhibit comparable levels of the classical T<sub>reg</sub> 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<sup>+</sup> 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<sup>+</sup>CD25<sup>High</sup> T<sub>reg</sub> cells, which we have shown to be differentially prone to downregulation of FOXP3 expression. We find that CD25<sup>High</sup> cells produce insignificant levels of cytokines when compared with CD25<sup>−</sup> T<sub>eff</sub> cells (maximum 3.7 versus 23.9% IL-2<sup>−</sup> and 1.37 versus 14.8% IFN-γ<sup>−</sup>), irrespective of their ability to maintain FOXP3 (Fig. 7C, 7D). Overall, we show that the memory FOXP3<sup>+</sup> TREG cell population is enriched for a subset of cells exhibiting activation-triggered FOXP3 instability, although this does not lead to loss of the T<sub>reg</sub> cell phenotype or the acquisition of inflammatory features.
Discussion

The characterization of human CD4+FOXP3+CD25\textsuperscript{High} nT\textsubscript{REG} 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 nT\textsubscript{REG} cells has to date been lacking. We addressed these questions by evaluating the nT\textsubscript{REG} 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 T\textsubscript{EFF} and nT\textsubscript{REG} cells in various phenotypic and functional microassays. Our assays with cloned cells mimic the functional property also seen in CD45RO\textsuperscript{+} nT\textsubscript{REG} cells directly ex vivo. Recently, important biological differences between naive and memory human CD4+CD25\textsuperscript{High} nT\textsubscript{REG} cells have been described, although the division of labor between these subsets in vivo remains unclear (17). Notably, memory CD45RO\textsuperscript{+}CD4+CD25\textsuperscript{High} T\textsubscript{REG} 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 T\textsubscript{REG} cells also comprise a greater proportion of less potent T\textsubscript{REG} 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\textsuperscript{+} and CD45RO\textsuperscript{+} nT\textsubscript{REG} cells in CFSE-based assays suggest that these two subsets may in fact 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 nT\textsubscript{REG} cell function.

Currently, the molecular pathways that enable and sustain FOXP3 expression in T\textsubscript{REG} 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 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). Differently, nT\textsubscript{REG} 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+ nT\textsubscript{REG} cells (40, 41). We have not assessed whether foxp3 locus remodelling is involved in the FOXP3 downregulation we observe, but it has been shown that dynamic remodelling 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 T\textsubscript{REG} cells. Furthermore, it remains to be assessed whether these unstable T\textsubscript{REG} cells exhibit this phenotype in a definitive or transient manner. Discriminating between these hypotheses requires being able to reassess FOXP3 expression and function on the same clones at a later point in time, a measure that imposes a number of technical challenges. Thus, this unstable T\textsubscript{REG} cell phenotype could be permanent and reflect an advanced, irreversible stage in the progression toward a total loss of T\textsubscript{REG} cell phenotype and conversion into T\textsubscript{EFF} cells. However, our preliminary data argue in favor of a transient, rather than permanent, loss in FOXP3 expression because clones that had lost FOXP3 upon restimulation seemingly recover FOXP3 expression levels by 80% 7 d later (data not shown).
Recent studies in mice show that nTREG cells can lose Foxp3 expression and suppressive capacity, and acquire an effector-like Th1/Th17 proinflammatory phenotype. In contrast, our data show that TREG cells that lack suppressive activity and downregulate FOXP3 expression after activation maintain a TREG cell phenotype and do not spontaneously produce inflammatory cytokines. This is surprising, considering that FOXP3+ 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 nTREG cell phenotype and regulatory function correspond to two distinct pathways in FOXP3+ T cells, suggesting they may require distinct molecular partners and signaling events (48–50). As such, AML/RuntX1 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 ifn-g (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+ TREG 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 TREG cell function (53). These cytokines are thought to contribute to the lack of function of tissue-infiltrating TREG 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 TREG cell phenotype, or their regulatory function, downstream of FOXP3.

We examined whether nonsuppressive FOXP3+ 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 TREG cells to be in direct correlation with their proliferation, suggesting that clones that are unable to suppress the proliferation of TREG cells do not act by shifting the cytokine pattern in TREG cells. Interestingly, our data also showed that, contrary to previous conclusions obtained in bulk studies, the suppression of IL-2 production by TREG cells in their targets is not correlated to the expression of FOXP3, suggesting that this particular TREG 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 TREG 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 TREG 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+CD25+High T cell pool. First, ~10% of clones found within the CD25+High group are FOXP3+, 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(CD127) 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+ TReg cells (23, 24, 57). Second, a small fraction (<5%) of the CD25(CD127) lines consists of suppressive CD25- FOXP3+ TReg cells and is reminiscent of the TReg I-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 nTReg cells can secrete IFN-γ.

In conclusion, we identify unique functional signatures within CD4+ nTReg cell subsets, and show that FOXP3 expression is not an absolute marker of T cell regulation in human peripheral blood. Rather, the function of nTReg 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-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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