Immunity
Resource

Functional Delineation and Differentiation Dynamics of Human CD4$^+$ T Cells Expressing the FoxP3 Transcription Factor

Makoto Miyara,[1,10] Yumiko Yoshioka,[1,9] Akihiko Kitoh,[1,9] Tomoko Shima,[1,9] Kajsa Wing,[1] Akira Niwa,[2] Christophe Parizot,[3] Cécile Taflin,[3] Toshio Heike,[2] Dominique Valeyre,[4] Alexis Mathian,[3] Tatsutoshi Nakahata,[2] Tomoyuki Yamaguchi,[1] Takashi Nomura,[1] Masahiro Ono,[1] Zahir Amoura,[5,6] Guy Gorochov,[3,6] and Shimon Sakaguchi[1,7,8,*]

[1]Department of Experimental Pathology, Institute for Frontier Medical Sciences
[2]Department of Pediatrics, Graduate School of Medicine
Kyoto University, Kyoto 606-8507, Japan
[3]Institut National de la Santé et de la Recherche Médicale (INSERM) UMR-S 945, Laboratoire AP-HP d'immunologie cellulaire et tissulaire, Hôpital Pitié-Salpêtrière, 75013 Paris, France
[4]Pneumology Department, AP-HP Hôpital Avicenne, 93000 Bobigny, France
[5]Internal Medicine Department, AP-HP Hôpital Pitié-Salpêtrière, 75013 Paris, France
[6]Pierre and Marie Curie University, UPMC Paris Universitas, 75005 Paris, France
[7]Core Research for Evolutional Science and Technology (CREST), Japan Science and Technology Agency, Kawaguchi 332-0012, Japan
[8]WPI Immunology Frontier Research Center, Osaka University, Suita 565-0871, Japan
[9]These authors contributed equally to this work
[10]Present address: Internal Medicine Department and Institut National de la Santé et de la Recherche Médicale (INSERM) UMR-S 945, Laboratoire AP-HP d'immunologie cellulaire et tissulaire, Hôpital Pitié-Salpêtrière, 75013 Paris, France
*Correspondence: shimon@frontier.kyoto-u.ac.jp
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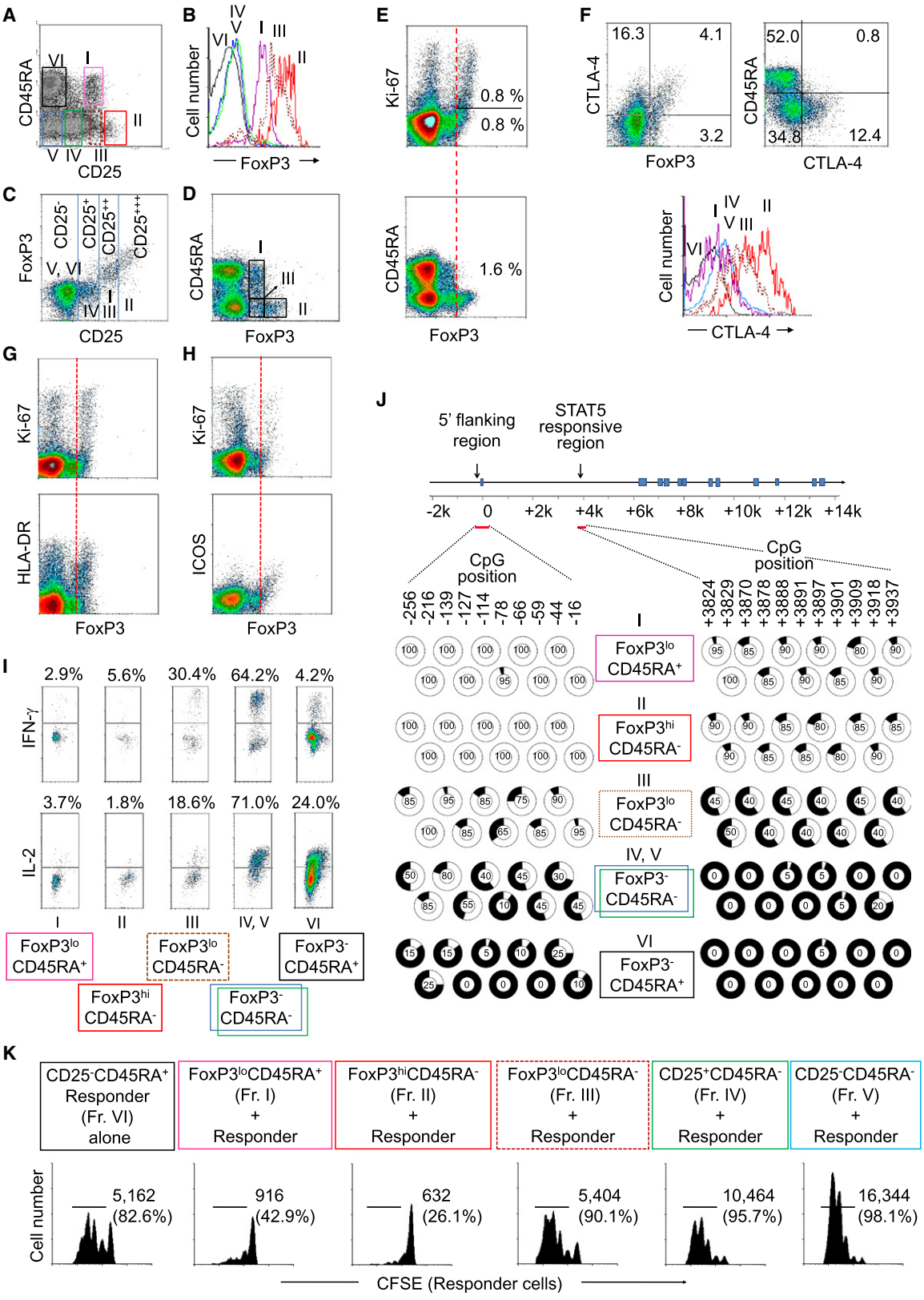
SUMMARY

FoxP3 is a key transcription factor for the development and function of natural CD4$^+$ regulatory T cells (Treg cells). Here we show that human FoxP3$^+$CD4$^+$ T cells were composed of three phenotypically and functionally distinct subpopulations: CD45RA$^+$FoxP3^{lo} resting Treg cells (rTreg cells) and CD45RA$^-$FoxP3^{hi} activated Treg cells (aTreg cells), both of which were suppressive in vitro, and cytokine-secreting CD45RA$^-$FoxP3^{lo} nonsuppressive T cells. The proportion of the three subpopulations differed between cord blood, aged individuals, and patients with immunological diseases. Terminally differentiated aTreg cells rapidly died whereas rTreg cells proliferated and converted into aTreg cells in vitro and in vivo. This was shown by the transfer of rTreg cells into NOD-scid-common γ-chain-deficient mice and by TCR sequence-based T cell clonotype tracing in peripheral blood in a normal individual. Taken together, the dissection of FoxP3$^+$ cells into subsets enables one to analyze Treg cell differentiation dynamics and interactions in normal and disease states, and to control immune responses through manipulating particular FoxP3$^+$ subpopulations.

INTRODUCTION

FoxP3-expressing CD4$^+$ thymus-derived naturally occurring regulatory T cells (Treg cells) play an indispensable role for the maintenance of self tolerance and immune homeostasis (Sakaguchi et al., 2008). They play crucial roles in human diseases, such as autoimmune disease, allergy, and cancer (Curiel et al., 2004; Ehrenstein et al., 2004; Kriegel et al., 2004; Miyara et al., 2005; Viglietta et al., 2004). Human natural Treg cells were initially defined according to their high expression of CD25 (Baecher-Allan et al., 2001; Dieckmann et al., 2001; Jonuleit et al., 2001; Levings et al., 2001; Ng et al., 2001; Taams et al., 2001), based on the finding that murine CD25$^+$CD4$^+$ T cells are highly suppressive (Sakaguchi et al., 1995). With the discovery of FoxP3 as a "master control gene" for CD4$^+$ Treg cell development and function (Fontenot et al., 2003; Hori et al., 2003; Khattri et al., 2003), detection of FoxP3 at the mRNA and protein level revealed that human CD25^{hi}CD4$^+$ T cells indeed express FoxP3 (Miyara et al., 2006; Roncador et al., 2005; Yagi et al., 2004). In contrast to murine FoxP3$^+$ Treg cells, however, human FoxP3$^+$ cells may not be functionally homogenous. For example, it has been reported that mere TCR stimulation can induce FoxP3 expression in apparently naive human FoxP3$^-$CD4$^+$ T cells without conferring suppressive activity (Allan et al., 2007; Gavin et al., 2006; Tran et al., 2007; Wang et al., 2007). Furthermore, some FoxP3$^+$ cells are phenotypically naive (e.g., CD45RA$^+$), present in cord blood as well as in peripheral blood of adults, and suppressive in vitro (Valmori et al., 2005), whereas other FoxP3$^+$ cells phenotypically resemble memory T cells (e.g., CD45RA$^-$) and are suggested to originate from peripheral memory FoxP3$^-$CD4$^+$ T cells (Vukmanovic-Stejic et al., 2006). To better understand the roles of FoxP3$^+$ T cells for the control of immune responses, it is necessary to determine whether FoxP3-expressing T cells in freshly isolated CD4$^+$ T cells are functionally heterogeneous, how functionally different subpopulations of FoxP3$^+$ cells can be reliably delineated, and how such


A
CD45RA
CD25
B
Cell number
FoxP3
C
FoxP3
CD25
D
CD45RA
FoxP3
E
Ki-67
0.8 %
0.8 %
CD45RA
1.6 %
FoxP3
F
CTLA-4
16.3
4.1
3.2
FoxP3
CD45RA
52.0
0.8
34.8
12.4
CTLA-4
Cell number
CTLA-4
G
Ki-67
HLA-DR
FoxP3
H
Ki-67
ICOS
FoxP3
I
IFN-γ
2.9%
5.6%
30.4%
64.2%
4.2%
IL-2
3.7%
1.8%
18.6%
71.0%
24.0%
I
II
III
IV, V
VI
FoxP3lo CD45RA+
FoxP3lo CD45RA-
FoxP3- CD45RA+
FoxP3hi CD45RA-
FoxP3- CD45RA-
J
5' flanking region
STAT5 responsive region
-2k
0
+2k
+4k
+6k
+8k
+10k
+12k
+14k
CpG position
-256 -216 -139 -127 -114 -78 -66 -59 -44 -16
+3824 +3829 +3870 +3878 +3888 +3891 +3897 +3901 +3909 +3918 +3937
I FoxP3lo CD45RA+
II FoxP3hi CD45RA-
III FoxP3lo CD45RA-
IV, V FoxP3- CD45RA-
VI FoxP3- CD45RA+
K
CD25-CD45RA+ Responder (Fr. VI) alone
FoxP3loCD45RA+ (Fr. I) + Responder
FoxP3hiCD45RA- (Fr. II) + Responder
FoxP3loCD45RA- (Fr. III) + Responder
CD25+CD45RA- (Fr. IV) + Responder
CD25-CD45RA- (Fr. V) + Responder
Cell number
5,162 (82.6%)
916 (42.9%)
632 (26.1%)
5,404 (90.1%)
10,464 (95.7%)
16,344 (98.1%)
CFSE (Responder cells)

subsets differentiate and interact in physiological and disease states.

In this report, we show that human FoxP3$^+$CD4$^+$ T cells can be separated into three functionally and phenotypically different subpopulations based on the expression of FoxP3, cell surface phenotype, the degree of DNA methylation of the FoxP3 gene, DNA microarray profile, proliferation status in the physiological state, cytokine secreting capacity, TCR repertoire, and in vitro suppressive activity. These populations are (1) CD45RA$^+$FoxP3^{lo} resting Treg cells, (2) CD45RA$^-$FoxP3^{hi} activated Treg cells, and (3) cytokine-secreting CD45RA$^-$FoxP3^{lo} non-Treg cells. With this dissection of FoxP3$^+$ T cells into subpopulations, we show the dynamics of Treg cell differentiation in vitro, in vivo, and ex vivo in normal and disease states. The results indicate that functional and numerical analysis of each FoxP3$^+$ subset is essential for assessing immunological states, and that manipulation of a particular subset, rather than whole FoxP3$^+$ cells, helps to dampen or augment a variety of physiological and pathological immune responses.

RESULTS

Separation of FoxP3$^+$CD4$^+$ T Cells into Three Subpopulations by the Expression of FoxP3, CD25, and CD45RA

The combination of CD25 and CD45RA staining of CD4$^+$ T cells in peripheral blood lymphocytes (PBL) of normal healthy individuals revealed six subpopulations (Fraction [Fr.] I–VI) that expressed the FoxP3 protein at different amounts (Figures 1A and 1B). Among them, Fr. I, II, and III were FoxP3$^+$ (Figure 1B) and the degree of FoxP3 expression in these fractions were proportional to CD25 expression (Figure 1C). Notably, these three FoxP3$^+$ populations could be distinctly separated by the combination of FoxP3 and CD45RA staining; i.e., FoxP3^{lo}CD45RA$^+$ cells, which were CD25^{++} (Fr. I), FoxP3^{hi}CD45RA$^-$ cells, which were CD25^{+++} (Fr. II), and FoxP3^{lo}CD45RA$^-$ cells, which were CD25^{++} (Fr. III) (Figure 1D). The fractions could be prepared as live cells by cell sorting as CD25^{++}CD45RA$^+$, CD25^{+++}CD45RA$^-$, and CD25^{++}CD45RA$^-$ cells, respectively (Figure S1A available online). Purified Fr. I, II, and III populations expressed FoxP3 transcripts to a similar degree irrespective of different amounts of FoxP3 protein expressed in each population (Figure 1B; Figure S1B). Fr. IV formed a distinct population as CD25$^+$FoxP3$^-$ cells (Figure 1C), but it was not well demarcated from Fr. V by CD45RA or CD25 staining (Figure 1A). Therefore, we analyzed Fr. IV and V together in the functional examination of FoxP3$^+$ subsets (see below).

Assessment of the proliferative status of each subpopulation in the physiological state by detecting the expression of Ki-67, a nuclear protein expressed in cells ready to proliferate and at a higher amount in actually proliferating cells (Figure S2), revealed that about half of the cells in Fr. II were proliferating whereas the cells in Fr. I and III were not (red dotted line in Figure 1E). Fr. II expressed intracellular CTLA-4 to the highest degree whereas Fr. I hardly expressed the molecule (Figure 1F). Furthermore, Fr. II corresponded to HLA-DR-expressing and also ICOS-expressing FoxP3$^+$ cells as reported by others (Figures 1G and 1H; Baecher-Allan et al., 2006; Ito et al., 2008).

Analysis of cytokine production by each fraction showed that Fr. II scarcely produced IL-2 or IFN-γ. Among FoxP3^{lo} cells, Fr. I was poor producer of IL-2 and IFN-γ whereas Fr. III produced high amounts of these cytokines (Figure 1I; Figure S3).

The 5′ flanking region and a STAT5-responsive region in the intron 1 of the *FOXP3* gene are critical for induction and enhancement of FoxP3 expression by TCR and IL-2 stimulation (Floess et al., 2007; Mantel et al., 2006; Zorn et al., 2006). Analysis of the DNA methylation status of these regions in each fraction prepared from a male donor showed that the CpG methylation sites in the regions were completely demethylated in Fr. I and Fr. II (Figure 1J). The 5′ flanking region of Fr. III was also highly demethylated, although the demethylation pattern was less uniform compared with Fr. I and II. In contrast, their STAT5-responsive region was less demethylated than other FoxP3$^+$ subsets. In addition, memory-like CD25$^+$ and CD25$^-$ CD45RA$^-$CD4$^+$ non-Treg cells (Fr. IV, V), which were FoxP3$^-$, had their 5′ flanking region moderately demethylated whereas the STAT5 responsive region was virtually completely methylated. Both regions were highly methylated in naive Fr. VI. These findings were confirmed by analysis of individual clones isolated from each subpopulation (Figure S4). The results collectively

Figure 1. Delineation of FoxP3$^+$CD4$^+$ T Cells into Subsets by Cell Surface Molecules, Proliferative State, Cytokine Production, Methylation Status of the *FOXP3* Gene, and In Vitro Suppressive Activity

(A–D) Six subsets of CD4$^+$ T cells defined by the expression of CD45RA and CD25: pink line (Fraction [Fr.] I), CD25^{++}CD45RA$^+$ cells; bold red line (Fr. II), CD25^{+++}CD45RA$^-$ cells; broken brown line (Fr. III), CD25^{++}CD45RA$^-$ cells; green line (Fr. IV), CD25$^+$CD45RA$^-$ cells; blue line (Fr. V), CD25$^-$CD45RA$^-$ cells; black line (Fr. VI), CD25$^-$CD45RA$^+$ cells. Expression of FoxP3 (B), CD25 and intracellular FoxP3 (C), and CD45RA and FoxP3 (D) in each fraction shown in (A). Data are representative of 19 blood donors.

(E) Flow cytometry of the expression of nuclear Ki-67 and FoxP3 in CD4$^+$ T cells. Red broken line separates Ki-67$^+$FoxP3^{hi} from Ki-67$^-$FoxP3^{lo} cells and CD45RA$^+$FoxP3^{lo} from CD45RA$^-$FoxP3^{hi} cells. The percentages of Ki-67$^+$ and Ki-67$^-$FoxP3^{hi} cells among CD4$^+$ cells are indicated in the top panel and the percentage of FoxP3^{hi}CD45RA$^-$ cells in the bottom panel.

(F) Flow cytometry of the expression of intracellular CTLA-4 and FoxP3 (top left); CD45RA and CTLA-4 (top right) by CD4$^+$ T cells; and expression of CTLA-4 by each fraction defined in (A)–(D) (bottom). Numbers indicate percent of cells in each quadrant.

(G and H) Expression of Ki-67 and FoxP3 (top) and of HLA-DR or ICOS and FoxP3 (bottom). Red broken line separates Ki-67$^+$FoxP3^{hi} from Ki-67$^-$FoxP3^{lo} cells and HLA-DR$^-$FoxP3^{lo} from HLA-DR$^+$FoxP3^{hi} cells (G) or ICOS$^-$FoxP3^{lo} from ICOS$^+$FoxP3^{hi} cells (H).

(I) Production of IFN-γ, IL-2 by each fraction after stimulation with PMA + ionomycin, and percent of cytokine-secreting cells in each fraction is shown. Data are representative of six independent experiments.

(J) Analysis of DNA methylation status at 5′ flanking region (left) and STAT5-responsive (right) region of the *FOXP3* gene in FoxP3-expressing or -nonexpressing CD4$^+$ T cell subsets (Figure S1) from PBMCs of one healthy male donor. Percentages of clones displaying demethylation of indicated CpG methylation sites are indicated and depicted in white in sector graphs. 19 to 20 clones were sequenced from each CD4$^+$ T cell subset.

(K) CFSE dilution by 10^4 labeled CD25$^-$CD45RA$^+$CD4$^+$ responder T cells assessed after 84–90 hr of TCR-stimulated coculture with indicated CD4$^+$ T cell subset at a 1 to 1 ratio. Cell number and percentage of dividing cells per well are indicated. Data are representative of 12 separate experiments.

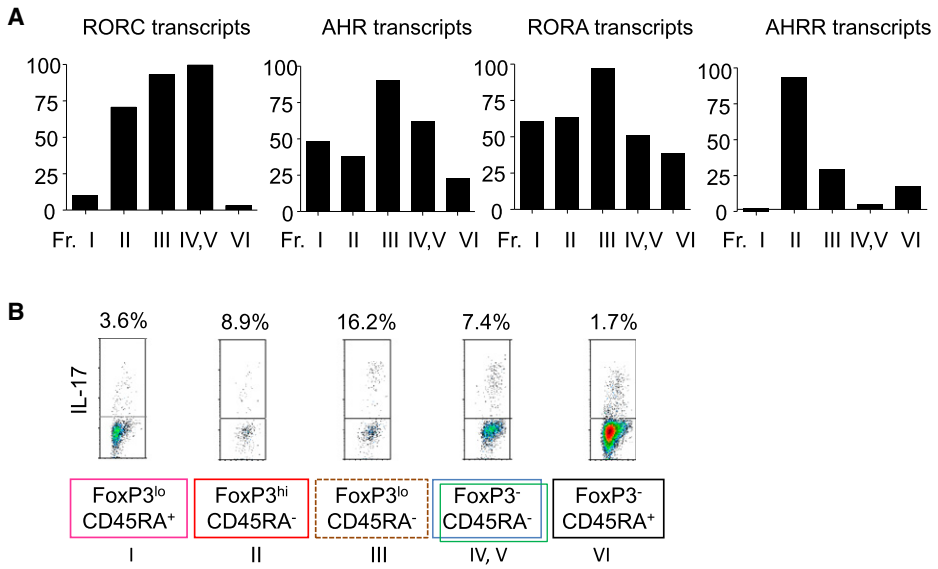


Figure 2. CD45RA$^-$FoxP3^{lo}CD4$^+$ T Cells Contain Cells with Th17 Cell Potential

(A) The amounts of transcripts of indicated genes in separated CD4$^+$ T cell subsets were assessed by quantitative PCR.

(B) Flow cytometry of the production of IL-17 by gated CD4$^+$ T cell subsets after stimulation with PMA + ionomycin for 5 hr. Percentages of cytokine-secreting cells are shown. Data are representative of six independent experiments.

indicate that Fr. I and II are active in FoxP3 gene transcription and close in their differentiation stage, and that, compared with these fractions, Fr. III may be less capable of maintaining FoxP3 expression in the presence of IL-2 and STAT5 signaling.

To assess the in vitro suppressive potency of each fraction, we measured the extent of CFSE dilution of labeled naive CD25$^-$CD45RA$^+$CD4$^+$ T cells (hereafter called responder cells) cocultured with an equal number of each fraction and stimulated for 4 days (Figure 1K). Fr. I and II (isolated as CD25^{++}CD45RA$^+$ and CD25^{+++}CD45RA$^-$ cells, respectively, as shown in Figure S1) potently suppressed the proliferation of responder cells, whereas Fr. III, IV, and V did not and even enhanced the responder proliferation. The inability of Fr. III (CD45RA$^-$Foxp3^{lo} cells) to suppress was confirmed by using CD127 as an additional marker for purifying FoxP3-expressing cells from CD4$^+$ T cells (Figure S5; Liu et al., 2006; Seddiki et al., 2006).

Taken together, three distinct subpopulations of FoxP3$^+$CD4$^+$ T cells can be defined in human PBL by the expression of CD45RA and FoxP3 as summarized in Table S1; i.e., Fr. I: CTLA-4^{lo}Ki-67$^-$CD45RA$^+$FoxP3^{lo} T cells; Fr. II: CTLA-4^{hi}CD45RA$^-$FoxP3^{hi} cells, both of which possess a fully functional *FOXP3* gene, hardly secrete cytokines, and potently suppress proliferation; and Fr. III: CTLA-4^{int}CD45RA$^-$FoxP3^{lo} T cells, which secrete cytokines, are much less active in the expression of the *FOXP3* gene under the control via STAT5, and do not suppress proliferation in vitro. Based on their phenotypic and functional characteristics, Fr. I and Fr. II can be designated as resting Treg cells (rTreg cells) and activated Treg cells (aTreg cells), respectively.

FoxP3^{lo}CD45RA$^-$ Nonregulatory T Cells Contain Cells with Th17 Cell Potential

DNA microarray analysis of each fraction showed that the gene expression patterns in the three FoxP3-expressing subpopulations were distinct (Figure S6A). Quantitative assessment of mRNA expression of differentially expressed genes revealed that the expression of RORC, a key transcription factor in Th17 cell lineage (Ivanov et al., 2006), was highly upregulated in Fr. II and III, indicating that RORC-FoxP3 double-positive population, which was recently described in mice (Yang et al., 2008a; Zhou et al., 2008), exists in humans as well in Fr. II and III (Figure S6B). Transcripts encoding RORα and AHR, both of which contribute to Th17 cell differentiation in mice (Veldhoen et al., 2008; Yang et al., 2008b), were highly upregulated in Fr.III, further indicating that this population contains cells with Th17 cell potential (Figure 2A). Also of note is that Fr. II specifically expressed high amounts of AHR repressor transcripts, suggesting that Treg cell differentiation might accompany an inhibition of Th17 cell differentiation via expression of AHR repressor. Assessment of cytokine production revealed that Fr. III was the highest producer of IL-17 even compared with naive FoxP3$^-$CD45RA$^+$ (Fr. VI) or memory-like FoxP3$^-$CD45RA$^-$CD4$^+$ non-Treg cells (Fr. IV and V) (Figure 2B; Figure S3).

Thus, DNA microarray profiling of FoxP3$^+$ subpopulations supports the relevance of separating FoxP3$^+$CD4$^+$ T cells into three subsets. Further, regarding the cell lineage relationship of FoxP3$^+$ cells and Th17 cells, Fr. III contains FoxP3-RORγ double-positive cells with a Th17 cell potential, in addition to IL-2- and/or IFN-γ-producing cells (Figure 1I).

Resting Treg Cells Proliferate whereas Activated Treg Cells Die while Suppressing In Vitro

As shown in Figure 1E, fresh Fr. I cells (rTreg cells) did not express Ki-67. However, when they were cocultured with responder cells and TCR stimulated, all the FoxP3-expressing cells became Ki-67$^+$ on day 4, indicating that rTreg cells proliferate (Figure S7). Assessment of proliferation by CFSE dilution during 4 days of culture also revealed that both aTreg cells

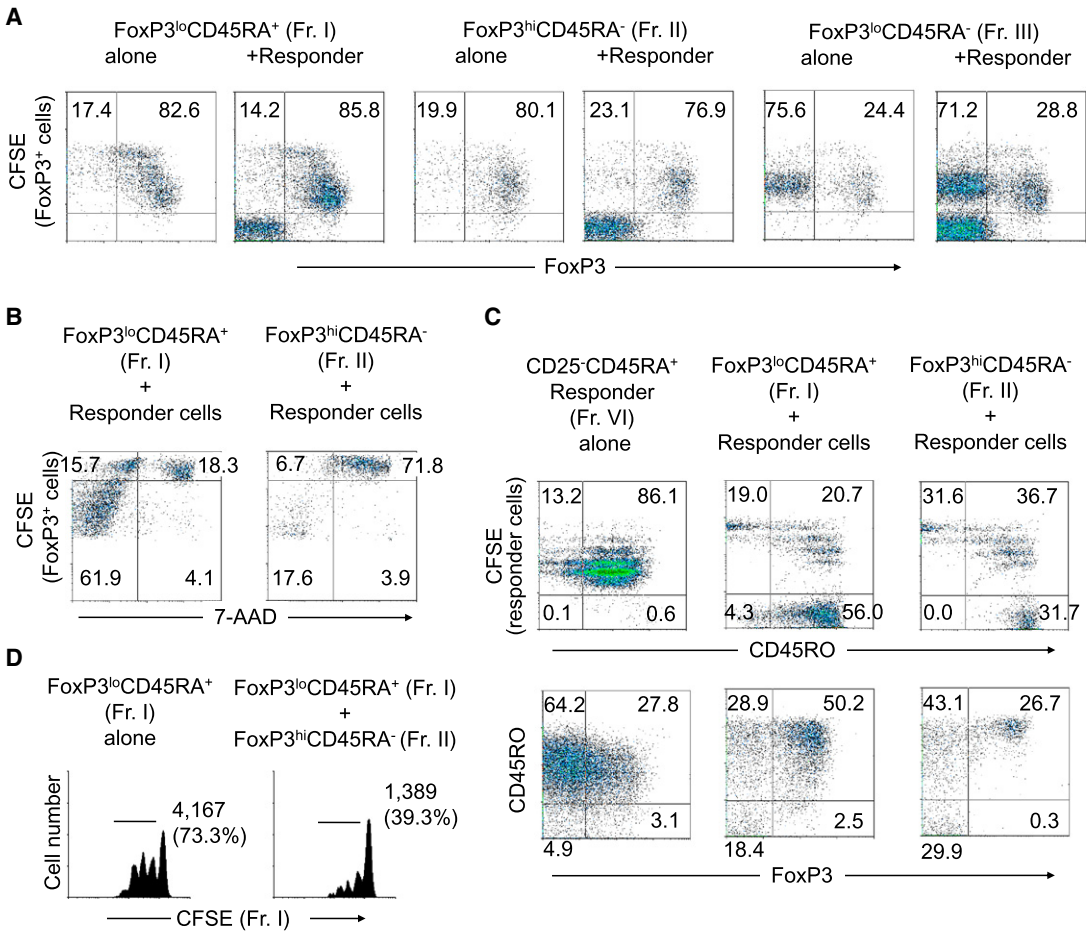


Figure 3. In Vitro Properties of FoxP3+ Subpopulations

(A) CFSE dilution by rTreg or aTreg cells and intracellular expression of FoxP3 were analyzed after 4 days TCR stimulation in the absence or presence of nonlabeled responder cells. Percentages of FoxP3$^-$ and of FoxP3$^+$ cells among CFSE-labeled cells are indicated.

(B) Viability assessed by 7-AAD staining of CFSE-labeled CD45RA$^+$FoxP3^{lo} (left) or CD45RA$^-$FoxP3^{hi} Treg cells (right) cocultured with nonlabeled responder cells for 4 days. Only CFSE-labeled cells are shown. Numbers indicate percentage in each quadrant. Data shown are representative of five independent experiments.

(C) CFSE dilution, surface CD45RO, and intracellular FoxP3 expression by CFSE-labeled responder cells cultured alone or cocultured with unlabeled Treg cell subsets for 4 days. Numbers indicate percentage in each quadrant.

(D) CFSE dilution of labeled rTreg cells cultured alone or with aTreg cells at a 1 to 1 ratio. Numbers and percentage of proliferating cells are indicated. Data shown in (B) and (D) are representative of five independent experiments.

(Fr. II) and rTreg cells (Fr. I) gave rise to CFSE-diluting FoxP3$^+$ cells when cultured alone (Figure 3A). In addition, rTreg cells showed more active proliferation than did aTreg cells in the presence of responder cells. In contrast with rTreg or aTreg cells, most (~70%) of CD45RA$^-$FoxP3^{lo}CD4$^+$ T cells (Fr. III) did not express FoxP3 during their proliferation, indicating that FoxP3 expression in the majority of Fr. III cells may not be stable in concordance with the methylation status of their *FOXP3* gene (Figure 3A).

Based on the finding that very few aTreg cells (Fr. II) were detectable after 4 days of culture (Figure S7), we assessed the viability of Treg cells by measuring incorporation of 7-AAD by CFSE-labeled rTreg or aTreg cells cultured with responder cells (Figure 3B). The majority (~75%) of aTreg cells were positive for 7-AAD. By contrast, although a fraction (~20%) of rTreg cells were nonproliferative and 7-AAD$^+$, the majority of proliferating rTreg cells (~60%) were 7-AAD$^-$ (Figure 3B). In addition to proliferation, rTreg cells showed increased expression of FoxP3 (Figure 3A), CD45RO (Figure 3C), and intracellular CTLA-4 (Figure S8). High expression of CD45RO was secondary to activation because rTreg cells, which were CD45RA$^+$, did not express CD45RO when freshly isolated from peripheral blood (Figure S9). Further, when CFSE-labeled rTreg cells (Fr. I) were cultured with nonlabeled aTreg cells (Fr. II), the latter substantially suppressed the proliferation of the former (Figure 3D).

Taken together, rTreg cells are not anergic and are able to proliferate upon TCR stimulation. They acquire a Ki-67$^+$FoxP3^{hi} aTreg cell phenotype and exert suppression during and after their proliferation and conversion to aTreg cells, which die after proliferation and exertion of suppression. Activated Treg cells also suppress the proliferation of resting Treg cells in a negative feedback fashion. Thus, in addition to different cell surface phenotypes, rTreg and aTreg cells possess different cell fates despite their comparable in vitro suppressive activity when assessed separately.

In Vivo Conversion of rTreg Cells to aTreg Cells and Differentiation of a Small Fraction of FoxP3− Cells to FoxP3+ Cells

Next, to investigate whether the in vitro conversion of rTreg cells to aTreg cells could also occur in vivo, we transferred human PBMCs containing CFSE-labeled $CD4^+$ T cells into NOG (Nod-scid-common γ-chain-deficient) mice and analyzed their splenocytes 5 days after transfer (Hiramatsu et al., 2003). $FoxP3^{hi}CD4^+$ T cells recovered in the recipients were largely $CD25^{hi}$ and $CD45RO^+$ (data not shown) and mostly confined to Ki-67^+CFSE-diluting cells, which had divided more than 6 times after transfer (Figure 4A). In addition, most $CD4^+$ T cells expressing low amounts of FoxP3 had not proliferated. These findings correspond to the in vitro findings that $FoxP3^{hi}$ aTreg cells found in PBMCs were highly proliferative and that $FoxP3^{lo}CD4^+$ T cells were Ki-67^- in PBMCs (Figure 1E), suggesting that, upon activation, rTreg cells upregulate FoxP3 expression and then proliferate. We also examined the behavior of Treg cells or whole $FoxP3^+$ cells when injected without other effector $CD4^+$ T cells. Neither population proliferated, indicating that the maintenance and proliferation of FoxP3-expressing cells requires the presence of other $CD4^+$ T cells in vivo (Figure S10).

Similar analysis of PBMCs containing CFSE-labeled rTreg cells, prepared as shown in Figure 1A and Figure S1, showed that they proliferated in vivo and upregulated the expression of FoxP3 and CD45RO along several cell divisions (Figure 4B and data not shown). Because most $FoxP3^{hi}$ cells were detected in CFSE-negative cells after transfer of CFSE-labeled $CD4^+$ T cells or rTreg cells (Figures 4A and 4B), we attempted to determine whether rTreg cells were the major source of CFSE-negative $FoxP3^{hi}$ cells. Injection of PBMCs containing CFSE-labeled $CD4^+$ T cells devoid of rTreg cells revealed a much lower number of $FoxP3^{hi}$ cells when compared with injection of whole $CD4^+$ T cells, indicating that most $FoxP3^{hi}$ aTreg cells derive from rTreg cells (Figure 4C).

Further, to investigate whether the conversion of $FoxP3^-$ to $FoxP3^+$ in $CD4^+$ T cells could occur in vivo, we transferred PBMCs containing CFSE-labeled $FoxP3^-$ $CD127^{hi}CD4^+$ T cells together with nonlabeled $FoxP3^+$ cells (as $CD25^{hi}CD127^{lo}$ cells) in NOG mice and examined whether $FoxP3^-CD4^+$ T cells could upregulate FoxP3 in vivo (Figure 4D). Although most CFSE-labeled cells remained $FoxP3^-$, a small number of cells upregulated FoxP3 from low to high amounts. Injection of only CFSE-labeled $CD4^+FoxP3^-$ cells confirmed that a small fraction (<1%) of $CD4^+FoxP3^-$ cells indeed divided at least 6 times to give rise to $FoxP3^{hi}$ cells (Figure 4E). Taken together, these results indicate that rTreg cells convert to aTreg cells and that only a small fraction of aTreg cells derives from $FoxP3^-CD4^+$ non-Treg cells in vivo.

In Vivo Conversion of rTreg Cells to aTreg Cells in a Normal Human Individual

To obtain further evidence for the in vivo rTreg to aTreg cell conversion in normal humans, we attempted to trace clonotypes of each Treg cell fraction in a single individual at separate time points. Cells in rTreg cells (Fr. I), aTreg cells (Fr. II), and also $FoxP3^-$ non-Treg $CD4^+$ T cells (Fr. IV, V, VI) that expressed the same TCRBV5 family were sorted from a single healthy individual at 18 month intervals. Single-cell RT-PCR and DNA sequencing of the amplicons was performed to compare TCRBV5 CDR3 regions in each sorted subset. Given the small size of the sample, the analysis was able to monitor dominant clones only. First, we observed that rTreg (Fr. I) and aTreg (Fr. II) cell subsets shared few dominant clonotypes at a given time point. Second, we found that a clonotype initially detected in the rTreg cell subset was found dominant 18 months later in the aTreg but not in the rTreg cell subset. The TCR repertoire being potentially so heterogeneous and the sample size being so limited (45 to 137 cells analyzed in each subset), it is highly improbable that T cell clones with identical TCR sequences could be found in the same subsets only by chance. Indeed, when random samples of conventional $CD4^+$ T cells were similarly compared, shared clonotypes were never found in this individual (Figure 5; Table S2). The analysis also revealed that none of the clonotypes found in $FoxP3^-$ cells was found in aTreg cells 18 months later, indicating that if conversion of $FoxP3^-CD4^+$ T cells ever occurs in vivo, it may not be a frequent phenomenon compared with the conversion of rTreg cells to aTreg cells (Figure 5).

Based on these observations in Figures 4 and 5, we conclude that most $FoxP3^{hi}$ aTreg cells are derived from recently activated and vigorously proliferating rTreg cells, and that only a minority of aTreg cells can develop from $FoxP3^-CD4^+$ non-Treg cells in vivo. The result also indicates that the TCR repertoire of Treg cells, in particular that of aTreg cells, adaptively changes in normal individuals.

Variations in Human rTreg and aTreg Cell Populations under Normal and Disease Conditions

We then attempted to determine whether the dissection of $FoxP3^+$ T cells in adult humans into Fr. I–III based on CD45RA and FoxP3 expression was pertinent to the analysis of the dynamics of Treg cell generation and differentiation in ontogeny, aging, and disease states.

Although rTreg cells were highly prevalent in cord blood, we could easily detect Ki-$67^+FoxP3^{hi}CD45RA^-CD4^+$ T cells that corresponded to aTreg cells in adults. We failed to confirm the previously reported finding that all $CD25^+CD4^+$ T cells were $FoxP3^+$ in cord blood (Fritzsching et al., 2006). However, $CD4^+$ T cells expressing the highest amounts of CD25 contained only $FoxP3^+$ cells and CD127 expression efficiently separated $FoxP3^+CD25^+$ from $FoxP3^-CD25^+CD4^+$ T cells (Figure 6A). IFN-γ production was barely detectable in whole $CD4^+$ T cells whereas IL-2 production was observed in $FoxP3^{lo}CD45RA^-CD4^+$ T cells as in adults (data not shown).

Analysis of the expression of CD31 (PECAM-1), which is known to be expressed in recent thymic emigrants but lost during their post-thymic peripheral expansion (Kimmig et al., 2002), revealed that almost all $CD31^+FoxP3^+CD4^+$ T cells in adult PBL were confined in the $CD45RA^+FoxP3^{lo}$ population (Fr. I). This finding suggests that the majority of rTreg cells may be recently derived from the thymus (Figure 6B).

The proportion of rTreg cells (Fr. I) among $CD4^+$ T cells was decreased in aged donors (1.1% ± 0.59%, n = 12 versus 2.40% ± 0.89% in healthy donors, n = 29; $p < 0.0001$) whereas that of aTreg cells (Fr. II) was increased (2.48% ± 1.07% versus 1.63% ± 0.53%; $p = 0.01$; Figures 6C and 6D).

We next applied our new definition of $FoxP3^+$ T cell subsets to the analysis of two pathological conditions that reportedly show

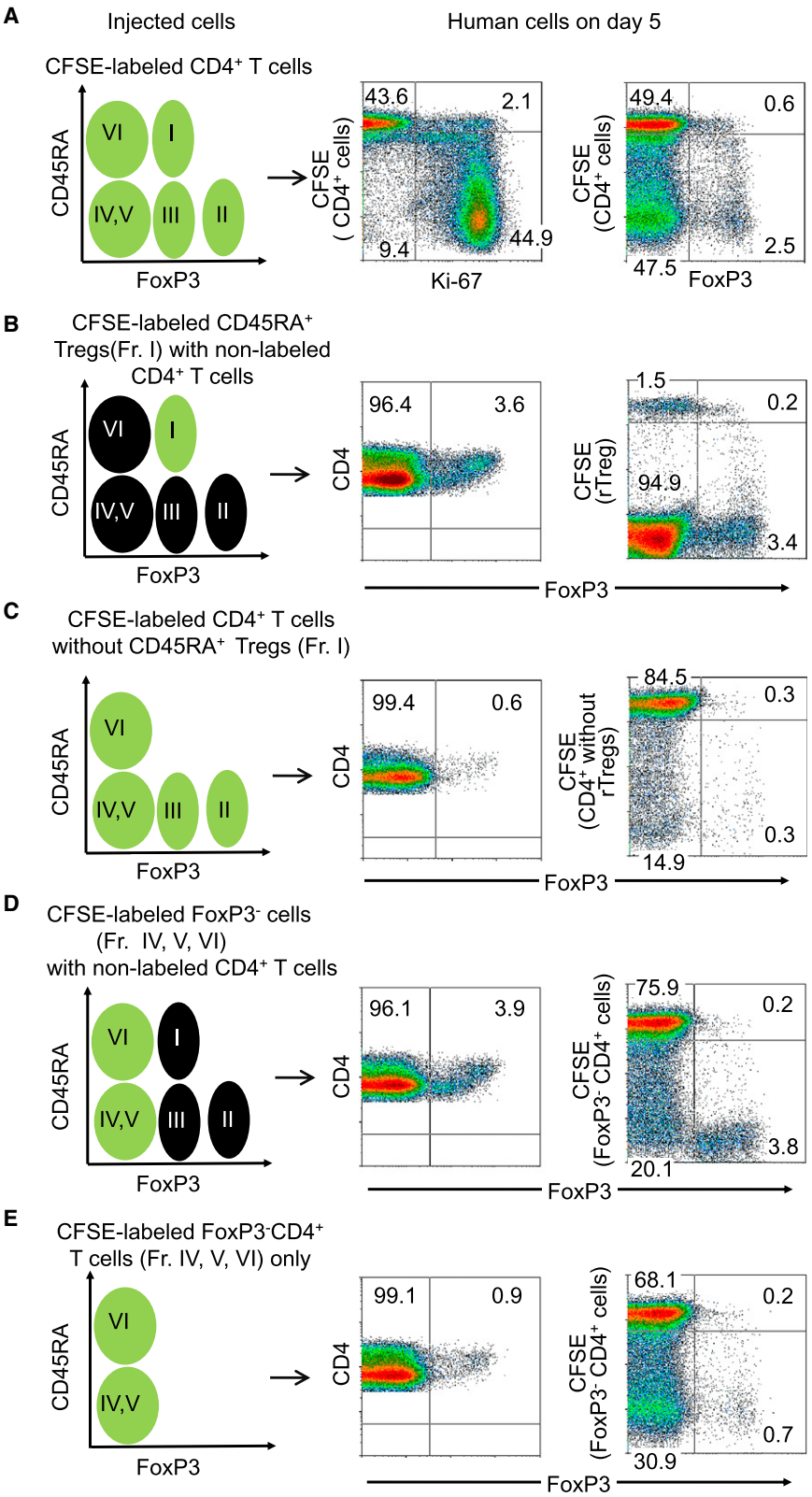


Figure 4. In Vivo Conversion of Treg Cell Phenotype in NOG Mice

PBMCs containing human CD4+ T cells were i.v. injected in NOG mice and collected in the spleen 5 days later. In schematic representations (left) of flow cytometric profiles of injected cells before transfer, CFSE-labeled CD4+ T subsets are depicted in green and injected with nonlabeled cells in black. Flow cytometry of human CD4+ T cells in the spleen after transfer of PBMCs containing CFSE-labeled human whole CD4+ T cells (A) or indicated CFSE-labeled CD4+ T cell subpopulations (B–E) into NOG mice. Numbers indicate percentage in each quadrant (right). Representative data of four mice transferred with PBMCs containing CFSE-labeled CD4+ T cells isolated from three different donors (A), and mice (two for each condition) transferred with PBMCs with indicated CFSE-labeled T cell populations obtained from two different donors (B–E).

different patterns of Treg cell involvement (Miyara et al., 2005, 2006). In sarcoidosis, a granulomatous disease of unknown origin, patients with active disease showed a considerable increase in the proportion of aTreg cells among CD4+ T cells (4.67% ± 3.35%, n = 41; $p < 0.0001$) combined with a high prevalence of Ki-67+FoxP3^{hi}CD4+ T cells and a decrease in the proportions of rTreg cells (1.48% ± 0.89%; $p < 0.0001$). In active systemic lupus erythematosus (SLE), a prototype of systemic autoimmune disease, there was a decrease in the proportion of aTreg cells (1.24 ± 0.72; n = 15; $p = 0.006$) and an increase in the proportions of rTreg cells (4.2 ± 1.86; $p = 0.0008$). Notably, CD45RA$^-$FoxP3^{lo} non-Treg cell fraction (Fr. III) increased to form a distinct population in active SLE (10.37% ± 9.3% versus 3.04 ± 1.1 in healthy donors; $p < 0.0001$; Figures 6C and 6D).

Thus, distinction of Treg cell subsets simply based on the combination of CD45RA and FoxP3 expression is highly informative in assessing the dynamics of Treg cell differentiation under physiological and disease conditions.

DISCUSSION

We have shown in this report that FoxP3+ cells in human PBL are heterogeneous in function and include not only suppressive T cells but also nonsuppressive ones that abundantly secrete proinflammatory cytokines such as IL-17. Further, Treg cells functionally and phenotypically differentiate within the FoxP3+ population. This functional heterogeneity and differentiation dynamics can be clearly shown by separating FoxP3+ cells into three subsets based on the expression of FoxP3 and CD45RA (or CD45RO), which

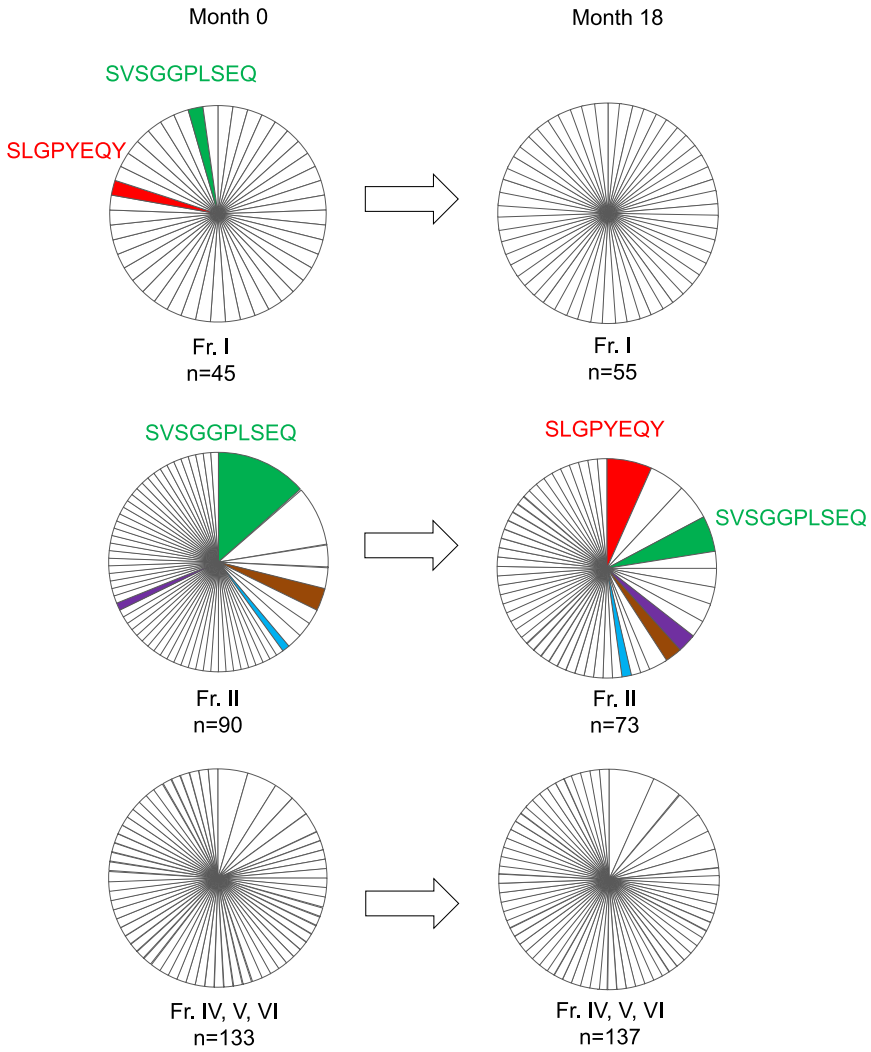


Figure 5. Longitudinal TCR Repertoire Analysis in FoxP3+ Cell Subpopulations

TCRBV5+CD4+ T cells belonging to indicated Treg or non-Treg cell fractions were FACS sorted as a single cell at indicated time points into wells of PCR plates. By RT-PCR and TCR sequencing, the frequencies of individual sequences were assessed. Empty slices correspond to sequences that were found only once in only one subset. Persistent clones are color highlighted. Slice size is proportional to the number of occurrences of the corresponding TCR sequences. The total number of sequences successfully analyzed in each subset is indicated.

controls signaling thresholds in lymphocytes, hence their state of differentiation and activation (Hermiston et al., 2003). The three subpopulations are CD45RA$^-$FoxP3^{hi}CD4$^+$ activated Treg cells and CD45RA$^+$FoxP3^{lo}CD4$^+$ resting Treg cells, both of which are potently suppressive in vitro, and cytokine-secreting nonsuppressive CD45RA$^-$FoxP3^{lo}CD4$^+$ non-Treg cells.

The distinction of FoxP3$^+$ subpopulations revealed the differentiation pathways of Treg cell subpopulations. First, most of FoxP3^{hi} aTreg cells originate from rTreg cells as shown in vitro and in vivo, although some FoxP3^{hi} Treg cells may arise from FoxP3$^-$CD4$^+$ non-Treg cells (Vukmanovic-Stejic et al., 2006). Second, a large proportion of FoxP3^{hi} aTreg cells is highly proliferative in vivo and appears to be recently activated, although most rTreg cells are in a resting state. Once rTreg cells are stimulated, they upregulate FoxP3 expression, differentiate to aTreg cells, and proliferate. In addition to these results obtained by direct ex vivo analysis of the subpopulations and by their transfer to NOG mice, our longitudinal study of the repertoire of a particular TCR Vβ subfamily in a single individual provides further evidence that the conversion of rTreg to aTreg cells physiologically occurs in vivo. In our current analysis, dominant clones found in the rTreg cell population were found 18 months later in the aTreg, but not rTreg, cell population. This indicates that the clones have preferentially expanded after differentiating to aTreg cells, as suggested by the high rate of Ki-67$^+$ cells in the aTreg cell population. The presence of such dominant T cell clones over a long period of time is a key feature of the aTreg cell population (C.P. and G.G., unpublished data). This also indicates that the TCR repertoire of Treg cells adaptively changes with clonal expansion especially in aTreg cells. Considering that aTreg cells rapidly die in vitro and that the immune system is constantly challenged by exogenous and endogenous antigens, it is highly likely that the maintenance of the pool of aTreg cells is the consequence of a tight balance between the constant development of aTreg cells from activated and proliferating rTreg cells and their death after exerting suppression. Further, aTreg cells control rTreg cell expansion in a feedback manner, contributing to the maintenance of the balance. FoxP3^{hi} aTreg cells thus appear to be terminally differentiated Treg cells; yet it remains to be determined whether the aTreg cell population contains a memory type long-living Treg cells. Given that aTreg cells die rapidly and that rTreg cells are highly proliferative upon stimulation, attempts to expand Treg cells ex vivo for cell therapy should be focused on rTreg cells as proposed by others (Hoffmann et al., 2006). It needs to be determined whether rTreg cells are constantly produced by the thymus, or whether they have a high renewal capacity in the periphery, or both.

Our study has clearly shown that human FoxP3$^+$CD4$^+$ T cells contain cytokine-secreting nonsuppressive effector T cells that display low expression of FoxP3. These nonsuppressive FoxP3^{lo}CD45RA$^-$CD4$^+$ T cells (Fr. III) may correspond to recently described activation-induced FoxP3-expressing cells that transiently express FoxP3 in vitro (Allan et al., 2007; Gavin et al., 2006; Tran et al., 2007; Wang et al., 2007). Supporting this notion, although the 5′ flanking region of the *FOXP3* gene in FoxP3^{lo}CD45RA$^-$CD4$^+$ T cells is highly demethylated, the STAT5-responsive region is poorly demethylated, suggesting that they may be unstable in maintaining FoxP3 expression through

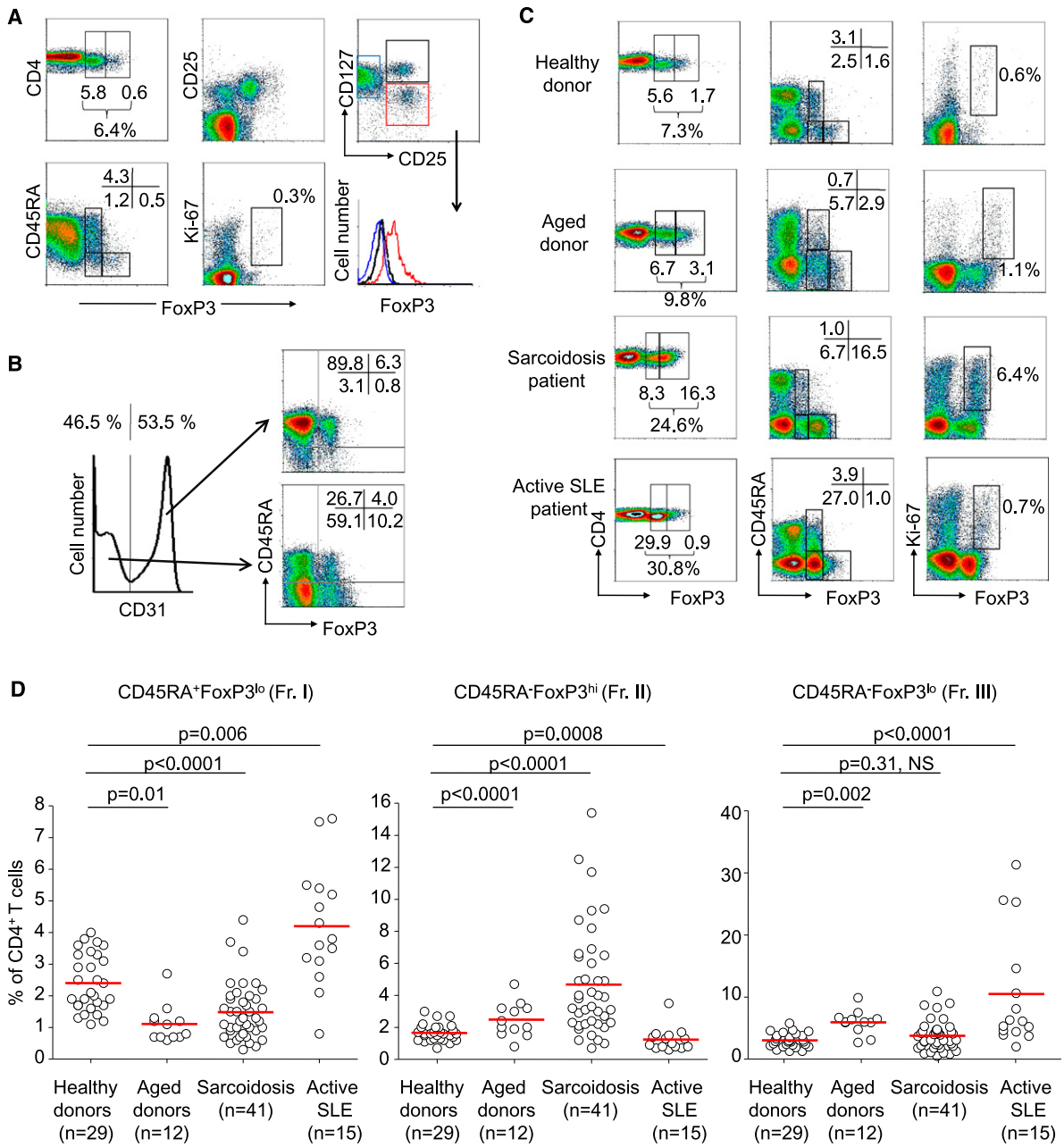


Figure 6. Variations in FoxP3+ Cell Subpopulations under Physiological and Disease Conditions

(A) Flow cytometry of PBMCs gated on $CD4^+$ T cells isolated from cord blood. A representative of four samples.

(B) Expression of CD45RA and FoxP3 by gated $CD31^+$ or $CD31^-$ $CD4^+$ T cells. Numbers indicate percentage in each quadrant. A representative of four independent experiments.

(C) Flow cytometry of PBMCs gated on $CD4^+$ T cells isolated from a 29-year-old healthy adult, an 88-year-old donor, and two patients with active sarcoidosis or active SLE. Percentage of each quadrant in each staining combination is shown.

(D) Percentages of each $FoxP3^+$ subset among $CD4^+$ T cells in indicated numbers of patients with active sarcoidosis, active SLE, aged donors (between 79 and 90 years old), and healthy donors (between 18 and 40 years old). Red horizontal bars represent mean percentage. Statistical comparisons were performed by nonparametric Mann-Whitney U test. NS, not significant.

STAT5 signaling. The notion is also supported by recent reports showing that activation-induced $FoxP3^+CD4^+$ T cells have *FOXP3* DNA significantly less demethylated than bona fide Treg cells (Baron et al., 2007; Janson et al., 2008). In addition, although CD127 is a convenient marker for isolating $FoxP3^+$ cells as $CD25^{hi}CD127^{lo}CD4^+$ T cells (Liu et al., 2006; Seddiki et al., 2006), it is of note that they also include $FoxP3^+$ non-Treg cells. We therefore propose that the combination of CD25 and CD45RA is so far the best markers for purifying human $FoxP3^+$ Treg cells as rTreg and aTreg cells.

We noticed in microarray analysis that RORC, the human ortholog of murine RORγt, a major transcription factor for Th17 cell differentiation (Ivanov et al., 2006), was highly upregulated in both Fr. II and III and much less in Fr. I. Indeed, FoxP3^{lo} memory-like non-Tregs (Fr. III) were the highest producers of IL-17 among CD4$^+$ T cells. These results support recent findings in mice that FoxP3-RORγt double-positive CD4$^+$ T cells can convert into either Treg cells or Th17 cells (Yang et al., 2008a; Zhou et al., 2008). AHR was recently shown in mice to be critical for the differentiation of naive T cells to Th17 versus FoxP3$^+$ Treg cells (Quintana et al., 2008; Veldhoen et al., 2008). Our finding of upregulated AHR repressor in aTreg cells therefore suggests that differentiation of FoxP3$^-$CD4$^+$ T cells to aTreg cells might be regulated through the modulation of AHR activity by AHR repressor. Further study is required to determine how the expression amount of FoxP3 in each FoxP3$^+$ subpopulation contributes to the function of each subset (e.g., suppression and IL-17 production) through interaction with other molecules including RORC.

This study has revealed several key features of Treg cell-mediated suppression in vitro. First, challenging the commonly accepted notion that Treg cells are anergic in vitro, human Treg cells proliferate and die, although the degree of their proliferation is much lower than that of non-Treg cells when Treg cells and non-Treg cells are separately stimulated and compared. Further, the hypoproliferation observed with CD25^{hi}CD4$^+$ T cells can be attributed, in part, to the suppression of rTreg cell proliferation by aTreg cells and also to the death of the latter. These findings mean that thymidine uptake by whole cocultured cells in Treg cell assay may not be accurate to monitor responder cell proliferation in the presence of Treg cells. Second, CTLA-4 expression in aTreg cells, but not in rTreg cells, suggests that aTreg cells are the main effectors of suppression as shown by the fact that Treg cell-specific deficiency impairs Treg cell suppressive function in vivo and in vitro in mice (Wing et al., 2008). Further, FoxP3$^+$ Treg cells out-compete naive T cells in in vitro aggregation around dendritic cells and downregulate their expression of CD80 and CD86 in a CTLA-4-dependent fashion (Onishi et al., 2008). It is likely in humans that, upon activation, rTreg cells differentiate to aTreg cells and exert suppression in vitro through these mechanisms. As another possibility, rTreg and aTreg cells might use different suppressive mechanisms by secreting different immunosuppressive cytokines such as IL-10 and TGF-β (Ito et al., 2008). Our microarray analysis indeed indicates that aTreg cells are more active in IL-10 transcription but less active in TGF-β transcription than rTreg cells. Further study is required to determine whether Treg cells use multiple suppressive mechanisms depending on their differentiation status (Sakaguchi et al., 2008).

Finally, supporting physiological and clinical relevance of distinguishing subpopulations of FoxP3$^+$ T cells, rTreg and aTreg cells can be clearly identified with different proportions in cord blood of healthy newborns, PBL of aged individuals, and patients with SLE or sarcoidosis. In cord blood, we unexpectedly found a small but always detectable population of CD45RA^{lo}Ki-67$^+$FoxP3^{hi}CD4$^+$ T cells that corresponded to adult aTreg cells. This finding suggests that even in fetuses, natural rTreg cells are constantly activated by endogenous self antigens and exogenous antigens derived from maternal circulation. An opposite trend exists in aged donors, who had high proportions of aTreg cells and low but still detectable proportions of rTreg cells. Because of thymus involution observed in aged individuals, one can speculate that, like conventional naive CD4$^+$ T cells (Vrisekoop et al., 2008), rTreg cells can be generated in the periphery in aged individuals to compensate for decreased thymic production of Treg cells; alternatively, but not exclusively, aTreg cells may homeostatically expand to counterbalance the lack of rTreg cells in the periphery. Under pathological conditions, a high prevalence of aTreg cells and a decrease in the rTreg cell population in sarcoidosis suggests that rTreg cells may be swiftly converted into aTreg cells immediately after having emigrated from the thymus or having been peripherally generated. In contrast, in active SLE, the number of aTreg cells decreased while that of rTreg cells remained normal or increased, with a notable increase in FoxP3^{lo}CD4$^+$ non-Treg cells. This also confirms the FoxP3^{lo}CD45RA$^-$ memory/effector-like non-Treg cell subset as a discrete population among FoxP3$^+$ CD4$^+$ T cells. Further functional analysis is required to interpret these anomalies and variations in disease states (Taflin et al., 2009). Yet, analysis of Treg cell function by dissecting FoxP3$^+$ cells into three subpopulations is instrumental for understanding pathophysiology of immunological diseases.

In conclusion, we propose a definition of human FoxP3$^+$ Treg cell subsets based on in vitro and in vivo features of FoxP3-expressing CD4$^+$ T cells. Functional and numerical analysis of each subset will help to understand and control immune responses in normal and disease states.

EXPERIMENTAL PROCEDURES

Human Samples

Blood samples were obtained from young healthy adult volunteers (18–40 years old), from aged control donors (79–90 years old), and from active sarcoidosis or active SLE patients and cord blood samples from full-term neonates who had no hereditary disorders, hematologic abnormalities, or infectious complications. Aged donors had no acute or chronic inflammatory or infectious disease, ongoing thrombosis, or neoplasia. Diagnosis of active SLE and sarcoidosis were made according to previously described criteria (Miyara et al., 2005, 2006). All patients were newly diagnosed and not medicated with steroid or immunosuppressant. The study was done according to the Helsinki declaration with the approval from the human ethics committee of the Institute for Frontier Medical Sciences, Kyoto University and from Comité Consultatif de Protection des Personnes dans la Recherche Biomédicale of Pitié-Salpétrière Hospital, Paris. Human peripheral blood PBMC were prepared by Ficoll gradient centrifugation. Lymphocyte subpopulations was isolated by a MoFLo cell sorter (Dako) after positive magnetic cell separation of CD4$^+$ T cells by CD4$^+$ T cell MACS beads (Miltenyi Biotec). Purity of isolated cells was always >95% (Figure S1). Autologous CD14$^+$ and CD19$^+$ cells positively selected by mixed MACS and irradiated (50 Gy) were used as accessory cells.

Mice

NOG mice described previously (Hiramatsu et al., 2003) were injected intravenously with 3.5–5 × 10^7 human PBMCs. The mice were maintained in our animal facility and treated in accordance with the guidelines of Kyoto University.

Flow Cytometry

Freshly obtained or in vitro cultured lymphocytes and human lymphocytes isolated from NOG mouse spleens were stained with anti-hCD4 (-PerCP-Cy5.5 from BD biosciences or -APC from R&D Systems), anti-hCD25 (-PE or -PE-Cy5 from BD), anti-hCD45RO (-PE from Beckman Coulter and PE-Cy7 from

BD), anti-hCD45RA (-PE-Cy7 from BD or -FITC from Beckman Coulter), anti-ICOS (-FITC from e-Bioscience), anti-HLA-DR (-PE from BD biosciences), anti-CD31 (-APC from e-Bioscience), anti-hCD127 (-PE from Beckman Coulter and -PE-Cy5 from e-bioscience), and 7-AAD (Dako). Intracellular detection of FoxP3 with anti-hFoxP3 (PE or Alexa Fluor 647, clone 236A/E7 [e-Bioscience] or clone 259D [BD biosciences]) and of Ki-67 antigen with Ki-67 antibody (FITC or PE from BD) was performed on fixed and permeabilized cells via Cytofix/Cytoperm (e-Bioscience). For detection of intracellular cytokine production, $CD4^+$ T cells were stimulated with 20 ng/ml PMA and 1 μM ionomycin in the presence of Golgi-Stop (BD Biosciences) for 5 hr and then stained with anti-hFoxP3-PE, Ki-67-FITC, anti-IL2-APC (BD Biosciences), anti-IFN-γ-APC (BD), or anti-IL-17-Alexa Fluor 647 (e-Bioscience) after fixation and permeabilization. Data acquired by FACSCalibur (Becton Dickinson) were analyzed with WinMDI 2.9 software (http://facs.scripps.edu/software.html). Statistical comparisons were performed with the nonparametric Mann-Whitney U test.

Cell Culture and Suppression Assay

RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin (Sigma, St. Louis, MO) was used for T cell culture. Cells were labeled with 1 μM CFSE (Dojindo and Invitrogen). In suppression assays, unless otherwise indicated, 1×10^4 CFSE-labeled responder $CD25^-CD45RA^+CD4^+$ T cells were cocultured with 1×10^4 unlabeled cells assessed for their suppressive capacity and 1×10^5 irradiated autologous accessory cells and were stimulated with 0.5 μg/mL plate-bound anti-CD3 (OKT3 mAb) in 96-well round-bottom plate in supplemented RPMI medium. Proliferation of CFSE-labeled cells was assessed by flow cytometry after 84–90 hr of culture. Percent suppression was calculated by dividing the number of proliferating CFSE-diluting responder cells in the presence of suppressor cells at a 1 to 1 ratio by the number of proliferating responder cells when cultured alone, and multiplied by 100.

FOXP3 Gene DNA Methylation

The genomic DNA from purified human $CD4^+$ T cell subsets was extracted by the Blood & Tissue Genomic DNA Extraction System (Viogene). Genomic DNA from purified cells was bisulfite converted by the EpiTect Bisulfite Kit (QIAGEN) according to the manufacturer's instructions. DNA was then subjected to PCR with primers for amplification of specific targets in bisulfite-treated DNA. The PCR products obtained were cloned into the pGEM-T Easy vector (Promega) and 20 individual clones from each sample were cycle sequenced by the BigDye Terminator kit (ver. 3.1; Applied Biosystems) and the ABI automated DNA sequencer (Applied Biosystems). Primers used: Fxpro-met_F1, 5′-TTTTTGTGGTGAGGGGAAGAAATTATATT-3′; Fxpro-met_R2, 5′-TACCATCTCCTCCAATAAAACCCACATC-3′; Fxint-met_F8, 5′-TTTGGGTTAAGTTTGTTGTAGGATAGGGTAGTTAG-3′; Fxint-met_R7, 5′-AAATCTACATCTAAACCCTATTATCACAACCCC-3′.

Single Cell Sorting, RT-PCR, and Vβ5 Sequence Analysis

PBLs were stained with anti-human CD4-FITC, anti-human CD25-PC7 (BD Biosciences), and anti-human BV5.1, BV5.2, BV5.3-PPE (Beckman Coulter). Single cells were sorted with the FACS Vantage (Becton Dickinson) into 96-well PCR plates (Abgene, Epsom). Single-cell RT-PCR conditions were as previously described (Miyara et al., 2006). In the first PCR round, BV5ext (5′-GATCAAAACGAGAGGACAGC-3′) and BC (5′-CGGGCTGCTCCTTGAGGGGCTGCG-3′) were used. Reactions were subjected after 5 min at 94°C to 8 cycles (94°C for 30 s, 60°C for 40 s, 72°C for 50 s), 32 cycles (94°C for 30 s, 55°C for 40 s, 72°C for 50 s), and a final elongation at 72°C for 5 min. In a second PCR round, nested primers BV5 (5′-AGCTCTGAGCTGAATGTGAACGCC-3′) and BC-int (5′-GCGGGTCYGTGCTGACCC-3′) were used. PCR was performed as in the first step.

Products were subjected to automated sequencing (ABI 3100, Applied Biosystems).

Specific questions regarding this repertoire analysis should be sent to guy.gorochov@upmc.fr.

Microarray and Real-Time PCR

RNA was extracted from FACS-sorted $CD4^+$ T cells according to their amounts of CD25 and CD45RA and analyzed by Affymetrix Human Genome U133 Plus 2.0 Arrays.

Real-time PCR was performed with a SYBR green assay on the LightCycler 480 system (Roche). Total RNA extracted from FACS-sorted T cells was reverse transcribed according to the manufacturer's instructions (RNeasy Micro kit, QIAGEN). In each reaction, hypoxanthine phosphoribosyltransferase-1 (HPRT-1) was amplified as a housekeeping gene to calculate a standard curve and to correct for variations in target sample quantities. Relative copy numbers were calculated for each sample from the standard curve after normalization to HPRT-1 by the instrument software. Primers used: FOXP3_F, 5′- CAGCACATTCCCAGAGTTCC-3′; FOXP3_R,5′-TGAGCGTGGCGTAGGTGAAAG-3′; RORA_F,5′- TCACCAACGGCGAGACTTC-3′; RORA_R,5′-GGCAAACTCCACCACATACTG-3′; RORC_F,5′-CGCTCCAACATCTTCTCC-3′; RORC_R,5′-CTAACCAGCACCACTTCC-3′; AHR_F,5′-AACAGATGAGGAAGGAACAGAGC-3′; AHR_R,5′-GAGTGGATGTGGTAGCAGAGTC-3′; AHRR_F,5′- AAGGCTGCTGTTGGAGTC-3′; AHRR_R,5′- TGGATGTAGTCATAAATGTTCTGG-3′; HPRT-1_F,5′-GCTGAGGATTTGGAAAGGGTG-3′; HPRT-1_R,5′-TGAGCACACAGAGGGCTACAATG-3′.

ACCESSION NUMBERS

Microarray data are available from the National Center for Biotechnology Information Gene Expression Omnibus (GEO) under accession number GSE15659.

SUPPLEMENTAL DATA

Supplemental Data include ten figures and two tables and can be found with this article online at http://www.cell.com/immunity/supplemental/S1074-7613(09)00202-7.

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