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Separation of human CD4⁺CD39⁺ T cells by magnetic beads reveals two phenotypically and functionally different subsets

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Abstract

Objective—The ectonucleotidase CD39 is an enzyme involved in adenosine production. Its surface expression on human regulatory T cells (Treg) allows for their flow-cytometry-based isolation from peripheral blood. To further develop and improve this method on a scale supporting translational studies, we introduced capture of CD39⁺ Treg on magnetic immunobeads.

Methods—Peripheral blood mononuclear cells (PBMC) obtained from healthy donors were used for negative selection of CD4⁺ T cells on AutoMACS using antibodies (Abs) specific for all lineage⁺ cells. CD4⁺CD39⁺ Treg were captured by biotin-conjugated anti-CD39 Abs and anti-biotin Ab-coated magnetic beads. Isolated CD4⁺CD39⁺ T cells were phenotyped by flow cytometry for Treg-associated markers: CD39, CD73, FOXP3, CD25, CTLA-4, CCR4, CD45RO and CD121a or for the absence of CD127 and CD49d. CFSE-based proliferation assays and ATP hydrolysis were used to measure Treg functions.

Results—The purity, recovery and viability of the separated CD4⁺CD39⁺ T cells were satisfactory. The isolated CD4⁺CD39⁺ T cell population consisted of FOXP3⁺CD25⁺ T cells which hydrolyzed exogenous ATP and suppressed autologous CD4⁺ T cell proliferation and of FOXP3^{neg}CD25^{neg} T cells without suppressor function. The same two subsets were detectable by flow cytometry in normal PBMC, gating on CD4⁺CD39⁺, CD4⁺CD127^{neg}, CD4⁺CD49d^{neg} or CD4⁺CD25^{high} Treg.

Conclusion—CD4⁺CD39⁺ Treg capture on immunobeads led to a discovery of two CD39⁺ subsets. Similar to CD39⁺ Treg in the peripheral blood, half of these cells are CD25⁺FOXP3⁺ active suppressor cells, while the other half are CD25^{neg}FOXP3^{neg} and do not mediate suppression.

Keywords

adenosine; cancer; CD39; CD73; FOXP3; Treg

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Introduction

The regulatory T cell (Treg) subset of human lymphocytes has attracted significant attention recently because of their key role in control of immune responses in health and disease (Sakaguchi et al., 2010). In human cancer, the frequency and suppressor activity of Treg are often upregulated, while in allergy and autoimmune diseases, they are decreased leading to over-reactive immune responses (Baecher-Allan and Hafler, 2006a; Miyara et al., 2009). Mechanisms utilized by Treg to induce suppression have been extensively examined and appear to be diverse as well as dependent on the environmental context (Sakaguchi et al., 2009). The major difficulty with Treg studies in humans, however, has been the lack of a cell marker which reliably defines Treg. While in mice, the intracellular forkhead transcription factor (FOXP3) has been widely accepted as a specific Treg marker, in humans expression of FOXP3 is not restricted to Treg (Gavin et al., 2006). FOXP3 may be transiently expressed in activated T cells, and its presence has also been reported in tumor cells (Ebert et al., 2008). Furthermore, FOXP3 is a nuclear protein, and its intracellular location limits its usefulness in Treg isolation, although it has often been applied to confirm the identity of Treg (Kryczek et al., 2009).

The isolation of human Treg from the peripheral blood or tissues has been largely dependent on negative immunoselection based on the lack of expression of CD127 (Peters et al., 2008; Liu et al., 2006) and CD49d on the cell surface (Kleinewietfeld et al., 2009). This type of isolation yields cells that are enriched in Treg but have low purity. We and others have used expression of CD25 to identify and isolate CD4⁺CD25^{high} T cells which express FOXP3 and mediate suppression (Baecher-Allan and Hafler, 2006b). However, a largely arbitrary selection of high CD25 expression leads to a loss of Treg, and the number of cells obtained by this method is significantly lower than the total number of Treg present in the peripheral blood. These problems with Treg isolation contribute to existing confusion about the identity and properties of human Treg subsets.

Recently, ectonucleotidases CD39 and CD73 have been found to be expressed on the surface of murine and human Treg (Dwyer et al., 2007; Mandapathil et al., 2009). Jointly, CD39 and CD73 are responsible for ATP conversion to AMP and, ultimately, to adenosine which is a well known immunosuppressive factor (Sitkovsky et al., 2008). Thus, CD39 and CD73 are functional markers linking Treg to ATP breakdown and adenosine production (Deaglio et al., 2007). In mice, adenosine producing CD39⁺ Treg cells promote liver metastasis and suppress NK cell functions (Sun et al., 2010) as well as contact hypersensitivity reactions (Ring et al., 2009). In humans, an increased frequency of CD4⁺CD39⁺ Treg has been reported in tuberculosis (Chiacchio et al., 2009), juvenile arthritis (Moncrieffe et al., 2010) and in tumor infiltrating T cells of lymphoma patients (Hilchey et al., 2009). A decreased frequency or function of CD39⁺ Treg has been reported in multiple sclerosis (Fletcher et al., 2009), ryegrass allergy (Mittag et al., 2010) and vascular inflammation after transplantation (Robson et al., 2005). These findings emphasize the importance of CD39⁺ Treg cells and their role in various pathologic conditions and a need for their isolation to provide sufficient cell numbers for potential therapies.

The relationship between the CD4⁺CD39⁺, CD4⁺CD127^{neg} and CD4⁺CD25^{high} Treg subsets has not been clear, and the overlap between these subsets contributes to the existing difficulties in a reliable identification and isolation of human Treg. As CD39 is both a surface marker and an enzyme directly involved in suppression, we considered it to be a superior candidate for the isolation of Treg. The isolation of human Treg based on CD39 expression by flow cytometry was previously described by us (Mandapathil et al., 2009). Here, we extend this method to positive immunoselection on magnetic beads of the CD39⁺ Treg subset from PBMC on a large scale to facilitate their further characterization.

Materials and Methods

Peripheral blood mononuclear cells

Peripheral venous blood samples used for flow cytometry studies were obtained from ten normal control (NC) subjects (University of Pittsburgh IRB approval #0403105). This cohort included 5 females and 5 males with a mean age of 50 ± 5 years (range: 39 – 69 years). Blood samples (30 – 40mL) were drawn into heparinized tubes and were processed on Ficoll-Hypaque gradients (GE Healthcare Bioscience). For functional studies, buffy coats were purchased from the Central Blood Bank of Pittsburgh, PA. Peripheral blood mononuclear cells (PBMC) were recovered, washed in AIM-V medium (Invitrogen), counted in a trypan blue dye, and immediately used for experiments.

Isolation of CD4⁺CD39⁺ T cells

Negative selection of CD4⁺ cells was performed using a biotin-conjugated antibody (Miltenyi, Auburn, CA) cocktail specific for the lineage antigens (CD8, CD14, CD16, CD19, CD36, CD56, CD123, TCR γ/δ , and Glycophorin A). Next, CD39⁺ T cells were isolated from the CD4⁺ T cell population using biotin-conjugated anti-CD39 Abs and magnetic beads coated with anti-biotin Abs (Miltenyi). The protocol was optimized for a cell number of $5 - 10 \times 10^7$ PBMC using the separation programs 'depl05' (step 1) and 'posseld2' (step 2) on an AutoMACS (Miltenyi) for cell separation. An additional washing step with phosphate buffered saline (PBS) was performed before applying anti-biotin Ab-labeled magnetic beads respectively, in order to reduce the number of false positive CD4^{neg} cells in the final population. Incubation times at 4°C were 10 min for the CD4 T cell antibody cocktail as well as the biotin-conjugated anti-CD39 Ab and 15 min for the magnetic beads coated with anti-biotin.

For functional assays, CD4⁺CD39⁺ T cells separated on AutoMACS were single-cell sorted using MoFlow (DAKO cytometry) into CD39⁺CD25⁺ and CD39⁺CD25^{neg} cells following staining for the relevant surface markers. A lymphocyte gate was applied based on the forward and sideward scatter, and viability of sorted cells was measured using a trypan blue dye.

Flow cytometry reagents

The following anti-human monoclonal antibodies (mAb) were used for staining: anti-CD3-ECD, anti-CD4-ECD, anti-CTLA-4-PC5, anti-CD25-PC5, anti-CD127-PE, anti-CD45RO-ECD, anti-CD45RA-ECD, anti-CD49d-FITC (all Beckman Coulter), anti-FOXP3-FITC and PE (clone PCH101), anti-CD127-PC5, anti-CD39-FITC and PE (all eBioscience), anti-CCR4-PE, anti-CD73-PE (all BD Pharmingen), and anti-CD25-PE (Miltenyi), including their respective isotypes, which served as negative controls for surface as well as intracellular staining. All Abs were pre-titrated using activated as well as non-activated PBMC to determine the optimal staining dilution for each.

Surface and intracellular staining

Freshly isolated cells to be used for flow cytometry, were incubated with mAbs specific for each surface marker in 50 μ L PBS for 30 min at room temperature in the dark. Cells were then fixed with 4% (w/v) paraformaldehyde in PBS for 15 min. For intracellular staining, cells were permeabilized with 0.1% (w/v) saponin in PBS for 5 min and stained with mAbs specific for intracellular markers for 30 min at 4°C. Cells were washed with 0.1% saponin in PBS, resuspended in paraformaldehyde, and immediately analyzed by flow cytometry. Appropriate isotype controls were included for each sample. For intracellular FOXP3 staining, a commercially available FOXP3 staining kit (eBioscience) was used, as previously described (Law et al., 2009).

Flow cytometry

Flow cytometry was performed using an EPICS XL-MCL flow cytometer equipped with Expo32 software (Beckman Coulter). The acquisition and analysis gates were restricted to the lymphocyte gate based on characteristic properties of the cells in the forward and side scatter. At least 1×10^5 events were acquired for analysis and, where applicable, gates were restricted to the CD4⁺ and CD4⁺CD39⁺ T cell subsets.

Suppression assays

The CD4⁺CD39⁺CD25⁺ and CD4⁺CD39⁺CD25^{neg} cell subsets were tested for suppression in co-cultures with proliferating autologous CD4⁺ responder cells (RS) as previously described (Strauss et al., 2007). CFSE-labeled autologous CD4⁺ cells (1×10^5 /well) were incubated in wells of flat-bottom 96-well plates at suppressor (S)/responder (R) cell ratios of 1:1, 1:2, and 1:5. To induce proliferation, RC were stimulated with soluble OKT3/anti-CD28 Ab-coated beads (ratio 2:1, Miltenyi) in the presence of 500 IU/mL IL-2 (Chiron) for 4 days. Using the same assay format, ARL67165 (250 μ mol/L, Sigma-Aldrich, Carlsbad, CA) was added to selected wells 30 min before adding the responder cells.

Statistics

All averages were calculated as geometric means. P-values were calculated by two-tailed Student's t test using JMP software from S.A.S. P values < 0.05 were considered to be significant.

Results

CD4⁺CD39⁺ Treg isolation by immunobeads

A two-step procedure was used for the isolation of CD4⁺CD39⁺ T cells using peripheral blood samples as a source of PBMC (Figure 1). In step 1, CD4⁺ T cells were isolated by negative selection from PBMC. Since no magnetic bead-conjugated anti-CD39 antibodies (Abs) are currently available, the biotin-anti-biotin Ab system was used in the second step to capture CD39⁺ Treg from negatively-isolated CD4⁺ T cells. The viability of isolated CD4⁺CD39⁺ T cells was generally >95% as determined using a trypan blue dye. Table I shows that the mean recovery was $93 \pm 5\%$ for CD4⁺ T cells and $85 \pm 5\%$ for CD4⁺CD39⁺ T cells. In normal donors, the mean absolute number of CD4⁺CD39⁺ T cells recovered from 50 mL of the peripheral blood was $2 \pm 0.5 \times 10^6$. This represents twice the number that we usually recover by single-cell sorting (Mandapathil et al., 2009). In cancer patients, this number is higher and varies depending on the frequency of CD4⁺CD39⁺ Treg in the blood or tumor tissue. From a buffy coat containing ~500 million PBMC, the recovery of CD4⁺CD39⁺ T cells is 15 million ± 3 (n=6). If necessary, buffy coats can be cryopreserved prior to Treg separation. However, we do not recommend cryopreservation of isolated CD4⁺CD39⁺ cells due to considerable losses in cell numbers and function.

Labeling of CD39⁺ cells with magnetic beads during positive selection had no visible effect on the characteristics of the cells in flow cytometry or on their function. These cells were immediately used for further experiments without any resting time. Expression of CD4, CD39, CD25 and FOXP3 was monitored by flow cytometry throughout the isolation process and was used to define the final product. Figure 2 and Table 2 show the frequency of CD4⁺CD39⁺ T cells in the PBMC used for isolation and in each cell subset after immunoselection on magnetic beads. After step 1, the recovered cell population contained $5 \pm 2\%$ of CD39⁺ T cells and 5% of CD4⁺ T cells that were CD25⁺FOXP3⁺ (Figure 2). After step 2, the separated CD4⁺CD39⁺ T cells constituted $82 \pm 4\%$ of the population. Contaminating cells included CD4⁺CD39⁺ monocytes ($4 \pm 2\%$), and CD4^{neg}CD39⁺ B cells ($3 \pm 1\%$).

Phenotype of isolated CD4⁺CD39⁺ T cells

The isolated CD4⁺CD39⁺ T cells were routinely phenotyped by flow cytometry. This revealed the existence of the two subsets within the CD4⁺CD39⁺ population, based on their CD25 and FOXP3 expression (Figure 2). One subset was FOXP3⁺CD25⁺, the other was FOXP3^{neg}CD25^{neg}, and each represented about half of the isolated CD4⁺CD39⁺ population (Table 2). These data indicating that the isolated Treg population was not phenotypically homogenous prompted us to perform functional studies with both subsets of the isolated T cells.

Suppressor function of isolated CD4⁺CD39⁺ T cells

The two subsets (CD4⁺CD39⁺CD25⁺ and CD4⁺CD39⁺CD25^{neg}) were next tested for suppressor function in CFSE-based proliferation assays with autologous CD4⁺ responder cells (RC) activated with OKT/anti-CD28 Abs. When CD39⁺CD25⁺ T cells were added to RC cultures, proliferation was significantly inhibited ($p < 0.003$). However, the addition of CD39⁺CD25^{neg} T cells did not suppress RC proliferation (Figure 3). These data suggested that only the CD39⁺CD25⁺ T cells which co-express FOXP3 are capable of mediating suppressor function as measured in the proliferation assays. Interestingly, this suppression was significantly ($p < 0.05$) inhibited when the ecto-ATPase inhibitor, ARL67156, was added to the co-cultures supporting the idea that adenosine production by CD39⁺CD25⁺ Treg is in part responsible for suppression of RC proliferation (Figure 3b). These results suggest that the two subsets of CD4⁺CD39⁺ T cells are functionally distinct, and that the loss of suppressor function might be related to the loss of CD25 and FOXP3 expression in the isolated Treg.

CD4⁺CD39⁺ Treg in the peripheral blood

In order to rule out the possibility that the magnetic bead separation is responsible for the loss of activation markers, FOXP3 and CD25, and function in CD4⁺CD39⁺ T cells, we resorted to a flow cytometry analysis of freshly-isolated PBMC obtained from NC. As shown in Figure 4, and similar to the immunobead separated cell population, two subsets of CD4⁺CD39⁺ T cells were found in fresh PBMC in about equal proportions. FOXP3 was expressed only by the CD39⁺CD25⁺ subset, while CD127 and CD49d were co-expressed on the CD39⁺CD25^{neg} T cell subset (Figure 4; Tables 3 and 4). The differential distribution of various markers between the CD4⁺CD39⁺ and the CD4⁺CD39^{neg} subsets in the peripheral blood indicated that the former can be phenotypically defined as Treg (Table 3). Further, as shown in Table 4, the CD4⁺CD39⁺CD25⁺ subset was phenotypically consistent with Treg while the CD25^{neg} subset was not. In aggregate, this analysis confirmed that among CD4⁺CD39⁺ T cells, only those expressing CD25 had the phenotypic markers previously described to characterize human Treg (Sakaguchi et al., 2010).

Treg subsets in the peripheral blood defined by phenotypic markers

Because Treg have been previously defined as CD4⁺CD25^{high}, CD4⁺CD127^{neg}, CD4⁺CD49d^{neg} T cell subsets (Peters et al., 2008; Kleinewietfeld et al., 2009), we also determined CD39 and FOXP3 expression by gating on these cell populations in freshly-isolated PBMC. As shown in Figure 5 and Table 5, the majority of CD4⁺CD25^{high} cells in the peripheral blood were FOXP3⁺ (up to 98%) and CD39⁺ (up to 73%) similar to the data previously reported by us (Mandapathil et al., 2009). However, CD4⁺CD25^{high} Treg accounted only for 1.5% of total CD4⁺ T cells in PBMC of NC. The subset of CD4⁺CD127^{neg} Treg accounted for about 4% of CD4⁺ T cells. Only half of these cells were FOXP3⁺CD25⁺ and a minority (~15%) expressed CD39. CD4⁺CD49d^{neg} T cells accounted for 7–8% of CD4⁺ T cells, but only about 10% of these cells co-expressed FOXP3, CD25 or CD39. In contrast, nearly all CD4⁺CD39⁺CD25⁺ T cells, which represent 2.5% of total

CD4⁺ T cell population, were FOXP3⁺. These data indicate that surface expression of CD39 and CD25 markers defines a distinct subset of human Treg from those defined by the absence of CD127 or CD49d markers. While the subset of CD4⁺CD25^{high} Treg contains only FOXP3⁺ T cells and is enriched in CD39⁺ Treg, it accounts for only 1.5% of all CD4⁺ T cells in PBMC of NC. Based on this type of phenotypic analysis, it appears the isolation of human Treg by immunobeads based on surface expression of CD39 and CD25 markers recovers the highest proportion of functionally active cells.

Discussion

The presence, frequency and activity of Treg in various human diseases have been of great interest because of evidence that this immune cell subset may play a role in pathogenesis, disease progression and responses to therapy (Sakaguchi et al., 2010). The development of future therapeutic strategies for Treg elimination or their adoptive transfers depends on the understanding of their biology, interactions with other cells and mechanisms used for suppression. To be able to study human Treg, which are a minor subset of CD4⁺ T lymphocytes, various methods for their isolation have been devised, all based on expression of surface markers. However, none of these markers are specific for Treg, and thus Treg identity is generally established by documenting their suppressor function.

We and others have previously defined Treg as CD4⁺CD25^{high} T cells (Strauss et al., 2007). However, due to the arbitrary cutoff for CD25 expression and the fact that the CD4⁺CD25^{high} population may include activated Teff cells, this definition has been problematic. More recently, we and others have reported that human Treg express the ectonucleotidase CD39 which is involved in the production of adenosine and thus mediating suppression of T effector cells (Borsellino et al., 2007). A consistently high level of CD39 expression on the surface of human Treg allows for positive isolation of CD4⁺CD39⁺ Treg from human PBMC by cell sorting as previously described (Mandapathil et al., 2009). To facilitate a larger scale CD4⁺CD39⁺ Treg isolation, we have developed and evaluated an immunobead-based method using a commercially available biotin-conjugated anti-CD39 Ab in combination with magnetic beads coated with anti-biotin Ab. This method allows for the isolation of CD4⁺CD39⁺ T cells with excellent purity and acceptably high recovery.

Considering the relatively low frequency of FOXP3⁺ and CD39⁺ cells in the CD127^{neg} and CD49d^{neg} populations, they appeared to include a high percentage of non-Treg cells, suggesting that Treg separation based on negative selection for these markers may not be optimal (Liu et al., 2006). The purity of Treg cells could be increased if both negative markers are combined for Treg isolation as previously suggested (Kleinewietfeld et al., 2009). Interestingly, the frequency of CD4⁺CD39⁺ cells in PBMC of normal donors is considerably higher than that of CD4⁺CD25^{high} Treg (i.e., 5% vs. 1%). But while the CD4⁺CD25^{high} subset contained a high frequency of FOXP3⁺ and CD39⁺ T cells, this population probably does not include all Treg because the stringent gating on CD25^{high} cells excludes Treg with the intermediate CD25 expression, as illustrated in Figure 4 and 5. Overall, the phenotypic analysis of the various Treg subsets in freshly-isolated PBMC based on the known Treg surface markers suggested that CD39 may be a promising molecule to consider for Treg isolation.

The phenotypic and functional characterization of the isolated CD4⁺CD39⁺ T cell population in the peripheral blood of normal donors revealed the presence of two distinct subsets: the CD4⁺CD39⁺CD25⁺ cells which were FOXP3⁺CD127^{neg}CD49d^{neg} and which mediated suppression of responder cell proliferation, and the CD4⁺CD39⁺CD25^{neg} subset which consisted of FOXP3^{neg}CD127⁺CD49d⁺ T cells unable to mediate suppression. The existence of similar two subsets of CD4⁺CD39⁺ T cells has been recently described in mice

(Zhou et al., 2009), in the specimens obtained from human juvenile joint inflammation (Moncrieffe et al., 2010) and in patients with renal allograft rejection (Dwyer et al., 2010). These subsets are present in the peripheral blood of NC and thus are not generated during cell isolation. The expression of ectonucleotidase CD39 in combination with CD25 reliably characterizes human FOXP3⁺ Treg able to mediate suppression.

It is possible, that the CD39⁺CD25^{neg} T cell subset represents a silenced form of Treg, which have down-regulated the activation markers CD25 and FOXP3. If these cells can be reactivated or if these cells are in a stage of irreversible apoptosis has to be determined. However, we failed to induce FOXP3 expression in these CD39⁺CD25^{neg} cells under various activation conditions (data not shown). Therefore, it is unlikely that these cells represent a form of precursor Treg. Studies are in progress to elucidate their biologic significance.

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Figure 1. Separation of CD4⁺CD39⁺ T cells by magnetic beads

Negative selection of CD4⁺ cells was performed using a biotin-conjugated antibody cocktail specific for the lineage antigens (CD8, CD14, CD16, CD19, CD36, CD56, CD123, TCR γ/δ , and Glycophorin A). Next, CD39⁺ T cells were isolated from the CD4⁺ T cell population using biotin-conjugated anti-CD39 Abs and magnetic beads coated with anti-biotin Abs. The protocol was optimized for a cell number of $5 - 10 \times 10^7$ PBMC.

Figure 2. Expression of surface markers CD4, CD39, CD25 and intracellular FOXP3 in PBMC before and after magnetic bead isolation

A) After negative selection for CD4 (step 1), nearly all cells were CD4⁺ ($93 \pm 5\%$). After positive selection for CD39, the majority of cells were CD4⁺CD39⁺ ($83 \pm 3\%$). Density plots show all acquired events, with no gates applied.

Figure 3. Suppression of autologous responder cell (RC) proliferation by CD4⁺CD39⁺CD25⁺ or CD4⁺CD39⁺CD25^{neg} cells measured in the CFSE-based assays

A) CD4⁺CD39⁺CD25⁺ T cells suppress RC proliferation, whereas proliferation was not suppressed by CD4⁺CD39⁺CD25^{neg} cells ($p < 0.003$). B) The ecto-ATPase inhibitor ARL67156 partly reversed suppression mediated by CD4⁺CD39⁺CD25⁺ Treg cells ($p = 0.05$).

Figure 4. Expression of various surface markers and intracellular FOXP3 as measured in the peripheral blood of a representative normal donor

Gates were set on the CD4⁺ T cell population (left column) and the CD4⁺CD39⁺ T cell population (right column). For each antibody, quadrants are set based on isotype control. Gate (A) includes all CD4⁺CD39⁺ T cells.

Figure 5. Flow cytometry analysis of PBMC for the presence of various Treg subsets based on expression of Treg-associated surface markers

The frequency of T cells expressing various markers are indicated in each panel. Density plots are representative for one normal control subject out of 10 studied. The lymphocyte gate is defined by the side and forward scatter. Density plots show the CD4⁺CD25^{high} (column A), CD4⁺CD127^{neg} (column B), and CD4⁺CD49^{neg} (column C) subsets.

Table I

Viability, frequency, recovery and purity of CD4⁺CD39⁺ T cells immunoselected by a two-step procedure from PBMC of normal donors^a.

	Step 1 CD4 ⁺ cells	Step 2 CD4 ⁺ CD39 ⁺ cells
Frequency^a	54 ± 10	3 ± 0.9
Viability	> 95	> 95
Recovery	93 ± 5	85 ± 5
Purity	94 ± 5	82 ± 4

^aPBMC separated from 10 NC were used for Treg isolation in the two-step procedure: (1) negative-selection of CD4⁺ T cells and (2) positive-selection of CD4⁺CD39⁺ Treg as described in Materials and Methods. The data are mean % ± SD of CD4⁺ or CD4⁺CD39⁺ T cells in the total PBMC as determined by flow cytometry performed prior to the isolation procedure.

Table II

Percentages of CD4⁺, CD4⁺CD39⁺, and CD25⁺FOXP3⁺ cells as determined during the isolation process performed on AutoMACS by immunobeads.^a

Population	PBMC	Step 1	Step 2
CD4 ⁺	31 ± 8	96 ± 3	94 ± 5
CD4 ⁺ CD39 ⁺	3 ± 1	5 ± 2	83 ± 3
CD25 ⁺ FOXP3 ⁺	4 ± 2	5 ± 2	44 ± 6

^aData are mean percentages ± SD of positive cells in the PBMC and in each subsequent isolation step. Peripheral blood samples used as a source of PBMC were obtained from 10 normal donors.

Table III

The frequency of CD4⁺CD39⁺ or CD4⁺CD39^{neg} T cells expressing FOXP3 and various Treg associated surface markers in the peripheral circulation of normal donors^a.

<u>Marker</u>	<u>CD4[±]CD39[±]</u>	<u>CD4[±]CD39^{neg}</u>	
	<u>mean % ± SD</u>		<u>p value</u>
FOXP3⁺	50 ± 15	5 ± 3	< 0.001
CD25⁺	49 ± 12	12 ± 9	< 0.001
CD127⁺	24 ± 3	89 ± 5	< 0.001
CD49d⁺	59 ± 9	61 ± 7	NSD
CCR4⁺	74 ± 7	29 ± 7	< 0.01
CD45RA⁺	3 ± 1	36 ± 6	< 0.001
CD45RO⁺	84 ± 4	53 ± 18	NSD
CD73⁺	65 ± 19	70 ± 10	NSD

^a The data were obtained by flow cytometry gating on CD4⁺CD39⁺ or CD4⁺CD39^{neg} subsets in the PBMC specimens obtained from 10 NC and stained for the markers listed above. The p values reflect differences in the percent of CD4⁺CD39⁺ or CD4⁺CD39^{neg} T cells.

Table IV

The frequency of CD39⁺CD25⁺ or CD39⁺CD25^{neg} T cells expressing FOXP3 and various Treg associated surface markers in the peripheral circulation of normal donors^a.

Marker	CD39⁺CD25[±]	CD39⁺CD25^{neg}	
	mean % ± SD		p value
CD25⁺	100	0	0
FOXP3⁺	89 ± 5	6 ± 3	< 0.001
CD127⁺	7 ± 3	54 ± 6	< 0.001
CD49d⁺	36 ± 7	73 ± 8	< 0.01
CCR4⁺	81 ± 8	23 ± 5	< 0.001
CD45RA⁺	3 ± 1	4 ± 1	NSD
CD45RO⁺	87 ± 8	81 ± 7	NSD
CD73⁺	64 ± 8	65 ± 18	NSD

^aThe data were obtained by flow cytometry gating on CD39⁺CD25⁺ or CD39⁺CD25^{neg} subsets in the PBMC specimens obtained from 10 NC and stained for the markers listed above. The p values reflect differences in the percent of CD39⁺CD25⁺ or CD39⁺CD25^{neg} T cells expressing the listed markers.

Table V

Percentages of Treg based on expression of FOXP3, CD39, and CD25 in the peripheral blood of normal donors^a.

Cell type/marker	CD4 ⁺ CD25 ^{high}	CD4 ⁺ CD127 ^{neg}	CD4 ⁺ CD49d ^{neg}	CD4 ⁺ CD39 ⁺ CD25 ⁺
Lymphocytes	1 ± 0.2	2 ± 1	4 ± 1	1.5 ± 1
CD4⁺ T cells	1.5 ± 0.3	3.4 ± 1.5	6.5 ± 1.5	2.5 ± 2
Co-expressing:				
FOXP3⁺	95 ± 1	48 ± 5	13 ± 3	89 ± 5
CD39⁺	70 ± 7	10 ± 4	7 ± 4	100
CD25⁺	100	52 ± 8	10 ± 3	100

^aThe data are mean percentages ± SD of lymphocytes or CD4⁺ T cells and CD4⁺ T cells co-expressing FOXP3, CD39 and CD25 on the surface in the normal peripheral blood. The four subsets of Treg were defined in the PBMC of 10 NC by flow cytometry based on the marker co-expression as indicated.