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*J Immunol* 2006; 177:840-851; doi: 10.4049/jimmunol.177.2.840

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Regulation of Trafficking Receptor Expression in Human Forkhead Box P3+ Regulatory T Cells

Hyung W. Lim,* Hal E. Broxmeyer, † and Chang H. Kim*

Forkhead Box P3+ (FOXP3+) T cells are regulatory cells important for maintaining immune tolerance. While chemokine- and other homing-receptors are important for T cell migration, it has been unclear how they are regulated in FOXP3+ T cells. We thoroughly investigated, ex vivo and in vitro, the regulation of chemokine receptor expression on human FOXP3+ T cells in neonatal cord blood, adult peripheral blood, and tonsils. We found that human FOXP3+ T cells undergo changes in trafficking receptors according to their stages of activation and differentiation. FOXP3+ T cells are divided into CD45RA+ (naive) type and CD45RO+ (memory type) FOXP3+ T cells in neonatal blood, adult blood, and tonsils. CD45RA+FOXP3+ T cells mainly express lymphoid tissue homing receptors (CD62L, CCR7, and CXCR4), while CD45RO+FOXP3+ T cells highly express both Th1 and Th2-associated trafficking receptors along with the lymphoid tissue homing receptors at reduced frequencies. Up-regulation of Th1/Th2-associated trafficking receptors begins with activation of CD45RA+FOXP3+ T cells and is completed after their differentiation to CD45RO+ cells. Some chemokine receptors such as CXCR5 and CXCR6 are preferentially expressed by many FOXP3+ cells at a specific stage (CD69+CD45RO+) in tonsils. Our in vitro differentiation study demonstrated that CD45RA+FOXP3+ T cells indeed undergo chemokine receptor switch from CD45RA+ (secondary lymphoid tissue homing) to CD45RO+ type (lymphoid and nonlymphoid tissue homing). The orderly regulation of trafficking receptors in FOXP3+ T cells according to stages of differentiation and activation is potentially important for their tissue-specific migration and regulation of immune responses in humans. *The Journal of Immunology, 2006, 177: 840–851.

Generally, conventional and unconventional T cells have different migratory behaviors. Conventional naive CD4+ T cells, generated in thymus, migrate to secondary lymphoid tissues but not to nonlymphoid tissues (1). They gain migratory capacity to nonlymphoid tissues when activated by Ags and dendritic cells in secondary lymphoid tissues (2). Upon stimulation by dendritic cells, human CD4+ T cells undergo chemokine receptor switch in two continuous steps (3). CXCR3 and CXCR5 are first up-regulated and then followed by down-regulation of lymphoid tissue-homing related receptors (CCR7 and CXCR5) and concomitant up-regulation of Th1/2 effector tissue-targeting receptors such as CCR4, CCR5, CXCR6, and CRTC2. However, unconventional T cells such as CD1d-restricted NKT cells precociously gain the memory and effector phenotype when generated in thymus without having to migrate to secondary lymphoid tissues (4). In line with this, most NKT cells express non-lymphoid tissue-homing receptors, and many NKT cells lack lymphoid tissue homing receptors such as CCR7 (5). Some γδ T cell subsets, generated early in development, directly migrate to skin and mucosal sites for immediate effector function (6, 7).

Forkhead Box P3+ (FOXP3+) regulatory T cells belong to the TCR-αβ T cell group along with conventional CD4+ or CD8+ T cells and unconventional CD1d-restricted NKT cells. CD4+FOXP3+ T cells are generated in thymus in response to self Ags (8–12). FOXP3+ T cells are unconventional in that they can suppress other immune cells and play a critical role in immune tolerance (13–15). FOXP3+ T cells are also different from conventional T cells in that they are functionally competent even in thymus (16). CD4+FOXP3+ T cells are resistant to thymic deletion, and express unusual Ags such as CD25, CD5, programmed death-1, CD152 (CTLA-4), OX40, and glucocorticoid-induced TNFR (17–19), which are often expressed by subsets of activated T cells. FOXP3+ or CD4+CD25+ T cells can directly suppress CD4+ T cells (20), CD8+ T cells (21), NKT cells (22), dendritic cells (23), monocytes/macrophages (24), and B cells (25) via largely unknown mechanisms that involve physical cell-cell contact. Trafficking receptors are thought to be important also for migration and function of FOXP3+ T cells. It has been reported that CD4+CD25+ T cells express high levels of CXCR4, CCR4, CCR5, CR5, E/P-selectin ligands, and CD103 (26–33). They also express homing receptors for lymphoid microenvironments such as CD62L, CCR7, and CXCR5 (34). CD18 (a subunit of the integrin LFA-1) is implicated in generation and function of regulatory T cells (35).

While chemokine receptors are important for T cell migration, it has been unclear how they are regulated in FOXP3+ T cells. We systematically investigated chemokine receptors and adhesion molecules of FOXP3+ T cell subsets in the human circulation and secondary lymphoid tissues using FOXP3 as the major marker. We found multiple subsets of FOXP3+ T cells, which characteristically express trafficking receptors according

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Received for publication December 30, 2005. Accepted for publication April 28, 2006.

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1 This study was supported, in part, by a grant from American Heart Association, National Institutes of Health-National Institute of Allergy and Infectious Diseases Grant AI065064, and a grant from Sidney Kimmel Foundation (to C.H.K.).

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3 Abbreviations used in this paper: FOXP3, Forkhead box P3; CLA, cutaneous lymphocyte Ag.
to their stage of differentiation and activation. We also demonstrated that human FOXP3+ T cells undergo a switch in trafficking receptors from lymphoid to more diverse lymphoid and nonlymphoid homing receptors upon activation and differentiation, a feature potentially important for tissue-specific migration of the FOXP3+ T cell subsets in humans.

Materials and Methods

Cell preparation and culture

Mononuclear cells were prepared by density gradient centrifuge on Histopaque 1077 (Sigma-Aldrich) from human peripheral blood and tonsils. T cells were enriched from blood or tonsil mononuclear cells by a sheep-RBC rosetting method (36). This enriched T cell fraction or CD4+ T cells isolated by CD4+ T cell isolation kit (Miltenyi Biotec) were used for fluorescent Ab staining, chemotaxis, and intracellular cytokine assays. The use of human peripheral blood and tonsils for this study has been approved by the institutional review board at Purdue University. In some experiments, neonatal cord blood CD45RA-CD4+ T cells (isolated by depletion of CD45RO+ T cells) were activated with PHA (5 μg/ml) and IL-2 (200 U/ml) for 3 days and further cultured in the presence of IL-2 for 3 more days for differentiation.

Chemotaxis of FOXP3+ and FOXP3- T cells

All chemokines were obtained from R&D Systems or PeproTech, and initially titrated to determine optimal concentrations. A total of 5 × 10^5 mononuclear cells in 100 μl was placed in each Transwell insert (5-μm pore, 24-well format; Corning Costar). Transwell inserts were placed in 24-well plates containing 600 μl of chemotaxis medium (RPMI 1640 with 0.5% BSA), with or without CCL2 (50 ng/ml), CCL4 (300 ng/ml), CCL17 (1000 ng/ml), CCL19 (2000 ng/ml), CCL20 (2000 ng/ml), CXCL10 (1000 ng/ml), CXCL12 (100 ng/ml), CXCL13 (3000 ng/ml), or CXCL16 (2500 ng/ml). Cells were allowed to migrate for 3 h in a 5% CO2 incubator at 37°C. After chemotaxis, cells that migrated to the lower chambers were harvested and stained with Abs to CD45RA (clone MEM-56; Caltag Laboratories), CD69 (clone CH/4; Caltag Laboratories) and/or CD4 (clone S3.5; Caltag Laboratories). Then, the cells were further stained with
anti-FOXP3 (clone PCH101 or 236A/E7; eBioscience) according to the manufacturer’s protocol. Stained cells were acquired on a FACSCalibur, and the data were analyzed with CellQuest software (BD Biosciences). The numbers of migrated cells to chemokines were subtracted by the numbers of cells that spontaneously migrated to control medium to calculate specific migration rates (%) as described previously (34).

Expression of trafficking receptors by FOXP3+ and FOXP3− T cells

T cells were stained with Abs to CCR2 (clone 48607.121; R&D Systems), CCR4 (clone 2055410; R&D Systems), CCR5 (clone 45531.111; R&D Systems), CCR6 (clone 53103.111; R&D Systems), CCR7 (clone 150503; R&D Systems), CCR9 (Millennium Pharmaceuticals), CCRX3 (clone 49801.111; R&D Systems), CCRX4 (clone 44717.111; R&D Systems), CCRX5 (clone 51505.111; R&D Systems), CCRX6 (clone 56811.111; R&D Systems), CD62L (BD Biosciences), CD62L (BD Biosciences), α4β7 (Act-1; BD Biosciences), CLA (BD Biosciences), or mouse control IgG2b (Caltag Laboratories) for 20 min on ice. Cells were further stained with biotinylated horse anti-mouse IgG (H+L) Ab (Vector Laboratories) for 20 min, followed by staining with PerCP-streptavidin (BD Biosciences) and Abs to CD45RA and CD4 in 10% mouse serum. The cells were further stained with anti-FOXP3 (PCH101 or 236A/E7) according to the manufacturer’s protocol. Stained cells were acquired on a FACSCalibur, and the CellQuest program was used for data analysis.

Intracellular cytokine staining

Intracellular staining was performed as described previously (37). T cells were prestained with Abs to CD4, CD69, and/or CD45RA before activation. Cells were activated for 4 h with phorbol myristate acetate (50 ng/ml; Sigma-Aldrich) and ionomycin (1 μM; Sigma-Aldrich) in the presence of monensin (Sigma-Aldrich). Activated T cells were fixed and permeabilized, followed by staining with PE-conjugated mAbs to cytokines (IL-2, IL-10, IL-13, INF-γ, TNF-α, and TNF-β; all from BD Biosciences) and FOXP3 (PCH101 or 236A/E7). Stained cells were examined with a FACSCalibur and CellQuest software.

Surface or intracellular Ag staining

T cells were first stained with Abs to CD4 and CD45RA along with one of the following Abs to surface Ags: CD25 (clone M-A251; BD Biosciences), CD44 (clone MEM-85; Caltag Laboratories), CD49f (integrin α5/β1; BD Biosciences), CD58 (LFA-3, clone 1C3; BD Biosciences), CD62L (clone Dreg 56; Immunotech), and CD103 (integrin αE, clone 2G5; Immunotech). Abs to CD152 (clone BNI3; BD

FIGURE 2. Surface phenotype and cytokine production capacity of FOXP3+ T cells in human circulation. FOXP3+ T cells in human, adult peripheral blood were examined for expression of various surface Ags. A, FOXP3+ and FOXP3− CD4+ T cell subsets (i, ii, iii, and iv) defined by expression of CD45RA. B, Expression of T cell differentiation/regulatory T cell-associated/adhesion molecules (CD45RO, CD45RB, CD44, CD62L, CD25, CD152, CD49f, CD58, and CD103) by the four FOXP3+ or FOXP3− T cell subsets. C, Cytokine production capacity of CD45RA+ and CD45RA− FOXP3+ T cells. Adult peripheral blood CD4+ T cells were stimulated by PMA and ionomycin before intracellular staining for cytokines. Representative or combined data with SEM from three independent experiments (five different donors) are shown. * Significant differences between CD45RA+FOXP3+ and CD45RA−FOXP3+ T cell subsets or CD45RA+FOXP3− and CD45RA−FOXP3− T cell subsets.
Results

Circulating FOXP3+ and FOXP3− T cell subsets in different tissue sources

<table>
<thead>
<tr>
<th>T cell activation and differentiation</th>
<th>CD45RA−</th>
<th>CD45RA+</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD45RO</td>
<td>10.3 ± 2.2</td>
<td>30.1 ± 3.5</td>
</tr>
<tr>
<td>CD45RB</td>
<td>13.2 ± 2.5</td>
<td>25.2 ± 4.6</td>
</tr>
<tr>
<td>CD69</td>
<td>9.2 ± 1.3</td>
<td>25.6 ± 4.0</td>
</tr>
<tr>
<td>Regulatory T cell associated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD152* (CTLA-4)</td>
<td>6.1 ± 0.7</td>
<td>93.6 ± 4.2</td>
</tr>
<tr>
<td>CD25</td>
<td>16.4 ± 23.6</td>
<td>36.2 ± 6.0</td>
</tr>
</tbody>
</table>

Adhesion

| CD49f (VLA-4)                        | 16.9 ± 4.1  | 34.2 ± 6.4 |
| CD58                                 | 23.1 ± 11.1 | 86.7 ± 5.5 |
| (LFA-3)                              | 17.3 ± 4.8  | 48.8 ± 6.4 |

Costimulation

| CD101                                | 0.5 ± 0.1   | 1.6 ± 0.5 |
| CD62L                                | 73.8 ± 7.0  | 66.7 ± 3.0 |
| αβT                                  | 63.3 ± 2.2  | 34.3 ± 4.5 |
| (CD40L)                              | 1.2 ± 0.4   | 1.4 ± 0.3 |
| CD28                                 | 95.8 ± 11.1 | 98.7 ± 12.2 |

Chemokine receptors

<table>
<thead>
<tr>
<th>Chemokine receptors (2° LT and homeostatic)</th>
<th>CD45RA−</th>
<th>CD45RA+</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCR5</td>
<td>95.1 ± 16.0</td>
<td>69.0 ± 13.2</td>
</tr>
<tr>
<td>(471.8 ± 108.1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CXCR4</td>
<td>98.0 ± 0.7</td>
<td>94.5 ± 0.6</td>
</tr>
<tr>
<td>(574.1 ± 120.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CXCR9</td>
<td>43.7 ± 3.5</td>
<td>17.9 ± 1.3</td>
</tr>
<tr>
<td>(664.8 ± 84.2)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Chemokine receptors (non-LT and inflammatory)</th>
<th>CD45RA−</th>
<th>CD45RA+</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCR2</td>
<td>2.8 ± 1.1</td>
<td>8.5 ± 3.5</td>
</tr>
<tr>
<td>(6.3 ± 0.6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCR4</td>
<td>4.8 ± 0.8</td>
<td>50.9 ± 13.1</td>
</tr>
<tr>
<td>(41.1 ± 8.8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCR5</td>
<td>0.9 ± 0.4</td>
<td>20.1 ± 6.1</td>
</tr>
<tr>
<td>(6.9 ± 0.4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCR6</td>
<td>2.8 ± 0.9</td>
<td>29.3 ± 10.9</td>
</tr>
<tr>
<td>(13.3 ± 21.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CXCR3</td>
<td>3.1 ± 0.6</td>
<td>9.6 ± 3.8</td>
</tr>
<tr>
<td>(19.2 ± 8.6)</td>
<td></td>
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<table>
<thead>
<tr>
<th>B follicle and others</th>
<th>CD45RA−</th>
<th>CD45RA+</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRX5</td>
<td>2.1 ± 0.7</td>
<td>3.2 ± 1.3</td>
</tr>
<tr>
<td>CRX6</td>
<td>1.5 ± 0.2</td>
<td>8.3 ± 2.5</td>
</tr>
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</table>

Regulatory T cells associated with FOXP3+ cells in different tissue sources

<table>
<thead>
<tr>
<th>FOXP3+ T cell subset</th>
<th>Neutrophil Blood</th>
<th>Tonsil Blood</th>
<th>Adult Peripheral Blood</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD45RA−</td>
<td>4.2 ± 0.3</td>
<td>21.6 ± 4.2</td>
<td>3.2 ± 1.3</td>
</tr>
<tr>
<td>CD45RA+</td>
<td>50.2 ± 14.9</td>
<td>155.3 ± 6.5</td>
<td>6.1 ± 3.0</td>
</tr>
</tbody>
</table>

* Averages and SEM of percent cells expressing each Ag are shown along with mean fluorescent intensity in parentheses. a, Intracellular expression.

Biosciences) and CD154 (clone TRAP-1; BD Biosciences) were used for intracellular staining. The cells were further stained with anti-FOX3P.

Immunohistochemistry

Frozen sections of tonsils were acetone-fixed and stained by monoclonal anti-FOX3P (236A/E7) or mouse control IgG1 (eBiosciences). Anti-mouse IgG1 (Vector Laboratories) and streptavidin-allophycocyanin (BD Biosciences) were used to access the expression of FOXP3. Frozen sections were further stained with anti-CD45RO-FITC or PE together with anti-IgD-FITC or anti-CD11c-PE (BD Biosciences) to visualize follicles vs T cell areas or dendritic cells respectively.

Statistical analyses

Student’s paired two-tailed t test was used. Values of p ≤ 0.05 were considered significant. All error bars shown in this paper are SEM.

Results

Circulating FOXP3+ and FOXP3− T cells: overall trafficking receptor phenotype

Trafficking receptors such as chemokine receptors and adhesion molecules regulate T cell migration to various organs. These receptors are also useful indicators for T cell differentiation and function (38, 39). We examined expression of chemokine receptors and adhesion molecules by FOXP3+ T cells in comparison with FOXP3− T cells in adult peripheral blood (Fig. 1). FOXP3− T cells expressed many chemokine receptors such as CCR2, CCR4, CCR5, CCR6, CCR7, CCR9, CXCR3, CXCR4, CXCR5, and CXCR6. Among these receptors, many more FOXP3+ T cells than FOXP3− T cells expressed CCR4, CCR5, CCR6, CXCR3, CXCR4, CXCR5, and CXCR6, chemokine receptors commonly expressed by memory/effector T cells. In contrast, CCR7 and CXCR4 (lymphoid tissue homing receptors expressed by all naive T cells) were expressed by 60–85% of FOXP3+ T cells, while they were expressed by almost all FOXP3− T cells. Many more FOXP3+ T cells, than FOXP3− T cells, expressed CD62L (a selectin molecule required for migration into secondary lymphoid tissues) and cutaneous lymphocyte Ag (CLA, a surface molecule expressed by skin-homing T cells). In contrast, αβT (an integrin expressed by gut homing lymphocytes) was expressed by many more FOXP3− T cells than FOXP3+ T cells. These data show that FOXP3+ T
cells in human adult peripheral blood basically have the trafficking receptors of FOXP3\(^{+}\) T cells, but the two populations appear to have different preference for trafficking receptors: FOXP3\(^{+}\) T cells (CCR4, CCR5, CCR6, CXCR3, CXCR6, and CLA) and FOXP3\(^{-}\) T cells (CCR7, CXCRC4, and \(\alpha_4\beta_7\)).

**Phenotype of CD45RA\(^{+}\) and CD45RA\(^{-}\) FOXP3\(^{+}\) T cell subsets in human peripheral blood**

Although not absolute, CD45RA is associated with the naive phenotype, while CD45RO is associated with the memory phenotype of human T cells. We fractionated FOXP3\(^{+}\) T cells in adult peripheral blood into CD45RA\(^{+}\) and CD45RA\(^{-}\) FOXP3\(^{+}\) T cells (Fig. 2A). While FOXP3\(^{+}\) conventional CD4\(^{+}\) T cells were almost equally divided into CD45RA\(^{+}\) and CD45RA\(^{-}\) T cells, the majority (on average 77%, \(n = 14\)) of FOXP3\(^{+}\) T cells were CD45RA\(^{+}\) T cells. Small but significant numbers (on average 23%, \(n = 14\)) of FOXP3\(^{+}\) T cells were CD45RA\(^{-}\) T cells. Almost all CD45RA\(^{-}\)FOXP3\(^{+}\) T cells were CD62L\(^{+}\). Most CD45RA\(^{-}\)FOXP3\(^{+}\) T cells were CD45RO\(^{-}\), and the majority of CD45RA\(^{-}\)FOXP3\(^{+}\) T cells were CD45RO\(^{-}\) (Fig. 2B).

However, some CD45RA\(^{-}\)FOXP3\(^{+}\) T cells (33.6 ± 16.5%, \(n = 6\)) were CD45RO\(^{+}\) (Table I). CD45RA\(^{-}\)FOXP3\(^{+}\) T cells were CD45RB\(^{+}\), while CD45RA\(^{-}\)FOXP3\(^{+}\) T cells were CD45RB\(^{+}\). However, not all FOXP3\(^{+}\) T cells expressed CD25: 14% of CD45RA\(^{-}\)FOXP3\(^{+}\) T cells and 16% of CD45RA\(^{-}\)FOXP3\(^{+}\) T cells lacked CD25 expression. Few FOXP3\(^{+}\) T cells in circulation were activated, lacking CD69 (data not shown). Expression of CD49f (a laminin receptor, also called integrin \(\alpha_6\beta_1\)) was atypically detected on both CD45RA\(^{+}\) and CD45RA\(^{-}\) FOXP3\(^{+}\) T cell subsets. However, expression of CD58 (LFA3, a CD2 ligand) on CD45RA\(^{-}\)FOXP3\(^{+}\) T cells was higher than that on CD45RA\(^{-}\)FOXP3\(^{-}\) T cells. Both CD45RA\(^{-}\) and CD45RA\(^{-}\) FOXP3\(^{-}\) T cells expressed regulatory T cell-associated receptors such as CD25 and CD152 (CTLA4). However, not all FOXP3\(^{+}\) T cells expressed CD25: 14% of CD45RA\(^{-}\)FOXP3\(^{+}\) T cells had CD62L expression, lacking CD25 (data not shown). Expression of CD49f (a laminin receptor, also called integrin \(\alpha_6\beta_1\)) was atypically detected on both CD45RA\(^{+}\) and CD45RA\(^{-}\) FOXP3\(^{+}\) T cell subsets. However, expression of CD58 (LFA3, a CD2 ligand) on CD45RA\(^{-}\)FOXP3\(^{+}\) T cells was higher than that on CD45RA\(^{-}\)FOXP3\(^{-}\) T cells. Both CD45RA\(^{-}\) and CD45RA\(^{-}\) FOXP3\(^{-}\) T cells expressed regulatory T cell-associated receptors such as CD25 and CD152 (CTLA4). However, not all FOXP3\(^{+}\) T cells expressed CD25: 14% of CD45RA\(^{-}\)FOXP3\(^{+}\) T cells had CD62L expression, lacking CD25 (data not shown). Expression of CD49f (a laminin receptor, also called integrin \(\alpha_6\beta_1\)) was atypically detected on both CD45RA\(^{+}\) and CD45RA\(^{-}\) FOXP3\(^{+}\) T cell subsets. However, expression of CD58 (LFA3, a CD2 ligand) on CD45RA\(^{-}\)FOXP3\(^{+}\) T cells was higher than that on CD45RA\(^{-}\)FOXP3\(^{-}\) T cells. Both CD45RA\(^{-}\) and CD45RA\(^{-}\) FOXP3\(^{-}\) T cells expressed regulatory T cell-associated receptors such as CD25 and CD152 (CTLA4). However, not all FOXP3\(^{+}\) T cells expressed CD25: 14% of CD45RA\(^{-}\)FOXP3\(^{+}\) T cells had CD62L expression, lacking CD25 (data not shown).
the expression of CD25, CD44, CD49f, CD45RO, and CD152 but are distinguished by differential expression of CD45RB and CD58.

**Cytokine production capacity of CD45RA⁺ and CD45RA⁻ FOXP3⁺ T cells in adult peripheral blood**

Effector cytokines such as IFN-γ, IL-13, and IL-10 are characteristically produced by distinct T cell subsets, namely Th1, Th2, and T regulatory type 1 (or Tr1) cells, respectively. Unlike other T cells, it is known that CD4⁺CD25⁺ regulatory T cells in humans and mice cannot produce IL-2, and the master transcription factor FOXP3 (called Foxp3 in mice) suppresses IL-2 expression in regulatory T cells (40–42). We examined the cytokine production capacity of CD45RA⁺, and CD45RA⁻ FOXP3⁺ T cells in human blood (Fig. 2C). As expected, FOXP3⁺ T cells, regardless of whether they were CD45RA⁺ or CD45RA⁻, were largely defective in production of IL-2. Few FOXP3⁺ T cells produced IFN-γ, a Th1 cytokine. In contrast, many CD45RA⁺, but not CD45RA⁻ FOXP3⁺ T cells, produced IL-2, IL-13, and IFN-γ. CD45RA⁺ FOXP3⁺ T cells were better than CD45RA⁻ FOXP3⁺ T cells in production of TNF-α. In contrast, many CD45RA⁺, but not CD45RA⁻ FOXP3⁺ T cells were able to produce TNF-β (also called LT-α, a subunit that is mainly produced by naïve T cells and forms LT-α β₂ and LT-α β₃ (43, 44)). The expression level of intracellular CD154 (CD40L, a costimulation molecule) was high in CD45RA⁻ FOXP3⁺ T cells (~54% are CD154⁺), but it was low in CD45RA⁺ FOXP3⁺ T cells (Table I). These results show that both CD45RA⁻ and CD45RA⁺ FOXP3⁺ T cell subsets largely lack the production capacity of IL-2 and Th1/2 cytokines, and they are different in the production capacity of TNF-β.

**Traffic receptors of CD45RA⁺ and CD45RA⁻ FOXP3⁺ T cells**

It has been established that CD45RA⁺ and CD45RA⁻ T cells express different chemokine receptors from each other. CD45RA⁺ CD4⁺ T cells express CCR7 and CXCR4, while CD45RA⁻ T cells express memory/effector type chemokine receptors such as CCR2, CCR4, CCR5, CCR6, CCR7, CXCR3, CXCR4, CXCR5, and CXC6. Among these, CCR4 is expressed by many Th2 cells, while CCR5, CXCR3, and CXCR6 are expressed by many Th1 cells. Many CD45RA⁻ T cells retain CCR7 and CXCR4, while some lose expression of these receptors for migration to nonlymphoid tissues. As shown in Fig. 1, FOXP3⁺ and FOXP3⁻ T cells differentially expressed trafficking receptors. This could be due to the CD45RA⁻ to CD45RA⁺ T cell ratio being much higher for FOXP3⁺ T cells than FOXP3⁻ T cells in adult human blood. Alternatively, it is possible that FOXP3⁺ T cells, whether they are CD45RA⁺ or CD45RA⁻ cells, preferentially express effector type chemokine receptors. To address this issue, we separately examined expression of trafficking receptors by CD45RA⁺, and CD45RA⁻ FOXP3⁺ T cells (Fig. 3). Almost all CD45RA⁺ FOXP3⁺ T cells expressed CCR7 and CXCR4. Small numbers (≤25%) of CD45RA⁻ FOXP3⁺ T cells atypically expressed also memory/effector type chemokine receptors such as CXCR5, CXCR3, CCR6, CCR5 and CCR4. As expected, many CD45RA⁻ FOXP3⁺ T cells expressed the memory/effector type chemokine receptors. FOXP3⁺ T cells lacking CCR7 and CXCR4 were present in CD45RA⁺, but not in CD45RA⁻, population in significant numbers (CCR7⁻ ~50%; CXCR4⁻ ~30%). These data show that CD45RA⁻ FOXP3⁺ T cells have memory/Th1/Th2 type, while CD45RA⁺ FOXP3⁺ T cells largely have lymphoid tissue homing receptors.
Chemotactic behavior of CD45RA⁺ and CD45RA⁻ FOXP3⁺ T cell subsets

We further determined the function of chemokine receptors expressed by CD45RA⁺ and CD45RA⁻ FOXP3⁺ T cells in chemotactic response (Fig. 4). CCL19 and CXCL12 induced migration of both CD45RA⁺ and CD45RA⁻ FOXP3⁺ T cells. CCL19 was more active for CD45RA⁺, while CXCL12 was more active for CD45RA⁻ FOXP3⁺ T cells. CCL4, CCL17, CCL20, CXCL10, CXCL13 and CXCL16 variably induced migration of CD45RA⁻ FOXP3⁺ T cells but not or only poorly of CD45RA⁺ FOXP3⁺ T cells. CCL2 induced migration of both CD45RA⁺ and CD45RA⁻ FOXP3⁺ T cells. Overall, there was a strong correlation between chemokine receptor expression and chemotactic behavior of the two subsets of FOXP3⁺ T cells. These results show that many chemokine receptors of FOXP3⁺ T cells such as CCR2, CCR4, CCR5, CCR6, CCR7, CXCR3, CXCR4, CXCR5, and CXCR6 are functional, and that CD45RA⁺ and CD45RA⁻ FOXP3⁺ T cells have chemotactic behaviors distinct from each other.

Both CD45RA⁺ and CD45RA⁻ FOXP3⁺ T cells circulate as early as in neonatal blood

Generally, all of the conventional T cells in circulation at birth are considered naïve T cells because they, most likely, have not been primed by Ags yet. Consistently, most FOXP3⁺ CD4⁺ T cells in neonatal cord blood are CD45RA⁺ (Fig. 5). 1.5–5% of CD4⁺ T cells were FOXP3⁺ T cells (n = 9). The majority (on average 79%, n = 9) of neonatal cord blood FOXP3⁺ T cells were CD45RA⁺, but unexpectedly, significant numbers (on average 21%, n = 9) of CD45RA⁻ FOXP3⁺ T cells were also present in neonatal blood. Both FOXP3⁺ T cell subsets were divided into CD62L⁺ and CD62L⁻ subsets. Neonatal CD45RA⁺ FOXP3⁺ T cells were CD45RO⁺CD45RB⁺CD44highCD58⁺, while CD45RA⁻ FOXP3⁺ T cells were CD45RO⁻CD45RBhighCD44mediumCD58⁻ (Fig. 5B). Although both types of FOXP3⁺ T cells expressed CD25 and CD122, most CD45RA⁺ FOXP3⁺ T cells expressed them at higher levels than CD45RA⁻ FOXP3⁺ T cells. Most FOXP3⁺ T cells were CD103⁻. Overall, neonatal Foxp3⁺ T cells are clearly divided into CD45RA⁺ and CD45RA⁻ subsets, and they share the expression of CD25, CD122, and CD49f but are distinguished by different expression levels of CD45RO, CD45RB, and CD58.

Regulation of lymphoid and nonlymphoid tissue trafficking receptors on CD45RA⁺ and CD45RA⁻ FOXP3⁺ T cells in neonatal blood

We examined expression of chemokine receptors and adhesion molecules on CD45RA⁺ and CD45RA⁻ FOXP3⁺ T cells in neonatal cord blood (Fig. 5C). CD45RA⁻ FOXP3⁺ T cells uniformly expressed CXCR4, CCR7, and αβ, along with CCR9 on some cells. CD45RA⁺ FOXP3⁺ T cells were similar to CD45RA⁻ FOXP3⁻ T cells in expression of the receptors, but many more of them had CCR9 while fewer had αβ, compared...
with CD45RA⁺ FOXP3⁺ T cells. Unlike the CD45RA⁺ T cell subsets, CD45RA⁻ FOXP3⁺ T cells expressed CCR4, CCR6, CCR5, CXCR3, CXCR6, CCR2, and CLA, trafficking receptors characterized by expression of memory and effector (Th1 and Th2) cells. Expression of CXCR4, CCR7, αβ⁺, and CCR9 on CD45RA⁻ FOXP3⁺ T cells was sharply decreased compared with the CD45RA⁺ subset.

**Traffic receptor expression and microenvironmental localization of CD45RA⁺ and CD45RO⁻ FOXP3⁺ T cells in tonsils**

Ag-dependent activation of T cells occurs in secondary lymphoid tissues such as tonsils. We further examined human tonsils to examine regulation of chemokine receptor expression in FOXP3⁺ T cells undergoing activation and differentiation. 5–10% of CD4⁺ T cells were FOXP3⁺ in human tonsils (n = 12). They were divided into CD45RA⁺ (CD45RO⁻) and CD45RA⁻ (CD45RO⁺) FOXP3⁺ T cells. CD45RA⁺ FOXP3⁺ T cells were CD45RBhighCD44medium, while CD45RA⁻ FOXP3⁺ T cells were CD45RBlowCD44high (Fig. 6A). Based on CD69 expression, we further divided the FOXP3⁺ T cells into four identifiable FOXP3⁺ T cell subsets at different stages of activation and CD45RA expression: CD45RA⁺ CD69⁺ (stage I), CD45RA⁺ CD69⁺ (stage II), CD45RA⁻ CD69⁺ (stage III), and CD45RA⁻ CD69⁻ (stage IV) subsets (Fig. 6B). All of the four FOXP3⁺ T cell subsets lacked the production capacity of IL-2 upon stimulation, while FOXP3⁻ T cells were able to produce IL-2 (Fig. 6C). In contrast, many IL-10⁺ T cells were detected within the FOXP3⁻ T cell population at stage III. Again, TNF-β was better produced by CD45RA⁺ FOXP3⁺ cell subsets.

Frozen tonsil sections were examined for microenvironmental localization of CD45RA⁺ and CD45RO⁻ FOXP3⁺ T cells. CD45RO and FOXP3 were used as cell markers to distinguish CD45RO⁻ FOXP3⁺ T cells from CD45RO⁺ (CD45RA⁻) FOXP3⁺ cells. As shown in Fig. 6, D–I, CD45RO⁺ and CD45RO⁻ FOXP3⁺ T cell subsets were frequently detected in the interfollicular area. Generally, CD45RO⁻ FOXP3⁺ T cells were found in the areas where CD45RO⁺ FOXP3⁻ T cells were located (area “a”). CD45RO⁺ FOXP3⁺ T cells were often found where few CD45RO⁻ (FOXP3⁺ or FOXP3⁻) T cells were found (area “b”). CD45RO⁻ FOXP3⁺ T cells were found close to where many CD11c⁺ dendritic cells were located (area “a”). In contrast, CD45RO⁻ FOXP3⁻ T cells were more frequently found where few CD11c⁺ dendritic cells were located (area “b”).

Most CD45RA⁺ CD69⁻ FOXP3⁻ T cells (stage I) expressed lymphoid tissue homing receptors, CD62L, CXCR4, and CCR7 (Fig. 7). Precocious up-regulation of CXCR5 by ~30% of CD45RA⁺ FOXP3⁻ cells was detected. Expression of memory type receptors, CCR4 and CXCR3 at low frequencies (<10%), was detected as at early as stage II (CD45RA⁻). At a CD45RA⁻ stage (III), the majority of FOXP3⁺ T cells expressed CCR4, CCR5, CCR6, CXCR3, CXCR4, and CXCR6. At CD45RA⁻ CD69⁺ stage (IV), many FOXP3⁺ T cells down-regulated CXCR5 (a homing receptor for B cell zone and germinal centers) and CXCR6, but retained CCR4, CCR5, CCR6, and CXCR3. Down-regulation of CCR7, CXCR4, and CD62L at stage III was obvious for FOXP3⁺ T cells.

We performed chemotaxis assays to chemokine ligands to assess the function of the chemokine receptors expressed by tonsil FOXP3⁺ cells (Fig. 8). CD45RA⁻ FOXP3⁺ T cells (stages III and IV) had strong chemotactic response to a group of memory/effector chemokine receptors: CCL2 (a CCR2 ligand), CCL4 (CCR5), CCL17 (CCR4), CCL20 (CCR6), CXCL10 (CXCR3), CXCL13 (CXCR5), and CXCL16 (CXCR6). The responses to lymphoid tissue chemoattractants, CCL19 (CCR7) and CXCL12 (CXCR4), were high at stage I and II but slightly weaker at stage III and IV. There were statistically significant differences between FOXP3⁺ and FOXP3⁻ T cells in chemotactic responses to CCL4, CCL17, CCL20, CXCL10, and CXCL16, where FOXP3⁻ T cells were more responsive to the memory/effector chemokines examined. Overall, the chemotactic response of FOXP3⁺ T cells is consistent with their expression pattern of chemokine receptors, and suggests that FOXP3⁻ T cells change their chemotactic behavior during activation and differentiation in secondary lymphoid tissues.

**Differential expression of trafficking receptors by FOXP3⁺ T cells**

Differential expression of trafficking receptors by CD45RA⁺, and CD45RA⁻ FOXP3⁺ T cell subsets in the (neonatal and adult) blood circulation and tonsils suggests the possibility of a switch in expression of trafficking receptors during FOXP3⁺ T cell differentiation. To demonstrate that FOXP3⁺ T cells acquire more diverse chemokine receptors beyond secondary lymphoid tissue-homing receptors during their differentiation, partially purified cord blood CD45RA⁺CD4⁺ T cells containing FOXP3⁺ and FOXP3⁻ cells were cultured for 3 days in the presence PHA and...
IL-2 followed by resting in IL-2 for 3 additional days. Anti-FOXP3 and anti-CD45RO Abs were used to identify CD45RO<sup>+</sup>FOXP3<sup>+</sup> T cells after culture. After culture, most cells became CD45RO<sup>+</sup> cells. While neonatal CD45RA<sup>+</sup>FOXP3<sup>+</sup> T cells did not express memory, Th1 or Th2 chemokine receptors, in vitro differentiated CD45RO<sup>+</sup>FOXP3<sup>+</sup> T cells up-regulated CCR2, CCR4, CCR5, CCR6, CXCR3, CXCR5, and CXCR6 (Fig. 9). Down-regulation of CCR9 and α<sub>4</sub>β<sub>7</sub> on memory FOXP3<sup>+</sup> T cells was detected in a manner similar to FOXP3<sup>+</sup> T cells circulating in adult and neonatal blood (Figs. 3 and 5). However, the in vitro differentiated CD45RO<sup>+</sup>FOXP3<sup>+</sup> T cells still retained CCR7 and CXCR4. Overall, there was clear evidence that human CD45RA<sup>+</sup>FOXP3<sup>+</sup> T cells undergo chemokine receptor switch during their differentiation to CD45RO<sup>+</sup> cells.

**Discussion**

The human CD4<sup>+</sup> TCR-αβ T cell population is composed of functionally distinct conventional T cell subsets such as naive, memory, Th1, Th2 and B cell-helping T cells, and regulatory T cells exemplified by FOXP3<sup>+</sup> T cells (8–12, 45). Functionally specialized T cell subsets express different trafficking receptors, which is important for their migration to appropriate tissue sites (46). We thoroughly examined, ex vivo, the existence of distinct subsets of FOXP3<sup>+</sup> T cells in expression of trafficking receptors in humans and demonstrated their change in expression of chemokine receptors during differentiation in vitro. Our results revealed that human FOXP3<sup>+</sup> cells undergo trafficking receptor switch from CCR4, CCR7 and CD62L (secondary lymphoid tissue homing receptors) to more diverse nonlymphoid tissue homing related receptors upon activation and differentiation.

We show in this study the presence of distinct subsets of human FOXP3<sup>+</sup> T cells defined by differentiation/activation-related surface Ags (CD45RA, CD45RO, CD69, and many others) and trafficking receptors. It was initially reported that CD4<sup>+</sup>CD25<sup>+</sup> T cells in adult blood expressed CD45RO but not CD45RA, and,
thus, the whole population was considered memory T cells (47, 48). This interpretation was probably due to the fact that CD25 was used instead of FOXP3 as the marker of regulatory T cells, and that CD45RA⁺FOXP3⁺ T cells constitute a minor population compared with CD45RA⁻FOXP3⁺ T cells in adult peripheral blood. Moreover, the presence of naive (CD45RA⁻) and memory (CD45RO⁺) regulatory T cells with comparable suppressor activity at various stages of human development was recently confirmed (49–51). The CD45RA⁻ FOXP3⁺ T cells in adult blood have all the features of memory/effector T cells in surface phenotype and trafficking receptors, but they largely lack the production capacity of CD154, IL-2 and Th1 and Th2 cytokines, as expected. We found that CD45RA⁺ and CD45RO⁺ FOXP3⁺ T cell subsets are different from each other in expression of CD58 and TNF-β (also called LT-α). While the functional significance of this difference remains to be determined, CD58 and TNF-β appear to be useful Ags for identification of different FOXP3⁺ T cell subsets. Most CD45RA⁻ FOXP3⁺ T cells in adult blood are CD45RBhigh and CD45RO⁻ as expected but some of these cells atypically coexpress CD45RO (Fig. 2). The double-positive FOXP3⁺ T cells are believed to be a transitional cell subset that would eventually become CD45RA CD45RO⁻ FOXP3⁺ cells. We noticed a wide variation in the frequency of CD45RA⁻ CD45RO⁻ FOXP3⁺ cells among different blood donors. The Ag priming status of CD45RA⁻ FOXP3⁺ cells in different individuals may determine the frequency of these cells in the blood circulation. Overall, the CD45RA⁻ FOXP3⁺ T cells in adult blood are closer to conventional CD45RA⁻ CD45RO⁺ T cells in trafficking receptor expression and chemotactic behavior. Adult blood CD45RA⁻ FOXP3⁺ T cells uniformly express CD62L, CCR7 and CXCR4, an indication for secondary lymphoid tissue homing phenotype. Small numbers of (∼20%) CD45RA⁻ FOXP3⁺ T cells in adult blood express certain memory/effector type trafficking receptors, which is different from conventional CD45RA⁻ CD45RO⁻ T cells and FOXP3⁺ T cells in neonatal cord blood. The unconventional expression of memory T cell chemokine receptors by CD45RA⁻ FOXP3⁺ T cells in adult blood is consistent with their expression of other memory cell-associated molecules.

It was reported that all CD4⁻ CD25⁺ T cells in human cord blood are naive cells with the CD45RA⁺ CD45RO⁻ phenotype (52). In this study, we show that roughly one of five FOXP3⁺ T cells in neonatal blood is a CD4⁺ T cell with nonlymphoid tissue trafficking receptors. This is highly unusual compared with conventional CD4⁺ T cells at birth, which have the trafficking potential only to secondary lymphoid tissues. CD45RO⁺ FOXP3⁺ T cells have the trafficking receptor phenotype for both lymphoid and nonlymphoid/inflamed tissue sites, while CD45RA⁺ FOXP3⁺ T cells have the trafficking receptor phenotype only for secondary lymphoid tissues. Therefore, the two (CD45RA⁻ and CD45RO⁻) FOXP3⁺ T cell subsets are thought to patrol different parts of the human body. CD45RA⁺ FOXP3⁺ T cells would migrate to secondary lymphoid tissues along with conventional T cells. On the other hand, CD45RO⁻ FOXP3⁺ T cells would migrate more comprehensively to lymphoid and nonlymphoid tissue sites.

We classified FOXP3⁺ T cells in tonsils into four subsets at different stages of CD45RA expression and activation. FOXP3⁺ T cells start to up-regulate memory type chemokines receptors and down-regulate lymphoid tissue homing receptors upon CD69 expression at the CD45RA⁺ stage. The up- or down-regulation peaks at the CD69⁻ CD45RA⁻ stage. Interestingly, only these FOXP3⁺ T cells can produce IL-10 (Fig. 6). While they up-regulate CXCR5 and other receptors, they down-regulate CCR7. This is consistent with the FOXP3⁺ cell migration away from the T cell zone to the B cell area or other microenvironments in secondary lymphoid tissues (34). Expression of CCR7 and CD62L is regained on CD69⁻ CD45RA⁻ cells, which is potentially important for reentry of FOXP3⁺ T cells into secondary lymphoid tissues from the circulation.
pressed on activated CD45RO modulation of trafficking receptors as they differentiate to CD45RO (or CD45RA) 

FOXP3+ T cells with memory/effector T cell phenotype. In neonatal cord blood, CD45RA+ FOXP3+ T cells are of the majority, but CD45RO+ FOXP3+ T cells become the majority in adult peripheral blood (PB). Lymphoid tissue-homing receptors such as CCR7, CXCR4 and CDS2L are down-regulated in some CD45RO+ FOXP3+ T cells. Nonlymphoid tissue (LT) homing, memory or inflammatory tissue-associated trafficking receptors are highly up-regulated as they become CD45RO+ FOXP3+ T cells. Some trafficking receptors are transiently expressed on activated CD45RO+ FOXP3+ T cells in secondary lymphoid tissues. The trafficking receptors of naive, Ag primed and memory-like FOXP3+ T cell subsets would help them find appropriate tissue sites in human beings.

In our immunofluorescence confocal microscopy analysis of FOXP3+ T cell distribution in tonsils, we noted that CD45RA+ FOXP3+ T cells are frequently colocalized with CD45RA+ FOXP3+ T cells, while CD45RO+ FOXP3+ T cells are colocalized with CD45RO+ FOXP3+ T cells and dendritic cells (Fig. 6). This is consistent with the similarity in trafficking receptor expression between CD45RA+ FOXP3+ and CD45RA+ FOXP3- T cells or between CD45RO+ FOXP3- and CD45RO+ FOXP3+ T cells (Fig. 7). CD45RA+ FOXP3+ T cells and CD45RO+ FOXP3+ T cells, found in dendritic cell-rich areas, are likely to be the FOXP3+ T cells undergoing Ag priming and trafficking receptor switch.

We were able to reproduce the trafficking receptor switch in vitro using cord blood T cells (Fig. 9). After in vitro differentiation, most CD45RA+ CD4+ T cells including FOXP3+ and FOXP3- cells became CD45RO+ T cells within 5–6 days. It is possible that some CD45RO+ FOXP3+ T cells may have been converted from FOXP3+ T cells, as it has been reported that FOXP3 expression can be induced in certain conditions (53). The trafficking receptor switch occurring in vitro mimics that occurring in vivo in up-regulation of memory and inflammatory tissue-homing related receptors. A slight deviation from the phenotype of FOXP3+ T cells in peripheral blood was the lack of down-regulation of CCR7 and CXCR4 on in vitro primed FOXP3+ T cells (Fig. 9). The FOXP3+ T cells, Ag-primed in vitro, were rather similar to resting CD45RO+ FOXP3+ T cells in tonsils which express both lymphoid and nonlymphoid tissue-associated trafficking receptors at high frequencies.

Taken together, our results revealed both unconventional and conventional characteristics of FOXP3+ T cells and provide insights into their differentiation and trafficking potentials in the human body. The fact that CD45RA- FOXP3+ T cells with nonlymphoid tissue trafficking receptors appear as early as at birth is unconventional. By expressing the complete set of lymphoid homing receptors (CD62L, CCR7, and CXCR4), CD45RA+ FOXP3+ T cells have the trafficking receptor phenotype for secondary/mucosal lymphoid tissues. CD45RA- FOXP3+ T cells have the trafficking receptor phenotype for nonlymphoid and inflamed tissue sites (CLA, CCR2, CCR4, CCR5, CXCR3, and CXCR6). While the majority of CD45RA- FOXP3+ T cells retain CCR7, the loss of CCR7 (and CXCR4) and up-regulation of CXCR5 on activated CD45RA- FOXP3+ T cells may be important for their positioning in secondary lymphoid tissues. We conclude that human FOXP3+ T cells undergo trafficking receptor switch during the differentiation from CD45RA+ to CD45RA- (Fig. 10). This differentiation program is potentially important for tissue-specific migration and function of FOXP3+ T cell subsets in humans.

Acknowledgments

We thank J. H. Lee, S. G. Kang, and C. W. Wang (Kim Lab, Purdue University) and S. Ito (Indiana University) for their helpful input.

Disclosures

The authors have no financial conflict of interest.

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