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Dynamic Development of Homing Receptor Expression and Memory Cell Differentiation of Infant CD4⁺CD25<sup>high</sup> Regulatory T Cells<sup>1</sup>

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Migration of CD4⁺CD25<sup>FOXP3</sup> regulatory T cells (Treg) is important for suppressing immune responses in different tissues. Previous studies show that the majority of Treg at birth express gut homing receptor α<sub>4</sub>β<sub>7</sub> and that only few express CCR4, while the reverse pattern is found in adults. The age at which homing receptor switch occurs in vivo is not known. In this study, we show, in a prospective study of human infants from birth to 3 years of age, that homing receptor switch from α<sub>4</sub>β<sub>7</sub> to CCR4 commences between 1 1/2 and 3 years of age and that Treg at that age also had started their switch to a memory phenotype. The majority of naive Treg express α<sub>4</sub>β<sub>7</sub> in infants but not in adults, while the majority of memory Treg express CCR4 both infants and adults. The homing receptor expression on Treg corresponds to their actual migration properties, because Treg from cord blood migrate foremost toward the gut-associated chemokine CCL25. CD4⁺FOXP3<sup>+</sup> T cell numbers increase rapidly in the circulation during the first days of life indicating conversion to suppressive Treg from CD25<sup>high</sup> Treg precursors. These findings suggest that the gut is the primary site of Treg stimulation to exogenous Ags during the first 18 mo of life and that homing receptor switch toward a more extra-intestinal phenotype occurs thereafter. The Journal of Immunology, 2009, 183: 4360 – 4370.

C D4⁺CD25<sup>FOXP3</sup> regulatory T cells (Treg)<sup>3</sup> play a central role in maintenance of self tolerance and immune homeostasis. Depletion of Treg leads to severe autoimmune and inflammatory conditions in mice (1, 2). In adults, Treg from both peripheral blood and tissues suppress in vitro proliferation and cytokine production of other T cells in response to both self and exogenous Ags (3–6). Treg in thymus and umbilical blood have a suppressive capacity (7). Treg express high levels of self and exogenous Ags (3–6). Treg in thymus and umbilical blood may be induced from organ-specific autoimmune diseases, severe dermatitis, enterodysregulation) (9, 10). This syndrome is characterized by several enteropathy X-linked syndrome/X-linked autoimmunity-allergic IPEX/XLAAD (immunodysregulation, polyendocrinopathy, and
Treg than conventional T cells in adults (3). In contrast, Treg in umbilical cord blood generally have a naive phenotype, as determined by the expression of CD45RA, and express more α4β7 and less CCR4 compared with Treg from adults (23, 25, 26). When naïve CD45RA−FOXP3+ Treg from cord blood were stimulated in vitro, the resulting CD45RO+ FOXP3+ Treg expressed little α4β7, but had up-regulated their expression of CCR4 (25). Taken together, these results imply that Treg mature and switch homing receptors at some time between birth and adulthood, but at what age this occurs in vivo is not known.

We hypothesized that the homing receptor switch on Treg would occur during the first years of life, when most foreign Ags are first encountered. We therefore in a prospective cohort of infants explored homing receptor expression by regulatory and conventional T cells in infants from birth until 3 years of age and compared this expression with that seen in corresponding cells from adults. The association between memory cell differentiation and homing receptor expression was examined as well as the capacity of cord and adult blood Treg to migrate toward various tissue-specific chemokine gradients. Our results demonstrate that homing receptor switch from α4β7 to CCR4 on Treg does not occur during the first 18 mo of life but has commenced at 3 years of age. Further, we show that the homing receptor switch is associated with the maturation of the Treg.

Materials and Methods
Subjects and collection of blood samples
Two different sources of infant blood samples were used in this study. In 38 gestational weeks) in rural areas of southwestern Sweden; 7, 8, and 11, cord blood samples were obtained totally different sources of infant blood samples were used in this study, aiming at investigating the relation between gut bacterial colonization pattern in infancy, maturation of the immune system, and the development of allergy. From these 66 children, we obtained blood samples at birth (n = 45), 3–5 days (n = 55), 1 mo (n = 55), 4 mo (n = 50), 18 mo (n = 54), and at 3 years (n = 11) of age. For the results in Figs. 2, C and D; 4, B and C; 5; 6D; 7; and 8, cord blood samples were obtained totally from 29 unselected healthy children born at the Sahlgrenska University Hospital, whereas the 18 mo samples (n = 8) were obtained from the prospective study described above. We also obtained peripheral blood samples from totally 30 healthy adults aged 25–55 years. All blood samples were collected in preservative-free heparinized tubes. Informed consent was obtained from the parents and volunteers, and the study was approved by the Human Research Ethics Committee of the Medical Faculty, University of Gothenburg, Sweden.

Flow cytometry
Phenotypic analysis of lymphocytes was performed by flow cytometry within 72 h of venepuncture. Initial experiments showed that proportions and numbers of different lymphocyte populations were unaffected by 72 h storage compared with freshly isolated samples. Flow cytometry analysis was either performed on whole blood (data in Figs. 1, 2, A and B; 3; 4A; and 6, A–C) or on lymphocytes separated by Lymphoprep (Nycomed) gradient centrifugation. For cell surface staining, whole blood (50 μl per tube) or separated PBMC were incubated with the respective mAbs for 20 min at 4°C in the dark, thereafter RBC were lysed (FACS lysing solution, BD Bioscience). Lymphocytes were washed twice and suspended in FACs buffer before analysis. Cells stained using biotinylated mAbs, were washed twice with FACs buffer before incubation with streptavidin-allophycocyanin (Fig. 4, B and C) or streptavidin-FITC for 20 min (Fig. 7A). After the cell surface staining was completed, the cells were fixed, permeabilized and stained for FOXP3 according to the manufacturer’s instructions (eBioscience). The following anti-human mAbs were used: allophycocyanin-conjugated; anti-CD25 (clone 2A3; BD Bioscience), FITC-conjugated; anti-CD45RA (clone L48; BD Bioscience), anti-CD62L (Dreg-56; BD Bioscience), anti-CD45RO (clone UCHL-1; BD Bioscience), anti-CD49d (clone 44H6; Se-rotech), anti-CCR7 (clone 150503; R&D Systems), anti-CCR4 (clone 205410; R&D Systems), anti-CD4 (clone SK3; BD Bioscience), PE-conjugated; anti-CD45RA (clone UCHL-1; BD Bioscience), anti-β7-integrin (clone FIB504; BD Bioscience), anti-CCR4 (clone IG1; BD Bioscience), PEcy7-conjugated; anti-CD25 (clone SA3; BD Bioscience), anti-FOXP3 (clone PCH101; eBioscience), biotinylated anti-α4β7 (clone ACT-1, provided by Dr. M. Briskin, previously Millenium Pharmaceuticals, Cambridge, MA), and streptavidin-allophycocyanin and -FITC (BD Bioscience). All isotype controls were purchased from BD Bioscience except for the isotype control for FOXP3 that was purchased from eBioscience. Samples were analyzed on a FACScalibur (BD Bioscience) equipped with CellQuestPro software or a FACScan (BD Bioscience) equipped with a FACSDiva software. Data were analyzed using the Flow Jo software (TreeStar).

Chemotaxis assay
Chemotaxis assays were performed to analyze the migration of adult and cord blood CD4+ T cells to a panel of chemokines using 12-well Corning Transwells (5 μm pore size, Corning). CD4+ cells were purified with Dynabeads (Dynal Biotech) from blood mononuclear cells separated by Lymphoprep (Nycomed) gradient centrifugation. Isolated CD4+ T cells were incubated in chemotaxis medium (RPMI 1640 supplemented with 0.5% BSA from Sigma-Aldrich) for 1.5h at 37°C, after which 3 x 105 cells were added to the top well of each Transwell insert. Cells were allowed to migrate toward CCL19, CCL22, and CCL25 diluted in chemotaxis medium to a final concentration of 1 μg/ml, 100 ng/ml, and 600 nM respectively, or chemotaxis medium alone for 90 min at 37°C. Cells in the starting population and migrating populations were stained for CD4, CD25, and FOXP3, and the percentage of indicated cell populations in the starting population (start) and in the population migrating to chemokine (chemokine), or chemotaxis medium alone (control) were determined by flow cytometry. Expression of integrin αβ7 and chemokine receptor CCR4 and CCR7 was determined on the starting population and performed as described earlier. The specific migration was calculated as percentage of indicated cell population (chemokine minus control)/percentage of indicated cell population (start).

Statistical analysis
Statistical analysis was performed using GraphPad Prism (GraphPad). Kruskal-Wallis test followed by Dunn’s multiple comparison test was used to analyze differences in cell marker expression between the different age groups. Friedman’s test followed by Dunn’s multiple comparison test was used to compare the difference between CD25low, CD25int, and CD25high T cell fractions within each age group. p ≤ 0.05 values were considered significant (∗, p < 0.05; ∗∗, p < 0.01; ∗∗∗, p < 0.001).

Results
Rapid increase of FOXP3 expression after birth
FOXP3 is expressed mainly in the CD4+CD25+ T cell subset and is a marker to identify regulatory T cells (2, 8, 27). We first wanted to determine whether the fraction of CD4+CD25+ FOXP3+ T cells in blood would increase during early infancy. Therefore, we prospectively collected blood samples at the ages 0 and 3–5 days; 1, 4, and 18 mo; and 3 years and compared the data with those of adult blood. As expected, FOXP3 was expressed mainly by CD4+CD25+ T cells and only rarely by CD4+CD25− T cells (Fig. 1, A and E). Interestingly, the percentage of CD4+CD25+ FOXP3+ T cells increased significantly between birth and 3–5 days of age, but after this timepoint no further increase in CD4+CD25+FOXP3+ T cell numbers occurred (Fig. 1B).

The intensity of CD25 expression on CD4+ T cells is shown in Fig. 1C. In children, two relatively clearly demarcated populations were observed, i.e., CD25+ and CD25low cells. CD25low were defined as the 2% CD4+ T cells with the brightest expression of CD25. Among the CD25low T cells, only very few in all age groups expressed FOXP3, suggesting that CD25+ T cells are nonregulatory T cells (Fig. 1, D and E) (27). In adults, there was no clear separation between CD4+CD25+ and CD4+CD25low T cells due to the presence of cells with intermediate CD25 expression (CD25int), assumed to be activated/memory T cells. Of the CD25low T cells identified in adults, only ~10% expressed FOXP3, suggesting that the majority of these cells were not Treg. In contrast, FOXP3 was expressed by most of the CD25int T cells (~80–90%) and by a large proportion (~65–70%) of the CD25+ T cells in...
blood from infants in all age groups, but only by 30 and 40% of
cord blood CD25^+ and CD25^{high} T cells, respectively (Fig. 1E).
Thus, significantly lower numbers of FOXP3^+ T cells were
present in the CD25^+ and CD25^{high} population in cord blood com-
pared with blood from infants collected already at 3–5 days of age
(Fig. 1E). We also noted a remarkable individual variation in
FOXP3 expression in both the CD25^+ and CD25^{high} T cell frac-
tions up to 4 mo of age, e.g., 10–90% at 4 mo of age (Fig. 1E). At
18 mo of age the variation between infants was less pronounced
and resembled the individual variation in adults.

Together these results indicate a rapid increase of FOXP3-ex-
pressing T cells in the circulation after birth and variability in
FOXP3 expression among T cells in the blood of young infants.
Owing to the large discrepancy between expression of FOXP3 and
high level expression of CD25 in CD4^+ T cells from cord blood,
we decided to use a CD25-based subsetting when analyzing the
expression of homing receptors.

Expression of CD62L and CCR7 on CD4^+ T cells during
postnatal development

CD62L and the chemokine receptor CCR7 direct naive cells and
central memory T cells to secondary lymphoid tissues (14). In
infants, CD25^+ T cells expressed significantly more (p < 0.01–
0.001) CD62L than both CD25^+ and CD25^{high} T cells (Fig. 2, A
and B). In adults, CD25^{high} T cells instead expressed significantly
higher (p < 0.001) proportions of CD62L than CD25^+ T cells
(Fig. 2, A and B). The largest proportion of CCR7^+ T cells was
detected in cord blood T cell fractions and the percentage

**FIGURE 1.** Expression of FOXP3 and CD25 in CD4^+ T cells at different ages. Cord blood and peripheral blood from children at different ages and from adults were costained with anti-CD4, anti-CD25 and anti-FOXP3. A, Representative dot plots showing the expression of CD25 vs FOXP3 on gated CD4^+ T cells at 0, 3–5 days and at 1, 4, and at 18 mo of age, and in adults. B, Percentage of CD25^+FOXP3^+ cells among all CD4^+ T cells (upper right quadrant in A) at different ages. C, Three gates were set based on the percentage of CD25 expression on CD4^+ T cells to identify CD25^-, CD25^{int} (only in adults), CD25^+ and CD25^{high} T cells. One representative contour plot is shown for each age group. D, Representative contour plots showing FOXP3 expression in CD25^-, CD25^+, and CD25^{high} T cells from cord blood and peripheral blood from infants and adults. E, Percentage of FOXP3^+ cells in the different cell fractions at the different ages. Each dot represents an individual at 0 days (n = 43), 3–5 days (n = 52), at 1 (n = 55), 4 (n = 50), and 18 mo of age (n = 34), and in adults (n = 20). Horizontal bars indicate median values. Statistical differences between ages within CD25^-, CD25^+, and CD25^{high} T cell fractions were calculated using Kruskal-Wallis test followed by Dunn's multiple comparison test. ***, p < 0.01; ****, p < 0.001.
CCR7$^+$ T cells was significantly reduced in all T cell fractions from adults (Fig. 2, A and D). Whereas CD25$^-$ T cells retained CCR7 expression during the first 18 mo, we observed down-regulation of CCR7 in CD25$^{high}$ T cells between birth and 18 mo of age (Fig. 2, A and D). Thus, a significantly lower ($p < 0.05$) proportion of CD25$^{high}$ T cells expressed CCR7 at 18 mo of age than at birth (Fig. 2, A and D). Together, these results demonstrate that both Treg and conventional T cells in infants have a phenotype that allow migration to lymphoid tissues, and that a more rapid down-regulation of homing receptors for secondary lymphoid tissues occurs in Treg cells than in the conventional T cells during postnatal development.

Expression of CCR4 and $\alpha$4$\beta$7 on CD4$^+$ T cells during postnatal development

Previous studies have shown that a majority of the Treg in cord blood express the gut-homing integrin $\alpha$4$\beta$7, while only a minor subset of Treg express CCR4, a chemokine receptor associated with extra-intestinal homing (23, 25). In contrast, the majority of Treg isolated from adult blood express CCR4, while only a minority of the cells express $\alpha$4$\beta$7 (23, 24). We therefore aimed at elucidating the postnatal changes in the expression of these homing markers on circulating T cells. When examining homing properties of T cells the circulating cells are highly relevant because the expression of homing receptors are often down-regulated when the cells have reached their target tissue (5). In Fig. 3A we combined $\beta_7$-integrin and CD49d ($\alpha4$) staining, enabling us to detect cells positive for the heterodimer $\alpha$4$\beta$7. Expression of $\alpha$4$\beta$7 was detected on a substantially larger proportion of T cells in infants up to 18 mo of age than in adults (Fig. 3, A and B). Conversely, a significantly smaller proportion of cells expressed CCR4 in infants up to 18 mo of age, relative to adults (Fig. 3, C and D). Thus, until this age no homing receptor switch had occurred. However, at 3 years of age the expression of $\alpha$4$\beta$7 was significantly reduced and the expression of CCR4 significantly increased on CD25$^+$ and CD25$^{high}$ T cells as compared with at birth (Fig. 3, A–D). Furthermore, the proportion of cells expressing $\alpha$4$\beta$7 in both infants and adults was significantly higher among the conventional CD25$^-$ T cells than among the CD25$^+$ T cells in cord blood while the fraction of CD25$^+$ and CD25$^{high}$ T cells expressing CCR4 was significantly higher in CD25$^+$ and CD25$^{high}$ T cells than in the CD25$^+$ and the CD25$^{high}$ T cells ($p < 0.05–0.001$).

Because it has previously been shown that naive conventional T cells express intermediate levels of $\alpha$4$\beta$7, allowing them to access the mesenteric lymph nodes and Peyer’s patches, while memory T cells express high levels of $\alpha$4$\beta$7, directing them toward the intestinal lamina propria, we also analyzed the proportion of high level $\alpha$4$\beta$7 expression on the CD25$^+$, CD25$^-$ and CD25$^{high}$ T cells. In Fig. 3A, the example shows that the $\alpha$4$\beta$7 is expressed at intermediate levels by CD25$^-$ T cells in cord blood while the fraction of $\alpha$4$\beta$7 expressed at high levels increases with age. As clearly shown...
in the summarizing Fig. 4A, these age-dependent changes in \(\alpha_4\beta_7\) T cells are not found in CD25\(^{high}\) T cell fraction. Although the \(\alpha_4\beta_7\) T cells reach a median level of 8% of all \(\alpha_4\beta_7\)-expressing CD25\(^{neg}\) T cells in adults, the proportion of \(\alpha_4\beta_7\) T cells in the CD25\(^{high}\) fraction is not increased in adults compared with in infants. To conclude, Tregs not only differ in the qualitative expression of \(\alpha_4\beta_7\) from conventional T cells, but also in the quantitative change of expression level of the homing receptor during growth.

Next, we explored whether the Treg and conventional T cells from cord and adult blood expressed only \(\alpha_4\beta_7\) or CCR4 or whether the receptors were coexpressed on the same cell. In Fig. 4B we show dot plots for staining with anti-CCR4 vs a biotinylated mAb to \(\alpha_4\beta_7\) on CD25\(^{-}\), CD25\(^{+}\), and CD25\(^{high}\) T cells from cord and adult blood. The majority of the CD25\(^{-}\), CD25\(^{+}\), and CD25\(^{high}\) T cells in cord blood as well as in adult blood expressed either \(\alpha_4\beta_7\) or CCR4 (Fig. 4B and C). At the most, 15% of the CD25\(^{+}\) T cells from cord blood coexpressed the receptors (Fig. 4C). Thus, Treg from infants express a gut-homing phenotype, whereas Treg from adults show an extra-intestinal homing phenotype. Furthermore, Treg from infants had started the switching of homing receptors from \(\alpha_4\beta_7\) to CCR4 by 3 years of age and the receptors \(\alpha_4\beta_7\) and CCR4 were not coexpressed.

**FIGURE 3.** Expression of \(\alpha_4\beta_7\) and CCR4 by CD25\(^{-}\), CD25\(^{+}\), and CD25\(^{high}\) T cells at different ages. A, One representative contour plot per age group showing \(\beta_7\)-integrin and \(\alpha_4\)-integrin staining in the different T cell fractions. B, Percentage of \(\alpha_4\beta_7\) cells in all individuals in the different fractions at birth (\(n = 41\)), at 4 mo (\(n = 48\)), at 18 mo (\(n = 33\)), at 3 years of age (\(n = 10\)), and in adults (\(n = 20\)). C, One representative contour plot per age group and cell fraction showing CCR4 and CD4 staining. D, Percentage of CCR4\(^{+}\) cells in all individuals in the different cell fractions at birth (\(n = 39\)), at 4 mo (\(n = 48\)) at 18 mo (\(n = 33\)), at 3 years of age (\(n = 11\)), and in adults (\(n = 20\)). Each dot represents an individual and horizontal bars indicate median values. Statistical differences were calculated using Kruskal-Wallis test (differences between age groups within each cell fraction) and Friedman’s test (differences between cell fractions within each age group), followed by Dunn’s multiple comparison test. ***, \(p < 0.001\).

**Treg from infants and adults display distinct migration properties**

To test whether the differential expression of homing receptors on Treg reflects their functional migratory properties, CD4\(^{+}\) T cells were isolated from cord and adult blood and their migration patterns were analyzed in a chemotaxis assay. Chemokines were titrated and the optimal concentrations of CCL19, CCL22, and CCL25 were used (data not shown). The specific migration of cord CD25\(^{high}\) and FOXP3\(^{+}\) T cells to CCL19 (the ligand for CCR7) was higher than the migration of the corresponding adult Treg cells (Fig. 5A), which is in agreement with a larger proportion of the cord cells expressing CCR7 (Fig. 2D). Interestingly, and in accordance with their gut homing receptor profile, cord Treg cells also demonstrated a more efficient migration to the gut-associated chemokine CCL25 compared with adult Treg cells (Fig. 5B). In contrast, adult CD25\(^{high}\) T cells as well as FOXP3\(^{+}\) T cells seemed to migrate somewhat more efficiently toward the CCR4 ligand CCL22 compared with cord T cells (Fig. 5C). Down-regulation of chemokine receptor expression was detected upon chemotaxis for both cord and adult CD4\(^{+}\) T cells (data not shown), indicating chemokine binding and active migration to the chemokine.
examined. Thus, the homing receptor expression on Treg cells seems to correspond to their actual migration properties.

Postnatal development of memory phenotype of CD4+ T cells

In humans, the cell surface marker CD45RA is associated with a naive phenotype of T cells, while CD45RO is associated with a memory phenotype (28). As expected, we detected higher CD45RA and lower CD45RO expression on all T cell subsets up to 18 mo of age compared with the corresponding T cells from adults (Fig. 6, A–C). However, at 3 mo, the expression of CD45RA was significantly reduced on the CD25high T cells compared with at 18 mo of age, while the expression of CD45RO was increased. Moreover, virtually all conventional CD25− T cells in infants were of a naive phenotype, while a median of 19–28% of the CD25+ and CD25high in infants were CD45RO positive already at birth (Fig. 6C). To confirm this finding, we analyzed CD4+CD25− FOXP3+ T cells isolated from cord blood and almost one third of the cells expressed CD45RO (Fig. 6D). However, when we divided these CD4+CD25− FOXP3+ T cells further into FOXP3low- and FOXP3high-expressing cells, the large majority of the FOXP3high T cells expressed CD45RO, while much fewer of the FOXP3low T cells expressed CD45RO (Fig. 6D). Thus, Treg with a bright expression of FOXP3 have a mature phenotype already in cord blood. In conclusion, except for the FOXP3high T cells that were of a memory phenotype already at birth, the CD25−, CD25+, and CD25high T cells were of a naive phenotype up to 18 mo of age but the CD25high T cells had started the switch to a memory phenotype by 3 years of age.

Expression of homing receptors αβγ, CCR4, or CCR7 on naive and memory T cells during postnatal development

As the pattern of αβγ expression in infants mirrored the CD45RA expression and as the CCR4 expression showed similarities to the expression of CD45RO, we continued to explore the homing receptor expression on CD45RA and CD45RO positive T cell fractions, respectively, in infants and adults. Accordingly, in infants, the majority of the naive CD25high T cells expressed αβγ, while only a minority of the memory CD25high T cells expressed αβγ (Fig. 7A). In adults, a low fraction of both naive and memory CD25high T cells expressed αβγ, (Fig. 7A). Moreover, both in infants and in adults, a minority of the CD25high T cells of naive phenotype expressed CCR4, whereas a majority of the CD25high T cells of memory phenotype expressed CCR4 (Fig. 7B). Almost all naive T cells expressed CCR7 both in infants and in adults, whereas a lower fraction of CD25high T cells from infants and adults expressed CCR7 (Fig. 7C). However in newborns, the majority of the CD25high T cells still expressed CCR7 (Fig. 7C). In conclusion, the majority of naive Treg express αβγ in infants but not in adults, while the majority of memory Treg express CCR4 in both infants and adults.

Age-dependent changes in the distribution of naive, central, and effector memory subsets in CD25−, CD25+, and CD25high T cells

Naive cells are only allowed to circulate between the blood and the secondary lymphoid tissues. Memory cells (CD45RO) are divided into two subpopulations: so-called central memory T cells that also circulate between blood and secondary lymphoid tissues, and effector memory cells that are allowed to exit into the tissues. We explored changes in the proportions of central memory and effector memory T cells in CD25−, CD25+, and CD25high T cells during early childhood. Naive (CCR7+CD45RO−), central memory (CCR7+CD45RO+), and effector memory (CCR7−CD45RO−) cells were identified in the different CD4 T cell fractions at birth, at 18 mo of age and in adults (Fig. 8). In cord blood the majority (93%) of conventional CD25− T cells were of a naive (CCR7−CD45RO−) phenotype (Fig. 8A). However, only 65% of the regulatory CD25high T cells were of a naive (CCR7−CD45RO−) phenotype, while 27% of the cells were of a central memory phenotype (CCR7+, CD45RO−), and 8% were of effector memory
CCR7 are linked to a naive phenotype of Treg. Furthermore, the maturation of the cells. We also demonstrate that in newborns and commenced at 3 years of age concomitantly with other signs of time that homing receptor switch from infants during the first 3 years of life. We show for the first time that homing receptor expression on central and effector memory CD25high T cells in the circulation as soon as 3–5 days after birth confirm and extend previous results showing in a smaller, cross-sectional study that the observed increase in CD25 expression of FOXP3-expressing T cells to the blood soon after birth or, alternatively, an up-regulation of FOXP3 in the circulating CD4+ T cells, and that the first days in life appear to provide the window during which this cell subset is most dynamically affected. Our results from infant peripheral blood are interesting in the light of mouse data showing that ~25% of CD4+CD25high Foxp3+ thymocytes represent Treg precursors that are rendered Foxp3pos and functional by IL-2 stimulation alone (30). Thus, stimulation of T cells from newborn infants might result in IL-2 production that would be sufficient for converting CD25high FOXP3+ T cells into CD25highFOXP3+ T cells.

The fetal intestine is sterile at birth, but colonization by a variety of microorganisms begins directly after delivery (31). Because murine studies show that exposure to bacterial Ags favor the generation and/or expansion of functional Treg it is likely that acquisition of commensal flora is necessary to kick-start the tolerogenic mechanisms in of the immune system (32-34). In addition, Treg from germ-free mice are less suppressive than those from conventional or colonized but pathogen-free mice, and they also express less FOXP3 than conventional mice (33, 34). Thus, it is possible that the observed increase in CD25+ Treg during the first days of life are due to the stimulatory effect of intestinal bacteria that induce IL-2 production from both intestinal and systemic T cells. In fact, we observed a large individual variation in the proportions of FOXP3 expression in both the CD25+ and CD25high T cell fractions during the first 4 mo of life. This variation might well reflect differences in colonizing bacterial strains as well as in the timing of colonization.

The large interindividual variation in FOXP3 expression that we observed in cord blood and up to 4 mo of age clearly shows that expression of high levels of CD25 does not always correlate with the expression of FOXP3, particularly not in newborns. These data together with observations of lower frequency of FOXP3+ T cells in cord blood and up to 4 mo of age show that increasing CD25 expression of FOXP3-expressing T cells from newborn infants might result in IL-2 production from both intestinal and systemic T cells. In CD4+CD25high T cells isolated from cord blood we observed similar proportions of T cells with central and effector memory phenotype, i.e., 46 and 44%, respectively, and only 6% with a naive phenotype (Fig. 8C). In summary, the majority of the memory CD25high T cells in newborns were of a central memory phenotype, while at 18 mo of age and in adults the memory CD25high T cells were equally distributed in the central memory and effector memory fractions.

**Discussion**

In this study, we provide information on the postnatal kinetics of FOXP3, homing receptor and memory cell expression in/on Treg from infants during the first 3 years of life. We show for the first time that homing receptor switch from α4β7 to CCR4 on Treg does not occur during the first 18 mo of life but that the switch has commenced at 3 years of age concomitantly with other signs of maturation of the cells. We also demonstrate that in newborns and infants CCR4 is linked to a mature phenotype, while α4β7 and CCR7 are linked to a naive phenotype of Treg. Furthermore, the majority of the memory Treg in newborns were of a central memory (CCR7+CD45RO−) phenotype, while they already at 18 mo of age were equally distributed between central and effector (CCR7−CD45RO+) memory fractions, as in adults. We also show that frequencies of CD4+ FOXP3+ T cells rapidly increase in the circulation during the first days after birth.

Our results demonstrating increased proportions of FOXP3+ T cells in the circulation as soon as 3–5 days after birth confirm and extend previous results showing in a smaller, cross-sectional study increased expression of FOXP3 in CD4+ T cells between birth and 4–14 days of age (29). Together, these results indicate a very rapid recruitment of FOXP3-expressing T cells to the blood soon after birth or, alternatively, an up-regulation of FOXP3 in the circulating CD4+ T cells, and that the first days in life appear to provide the window during which this cell subset is most dynamically affected.

The fetal intestine is sterile at birth, but colonization by a variety of microorganisms begins directly after delivery (31). Because murine studies show that exposure to bacterial Ags favor the generation and/or expansion of functional Treg it is likely that acquisition of commensal flora is necessary to kick-start the tolerogenic mechanisms in of the immune system (32-34). In addition, Treg from germ-free mice are less suppressive than those from conventional or colonized but pathogen-free mice, and they also express less FOXP3 than conventional mice (33, 34). Thus, it is possible that the observed increase in CD25+ Treg during the first days of life are due to the stimulatory effect of intestinal bacteria that induce IL-2 production from both intestinal and systemic T cells. In fact, we observed a large individual variation in the proportions of FOXP3 expression in both the CD25+ and CD25high T cell fractions during the first 4 mo of life. This variation might well reflect differences in colonizing bacterial strains as well as in the timing of colonization.

The large interindividual variation in FOXP3 expression that we observed in cord blood and up to 4 mo of age clearly shows that expression of high levels of CD25 does not always correlate with the expression of FOXP3, particularly not in newborns. These data together with observations of lower frequency of FOXP3+ T cells in cord blood and up to 4 mo of age show that increasing CD25 expression of FOXP3-expressing T cells from newborn infants might result in IL-2 production from both intestinal and systemic T cells. In CD4+CD25high T cells isolated from cord blood we observed similar proportions of T cells with central and effector memory phenotype, i.e., 46 and 44%, respectively, and only 6% with a naive phenotype (Fig. 8C). In summary, the majority of the memory CD25high T cells in newborns were of a central memory phenotype, while at 18 mo of age and in adults the memory CD25high T cells were equally distributed in the central memory and effector memory fractions.

**Discussion**

In this study, we provide information on the postnatal kinetics of FOXP3, homing receptor and memory cell expression in/on Treg from infants during the first 3 years of life. We show for the first time that homing receptor switch from α4β7 to CCR4 on Treg does not occur during the first 18 mo of life but that the switch has commenced at 3 years of age concomitantly with other signs of maturation of the cells. We also demonstrate that in newborns and infants CCR4 is linked to a mature phenotype, while α4β7 and CCR7 are linked to a naive phenotype of Treg. Furthermore, the majority of the memory Treg in newborns were of a central memory (CCR7+CD45RO−) phenotype, while they already at 18 mo of age were equally distributed between central and effector (CCR7−CD45RO+) memory fractions, as in adults. We also show that frequencies of CD4+ FOXP3+ T cells rapidly increase in the circulation during the first days after birth.

Our results demonstrating increased proportions of FOXP3+ T cells in the circulation as soon as 3–5 days after birth confirm and extend previous results showing in a smaller, cross-sectional study increased expression of FOXP3 in CD4+ T cells between birth and 4–14 days of age (29). Together, these results indicate a very rapid recruitment of FOXP3-expressing T cells to the blood soon after birth or, alternatively, an up-regulation of FOXP3 in the circulating CD4+ T cells, and that the first days in life appear to provide the window during which this cell subset is most dynamically affected. Our results from infant peripheral blood are interesting in the light of mouse data showing that ~25% of CD4+CD25high Foxp3+ thymocytes represent Treg precursors that are rendered Foxp3pos and functional by IL-2 stimulation alone (30). Thus, stimulation of T cells from newborn infants might result in IL-2 production that would be sufficient for converting CD25high FOXP3+ T cells into CD25highFOXP3+ T cells.

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The large interindividual variation in FOXP3 expression that we observed in cord blood and up to 4 mo of age clearly shows that expression of high levels of CD25 does not always correlate with the expression of FOXP3, particularly not in newborns. These data together with observations of lower frequency of FOXP3+ T cells in cord blood may explain previous contradictory results regarding the suppressive function of cord Treg. Cells that have been isolated with anti-CD25 coated magnetic beads have had weaker suppressive function than CD4+CD25high Foxp3+ T cells isolated by flow cytometry (6, 7, 35, 36). This may not be surprising because ~90% percent of the CD4+CD25−CD127low T cells isolated by flow cytometry (6, 7, 35, 36). This may not be surprising because ~90% percent of the CD4+CD25−CD127low T cells isolated by flow cytometry (6, 7, 35, 36). This may not be surprising because ~90% percent of the CD4+CD25−CD127low T cells isolated by flow cytometry (6, 7, 35, 36).
Regulation of immune responses by Treg is likely to occur both in lymph nodes, where immune responses are initiated and in target tissues where effector cells may cause tissue damage if not adequately controlled (39, 40). Chemokines and their receptors direct tissue-specific homing in combination with adhesion molecules on endothelial cells and their ligands on leukocytes (12). Thus, CD62L and CCR7 permit lymphocytes to circulate through secondary lymphoid organs. In this study, we demonstrate that most CD25high T cells in from both infants and adults are CD62L−/H11001 and CCR7−/H11001. Treg-expressing CCR7 and high levels of CD62L have been shown to prevent the development of autoimmune diabetes in mice, suggesting suppression of self-reactive T cells in the regional lymph nodes (41). Upon encounter with Ag in secondary lymphoid tissues, effector T cell gain distinct homing characteristics, mediated by de novo expression of organ-specific adhesion molecules and chemokine receptors (16). T cells activated in Peyer's patches or lymph nodes draining the gut start to express CCR9 and integrin $\alpha_4\beta_7$, whereas activation in peripheral lymph nodes draining the skin results in up-regulation of CCR4, cutaneous lymphocyte-associated Ag, and P-selectin ligand (15–18, 20). It has been shown that Treg are also important in down-regulating the activity of effector T cells in peripheral tissues and at sites of ongoing immune responses. For example, mice whose Treg lack CCR4 develop lymphocytic infiltration and severe inflammatory disease in the skin and lungs (22). Recently, it was shown that FOXP3+ T cells in mice that were CD62L−/CCR7−/CXCR4low when leaving the thymus preferentially migrated to secondary lymphoid tissues and that switch in homing receptors enabled migration to nonlymphoid tissues after Ag stimulation (42). This switch involved down-regulation of CCR7 and up-regulation of different memory/effector type homing receptors and occurred more rapidly in Foxp3+ T cells than in FoxP3−T cells (42).

In the current study, we found that the majority of the Treg in cord and infant blood up to 18 mo of age expressed $\alpha_4\beta_7$, but not CCR4 and would therefore migrate to intestinal secondary lymphoid tissues. This points to the crucial importance of the gut as primary site of Ag exposure for T cells in early life. However, at 3 years of age, the homing receptor switch from $\alpha_4\beta_7$ to CCR4 had started, ending with an almost complete switch in adults in whom the majority of Treg express CCR4 and only a minority $\alpha_4\beta_7$ (23, 24). Interestingly, Treg also seemed to more rapidly down-regulate CCR7 and $\alpha_4\beta_7$ and up-regulate CCR4 compared with conventional T cells. Thus, our results suggest that Treg may migrate to the tissues before conventional T cells, which is important to avoid tissue pathology when Ag and infectious agents are encountered. The differential homing receptor expression of $\alpha_4\beta_7$ or CCR4 develop lymphocytic infiltration and severe inflammatory disease in the skin and lungs (22). Recently, it was shown that FOXP3+ T cells in mice that were CD62L−/CCR7−/CXCR4low when leaving the thymus preferentially migrated to secondary lymphoid tissues and that switch in homing receptors enabled migration to nonlymphoid tissues after Ag stimulation (42). This switch involved down-regulation of CCR7 and up-regulation of different memory/effector type homing receptors and occurred more rapidly in Foxp3+ T cells than in FoxP3− T cells (42).

![Figure 6](http://www.jimmunol.org/)

**FIGURE 6.** Expression of CD45RA and CD45RO by CD25−, CD25+, and CD25high CD4+ T cells at different ages. **A**, One representative contour plot of CD45RA and CD45RO staining per age group and cell fraction. B and C, Percentage of CD45RA−/CD45RO− (B) and CD45RA−/CD45RO+ (C) cells in CD25−, CD25+, and CD25high T cell fractions from cord blood ($n = 41$), and peripheral blood of children aged 3–5 days ($n = 53$), 1 ($n = 53$), 4 ($n = 50$), 18 mo ($n = 34$), at 3 years of age ($n = 11$), and in adults ($n = 20$). D, Expression of CD45RO by FOXP3+ CD4+ T cells from cord blood ($n = 11$) gated into FOXP3high and FOXP3low populations. Data are depicted as a representative dot plot and a summarizing graph. Each dot represents an individual and horizontal bars indicate the median. Statistical significances are calculated using Kruskal-Wallis test followed by Dunn’s multiple comparison test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

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CCR4 in infants and adults also reflected their functional migratory properties. Thus, cells from cord blood migrated better than adult cells toward the gut-associated CCR9 ligand CCL25, demonstrating that Treg in infants have gut homing properties and supporting the notion that the intestine may be central in tolerance development. Our results also suggest that the recently developed method of isolating pure FOXP3$^{+}$ Treg from adult human peripheral blood by using Abs directed against CD49d ($\alpha_4$-integrin) to

![FIGURE 7. Expression of $\alpha_4\beta_7$, CCR4 and CCR7 by naive and memory CD25$^+$, CD25$^-$, and CD25$^{high}$ CD4$^+$ T cells at different ages. A, Percentage of $\alpha_4\beta_7$ on CD45RA$^-$CD45RO$^+$ and CD45RA$^+$CD45RO$^+$ cells in CD25$^+$, CD25$^-$, and CD25$^{high}$ CD4$^+$ T cell fractions from cord blood (n = 7), 18 mo of age (n = 7) and in adults (n = 6). B, Percentage of CCR4 on CD45RA$^-$CD45RO$^+$ and CD45RA$^+$CD45RO$^+$ cells in CD25$^+$, CD25$^-$, and CD25$^{high}$ CD4$^+$ T cell fractions from cord blood (n = 7), 18 mo of age (n = 7) and in adults (n = 6). C, Percentage of CCR7 on CD45RA$^-$CD45RO$^+$ and CD45RA$^+$CD45RO$^+$ cells in CD25$^+$, CD25$^-$, and CD25$^{high}$ CD4$^+$ T cell fractions from cord blood (n = 7), 18 mo of age (n = 7) and in adults (n = 6). Each dot represents an individual and horizontal bars indicate the median.](http://www.jimmunol.org/)

![FIGURE 8. T cell subsets with naive, central, and effector memory phenotype within CD25$^+$, CD25$^-$, and CD25$^{high}$ T cell fractions at different ages. Percentages of cells with naive (CCR7$^+$, CD45RO$^-$) (A), central memory (CCR7$^-$, CD45RO$^+$) (B), and effector memory (CCR7$^-$, CD45RO$^+$) (C) phenotype in CD25$^+$, CD25$^-$, and CD25$^{high}$ CD4$^+$ T cell fractions from cord blood (n = 9), from peripheral blood of children aged 18 mo (n = 8), and from adults (n = 9). Each dot represents an individual and horizontal bars indicate the median values. Statistical differences were calculated using Kruskal-Wallis test (differences between ages within each cell fraction) and Friedman’s test (differences between cell fractions within each age group), followed by Dunn’s multiple comparison test, *, p < 0.05; **, p < 0.01; ***, p < 0.001.](http://www.jimmunol.org/)
deplete non-Treg is not suitable in human infants and children because the vast majority of the CD25high T cells up to 18 mo of age express CD49d, as well as a large fraction of the CD25high T cells in the 3 year olds (43). It is very unlikely that these cells are all activated proinflammatory T cells, particularly in newborns.

Most Treg and conventional T cells remained naive until 18 mo of age. At 3 years of age, a significant fraction of Treg had become memory cells as determined by CD45RO expression. The majority of naive CD25high T cells express αβ, in infants but not in adults, while memory CD25high T cells express CCR4 in both infants and adults. Thus, maturation of Treg in infants is thus coupled to a reduced expression of αβ, and increased expression of CCR4. In agreement with our results, a previous study showed that CD45RA+ FOXP3+ T cells in cord blood express αβ, while they found that that CD45RA– FOXP3– cells expressed CCR4 (25). After strong polyclonal in vitro stimulation, naive CD45RA+ T reg from cord blood converted to CD45RO+ with a subsequent decrease in αβ, expression and increase in CCR4 expression (25). Altogether, these results suggest that Treg undergo homing receptor switch during their differentiation to a memory CD45RO phenotype both in vitro and in vivo.

In line with previous studies, we also demonstrate that Treg with a memory phenotype are present already at birth because approximately a quarter of both CD25+ and CD25high T cells were CD45RO+ (25, 44). Differential expression of the chemokine receptor CCR7 can further divide human memory T cells into two functionally distinct subset (14). CCR7+ central memory cells controls T cell homeostasis and the onset of immune responses in lymphoid organs, while CCR7– effector memory cells are able to migrate toward inflamed tissues to exert effector functions (14).

We found that the majority of memory Treg are of a central memory (CCR7 CD45RO+) phenotype at birth. These central memory Treg have most likely been generated in the thymus and are therefore probably specific for self-Ags. Interestingly, we observed a decrease in the proportion of central memory T cells and a concomitant increase of the proportion of effector memory T cells in the CD25+ T cell fraction at 18 mo of age compared with newborns. This indicates that not only self-Ag-specific Treg cells are present at 18 mo of age but also Treg specific for exogenous Ags.

In accordance with our results, a previous study reported a 2-fold increase in the proportion of central memory T cells and a 5-fold increase in the proportion of effector memory T cells throughout life (36). However, while we show similar proportions of central and effector memory T cells at 18 mo of age in adults, these authors observed a larger proportion of effector T cells than central memory T cells in the CD4+ FOXP3+ T cell fraction in adults.

In conclusion, this study demonstrates a rapid increase in the proportions of FOXP3-expressing T cells in the blood soon after birth, which might reflect stimulation by environmental Ags leading to IL-2 production during the first days of life. We also demonstrate that Treg cells in infants up to 18 mo of age preferentially migrate toward gut-homing chemokines which points toward the gut as an important organ where stimulation occurs. However, at 3 years of age, the Treg had both started to mature and to switch into extra-intestinal homing receptors. This may indicate that as children grow older the gut is less important for activating naive T cells. The large interindividual variation in expression of homing receptors and maturation of Treg could be due to environmental factors, for example differential patterns of intestinal bacterial colonization. It will be of great interest to investigate whether early colonization with any particular bacterial species or groups will be able to induce maturation and change of homing receptor expression on Treg.


