Postthymic Expansion in Human CD4 Naive T Cells Defined by Expression of Functional High-Affinity IL-2 Receptors


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Postthymic Expansion in Human CD4 Naive T Cells Defined by Expression of Functional High-Affinity IL-2 Receptors


As the thymus involutes with age, the maintenance of peripheral naive T cells in humans becomes strongly dependent on peripheral cell division. However, mechanisms that orchestrate homeostatic division remain unclear. In this study we present evidence that the frequency of naive CD4 T cells that express CD25 (IL-2 receptor α-chain) increases with age on subsets of both CD31+ and CD31– naive CD4 T cells. Analyses of TCR excision circles from sorted subsets indicate that CD25+ naive CD4 T cells have undergone more rounds of homeostatic proliferation than their CD25– counterparts in both the CD31+ and CD31– subsets, indicating that CD25 is a marker of naive CD4 T cells that have preferentially responded to survival signals from self-Ags or cytokines. CD25 expression on CD25– naive CD4 T cells can be induced by IL-7 in vitro in the absence of TCR activation. Although CD25+ naive T cells respond to lower concentrations of IL-2 as compared with their CD25– counterparts, IL-2 responsiveness is further increased in CD31+ naive T cells by their expression of the signaling IL-2 receptor β-chain CD122, forming with common γ-chain functional high-affinity IL-2 receptors. CD25 plays a role during activation: CD25+ naive T cells stimulated in an APC-dependent manner were shown to produce increased levels of IL-2 as compared with their CD25– counterparts. This study establishes CD25+ naive CD4 T cells, which are further delineated by CD31 expression, as a major functionally distinct immune cell subset in humans that warrants further characterization in health and disease. The Journal of Immunology, 2013, 190: 000–000.

Peripheral expansion of human naive T cells is vital to maintain the naive T cell pool, particularly after thymic involution. Naive T cell expansion in the periphery preserves a diverse naive TCR repertoire that is critical to provide immunity to foreign Ags and to maintain peripheral tolerance when the thymus, owing to progressive involution with increasing age, is no longer able to generate sufficient naive TCR repertoire diversity. Recent quantitative studies of naive CD4 T cell expansion provided evidence that, in contrast to mice, naive T cells in healthy human adults are sustained almost exclusively by peripheral proliferation (1). Postthymic naive T cell expansion, which depends on various degrees of stimulation with cytokines such as IL-7 and interactions with APCs, creates a heterogeneous pool of naive T cells (2). Naive CD4+ T cells can be subdivided based on CD31 (PECAM-1) expression (3). CD31+ naive CD4+ T cells have undergone a minimal number of divisions after exiting the thymus whereas CD31– naive T cells have undergone multiple rounds of division since emigrating from the thymus. During naive CD4 T cell expansion, signals received through the TCR appear to drive CD31 downregulation, thereby forming the central naive T cell subset (2, 4). Because naive T cells are thought to downregulate the expression of CD31 after stimulation in the context of MHC class II molecules, their bona fide Ag inexperienced naive T cell status has been questioned. Although the TCR signals that drive loss of CD31 expression on central naive T cells are not strong enough to lead to naive T cell activation and loss or acquisition of markers characterizing effector or memory cells, that is, loss of CD45RA and CCR7 and gain of CD45RO, the signals are suffi-

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Abbreviations used in this article: DC, dendritic cell; MEF, molecules of equivalent fluorescence; SEB, staphylococcal enterotoxin B; TREC, TCR excision circle; Treg, regulatory T cell.

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cient to induce peripheral expansion, as manifested by loss of TCR excision circles (TRECs) and a reduction in the TCR repertoire of the expanding naïve CD4 T cell subset (2, 3). CD25 has long been categorized as a T cell activation marker. As a consequence, the functional significance of homeostatic CD25 expression on unstimulated T cells has been largely ignored, except in the case of FOXP3 regulatory CD4 T cells (Tregs) (5, 6). CD25 is the α-chain of the high-affinity trimeric IL-2 receptor; high levels of the high-affinity IL-2 receptor on Tregs enable them to respond to low concentrations of IL-2 that are critical for Treg survival and the maintenance of their suppressive function. In addition to Tregs, most resting memory CD4 T cells express CD25 in a constitutive fashion, albeit at lower levels than Tregs (7) (see Fig. 1A). We were, therefore, surprised to discover a subset of naïve CD4(+)CD45RA(+) T cells that expressed CD25 (7). This subpopulation increased in frequency with age, reaching as much as 20% of naïve CD4(+) by 40 y of age. In this study, we have confirmed and extended the evidence for the age-dependence of this expansion of CD25(+) naïve T cells, their relationship to loss of CD31 and TRECs, a role for IL-7, and the coexpression of the β-chain of the IL-2 receptor to form functional, high-affinity receptors on these naïve CD4(+) T cells that correlates with their increased responsiveness to IL-2.

Materials and Methods

Donors

Cambridge BioResource donors were collected with the prior approval of the National Health Service Cambridgeshire Research Ethics Committee. Donors were selected as part of three studies: genes and mechanisms of type 1 diabetes; genotype/phenotype study of newly diagnosed children with type 1 diabetes and siblings; and investigating genes and phenotypes associated with type 1 diabetes. Diabetes—Genes, Autoimmunity, and Prevention was approved by the National Research Ethics Committee London—Hampstead.

Genotyping

Single nucleotide polymorphisms were genotyped using custom TaqMan single nucleotide polymorphism genotyping assays or TaqMan (Applied Biosystems) according to the manufacturer’s protocols.

Cytokines

Recombinant human IL-7 and recombinant human IL-15 were obtained from R&D Systems and diluted according to the manufacturer’s instructions. Recombinant human IL-2 (Proleukin) was obtained from Novartis. Complete media for functional studies was X-VIVO 15 media (Lonza) supplemented with 5% heat-inactivated, filtered human AB serum (Sigma-Aldrich).

Whole blood and PBMC immunostaining

Blood samples were directly immunophenotyped within 5 h following donation. Samples were blocked for 10 min with mouse IgG (20 μg/ml), stained for 40 min at room temperature with appropriate Abs (Supplemental Table 1), and then lysed with freshly prepared 1× BD FACS lysing solution (Becton Dickinson Biosciences). After lysis of RBCs, samples were washed with BD CellWash (Becton Dickinson Biosciences). Finally, the samples were fixed with freshly prepared 1× BD CellFix (Becton Dickinson Biosciences). The samples were stored at 4°C in the dark until analysis using a BD Fortessa flow cytometer. PBMC samples, prepared as previously described by Dendrou et al. (7), were blocked for 10 min, stained for 1 h at 4°C, washed twice, and fixed as described for peripheral blood immunophenotyping except for intracellular staining when surface-stained cells after the wash step were placed in FOXP3 Fix/Perm buffer (eBioscience). The isotype control Abs used were allophycocyanin-conjugated mouse IgG1 (Becton Dickinson Biosciences), Alexa Fluor 488-conjugated mouse IgG1 (BioLegend), FITC-conjugated mouse IgG2 (BioLegend), PE20-conjugated IgG1 and IgG2 (Becton Dickinson Biosciences, BioLegend, eBioscience), and PerCP-Cy5.5-conjugated mouse IgG1 and IgG2 (BioLegend). As described by Dendrou et al. (7), CD25 detection sensitivity was increased by simultaneous application of two anti-CD25 mAbs labeled with the same fluorochrome (clones 2A3 and M-A251, Becton Dickinson Biosciences). Ab concentrations used were based on the manufacturers’ instructions as well as on optimization studies.

Intracellular detection of Ki-67 and FOXP3

Intracellular staining was performed on freshly surface-stained washed PBMCs that were placed in FOXP3 Fix/Perm buffer (eBioscience) and further processed according to the manufacturer’s protocol (to achieve high resolution of the FOXP3 staining, extensive washing steps were essential). The surface anti-human FOXP3 PE-Cy7–conjugated anti-CD25, allophycocyanin-conjugated anti-CD25 (clones M-A251 and 2A3; Becton Dickinson Biosciences), PE-Cy7–conjugated anti-CD127 (eBioscience), and eFluor 605–conjugated anti-CD45RA (eBioscience). The anti-human mAbs used for intracellular T cell immunophenotyping were Pacific Blue–conjugated anti-FOX3 (BioLegend) and PerCP-Cy5.5–conjugated anti-Ki-67 (Becton Dickinson Biosciences). Stained cells were washed, acquired, and analyzed using FlowJo (Treestar).

Flow cytometry detection of pSTAT5

For simultaneous detection of pSTAT5a, FOXP3, CD4, CD25, CD127, CD45RA, and CD31, 500 μl fresh blood was incubated with 500 μl X-VIVO medium with various concentrations of IL-2, IL-7, and IL-15 for 10 min at 37°C. Phosphorylation was preserved by adding 30 ml warm BD Lyse/Perm buffer (Becton Dickinson Biosciences). Cells were then washed with PBS containing 0.2% BSA and treated with 1 ml fresh ice-cold methanol for 20 min on ice (pSTAT5a-dimer disruption). After extensive washing with PBS containing 0.2% BSA, cells were stained for 1 h at 4°C in the dark. The following Abs were used: Pacific Blue–conjugated anti-FOXP3 (BioLegend), Alexa Fluor 488–conjugated anti-pSTAT5 (pY694) (Becton Dickinson Biosciences), Alexa Fluor 700–conjugated anti-CD4, allophycocyanin-conjugated anti-CD25 (clones MA251 and 2A3; Becton Dickinson Biosciences), PE-Cy7–conjugated anti-CD127 (eBioscience), eFluor 605–conjugated anti-CD45RA (eBioscience), and PE-conjugated anti-CD31 (eBioscience). Stained cells were washed and analyzed as above.

Flow cytometry and data analysis

Immunostained samples were analyzed as previously described by Dendrou et al. (7, 8). Calibration beads were used to calculate molecules of equivalent fluorescence (MEF) values.

PBMC activation

PBMCs stimulated with 5 μg/ml staphylococcal enterotoxin B (SEB; Sigma-Aldrich) for 4 h were cultured in 24-well flat-bottom plates as described previously by Dendrou et al. (7) except that PBMCs were cultured overnight before the addition of SEB, thereby allowing more robust IL-2 secretion. Naïve T cells responding to SEB were identified as CD69(+) using Pacific Blue–conjugated anti-CD69 (BioLegend), allophycocyanin-conjugated anti-CD25 (clones M-A251 and 2A3; Becton Dickinson Biosciences), FITC–conjugated anti-CD31 (eBioscience), Alexa Fluor 700–conjugated anti-CD4 (BioLegend), and PerCP-Cy5.5–conjugated anti-CD45RO (BioLegend). CD69(+) naïve T cell subsets secreting IL-2 were identified using an IL-2 PE secretion assay (Miltenyi Biotec).

PBMC and CD4(+) lymphocytes preparation

PBMC isolation, cryopreservation, and thawing were performed as previously described (7). Briefly, PBMC isolation was carried out using Lympholyte (Cedarlane Laboratories). PBMCs were cryopreserved in heat-inactivated, filtered human AB serum (Sigma-Aldrich) and 10% DMSO (Hybri-MAX, Sigma-Aldrich) at a final concentration of 10 × 10^6/ml and were stored in liquid nitrogen. Cells were thawed in a 37°C water bath for 2 min. PBMCs were subsequently washed by adding the cells to 10 ml cold (4°C) X-VIVO (Lonza) containing 10% AB serum per 10 × 10^6 cells, in a dropwise fashion. PBMCs were then washed again with 10 ml cold (4°C) X-VIVO containing 1% AB serum per 10 × 10^6 cells. CD4(+) T cells were purified using CD4(+) RosetteSep (Stemcell Technologies) according to the manufacturer’s instructions.

FACS

CD4(+) T cells were washed and immediately incubated with Abs (Supplemental Table I) for 40 min at 4°C, washed and followed by flow cytometry sorting (BD FACSAria III).

RNA and DNA isolation

Following cell sorting, cell subsets were collected for purity and placed in TRIzol reagent (Life Technologies) at −80°C. Total RNA was isolated using the phenol-chloroform method according to the manufacturer’s instructions. To isolate DNA, sorted cell subsets were collected for purity and DNA was isolated using DNA extraction reagent (Qiagen).
**cDNA synthesis and microarray gene expression analysis**

Single-stranded cDNA was synthesized from 200 ng total RNA using the Ambion whole transcript expression kit according to the manufacturer’s instructions. Labeled cDNA (GeneChip terminal labeling and hybridization kit; Affymetrix) was hybridized to a 96-sample Titan Affymetrix human gene 1.1 ST array. Data are deposited with ArrayExpress (http://www.ebi.ac.uk/arrayexpress/, accession no. E-MTAB-1138).

**TREC assay**

TREC assay was performed as described previously (9). Briefly, a quantitative PCR assay was purchased from Sigma-Genosys for the single joint TCR excision circle that arises through an intermediate rearrangement in the TCRD/TCRA locus in developing TCR\(\alpha\beta\)+ T lymphocytes. An assay for the gene encoding albumin was used to normalize the data. Sequences were as follows: single joint TREC, forward, 5’-TCGTGAGAACGGTGAATGAAG-3’, reverse, 5’-CCATGCTGACACCTCTGGTT-3’, probe, FAM-5’-CACGGTGATGCAATGGACCTGC-3’-TAMRA; albumin, forward, 5’-GCTGTCACTCTTGTGGGCTGT-3’, reverse, 5’-ACTCATGGAGCTGCTGGTTC-3’, probe, FAM-5’-CCTGTCATGCCCACACAAAATCCTCCC-3’-TAMRA.

For each sample, 24 ng DNA was incubated in duplicate with both primers (700 nM), probe (150 nM), and 12.5 \(\mu\)l TaqMan master mix (Applied Biosystems) on the 7900HT system (Applied Biosystems).

**Statistical analysis of flow cytometry data versus age**

We included 138 healthy individuals in the analysis in Fig. 1B. Statistical tests and model selections were implemented using R software (http://www.R-project.org). Age and sex effects were tested by linear regression analysis, with the phenotype, percentage of CD25+ naive T cells, and logarithm transformed to base 10. ANOVA and \(\chi^2\) tests were used for model selections.

**Statistical analysis of flow cytometry data interrogating T cell subsets**

Statistical analyses of the percentage of cells expressing Ki-67, CD31, IL-2, or pSTAT5 and the mean CD122 MEF were performed and presented using Prism 5 software (Graphpad Software). Comparisons between cell subsets were performed using a paired Student t test. A \(p\) value <0.05 was considered significant; error bars show the SD of the samples at each test condition.

**Results**

**Frequency of CD25+ naive T cells in humans increases with age**

We carried out an immunophenotyping study using 139 donors, including samples from young children and umbilical cords. The percentage of CD4+CD25+ naive T cells (gating strategy, Fig. 1A) was strongly associated with increasing age (\(p = 1.41 \times 10^{-28}\); Fig. 1B): over the age of 40 y \(>20\%\) of CD4+ naive T cells are CD25+. CD25+ naive CD4 T cells were also detected in cord blood, indicating that acquisition of CD25 expression by naive CD4 T cells begins prior to birth (Supplemental Fig. 1A).

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**FIGURE 1.** Frequency of human CD25+ naive CD4 T cells is determined by age. (A) CD4+ naive T cells were gated as CD4+CD127+CD25−/−CD45RO−CD45RA+; CD4+ memory cells were gated as CD4+CD127+CD25−/−CD45RO−CD45RA+; and Tregs were gated as CD4+CD127−/low/CD25+high. Dot plot inserts in the naive, memory, and Treg plots represent isotype controls for both fluorochromes. (B) The proportion of naive CD4 T cells that are CD25+ increases with age. \(p = 1.41 \times 10^{-28}\), \(n = 139\).
CD25+ CD45RA+CD4+ T cells: confirmation of the naive phenotype

Given the high prevalence of CD25+ naive CD4+ T cells, we wanted to obtain further evidence that they are in fact naive. CD25+ naive CD4+ T cells were immunophenotyped in detail using established markers that differentiate naive from memory CD4 T cells and characterize their activation, differentiation, or exhaustion status: CD27, CD28, CD45RA, CD45RO, CD38, CD57, CD44, CD62L, CD69, CD95, CD137, CD194, CD195, CD197, and PD-1. Expression of these molecules did not discriminate CD25+ naive CD4+ T cells from their CD25- counterparts (Fig. 2A, 2B). CD25+ and CD25- naive CD4 T cells expressed equal amounts of CCR7, CD62L, and CD27, indicating that CD25+ naive T cells do not belong to a memory subset that has reverted to CD45RA expression (10). We also detected a rare subset of memory cells with stem cell-like properties that display in general a naive phenotype but express higher levels of CD95 and CD122 as compared with naive T cells (15). These cells are also characterized by heterogeneous CD25 expression (Supplemental Fig. 2).

Homeostatic proliferation of naive T cells averages ~0.4% measured using an Ab against the proliferation marker Ki-67 and distinguishes the naive CD4+ T cell subset from CD4+ memory cells and Tregs that proliferate at higher rates (16, 17). To investigate a potential relationship between CD25 expression and naive T cell proliferation, naive T cells were stained for Ki-67. The analysis of Ki-67+ T cells indicated that CD25+ naive T cell proliferation was not significantly different from the proliferation rate of CD25- naive T cells (p = 0.06) (Fig. 2C, 2D). Importantly, however, note that the percentage of a cell population that is currently in cycle as detected by Ki-67 does not assess the proliferative history of the cells.

CD25+ naive T cells display naive T cell transcription profile

Because neither an obvious phenotypic difference nor a difference in ex vivo proliferation was observed between CD25+ and CD25- naive CD4+ T cells, we next compared the gene expression of sorted CD4 T cell subsets. For each of seven individual donors, we sorted five CD4 T cell populations (Fig. 3A, Supplemental Fig. 3C): CD25+ and CD25- naive T cells, CD25+ and CD25- memory T cells, and CD4 T cells, and gene expression were compared by microarray. A principal component analysis of the five studied populations demonstrated that the CD25+ naive CD4 T cell subset could not be distinguished from the CD25- naive subset but was clearly distinct from both of the memory and the Treg subsets (Fig. 3B). Specific analysis of Affymetrix human gene 1.1 ST probe sets mapping to IL2RA demonstrated that CD25+ naive T cells contain on average 5-fold more mRNA encoding IL2RA than their CD25- counterparts (Supplemental Fig. 3A, 3B), validating the approach.

Functional consequences of CD25 expression by naive T cells

To assess the functional significance of CD25 expression on naive CD4 T cells, peripheral blood was incubated with IL-2 in vitro and the phosphorylation of STAT5a was monitored by flow cytometry (Fig. 4A, 4B). The percentage of pSTAT5a CD4+ CD45RA+FOXP3- naive T cells increased with the dose of IL-2. At 1 U/ml IL-2, STAT5 was preferentially phosphorylated in naive CD4 T cells expressing the highest level of the α-chain (CD25) of the IL-2R, which is consistent with the α-chain’s ability to confer a high-affinity status to the receptor. A large proportion of all CD25+ naive CD4 T cells had increased pSTAT5 levels at a 10 U/ml dose of IL-2 whereas only a minimal response was observed with CD25- naive T CD4 cells (p = 0.042). Most of both CD25+ and CD25- naive CD4 T cells phosphorylated STAT5 in response to 1000 U/ml IL-2, although fewer of the CD25+ naive CD4 T cells responded at this relatively high dose of IL-2 (p = 0.041). Notably, the CD25+ naive CD4 T cells had an IL-2 dose response more comparable to that of CD25- memory CD4 T cells than to their CD25+ naive CD4 T cell counterparts. Similarly, CD25- memory CD4 T cells resembled CD25+ naive CD4 T cells more than CD25+ memory CD4 T cells at the 1 and 10 U/ml IL-2 doses. This similarity disappeared at 100 and 1000 U/ml where a higher proportion of the CD25- memory cells phosphorylated STAT5 as compared with the CD25+ naive cells (p = 0.008 and 0.0006, respectively) (Fig. 4B). We hypothesized that differential expression of CD122 (the β-chain of the IL-2 receptor, which is encoded by IL2RB) by naive and memory CD4 T cell subsets stratified by CD25 expression (Fig. 4C) could account for the observed IL-2 sensitivity differences. Indeed, CD122 cell surface expression analysis revealed that CD25+ memory T cells express 2-fold more CD122 than do CD25+ naive T cells (mean MEF of 767 and 342, respectively; p = 0.048) and CD25- memory T cells express 8-fold more surface CD122 than do CD25+ naive T cells (mean MEF of 1558 and 184, respectively; p = 0.0056) (Figs. 4D, 4E). The relatively high level of CD122 expression on CD25- memory CD4 T cells likely accounts for the fact that nearly all of the cells phosphorylated STAT5 at 1000 U/ml IL-2.

CD25 is expressed on CD31+ and CD31- naive CD4 T cells

The association of the frequency of CD25+ naive CD4 T cells with age led us to investigate its relationship to another molecule expressed on naive CD4 T cells that decreases with age: CD31. CD31 is thought to mark naive CD4 T cells that have undergone a minimal number of divisions since leaving the thymus; naive cells lacking CD31 expression have undergone homeostatic proliferation and are characterized by a less diverse TCR repertoire, leading to the hypothesis that CD31+ naive T cells may be more likely to respond to peptide/MHC complexes (2, 4). We therefore hypothesized that expanded naive CD31+ cells should express CD25 whereas CD31- cells should lack CD25 expression. However, dual parameter analysis of CD31 and CD25 on naive CD4 T cells demonstrated that CD25 is expressed on both CD31+ and CD31- naive CD4+ T cells (Fig. 5A, representative example of 4 donors of 128 tested).

Nevertheless, as compared with CD31+ naive CD4+ T cells, the frequency of CD25+ cells in the CD31- naive subset is increased 1.6-fold (mean CD25+ frequency of 23 and 37% for the CD31+ and CD31- subsets, respectively; p = 4.1 × 10-14) (Fig. 5B). The same relationship of CD31 and CD25 expression among naive CD4 T cells was observed within umbilical cord blood (Fig. 5A, Supplemental Fig. 1B). Previous studies reporting that the percentage of naive CD4 T cells expressing CD31 decreases with age (1) were confirmed (p = 0.0005) using previously frozen PBMCs from 92 Cambridge BioResource donors (Fig. 5C). Consistent with our observation that both the CD31+ and CD31- subsets express CD25 (Fig. 5A, 5B) and that there is an age-dependent increase in the percentage of CD25+ cells in the naive CD4 T cell subset (Fig. 1B), we observed an age-dependent increase among the 92 donors in the percentage of CD25+ cells for both subsets of naive cells: CD31+ (p = 0.0072) and CD31- (p = 0.0019) (Fig. 5D, 5E).

CD25 expression identifies homeostatically expanded naive CD4 T cells within the CD31+ subset

It has been reported that TREC levels are reduced among CD31+ naive T cells with age (1, 18), demonstrating that, as for the CD31- subset, CD31+ naive CD4 T cells are in part produced by peripheral division. However, no phenotypic marker has been de-
FIGURE 2. Immunophenotyping of CD25+ and CD25- naive CD4+ T cells. (A) Gating strategy used to define the CD25+ and CD25- subsets of human peripheral blood CD4+, CD45RA+, CD45RO-, CCR7+, and CD62L+ naive T cells (representative of 20 donors). (B) Representative examples of flow cytometric analyses of CD25+ and CD25- naive CD4+ T cell subsets: overlaid histograms show the expression of the indicated molecule in three CD4+ T cell subsets defined as memory CD45RA-CD45RO+ (gray solid histogram), naive CD45RA+CD45RO-CD62L+CD25+ (blue (Figure legend continues))
scribed to identify CD31+ cells that had undergone homeostatic proliferation. To test the hypothesis that CD25 expression identifies naive CD31+CD4 T cells that have divided more extensively than their CD25+CD31+ counterparts, we sorted naive CD4 T cells based on CD31 and CD25 expression and quantified the TREC content (Fig. 6). Importantly, when comparing sorted CD31+CD25+ and CD31+CD25- naive CD4 T cells obtained from the same donor, the difference in TREC levels can be accounted for by on average fewer than three cell divisions (2.37- to 5.18-fold TREC reduction) in the CD31+CD25+ population. TREC values in the most naive CD4 T cell subset (CD25-CD31+) varied only 2.27-fold among the donors whereas the TREC values varied 4.98-fold in the CD31+CD25+ cells, suggesting that the age-dependent decrease in TREC content observed by others in CD31+ naive CD4 T cells (1, 18) is predominantly accounted for by the CD31+CD25+ subpopulation. Despite the fact that CD31+CD25+ naive T cells have diluted their TREC content more than CD31+CD25- cells, within an individual’s naive CD4 T cell populations, CD31+CD25+ naive CD4 T cells still represent a population that has expanded less than either subset of CD31- naive CD4 T cells (Fig. 6). Finally, in the three donors in whom sufficient numbers of both CD31+ subsets could be isolated for TREC analysis, the CD31+/CD31- naive CD4 T cell subset was consistently enriched for cells having undergone the highest number of divisions (Fig. 6).

**IL-7 induces CD25 on CD31+ and CD31-CD25- naive T cells**

Azevedo et al. (19) have shown that IL-7 sustains CD31 expression on naive CD31+CD4 T cells without inducing substantial proliferation limited only to the CD31+ subset. We tested the hypothesis that IL-7 also influences the expression of CD25 by culturing the four sorted naive CD4 T cell subsets (Fig. 7A, 7B) in the presence or absence of IL-7 and assessing CD31 and CD25 expression 7 d later (Fig. 7C, 7D). In agreement with the previous study (19), sorted CD31+ naive CD4 T cells remained CD31+ with (10 ng/ml) or without the addition of IL-7, whereas CD31- naive CD4 T cells remained CD31- (Fig. 7C). Notably, most of both CD31+ and CD31- naive CD4 T cells sorted as CD25- and when cultured for 7 d expressed CD25 in an IL-7-dependent manner (Fig. 7D).

**Differential IL-2 responsiveness by the four naive CD4 T cell subsets**

Although a portion of both CD31+ and CD31- naive T cell subsets express CD25, the potential of the CD25+CD31+ and CD25-CD31- subsets to respond to IL-2 may vary owing to their distinct immune histories, as defined by CD31 expression differences. To test this hypothesis, we stimulated blood samples with IL-2 and monitored pSTAT5a phosphorylation in naive T cells by flow histogram), and naive CD45RA+CD45RO-CD62L-CD25- (red histogram), with representative examples of between 5 and 20 donors. (C) Gating strategy to assess the proportion of cells in cycle (Ki-67+) within various CD4+ T cell subsets: CD25+/CD45RA+/FOXP3- naive CD4 T cells, CD25+/CD45RA-FOXP3- memory CD4 T cells, and CD25high Tregs. (D) Combined analysis of Ki-67+ T cell frequency in five CD4 T cell subsets in 16 donors.
**A** Gated on Naïve CD4 T cells:

No IL-2 | 1 U/ml IL-2 | 10 U/ml IL-2 | 100 U/ml IL-2 | 1000 U/ml IL-2

Donor 1

Donor 2

**FIGURE 4.** CD25 and CD122 expression modulates IL-2 responsiveness on CD4+ T cell subsets. (A) PBLs were stimulated with various doses of IL-2 for 10 min followed by pSTAT5 staining. Flow cytometry analysis is shown of CD4+CD127+CD45RA+FOXP3+ naïve T cells from two representative donors defined by a high (top panel) and low frequency (lower panel) of naïve T cells expressing CD25. (B) Dose-dependent induction of pSTAT5 among CD4+CD25+ versus CD25- subsets of both naïve and memory T cells as well as FOXP3+ Tregs (representative example of 20 donors; analysis performed on 4 donors). (C) Representative flow cytometry histograms of CD25 expression among CD4+ naïve, memory, and Treg subsets. (D) Parallel flow cytometry analysis of CD25 and CD122 expression on the CD4+ naïve, memory, and Treg subsets (representative of 20 donors, appropriate CD4 T cell subsets are overlaid on the total CD4 density plots). (E) Analysis of CD122 MEF from 5 representative donors of 20 tested indicates that the β-chain of the IL-2 receptor is expressed on naïve T cells; however, its expression is increased on memory T cells and is the highest on the Treg subset. Note that memory cells that express the highest level of cell surface CD122 belong to the CD25- memory T cell subset.
cytometry. Fig. 8A shows that CD25+ naive T cells phosphorylated increased amounts of STAT5 in response to 100 U/ml IL-2 compared with their CD25− counterparts and this IL-2 responsiveness was further increased in the CD31− subset. We then compared STAT5a phosphorylation in response to increasing doses of IL-2 between the CD25− and CD25+ subsets of CD31+ and CD31− naive CD4 T cells. CD25+ cells showed increased levels of pSTAT5a as compared with CD25− cells in both CD31 subsets (for the CD31+ fraction, p = 0.009, 0.0002, and 0.0098 for 10, 100, and 1000 U/ml IL-2, respectively, and for the CD31− fraction, p = 0.0024, 0.0065, and 0.0029 for 10, 100, and 1000 U/ml IL-2, respectively; Fig. 8B). Interestingly, further analysis of STAT5a phosphorylation indicated that the CD31+ naive CD4 T cell fraction that expresses CD25 is less responsive to IL-2 than is the CD31− naive T cells (p = 0.0014, 0.0002, and 0.0038 for 10, 100, and 1000 U/ml IL-2, respectively; Fig. 8B). The difference in responsiveness between the CD25+CD31+ and CD25+CD31− subsets was not attributable primarily to a difference in CD25 expression (mean CD25 MEF for the CD25+CD31+ and CD25+CD31− subsets were 180 ± 6 and 228 ± 46, respectively), but rather to the difference in the expression levels of the β-chain of the IL-2 receptor (CD122; mean MEF of 70 and 166 for the CD25+CD31+ and CD25+CD31− subsets, respectively; Fig. 8C, 8D).

Similar to the CD25− subsets, the CD25+ fraction of CD31− naive CD4 T cells stimulated with IL-2 responded to a greater extent than did the CD25− fraction of CD31− naive T cells (p = 0.0072 and 0.0003 for 100 and 1000 U/ml, respectively; Fig. 8B). Consistent with the previous results, higher IL-2 responsiveness in the CD31−/CD25+ subset corresponded to a higher expression of CD122 as compared with the CD31+CD25− fraction (Fig. 8C, 8D).
Because the difference in IL-2 responsiveness by CD31+ and CD31− naive CD4 T cells appeared to be attributable to CD122 levels, we next examined the pSTAT5α response of the four naive CD4 T cell subsets to IL-15 because the IL-15 receptor shares both the β-chain (CD122) and γ-chain (CD132) with the IL-2 receptor (Fig. 8E). As predicted by the pattern of β-chain expression (Fig. 8D), the responses by the CD25− and CD25+CD31− cell subsets were lower than their CD31− counterparts (\( p = 0.0025 \) and 0.0559, respectively, for 0.5 ng/ml IL-15; \( p = 0.0015 \) and 0.0378, respectively, for 5 ng/ml IL-15; and \( p = 0.035 \) and 0.0063, respectively, for 50 ng/ml IL-15; Fig. 8E). As a further control to demonstrate that all naive cells, regardless of CD31 and CD25 expression, are capable of phosphorylating STAT5α, we incubated blood with IL-7. The response to IL-7 is CD122-independent but requires the common γ-chain, CD132, and IL-7Rα. All four naive CD4 T cell subsets produced identical responses (Fig. 8F).

**Differential IL-2 production by the four naive CD4 T cell subsets upon APC-dependent activation**

Having demonstrated that four naive T cell subsets defined by CD25 and CD31 expression display different IL-2 responsiveness (Fig. 9), we sought evidence for a functional role of CD25 during naive CD4 T cell activation. Based on the previous study by Wuest et al. (20), who demonstrated a role for CD25 during early APC/T cell interactions, we hypothesized that CD25 on naive CD4 T cells would increase the likelihood that a peptide/MHC complex presented by immunostimulatory APCs causes cell activation and IL-2 production. PBMCs were stimulated for 4 h with SEB, which is an APC-dependent response (21), and CD69+ naive T cells were analyzed for IL-2 production (Fig. 8G). In agreement with our initial hypothesis, we have observed increased IL-2 production among CD31− naive T cells as compared with CD31+ naive T cells in both the CD25− and CD25+ subsets (\( p = 0.0027 \) and 0.0031, respectively; Fig. 8H).

**Discussion**

The production of thymically derived human naive CD4 T cells decreases with age owing to the involvment of the thymus. As thymic involution progresses, the maintenance of an adequate number of naive CD4 T cells having a sufficiently broad TCR repertoire in the periphery becomes increasingly dependent on the homeostatic expansion of naive CD4 T cells that exited the thymus years or decades earlier (1). In this study we have obtained evidence supporting the hypothesis that CD25 expression identifies naive CD4 T cells that have undergone homeostatic expansion in vivo. Together with CD31, a previously reported marker differentiating naive CD4 T cells with a limited proliferative history outside the thymus (2), CD25 expression defines four subsets of naive CD4 T cells: 1) CD31+CD25−, 2) CD31+CD25+, 3) CD31−CD25−, and 4) CD31−CD25+ (Fig. 9). Analysis of IL-2 responsiveness indicated that the four populations of naive CD4 T cells differ in their responsiveness to IL-2 consistent with each subset’s CD25 and CD122 levels. Notably, even though CD31−CD25− cells do not express CD25 constitutively, increased CD122 co-expression to form the high-affinity trimeric receptor with γ-chain on this subset of naive cells allows responsiveness to low doses of IL-2 (Fig. 8B). The previously uncharacterized increased sensitivity and responsiveness to IL-2 in naive CD4 T cells that have undergone postthymic rounds of division is likely to play a modulatory role in these cells during their further homeostatic proliferation and in their encounters with Ag.

The previous description of an ~2-fold dilution of TREC in CD31+ naive CD4 T cells isolated from donors aged 20–60 y provided evidence for homeostatic proliferation within the CD31+ subset (1). However, no markers were defined identifying CD31+ cells that had divided (1, 18). In this study, TREC analyses of
sorted subsets indicated that the CD31\(^+\)CD25\(^-\) naive CD4 T cell subset was enriched with cells that have undergone more rounds of peripheral division than their CD31\(^-\)CD25\(^+\) counterparts, thereby making CD25 a cell surface marker that can be used to identify and study this expanded population. Thus, much of the \(~2\)-fold dilution of TREC content in CD31\(^-\) naive CD4 T cells that occurs in adulthood is likely accounted for by the increase of CD25\(^+\) cells in the CD31\(^-\) population, which on average represent 10–20% of this subset (Fig. 5D). As noted in the previous age-dependent TREC analyses of CD31\(^+\) cells, there is a large variation in the percentage of naive CD31\(^+\) cells that are CD25\(^-\) within individuals of the same age, which likely reflects genetic and environmental influences on the regulation of homeostatic proliferation. Although the CD31\(^+\)CD25\(^-\) subset contains the highest number of TREC content among the four naive populations that we tested (Fig. 6), it remains likely that the CD31\(^+\)CD25\(^-\) subset still contains some cells that have divided after exiting the thymus.

It is clear that CD31\(^-\) naive cells that lack constitutive CD25 expression have diluted their TREC content more than their CD31\(^+\)CD25\(^-\) counterparts (Fig. 6). Thus, cell division by naive CD4 cells does not necessarily lead to stable CD25 expression. One explanation for this apparent paradox is that the signals that stimulate the loss of CD31 and the several rounds of proliferation that ensue do not lead to the stable expression of CD25. It has been proposed previously that CD31 downregulation marks cells that have been stimulated in the context of self-peptide MHC complexes resulting in a reduced TCR repertoire, a finding that has important implications because CD31\(^-\) cells constitute a progressively larger proportion of the naive CD4 T cell pool with age (3).

Because CD31\(^-\)CD25\(^-\) naive CD4 T cells have the greatest dilution of their TREC content, it is logical to hypothesize that this subset represents the “least naive” of naive CD4 T cells. However, because of our limited understanding about the selection

**FIGURE 7.** IL-7 induces CD25 expression on naive CD4 T cells in a TCR-independent manner. (A) TCRβ\(^+\), CD4\(^+\), CD127\(^+\), CD45RA\(^+\), and CD62L\(^+\) naive T cells were divided into four subsets on the basis of CD25 and CD31 expression followed by FACS as: CD31\(^+\)CD25\(^-\), CD31\(^-\)CD25\(^+\), CD31\(^-\)CD25\(^-\), CD31\(^-\)CD25\(^-\). (B) Sorted cells were placed in culture for 7 d in the presence (10 ng/ml) or absence of IL-7; a representative example includes four experiments showing the gating strategy for analyzing sorted naive T cell subsets cultured for 7 d in the presence of IL-7. Flow cytometry analysis is shown of CD31 (C) and CD25 (D) expression of four sorted naive T cell subsets cultured for 7 d in the presence (blue histogram) or absence (gray histogram) of IL-7 (representative example of three independent experiments).
FIGURE 8. Expression of high- and low-affinity IL-2 receptors as well as IL-2 responsiveness is differentially regulated on CD31\(^+\) and CD31\(^-\) naive T cells. (A) Representative example of pSTAT5 versus CD31 from CD25\(^-\) and CD25\(^+\) naive CD4\(^+\)CD127\(^-\)CD45RA\(^+\)FOXP3\(^-\) T cells after 10 min stimulation with 100 U/ml IL-2 (\(n=4\)). (B) Combined, dose-dependent IL-2 responsiveness measured by flow cytometry analysis of pSTAT5 in CD4\(^+\) naive T cells that were divided into four subsets on the basis of their CD25 and CD31 expression (\(n=4\)). (C) Representative flow cytometry analysis of CD122 expression in four different CD4\(^+\) naive T cell subsets defined on the basis of the CD25 and CD31 expression (CD45RA\(^+\)CD122\(^{high}\) cells were excluded from the analysis; see Supplemental Fig. 2). (D) Combined analysis of CD122 MEF determined that the CD31\(^-\) central naive T cells express increased levels of CD122 (\(n=5\)). CD122\(^{high}\) cells were excluded from the analysis; see (C) and Supplemental Fig. 2. CD4 T cells present within the peripheral blood were independently stimulated with increasing doses of IL-15 or IL-7 and cytokine responsiveness was measured by a flow cytometric pSTAT5 assay. Analysis of pSTAT5 present in the peripheral blood CD4\(^+\)CD127\(^-\)CD45RA\(^+\)FOXP3\(^-\) naive T cells that were divided into the four (Figure legend continues)
processes acting on CD31^−CD25^− cells, it is not possible to understand which naive cell subset or subsets are the direct precursors of CD31^−CD25^+ naive CD4 T cells. Indeed, it is possible that all three non-CD31^−CD25^+ subsets have direct progenitors for this subset that has expanded to the greatest extent. Deep sequencing of the TCR repertoire of the four subsets could help determine the origin of the CD31^−CD25^+ subset. Of particular interest is the question of whether the TCR diversity among CD31^−CD25^+ cells is more similar to that within CD31^−CD25^− or CD31^+CD25^+ cells.

The expression of CD25 on naive CD4 T cells could increase the likelihood of their long-term survival into old age, mediated by increased survival signals transmitted through the IL-2 receptor. To test this possibility, we have compared the survival of CD25^+ versus CD25^− naive cells with and without IL-2 during extended in vitro cultures but we have not obtained evidence that IL-2 alters naive T cell survival even when CD25 is expressed (data not shown). However, we have demonstrated that the presence of functional, high-affinity IL-2 receptors on naive CD4 T cells is likely to influence immune responses during early activation events because upon APC-dependent stimulation with SEB, CD25^+ naive CD4 T cells produce more IL-2 as compared with their CD25^− counterparts (Fig. 8H). The importance of IL-2 signaling during the early phases of activating allo- and Ag-specific T cells has been highlighted by the observations that CD25 copolarizes with the TCR at the immunological synapse (22) and that immunostimulatory dendritic cells (DCs) express CD25 and secrete IL-2 toward the DC/T cell interface (23–26). The observation that daclizumab (anti-CD25), a potent immunosuppressive agent in humans, is a poor inhibitor of polyclonal T cell proliferation but a potent inhibitor of Ag-specific responses is consistent with the hypothesis that IL-2 signaling is a key early event in T cell activation stimulated by peptide MHC complexes (20). From the CD25 expression data presented in this study, we suggest that naive CD25^+ CD4 T cells having constitutive expression of CD25 will be less dependent on IL-2 transpresentation by DCs.

The expression of CD25 on naive T cells could also directly alter the immune response by influencing the differentiation of naive cells toward effector phenotypes: STAT5 signaling has been shown to regulate naive T cell differentiation toward effector T cell subsets, including induced Tregs (5) and Th17 (27, 28), Th2 (29), and T follicular helper cells (30, 31). Thus, the high-affinity IL-2 receptors on naive T cells that are exposed to foreign or self Ags could make a substantial contribution to the numbers and quality of the effector cells that are generated.

The dynamics of naive T cell expansion could influence susceptibility and development of autoimmune diseases such as multiple sclerosis (32–37). In this context it is interesting that single nucleotide polymorphisms in IL2RA associated with multiple sclerosis and type 1 diabetes also control the frequency of CD25^+ naive T cells (7, 38). Polymorphisms in the IL7R have also been associated with the risk of multiple sclerosis (39).

In summary, the upregulation of CD25 (and CD122) on naive CD4 T cell subsets in humans not only marks cells that have undergone more divisions in vivo but also provides a more heterogeneous naive CD4 T cell pool based on differing IL-2 sensitivities and differing TCR diversities. The ability of each of the four naive T cell subsets defined in this study to contribute precursors to the even more heterogeneous groups of differentiated effector and memory T cells as well as their clinical significance will be investigated in future studies.

**FIGURE 9.** Model of post-thymic naive T cell expansion.
Acknowledgments

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Disclosures

The authors have no financial conflicts of interest.

References


Supplemental Figure Legends

Supplemental Figure 1. Flow cytometric analysis of umbilical cord blood CD25⁺ naïve T cells

(A) Naïve CD4⁺ CD127⁺ T cells were gated as CD45RA⁺ CD45RO⁻ CD62L⁺ and analysed for CD25 expression. (B) CD4⁺ naïve T cells were analysed by flow cytometry and divided into four subsets on the basis of CD25 and CD31 expression. Two representative examples (UCB-1 and UCB-2) of 9 cord blood samples (UCB: umbilical cord blood) are shown.

Supplemental Figure 2. Flow cytometric analysis of CD25 expression from the CD4⁺ CD45RO⁻ CD62L⁺ CD122⁺ CD95⁺ subset of memory cells with stem cell-like properties
Flow cytometry analysis of peripheral blood CD4 memory cells with stem cell-like properties; (A) Gating strategy of naïve and memory CD4 T cells with stem cell-like properties. Combined analysis of CD31 and CD25 or CD31 and CD122 expression among naïve (B) and memory cells with stem cell-like properties (C), (Representative of 3 donors).

Supplemental Figure 3. Analysis of IL2RA expression differences on naïve CD25⁻ vs naïve CD25⁺ T cell subsets purified from 7 donors delineated by 96-sample titan Affymetrix Human Gene 1.1 ST array
(A) Graphical illustration of Affymetrix GeneST Probesets mapping uniquely to IL2RA gene (t1dbase.org). (B) Owing, to the limited sample size tested in the microarray analysis (seven independent donors), modest expression differences observed between the CD25⁺ and CD25⁻ naïve cells did not reach genome-wide significance. To test the hypothesis that the naïve CD4 T cells sorted on the basis of CD25 protein expression have different IL2RA mRNA expression levels accounting for the protein expression difference, results from IL2RA probesets were evaluated without a correction for multiple testing and a 5-fold difference in expression was observed using 10 probesets in all seven donors. Analysis of IL2RA expression for each IL2RA Affymetrix GeneST Probesets demonstrated as fold change in gene expression of naïve CD25⁺ vs naïve CD25⁻ T cells, n=7. (C) Purity check of sorted TCRαβ⁺ CD4⁺, CD127⁺, CD45RA⁺, CD62L⁺ CD25⁺ and TCRαβ⁺ CD4⁺, CD127⁺, CD45RA⁺, CD62L⁺ CD25⁻ naïve T cells.
Supplemental Table 1.
Detailed information about antibodies used for immunophenotyping
S-Fig. 1.

CD25^+ naive T cells are present in cord blood

A

UCB-1

UCB-2

B

UCB-1

UCB-2
S-Fig. 2. CD4+ stem cell-like memory display heterogeneous CD25 expression

A

CD4+ T cells

Non-Tregs

CD62L+ CD45R0-

stem cell-like memory

naive

B

Gated on CD4+ CD45RO- CD62L+ CD122-flow CD95-

Naive CD4+ T cells

C

Gated on CD4+ CD45RO- CD62L+ CD122+ CD95+

Memory cells with stem cell-like properties
S-Fig. 3.

A  **IL2RA**

B

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C  Flow cytometric analysis of sorted CD25 +/- Naïve CD4 T cell populations (CD4+ CD127+ CD45RA+ CD45RO- CD62L+).

before sort          after sort: CD25-          CD25+
### Antibodies used for immunophenotyping

#### The anti-human monoclonal abs antibodies used for T-cell surface immunophenotyping

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#### The anti-human monoclonal abs used for intracellular T-cell immunophenotyping

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#### The anti-human monoclonal abs antibodies used for T-cell sorting

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