

Two Subsets of Naive T Helper Cells with Distinct T Cell Receptor Excision Circle Content in Human Adult Peripheral Blood

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Abstract

During ageing thymic function declines and is unable to meet the demand for peripheral T helper (Th) cell replenishment. Therefore, population maintenance of naive Th cells must be at least partly peripherally based. Such peripheral postthymic expansion of recent thymic emigrants (RTEs) during ageing consequently should lead to loss or dilution of T cell receptor excision circles (TRECs) from a subset of naive T cells. We have identified two subsets of naive Th cells in human adult peripheral blood characterized by a striking unequal content of TRECs, indicating different peripheral proliferative histories. TRECs are highly enriched in peripheral naive CD45RA⁺ Th cells coexpressing CD31 compared with peripheral naive CD45RA⁺ Th cells lacking CD31 expression, in which TRECs can hardly be detected. Furthermore we show that CD31⁻CD45RA⁺ Th cells account for increasing percentages of the naive peripheral Th cell pool during ageing but retain phenotypic and functional features of naive Th cells. As CD31 is lost upon T cell receptor (TCR) engagement *in vitro*, we hypothesize that TCR triggering is a prerequisite for homeostatically driven peripheral postthymic expansion of human naive RTEs. We describe here the identification of peripherally expanded naive Th cells in human adult blood characterized by the loss of CD31 expression and a highly reduced TREC content.

Key words: CD31 • naive Th cells • Th cell homeostasis • recent thymic emigrants • peripheral Th cell pool

Introduction

The size of the recirculating naive T cell pool remains relatively constant throughout adult life in spite of continuous environmental antigenic stimulation and a dramatic reduction in production of naive T cells in the thymus with age (1–3). The presence of a great number of naive T cells within CD4⁺ and CD8⁺ T cell subsets even in the peripheral blood of centenarians is thus puzzling. Extensive studies in the murine system revealed that subsets of recent thymic emigrants (RTEs) proliferate in response to triggering via MHC molecules presenting self-peptides (4–6) depending on the TCR affinity for the MHC–peptide complexes. In this manner, subsets of naive RTEs are positively selected a second time before they are allowed to enter a

“second” pool of naive Th cells. In addition, cytokines such as IL-7 and IL-15 play an important role in the post-thymic expansion of peripheral naive Th cells (7–10).

RTEs have been identified recently by analyzing T cell receptor excision circles (TRECs), which are stable DNA episomes formed during T cell receptor rearrangement in at least 70% of $\alpha\beta^+$ Th cells (2,3, 11–14). As TRECs are not replicated during mitosis they are diluted out during cell divisions (11, 15), which include priming of RTEs to become memory Th cells but also homeostatic cell division of naive Th cells. Interestingly, TREC numbers among CD4⁺ Th cells decrease 50 to 100 times during ageing (2, 3), while absolute numbers of naive CD4⁺ Th cells, as characterized by expression of CD45RA, decrease only by a factor of 2 or 3 with age (16, 17). This implies extensive peripheral expansion of naive Th cells during ageing. Such homeostasis-driven expansion of naive T cells, which is

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most obvious in lymphopenic environments, requires specific TCR interaction with self-peptide/MHC.

Here we addressed the issue whether homeostasis driven proliferation results in a second subset of naive Th cells. To distinguish RTEs from peripherally expanded naive Th cells we searched for cell surface molecules with an altered expression pattern after TCR triggering on Th cells. Apparently such TCR engagement does not alter the expression of molecules, which have been used widely to characterize human naive Th cells as CD27 and CD62L, as these markers are coexpressed by the majority of CD45RA⁺ Th cells in adult peripheral blood lacking CD45RO expression (data not shown). Only CD31 (platelet endothelial cell adhesion molecule-1 [PECAM-1]) meets these criteria: CD31 is lost after TCR triggering *in vitro* and has been shown to be differentially expressed among peripheral Th cells in healthy donors (18–21). Hence, we examined whether frequencies of CD45RA⁺ Th cells lacking CD31 expression change during ageing and whether they differ in phenotypic or functional characteristics from CD31⁺ CD45RA⁺ Th cells. We finally assessed directly the TREC content in CD31-expressing and CD31-nonexpressing CD45RA⁺ Th cells highly purified from adult peripheral blood in order to determine the frequency of recent thymic emigrants among these two subsets of naive Th cells in human adult peripheral blood.

Materials and Methods

Blood Samples. Peripheral blood samples were obtained from adult healthy donors (blood bank Virchow Klinikum, Humboldt Universität Berlin, Berlin, Germany). Umbilical cord blood samples were obtained directly after birth (Virchow Klinikum, Humboldt Universität Berlin).

Isolation of PBMCs and Cord Blood Mononuclear Cells. PBMCs were obtained from peripheral blood and cord blood mononuclear cells (CBMCs) were obtained from cord blood by density gradient sedimentation using Ficoll/Hypaque (Sigma-Aldrich). Cells were washed twice with PBS before staining with mAbs for flow cytometry or cell sorting.

Magnetic Activated Cell Sorting. CD4⁺ Th cells were enriched to high purity (>98%) from PBMCs or CBMCs by magnetic separation using releasable CD4-MultiSort-Microbeads (Miltenyi Biotec). After release of the CD4-MultiSort-Microbeads the CD4⁺ T cells were depleted with CD45RO-Microbeads (Miltenyi Biotec) to obtain pure CD45RA⁺CD45RO⁻ T cells. These cells were then separated in a third magnetic separation step into CD31⁺ and CD31⁻CD45RA⁺ Th subpopulations after staining with anti-CD31-FITC (Becton Dickinson) and subsequent labeling with anti-FITC-Microbeads (Miltenyi Biotec). The purities of these two populations were at least 95%.

Immunophenotypic Analysis and Cell Sorting by Flow Cytometry. For immunophenotypic analysis, PBMCs or CD4⁺ Th cells (isolated by magnetic activated cell sorting [MACS]) were quadruple stained with fluorescent-conjugated mAbs specific for cell surface markers and analyzed by flow cytometry using a FACSCalibur™ (Becton Dickinson). FITC-labeled anti-CD7, anti-CD11a, anti-CD26, anti-CD44, anti-CD45RA, anti-CD45RB, anti-CD62L, anti-CD95, anti-HLA-DR, PE-labeled anti-CD31, PerCP-labeled anti-CD4, and allophycocyanin (APC)-labeled anti-

CD45RO were purchased from BD PharMingen. Monoclonal antibody 2E4 (anti-CD27) was a generous gift from René van Lier (Clinical Immunology Laboratory, Academic Medical Center, Amsterdam, Netherlands). Purified anti-CD27 was conjugated to Cy5 (Amersham Pharmacia Biotech). After gating on CD4⁺ or CD45RA⁺ CD45RO⁻ Th cells lymphocyte data was analyzed with CELLQuest™ software (Becton Dickinson). For purification of CD31⁺ and CD31⁻CD45RA⁺CD45RO⁻ and CD31⁻CD45RA⁻CD45RO⁺ Th subsets CD4⁺ Th cells were isolated from PBMCs by MACS as described above. The CD4⁺ Th cells were stained with mAbs specific for CD31, CD45RA, and CD45RO and further separated by a FACSVantage™ (Becton Dickinson) into CD31⁺ and CD31⁻CD45RA⁺CD45RO⁻ and CD31⁻CD45RA⁻CD45RO⁺ Th cells. Reanalysis of the isolated subsets using a FACSCalibur™ (Becton Dickinson) showed that the purities were at least 91% (data not shown).

Polyclonal *In Vitro* Stimulation and Intracellular Cytokine Staining. Highly purified CD31⁺ and CD31⁻CD45RA⁺CD45RO⁻ and CD31⁻CD45RA⁻CD45RO⁺ Th cells were counted and 10⁶ cells/ml were cultured for 6 h in the presence of 5 ng/ml PMA (Sigma-Aldrich) and 1 ng/ml ionomycin (Sigma-Aldrich), with the secretion inhibitor brefeldin-A (Sigma-Aldrich) added during the last 2 h. The cells were then fixed with 2% formalin, stained with mAbs specific for IL-2, IFN- γ , and IL-4 (BD PharMingen), and cytokine positive cells were quantified by flow cytometry using a FACSCalibur™ (Becton Dickinson).

Quantification of TRECs by Real-time PCR. The number of TRECs was determined by quantitative real-time PCR using the ABI PRISM 7700 Sequence Detector TaqMan (PE Biosystems). In this PCR binding of a specific probe, containing a quencher (TAMRA) and a reporter (6FAM) dye, to the amplification products results in Taq DNA polymerase mediated cleavage of the probe. This leads to separation of the quencher from the reporter, thereby inducing fluorescence of the reporter dye. The amount of target in analyzed samples is established by measuring the threshold cycle (C_T), defined as the fractional cycle number at which the fluorescence generated by cleavage of the probe passes a fixed threshold above baseline, and using the standard curve to determine starting copy number. To precisely determine the percentage of cells carrying TREC a duplex vector was constructed, containing a fragment of the δ Rec- ϕ J α signal joint (TREC) and a fragment of the RAG2 gene, used as a reference. The RAG2 was cloned first in the T-A acceptor site and subsequently the TREC was cloned into the EcoRV restriction site of the TOPO TA Vector (Invitrogen). Based on the DNA concentration, measured by spectrophotometry and confirmed by a quantitative gel electrophoresis, standard dilutions of the vector from 10⁷ to 10¹ copies were prepared. In brief, PCR of 50 μ l total volume was performed with \sim 100 ng of genomic DNA, 25 pmol of each primers, 10 nmol each dNTP (PerkinElmer), 1.25 U Platinum Taq polymerase (Life Technologies), 5 pmol of 6FAM-TAMRA probe, and PCR buffer including 4.5 mM MgCl₂ (Life Technologies). After the initial denaturation at 95°C for 5', 45 cycles consisting of 95°C for 30 s and 66°C for 30 s were performed. For TREC analysis the 5' primer ϕ J α (-258): AAC AGC CTT TGG GAC ACT ATC G and the 3' primer δ Recsj(+104): AAC AGC CTT TGG GAC ACT ATC G, amplifying the signal joint sequence generated by the δ Rec- ϕ J α rearrangement, were used together with the TREC probe: 6FAM-CCA CAT CCC TTT CAA CCA TGC TGA TGA CAC CTC T-TAMRA. For RAG2 analysis the 5' primer: RAG2(2160) GCA ACA TGG GAA ATG GAA CTG, the 3' primer: RAG2(2404) GGT GTC AAA TTC ATC ATC ACC ATC and the RAG2 probe:

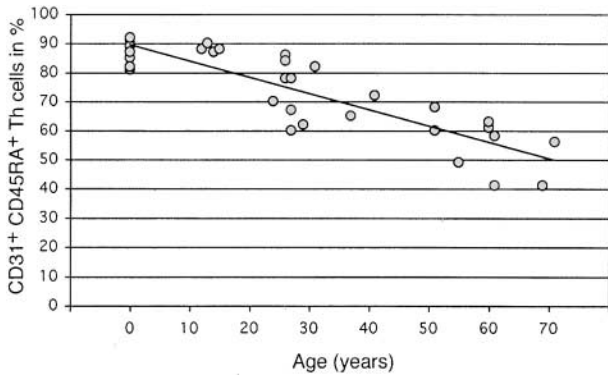


Figure 1. Frequencies of CD31⁺ Th cells among the CD45RA⁺CD45RO⁻ Th cells during ageing. CD4⁺ Th cells were isolated from PBMCs by MACS. The CD4⁺ Th cells were stained with mAbs specific for CD31, CD45RA, and CD45RO and analyzed by flow cytometry. Gray points indicate the relative frequency of CD31⁺ Th cells among the CD45RA⁺CD45RO⁻ Th cells in individual samples. The linear regression curve is shown. There is a significant negative correlation between the age and the frequency of CD31⁺ Th cells among the CD45RA⁺CD45RO⁻ Th cell subset ($r = -0.875$).

6FAM-CCC CTG GAT CTT CTG TTG ATG TTT GAC
TGT TTG TGA-TAMRA were used.

Results

Frequencies of CD31⁺ and CD31⁻CD45RA⁺ Th Cells during Ageing. At first, we examined whether frequencies

of CD45RA⁺ Th cells coexpressing the CD31 molecule change with ageing. We observed drastic changes: CD31 coexpression was high (85–90%) in CD45RA⁺ Th-cells from children (younger than 15 yr of age) but decreased constantly during ageing, from 60–80% in 20–30-yr-old adults down to 40–60% in adults older than 60 yr (Fig. 1). The concomitant age dependent expansion of peripheral CD45RA⁺ Th cells lacking CD31 expression correlates with the increased requirement for thymus independent peripheral expansion of naive Th cells in response to the reduced output of RTEs in elderly people. However, it would also correlate with increasing frequencies of memory/effector Th cells with regaining CD45RA expression.

Phenotypic Analysis of CD31⁺ and CD31⁻CD45RA⁺ Th Cells. To investigate whether these expanded CD45RA⁺ Th cells could represent increasing frequencies of memory/effector Th cells with regaining CD45RA expression, we next analyzed the expression of CD7, CD11a, CD26, CD27, CD44, CD45RB, CD62L, CD95, HLA-DR on purified indicated CD4⁺ Th cell subsets. CD31⁻CD45RA⁺ Th cells phenotypically resemble naive Th cells: they do not coexpress the CD45RO and they are not characterized by elevated levels of CD11a or CD44. Only a few cells (<1%) have lost CD7, CD26, CD27, or CD62L expression (Fig. 2) and thus represent memory/effector Th cells with regaining CD45RA expression. As expected such specialized CD45RA⁺ memory/effector Th cells are rare in peripheral blood of healthy adults. Hence, CD31⁺ and CD31⁻CD45RA⁺ Th cells both correspond phenotypically to naive Th cells.

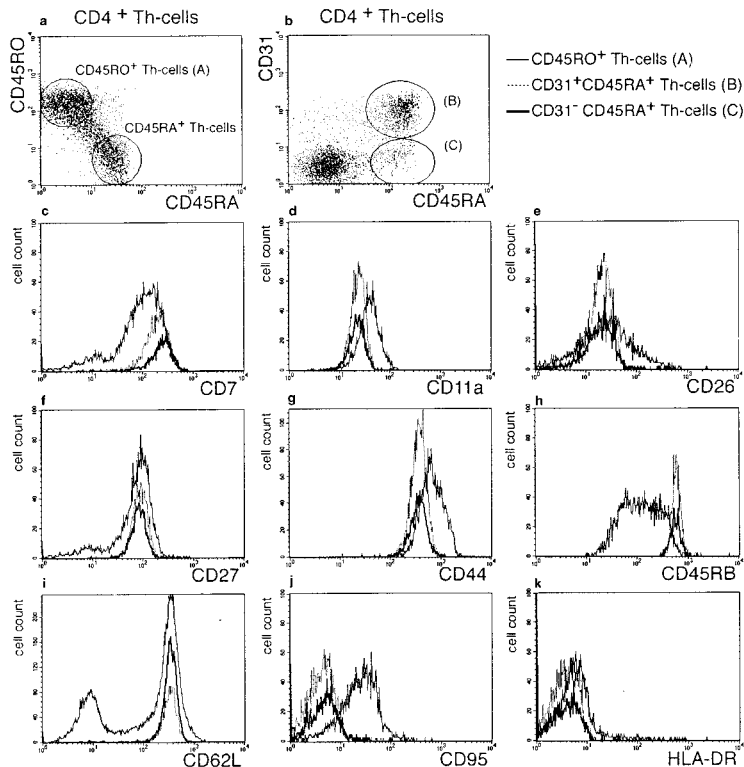


Figure 2. Phenotypic characterization of CD31⁺ and CD31⁻CD45RA⁺ Th cells (B and C) in comparison to CD45RO⁺ Th cells (A). The dot plot (a) shows the expression of the cell surface molecules CD45RO and CD45RA, the dot plot (b) shows the expression of the cell surface molecules CD31 and CD45RA among peripheral CD4⁺ Th cells. PBMCs from a healthy donor were stained with anti-CD4, anti-CD45RA, anti-CD45RO, and anti-CD31 mAbs and examined by flow cytometry. The cells in a and b are gated on scatter and CD4⁺ Th cells. The histograms (c–k) show the expression of the cell surface molecules CD7, CD11a, CD26, CD27, CD44, CD45RB, CD62L, CD95, HLA-DR by peripheral blood CD45RO⁺ Th cells (fine line A), CD31⁺CD45RA⁺ Th cells (dotted line B) and CD31⁻CD45RA⁺ Th cells (bold line C) in one healthy donor (representative for three analyzed healthy individuals). PBMCs were stained with mAbs against CD4, CD45RO, CD31, and one of the surface markers (indicated in each plot) and examined by flow cytometry.

Cytokine Program of CD31⁺ and CD31⁻CD45RA⁺ Th Cells. We also analyzed the functional potential of both CD31⁺ and CD31⁻CD45RA⁺ Th cells according to their cytokine secretion profiles after polyclonal in vitro stimulation. CD31⁺ and CD31⁻CD45RA⁺ Th cells showed functional capabilities of naive Th cells, in that they are both able to produce considerable amounts of IL-2 (Fig. 3). In addition, CD31⁺ and CD31⁻CD45RA⁺CD4⁺ T cell subsets of all analyzed samples showed very low frequencies (<1%) for the Th1 and Th2 effector cytokines IFN- γ and IL-4 (Fig. 3, donor #1: a and b, donor #2: g and h), whereas in the CD45RO⁺ memory/effector Th cells isolated from the same donors frequencies of 11% (donor #1: Fig. 3 c) and 24% (donor #2: Fig. 3 i) for IFN- γ +IL-4⁻ cells and 1.2% (donor #1: Fig. 3 c) and 1.5% (donor #2: Fig. 3 i) for IL-4⁺IFN- γ ⁻ cells were detectable. Thus CD45RA⁺CD45RO⁻ Th cells lacking CD31 expression are naive Th cells with respect to both their phenotypic and functional features. Consequently only an augmented peripheral naive Th cell homeostasis during ageing can account for the age dependent increase of peripheral CD45RA⁺ Th cells lacking CD31.

TREC Analysis in CD31⁺ and CD31⁻CD45RA⁺ Th Cells. We tested this hypothesis directly by performing quantitative PCR analysis of TREC numbers in highly pu-

rified peripheral CD31⁺ and CD31⁻CD45RA⁺CD45RO⁻ Th cells from healthy adults. The isolation procedure involved magnetic purification of CD4⁺ Th cells, and separation of CD31⁺ and CD31⁻CD45RA⁺CD45RO⁻ Th cells and CD31⁻CD45RA⁻CD45RO⁺ memory/effector Th cells by FACS[®]. Reanalysis of isolated subsets revealed purities higher than 94% for all samples (data not shown). Samples were taken from PBMCs of six healthy adult donors between 24 and 52 yr. While ~40 TRECs per 10³ Th cells were detected in cord blood Th cells, no TRECs were measured in highly purified peripheral blood B cells, as shown in Fig. 4. Naive CD45RA⁺ Th cells coexpressing CD31 showed a TREC content on average 8 times higher than the naive CD45RA⁺ Th cells lacking CD31 isolated from the same donor (range: 3.9–39.5 times). As expected, only very few TRECs were detected among CD45RO⁺ Th cells in all analyzed samples (Fig. 4). In two samples (donor #1, donor #3), the decrease in TRECs measured among the CD31⁻ subset was analogous to that measured among CD45RO⁺ memory/effector Th cells (Fig. 4). Absolute TREC content in CD31⁺CD45RA⁺ Th cells was an order of magnitude lower than TREC measurements in thymic cells (data not shown). This reduction can be ascribed to proliferation during thymocyte maturation before emigration into the periphery. Similar findings for TREC levels have been obtained recently within sorted monkey thymocyte and peripheral blood Th cell populations (22).

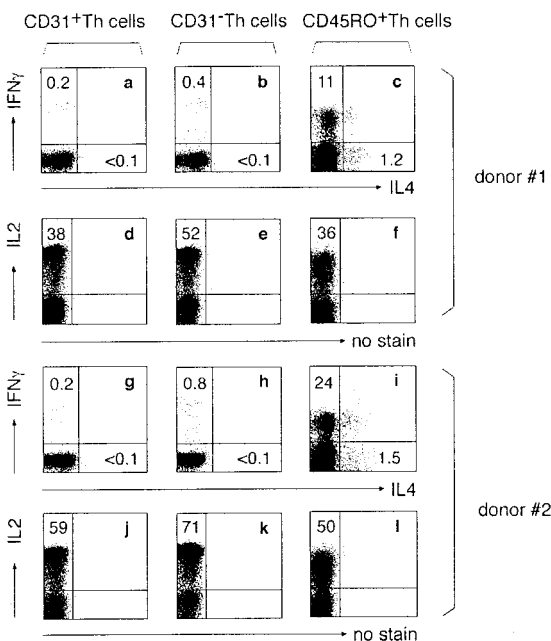


Figure 3. Cytokine production of CD45RO⁺CD45RA⁻CD4⁺ Th cells, CD31⁺ and CD31⁻CD45RA⁺CD45RO⁻ CD4⁺ Th cell subsets upon polyclonal in vitro stimulation. The CD45RO⁺CD45RA⁻ Th cells, CD31⁺ and CD31⁻CD45RA⁺CD4⁺ Th cell subsets were isolated using MACS, stimulated for 6 h with PMA/Ionomycin, fixed with 2% formalin, and stained for intracellular cytokines (IL-2, IFN- γ , IL-4). The dot plots show the results of examined CD45RO⁺CD45RA⁻, CD31⁺ and CD31⁻CD45RA⁺CD4⁺ T cell subsets isolated from PBMCs of two healthy individuals (a–f: donor #1, g–i: donor #2) representative for six analyzed healthy individuals. The percentages of cytokine-positive cells are given in each plot.

Discussion

We have identified here for the first time a subset of human naive Th cells within in adult peripheral blood which has undergone extensive peripheral postthymic expansion.

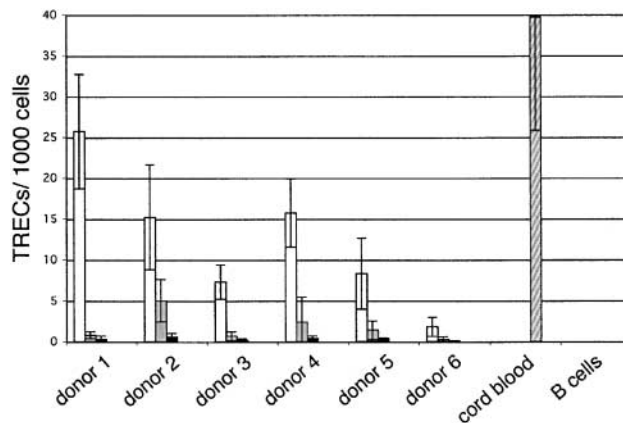


Figure 4. Quantification of TREC content among highly purified peripheral Th cell subsets from six healthy adults. Genomic DNA of sorted subpopulations from six healthy adults was isolated and the number of TRECs was determined by quantitative real-time PCR. White bars represent TREC content in the CD31⁺CD45RA⁺CD45RO⁻ Th cells, gray bars represent TREC content in the CD31⁻CD45RA⁺CD45RO⁻ Th cells and black bars represent the TREC content among CD31⁻CD45RA⁻CD45RO⁺ Th cells. The highest TREC content was detected in cord blood (striped bar) and no TRECs were detected in B cells.

We show directly that CD45RA⁺ Th cells lacking CD31 are characterized by a drastically reduced content of TRECs. Hence, CD31 is expressed on RTEs but apparently lost during postthymic peripheral expansion of RTEs. We also show that naive Th cells lacking CD31 increase in frequency during ageing. However, these peripherally expanded Th cells retain all phenotypic and functional features of naive Th cells. Based on our results it is now possible to distinguish peripherally postthymically expanded naive Th cells (^{central}naive Th cells) from true recent thymic emigrants (^{thymic}naive Th cells).

^{thymic}Naive Th cells probably use CD31 for transendothelial migration to enter secondary lymphoid organs, a process that has recently been suggested to be essential for homeostatic proliferation of T cells (23). Interestingly, more recent investigations have shown that CD31 triggering leads to inhibition of TCR signaling via immunoreceptor tyrosine-based inhibitory motifs (ITIMs; reference 24). It remains to be clarified whether CD31 indeed transduces negative signals during postthymic naive T cell proliferation to control T cell activation after tickling of the TCR with specific MHC/self-peptide ligands.

Taken together, the age-dependent increasing percentages of peripheral CD31⁻CD45RA⁺CD45RO⁻ Th cells (and concomitant age-dependent decrease of CD31⁺CD45RA⁺CD45RO⁻ Th cells) and the drastic reduction in absolute TREC numbers among the CD31⁻ subset in all analyzed donors, indicate that CD45RA⁺ Th cells lacking the CD31 molecule must have undergone extensive peripheral proliferation. We propose that after emigrating from the thymus, ^{thymic}naive CD31⁺ Th cells may undergo a peripheral, second positive selection to counterbalance the age-dependent decreased thymic output and enter a second pool of ^{central}naive CD31⁻ Th cells (Fig. 5). Strikingly, up to half of the naive Th cells in normal healthy adults are characterized by the loss of CD31 expression and since they are almost void of TRECs compared with their CD31⁺ counterparts represent peripherally expanded naive Th cells. What are the signals responsible for peripheral expansion of naive Th-cells? MHC II molecules presenting self peptides have been shown to trigger peripheral expansion of naive Th cells (4–6, 25–28). Such a second postthymic peripheral positive selection must be tightly regulated as potentially autoreactive T cells have to be eliminated or controlled efficiently. As CD31 is lost during in vitro stimulation of Th cells after TCR triggering (20), we hypothesize that down-regulation of CD31 expression on peripherally expanded naive Th cells could in fact be a consequence of the TCR ligation with endogenous MHC and self-peptides.

To summarize, we could show for a first time that the human peripheral naive Th cell pool in adults consists of two major parts. ^{thymic}Naive Th cells, characterized by high TREC levels exist among CD31⁺CD45RA⁺ Th cells. In contrast, ^{central}naive Th cells lacking CD31 expression are characterized by TREC levels which are lower by a factor of up to 0.025 compared with their CD31⁺ counterparts, implying that ^{central}naive Th cells have extensively prolifer-

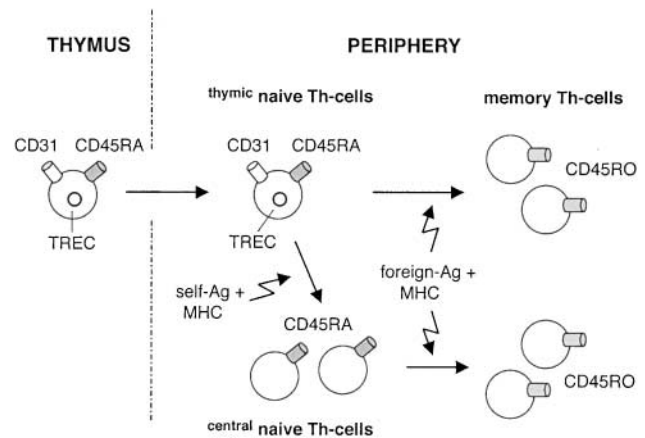


Figure 5. Model of peripheral naive Th cell homeostasis. Full thymic activity in young healthy individuals leads to continuous replenishment with naive Th cells with high TREC content. These recent thymic emigrants coexpress CD45RA and CD31. Upon activation with foreign antigens thymic naive Th cells (^{thymic}naive Th cells) differentiate into CD31⁻CD45RA⁻CD45RO⁺ memory/effector Th cells. During ageing thymic activity decreases resulting in reduced absolute numbers of ^{thymic}naive Th cells. Upon triggering with self peptides and MHC ^{thymic}naive Th cells proliferate and differentiate into central naive Th cells (^{central}naive Th cells) to compensate for the reduced thymic output. ^{central}Naive Th cells have reduced TREC numbers and express CD45RA while they lack CD31 expression. Both ^{thymic}naive and ^{central}naive Th cells are able to differentiate into CD31⁻CD45RA⁻CD45RO⁺ memory/effector Th cells.

ated. Our results should facilitate further studies investigating how a naive TCR repertoire is maintained through peripheral expansion. It remains to be clarified how the processes during which Th cells are triggered by endogenous MHC and self-peptides are controlled to avoid expansion and activation of autoreactive Th cells. Wagner et al. have reported a possible perturbation of the naive Th cell repertoire in rheumatoid arthritis patients (29). Previously it has also been reported that TRECs are substantially reduced in naive Th cells from these patients, suggesting a decreased thymic output or/and an increased self-replication of naive Th cells (30). In this study we defined phenotypic features distinguishing naive human Th cells emigrating from the thymus from those naive human Th cells which have undergone a further secondary peripheral expansion. We have shown that the subset of human peripheral CD45RA⁺ Th cells coexpressing CD31 contains recent thymic emigrants, while the counterpart population lacking CD31 expression represents Th cells which have extensively proliferated most likely to compensate for the reduced thymic output during ageing. Our results enable direct access and thereby further functional and molecular analysis of these two human peripheral naive Th cell subsets.

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