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Lymphopenia-Driven Homeostatic Regulation of Naive T Cells in Elderly and Thymectomized Young Adults

Delphine Sauce,* Martin Larsen,* Solène Fastenackels,* Antoine Roux,* Guy Gorochov,*† Christine Katlama,‡§ Daniel Sidi,‖ Joyce Sibony-Prat,† and Victor Appay*‡†

Reduced thymopoiesis and continuous mobilization of naive T cells into the effector–memory pool can lead to severe alterations of the naive T cell compartment. However, maintenance of the naive T cell population is essential to mount effective immune responses. Evidence of homeostatic regulation of naive T cells is currently debated in animal models. In humans, the situation remains unresolved, in particular with advanced age. In this study, we analyzed the CD4+ and CD8+ naive T cell compartments from elderly, young adults thymectomized during early childhood, and HIV-1–infected patients, which are characterized by T lymphocytopenia. We show a direct association between increased turnover and decreased frequency of naive T cells. Moreover, the IL-7–induced pathway was fully functional in naive T cells from elderly and young adults thymectomized during early childhood, who are characterized by elevated IL-7 plasma levels. Our findings support the establishment of homeostatic regulation of naive T cell proliferation in humans. This regulation is particularly active in lymphopenic hosts, such as elderly and thymectomized patients. The Journal of Immunology, 2012, 189: 5541–5548.

C ontrol of viral infections by Ag-specific effector cells and establishment of memory cell pools depend on the availability and differentiation of naive T cells upon successful priming with their cognate Ag. The naive T cell population is polyclonal, characterized by a large TCR repertoire diversity, which confers to the immune system its capacity to recognize a quasi-limitless number of foreign Ags. Preservation of the naive T cell compartment is therefore essential to mount effective immune responses against newly encountered pathogens over time. However, during a lifetime, the naive T cell pool is subject to two major effects: a reduced production, associated with the decreased activity of the thymus (i.e., the primary organ of T cell production), and a recurrent transition into the effector–memory pool, due to the exposure to various Ags. This can eventually result in manifest alterations, including the predominant representation of memory over naive T cells within the peripheral T cell pool, low naive T cell numbers, and loss of T cell diversity (1). Several settings in humans are characterized by such alterations of the T cell compartment. This is particularly noticeable with advanced age, which cumulates the consequences of the progressive atrophy or involution of the thymus together with a lifetime of challenges with pathogens (2). Young adults thymectomized during early childhood (YATEC) can also exhibit very similar characteristics, as a result of the poor thymic activity eventually affecting the size of the naive T cell pool (3, 4). A scarce naive T cell compartment is also one of the primary characteristics of the immune system of HIV-1–seropositive patients, and is the most likely consequence of chronic immune activation and suboptimal thymic output due to HIV infection (5–11).

Although thymic production and antigenic stimulation are the two principal determinants of the T cell compartment fate, the immune system also has the capacity to regulate its own environment to maintain relatively stable numbers of T cells in the peripheral lymphoid organs. This capacity refers to T cell homeostasis. This relies generally on the stimulatory effects on lymphocytes of common cytokine receptor γ-chain–dependent cytokines, in particular IL-7 [reviewed in (12)]. IL-7 is a key regulator of the size of the T cell pool, driving proliferation of naive and memory cells in the vacuum of lymphopenia (13, 14). Homeostasis was first demonstrated for the memory T cell compartment (15). It is essential for the long-term maintenance of immunological memory, and for reconstituting the T cell compartment in transplanted patients. Evidence of the homeostatic regulation of the naive T cell pool has been provided in animal models, such as mice or nonhuman primates (16–19). However, this has been more difficult to demonstrate in humans. Naive T cell homeostasis may be particularly relevant in the context of advanced age.

In this study, our aim is to gather evidence for the homeostatic regulation of the naive T cell compartment in humans. To this end, the relationship between proliferation and frequency of CD4+ and CD8+ naive T cells was analyzed in different contexts with T lymphocytopenia: in elderly, in YATEC, as well as in HIV-1–infected patients. We also studied IL-7 levels and induced pathway in elderly and YATEC. We provide evidence for the functional

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Abbreviations used in this article: PBSE, Pacific Blue succinimidyl ester; YATEC, young adults thymectomized during early childhood.

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homeostatic regulation of the naïve T cell compartment, independently from age.

**Materials and Methods**

**Study subjects and samples**

The study concentrated on three main groups of donors, as follows: elderly healthy adults, YATEC, and HIV-1–infected patients (Table I). YATEC had complete removal of the thymus within 15 d after birth during open-heart surgery due to transposition of great vessels. Thymectomy was performed by total resection of both lobes for ease of surgical access to the heart and major vessels. Included donors (healthy or thymectomized) had no residual cyanosis, transplantation or immunosuppressive therapy, cortisone therapy, hemodynamic or hemorrhagic disorders, and no medication with drugs known to influence blood production in the bone marrow or the immune system. We excluded elderly individuals with malignancies, acute diseases, or advanced stages of severe chronic diseases, such as chronic inflammatory disease, atherosclerotic disease, congestive heart failure, diabetes mellitus, renal or hepatic disease, or chronic obstructive pulmonary disease, as well as in individuals under immunosuppressive therapy. HIV-1–infected patients were divided into two groups, as follows: 1) treatment of naïve patients infected with HIV-1 for >3 y (positive for p24 ELISA and Western blot), and 2) HIV-1–infected patients receiving antiretroviral therapy for >3 y. Young and middle-aged healthy adults were also studied for comparison. All participants gave their written informed consent. The study was approved by the local institutional ethics committee (i.e., Comité de Protection des Personnes of the Pitie® Salpétrie®re Hospital, Paris, France).

Blood samples were obtained from all donors; mononuclear cells were isolated over a Lymphoprep gradient and cryopreserved until use.

**Flow cytometry reagents and staining**

Directly conjugated Abs were obtained from the following vendors: BD Biosciences (San Jose, CA), CD4 (allophycocyanin-cyanin 7), CCR7 (PE-Cy7), CD38 (allophycocyanin), and Ki67 (FITC); Beckman Coulter (Villepinte, France), CD45RA (ECD); Caltag (Burlingame, CA), CD8 (Alexa405); Dako (Glostrup, Denmark), CD3 (Cascade Yellow); BioLegend (San Diego, CA), CD27 (AlexaFluor700) and CD31 (AlexaFluor647). Cell surface marker stainings were performed by addition of the respective Abs for 15 min at room temperature. After incubation, cells were washed in PBS and then permeabilized with Perm/Fix kit (eBiosciences, San Diego, CA) before the addition of Ki67 Ab. Of note, our gating strategy, based on CCR7, CD27, and CD45RA coexpression, stringently defines naïve T cells as they do not rapidly produce cytokines in response to polyclonal stimulation (Supplemental Fig. 1S).

Stainings were analyzed on an LSR2 flow cytometer (BD Biosciences) with appropriate isotype controls and color compensation. Data were analyzed using FlowJo v8.2 (Tree Star) and DIVA software (BD Biosciences).

**In vitro IL-7–induced proliferation**

PBMC were stained with a cell proliferation dye (Pacific Blue succinimidyl ester [PBSE]; Invitrogen Life Technologies, Paisley, U.K.), according to the provider’s recommendations. Labeled cells were then cultured in presence of IL-7 at 10 ng/ml (R&D Systems, Abingdon, U.K.) or CD3/CD28-coated beads (Dynabeads; Invitrogen) during 5 d. Negative controls were obtained in absence of stimulation. The frequency of proliferating T cells (PBSE low) was determined by flow cytometry.

**IL-7 levels and STAT5 phosphorylation**

Measurement of IL-7 in the plasma of donors was performed by Quantikine ELISA (R&D Systems). To assess IL-7–induced STAT5 phosphorylation, PBMCs were exposed to rIL-7 (R&D Systems) at doses up to 20 ng/ml, then washed and fixed in Cytofix buffer (BD Biosciences) for 10 min at room temperature. Cells were subsequently stained with T cell differentiation surface markers for 15 min at 4°C, then permeabilized with Phosphoflow Perm buffer (BD Biosciences). After washing, cells were stained intracellularly for 30 min at room temperature using anti-human phosphoSTAT5 mAb-AF647 conjugated (BD Biosciences) and analyzed by flow cytometry.

**Statistical analysis**

Univariate statistical analysis was performed using GraphPad prism software. Groups were compared using the nonparametric Mann–Whitney U test. Spearman’s rank test was used to determine correlations. Multivariate statistical analysis was performed using JMP software. The p values <0.05 were considered significant.

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**Table I. Donor characteristics**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Age (y)</th>
<th>Gender (% male)</th>
<th>CD4+ T Cell Count (cells/μl)</th>
<th>CD8+ T Cell Count (cells/μl)</th>
<th>Viral Load (copies/ml)</th>
<th>Viral Load Nadir (copies/ml)</th>
<th>Time Since Diagnosis (y)</th>
<th>Duration of Treatment (y)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy Adults, YATEC</td>
<td>21-26</td>
<td>36</td>
<td>[96.4–103.8]</td>
<td>35-128.5</td>
<td>22-51.7</td>
<td>196-28.65</td>
<td>18-26.2</td>
<td>18-26.2</td>
</tr>
<tr>
<td>HIV infection</td>
<td>21-26</td>
<td>44.2</td>
<td>[26.5–54.99]</td>
<td>25</td>
<td>25</td>
<td>15.6-25.05</td>
<td>18-26.2</td>
<td>15.6-25.05</td>
</tr>
<tr>
<td>HIV+ ART (&gt;3 y of treatment)</td>
<td>21-26</td>
<td>44.3</td>
<td>[33.16-56.65]</td>
<td>70.5</td>
<td>70.5</td>
<td>3.59-9.4</td>
<td>3.59-9.4</td>
<td>3.59-9.4</td>
</tr>
</tbody>
</table>
**Results**

*Increased cell cycling of human naïve T cells in lymphopenic donors*

To seek evidence of homeostatic regulation of the naïve T cell pool in humans, we studied two independent settings characterized by suboptimal T cell production due to: 1) thymic involution with advanced age (elderly compared with middle-aged donors), or 2) thymectomy during early childhood (YATEC compared with age-matched young adults) (Table I). Assessment of proliferation within the naïve CD4+ and CD8+ T cell compartments was based on the expression of Ki67 in CCR7+CD27−CD45RA+ T cells. The expression of nuclear protein, Ki67, is upregulated as cells enter the cell cycle (20) and usually associated with the expression of CD38, known as a marker of cell activation (21–23). In contrast to memory T cell populations, naïve T cells are usually quiescent and express only low levels of Ki67 (Fig. 1A). Nonetheless, we observed that CD4+ and CD8+ naïve T cells from elderly and YATEC donors were inversely correlated with the percentages of CD4+ or CD8+ naïve T cells from our donors (Fig. 2A), as this is usually observed for the memory T cell compartment, most likely through homeostatic regulation, which confirms our observations in elderly and YATEC.

*Functional IL-7 pathway in naïve T cells from elderly and YATEC*

We next aimed to provide further indication for the establishment of naïve T cell homeostatic regulation by studying the potential role of IL-7 using in vitro and ex vivo assays. IL-7 is an established key factor for the maintenance and proliferation of naïve T cells (29, 30). First, we performed in vitro experiments, in which PBMC from healthy individuals were cultured in presence of IL-7 for 5 d. Using a cell proliferation dye (illustrated in Fig. 3A), we show that both CD4+ and CD8+ naïve T cells are able to proliferate in response to IL-7 stimulation (Fig. 3B). This confirms that the IL-7 signal plays an important role in naïve T cell homeostatic proliferation in humans, as previously published (14). Next, we analyzed IL-7 plasma levels and signaling in elderly and YATEC. IL-7 plasma levels are known to be elevated in HIV-infected patients with lymphopenia (7) as well as in children following thymectomy (31, 32). The situation in older thymectomized patients and in elderly is less clear. We therefore measured the plasma level of IL-7 in these donors in comparison with their respective control groups. Our results show that circulating IL-7 levels are higher in both groups of lymphopenic subjects (p = 0.03) (Fig. 3C). This supports an active homeostatic role of the cytokine in these settings. STAT5 is a pivotal factor of IL-7 signaling transduction (33, 34). To assess the capacity of CD4+ or CD8+ naïve T cells from our donors to respond to IL-7, we thus analyzed the level of STAT5 phosphorylation induced ex vivo in these cells by exogenous IL-7 stimulus (Fig. 3D). Basal (obtained on unstimulated cells) and IL-7–induced levels of STAT5 phosphorylation were similar between lymphopenic and control groups (Fig. 3E). This indicates that naïve T cells from both elderly and YATEC are fully capable of responding to IL-7 stimuli. Altogether, these results support further the possibility of establishing naïve T cell homeostasis in a T lymphopenic environment, independently from age.

**Discussion**

The present study of long-term consequences of age, thymectomy, and HIV infection provides evidence for a homeostatic regulation of the naïve T cell compartment in humans. These different settings are characterized by a significant reduction of CD8+ and CD4+ naïve T cell frequencies. Moreover, we observed that the degree of cell cycling in this compartment is directly related to the frequency of donors’ naïve T cells: the lower their numbers, the higher their turnover.

The homeostatic regulation of naïve T cells in humans, demonstrated in the current study, concurs with a recent report from den Braber et al. (35), showing that the maintenance of peripheral naïve T cells occurred through peripheral cell division in healthy individuals. In addition to these findings, our work provides new insights into the underlying mechanism driving such peripheral...
We propose that homeostatic regulation of cell proliferation is established to oppose the development of naive T lymphocytopenia, resulting from both reduced thymopoiesis and elevated Ag-driven T cell stimulation. Suboptimal production of naive T cells due to thymectomy in YATEC appears indeed to be compensated by homeostatic proliferation of naive T cells, which maintain the integrity of the peripheral T cell compartment.

Remarkably, this T lymphocytopenia feedback mechanism acts independently of both antigenic context and age of the donor. Despite an advanced age, the homeostatic machinery and regulation of CD8⁺ and CD4⁺ naive T cell frequencies appear to be essentially intact in elderly. Prominent homeostatic proliferation of naive T cells in elderly is concordant with shorter telomere length and lower TCR excision circle content reported for this

**FIGURE 1.** Cell cycling of naive T cells from elderly and YATEC. (A) Representative FACS staining illustrating the gating strategy of T cell differentiation (based on CCR7, CD27, and CD45RA markers) and turnover (based on Ki67 and CD38 markers) obtained on naive and memory T cells for both CD4⁺ (on the left) and CD8⁺ (on the right) T cells. (B) Percentage of Ki67⁺ cells among CD4⁺ and CD8⁺ naive T cells in young (Young, n = 20), middle-aged (Mid, n = 34), or old (Old, n = 25) adults, and in YATEC (n = 25). Bars indicate the median. The Mann–Whitney U test was used for comparison. The p values are indicated on the graph. (C) Inverse correlations between the percentage (lower panel) or count (upper panel) of CD4⁺ and CD8⁺ naive T cells and the frequency of proliferating Ki67⁺. Similar symbols in (B) and (C) are used to discriminate groups of patients. (D) Inverse correlation between the percentage of CD31 high CD4⁺ naive T cells and the frequency of proliferating Ki67⁺ CD4⁺ naive T cells. The Spearman’s rank test was used to determine correlations. The p and r values are indicated on the graph.
cellular subset (36). Nonetheless, the reduced naive T cell counts in lymphopenic environments, such as aging and thymectomy (1–4), indicate that the peripheral homeostatic proliferation of naive T cells is insufficient to compensate the loss of thymic output.

Although cytokines like IL-21 (37–39) or thymic stromal lymphopoietin (40, 41) could play a role in the homeostatic regulation of naive T cells, IL-7 has emerged as the primary factor for the proliferation of both CD8+ and CD4+ naive T cells in lymphopenic hosts, as initially demonstrated by Schluns et al., Tan et al., and Goldrath et al. (24, 42, 43) in mice. In humans, levels of circulating IL-7 are increased in the context of T lymphocytopenia. The first study demonstrating a negative correlation between absolute lymphocyte counts and IL-7 levels was performed in pediatric patients undergoing bone marrow transplantation (44). Thereafter, it was shown that low T cell frequencies were associated with high levels of IL-7 in sera from HIV patients (45, 46)

**FIGURE 2.** Cell cycling of naive T cells from HIV-1–infected patients. (A) Percentages of Ki67+ cells among CD4+ and CD8+ naive T cells in middle-aged donors (Mid, n = 34), treatment naive (antiretroviral therapy [ART], n = 57), or treated (ART+, n = 44) HIV-1–infected patients. Bars indicate the median. The Mann–Whitney U test was used for comparison. The p values are indicated on the graph. (B) Correlation between the viral load (upper panel) and the frequency of proliferating Ki67+ cells in untreated patients. Inverse correlations between the percentages of CD4+ and CD8+ naive T cells (lower panel) and the frequency of proliferating Ki67+ in untreated patients. (C) Lack of correlation between the viral load (upper panel) and the frequency of proliferating Ki67+ cells in treated patients. Inverse correlations between the percentages of CD4+ and CD8+ naive T cells (lower panel) and the frequency of proliferating Ki67+ in treated patients. As in Fig. 1C, the correlations between the count of CD4+ and CD8+ naive T cells and the frequency of proliferating Ki67+ are shown in a reduced size both for untreated patients (B, lower panel) and for treated patients (C, lower panel). The Spearman’s rank test was used to determine correlations.
as well as patients with cancer chemotherapy-associated lymphopenia (29), and children following thymectomy (31). In this study, we extend these observations to elderly and YATEC, who thus present elevated circulating IL-7 levels. T cell responsiveness to IL-7 depends largely on the surface expression of IL-7Rα (CD127), which is a hallmark of naive T cells, as well as IL-7 intracellular signal transduction pathway (47). HIV infection is known to perturb IL-7 signaling in T cells (7, 48–50). Moreover, alterations in IL-7 expression, signaling, and survival responses have been reported in effector–memory cells from elderly (51). The present analysis of STAT5 phosphorylation upon IL-7 stimulation indicates that the long-term consequence of thymectomy or age does not result in aberrant IL-7 signal transduction in naive T cells, so that the homeostatic drive of this subset is preserved. In line with our findings, Alves et al. (52) reported on the presence in elderly, HIV-infected donors, and hematopoietic stem cell trans-
plant patients of IL-7Rαlow naive T cells, which may reflect cells that have recently received homeostatic IL-7 signals.

Collectively, our results provide evidence for the active homeostatic regulation of naive T cells, particularly in the case of T lymphopoeisis in human lymphoid tissues, with an advanced age. Although not addressed in the current study, the preservation of naive T cell homeostasis is likely to be important for the long-term maintenance of immunity in these settings.

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Disclosures

The authors have no financial conflicts of interest.

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