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

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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

homeostatic regulation of the naive 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, hematologic 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 individuals under immunosuppressive therapy. HIV-1–infected patients were divided into two groups, as follows: 1) treatment of naive 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 Pitié Salpétrière Hospital, Paris, France). Blood samples were obtained from all donors; mononuclear cells were then 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); Bio-Legend (San Diego, CA), CD27 (AlexaFluor700) and CD31 (Alexa-Fluor647). 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 naive 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.

Table I. Donor characteristics

Lymphopenic Contexts	Groups	Age (y)	Gender (% male)	CD4+ T Cell Count (cells/μl)	CD8+ T Cell Count (cells/μl)	Viral Load (copies/ml)	CD4+ T Cell Nadir (cells/μl)	Time Since Diagnosis (y)	Duration of Treatment (y)
Age	Old (n = 25)	86.1 [76.1–96.5]	36	584 [209–890]	348 [55–1,224]	n.a.	n.a.	n.a.	n.a.
	Middle age (n = 34)	35.28 [26.4–54.99]	44.2	843 [525–1,509]	492 [241–1,149]	n.a.	n.a.	n.a.	n.a.
	Young (n = 20)	22.17 [19.05–25.65]	25	925 [314–1,562]	491 [215–3,153]	n.a.	n.a.	n.a.	n.a.
Thymectomy	YATEC (n = 25)	22.05 [18–26.2]	72	741 [252–1,248]	388 [129–810]	n.a.	n.a.	n.a.	n.a.
HIV infection	HIV+ (untreated) ART− (n = 57)	39.94 [25.9–57.5]	72	326 [2–1,084]	770 [103–2,900]	23,208 [40–2.e+6]	294 [3–973]	5.10 [0.01–25.68]	n.a.
	HIV+ (>3 y of treatment) ART+ (n = 44)	44.43 [33.16–56.45]	70.5	348 [55–1,224]	832 [191–1,831]	40 [40–172]	73 [2–180]	14.56 [3.24–23.10]	10.26 [2.92–18.96]

Age (years), gender (% male), CD4 T cell count (cell/μl), and CD8 T cell count (cell/μl) are parameters shown for four groups of individuals: healthy controls (divided according to age: young, middle age, old), thymectomized patients (YATEC), and HIV-1–infected patients. Viral load (copies/ml), nadir values, time since diagnosis (years), as well as the duration of treatment (years) are also indicated for HIV-infected patients. HIV-seropositive untreated patients (ART−), and HIV-seropositive treated (ART+) patients. Data ranges are indicated in brackets.

Results

Increased cell cycling of human naive T cells in lymphopenic donors

To seek evidence of homeostatic regulation of the naive 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 naive 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, naive T cells are usually quiescent and express only low levels of Ki67 (Fig. 1A). Nonetheless, we observed that CD4$^+$ and CD8$^+$ naive T cells from elderly and YATEC displayed significantly higher Ki67 expression than their respective control groups (Fig. 1B). Increased cell cycling in this compartment may be the consequence of a sustained proliferation of naive T cells upon stimulation with Ags to differentiate into effector–memory cells (24), which is known to rise with age, as well as in certain YATEC (i.e., due to infection by CMV) (4). It may also reflect homeostatic proliferation of these cells to regulate their frequency. In line with this possibility, we found that Ki67 expression levels in CD4$^+$ or CD8$^+$ naive T cells from these donors were inversely correlated with the percentages of CD4$^+$ or CD8$^+$ naive T cells, respectively, as well as their absolute counts (Fig. 1C). Using multivariate analyses, both the frequency of naive T cells ($p = 0.0001$) as well as the age of donors ($p = 0.03$) came out as independent predictive factors of Ki67 expression levels in CD4$^+$ or CD8$^+$ naive T cells. Of note, gender did not appear to influence the frequency of proliferating naive T cells.

To support the possibility that homeostatic regulation of naive T cells may be related to low T cell production and lymphopenia, we studied the relationship between naive T cell production and turnover. For this purpose, we assessed CD31 expression on naive CD4$^+$ T cells, which has been proposed to be a marker of recent thymic emigrants (25, 26). We found an inverse correlation between CD31 expression on naive CD4$^+$ T cells and their respective turnover as measured by Ki67 expression (Fig. 1D).

Overall, these observations suggest that T lymphocytopenia can result in naive T cell homeostatic regulation in humans.

Homeostatic cell cycling of naive T cells in treated HIV-infected patients

To differentiate further between Ag-driven differentiation and homeostatic proliferation of the naive T cell compartment, we decided to analyze CD4$^+$ or CD8$^+$ naive T cells from HIV-infected donors, comparing patients receiving antiretroviral treatments and those who did not (Table I). Most of untreated HIV-infected patients are characterized by an important lymphopenia (mainly of CD4$^+$ T cells) as well as chronic immune activation, associated with viral replication. In this context, antiretroviral therapy results in potent HIV replication suppression and diminished antigenic stimulation. Patients in both groups were selected to display a large range of CD4$^+$ and CD8$^+$ T cell counts. Untreated HIV patients (i.e., characterized by viral replication) presented significantly higher CD4$^+$ or CD8$^+$ naive T cell Ki67 expression levels compared with both healthy controls and treated HIV-seropositive donors (Fig. 2A), as this is usually observed for the memory compartment (data not shown) (27, 28). Ki67 expression levels were associated with the frequency or counts of naive T cells in

these patients. However, they also correlated strongly with their plasma viral load (Fig. 2B). The latter suggests that viral replication and therefore the Ag load can indeed drive the proliferation of CD4$^+$ or CD8$^+$ naive T cells, thus explaining high levels of naive T cell proliferation in untreated patients. In contrast, when viral replication was suppressed in treated patients, only the inverse correlation between the percentage (or counts) of Ki67$^+$ CD4$^+$ or CD8$^+$ T cells and the frequency of respective naive T cells remained highly significant (Fig. 2C). Therefore, regardless of antigenic stimulation and naive T cell differentiation, the naive T cell frequency impacts strongly on cell cycling in this compartment, most likely through homeostatic regulation, which confirms our observations in elderly and YATEC.

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

We next aimed to provide further indication for the establishment of naive 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 naive 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$^+$ naive 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 naive 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$^+$ naive 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 naive 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 naive T cell homeostasis in a T lymphocytopenic 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 naive T cell compartment in humans. These different settings are characterized by a significant reduction of CD8$^+$ and CD4$^+$ naive T cell frequencies. Moreover, we observed that the degree of cell cycling in this compartment is directly related to the frequency of donors' naive T cells: the lower their numbers, the higher their turnover.

The homeostatic regulation of naive 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 naive 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
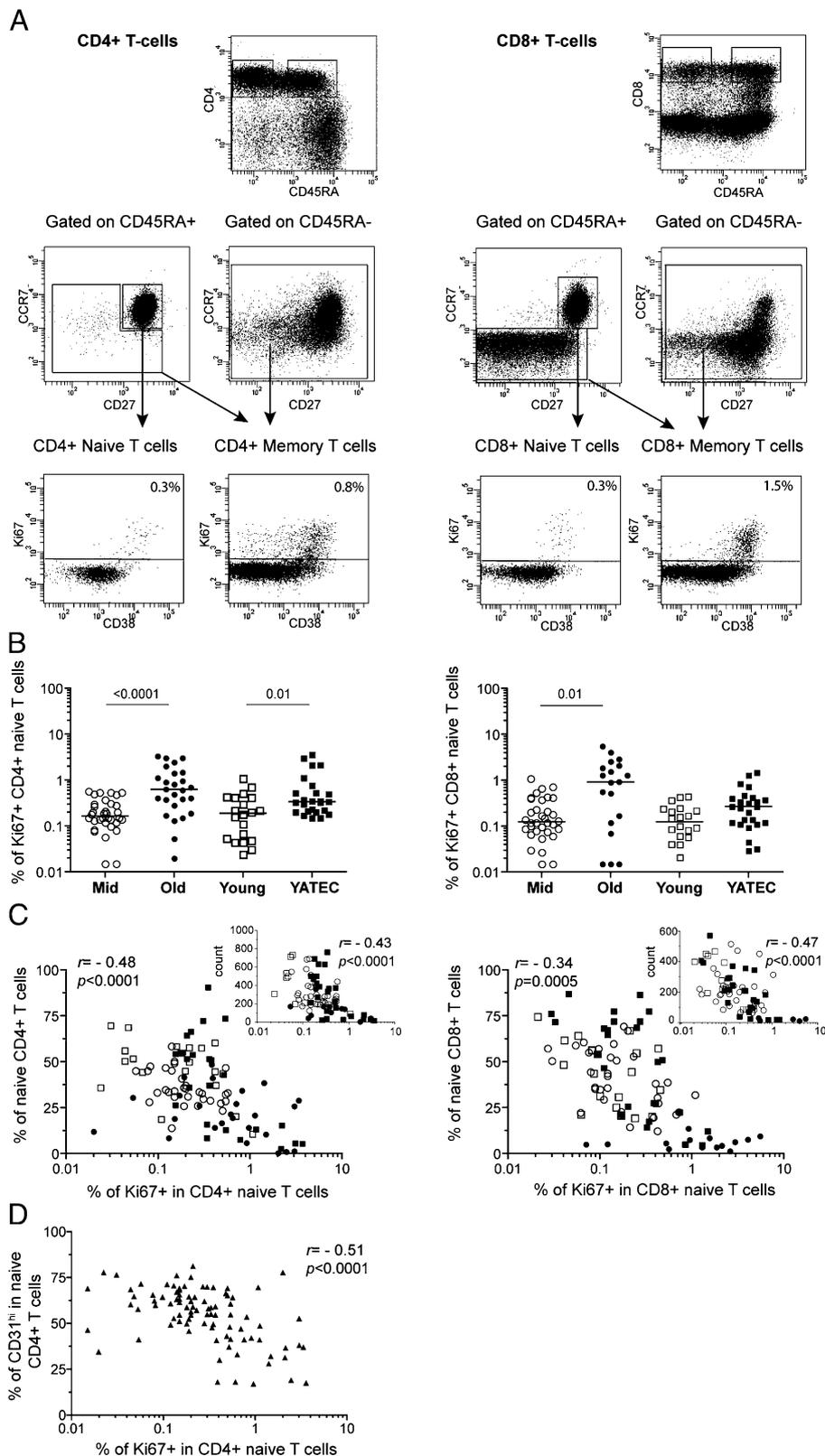
FIGURE 1. Cell cycling of naive T cells from elderly and YATEC. (**A**) Representative FACS staining illustrating the gating strategy of T cell differentiation (based on CCR7, CD27, and CD45RA markers) and turnover (based on Ki67 and CD38 markers) obtained on naive and memory T cells for both CD4$^+$ (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.

cell division. 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
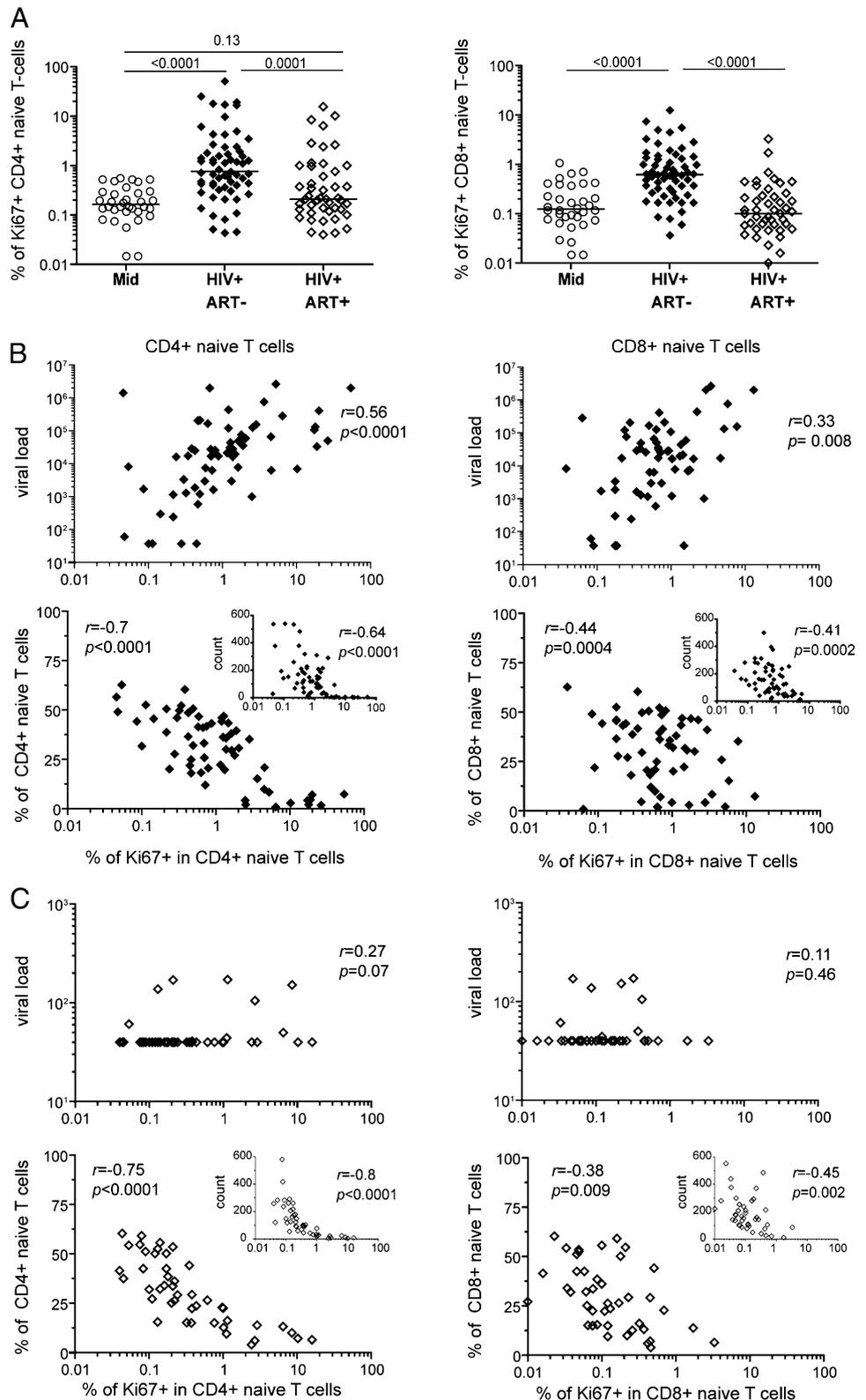
FIGURE 2. Cell cycling of naive T cells from HIV-1–infected patients. (**A**) Percentages of Ki67+ cells among CD4+ and CD8+ naive T cells in middle-aged donors (Mid, $n = 34$), treatment naive (antiretroviral therapy [ART]−, $n = 57$), or treated (ART+, $n = 44$) HIV-1–infected patients. Bars indicate 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.

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 3. IL-7–mediated proliferation, plasmatic IL-7 concentrations, and levels of cytokine-induced STAT5 phosphorylation. (**A**) Representative dot plots gated on naive CD8$^+$ T cells (on the *left*) and on naive CD4$^+$ T cells (on the *right*), illustrating the cell proliferation dye (PBSE) on unstimulated PBMC after 5 d of culture (US, *upper panel*), post–IL-7 stimulation at 10 ng/ml (*middle panel*), and postpolyclonal stimulation with CD3/CD28-coated beads (*bottom panel*). Percentages of proliferating naive T cells (PBSE low) are indicated for each culture condition. (**B**) Frequency of proliferating naive CD8$^+$ or CD4$^+$ T cells after in vitro culture of PBMC unstimulated (US) or in the presence of 10 ng/ml IL-7. (**C**) Level of IL-7 in plasma from young (Young, $n = 25$), middle-aged (Mid, $n = 30$), or old (Old, $n = 29$) 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. (**D**) Representative flow cytometry profiles obtained from one healthy individual showing the gating strategy combining both CD4/CD8 phenotyping (CCR7/CD27/CD45RA markers) and STAT5 threshold (unstimulated cells in gray versus IL-7–stimulated cells in black). (**E**) Level of STAT5 phosphorylation in CD4$^+$ and CD8$^+$ naive T cells from young (Young, $n = 18$), middle-aged (Mid, $n = 19$), or old (Old, $n = 10$) adults, and in YATEC ($n = 15$). Mean fluorescence intensity (MFI) values of phosphorylated STAT5 obtained after exposure to 10 ng/ml IL-7 or at baseline (unstimulated [US]) are plotted. Bars indicate the median.
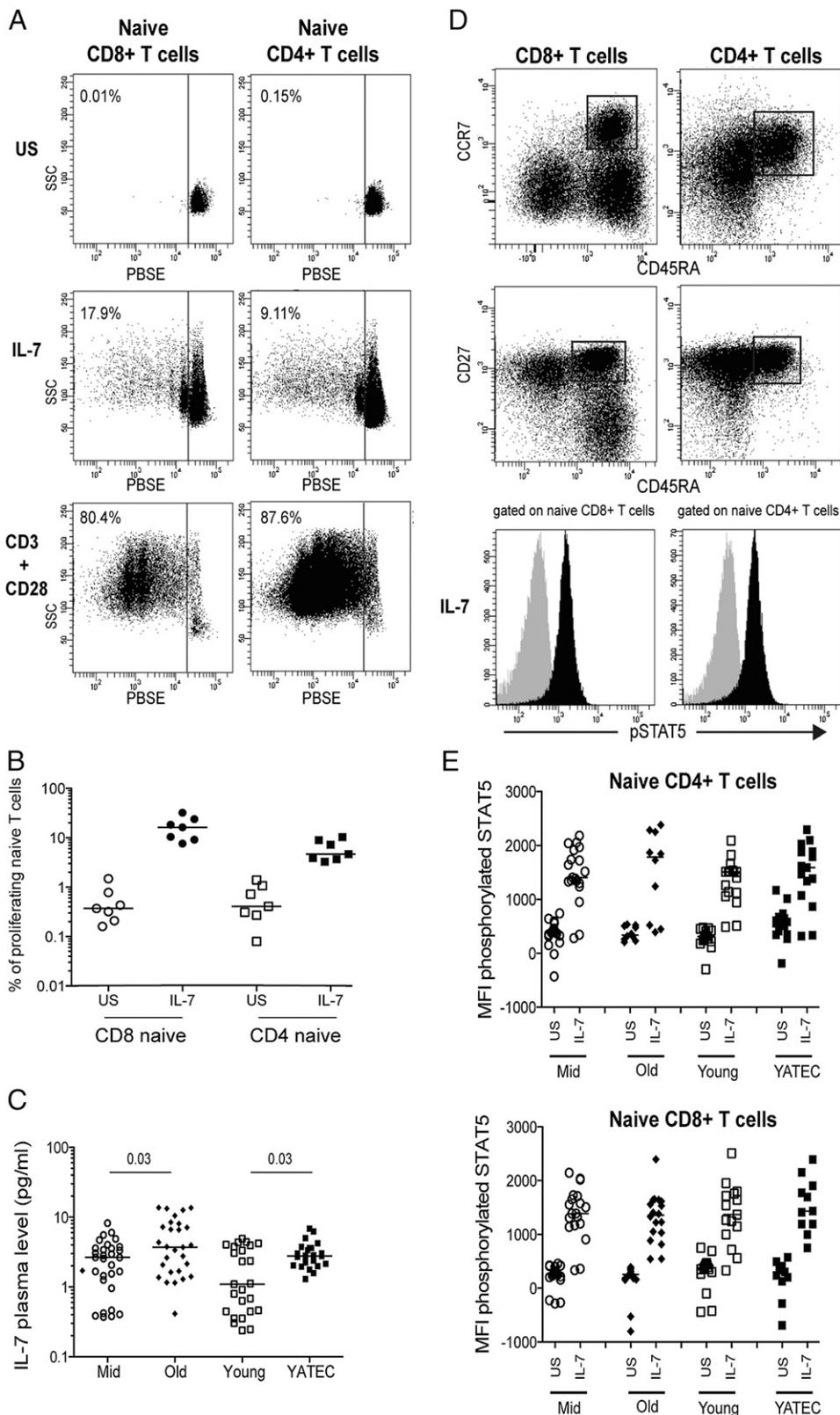
as well as patients with cancer chemotherapy-associated lymphopenia (29), and children following thymectomy (31). In this study, we extend these observations to elderly and YATEC, who thus present elevated circulating IL-7 levels. T cell responsiveness to IL-7 depends largely on the surface expression 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-7Ra^{low} 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 lymphopenia in humans, including with 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.

Acknowledgments

Disclosures

The authors have no financial conflicts of interest.

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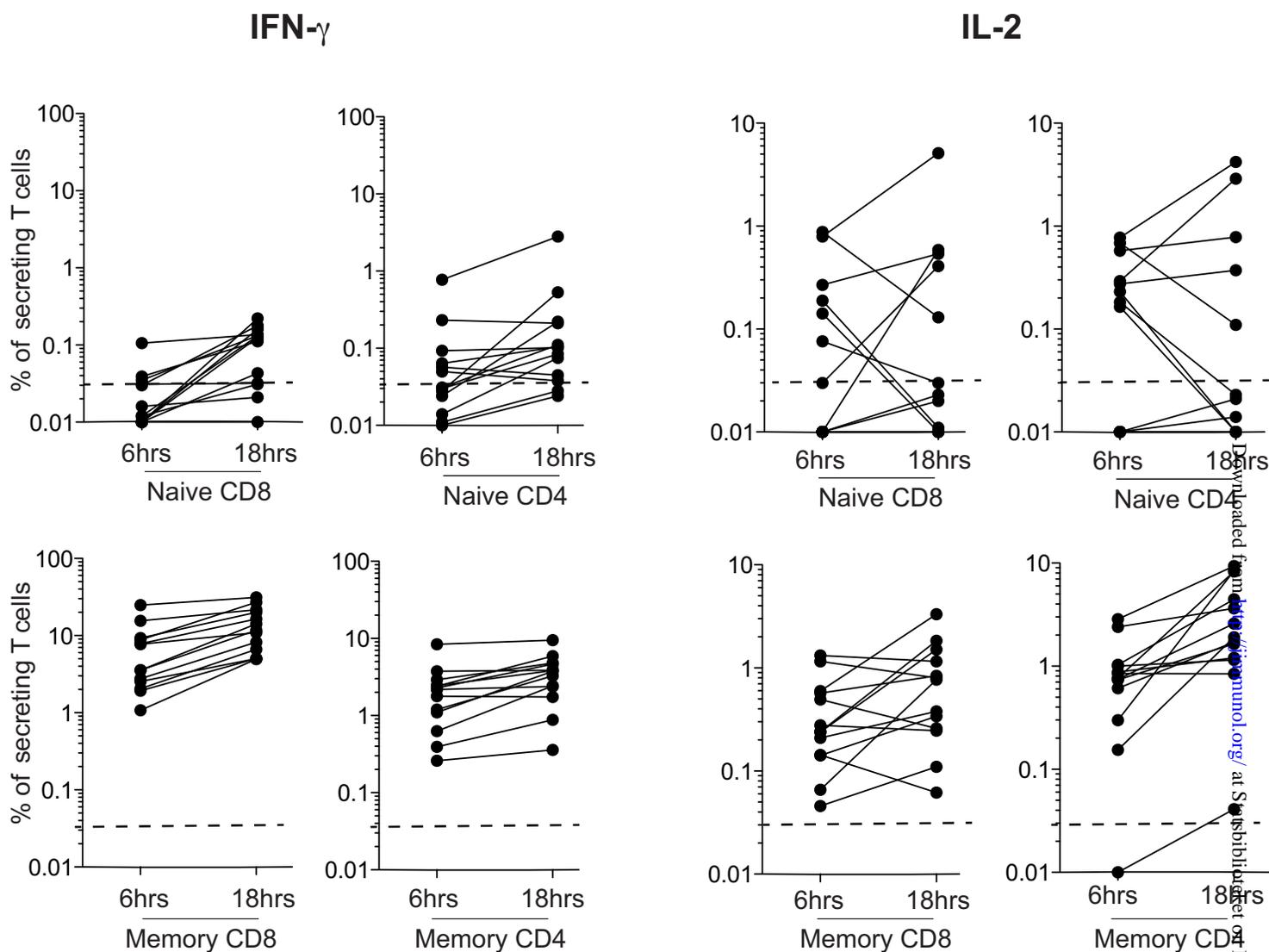
IFN-γ

IL-2

Supplementary Figure 1S: Cytokine-secretion assay
PBMC from lymphopenic patients were stimulated with CD3/CD28 coated beads during 6hrs or 18hrs
and then stained for phenotypic markers (CCR7, CD27, CD45RA) as well as for intra-cellular cytokines
(such as IFN-γ (on the left side of the figure) and IL-2 (on the right side)). The percentage of secreting T-cells
is plotted for each individual subset (naïve (upper panel) or memory (bottom panel) CD8+ and CD4+ T-cells)
and at both time points (6hrs or 18hrs post polyclonal stimulation). The limit of detection for cytokine secretion
was 0.03% (defined as twice the background level and represented by a dashed line) in CD8+ or CD4+ T-cell
populations.