In vivo expansion of naive and activated CD4⁺CD25⁺FOXP3⁺ regulatory T cell populations in interleukin-2-treated HIV patients

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HIV-1 infection is characterized by a progressive decline in CD4⁺ T cells leading to a state of profound immunodeficiency. IL-2 therapy has been shown to improve CD4⁺ counts beyond that observed with antiretroviral therapy. Recent phase III trials revealed that despite a sustained increase in CD4⁺ counts, IL-2-treated patients did not experience a better clinical outcome [Abrams D, et al. (2009) *N Engl J Med* 361(16):1548–1559]. To explain these disappointing results, we have studied phenotypic, functional, and molecular characteristics of CD4⁺ T cell populations in IL-2-treated patients. We found that the principal effect of long-term IL-2 therapy was the expansion of two distinct CD4⁺CD25⁺ T cell populations (CD4⁺CD25^{lo}CD127^{lo}FOXP3⁺ and CD4⁺CD25^{hi}CD127^{lo}FOXP3^{hi}) that shared phenotypic markers of Treg but could be distinguished by the levels of CD25 and FOXP3 expression. IL-2-expanded CD4⁺CD25⁺ T cells suppressed proliferation of effector cells in vitro and had gene expression profiles similar to those of natural regulatory CD4⁺CD25^{hi}FOXP3⁺ T cells (Treg) from healthy donors, an immunosuppressive T cell subset critically important for the maintenance of self-tolerance. We propose that the sustained increase of the peripheral Treg pool in IL-2-treated HIV patients may account for the unexpected clinical observation that patients with the greatest expansion of CD4⁺ T cells had a higher relative risk of clinical progression to AIDS.

molecular profiling | immune-based therapy

HIV infection is mainly associated with a progressive decrease in the number of CD4⁺ T lymphocytes and defective CD4- and CD8-specific T cell responses against HIV and other pathogens. Quantitative and qualitative CD4 T cell reconstitution following introduction of antiretroviral therapy (ART) in HIV-infected patients is often incomplete, leading one to evaluate the impact of immune-based therapy in combination with ART. In the past 20 years, a large set of clinical trials demonstrated that intermittent interleukin (IL)-2 therapy increased the CD4 T cell pool in HIV-infected patients and induced, in a large majority of patients, significant and sustained increases in CD4 T cell count beyond that seen in patients treated with antiviral drugs alone. In particular, recombinant IL-2 therapy raised naive and central memory CD4⁺ cells expressing CD25, the α -chain of the IL-2 receptor, more than antiretroviral drugs alone (1–6). This population accounted for up to 70% of the total CD4⁺ T cell pool of IL-2-treated patients and persisted for a long time.

The clinical impact of IL-2 was evaluated in two long-run large phase III trials (SILCAAT and ESPRIT) involving almost 6,000 chronically HIV-1-infected patients. Results recently reported showed that, despite a sustained increase in CD4⁺ T cell counts, over a median follow-up of 7–8 years, IL-2-treated patients did

not experience a better clinical outcome (7). These disappointing results have remained unexplained so far.

In mice, IL-2 has been shown to be essential for the maintenance and function of a regulatory T cell subset characterized by the expression of CD25 and of the transcription factor FOXP3 (CD4⁺CD25^{hi}FOXP3⁺ T cells, Treg) (8). Neutralization of IL-2 resulted in a decrease in peripheral Treg and the development of autoimmune pathologies (9). Furthermore, analysis of IL-2- or CD25-deficient mice revealed that IL-2 signaling was essential for peripheral maintenance and function of Treg, but not for their generation in the thymus (10, 11). These data argued for an important role of IL-2 in the homeostasis of Treg in mice; however, it is still debated to which extent IL-2 affects the generation, maintenance, and/or function of human Treg. It was shown that IL-2 therapy expanded CD4⁺CD25^{hi}FOXP3⁺ regulatory T cells that displayed suppressive activity in vitro in cancer patients (12, 13). On the other hand, IL-2-expanded CD4⁺CD25⁺ T cells in HIV patients were not considered to be Treg (14, 15).

Here, we have determined the long-term effects of IL-2 therapy on CD4⁺ T cell homeostasis and function in HIV-infected patients. We focused our analysis on phenotypic, functional, and molecular characteristics of the IL-2-expanded CD4⁺CD25⁺ T cell subset. We found that the main effect of long-term IL-2 therapy was the expansion of two distinct CD4⁺CD25⁺ T cell populations (CD4⁺CD25^{lo}CD127^{lo}FOXP3⁺ and CD4⁺CD25^{hi}CD127^{lo}FOXP3^{hi}) that shared phenotypic markers of Treg but could be distinguished by the levels of CD25 and FOXP3 expression. IL-2-expanded CD4⁺CD25⁺ T cells suppressed proliferation of effector cells in vitro and therefore shared functional characteristics of Treg from healthy donors. Finally, to assess the molecular characteristics of IL-2-expanded CD4⁺CD25⁺ T cells, we performed a transcriptome analysis of CD4⁺CD25⁺ T cells from HIV patients before and after IL-2 treatment. We found that the gene expression signatures before and after IL-2 treatment cycles are closely related

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and resemble the gene expression profiles generated from sorted CD4⁺CD25^{hi} Treg from healthy donors. Together, our data suggest that IL-2 treatment expands CD4⁺CD25⁺FOXP3⁺ T cell populations in HIV patients that share phenotypic, functional, and molecular characteristics with Treg.

Results

IL-2 Therapy Expands Distinct Populations of CD4⁺CD25⁺ T Cells Sharing Phenotypic Markers of Treg. To determine the effects of intermittent IL-2 therapy on CD4⁺ T cell homeostasis we first examined phenotypic characteristics of IL-2-expanded CD4⁺CD25⁺ in a cohort of 31 IL-2-treated patients. CD4⁺ T cell counts were 445/μL (95–919) before IL-2 treatment and 843/μL (422–2348), at the time of sampling. Patients received a median (ranges) of 7 (3–18) IL-2 cycles (Table 1). IL-2 recipients had a median 29.5% ($n = 15$; ranges, 12.2–69.0) and 167/μL CD4⁺CD25⁺ T cells and differed significantly from patients treated with combination antiretroviral therapy (cART) alone ($n = 20$) who had 16.6% (9.0–34.0) ($P = 0.002$) and 94 cells/μL ($P = 0.012$).

Because CD4⁺CD25⁺ T cells in HIV-infected patients may include activated T cells or cells that up-regulated CD25 in response to IL-2 treatment, we sought to quantify Treg by analyzing the proportion of CD4⁺CD25^{lo} and CD4⁺CD25^{hi} T cells expressing low levels of CD127 and the transcription factor FOXP3 (16, 17) (Fig. 1A).

The proportion of cells expressing Treg characteristics (FOXP3⁺CD127^{lo}) among CD4⁺CD25^{hi} subsets was identical in IL-2 and cART patients (patients 1–6, Table 1) (Fig. 1A for representative cases and Fig. 1B; see Fig. S1 for gating strategy). CD4⁺ T cells expressing intermediate levels of CD25 (CD4⁺CD25^{lo}) were previously reported as the main CD4⁺ T cell population expanded in IL-2-treated patients (6, 15, 18). We found that a higher proportion of CD4⁺CD25^{lo} T cells were characterized by a Treg phenotype (FOXP3⁺CD127^{lo}) in IL-2-treated patients compared to patients treated by cART alone (Fig. 1A and B). Furthermore,

absolute counts of CD4⁺CD25^{hi}FOXP3⁺CD127^{lo} and CD4⁺CD25^{lo}FOXP3⁺CD127^{lo} T cells were significantly increased in IL-2 recipients ($n = 6$, 48.7 ± 11.8 cells/μL and 49 ± 14 cells/μL) compared with cART-treated patients ($n = 5$, 10.4 ± 2.4 cells/μL and 9.6 ± 2.2 cells/μL; $P = 0.006$ for both comparisons; Fig. 1C). We also noted that the level of FOXP3 expression was lower in the CD4⁺CD25^{lo}CD127^{lo} population [mean fluorescent intensity (MFI) FOXP3: 83.8 ± 11.3] than in the CD4⁺CD25^{hi}CD127^{lo} T cell population (MFI FOXP3: 267.7 ± 78.7). These results indicate that IL-2 therapy expands two distinct CD4⁺CD25⁺ T cell populations that share phenotypic markers of Treg but can be distinguished by the levels of CD25 and FOXP3 expression.

A previous report suggested that the majority of IL-2-expanded CD25⁺ T cells have phenotypic and functional characteristics that are distinct from Treg (14). More recent reports have established heterogeneity within the human Treg compartment (19–21). In light of these findings, we decided to better characterize IL-2-expanded CD4⁺CD25⁺ T cells in HIV patients. We first compared phenotypic markers of CD4⁺CD25^{lo} and CD4⁺CD25^{hi} T cells in 15 IL-2-treated patients (Table 1, patients 7–21). Constitutive expression of intracytoplasmic cytotoxic T lymphocyte-associated antigen-4 (CTLA-4), a negative regulator of T cell activation, of CD103, the α -chain of the α E β 7 integrin, and of CD122 (the β -chain of the IL-2 receptor) was significantly higher in CD25^{hi} T cells compared with CD25^{lo} T cells. Also, the expression of the activation markers CD69, CD95, CD40L, and HLA-DR was significantly higher in CD4⁺CD25^{hi} T cells compared with CD4⁺CD25^{lo} T cells. In contrast, the proportion of CD4⁺ T cells that express CD54RA and/or CD45RO isoforms was similar in both cell populations. Most of CD4⁺CD25^{hi} and CD4⁺CD25^{lo} T cells exhibited high levels of CD62L, a characteristic of Treg. Among CD4⁺CD25^{hi} T cells, a mean of 25.7% of cells expressed HLA-DR, a marker of mature Treg (19). Compared to CD25⁺ T cells, a higher proportion of CD25^{lo} T cells expressed CD122 ($P = 0.005$), CD103 ($P = 0.046$), and high levels of CD62L ($P = 0.018$) (Fig. 2).

Fig. 1. IL-2 therapy expands CD4⁺CD25^{hi} and CD4⁺CD25^{lo} T cells that share phenotypic characteristics of natural regulatory T cells. (A) Proportion of FOXP3⁺CD127^{lo} cells among CD4⁺CD25^{hi} (Left) and CD4⁺CD25^{lo} T cells (Right) in IL-2 recipients (Upper) and patients treated with retroviral therapy alone (cART, Lower). Purified CD3⁺ T cells isolated from PBMC were stained with anti-CD4, anti-CD25, anti-CD127, and anti-FOXP3. Cells were gated according to forward scattering properties and CD25 expression. Coexpression of FOXP3 and CD127 is illustrated for three representative IL-2-treated HIV patients (patients 1, 2, and 3) and three representative patients treated with cART alone (C1, C2, and C3). (B) Proportion of FOXP3⁺CD127^{lo} cells among the CD4⁺CD25^{hi} (Left) and the CD4⁺CD25^{lo} population (Right) in IL-2 recipients ($n = 6$) and cART-treated controls ($n = 5$); **, $P < 0.01$ (Mann–Whitney test). (C) Absolute numbers of CD4⁺FOXP3⁺CD127^{lo}CD25^{hi} (conventional Treg cells) and CD4⁺FOXP3⁺CD127^{lo}CD25^{lo} in peripheral blood of IL-2 recipients ($n = 6$) and cART-treated controls ($n = 5$); **, $P < 0.01$ (Mann–Whitney test).

Table 1. Clinical characteristics of IL-2-treated patients

Patients	Viral load, copies/mL	CD4 cell count/mm ³	CD4 cell count/mm ³	Total no. of IL-2 cycles	Interval from last IL-2 cycle (mo)
		before IL-2 treatment	after IL-2 treatment		
1	56	197	656	17	2
2	<50	137	607	12	2
3	<50	95	479	9	2
4	<50	329	961	8	2
5	65	250	684	11	2
6	<50	146	290	11	2
7	<50	130	560	14	11
8	<50	97	490	18	12
9	<50	177	860	12	10
10	<50	300	895	11	18
11	<50	209	422	12	6
12	<50	102	645	13	14
13	<50	302	429	10	11
14	<50	363	520	12	6
15	<50	477	872	7	4
16	<50	590	633	9	4
17	<50	632	682	6	8
18	<50	324	1,194	5	8
19	700	445	687	6	8
20	<50	556	880	6	59
21	<50	691	843	3	2
34,001	<50	805	1,049	3	2
34,002	<50	772	865	3	2
59,002	<50	527	2,348	3	2
59,003	<50	631	997	3	2
59,004	<50	765	1,563	3	2
59,008	<50	897	1,236	3	2
59,011	<50	805	1,543	3	2
59,013	<50	645	830	3	2
59,015	<50	579	902	3	2
59,019	<50	919	1,375	3	2

CD4⁺CD25⁺ IL-2-Expanded T Cells Exhibit Functional Characteristics of Treg. Next, we explored the proliferative potential of IL-2-expanded CD4⁺CD25⁺ T cells. Upon stimulation with immobilized anti-CD3 mAb, the proliferation of CD4⁺CD25⁺ T cells was significantly reduced compared to autologous CD4⁺CD25⁺ T cells ($P = 0.002$). Addition of soluble anti-CD28 mAb restored only partially the proliferative capacity of these cells as described for Treg in healthy individuals and cART-treated patients (22) (Fig. 3A). Interestingly, in contrast to CD4⁺CD25⁺ T cells, purified CD4⁺CD25⁺ T cells containing both the CD4⁺CD25^{hi} and CD4⁺CD25^{lo} T cell populations did not proliferate in response to stimulation with purified tuberculin (PPD), CMV, or HIV-p24 antigens (Fig. 3B). These data demonstrate that IL-2-expanded CD4⁺CD25⁺ cells that exhibit functional characteristics of Treg.

Then, we investigated the capacity of CD4⁺CD25⁺ T cells expanded in IL-2-treated patients ($n = 13$) to suppress effector functions of CD4⁺ T cells. First, we found that depletion of these cells led to a significant increase in CD4 T cell proliferation in response to PPD and HIV-p24 antigens ($P < 0.05$ for both comparisons) (Fig. 3C). Next, addition of purified CD4⁺CD25⁺ T cells (10^4 – 10^5 cells) resulted in ≈ 50 and 80% inhibition of CD4⁺CD25⁺ T cell proliferation to PPD and HIV-p24 antigens at a 1:4 and a 1:1 ratio (Treg:Effector), respectively (Fig. 3D). It is noteworthy that the levels of inhibition of the CD4⁺CD25⁺ population from IL-2 patients were similar to those observed with CD25^{hi} T cells from cART-treated patients (22). This suggests that CD4⁺CD25^{hi} cells from IL-2-treated patients are highly suppressive or alternatively that expanded CD4⁺CD25^{lo} T cells exert also regulatory functions.

Fig. 2. Phenotype of the CD4⁺CD25^{hi}, CD4⁺CD25^{lo} and CD4⁺CD25⁺ T cell subsets in IL-2-treated HIV-infected patients ($n = 15$). The membrane or intracellular (CTLA-4) expression of the different molecules was determined in whole blood cells by four-color direct flow cytometry after successively combining the FSC/SSC, CD3⁺, CD4⁺, and CD25 gates. Analysis was performed on CD25^{hi} (solid bars), CD25^{lo} (shaded bars), and CD25⁺ (open bars) T cells. Data are presented as mean percentage of positive cells $\pm$ SEM (*, $P < 0.05$; **, $P < 0.01$, using Wilcoxon's rank test).

CD4⁺CD25⁺ IL-2-Expanded T Cells Exhibit Molecular Characteristics of Treg. Finally, to assess the molecular characteristics of IL-2-expanded CD4⁺CD25⁺ T cells, we performed a longitudinal analysis of the gene expression profiles of enriched CD4⁺CD25⁺ and CD4⁺CD25⁺ T cells from 10 HIV-infected patients (patients 34001–59019; Table 1) before the first (week 0) and 8 weeks after the third IL-2 cycle (week 24), using microarrays. We found that 206 genes were differentially expressed ($P < 0.001$) between enriched CD4⁺CD25⁺ and CD4⁺CD25⁺ T cells before IL-2 treatment (week 0) (Dataset S1), whereas 60 genes were differentially expressed ($P < 0.001$) after three IL-2 cycles at week 24 (Dataset S2).

As expected, many of the genes differentially expressed at week 0 (*FOXP3*, *CTLA4*, *IL10*, *FAS*, *LRRC32* (GARP), *CCR8*, and *IL1R1*) have been associated with phenotype or function of Treg (23–26) (Fig. 4). Analysis of the gene expression signature after three IL-2 treatment cycles at week 24 revealed that of the 60 genes differentially expressed at this time point ($P < 0.001$), 50 were also differentially expressed at week 0 (Fig. 4 and Dataset S2). We noted, however, also several differences between CD4⁺CD25⁺ T cells before IL-2 treatment and CD4⁺CD25⁺ T cells after IL-2 treatment. The chemokine receptor *CCR8* (27) was expressed at lower levels after three treatment cycles, whereas the dual-specificity phosphatase 6 (*DUSP6*), a negative regulator of ERK2 activity involved in tuning T cell excitation thresholds (28), was up-regulated in CD4⁺CD25⁺ T cells after IL-2 treatment (Fig. 4). Then, we used the 60 genes differentially expressed between CD4⁺CD25⁺ and CD4⁺CD25⁺ T cells from IL-2-treated HIV patients as a “signature” to analyze sorted CD4⁺CD25^{hi} and CD4⁺CD25⁺ T cells from peripheral blood of healthy donors. Clustering according to this gene set could accurately discriminate between CD4⁺CD25^{hi} and CD4⁺CD25⁺ T cells (Fig. 5). In contrast, this signature could not discriminate between CD4⁺CD25⁺ T cells from patients before and after IL-2 treatment using hierarchical clustering (Fig. S2), suggesting that Treg from HIV patients before and after IL-2 treatment are related and are similar to Treg from healthy donors.

IL-2 Expands Preexisting CD4⁺CD25⁺ Populations Sharing Molecular Markers of Treg. To visualize quantitative differences in gene expression between CD4⁺CD25⁺ and CD4⁺CD25⁺ T cells before and after IL-2 treatment, we plotted the “log₂ fold-change” values for differentially expressed genes ($P < 0.001$) at week 24 (y axis) against those at week 0 (x axis) (Fig. 6). In this representation, genes that are up-regulated in CD4⁺CD25⁺ versus CD4⁺CD25⁺ T cells both before (week 0) and after IL-2 treatment (week 24) are located in the upper-right part of the diagram, whereas genes that are down-

Fig. 5. Genes differentially expressed between IL-2-expanded CD4⁺CD25⁺ and CD4⁺CD25[−] T cells in HIV patients discriminate between sorted Treg and CD4⁺CD25[−] T cells from healthy donors. Treg and CD4⁺CD25[−] T cells from peripheral blood of healthy donors were isolated by cell sorting and stimulated for 24 h with anti-CD3 and anti-CD28 antibodies. Gene expression profiling using ABI 1700 technology of six independent donors was performed as described for samples from HIV patients (*Materials and Methods*). The 60 genes differentially expressed ($P < 0.001$) between CD4⁺CD25⁺ and CD4⁺CD25[−] T cells from HIV patients after IL-2 treatment (*Dataset S2*) were used to analyze the dataset of healthy donors using a hierarchical clustering tool (Spotfire). This algorithm groups samples (in columns) and genes (in rows) according to their Euclidean distances. The dendrogram on top shows that the 60-gene “signature” derived from IL-2-expanded CD4⁺CD25⁺ and CD4⁺CD25[−] T cells from HIV patients groups together samples from CD4⁺CD25[−] T cells (left side of the diagram) and Treg (right side) from healthy donors.

patients (14, 15). These studies were mainly focused on expanded naive CD4⁺CD25^{lo}, the so-called “cytokine expanded naive (CEN)” cells (14). We found that ~70–75% of CD4⁺CD25^{lo} and CD4⁺CD25^{hi} express CD45RO or both CD45RA/CD45RO, a phenotype of memory T cells. We also noted some phenotypic differences between expanded CD4⁺CD25^{lo} and CD4⁺CD25^{hi} cells. The expression of the activation markers CD69, CD95, CD40L, and HLA-DR was significantly higher in CD4⁺CD25^{hi} T cells compared with CD4⁺CD25^{lo} T cells. We also found that constitutive expression of intracytoplasmic CTLA-4, a negative regulator of T cell activation recently described as a marker of progression, was significantly higher in CD25^{hi} T cells compared with CD25^{lo} T cells.

Attempts to better characterize the origin and function of IL-2-expanded CD4⁺CD25⁺ T cells generated several studies in the past years (6, 14, 15, 18, 29). Results reported here are highly reminiscent of recent reports that have established heterogeneity within the Treg compartment. Baecher-Allan et al. reported two distinct Treg populations that can be distinguished on the basis of the expression of MHC class II molecules (19) and Miyara et al. very recently demonstrated the presence of “resting” and “activated” Treg in the periphery (20). Resting Treg (CD25⁺CD45RA⁺FOXP3^{lo}) converted in vitro and in vivo into activated Treg (CD25^{hi}CD45RA[−]FOXP3^{hi}), establishing a developmental link between these two populations (20). Of note, the majority of IL-2-expanded CD4⁺CD25^{lo}CD127^{lo}FOXP3⁺ T cells in HIV patients share phenotypic characteristics with HLA-DR-negative (19) or resting (20) Treg. However, we also observed an increase in fre-

setting, intermittent IL-7 therapy in HIV and non-HIV conditions also increases CD4 T cells and perturbs significantly T cell homeostasis without any apparent effect on Treg populations, which shows promise. Results of large trials with clinical endpoints are eagerly awaited (34).

Materials and Methods

A detailed description of procedures is given in *SI Text*.

Patients. Thirty-one HIV-infected patients who received, while on antiretroviral therapy, at least three cycles of IL-2 in the frame of Agence Nationale de Recherches sur le SIDA (ANRS) trials or expanded open access to IL-2 were included in the study. Blood samples collected on EDTA tubes were processed within 3 h. The clinical characteristics of the patients are depicted in Table 1. Median (ranges) CD4 T cell counts were 445/ μ L (95–919) before IL-2 treatment and 843/ μ L (422–2,348) at the time of sampling. All patients but three had plasma HIV-RNA levels <50 copies/mL. Patients received a median (ranges) of 7 (3–18) IL-2 cycles. The median time between testing and the last IL-2 cycle was 2 months (ranges: 2–59). Written informed consent was obtained from all of the patients, according to human experimentation guidelines from national ethical committees.

Cell Isolation. CD4⁺ T lymphocytes were purified from peripheral blood as previously described (22, 35–37).

Flow Cytometric Analysis. Phenotyping of cell subsets was performed on fresh PBMC by four-color flow cytometry, using the mAbs listed in *SI Materials and Methods*. Analyses were performed using FACScalibur or LSRII cytometer and CellQuest software (Becton Dickinson) on at least 1,000 events. Gating was restricted to the population of lymphocytes according to their light-scattering properties.

Proliferation and Suppression Assays. The different subpopulations (unfractionated CD4⁺ cells, CD4⁺CD25[−] cells, and CD4⁺CD25⁺ cells) were assessed for their proliferative capacities in response to polyclonal stimulation and to recall antigens and p24 protein, as described previously (22).

Microarray Procedures. Gene expression profiling of enriched CD4⁺CD25⁺ and CD4⁺CD25[−] T cells from HIV patients and healthy donors was performed using an Applied Biosystems 1700 microarray platform following protocols provided by the supplier as previously described (38). Microarray data were deposited in the MACE database (<http://mace.ihes.fr/>) with the accession numbers 2560865806 (data from HIV patients) and 2828616462 (healthy donors).

Statistical Analyses. Data are expressed as mean $\pm$ SEM for percentages and median and ranges for absolute values. Statistical comparisons were performed using a two-tailed unpaired nonparametric Mann–Whitney test for comparisons between groups (IL-2 vs. controls) and Wilcoxon's rank test for comparisons between CD4 cell subsets. Significance was considered for $P \leq 0.05$.

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