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Preserved central memory and activated effector memory CD4+ T cell subsets in HIV controllers: an ANRS EP36 study

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ABSTRACT

HIV controllers are rare individuals who spontaneously control HIV-1 replication for 10 years or more in the absence of antiretroviral treatment. HIV controllers (n=11) maintained potent HIV-specific CD4 responses in spite of very low antigenic load. Their central memory (CM) CD4+ T cells were characterized by near normal numbers, preserved IL-2 secretion in response to HIV antigens, and uniformly high expression of the survival receptor IL-7Rα. Controllers expressed CCR7 at higher levels than uninfected controls, suggesting differences in TCM cell homing patterns. Effector memory (EM) CD4+ T cell responses were polyfunctional in HIV controllers, while IL-2 secretion was lost in viremic patients. Cytokine production was 3 times higher in controllers than in HAART-treated patients with undetectable viral load, suggesting an intrinsically more efficient response in the former group. The total CD4+ TEM cell pool underwent immune activation in controllers, as indicated by increased HLA-DR expression, decreased IL-7Rα expression, a bias toward IFN-γ production upon polyclonal stimulation, and increased MIP-1β secretion associated with chronic CCR5 down-regulation. Thus, HIV controllers showed a preserved CD4+ TCM cell compartment and signs of a potent functional activation in the CD4+ TEM cell compartment. While controllers did not show the generalized immune activation pattern associated with disease progression, they had signs of immune activation restricted to the effector compartment. These findings suggest the induction of an efficient, non-detrimental type of immune activation in patients who spontaneously control HIV.
INTRODUCTION

HIV controllers are rare individuals who spontaneously control HIV replication to undetectable levels in the absence of therapy. Controllers account for less than 1% of seropositive individuals (33) and can be distinguished from long-term non progressors (LTNP), who were defined on the basis of persistently elevated CD4+ T cell counts. While LTNP have a significant risk of progressing to disease even after 10 years or more of asymptomatic infection, the HIV controller status is associated with a very low risk of progression (10, 11, 37). Controllers have HIV DNA loads significantly lower than those of LTNP, emphasizing the importance of limited viral dissemination in maintaining a healthy status in the long term (33, 52). Attenuated virus, characterized by major genetic defects such as deletions in the nef gene, have been isolated in a few cases of controlled HIV infection (15, 31). However, evidence for viral attenuation remains uncommon. Virologic analyses of 8 of the HIV controllers included in the present study showed that replication competent HIV could be isolated in each case, and suggested a degree of ongoing viral replication in vivo based on phylogenetic evidence (34). Full-length sequencing of HIV isolates obtained from another cohort of controllers did not reveal inactivating deletions or mutations in the viral genome (7). In addition, viral fitness of HIV clones obtained from these isolates remained in the normal range (7). Based on these studies, HIV controllers appear infected with fully replication competent virus in the vast majority of cases. Thus, host factors, genetic or immunologic in nature, most frequently account for HIV control.

While the role of CD8+ T cells in clearing HIV-infected cells is clearly established (1, 3, 5, 6, 42, 43, 51), several factors also point to the contribution of CD4+ T cells in HIV control. A hallmark of non-progressive HIV infection is the presence of CD4+ T cells that proliferate in response to HIV antigens, while this response is weak or absent in viremic patients (41, 50, 58). Evidence from murine models suggest that CD4+ T cells are needed to sustain an efficient CD8 antiviral responses in the long term (13, 39). In rhesus macaques, attenuated simian
immunodeficiency virus (SIV) infection induces a particularly high frequency of virus-specific CD4+ T cells that have direct effector function (20). Seronegative individuals who have been repeatedly exposed to HIV also show signs of efficient CD4+ T cell proliferative responses to HIV antigens (2). However, the causative link between strong CD4+ T cell responses and HIV control is still under debate (38, 47). Strong CD4+ T cell proliferative responses in treated patients do not always predict a better virological outcome upon structured treatment interruption (28), a possible reason being the rapid and preferential infection of HIV-specific CD4+ T cells in the presence of replicating virus (18). Most patients with progressive HIV disease still maintain significant numbers of cytokine-producing CD4+ T cells when stimulated with HIV antigens (48). The quality rather than the quantity of HIV-specific CD4+ T cells distinguishes slowly progressive infection. It is associated with a particular cytokine-secretion profile, dominated by CD4+ T cells that produce IL-2, either exclusively or in conjunction with IFN−γ. In contrast, high viremia is associated with the loss of IL-2 secretion and a predominant IFN−γ response (8, 14, 19, 24, 25, 56, 60).

A specific subset of memory CD4+ T cells is thought to be responsible for the long term maintenance of immune memory. These cells, termed central memory (CM) CD4+ T cells, are endowed with a self-renewal capacity which is thought to depend on autocrine IL-2 secretion (30, 35). In addition, CD4+ TCM cells express homing molecules such as CCR7 and CD62L that allow their recirculation into secondary lymphoid organs, where recall responses are initiated. Younes et al. (60) have proposed that persistent HIV antigenemia drives the differentiation of CD4+ TCM cells into effector memory (EM) cells, that lose IL-2 secretion and thus self-renewal capacity. Whether the loss of IL-2 secretion is a mere consequence of exposure to high antigenemia or is the underlying cause for uncontrolled viral replication is not yet understood.
In this context, it is important to characterize CD4 responses in HIV controllers, since these responses are presumably optimal. Few studies have focused on CD4 responses in patients with spontaneously undetectable viral load. We found that controllers had intrinsically higher cytokine responses to HIV antigens than treated patients with undetectable viral load. HIV controllers showed a unique CD4+ T cell activation pattern, characterized by the co-existence of a preserved central memory compartment and an activated effector memory compartment. Such activation status may account for both the persistence and potency of anti-HIV CD4 responses in these rare patients.

MATERIALS AND METHODS

Study design

HIV controllers were defined as HIV-infected patients who were seropositive for > 10 years, who had received no antiretroviral treatment, and for whom >90% of the plasma viral load measurements were <400 copies HIV RNA/ml. Controllers were identified among 1300 patients followed at the Centre Hospitalo-Universitaire de Bicêtre (CHU Bicêtre, France) and 1500 patients of the ANRS SEROCO-HEMOCO cohort, with a frequency of 0.6 % in both cohorts. Ten HIV controllers included in the present study have been described elsewhere (33) and two were newly recruited (patients A9 and A10) (Table I). In the course of the study, one patient (A8) showed a viremic episode associated with bronchitis, but subsequently spontaneously controlled his viremia to 100 copies /ml. The CCR5 Δ32 genotype was determined for all controller patients by sequencing (I. Théodorou, INSERM U543).

Control groups included: (1) non-infected healthy blood donors from the Etablissement Français du Sang (Paris, France); (2) viremic patients with a viral load >2,000 copies HIV RNA/ml who had been infected with HIV for at least 6 months and did not receive antiretroviral therapy; (3) HIV-infected patients successfully treated with antiretroviral therapy for more than 3
years and with <50 copies HIV RNA/ml. Patients from the last two groups were followed at the CHU Bicêtre. Summaries of patients characteristics, CD4+ T cell counts and viral load data are reported in Table II. The study was approved by Comité Consultatif de Protection des Personnes dans la Recherche Biomédicale de l'Hôpital Bicêtre. All participants gave written informed consent prior to blood sampling.

**Intracellular cytokine staining (ICS) assays**

Cryopreserved PBMC stored in liquid nitrogen were thawed and left to recover at 37 °C in complete medium (RPMI 1640 supplemented with glutamine 2 mM, antibiotics and 10% human AB serum) for 8-9 hours. HIV-specific stimulation was performed by adding baculovirus-derived recombinant p24 Gag protein (Protein Sciences) at 5 µg/ml to 2 x 10^6 PBMC. Cells were stimulated for 14 hours, with brefeldin A (5 µg/ml; Sigma) added after the first 2 hours of stimulation. Polyclonal stimulation was performed by incubating 0.6 x10^6 PBMC in a 96-well plate pre-coated with anti-CD3 antibody (0.6 µg/ml; clone UCHT1, Immunotech) in the presence of 0.5 µg/ml soluble anti-CD28 antibody (clone CD28.2, Immunotech), with brefeldin added after the first 2 hours. Following stimulation, cells were harvested from wells and stained for surface antigens with the following combination of antibodies (all purchased from BD Biosciences unless otherwise specified): CD3-PerCP, CD45RA-PE-Cy7, CCR7-APC (R&D Systems), and CD4-APC-Cy7. Cells were fixed and permeabilized using an Intraprep permeabilization kit (Beckman Coulter) according to the manufacturer’s instructions. Intracellular cytokine staining was detected with IFN-γ-Carboxyfluorescein (clone 25723.11, R&D Systems) and IL-2-PE (clone MQ1-17H12). Fluorescence was measured on a 6-color flow cytometer (FacsCanto) using the FACSDiva software (BD Biosciences). Data were analyzed with FACSDiva and FlowJo 8.1 (Tree Star).

For p24-stimulated cultures and unstimulated control cultures, a minimum of 750,000 lymphocyte–gated events were acquired. The percentage of cytokine-producing CD4+ T cells...
was determined after subtracting the percentage of cytokine-positive events in unstimulated controls. Responses that were below 0.01% cytokine-producing cells were considered negative. None of the 10 HIV-seronegative donors tested showed a positive cytokine response upon p24 stimulation.

To detect chemokine MIP−1β (CCL4) production, PBMC were stimulated either with p24 Gag (5 µg/ml) or with the superantigen Staphylococcus Enterotoxin B (SEB, 1 µg/ml, Toxin Technology). ICS was performed as described above, except that the IL-2 antibody was replaced with a MIP−1β-PE antibody (clone D21-1351, BD Biosciences).

**Phenotyping**

Phenotyping was carried out on fresh PBMC samples. Blood (20-30ml) was collected in heparinized tubes and PBMC separated by Ficoll-Paque (Pharmacia) density centrifugation. Four-colour antibody staining was performed on $10^6$ PBMC at 4 °C for 30min. CD4+ T cell subsets were defined using antibodies CD45RA-FITC, CD4 PE-Cy7 (BD Biosciences) and CCR7-APC (R&D Systems). A fourth antibody was used to examine the expression of specific activation markers or receptors within each subset: HLA-DR-PE, CD25-PE, CD69-PE (BD Biosciences), or CD127-PE (R&D Systems). A minimum of 250,000 lymphocyte–gated events were acquired on a FACSCalibur flow cytometer and analyzed with the CellQuest software (BD Biosciences). To standardize analyzes of Mean Fluorescence Intensity (MFI), the same settings were used on the same flow cytometer across experiments. Titration of each new lot of antibody was carried out on cryopreserved PBMC samples from the same donor.

Expression of CCR5 was assayed on whole blood samples since this receptor can be down-modulated on PBMC after Ficoll gradient purification (36). Briefly, 200 µl blood were diluted in 4 ml PharmLyse buffer (BD Biosciences) and incubated for 10 min at room temperature. Upon completion of red cell lysis, cells were washed, stained with CD45RA-FITC, CD4-PE-Cy7, CCR7-APC and CCR5-PE, and analyzed by flow cytometry as described above.
**Proliferation assay**

Proliferation was assayed by [3H]-thymidine incorporation as described previously (32). Briefly, PBMC at $10^5$ cells per well were treated in quadruplicate with p24 Gag protein at 5 µg/ml. Positive controls were stimulated with 5 µg/ml phytohemagglutinin and negative controls were incubated with complete medium alone. On day 5, each well was pulsed with 1 µCi [3H]-thymidine (Amersham). Sixteen hours later, cells were harvested onto glass fiber filters, and radioactivity was quantitated with a b-scintillation counter (Wallac, Perkin Elmer). The stimulation index (SI) was computed as median [3H]-thymidine incorporation in stimulated cells/median [3H]-thymidine incorporation in unstimulated cells. A positive response was defined as both an SI >3 and a median count >3000 in the stimulated wells.

**Statistical analysis**

Analyses were performed with GraphPad Prism v4.0 software using non-parametric statistical tests in all cases. Differences in variables between patient groups were analyzed with the Mann-Whitney U test. Horizontal bars on scatter data plots indicate median values. All significant differences between groups (p<0.05) are reported on the scatter data plots.

**RESULTS**

**Potent HIV-specific CD4+ T cell responses in HIV controllers**

CD4+ T cell cytokine responses were analyzed in a group of HIV controllers (HIC, n=11) with spontaneous control of viremia in the absence of therapy. Detailed characteristics of these patients are reported in Table I. Control groups included untreated viremic patients (VIR, n=10), patients responding to antiretroviral therapy (HAART, n=10), and healthy blood donors (NI, not infected, n=10).
CD4+ T cell responses were tested by ICS on cryo-preserved PBMC. In a representative experiment, unstimulated CD3+ CD4+ T cells produced very little but detectable amounts of cytokines, while cells stimulated with p24 HIV Gag protein produced IL-2 and IFN-γ, either alone or in combination (Fig. 1A, left and middle panels). Polyclonal stimulation with anti-CD3/anti-CD28 antibodies induced massive cytokine secretion (Fig. 1A, right panel). The analysis of p24 Gag-induced cytokine production indicated that HIV-specific CD4 responses were generally high in the HIV controllers group, though individually variable. Production of IFN-γ was comparable in CD4+ T cells from controllers and viremic patients, even though in vivo the amount of viral antigen responsible for the induction of these responses was 2 to 3 log higher in the latter group (Fig. 1B, left panel). Interestingly, IFN-γ responses were significantly higher in the controllers than in the HAART group, while both groups were characterized by undetectable viral load. This observation suggested that the efficiency of the IFN-γ response, as measured by the level of IFN-γ secretion reported to the HIV antigenic load, was intrinsically high in HIV controllers.

Differences in p24 Gag-induced IL-2 production were marked between the different patient groups (Fig. 1B right panel). IL-2 responses were abolished in viremic patients, low in HAART-treated patients, but remained as high as the IFN-γ responses in the controller group. Thus, HIV controllers were characterized by the persistence of potent IL-2 and IFN-γ CD4 responses in spite of low antigenic load.

Proliferative responses to the p24 Gag protein were positive for all the patients in the controller group, as measured by a stimulation index (SI) >3 (median SI = 11.1). In contrast, proliferative responses were low or undetectable in the viremic group (median SI = 2.4) and intermediate in the HAART group (median SI = 3.2) (Fig. 2). Thus, CD4+ T cells from controllers
retained the capacity for clonal expansion in response to HIV antigens, a finding compatible with their preserved IL-2 secretion capacity (56, 60).

**Preserved central memory compartment with high CCR7 expression in HIV controllers**

CD4+ T cell subsets were defined according to the expression of the CD45RA and CCR7 markers (Fig. 3A). The percentage of naive CD4+ T cells (CD45RA+ CCR7+) was variable and did not distinguish the different groups studied (Fig. 3B). The percentage of CD4+ central memory (CM) CD4+ T cells (CD45RA- CCR7+) was heterogeneous in the controller group, but did not differ significantly from that of non-infected controls (Fig. 3C). In contrast, the CD4+ TCM cell compartment was decreased in viremic and HAART-treated patients. An opposite trend was observed in the effector memory (EM) CD4+ T cell compartment (CD45RA- CCR7-), since controllers had significantly less CD4+ TEM cells than viremic and HAART-treated patients (Fig. 3D). These data suggested that the differentiation state of peripheral CD4+ T cells varied between the groups, the reduction in central memory cells being associated with progressive HIV infection.

CCR7 expression is a key determinant of the capacity of T cells to migrate to secondary lymphoid organs (16, 44). Interestingly, the expression level of CCR7 per cell, as measured by the mean fluorescence intensity (MFI), was markedly increased in CD4+ T cells of controllers (Fig. 3E). This increase was observed in both naive and CD4+ TCM cell (not shown). Of note, high CCR7 expression was not observed in uninfected donors, HAART-treated patients, or viremic patients, but appeared to be specific to the controller group. High expression of this homing receptor may alter the recirculation pattern of controller CD4+ T cells and increase their chances of recognizing HIV antigens within lymphoid organs.
Polyfunctional effector memory responses and preserved central memory responses in HIV controllers

Cytokine responses were evaluated in the different CD4+ T cell subsets using 6-color flow cytometry. HIV-specific cytokine production by naive CD4+ T cells was below detection threshold (not shown). Cytokine production was 6 to 10 times more abundant in CD4+ TEM than TCM cells (compare Fig. 4A and B). The percentage of TEM cells that produced IFN-γ only was equivalent in the controller and viremic groups, while HIV controllers were characterized by the presence of additional populations of TEM cells that produced IL-2 only, or both IL-2 and IFN-γ. HIV-specific cytokine production was approximately 3 times lower in CD4+ TEM cells of HAART-treated patients compared to controllers (Table III). IL-2 secretion by TEM cells remained detectable in the HAART group, although the proportion of IL-2+ cells, including dual producers, was slightly reduced as compared to the HIC group (Fig. 4B, 37% versus 52%, respectively).

HIV-specific cytokine responses were markedly different in the central memory compartment. CD4+ TCM cells of HIV controllers maintained the capacity to produce cytokines upon p24 Gag stimulation, with a predominant IL-2 secretion pattern (Fig. 4A). In contrast, these responses were minimal in viremic and HAART-treated patients. Notably, the bulk of IL-2 secretion was not carried out by TCM cells but rather by TEM cells, the percentage of IL-2+ cells being 4 times higher in TEM compartment (ratio TEM/TCM= 4.7 in the HIC group and = 4.2 in the HAART group; Table III). IL-2 production by HIV-specific CD4+ TCM cells is not quantitatively the most important, but may nevertheless contribute to HIV control due to the long half-life and self-renewing capacity of TCM cells. Thus, it was important to note that the TCM response appeared qualitatively different in the HIV controller group.

Skewed polyclonal responses in HIV controllers

Polyclonal responses in CD4+ T cell memory subsets were analyzed by measuring cytokine
production upon stimulation with anti-CD3/anti-CD28 antibodies. CD4+ T CM cells responded to polyclonal stimulation by a predominant IL-2 production, while CD4+ TEM cells produced a mixed response, with both IL-2 and IFN−γ production (compare Fig. 4C and 4D). The intensity of polyclonal responses was of the same order of magnitude in TCM and TEM cells, indicating that TCM cells were not inherently limited in their cytokine secretion capacity.

The intensity of total polyclonal responses did not differ markedly between the HIC, HAART and uninfected groups (Table III). In particular, CD4+ T cells from HIV controllers did not appear endowed with an intrinsically high IL-2 secretion capacity that could have accounted for the maintenance of HIV-specific TCM responses. Polyclonal responses were significantly decreased in the viremic group, both in the TEM and the TCM compartment (Table III). These observations underscored a generalized defect of CD4+ T cell-dependent immunity in viremic patients, as previously reported (54). Interestingly, the proportion of TEM cells producing IFN−γ only upon polyclonal stimulation was significantly higher in HIV controllers than in uninfected blood donors (p=0.01; Table III). This finding suggested that global CD4+ T cell responses were somehow altered in HIV controllers, even though these patients appeared healthy and suppressed HIV replication efficiently. A similar phenomenon was observed for viremic and HAART-treated patients, who showed an increased proportion of IFN−γ producers upon anti-CD3/CD28 stimulation as compared to uninfected donors (p<0.001 and p<0.05, respectively). Thus, HIV infection primed the pool of CD4+ TEM cell for IFN−γ production in controllers as well as in other patients groups.

Activation of the effector memory compartment in HIV controllers

The observation of a priming for IFN−γ production suggested that the CD4+ T cell compartment may not be in a resting state in HIV controllers. We therefore assessed the expression of
activation markers at the surface of CD4+ T cells. Phenotyping studies were carried out for a subset of HIV controllers (patients A1 to A8, n=8) and for patients from control groups (n=8 for each group; Table II). As expected, the expression of HLA-DR was increased in viremic patients, both in the CD4+ TCM and TEM cell compartments (Fig. 5A). HIV controllers showed an intermediate pattern, with an increase of HLA-DR expression in CD4+ TEM cells, but no significant changes in CD4+ TCM cells. The increase of HLA-DR expression in TEM cells was heterogeneous, suggesting variable activation levels in the controller group. However, individual variability was also observed in the viremic group, and median HLA-DR expression was comparable between the two groups (12.3% vs 13.2% for HIC and VIR, respectively). The percentage of CD25 expression was not increased in controllers or viremic patients (not shown).

The CD69 molecule is an early activation marker expressed at low levels in peripheral blood, and which is thought to play a role in the retention of recently activated lymphocytes in lymphoid organs (55). The percentage of CD69+ cells did not show significant changes between groups (median % CD69+ cells in CD4+ TEM: 1.9 % in HIC, 4.3 % in VIR, 2.4 % in NI, 1.5 % in HAART). However, it was interesting that the level of CD69 expression per positive CD4+ T cell, as measured by the mean fluorescence intensity (MFI), was significantly higher in controllers than in viremic patients and uninfected controls (Fig. 5B). This suggested a particular activation status of recently primed CD4+ T cells in the controller group.

The alpha chain of the IL-7 receptor (CD127) is lost upon antigen exposure, and re-expressed in CD4+ memory T cells that survive in the long term (17). CD127 expression was uniformly high in CD4+ TCM cells of controllers, while it was decreased in those of viremic and HAART-treated patients (Fig. 5C). Interestingly, CD127 showed a trend to decrease in CD4+ TEM cells of controllers as well as other HIV+ patients, suggesting a degree of ongoing immune
activation in this compartment. Thus, the phenotyping analysis showed signs of a preferential activation of the TEM compartment in HIV controllers. Given that the level of T cell activation has been described as a surrogate marker for HIV disease progression (21), it was striking to find that, within the TEM compartment, levels of HLA-DR expression were as high in HIV controllers as in progressors.

**HIV controller CD4+ T cells are primed for MIP−1β production**

The chemokine receptor CCR5 is considered to be an activation marker, since it is expressed at minimal levels in naive CD4+ T cells, at intermediate levels in CD4+ TCM cells, and at high levels in CD4+ TEM cells (mean of 1.1%, 9.5% and 48.8% CCR5+ cells, respectively, for uninfected donors). Thus, it was unexpected that CCR5 expression appeared to be decreased, rather than increased, in CD4+ TEM cells of HIV controllers and other HIV-infected patients (Fig. 6A). A similar decrease was observed in CD4+ TCM cells. The expression of CCR5 per cell was also decreased, as indicated by a lower CCR5 MFI in CD4+ TCM cells of the controller group compared to uninfected donors (p<0.05; not shown). A possibility was that chronic secretion of CCR5 ligands led to downregulation of the CCR5 receptor, a phenomenon classically associated with chemokine receptor desensitization (4).

Further analyses were performed to evaluate the production of the chemokine MIP−1β, a high affinity ligand for CCR5, in response to HIV-specific and polyclonal stimulation. Stimulation with p24 Gag induced MIP−1β production to comparable levels in CD4+ TEM cells of controllers and viremic patients, and to lower levels in the HAART group, again emphasizing the strength of HIV-specific responses in the controller group (Fig. 6B, right panel). MIP−1β was predominantly produced by CD4+ TEM cells, the frequency of MIP−1β+ cells being about ten times higher in the TEM than in the TCM subset. Thus, MIP−1β secretion represented an effector response, a
notion supported by the fact that a significant fraction of CD4+ TEM cells that produced MIP−1β also produced IFN−γ.

Polyclonal stimulation with the superantigen SEB induced MIP−1β responses that were twenty times higher in the CD4+ TEM than in the TCM cell compartment (not shown). Since the CD4+ TCM cell contribution was minimal, total CD4+ T cell responses are reported (Fig. 6C, left panel). Of note, HIV controller showed a twofold increase in polyclonal MIP−1β responses as compared to uninfected donors. A threefold increase was observed for viremic patients. A large fraction of MIP−1β+ CD4+ T cells also produced IFN−γ, emphasizing the effector phenotype of the cells involved in the polyclonal response. We also evaluated MIP−1β responses in the CD8+ T cell compartment, since CD8+ T cells are known to produce CCR5 ligands at high levels (57).

Upon polyclonal stimulation, CD8+ T cells from all patients groups produced MIP−1β at higher levels than CD8+ T cells from uninfected donors (Fig. 6C, right panel). Thus, HIV infection primed the total T cell pool for increased MIP−1β production, a finding that may account for the downregulation of CCR5.

It was striking that the intensity of MIP−1β responses, both HIV-specific and polyclonal, were comparable between HIV controllers and viremic patients, while these two groups differed markedly in terms of viral load. These findings suggested that, for certain parameters, the level of functional T cell activation was comparable in both groups. Taken together, the intensity of HIV-specific cytokine responses, the increased expression of HLA-DR, and the priming of the CD4+ T cell pool for IFN−γ and MIP−1β production pointed to a potent activation of the CD4+ TEM cell compartment in HIV controllers.
DISCUSSION

HIV controllers were characterized by efficient antiviral responses in both the central and the effector memory CD4+ T cell compartments. CD4+ TCM cells were present at near normal numbers, maintained the capacity to produce IL-2 in responses to HIV antigens, and expressed high levels of the survival and homing receptors IL-7R and CCR7, respectively. CD4+ TEM cells were responsible for potent, polyfunctional HIV-specific responses, with half of the responding cells secreting IL-2. In addition, HIV controllers showed signs of ongoing activation within the whole CD4+ TEM cell compartment, as indicated by increased expression of HLA-DR, decreased expression of CD127, and a bias in cytokine and chemokine responses to polyclonal stimulation. These findings pointed to an involvement of the whole immune system in controlling HIV infection. Importantly, this set of characteristics was not shared by other patients groups. Viremic patients showed clearly defective CD4+ TCM cell responses, but had an activated CD4+ TEM compartment, and showed HIV-specific cytokine responses of high level but of limited functionality. CD4+ T cell responses in controllers also differed from those of patients successfully treated with antiretroviral therapy, especially in terms of strength of HIV-specific responses. CD4+ TCM cells of HAART-treated patients showed signs of dysfunction, such as a decreased expression of the survival receptor CD127. CD4+ TEM cells from the same patients showed some but not all signs of immune activation, and produced low amounts of cytokines in response to HIV antigens. Thus, HIV controllers were unique in showing both a preserved CD4+ TCM cell compartment and an activated CD4+ TEM cell compartment. We propose that the combination of these two characteristics underlies the remarkable capacity of HIV controllers to suppress HIV replication in the long term.

It was noteworthy that levels of HIV-specific CD4+ T cell responses were higher in HIV controllers than in HAART-treated patients. The strength of CD4 responses has been proposed...
to follow a bell-shaped curve, with a positive association between CD4 responses and viral load in the low viral load range (29, 59). HIV controllers were exposed to very low levels of HIV antigens, given their particularly low level of viral DNA (median = 43 copies/10^6 PBMC; Table I) and the large amount of CD4+ T cells needed to isolate replication competent virus from their PBMC (34). However, cytokine responses were 3 times higher in the controller than in the HAART group, pointing to a disparity between the strength of CD4 responses and viral load in the controller group. Specifically, it was the ratio of cytokine producing cells to the amount of stimulating antigen that was high in this particular group of patients. It is possible that CD4+ T cells responses appear comparatively high in controllers because they are actually decreased in HAART treated patients. Studies of immune reconstitution indicate that patients who reached a low CD4+ T cell nadir prior to treatment show a poor recovery of HIV-specific CD4+ T cell responses (59). The median CD4+ T cell nadir in the HAART group was 200 cells/mm^3 (Table II), which is not unusually low, but is still associated with a degree of long-term immune damage.

On the other hand, HIV controllers may share genetic traits that promote the development of efficient CD4 specific responses, or that help contain viral replication during acute infection so that CD4 responses develop optimally. Whether the potent CD4 responses observed in controllers directly contribute to viral control through cytotoxic mechanisms, as suggested for CMV and attenuated SIV infections, warrants further investigation (12, 20, 61).

Central Memory T cells are thought to ensure the long-term maintenance of antiviral responses due to their long half-life and self-renewal capacity (35, 39). CD4+ TCM cells of controllers showed characteristics associated with cell survival, particularly evident in their high expression of the alpha-chain of the IL-7 receptor, CD127. Studies of the basis for immunological memory in murine models indicate that T cells that have encountered their cognate antigen and re-express CD127 will preferentially survive the encounter, while CD127-negative cells will preferentially die by activation-induced apoptosis (39). According to a recent
study, expression of CD127 on antigen-exposed CD4+ T cells is dependent on the presence of IL-2 (17). Thus, the IL-2 secretion capacity of HIV-specific T cell in controllers may contribute to the high CD127 expression and long-term antiviral memory in these patients. The reduced expression of CD127 in viremic and HAART-treated patients suggests a shorter half-life of CD4+ TCM cells in these groups. Further studies aimed at directly measuring TCM turnover will be needed to confirm this point. Of note, the differences in CD127 expression did not extend to the CD4+ TEM cell population. The three groups of patients showed a trend towards low CD127 expression in CD4+ TEM cells, consistent with an activation of the effector memory compartment in all cases of HIV infection.

Another finding specific to the central memory compartment of HIV controllers was the increased expression of the homing receptor CCR7. This chemokine receptor binds ligands expressed at the surface of high endothelial venules (CCL19) and within lymphoid tissues (CCL19 and CCL21) and thus plays a central role in the recirculation of naive and TCM cells within lymphoid organs (44). CCR7 also drives the migration of TCM cells from peripheral tissues to draining lymph nodes via the lymphatics (16). A high expression of CCR7 may facilitate the entry and increase the residence time of CD4+ TCM cells within lymphoid organs. An increase in CD69 expression may also prolong their retention within lymphoid organs (55). Such a mechanism may favor the encounter with rare antigen presenting cells loaded with HIV antigens and contribute to the efficiency of antiviral responses in HIV controllers. It was interesting that CCR7 expression levels in controller CD4+ T cells exceeded those of CD4+ T cells obtained from uninfected blood donors. As CCR7 expression is known to be regulated by the cytokine milieu (49, 53), it is possible that the cytokine expression profile particular to HIV controllers influences CCR7 expression, and hence the recirculation pattern of CD4+ T cells. Taken together, CD4+ TCM cells of HIV controllers exhibited a set of characteristics that were
compatible with a fully functional and long-lived central memory response.

The presence of signs of immune activation in the whole CD4+ TEM cell population of HIV controllers was an unexpected finding. In the current model of HIV pathogenesis, abnormal immune activation is viewed as the major force driving progression to disease, by constantly replenishing the pool of activated CD4+ T cells that can be targeted by HIV and by progressively exhausting the renewal capacity of the immune system (23, 27). The degree of CD8+ T cell activation correlates positively with the risk of progression to AIDS (21). It was intriguing that HIV controllers, who do not progress to disease, expressed similar levels of the HLA-DR activation marker as viremic patients within the CD4+ TEM cell compartment. The increase in HLA-DR expression was minimal within the CD4+ TCM cell compartment for controllers, but significant for viremic patients, suggesting that immune activation was confined to TEM cells in controllers and was generalized in viremic patients. Another element pointing to the activation of the TEM compartment in controllers was the increased production of the chemokine MIP-1β upon polyclonal stimulation, as compared to responses from uninfected blood donors. The propensity of CD4+ TEM cells of controllers to produce IFN-γ rather than IL-2 after CD3/CD28 stimulation supports the notion of a bias towards effector responses. Thus, the CD4+ TEM cell compartment in controllers was not resting, and produced chemokines and cytokines to levels similar to those seen in viremic patients. These signs of a potent immune activation may either reflect the bystander effects of the ongoing HIV-specific immune response or may be intrinsic to HIV controllers. Determining the capacity of HIV controllers to cope with other chronic viral infections may help distinguish between these possibilities.

We cannot rule out that efficient activation of CD4 responses is a consequence, rather than an initial cause, of HIV control, and that viral replication was contained early on due to other
intrinsic or immune factors. However, once established, the effector CD4 response is likely to reinforce HIV control at several levels, through secretion of chemokines that inhibit HIV replication, helper function, and possibly direct cytotoxic function. The finding of a reduced CCR5 expression at the surface of CD4+ T cells suggests that HIV controllers are indeed exposed chronically to high levels of beta-chemokines such as MIP–1β. Alternate explanations such as genetic polymorphisms that would reduce basal CCR5 expression levels are also possible but less likely, since the CCR5 coding sequence was wild-type in all the patients studied. Further analyses focused on CCR5 promoter polymorphisms and CCR5 ligand gene copy numbers are needed to evaluate the genetic component of CCR5 expression level in controllers (22, 26). Preferential infection and deletion of CCR5+ cells may also contribute to decrease CCR5 expression (40), but is unlikely in the case of controller patients who by definition show very limited HIV replication. High levels of beta-chemokines can lead to chronic down-regulation of CCR5 through activation-induced receptor internalization (4, 45). Since CCR5 expression levels condition the infectability of HIV target cells, CCR5 downregulation may contribute to the low-levels of infection in controllers. In particular, this mechanism may play a role in protecting cells that express already limiting amounts of CCR5, such as CD4+ TCM cells. In controllers, the combination of decreased CCR5 expression and low cellular activation would make CD4+ TCM cells suboptimal targets for HIV replication.

Of note, high MIP–1β production was also detected in the CD8+ T cell population (Fig. 6C). Analysis of CD8+ T cell responses in the same cohort of controllers confirmed that activation of the effector compartment, as measured by HLA-DR expression, extends to CD8+ T cells (51). Dosage of soluble LPS in plasma samples from this cohort showed raised levels in HIV controllers, another indication of chronic immune activation (9). These observations suggest that activation of the effector compartment involves CD8+ T cells and possibly to other immune
cells. Importantly, the immune activation evident in HIV controllers was not equivalent in every aspect to that seen in viremic patients. T cells from controllers showed a lower susceptibility to apoptosis in vitro than those of viremic patients (S.P.P., unpublished observation). Plasma levels of soluble CD14, another indicator of inflammation, were intermediate in controllers and significantly higher in progressors (9). Conversely, CD69 was expressed to higher levels in recently activated CD4+ T cells of controllers than of viremic patients. A marked difference was noted in the activation status of HIV-specific CD8+ T cells, which expressed HLA-DR but not CD38 in controllers, while both activation markers were expressed in viremic patients (51). These findings point to a differential pattern of immune activation in HIV controllers and viremic patients. Chronic immune activation may not be detrimental in controllers because it spares the central memory compartment, and thus preserves the regenerative capacity of the immune system. Activation would then benefit controller patients, by ensuring the efficacy of effector responses. In contrast, as shown in a recent study of SIV infection (46), chronic activation in the presence of viremia leads to a progressive loss of CD4+ TCM cells, with ensuing immune exhaustion.

In conclusion, HIV controllers were characterized by the maintenance of a functional and long-lived pool of CD4+ TCM cells, in parallel with a chronically activated pool of CD4+ TEM cells. The conjunction of these two types of immune memory may generate optimal responses against chronic HIV infection, and represent a goal to be achieved by candidate HIV vaccines.
ACKNOWLEDGMENTS

We thank Marie-Thérèse Rannou and the participating nurses from Bicêtre Hospital for their cooperation, Laurence Meyer for advice on the SEROCO/HEMOCO cohort, Ioannis Theodorou for CCR5 genotyping, Christine Rouzioux for contributing viral DNA data, and Florence Bugault for help with flow cytometry analysis. We are especially grateful to the patients who participated to the study.

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The authors declare no competing financial interests.
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producing HIV-specific memory CD4+ T cells endowed with proliferative capacity. J Exp 

Identification of circulating antigen-specific CD4+ T lymphocytes with a CCR5+, cytotoxic 
phenotype in an HIV-1 long-term nonprogressor and in CMV infection. Blood 103:2238-47.
**LEGEND TO THE FIGURES**

**Figure 1: Cytokine responses in CD4+ T cells**

(A) Representative Intracellular Cytokine Staining (ICS) analysis: PBMC of controller patient A4 were left unstimulated (left panel), stimulated with the HIV p24 Gag core protein (middle panel), or with anti-CD3/CD28 antibodies (right panel) for 14h. For flow cytometry analysis, $10^6$ events were acquired in the lymphocyte gate. In this example, IFN-$\gamma$ and IL-2 production are analyzed within the CD3+ CD4+ population. Flow cytometric data are represented by pseudo-color density plots. Percentages of cytokine+ cells are reported in each quadrant.

(B) Interferon-$\gamma$ responses (left panel) and interleukin-2 responses (right panel) to HIV-1 Gag p24 protein in CD4+ T cells: percentages of cytokine+ cells are reported for the different groups: HIC: HIV controllers; VIR: viremic patients; NI: non-infected; HAART: HAART-treated patients. Responses that were below 0.01% were considered negative. P values were determined by the non-parametric Mann-Whitney U test. Horizontal bars indicate median values.

**Figure 2: Proliferative responses**

PBMC were stimulated with the HIV p24 Gag protein for 5 days. Stimulation indexes obtained in the $[^3]$H-thymidine incorporation assay are reported. P values were determined by the non-parametric Mann-Whitney U test.

**Figure 3: Phenotyping of naive and memory CD4+ T cell subsets**

(A) representative gating strategy used to define CD4+ T cell subsets: CD3+ CD4+ T cells were classified according to CD45RA and CCR7 expression. Naive cells were defined as CCR7+ CD45RA+; Central Memory cells were defined as CD45RA- CCR7+; Effector memory cells were defined as CD45RA- CCR7-; The CD45RA+ CCR7- subset remained below 2.6 % within the CD3+ CD4+ population and was not analyzed further. (B) Frequency of naive CD4+ T cells. (C)
Frequency of central memory CD4+ T cells. (D) Frequency of effector memory CD4+ T cells. (E) Expression of the chemokine receptor CCR7 at the surface of CD4+ T cells: the mean fluorescence intensity (MFI) per cell is reported. P values were determined by the non-parametric Mann-Whitney U test.

Figure 4: Cytokine responses in CD4+ T cell memory subsets

The mean percentage of cells secreting IFN-γ only (black bars), IL-2 only (white bars) or both cytokines (grey bars) is reported. (A) HIV-specific response to p24 Gag stimulation in the CD4+ TCM cell subset. (B) HIV-specific response to p24 Gag in the CD4+ TEM cell subset. (C) Polyclonal responses to anti-CD3/CD28 stimulation in the CD4+ TCM cell subset. (D) polyclonal responses in the CD4+ TEM cell subset. Statistics used to compared cytokine production between the different patient groups are reported in Table III.

Figure 5: Expression of activation markers in CD4+ T cell memory subsets

(A) Proportion of cells expressing the HLA-DR marker in CD4+ TCM cells (left panel) and CD4+ TEM cells (right panel). (B) Mean fluorescence intensity (MFI) of the early activation marker CD69 in CD69+ CD4+ TCM cells (left panel) and CD69+ CD4+ TEM cells (right panel). (C) Proportion of cells expressing the alpha chain of the IL-7 receptor, CD127, in CD4+ TCM cells (left panel) and CD4+ TEM cells (right panel). P values were determined by the non-parametric Mann-Whitney U test.

Figure 6: Expression of the chemokine receptor CCR5 and production of the CCR5 ligand MIP−1β

(A) Proportion of cells expressing the CCR5 chemokine receptor in CD4+ TCM cells (left panel) and CD4+ TEM cells (right panel). (B) HIV-specific MIP−1β response in the CD4+ TCM and TEM
1 cell subsets (left and right panels, respectively). The mean percentage of cells secreting MIP−1β
2 only (white bars) or both MIP−1β and IFN−γ (grey bars) is reported. Note the scale difference
3 between the two graphs (C) Polyclonal MIP−1β responses to SEB stimulation in the total CD4+
4 T cell pool (left panel) and in the total CD8+ T cell pool (right panel). P values were determined
5 by the non-parametric Mann-Whitney U test.
Table I: Characteristics of patients in the HIV controller group

<table>
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<th>Patient</th>
<th>Sex</th>
<th>Age</th>
<th>HIV diagnosis</th>
<th>Median CD4 count</th>
<th>last CD4 count</th>
<th>median HIV RNA</th>
<th>last HIV RNA</th>
<th>HIV DNA</th>
<th>CCR5 genotype</th>
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</table>

Note: (1) CD4 T cells/mm3 blood; (2) HIV RNA copies/ml plasma; (3) HIV DNA copies/10^6 PBMC; (4) Intracellular Cytokine Staining assay: (+) test positive (ND) not done.
**Table II: Summary of patients' characteristics**

<table>
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<th>Patient Group</th>
<th>Age median (range)</th>
<th>CD4 count(^1) median (range)</th>
<th>Viral Load(^2) median (range)</th>
<th>CD4 nadir(^1) median (range)</th>
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<td>800 (522-1159)</td>
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<td>349 (35-789)</td>
<td>25,150 (2,930-920,674)</td>
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<td>HAART (n=10)</td>
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<td>578 (147-1184)</td>
<td>&lt;50 (&lt;50-&lt;50)</td>
<td>204 (29-282)</td>
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<tr>
<td>Phenotyping</td>
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<td>HIC (n=8)</td>
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<td>768 (524-1,159)</td>
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<td>462 (272-570)</td>
<td>18,300 (10,800-180,000)</td>
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<td>587 (441-862)</td>
<td>&lt;50 (&lt;50-&lt;50)</td>
<td>190 (18-376)</td>
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Note: \(^1\)(CD4 T cells/mm\(^3\) blood); \(^2\)(HIV RNA copies/ml plasma); \(^3\)(Intracellular Cytokine Staining Assay); \(^4\)(Not Applicable)
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<tr>
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<tr>
<td>% IL-2+</td>
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**Table III: Comparison of the percentage of cytokine secreting CD4+ T cells following HIV-specific or polyclonal stimulation**

HIC: HIV controllers; VIR: viremic patients; NI: non infected; HAART:treated patients
CM: central memory; EM: effector memory; -: not significant
Cytokine+: sum of IFN-γ+, IL-2+, and IFN-γ+IL-2+ cells

Potter et al.
Figure 1: Potter et al.
Fig. 2 Potter et al.

HIC VIR HAART

Stimulation index

$\text{p} < 0.001$

$\text{p} = 0.01$

Hal-Pasteur author manuscript    pasteur-00193768, version 1
Fig. 3 Potter et al.
Fig. 4 Potter et al.
Fig. 5 Potter et al.
A  CCR5 expression

B  p24 Gag-specific responses

C  Polyclonal responses

Figure 6 Potter et al.