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immunodeficiency virus controllers: an ANRS EP36
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3 ***Preserved central memory and activated effector memory CD4+ T cell***
4 ***subsets in HIV controllers: an ANRS EP36 study***

5

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1 **ABSTRACT**

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

20

1 **INTRODUCTION**

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

20
21 While the role of CD8+ T cells in clearing HIV-infected cells is clearly established (1, 3, 5,
22 6, 42, 43, 51), several factors also point to the contribution of CD4+ T cells in HIV control. A
23 hallmark of non-progressive HIV infection is the presence of CD4+ T cells that proliferate in
24 response to HIV antigens, while this response is weak or absent in viremic patients (41, 50, 58).
25 Evidence from murine models suggest that CD4+ T cells are needed to sustain an efficient CD8
26 antiviral responses in the long term (13, 39). In rhesus macaques, attenuated simian

1 immunodeficiency virus (SIV) infection induces a particularly high frequency of virus-specific
2 CD4⁺ T cells that have direct effector function (20). Seronegative individuals who have been
3 repeatedly exposed to HIV also show signs of efficient CD4⁺ T cell proliferative responses to
4 HIV antigens (2). However, the causative link between strong CD4⁺ T cell responses and HIV
5 control is still under debate (38, 47). Strong CD4⁺ T cell proliferative responses in treated
6 patients do not always predict a better virological outcome upon structured treatment interruption
7 (28), a possible reason being the rapid and preferential infection of HIV-specific CD4⁺ T cells in
8 the presence of replicating virus (18). Most patients with progressive HIV disease still maintain
9 significant numbers of cytokine-producing CD4⁺ T cells when stimulated with HIV antigens (48).
10 The quality rather than the quantity of HIV-specific CD4⁺ T cells distinguishes slowly progressive
11 infection. It is associated with a particular cytokine-secretion profile, dominated by CD4⁺ T cells
12 that produce IL-2, either exclusively or in conjunction with IFN- γ . In contrast, high viremia is
13 associated with the loss of IL-2 secretion and a predominant IFN- γ response (8, 14, 19, 24, 25,
14 56, 60).

15
16 A specific subset of memory CD4⁺ T cells is thought to be responsible for the long term
17 maintenance of immune memory. These cells, termed central memory (CM) CD4⁺ T cells, are
18 endowed with a self-renewal capacity which is thought to depend on autocrine IL-2 secretion
19 (30, 35). In addition, CD4⁺ TCM cells express homing molecules such as CCR7 and CD62L that
20 allow their recirculation into secondary lymphoid organs, where recall responses are initiated.
21 Younes et al. (60) have proposed that persistent HIV antigenemia drives the differentiation of
22 CD4⁺ TCM cells into effector memory (EM) cells, that lose IL-2 secretion and thus self-renewal
23 capacity. Whether the loss of IL-2 secretion is a mere consequence of exposure to high
24 antigenemia or is the underlying cause for uncontrolled viral replication is not yet understood.

25

1 In this context, it is important to characterize CD4 responses in HIV controllers, since
2 these responses are presumably optimal. Few studies have focused on CD4 responses in
3 patients with spontaneously undetectable viral load. We found that controllers had intrinsically
4 higher cytokine responses to HIV antigens than treated patients with undetectable viral load. HIV
5 controllers showed a unique CD4+ T cell activation pattern, characterized by the co-existence of
6 a preserved central memory compartment and an activated effector memory compartment. Such
7 activation status may account for both the persistence and potency of anti-HIV CD4 responses
8 in these rare patients.

9 10 **MATERIALS AND METHODS**

11 ***Study design***

12 HIV controllers were defined as HIV-infected patients who were seropositive for > 10 years, who
13 had received no antiretroviral treatment, and for whom >90% of the plasma viral load
14 measurements were <400 copies HIV RNA/ml. Controllers were identified among 1300 patients
15 followed at the Centre Hospitalo-Universitaire de Bicêtre (CHU Bicêtre, France) and 1500
16 patients of the ANRS SEROCO-HEMOCO cohort, with a frequency of 0.6 % in both cohorts. Ten
17 HIV controllers included in the present study have been described elsewhere (33) and two were
18 newly recruited (patients A9 and A10) (Table I). In the course of the study, one patient (A8)
19 showed a viremic episode associated with bronchitis, but subsequently spontaneously controlled
20 his viremia to 100 copies /ml. The CCR5 $\Delta 32$ genotype was determined for all controller patients
21 by sequencing (I. Théodorou, INSERM U543).

22
23 Control groups included: (1) non-infected healthy blood donors from the Etablissement
24 Français du Sang (Paris, France); (2) viremic patients with a viral load >2,000 copies HIV
25 RNA/ml who had been infected with HIV for at least 6 months and did not receive antiretroviral
26 therapy; (3) HIV-infected patients successfully treated with antiretroviral therapy for more than 3

1 years and with <50 copies HIV RNA/ml. Patients from the last two groups were followed at the
2 CHU Bicêtre. Summaries of patients characteristics, CD4+ T cell counts and viral load data are
3 reported in Table II. The study was approved by Comité Consultatif de Protection des Personnes
4 dans la Recherche Biomédicale de l'Hôpital Bicêtre. All participants gave written informed
5 consent prior to blood sampling.

7 ***Intracellular cytokine staining (ICS) assays***

8 Cryopreserved PBMC stored in liquid nitrogen were thawed and left to recover at 37 °C in
9 complete medium (RPMI 1640 supplemented with glutamine 2 mM, antibiotics and 10% human
10 AB serum) for 8-9 hours. HIV-specific stimulation was performed by adding baculovirus-derived
11 recombinant p24 Gag protein (Protein Sciences) at 5 µg/ml to 2 x 10⁶ PBMC. Cells were
12 stimulated for 14 hours, with brefeldin A (5 µg/ml; Sigma) added after the first 2 hours of
13 stimulation. Polyclonal stimulation was performed by incubating 0.6 x10⁶ PBMC in a 96-well
14 plate pre-coated with anti-CD3 antibody (0.6 µg/ml; clone UCHT1, Immunotech) in the presence
15 of 0.5 µg/ml soluble anti-CD28 antibody (clone CD28.2, Immunotech), with brefeldin added after
16 the first 2 hours. Following stimulation, cells were harvested from wells and stained for surface
17 antigens with the following combination of antibodies (all purchased from BD Biosciences unless
18 otherwise specified): CD3-PerCP, CD45RA-PE-Cy7, CCR7-APC (R&D Systems), and CD4-
19 APC-Cy7. Cells were fixed and permeabilized using an Intraprep permeabilization kit (Beckman
20 Coulter) according to the manufacturer's instructions. Intracellular cytokine staining was detected
21 with IFN-γ-Carboxyfluorescein (clone 25723.11, R&D Systems) and IL-2-PE (clone MQ1-
22 17H12). Fluorescence was measured on a 6-color flow cytometer (FacsCanto) using the
23 FACSDiva software (BD Biosciences). Data were analyzed with FACSDiva and FlowJo 8.1
24 (Tree Star).

25 For p24-stimulated cultures and unstimulated control cultures, a minimum of 750,000
26 lymphocyte-gated events were acquired. The percentage of cytokine-producing CD4+ T cells

1 was determined after subtracting the percentage of cytokine-positive events in unstimulated
2 controls. Responses that were below 0.01% cytokine-producing cells were considered negative.
3 None of the 10 HIV-seronegative donors tested showed a positive cytokine response upon p24
4 stimulation.

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

9

10 ***Phenotyping***

11 Phenotyping was carried out on fresh PBMC samples. Blood (20-30ml) was collected in
12 heparinized tubes and PBMC separated by Ficoll-Paque (Pharmacia) density centrifugation.
13 Four-colour antibody staining was performed on 10⁶ PBMC at 4 °C for 30min. CD4+ T cell
14 subsets were defined using antibodies CD45RA-FITC, CD4 PE-Cy7 (BD Biosciences) and
15 CCR7-APC (R&D Systems). A fourth antibody was used to examine the expression of specific
16 activation markers or receptors within each subset: HLA-DR-PE, CD25-PE, CD69-PE (BD
17 Biosciences), or CD127-PE (R&D Systems). A minimum of 250,000 lymphocyte-gated events
18 were acquired on a FACSCalibur flow cytometer and analyzed with the CellQuest software (BD
19 Biosciences). To standardize analyzes of Mean Fluorescence Intensity (MFI), the same settings
20 were used on the same flow cytometer across experiments. Titration of each new lot of antibody
21 was carried out on cryopreserved PBMC samples from the same donor.

22 Expression of CCR5 was assayed on whole blood samples since this receptor can be
23 down-modulated on PBMC after Ficoll gradient purification (36). Briefly, 200 μ l blood were
24 diluted in 4 ml PharmLyse buffer (BD Biosciences) and incubated for 10 min at room
25 temperature. Upon completion of red cell lysis, cells were washed, stained with CD45RA-FITC,
26 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 $\mu\text{g/ml}$. Positive controls were stimulated with 5 $\mu\text{g/ml}$ 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, $n=10$).

1 CD4+ T cell responses were tested by ICS on cryo-preserved PBMC. In a representative
2 experiment, unstimulated CD3+ CD4+ T cells produced very little but detectable amounts of
3 cytokines, while cells stimulated with p24 HIV Gag protein produced IL-2 and IFN- γ , either alone
4 or in combination (Fig. 1A, left and middle panels). Polyclonal stimulation with anti-CD3/anti-
5 CD28 antibodies induced massive cytokine secretion (Fig. 1A, right panel). The analysis of p24
6 Gag-induced cytokine production indicated that HIV-specific CD4 responses were generally high
7 in the HIV controllers group, though individually variable. Production of IFN- γ was comparable in
8 CD4+ T cells from controllers and viremic patients, even though *in vivo* the amount of viral
9 antigen responsible for the induction of these responses was 2 to 3 log higher in the latter group
10 (Fig. 1B, left panel). Interestingly, IFN- γ responses were significantly higher in the controllers
11 than in the HAART group, while both groups were characterized by undetectable viral load. This
12 observation suggested that the efficiency of the IFN- γ response, as measured by the level of
13 IFN- γ secretion reported to the HIV antigenic load, was intrinsically high in HIV controllers.

14
15 Differences in p24 Gag-induced IL-2 production were marked between the different
16 patient groups (Fig. 1B right panel). IL-2 responses were abolished in viremic patients, low in
17 HAART-treated patients, but remained as high as the IFN- γ responses in the controller group.
18 Thus, HIV controllers were characterized by the persistence of potent IL-2 and IFN- γ CD4
19 responses in spite of low antigenic load.

20
21 Proliferative responses to the p24 Gag protein were positive for all the patients in the
22 controller group, as measured by a stimulation index (SI) >3 (median SI = 11.1). In contrast,
23 proliferative responses were low or undetectable in the viremic group (median SI = 2.4) and
24 intermediate in the HAART group (median SI = 3.2) (Fig. 2). Thus, CD4+ T cells from controllers

1 retained the capacity for clonal expansion in response to HIV antigens, a finding compatible with
2 their preserved IL-2 secretion capacity (56, 60).

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

5 CD4⁺ T cell subsets were defined according to the expression of the CD45RA and CCR7
6 markers (Fig. 3A). The percentage of naive CD4⁺ T cells (CD45RA⁺ CCR7⁺) was variable and
7 did not distinguish the different groups studied (Fig. 3B). The percentage of CD4⁺ central
8 memory (CM) CD4⁺ T cells (CD45RA⁻ CCR7⁺) was heterogenous in the controller group, but
9 did not differ significantly from that of non-infected controls (Fig. 3C). In contrast, the CD4⁺ TCM
10 cell compartment was decreased in viremic and HAART-treated patients. An opposite trend was
11 observed in the effector memory (EM) CD4⁺ T cell compartment (CD45RA⁻ CCR7⁻), since
12 controllers had significantly less CD4⁺ TEM cells than viremic and HAART-treated patients (Fig.
13 3D). These data suggested that the differentiation state of peripheral CD4⁺ T cells varied
14 between the groups, the reduction in central memory cells being associated with progressive
15 HIV infection.

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

1 ***Polyfunctional effector memory responses and preserved central memory responses in***
2 ***HIV controllers***

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

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

23
24 ***Skewed polyclonal responses in HIV controllers***

25 Polyclonal responses in CD4⁺ T cell memory subsets were analyzed by measuring cytokine

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

6
7 The intensity of total polyclonal responses did not differ markedly between the HIC,
8 HAART and uninfected groups (Table III). In particular, CD4⁺ T cells from HIV controllers did not
9 appear endowed with an intrinsically high IL-2 secretion capacity that could have accounted for
10 the maintenance of HIV-specific TCM responses. Polyclonal responses were significantly
11 decreased in the viremic group, both in the TEM and the TCM compartment (Table III). These
12 observations underscored a generalized defect of CD4⁺ T cell-dependent immunity in viremic
13 patients, as previously reported (54). Interestingly, the proportion of TEM cells producing IFN- γ
14 only upon polyclonal stimulation was significantly higher in HIV controllers than in uninfected
15 blood donors ($p=0.01$; Table III). This finding suggested that global CD4⁺ T cell responses were
16 somehow altered in HIV controllers, even though these patients appeared healthy and
17 suppressed HIV replication efficiently. A similar phenomenon was observed for viremic and
18 HAART-treated patients, who showed an increased proportion of IFN- γ producers upon anti-
19 CD3/CD28 stimulation as compared to uninfected donors ($p<0.001$ and $p<0.05$, respectively).
20 Thus, HIV infection primed the pool of CD4⁺ TEM cell for IFN- γ production in controllers as well
21 as in other patients groups.

22

23 ***Activation of the effector memory compartment in HIV controllers***

24 The observation of a priming for IFN- γ production suggested that the CD4⁺ T cell compartment
25 may not be in a resting state in HIV controllers. We therefore assessed the expression of

1 activation markers at the surface of CD4⁺ T cells. Phenotyping studies were carried out for a
2 subset of HIV controllers (patients A1 to A8, n=8) and for patients from control groups (n=8 for
3 each group; Table II). As expected, the expression of HLA-DR was increased in viremic patients,
4 both in the CD4⁺ TCM and TEM cell compartments (Fig. 5A). HIV controllers showed an
5 intermediate pattern, with an increase of HLA-DR expression in CD4⁺ TEM cells, but no
6 significant changes in CD4⁺ TCM cells. The increase of HLA-DR expression in TEM cells was
7 heterogeneous, suggesting variable activation levels in the controller group. However, individual
8 variability was also observed in the viremic group, and median HLA-DR expression was
9 comparable between the two groups (12.3% vs 13.2% for HIC and VIR, respectively). The
10 percentage of CD25 expression was not increased in controllers or viremic patients (not shown).

11
12 The CD69 molecule is an early activation marker expressed at low levels in peripheral blood,
13 and which is thought to play a role in the retention of recently activated lymphocytes in lymphoid
14 organs (55). The percentage of CD69⁺ cells did not show significant changes between groups
15 (median % CD69⁺ cells in CD4⁺ TEM: 1.9 % in HIC, 4.3 % in VIR, 2.4 % in NI, 1.5 % in
16 HAART). However, it was interesting that the level of CD69 expression per positive CD4⁺ T cell,
17 as measured by the mean fluorescence intensity (MFI), was significantly higher in controllers
18 than in viremic patients and uninfected controls (Fig. 5B). This suggested a particular activation
19 status of recently primed CD4⁺ T cells in the controller group.

20
21 The alpha chain of the IL-7 receptor (CD127) is lost upon antigen exposure, and re-
22 expressed in CD4⁺ memory T cells that survive in the long term (17). CD127 expression was
23 uniformly high in CD4⁺ TCM cells of controllers, while it was decreased in those of viremic and
24 HAART-treated patients (Fig. 5C). Interestingly, CD127 showed a trend to decrease in CD4⁺
25 TEM cells of controllers as well as other HIV⁺ patients, suggesting a degree of ongoing immune

1 activation in this compartment. Thus, the phenotyping analysis showed signs of a preferential
2 activation of the TEM compartment in HIV controllers. Given that the level of T cell activation has
3 been described as a surrogate marker for HIV disease progression (21), it was striking to find
4 that, within the TEM compartment, levels of HLA-DR expression were as high in HIV controllers
5 as in progressors.

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

8 The chemokine receptor CCR5 is considered to be an activation marker, since it is expressed at
9 minimal levels in naive CD4+ T cells, at intermediate levels in CD4+ TCM cells, and at high
10 levels in CD4+ TEM cells (mean of 1.1 %, 9.5% and 48.8% CCR5+ cells, respectively, for
11 uninfected donors). Thus, it was unexpected that CCR5 expression appeared to be decreased,
12 rather than increased, in CD4+ TEM cells of HIV controllers and other HIV-infected patients (Fig.
13 6A). A similar decrease was observed in CD4+ TCM cells. The expression of CCR5 per cell was
14 also decreased, as indicated by a lower CCR5 MFI in CD4+ TCM cells of the controller group
15 compared to uninfected donors ($p < 0.05$; not shown). A possibility was that chronic secretion of
16 CCR5 ligands led to downregulation of the CCR5 receptor, a phenomenon classically
17 associated with chemokine receptor desensitization (4).

18
19 Further analyses were performed to evaluate the production of the chemokine MIP-1 β , a
20 high affinity ligand for CCR5, in response to HIV-specific and polyclonal stimulation. Stimulation
21 with p24 Gag induced MIP-1 β production to comparable levels in CD4+ TEM cells of controllers
22 and viremic patients, and to lower levels in the HAART group, again emphasizing the strength of
23 HIV-specific responses in the controller group (Fig. 6B, right panel). MIP-1 β was predominantly
24 produced by CD4+ TEM cells, the frequency of MIP-1 β + cells being about ten times higher in
25 the TEM than in the TCM subset. Thus, MIP-1 β secretion represented an effector response, a

1 notion supported by the fact that a significant fraction of CD4⁺ TEM cells that produced MIP-1 β
2 also produced IFN- γ .

3
4 Polyclonal stimulation with the superantigen SEB induced MIP-1 β responses that were
5 twenty times higher in the CD4⁺ TEM than in the TCM cell compartment (not shown). Since the
6 CD4⁺ TCM cell contribution was minimal, total CD4⁺ T cell responses are reported (Fig. 6C, left
7 panel). Of note, HIV controller showed a twofold increase in polyclonal MIP-1 β responses as
8 compared to uninfected donors. A threefold increase was observed for viremic patients. A large
9 fraction of MIP-1 β ⁺ CD4⁺ T cells also produced IFN- γ , emphasizing the effector phenotype of
10 the cells involved in the polyclonal response. We also evaluated MIP-1 β responses in the CD8⁺
11 T cell compartment, since CD8⁺ T cells are known to produce CCR5 ligands at high levels (57).
12 Upon polyclonal stimulation, CD8⁺ T cells from all patients groups produced MIP-1 β at higher
13 levels than CD8⁺ T cells from uninfected donors (Fig. 6C, right panel). Thus, HIV infection
14 primed the total T cell pool for increased MIP-1 β production, a finding that may account for the
15 downregulation of CCR5.

16
17 It was striking that the intensity of MIP-1 β responses, both HIV-specific and polyclonal,
18 were comparable between HIV controllers and viremic patients, while these two groups differed
19 markedly in terms of viral load. These findings suggested that, for certain parameters, the level
20 of functional T cell activation was comparable in both groups. Taken together, the intensity of
21 HIV-specific cytokine responses, the increased expression of HLA-DR, and the priming of the
22 CD4⁺ T cell pool for IFN- γ and MIP-1 β production pointed to a potent activation of the CD4⁺
23 TEM cell compartment in HIV controllers.

24
25

1 **DISCUSSION**

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

23
24 It was noteworthy that levels of HIV-specific CD4⁺ T cell responses were higher in HIV
25 controllers than in HAART-treated patients. The strength of CD4 responses has been proposed

1 to follow a bell-shaped curve, with a positive association between CD4 responses and viral load
2 in the low viral load range (29, 59). HIV controllers were exposed to very low levels of HIV
3 antigens, given their particularly low level of viral DNA (median = 43 copies/ 10^6 PBMC; Table I)
4 and the large amount of CD4+ T cells needed to isolate replication competent virus from their
5 PBMC (34). However, cytokine responses were 3 times higher in the controller than in the
6 HAART group, pointing to a disparity between the strength of CD4 responses and viral load in
7 the controller group. Specifically, it was the ratio of cytokine producing cells to the amount of
8 stimulating antigen that was high in this particular group of patients. It is possible that CD4+ T
9 cells responses appear comparatively high in controllers because they are actually decreased in
10 HAART treated patients. Studies of immune reconstitution indicate that patients who reached a
11 low CD4+ T cell nadir prior to treatment show a poor recovery of HIV-specific CD4+ T cell
12 responses (59). The median CD4+ T cell nadir in the HAART group was 200 cells/mm³ (Table
13 II), which is not unusually low, but is still associated with a degree of long-term immune damage.
14 On the other hand, HIV controllers may share genetic traits that promote the development of
15 efficient CD4 specific responses, or that help contain viral replication during acute infection so
16 that CD4 responses develop optimally. Whether the potent CD4 responses observed in
17 controllers directly contribute to viral control through cytotoxic mechanisms, as suggested for
18 CMV and attenuated SIV infections, warrants further investigation (12, 20, 61).

19
20 Central Memory T cells are thought to ensure the long-term maintenance of antiviral
21 responses due to their long half-life and self-renewal capacity (35, 39). CD4+ TCM cells of
22 controllers showed characteristics associated with cell survival, particularly evident in their high
23 expression of the alpha-chain of the IL-7 receptor, CD127. Studies of the basis for
24 immunological memory in murine models indicate that T cells that have encountered their
25 cognate antigen and re-express CD127 will preferentially survive the encounter, while CD127-
26 negative cells will preferentially die by activation-induced apoptosis (39). According to a recent

1 study, expression of CD127 on antigen-exposed CD4⁺ T cells is dependent on the presence of
2 IL-2 (17). Thus, the IL-2 secretion capacity of HIV-specific T cell in controllers may contribute to
3 the high CD127 expression and long-term antiviral memory in these patients. The reduced
4 expression of CD127 in viremic and HAART-treated patients suggests a shorter half-life of CD4⁺
5 TCM cells in these groups. Further studies aimed at directly measuring TCM turnover will be
6 needed to confirm this point. Of note, the differences in CD127 expression did not extend to the
7 CD4⁺ TEM cell population. The three groups of patients showed a trend towards low CD127
8 expression in CD4⁺ TEM cells, consistent with an activation of the effector memory
9 compartment in all cases of HIV infection.

10

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

1 compatible with a fully functional and long-lived central memory response.

2

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

23

24 We cannot rule out that efficient activation of CD4 responses is a consequence, rather
25 than an initial cause, of HIV control, and that viral replication was contained early on due to other

1 intrinsic or immune factors. However, once established, the effector CD4 response is likely to
2 reinforce HIV control at several levels, through secretion of chemokines that inhibit HIV
3 replication, helper function, and possibly direct cytotoxic function. The finding of a reduced
4 CCR5 expression at the surface of CD4+ T cells suggests that HIV controllers are indeed
5 exposed chronically to high levels of beta-chemokines such as MIP-1 β . Alternate explanations
6 such as genetic polymorphisms that would reduce basal CCR5 expression levels are also
7 possible but less likely, since the CCR5 coding sequence was wild-type in all the patients
8 studied. Further analyses focused on CCR5 promoter polymorphisms and CCR5 ligand gene
9 copy numbers are needed to evaluate the genetic component of CCR5 expression level in
10 controllers (22, 26). Preferential infection and deletion of CCR5+ cells may also contribute to
11 decrease CCR5 expression (40), but is unlikely in the case of controller patients who by
12 definition show very limited HIV replication. High levels of beta-chemokines can lead to chronic
13 down-regulation of CCR5 through activation-induced receptor internalization (4, 45). Since
14 CCR5 expression levels condition the infectability of HIV target cells, CCR5 downregulation may
15 contribute to the low-levels of infection in controllers. In particular, this mechanism may play a
16 role in protecting cells that express already limiting amounts of CCR5, such as CD4+ TCM cells.
17 In controllers, the combination of decreased CCR5 expression and low cellular activation would
18 make CD4+ TCM cells suboptimal targets for HIV replication.

19
20 Of note, high MIP-1 β production was also detected in the CD8+ T cell population (Fig.
21 6C). Analysis of CD8+ T cell responses in the same cohort of controllers confirmed that
22 activation of the effector compartment, as measured by HLA-DR expression, extends to CD8+ T
23 cells (51). Dosage of soluble LPS in plasma samples from this cohort showed raised levels in
24 HIV controllers, another indication of chronic immune activation (9). These observations suggest
25 that activation of the effector compartment involves CD8+ T cells and possibly to other immune

1 cells. Importantly, the immune activation evident in HIV controllers was not equivalent in every
2 aspect to that seen in viremic patients. T cells from controllers showed a lower susceptibility to
3 apoptosis *in vitro* than those of viremic patients (S.P.P., unpublished observation). Plasma levels
4 of soluble CD14, another indicator of inflammation, were intermediate in controllers and
5 significantly higher in progressors (9). Conversely, CD69 was expressed to higher levels in
6 recently activated CD4+ T cells of controllers than of viremic patients. A marked difference was
7 noted in the activation status of HIV-specific CD8+ T cells, which expressed HLA-DR but not
8 CD38 in controllers, while both activation markers were expressed in viremic patients (51).
9 These findings point to a differential pattern of immune activation in HIV controllers and viremic
10 patients. Chronic immune activation may not be detrimental in controllers because it spares the
11 central memory compartment, and thus preserves the regenerative capacity of the immune
12 system. Activation would then benefit controller patients, by ensuring the efficacy of effector
13 responses. In contrast, as shown in a recent study of SIV infection (46), chronic activation in the
14 presence of viremia leads to a progressive loss of CD4+ TCM cells, with ensuing immune
15 exhaustion.

16
17 In conclusion, HIV controllers were characterized by the maintenance of a functional and
18 long-lived pool of CD4+ TCM cells, in parallel with a chronically activated pool of CD4+ TEM
19 cells. The conjunction of these two types of immune memory may generate optimal responses
20 against chronic HIV infection, and represent a goal to be achieved by candidate HIV vaccines.

21 _____

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10

1 **LEGEND TO THE FIGURES**

2
3 **Figure 1: Cytokine responses in CD4+ T cells**

4 (A) Representative Intracellular Cytokine Staining (ICS) analysis: PBMC of controller patient A4
5 were left unstimulated (left panel), stimulated with the HIV p24 Gag core protein (middle panel),
6 or with anti-CD3/CD28 antibodies (right panel) for 14h. For flow cytometry analysis, 10⁶ events
7 were acquired in the lymphocyte gate. In this example, IFN- γ and IL-2 production are analyzed
8 within the CD3+ CD4+ population. Flow cytometric data are represented by pseudo-color density
9 plots. Percentages of cytokine+ cells are reported in each quadrant.

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

15
16 **Figure 2: Proliferative responses**

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

20
21 **Figure 3: Phenotyping of naive and memory CD4+ T cell subsets**

22 (A) representative gating strategy used to define CD4+ T cell subsets: CD3+ CD4+ T cells were
23 classified according to CD45RA and CCR7 expression. Naive cells were defined as CCR7+
24 CD45RA+; Central Memory cells were defined as CD45RA- CCR7+; Effector memory cells were
25 defined as CD45RA- CCR7-; The CD45RA+ CCR7- subset remained below 2.6 % within the
26 CD3+ CD4+ population and was not analyzed further. (B) Frequency of naive CD4+ T cells. (C)

1 Frequency of central memory CD4⁺ T cells. (D) Frequency of effector memory CD4⁺ T cells. (E)
2 Expression of the chemokine receptor CCR7 at the surface of CD4⁺ T cells: the mean
3 fluorescence intensity (MFI) per cell is reported. P values were determined by the non-
4 parametric Mann-Whitney U test.

5

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

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

13

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

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

21

22 **Figure 6: Expression of the chemokine receptor CCR5 and production of the CCR5 ligand**
23 **MIP-1 β**

24 (A) Proportion of cells expressing the CCR5 chemokine receptor in CD4⁺ TCM cells (left panel)
25 and CD4⁺ TEM cells (right panel). (B) HIV-specific MIP-1 β response in the CD4⁺ TCM and TEM

1 cell subsets (left and right panels, respectively). The mean percentage of cells secreting MIP-1 β
2 only (white bars) or both MIP-1 β and IFN- γ (grey bars) is reported. Note the scale difference
3 between the two graphs (C) Polyclonal MIP-1 β 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.

6
7

Table I: Characteristics of patients in the HIV controller group

Patient	Sex	Age	HIV diagnosis	Median CD4 count¹	last CD4 count¹	median HIV RNA²	last HIV RNA²	HIV DNA³	CCR5 genotype	ICS assay⁴
A1	F	49	1988	982	1010	<50	<50	3	WT	+
A2	M	48	1985	753	786	<50	<50	7	WT	+
A3	F	42	1993	876	1159	<50	<50	Neg	WT	+
A4	M	47	1987	592	749	<50	<50	80	WT	+
A5	M	42	1991	728	740	<50	<50	65	WT	ND
A6	M	69	1985	706	524	<50	150	40	WT	+
A7	M	38	1983	609	580	<50	<50	15	WT	+
A8	M	41	1992	1207	939	200	100	50	WT	+
A9	M	44	1992	844	522	<50	<50	60	WT	+
A10	F	48	1985	1033	1036	<50	<50	47	WT	+
B2	F	40	1987	928	860	<50	180	170	WT	+
B5	M	57	1989	799	800	<50	<50	33	WT	+

Note: (1) CD4 T cells/mm³ blood; (2) HIV RNA copies/ml plasma; (3) HIV DNA copies/10⁶ PBMC; (4) Intracellular Cytokine Staining assay:

(+) test positive (ND) not done.

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Table II: Summary of patients' characteristics

Patient Group	Age median (range)	CD4 count¹ median (range)	Viral Load² median (range)	CD4 nadir¹ median (range)
ICS Assay³				
HIC (n=11)	47 (38-69)	800 (522-1159)	<50 (<50-180)	N/A ⁴
Viremic (n=10)	39 (29-46)	349 (35-789)	25,150 (2,930-920,674)	N/A
HAART (n=10)	48 (21-53)	578 (147-1184)	<50 (<50-<50)	204 (29-282)
Phenotyping				
HIC (n=8)	44.5 (38-69)	768 (524-1,159)	<50 (<50-100)	N/A
Viremic (n=8)	45 (27-58)	462 (272-570)	18,300 (10,800-180,000)	N/A
HAART (n=8)	41 (38-68)	587 (441-862)	<50 (<50-<50)	190 (18-376)

Note: ¹(CD4 T cells/mm³ blood); ²(HIV RNA copies/ml plasma); ³(Intracellular Cytokine Staining Assay); ⁴(Not Applicable)

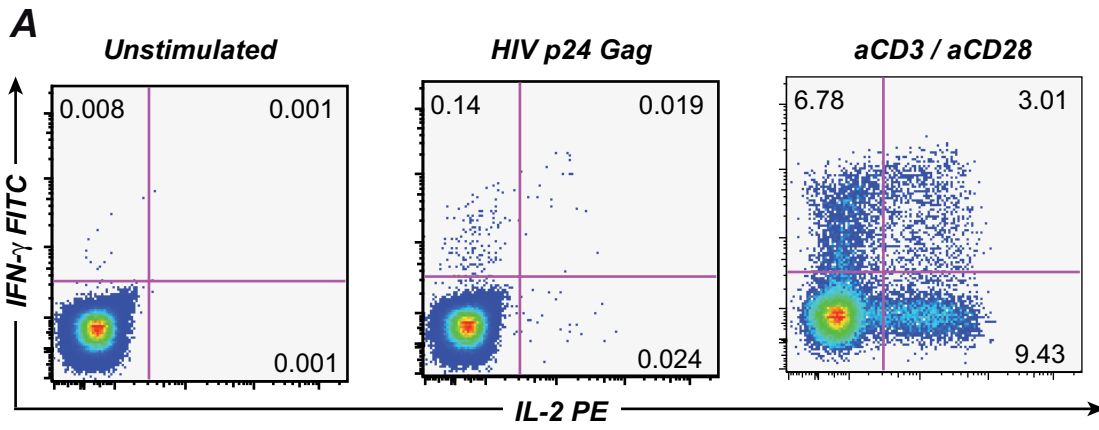
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p24 Gag-specific responses	Mean Percentage of Positive Cells				P-value			
	HIC	VIR	NI	HAART	HIC vs VIR	HIC vs NI	HIC vs HAART	VIR vs NI
% IFN-γ+								
CM CD4+T cells	0,010	0,008	0,000	0,001	-	p<0.01	p<0.01	-
EM CD4+T cells	0,148	0,170	0,000	0,064	-	p<0.001	p<0.05	p<0.01
% IFN-γ+ IL-2+								
CM CD4+T cells	0,003	0,000	0,000	0,000	-	-	-	-
EM CD4+T cells	0,094	0,005	0,000	0,019	p<0.001	p<0.001	p<0.001	-
% IL-2+								
CM CD4+T cells	0,032	0,000	0,000	0,009	p<0.01	p<0.01	-	-
EM CD4+T cells	0,069	0,005	0,000	0,019	p<0.001	p<0.001	p<0.05	-
% Cytokine+								
CM CD4+T cells	0,045	0,008	0,000	0,010	p<0.01	p<0.001	p=0.01	-
EM CD4+T cells	0,310	0,180	0,000	0,103	-	p<0.001	p<0.01	p=0.001
Polyclonal responses								
Polyclonal responses	Mean Percentage of Positive Cells				P-value			
	HIC	VIR	NI	HAART	HIC vs VIR	HIC vs NI	HIC vs HAART	VIR vs NI
% IFN-γ+								
CM CD4+T cells	2,39	1,37	1,67	1,85	p<0.05	-	-	-
EM CD4+T cells	17,27	20,95	7,52	17,91	-	p=0.01	-	p<0.001
% IFN-γ+ IL-2+								
CM CD4+T cells	3,16	0,65	2,49	1,88	p<0.001	-	-	p=0.001
EM CD4+T cells	13,72	3,27	12,78	8,17	p<0.001	-	-	p<0.001
% IL-2+								
CM CD4+T cells	27,08	14,98	24,24	18,49	p<0.05	-	-	p<0.05
EM CD4+T cells	14,64	8,83	22,27	14,10	p<0.05	-	-	p=0.001
% Cytokine+								
CM CD4+T cells	32,63	17,00	28,39	22,21	p<0.01	-	-	p=0.01
EM CD4+T cells	45,64	33,04	42,57	40,18	-	-	-	-

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

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B IFN- γ response in CD4+ T cells

C IL-2 response in CD4+ T cells

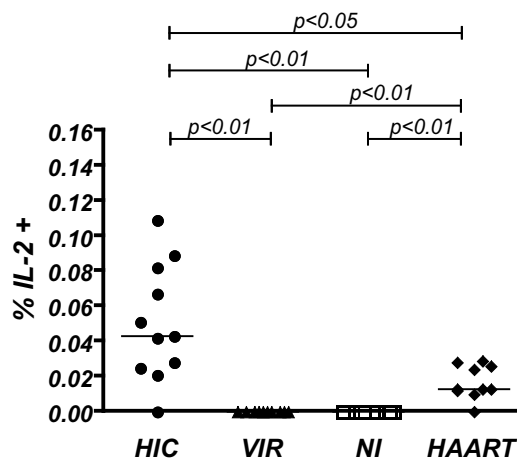
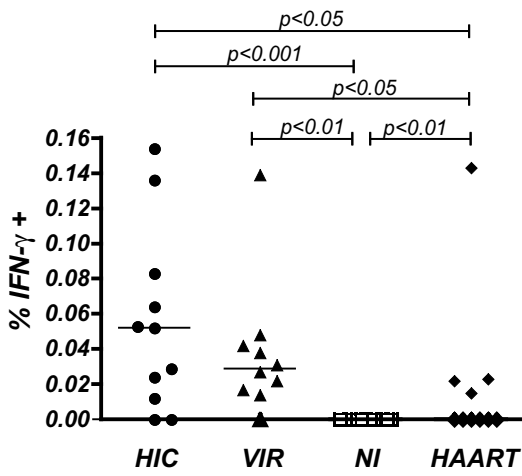


Fig. 1 Potter et al.

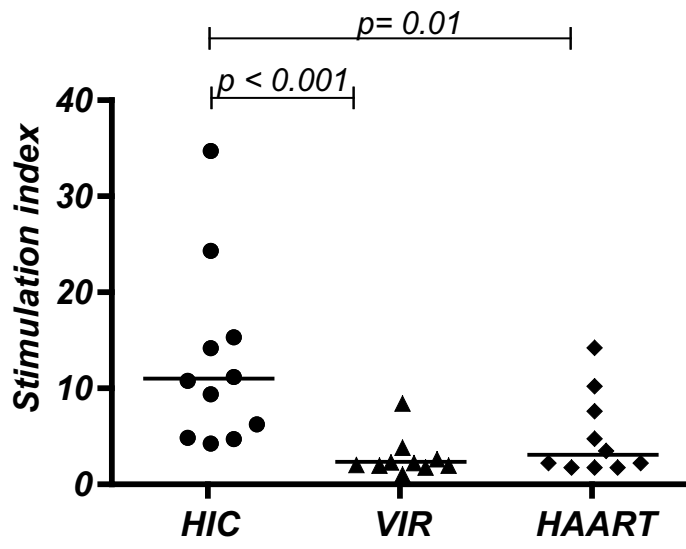


Fig. 2 Potter et al.

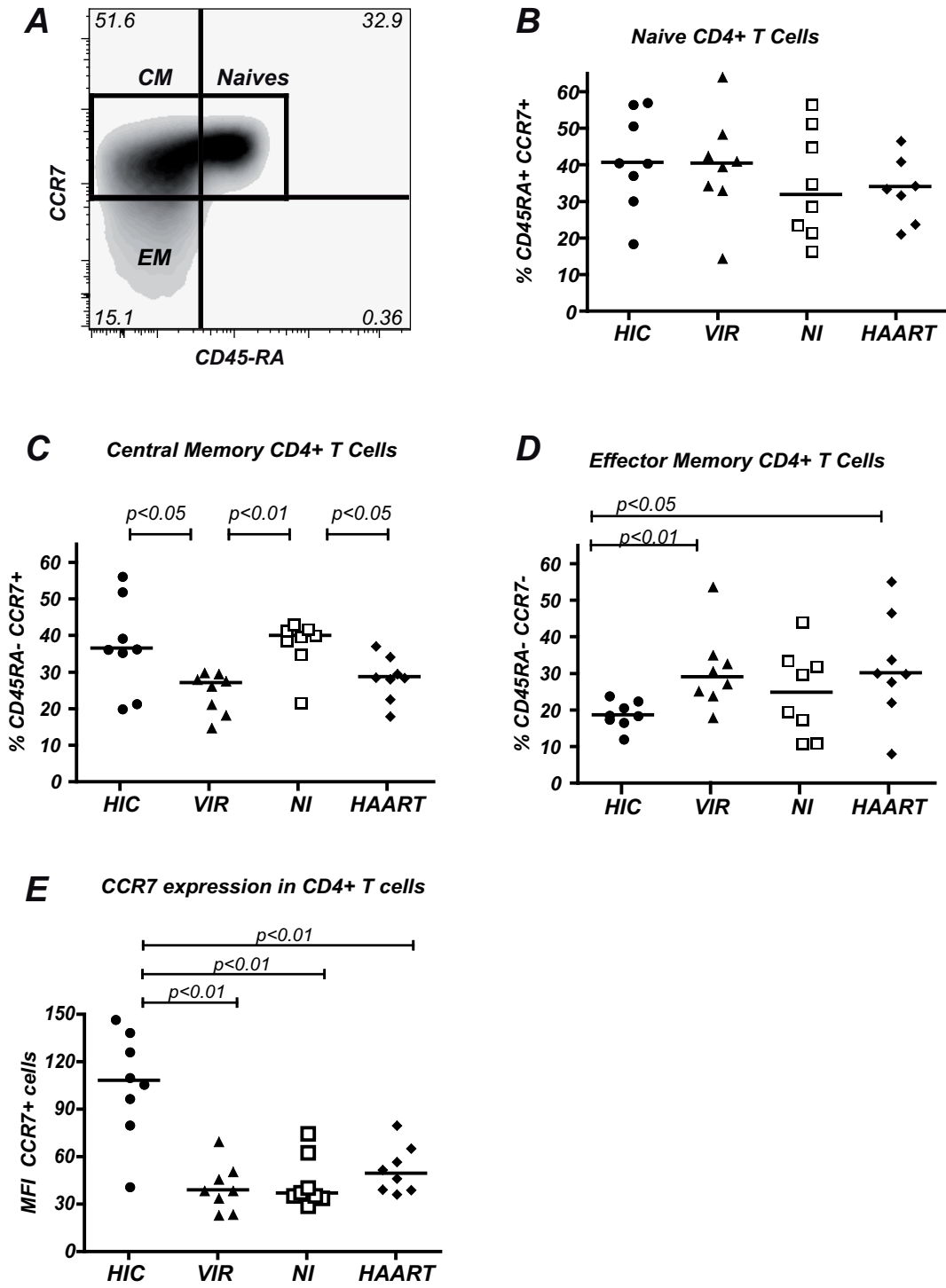


Fig. 3 Potter et al.

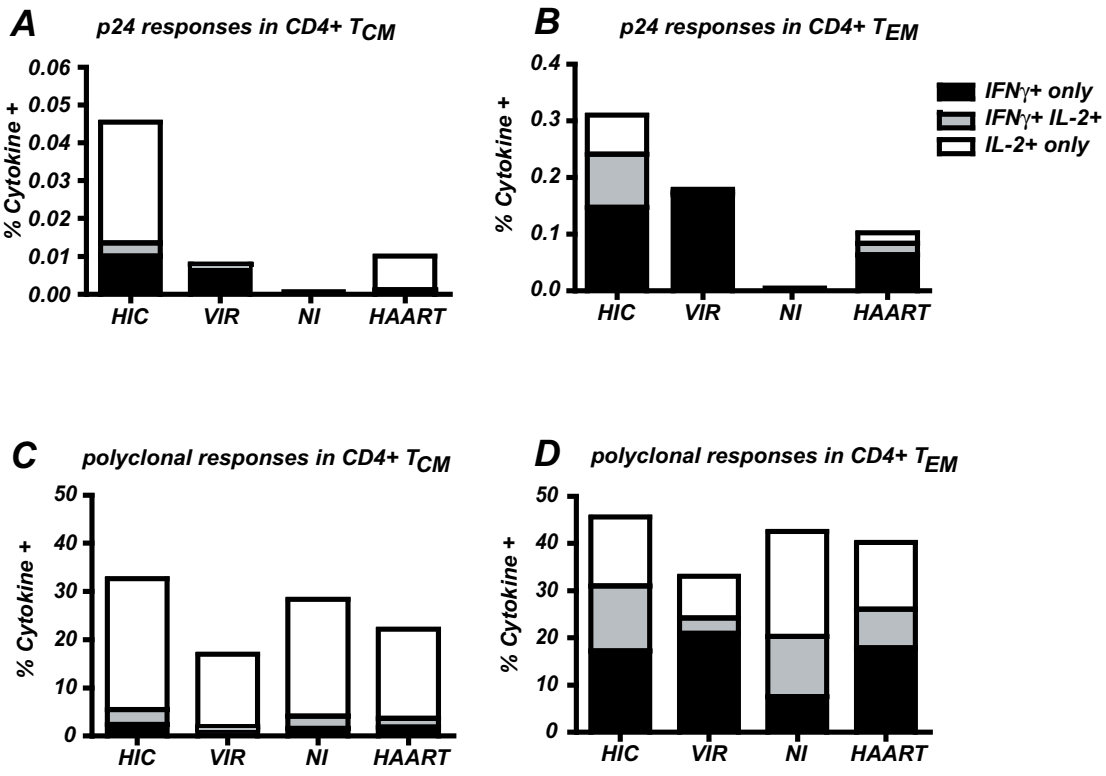


Fig. 4 Potter et al.

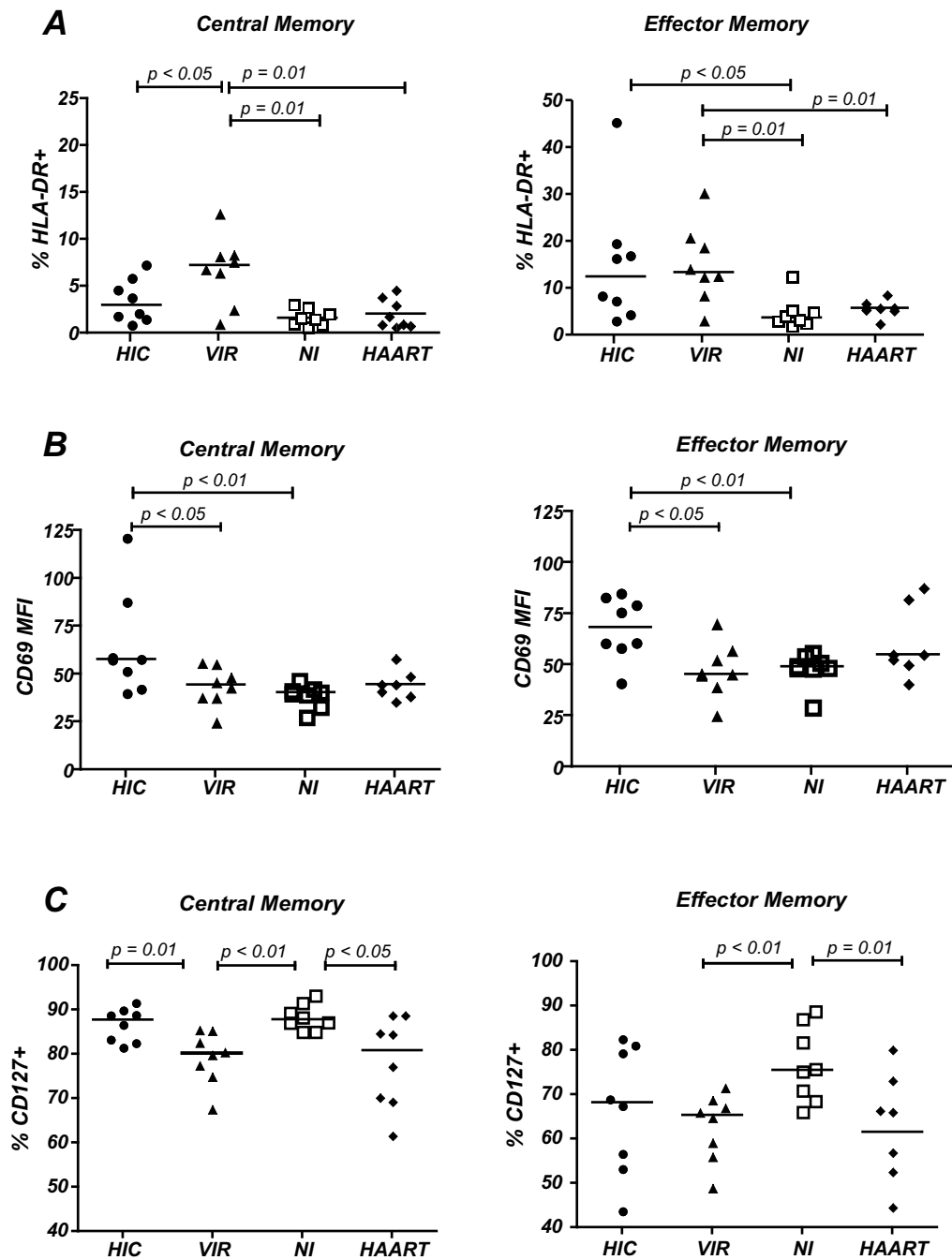
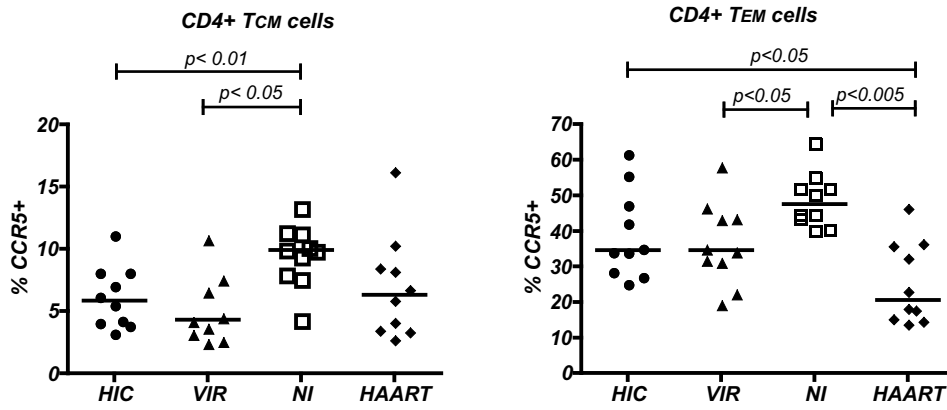
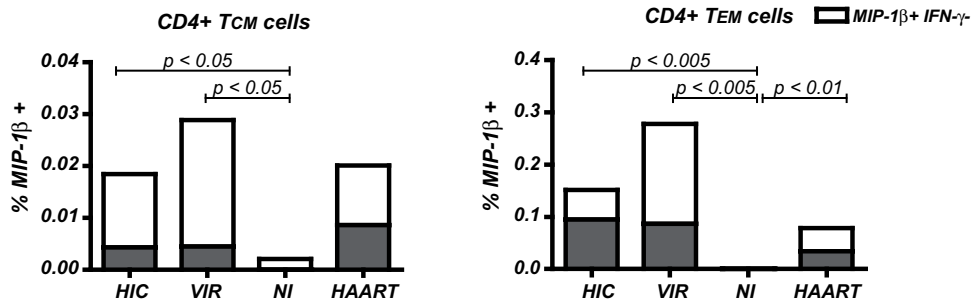


Fig. 5 Potter et al.

A CCR5 expression



B p24 Gag-specific responses



C Polyclonal responses

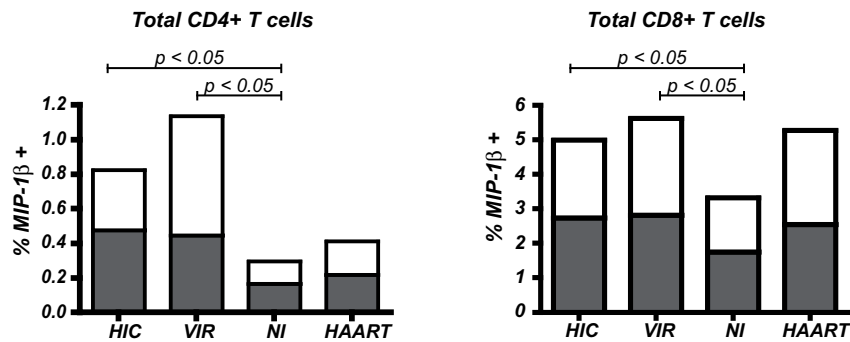


Figure 6 Potter et al.