A Lower Viral Set Point but Little Immunological Impact After Early Treatment During Primary HIV Infection

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Abstract

The Primo-SHM trial, a multicenter randomized trial comparing no treatment with 24 or 60 weeks of combination antiretroviral therapy (cART) during primary human immunodeficiency virus (HIV) infection (PHI), recently demonstrated that temporary early cART lowered the viral set point and deferred the need for re-initiation of cART during chronic HIV infection. This study examined whether the beneficial effect of early treatment was caused by preservation of immunological responses. Twenty-seven treated and 20 untreated PHI individuals participating in the Primo-SHM trial were compared at viral set point, that is, 36 weeks after baseline or after treatment interruption, respectively, for a diverse set of immunological parameters. The results show no differences between treated and untreated individuals at the level of effector T-cell formation or replication capacity of the T-cells; regulation of various T, B, natural killer, or dendritic cells; polyfunctionality of the CD8 T-cells; preservation of CD4 T-cells in the gut associated lymphoid tissue; or immune activation. There were subtle differences in the quality of the cytolytic CD4 T-cell response: 11% (median) of CD4 T-cells of the early treated individuals produced the cytolytic molecule perforin compared to 5% in untreated individuals ($p=0.046$), and treatment caused a modest increase in CD4 T-cells expressing both perforin and granzyme B (median 9% vs. 4% of CD4 T-cells; $p=0.045$). Early treatment had a modest positive effect on the quality of the CD4 T-cell response. It remains unclear, however, whether these subtle immunological differences were the cause or a result of the lower viral set point in patients who received early treatment.

Introduction

IN HUMAN IMMUNODEFICIENCY VIRUS (HIV) infection, a low viral set point and low levels of immune activation correlate with slower disease progression (10,21). The Primo-SHM trial, a multicenter randomized study comparing no treatment with 24 or 60 weeks of combination antiretroviral therapy (cART) during primary HIV infection (PHI), recently demonstrated that temporary early cART transiently lowered the viral set point and deferred the need for re-initiation of cART during chronic HIV infection (13). Factors such as baseline CD4 T-cell count, stage of PHI, and HLA background could not explain the differences observed in viral set point (13,31).

There are many immunological parameters that play a role in controlling HIV disease progression. Foremost, there is compelling evidence that (gag-specific) CTL responses correlate with HIV viral load and the rate of HIV-disease progression (1–3,18,19,22,25,29,30). Current consensus in the field is that such a “protective” T-cell response in HIV infection is comprised of two elements: effective cytolytic killing and production of multiple cytokines. Recent work from Soghoian *et al.* highlights the importance of vigorous cytolytic activity by HIV-specific CD4 T-cells in controlling disease progression (30). The presence during PHI of HIV-specific CD4 T-cells, which are able to express both perforin and high levels of granzyme A, was found to be highly predictive of slower disease progression and good

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clinical outcome. Likewise, HIV nonprogressors preferentially maintain more polyfunctional HIV-specific T-cells, that is, T-cells with the capacity to secrete multiple cytokines and chemokines upon antigenic stimulation (2). Almeida *et al.* demonstrated that superior control of HIV infection by CD8 T-cells was reflected by a polyfunctional and high avidity phenotype (1).

The effectivity of HIV-specific immune responses can be severely limited during disease progression, as persistent viremia has deleterious effects on HIV-specific CD4 and CD8 T-cell immunity (22). For instance, chronic antigen exposure leads to a terminally differentiated phenotype of the CD4 and CD8 T-cells, with upregulated CD57 expression and a diminished functional proliferative capacity (4). Also, HIV viremia prevents the establishment of highly functional memory CD4 T-cells that retain the capacity to proliferate upon antigen stimulation (15,20). Finally, chronic antigenic stimulation induces upregulation of inhibitory receptors, most notably PD-1 and CTLA-4, which may interfere with HIV-specific T-cell responses and ultimately leads to T-cell anergy and loss of HIV-specific T-cells (9,17).

One of the earliest effects of HIV infection is a massive depletion of central memory CD4 T-cells from the gut-associated lymphoid tissue (GALT) (14). The instant and massive early injury to the gut immune system, together with the subsequent damage exacted to the gut epithelial cells, is thought to induce gut permeability and the translocation of microbial products such as lipopolysaccharides (LPS). This is thought to contribute to systemic immune activation, which characterizes HIV infection (5). It is widely accepted that chronic immune activation drives progression to acquired immune deficiency syndrome (AIDS) (10,12,16,35). In fact, immune activation is more strongly associated with the rate of CD4 T-cell loss in HIV-infected individuals than with viral load (10).

It is hypothesized here, as well as by others, that viral suppression in PHI might prevent the excessive depletion of the GALT, thereby reducing the level of immune activation and delaying the CD4 T-cell loss. Depletion of CD4 T-cells in the GALT can be assessed indirectly in the blood by measuring the level of $\alpha 4\beta 7$ expression on T-cells. Indeed, in primary simian immunodeficiency virus (SIV) infection, the reduction of $\alpha 4\beta 7^{\text{high}}$ CD4 T-cells in the peripheral blood was shown to parallel the reduction of CD4 T-cells in intestinal tract biopsies (26,34). Likewise, the finding that $\alpha 4\beta 7^{\text{high}}$ CD4 T-cells are preferentially depleted in the blood in untreated PHI (8) likely reflects the depletion of CD4 T-cells from the GALT.

To determine the cause of the lower viral set point in individuals treated during PHI, this study evaluated whether (a) effector T-cell formation, (b) effectivity of the T-cell response (in terms of cytolytic function and polyfunctionality), and (c) inhibitory receptor expression on immune cells were altered in individuals treated during PHI compared with untreated PHI individuals. To investigate whether viral suppression in PHI caused preservation of CD4 T-cells in the GALT, this study evaluated whether expression of the gut homing receptor $\alpha 4\beta 7$ on CD4 T-cells from the blood was altered in individuals treated during PHI. Finally, the study assessed whether early treatment contributed to delayed disease progression by preventing excessive systemic T-cell activation.

Materials and Methods

Study population

Blood samples were obtained from participants of the Primo-SHM trial. The Primo-SHM trial was a multicenter, open-label, randomized controlled trial comparing temporary early cART (for 24 or 60 weeks) with no treatment. Detailed procedures have been described elsewhere (2). Briefly, inclusion criteria were older than 18 years of age and laboratory evidence of PHI, defined as a negative or indeterminate Western blot in combination with detectable plasma HIV-1 RNA (Fiebig stage I–IV) or, in case of a positive Western blot, a documented negative HIV screening test in the previous 180 days (Fiebig stage V–VI) (21).

Twenty untreated and 27 treated PHI individuals were selected based on sample availability. Patients treated for both 24 and 60 weeks were included. Immunological parameters were measured at inclusion (baseline) and “viral set point,” defined as the plasma viral load 36 weeks after baseline or treatment interruption (TI), respectively (see Fig. 1). In addition, blood samples of 18 healthy donors were obtained through the UMC Utrecht. For the studies of both patients and healthy donors, approval was obtained from the Medical Ethics Committee of each participating site, and written informed consent was obtained from all participants.

T-cell activation and phenotype

Expression of activation markers on CD4 and CD8 T-cells was measured after staining of the cells with anti-CD4-PB, anti-CD8-Amcyan, anti-CD38-PE, and anti-HLA-DR-APC monoclonal antibodies (BD Biosciences). The phenotype of the cells was determined using anti-CD27-APC-Cy7 (Biolegend) and anti-CD45RO-PECy7 (BD Biosciences). In the same sample, cells were also stained with anti-PD-1-PerCPcy5.5. All incubations were performed at 4°C (20 min) after which cells were fixed in cellfix (BD Biosciences) and analyzed with the LSRII flow cytometer.

Ex vivo T-cell function

Ex vivo surface staining was performed with anti-CD3-eFluor450 (eBioscience), anti-CD8-V500, anti- $\alpha 4\beta 7$ -APC, anti-CD57-FITC, anti-CD45RO-PE-Cy7 (all BD), and anti-CD27-APC-Cy7 (Biolegend) monoclonal antibodies. After fixation and permeabilization (permeabilization reagents; BD Biosciences) for 10 min, cells were stained for cytotoxic molecules with antigranzyme A-Pe or antigranzyme B-Pe (Sanquin) and antiperforin-PerCP-Cy5.5. Hereafter, cells were fixed in cellfix (BD Biosciences), and flow cytometry was performed.

CD8 T-cell stimulation and intracellular cytokine staining

Cryopreserved peripheral blood mononuclear cells were thawed and aliquoted at 2×10^6 cells/mL in round-bottom tubes (Becton Dickinson). CD8 T-lymphocytes were stimulated for 6 h with a gag-peptide pool (15mers with 11 overlap, final concentration of the individual peptides was 2 $\mu\text{g/mL}$, Consensus B 2007, NIH AIDS Research and Reagent program). As a positive control, PMA and ionomycin (Sigma-Aldrich; 5 ng/mL and 1 $\mu\text{g/mL}$ respectively) were

FIG. 1. Schematic overview of timeline. All immunological parameters were measured at the time of patient inclusion and randomization (baseline) and/or 36 weeks after inclusion or treatment interruption (viral load [VL] set point).

used. After 1.5 h, Brefeldin A (3 μ M; Sigma-Aldrich) was added. Surface staining was performed with anti-CD3-PerCP, anti-CD8-V500, anti- α 4 β 7-APC (all BD Biosciences), and anti-CD27-APC-Cy7 (Biolegend) monoclonal antibodies for 20 min at 4°C. After fixation and permeabilization (permeabilization reagents; BD) for 10 min, cells were stained with anti-IFN- γ -Pe-Cy7 (eBioscience), anti-TNF- α -FITC, anti-MIP1- β -PE, and anti-IL-2-PB (BD Biosciences) for 20 min at 4°C. Cells were fixed in cellfix (BD Biosciences), and flow cytometry was performed.

Characterization of inhibitory markers

Expression of inhibitory markers was assessed on CD4 and CD8 T-cells, B-cells, natural killer (NK) cells, and dendritic cells. Surface staining was performed for CD4 and CD8 T-cells (anti-CD3-eFluor450, eBioscience; antiCD8-V500, BD Biosciences), B-cells (anti-CD19-PerCP, BD Biosciences), NK cells (anti-CD56-APC, BD Biosciences), and cells (anti-HLA-DR-APC-Cy7, BD Biosciences; anti-CD11c-PE-Cy7, BD Biosciences). These sets were completed with either anti-CD31-PE (BD Biosciences)/3D3 anti-sir1-FITC or anti-LAIR-PE/anti-ILT4-FITC or anti-IREM-1-PE/anti-KLRG-1-FITC or isotype controls. After staining for 20 min at 4°C, cells were fixed in cellfix (BD Biosciences), and flow cytometry was performed.

Flow cytometry analysis

At least 100,000 events were acquired after phenotypical staining, and at least 300,000 events were acquired after intracellular cytokine staining, using the LSRII flow cytometer (BD Biosciences). Data were analyzed using the DIVA software (BD Biosciences). The events were gated for either lymphocytes or monocytes in a FSC-A versus SSC plot. Following this, events were gated using the markers described above. T-cell polyfunctionality was analyzed by Flowjo software (v9.2). After determining the lymphocyte gate in a FSC-A versus SSC plot, cells were sequentially gated for CD3 and CD8. Subsequently, within the CD8 T-cell population, a gate was created for the four respective functions: IFN- γ , TNF- α , MIP1- β , and IL-2. Herein, a

Boolean gating was performed, resulting in 20 different combinations. All data were background-subtracted using the unstimulated samples.

Statistical analysis

Differences between treated and untreated individuals and between healthy donors and patients were analyzed using the Mann-Whitney *U*-test because a normal distribution of the data could not be assumed. Whenever more than two groups were compared, the Kruskal-Wallis test was used. All statistical analyses were performed using the software program SPSS Statistics for Windows v19.0 (IBM Corp.).

Results

Patient characteristics

In the patients selected for the present study, the median viral loads of treated and untreated patients were not significantly different at baseline (for all patient characteristics, see Table 1). Comparable with the complete study group (13), at set point, the pVL was significantly lower in patients who were treated during PHI: median 4.2 versus 5.0 ¹⁰log copies/mL for treated and untreated individuals, respectively ($p=0.002$). CD4 and CD8 T-cell counts at viral set point also resembled those of the patients in the complete Primo-SHM trial (13). CD8 T-cell counts were not significantly different between treated and untreated individuals, both at baseline and at viral set point. CD4 numbers were comparable at baseline ($p=0.26$), but at viral set point, median CD4 T-cell counts were significantly higher in treated than in untreated patients [630 [range 110–1,280] vs. 430 [180–940] CD4 T-cells/mm³; $p=0.03$].

No change in T-cell subsets by early treatment

To investigate a possible immunological cause of the lower viral set points in individuals treated during PHI, this study examined whether T-cells of treated individuals had a different maturation state and/or a less exhausted phenotype, with a resulting more effective immune response at viral set point. The level of terminal differentiation (based on the

TABLE 1. PATIENT CHARACTERISTICS

	Treated ^a (n=27)	Not treated (n=20)	p-Value
<i>Patient characteristics at baseline</i>			
Age (years), median (range)	41 (30–59)	41 (25–55)	0.39
Male	25 (92%)	20 (100%)	0.22
MSM	21 (78%)	17 (85%)	0.76
Caucasian	23 (85%)	18 (90%)	0.63
Stage of PHI			
Fiebig I–IV	21 (78%)	17 (85%)	0.54
Fiebig V–VI	6 (22%)	3 (15%)	
CD4 cell count (cells/mm ³), median (range)	570 (150–1,050)	520 (200–1,110)	0.26
CD8 cell count (cells/mm ³), median (range)	1129 (260–2,570)	1057 (160–2,740)	0.98
Plasma HIV-1 RNA (log ₁₀ copies/ml), median (range)	4.8 (1.7–6.7)	5.4 (1.8–6.5)	0.42
<i>Patient characteristics at viral set point</i>			
CD4 cell count (cells/mm ³), median (range)	630 (110–1,280)	430 (180–940)	0.03
CD8 cell count (cells/mm ³), median (range)	860 (380–2,220)	860 (310–2,540)	0.29
Plasma HIV-1 RNA (log ₁₀ copies/ml), median (range)	4.2 (1.8–5.5)	5.0 (3.0–5.4)	0.002

Numbers are *n* (%), unless otherwise indicated.

^aTreatment consists of either 24 or 60 weeks of combination antiretroviral therapy.

MSM, men who have sex with men; PHI, primary human immunodeficiency virus (HIV) infection; HIV, human immunodeficiency virus.

expression of CD45RO and CD27) and replicative senescence (based on the marker CD57) of the CD4 and CD8 T-cells of treated and untreated individuals was analyzed at viral set point (Fig. 2). It was found that irrespective of early treatment, CD4 T-cells of HIV-infected individuals had a

more terminally differentiated phenotype (CD45RO–CD27–) and increased replicative senescence (CD57+) than healthy volunteers ($p=0.0002$ and $p<0.0001$, respectively). The level of senescence of CD8 T-cells was also higher in HIV-infected individuals compared to healthy volunteers

FIG. 2. No change in T-cell subsets after early treatment. Markers of maturation (CD45RO–CD27–) and senescence (CD57+) on CD4 (A) and CD8 T-cells (B) are shown for treated (T, $n=25$, square), untreated (UT, $n=13$ for terminally differentiated and $n=8$ for senescent T-cells respectively, open circle), and healthy donor (HD, $n=12$, triangle). Dots represent individuals, with a bar at the median. ** $p=0.01$ – 0.001 ; *** $p<0.001$.

($p=0.008$). However, no differences could be observed between treated and untreated PHI individuals.

To reveal whether HIV-specific cells displayed differences in replicative capacity between treated and untreated patients, an *in vitro* proliferation assay was performed. Cells were stimulated with an overlapping gag-peptide pool, and after 6 days the stimulation index was determined. Early treatment had no effect on the gag-specific proliferative capacity of either CD4 or CD8 T-cells (data not shown).

Ex vivo CD4 cytolytic T-cell activity is enhanced by early treatment

To assess the direct *ex vivo* cytolytic functionality of T-cells from treated and untreated patients, the levels of granzyme A, granzyme B, and perforin expression of total CD4 and CD8 T-cells were measured at viral set point. The percentage of CD4 and CD8 T-cells expressing granzyme A, perforin, and/or granzyme B was elevated in both treated and untreated HIV-infected individuals compared to healthy individuals (Fig. 3). At viral set point, early treated individuals showed an increased level of CD4 T-cells expressing perforin compared to untreated individuals (median of 11% vs. 5% of CD4 T-cells; $p=0.045$). In addition, treatment caused a modest increase of CD4 T-cells expressing both perforin and granzyme B (median of 9% vs. 4% of CD4 T-cells; $p=0.045$).

Treatment does not preserve a more polyfunctional T-cell response

Another measure for an effective T-cell response is its polyfunctionality. A stimulation assay was performed with an overlapping gag-peptide pool (Fig. 4), and the study measured which proportion of CD4 and CD8 T-cells produced one or more of the cytokines TNF- α , MIP1 β , IFN- γ

and IL-2. No differences were observed between treated and untreated PHI individuals at viral set point in the total amount of CD8 T-cells that produced these cytokines/chemokines in response to HIV gag (median 1.0% vs. 0.7% of CD8 T-cells, respectively; $p=0.74$, data not shown). The CD4 T-cell response to stimulation was either absent or too low to interpret. When the functional profiles of the CD8 T-cell responses were compared by expressing each functional component as a proportion of the total response (2), it was found that treated individuals did not exhibit a more polyfunctional response than untreated PHI individuals (Fig. 4A). In treated individuals, a median of 24% of the gag-specific CD8 T-cells displayed more than one function compared to 13% of CD8 T-cells in the untreated individuals ($p=0.59$). Likewise, no significant differences could be found in the type of cytokine that was produced by the CD8 T-cells in response to gag-antigen. For instance, in untreated individuals IFN- γ production by HIV-specific CD8 T-cells made up approximately 21% of the total response, while its median contribution did not exceed 17% in treated individuals ($p=0.72$; Fig. 4B).

No changes in expression of regulatory and inhibitory molecules after early treatment

Markers of regulation of the immune response were analyzed, and a panel of “well-recognized” but also recently characterized and largely unexplored inhibitory receptors (33) was selected. Their level of expression on CD4 and CD8 T-cells, B-cells, NK cells, and dendritic cells at viral set point was analyzed (see Supplementary Fig. S1 for gating strategy; Supplementary Data are available online at www.liebertpub.com/vim).

Pecam-1 (CD31), which is known to inhibit apoptosis (11), was expressed less in HIV-infected individuals compared to healthy controls in all measured cell types (Fig. 5

FIG. 3. *Ex vivo* cytolytic T-cell activity is not enhanced at viral set point after treatment. The *ex vivo* effector functions of CD4 (A) and CD8 T-cells (B) in terms of granzyme A, granzyme B, and perforin production in treated ($n=5$, square), untreated ($n=8$, open circle), and healthy ($n=6$, triangle) individuals was assessed. Individual production of granzyme B by CD4 and CD8 T-cells was assessed in a larger group of individuals ($n=10$, 14, and 18 for T, UT, and HD, respectively). Dots represent individuals, with a bar at the median. * $p=0.05$ –0.01; ** $p=0.01$ –0.001; *** $p=0.001$ –0.0001; **** $p<0.0001$.

FIG. 4. Preservation of a more polyfunctional T-cell response due to treatment. PHI individuals with treatment ($n=10$) and no treatment ($n=8$) were compared. The pie charts depict the relative contribution of the number of functions, that is, cytokines/chemokines secreted, which are produced by CD8+ T-cells in response to HIV gag (A). The graphs zoom in on the relative contribution of the cytokine (MIP1 β , IFN- γ , TNF- α , and IL-2) combinations to the total CD8 T-cell response (B). The bars represent the frequency of the CD8 T-cell response expressing the particular combination of functions shown. Each dot indicates MIP1 β , IFN- γ , TNF- α , and/or IL-2 positivity. In all graphs, medians (with range) are shown, and the number of functions (1 function = white; 2 functions = gray; 3 functions = dark gray; 4 functions = black) are depicted.

and Supplementary Fig. S2 first panel). Similarly, in (most) HIV-infected individuals, a distinct downregulation of LAIR-1 (known to inhibit cytolytic function) was seen on T-, B-, and dendritic cells (Fig. 5 and Supplementary Fig. S2 second panel). KLRG-1, an NK cell inhibitor, was increased on CD4 T-cells in HIV-infected individuals, reflecting an exhausted phenotype (Fig. 5 and Supplementary Fig. S2 third panel). On dendritic cells, no difference was seen in the expression of ILT4, a molecule that inhibits CTL function (Fig. 5 and Supplementary Fig. S2 fourth panel). Finally, IREM-1, which inhibits TLR signaling in dendritic cells (Fig. 5 and Supplementary Fig. S2 fifth panel), was the only receptor that showed a significant effect of early treatment. However, instead of mirroring the healthy controls, the expression of this receptor was decreased even more in treated compared to untreated individuals (median 78% vs. 88% of dendritic cells positive for IREM-1, respectively). The significance of this finding was lost after

correcting for multiple testing. Also the level of PD-1 expression did not differ between patients who did and those who did not receive treatment during PHI ($p=0.30$, data not shown).

No effect on GALT T-cell depletion and T-cell activation after early treatment

To study whether treatment in PHI could overcome the rapid depletion of T-cells from the GALT that is typically observed in untreated HIV infection, the level of expression of the gut homing receptor $\alpha 4\beta 7$ on CD4 and CD8 T-cells of treated and untreated individuals was evaluated at viral set point (Fig. 6A and B). Both treated and untreated HIV-infected individuals exhibited a marked decrease in the levels of $\alpha 4\beta 7^{\text{high}}$ cells in the naive CD4 T-cell compartment compared to healthy controls. Also, in the effector CD4 T-cell compartment, the percentage of $\alpha 4\beta 7$ expression

FIG. 5. No changes in regulatory and inhibitory molecules after early treatment. To assess the level of inhibitory receptor expression, the % (y-axis) of inhibitory receptors on CD4 T-cells, CD8 T-cells, B-cells, natural killer (NK) cells, and dendritic cells was determined. The parameters were compared between healthy individuals (HD, $n=13$, black), treated individuals (T, $n=8$, dark gray), and untreated individuals (UT, $n=10$, white). Bars represent the median with range. * $p=0.01-0.05$; ** $p=0.01-0.001$; *** $p<0.001$.

FIG. 6. No differences in gut homing marker expression between treated and untreated patients. The expression of gut homing marker a4b7 within the T-cell compartments (naïve, central memory, effector memory, and effector) on CD4 T-cells (A) and CD8 T-cells (B) is depicted. The mean fluorescent intensity of a4b7 was compared for treated (T, $n=8$, light gray), untreated (UT, $n=10$, dark gray), and healthy (HD, $n=12$, black) individuals. Bars represent the median with range. * $p=0.05-0.01$; ** $p=0.01-0.001$.

was lower in the treated HIV-infected individuals. Despite the lower viral set point in patients treated during PHI, there were no significant differences between treated and untreated individuals in the levels of $\alpha 4\beta 7^{\text{high}}$ cells, suggesting that treatment did not lead to the preservation of $\alpha 4\beta 7^{\text{high}}$ CD4 T-cells. In contrast, no significant differences were found between HIV-infected individuals and healthy donors in $\alpha 4\beta 7$ expression on CD8 T-cells.

Because immune activation levels are known to be even more predictive of the rate of disease progression than plasma viral load, the study analyzed whether early treatment affected the overall level of immune activation by measuring CD38 and HLA-DR expression on T-cells. No differences were found between treated and untreated individuals in the expression of CD38 and HLA-DR on CD4 or CD8 T-cells at viral set point (data not shown).

Discussion

This study investigated whether the reported beneficial effect of treatment during PHI (13) was caused by preservation of immunological responses, and whether early treatment could overcome the rapid depletion of CD4 T-cells from the GALT, and its immune activating effect. The data suggest that treatment during PHI does not induce differences at the level of (a) effector T-cell formation or replication capacity of the T-cells; (b) polyfunctionality of

HIV-specific CD8 T-cells; (c) expression of inhibitory receptors (Pecan-1, LAIR, ILT4, IREM-1, KLRG-1) on CD4 and CD8 T-cells, B-cells, NK cells, and dendritic cells; (d) preservation of CD4 T-cells in the GALT; and (e) immune activation. A modestly higher cytolytic capacity of CD4 T-cells was observed in patients at viral set point receiving early treatment.

The observation that early treatment did not decrease the level of senescence of the effector T-cells at viral set point is quite surprising, as previously chronic antigen exposure was shown to lead to a terminally differentiated phenotype of CD4 and CD8 T-cells, with a diminished functional proliferative capacity (4). In addition, after correction for multiple testing, also inhibitory receptor expression levels on immune cells were not significantly altered in treated compared to untreated individuals. Formally, the possibility cannot be excluded that the immune parameters that were measured were initially preserved by early treatment but regained the features of a disrupted immune response rapidly after TI.

Along the same line, the data suggest that treatment in PHI individuals did not preserve the quality of HIV-specific CD8 T-cell responses in terms of their cytokine polyfunctionality. So far, not *one single* function of HIV-specific T-cells has been proven to correlate with control of HIV infection (23). The current consensus is that the more HIV-specific functions a CD8 T-cell performs, the more

protective it is. Indeed, HIV-specific CD8 T-cells of long-term nonprogressors have been shown to be more prone to perform five functions (IL-2, IFN- γ , TNF- α , MIP-1 β , and CD107a) simultaneously than HIV progressors (2). However, neither the separate functions nor the combined functions of CD8 T-cells were better preserved in individuals treated during PHI.

Finally, early treatment had no effect on preservation of CD4 T-cells in the GALT. As there is an acute and severe depletion of CD4 T-cells in the GALT in PHI, it was hypothesized that treatment during PHI would prevent this. All individuals in the Primo-SHM trial were recruited early after infection, at least within 100 days of HIV infection and 73% of the individuals even within 30 days. However, previous work in SIV infection has shown that severe depletion of CD4 T-cells in the GALT was seen within days and not weeks (32). Therefore, it is possible that in the present study population, depletion may already have occurred before the start of treatment, which could explain why no differences were seen between treated and untreated PHI.

Jointly, gut permeability with subsequent translocation of microbial products such as LPS and HIV itself are thought to be responsible for systemic immune activation in untreated HIV infection (16). Indeed, immune activation has been shown to be an even stronger predictor of disease progression than viral load. Thus, it was hypothesized that the delayed re-initiation of cART, as was seen in the Primo-SHM trial (13), might be caused by a diminished level of immune activation. However, neither the CD4 nor CD8 T-cell activation was diminished 36 weeks after TI in the study population.

The only significant effect of early treatment that was identified was a slightly higher cytolytic functionality of CD4 T-cells in early treated individuals. In the existing literature, increased granzyme A production after HIV-specific stimulation was shown to be predictive of better clinical outcome (30). Moreover, the cytolytic response is thought to be more antiviral when producing a combination of various granzymes and perforin against HIV gag (30). In addition, proliferated HIV-specific CD8 T-cells, but not CD4 T-cells of long-term nonprogressors, have previously been shown to be more prone to produce perforin in comparison with CD8 T-cells of progressors (22). In contrast, elevated levels of perforin and granzyme B expression were found in the CD4 but not in the CD8 T-cell pool. Though not completely in line with existing data, similarly the present findings hint at the importance of highly functional cytotoxic CD4 T-cells.

While a modest impact of treatment in PHI was found, recently other groups have found far more profound differences in immunological results after early treatment (6,28). There are two possible explanations. First, it might be the timing of the treatment during PHI. Paci *et al.* showed that the progression of HIV markers corresponded to the timing—that is, Fiebig's stage—whereat the treatment was initiated (23,24). Second, early treatment that persists over a longer period of time has been shown to be more effective (6,28). In the VISCONTI trial, around 15% of individuals treated during PHI maintained viral control 12 months after TI. These individuals had been treated for a median of 36 months. It has been shown that immunological characteristics of predominantly CD4 and CD8 T-cells are markedly

improved after prolonged periods of treatment initiated during PHI (7,27). Therefore, even though a 24–60-week treatment period during PHI caused a one log decrease in viral load at viral load set point in the PRIMO-SHM trial, the effect was too modest to find more than moderate effects on the immune functions.

To broaden even further the scope of the innate and adaptive immune mechanism explored here, it would be fascinating to elucidate the effect of early treatment on cellular factors such as APOBEC3G, TRIM5 α , and tetherin, which also play an important role in the restriction of HIV-1. Another question that should be addressed in further research is whether the observed preservation of the cytotoxic capabilities of the CD4 T-cells after early treatment is the cause of a lower viral load set point in these individuals, or whether a lower viral load allows for the preservation of the cytotoxic capabilities of the CD4 T-cells.

In conclusion, these results show that early treatment during PHI has a limited impact on the immunological parameters measured. The observation that early treated individuals had CD4 T-cells with modestly higher cytolytic capabilities may be a result of the reduced viral load rather than the cause of the lower viral set point.

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