Proliferation responses to HIVp24 during antiretroviral therapy do not reflect improved immune phenotype or function

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Objective: To ascertain whether lymphoproliferation (LP) responses to HIVp24 in chronically infected patients treated with antiretroviral therapy (ART) predict an improved cytolytic T-cell phenotype or better *in vivo* immune function as measured by immunization responses.

Methods: HIV-infected patients who started ART during chronic infection and who achieved viral suppression (HIV-RNA < 400 copies/ml for > 12 months) were grouped by the presence of strong [stimulation index (SI) > 10; $n = 21$] or absent (SI < 3; $n = 18$) LP to HIV-core antigen. The two groups were compared for functional immune responses to vaccination with diphtheria-toxoid, tetanus-toxoid and keyhole-limpet-hemocyanin, frequency of circulating naive and memory CD4+ and CD8+ T lymphocytes, maturation phenotype and expression of cytolytic molecules on total and HIV-specific CD8+ T cells, and frequency of memory CD4+ T cells with intracellular HIV-mRNA. Group comparisons were analyzed by non-parametric Mann–Whitney tests. Proportions were estimated by Pearson's χ^2 analysis.

Results: There were no differences between the groups in immune responses to vaccination or in the numbers or phenotype of circulating T cells. In a subgroup of HLA-A2+ or B8+ patients, HIV-reactive CD8+ T cells in both groups had similar expression of perforin, granzyme A and T-cell maturation markers (CD27, CD28, CCR7, CD62L). However, patients with SI > 10 in response to HIVp24 tended to more often have high levels of circulating CD4+ T cells with intracellular HIV-1 mRNA than did patients with SI < 3.

Conclusion: Following long-standing suppression of viral replication on ART, the presence of HIV-1 specific T-helper proliferation responses does not correlate with improved indices of immune phenotype or function but may reflect relatively higher levels of HIV-expression.

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Introduction

In human-immunodeficiency-virus type-1 (HIV-1) infection, CD4+ T lymphocytes are both targets of viral infection and also may be an important component of the host immune defense against viral replication [1–3]. HIV-specific CD4+ T cells are preferentially infected by HIV-1 *in vivo* [4] and sustained viral replication is associated with diminished proliferation capacity [5] or depletion of HIV-specific CD4+ T cells [6]. HIV-1 specific immune responses in most patients are unable to control viral replication completely and prevent the progression to AIDS [7]. In contrast, the few chronically HIV-1 infected individuals who suppress viral replication for many years in the absence of antiviral drug therapy [referred to as long-term non-progressors (LTNP)], often exhibit strong CD4+ T-cell lymphoproliferative (LP) responses to HIV-core antigens [8]. These patients also maintain a high proliferative capacity of HIV-specific CD8+ T cells that is linked to enhanced effector cytotoxic T lymphocyte (CTL) function [9]. In most individuals with HIV-1 infection, there is an abundance of circulating HIV-specific CD8+ T cells [10]. As these CD8+ T cells are serial killers, it has been perplexing that at high frequency (estimated to be many fold greater than the numbers of infected cells actively replicating virus), they fail to control HIV replication and prevent disease progression in the absence of antiviral therapy. Most HIV-specific CD8+ T cells, as identified by tetramer labeling, are not cytotoxic, however, when tested directly *ex vivo*, and lack perforin, which is required for lysis of HIV-infected target cells [11–14]. In animal models of viral infection, CD4+ T cells may be required to sustain CD8+ cytolytic T-cell function [15]. CD4+ T-cell dysfunction has been proposed as a potential underpinning of cytolytic cell failure in HIV-1 disease [16–18].

Based on the characteristics of LTNPs, it has been widely inferred that restoring HIV-specific LP responses is key to restoring immune control over HIV-replication [8,17–19]. Some patients receiving antiretroviral therapy (ART) develop strong CD4+ T-cell LP responses to HIV-antigens after viral replication is suppressed on therapy [6,20,21]. The prognostic significance of developing strong LP responses on ART is unclear however.

This study was undertaken to ascertain if developing HIV-specific LP responses in patients with suppressed viral replication on ART is linked to improved immune function. We found that emerging LP responses to HIV-1 core antigen in persons with ART-induced control of HIV-1 replication were not associated with demonstrable enhancements of immune phenotype or function, but were associated with higher levels of intracellular HIV-1 RNA.

Patients and methods

Patients

HIV-1 infected patients followed at the John T. Carey Special Immunology Unit of University Hospitals of Cleveland with recent CD4+ T-cell counts above 250×10^6 cells/l and plasma HIV-RNA consistently below 400 copies/ml during the previous 12 months while treated with ART were eligible to participate if they exhibited either strong [stimulation index (SI) > 10] or absent (SI < 3) LP responses to HIVp24 antigen [22]. Individuals with an acute febrile illness, a history of adverse events after immunization, a history of cytokine therapy or anti-neoplastic chemotherapy and pregnant or nursing women were excluded. Informed consent was obtained from all patients in accordance with US Department of Health and Human Services guidelines. This study was approved by the institutional review boards of University Hospitals of Cleveland and the Center for Blood Research in Boston.

Immune phenotyping

Lymphocyte subsets were enumerated in freshly obtained whole blood using directly labeled murine monoclonal antibodies against CD3, CD4, CD8, CD28, CD45RA, CD45RO, CD62L, CD95, HLA-DR and CD38 (PharMingen, San Diego, California, USA) by three-color flow cytometry using AIDS Clinical Trials Group (ACTG) consensus methods [22]. Absolute lymphocyte counts were derived from complete blood counts and leukocyte differential counts. For phenotyping of HIV-specific CD8+ T cells, peripheral blood mononuclear cells (PBMC) were either freshly prepared from venous blood by Ficoll-Hypaque density sedimentation and were shipped frozen to the Center for Blood Research in Boston or cooled whole venous blood was shipped overnight from Cleveland to Boston (Z.X., B.H and J.L.) for isolation of PBMCs and further analysis. At the beginning of the study, staining of several paired

fresh and frozen samples was compared. The proportion of cells that stained above background for each of the markers was not significantly different in samples immediately frozen or shipped. HLA-A2 and B8 expressing subjects were identified by flow cytometric analysis with HLA-A2.1-specific mAb PA2.1 (kind gift of Herman Eisen, Massachusetts Institute of Technology) or HLA-B8 mAb from One Lambda, Inc. (Canoga Park, California, USA). Tetramers specific for A2.1 or B8-restricted epitopes in HIV*gag*, *pol* and *env*, cytomegalovirus (CMV) pp65 and Epstein–Barr virus (EBV) *bmlf1* and *bzlf1* were produced as described [23] or obtained from the NIAID Major Histocompatibility Complex (MHC) Tetramer Core Facility (Yerkes Regional Primate Facility, Atlanta, Georgia, USA) [24]. Tetramer-staining populations were analyzed for phenotypic properties if they represented at least 0.05% of CD8+ T cells and formed a clearly separated population on dot plots to allow unambiguous characterization. Tetramer-labeled PBMCs were externally stained with fluorescently conjugated monoclonal antibodies against CD8, CCR7, CD62L, CD27, CD28 or internally stained for granzyme A and perforin and analyzed by flow cytometry as previously described [11].

Antibody concentrations

Anti-tetanus, anti-diphtheria and anti-keyhole limpet haemocyanin (KLH) IgG antibodies were measured by enzyme immunoassays as described elsewhere [25].

Lymphoproliferation assays

Unfractioned PBMCs, prepared by Ficoll-Hypaque density sedimentation, were assayed for LP in response to antigens of HIVp24 (3.35 μg/ml, Protein Sciences Corporation, Meriden, Connecticut, USA), tetanus-toxoid (TT) (2 LFU/ml; Wyeth-Ayerst, Marieta, Pennsylvania, USA), diphtheria-toxoid (DT) (2 LFU/ml; Wyeth-Ayerst), *Candida albicans* (CASTA) (20 μg/ml; Greer Laboratories, Lenoir, North Carolina, USA) and KLH (10 μg/ml, KLH-ImmuneActivator; Intracel Corp., Rockville, Maryland, USA) by measurement of ^{3}H-thymidine incorporation after 5 days in culture (when LP to soluble antigen is virtually all attributable to CD4+ T cells; M. Lederman, unpublished). Results are expressed as the stimulation index (SI), defined as the ratio of the median counts per minute of quadruplicate cultures with antigen to the median counts per minute in culture medium without antigen. SI values < 1 were imputed a value of 1. The control wells for the HIVp24 assay contained culture supernatants from cells infected with baculovirus that do not express HIV-*gag* (Protein Sciences Corporation).

Delayed type hypersensitivity

Delayed type hypersensitivity (DTH) responses to antigens of tetanus-toxoid (0.08 LFU/0.1 ml; Tetanus Toxoid USP; Aventis Pasteur, Swiftwater, Pennsylvania, USA), KLH (0.05 mg/0.1 ml, KLH-ImmuneActivator) and *Candida albicans* (1 : 5000/0.1 ml; Candin Skin Test Antigen for Cellular Hypersensitivity, Allermed Laboratories, San Diego, California, USA), were measured 48–72 h after intradermal administration using the ballpoint-pen technique [26].

Immunizations

After baseline evaluations subjects were immunized with diphtheria/tetanus-toxoid (0.5 ml, Diphtheria/Tetanus Toxoid Fluid; Aventis Pasteur) by intramuscular injection and with KLH (0.1 mg/0.1 ml, KLH-ImmuneActivator; Intracel Corp.) by intradermal injection on days 3 and 31. Serologic responses and LP were measured twice before immunization and on days 17, 31 and 59. For baseline values, pre-immunization data from days 1 and 3 were combined. As previously defined and reported in a group that includes some of these subjects [27], responses to immunization were scored for LP, serum antibody, and DTH to each antigen. An LP response was defined if both the SI was $= 10$ and increased by 0.67 $\log_{10}$ above baseline, since only 11% of subjects had a stimulation index to the presumed neoantigen KLH > 10 prior to immunization and the standard deviation (SD) in SI to a control *Candida* antigen was 0.67 $\log_{10}$ in the absence of intervention. An antibody response required serum antibody levels above 0.1 U/ml and at least a four-fold increase above baseline. A DTH response was defined by at least 10 mm induration at the skin test site and at least a 6 mm increase from baseline, since the induration response to the control antigen *Candida* changed a mean (SD) of 2 (6) mm between the first and the second measurement.

Immune response score

To summarize functional immune responses to immunization with non-HIV antigens, an immune response score was calculated as described [27]. The score included the results of all evaluations (serum antibody, LP and DTH) after immunization. A response received one point and a non-response, zero points. All points were added to derive the score. For this analysis, all positive baseline responses (SI > 10, DTH > 10 mm, antibody > 0.1 U/ml) were excluded from consideration to generate a fractional response. The denominator represented the total possible number of responses a given participant could have developed and the numerator, the total number of responses that participant actually developed after immunization.

Plasma HIV-RNA

Plasma HIV-1 RNA was measured using quantitative HIV-1 RNA polymerase chain reaction assays (Amplicor HIV-1 MONITOR; Roche Molecular System, Branchburg, New Jersey, USA) with a lower limit of detection of 400 copies/ml.

Intracellular HIV-RNA staining

PBMC were separated from whole venous blood by Ficoll-Hypaque density sedimentation and were shipped frozen to Chicago (B.P.) for a blinded analysis. Cells (1×10^6) in duplicate samples were labelled with CD45RO-phycoerythrin and CD4-CyChrome (BD; PharMingen). Expression of intracellular HIV-1 mRNA was performed using the ViroTect In Cell HIV-1 detection System (Invirion Inc. Frankfort, Michigan, USA) [28]. We used a cut-off point of 1.5% infected CD4+ CD45RO+ T cells.

Statistical methods

Group comparisons were analyzed by non-parametric Mann–Whitney tests. Proportions were estimated by Pearson's χ^2 analysis.

Results

Patient characteristics

The median SI in response to HIVp24 was 1.4 [interquartile range (IQR), 1.3–2.1] in the group of patients with absent LP responses (group A; n = 18) versus a median SI of 22.0 (IQR, 14–30) in the group of patients with strong LP responses (group B; n = 21) to HIVp24 (Table 1). There was no difference between the groups in age, CD4+ T-cell nadirs, duration of treatment with highly antiretroviral therapy, duration of undetectable plasma HIV-RNA, history of previous AIDS-defining illnesses [29] or distribution of nucleoside reverse transcriptase inhibitor, non-nucleoside reverse transcriptase inhibitor or protease inhibitor therapies. According to our records, which include some patient-reported estimates, the median duration of HIV-infection prior to the initiation of ART was 4 years in group A patients and 4 years and 3 months in group B patients.

Median numbers of circulating absolute CD4+ and CD8+ T lymphocytes and T-lymphocyte subpopulations [naive CD4+ T cells (CD45RA+CD62L), memory CD4+ T cells (CD45RA-RO+) T cells, CD28+ CD4+ T cells and activated CD8+ T cells (HLA DR+ CD38+)] were also comparable (Table 1).

Phenotypes of HIV-reactive CD8+ T cells in the two groups are similar

To determine whether the development of LP responses to HIV-antigens influences the functional properties of CD8+ T cells and HIV-reactive CD8+ T cells, a detailed phenotypic analysis of total CD8+ T cells and HIV-peptide/tetramer-stained CD8+ T cells was performed by flow cytometry examining cytotoxic effector molecules (granzyme A, perforin) and cell surface markers (CD28, CD27, CCR7 and CD62L) of CD8+ T-cell differentiation. There were no significant differences in any of these indices between the groups (Table 2).

CD8+ T cells binding to HIV *gag*, *env*, or RT tetramers were identified in three Group A and four Group B patients with frequencies of 0.07–0.2% and 0.13–0.53% of total CD8+ T cells, respectively. Frequencies of circulating antiviral CD8+ T cells were similar in the two groups and were comparable in magnitude to results described elsewhere [30,31]. Proportions of total CD8+ T cells and HIV-peptide/tetramer binding CD8+ T cells expressing surface CD28, CD27, CCR7, and CD62L were similar in the two patient groups. The majority of HIV peptide-specific CD8+ T cells showed signs of past activation because they no longer expressed the cell surface

Table 1. Group characteristics and immune phenotypes of HIV-infected patients with absent or strong lymphoproliferation (LP) in response to HIVp24 antigen.

	HIVp24 LP SI < 3	HIVp24 LP SI > 10	*P*
n	18	21	
Median SI (HIVp24)	1.5 (1.3–2.1)	22 (14–30)	
Age (years)	41 (39–46)	38 (35–45)	NS
CD4+ nadir (cells × 10^6/l)	266 (105–498)	299 (197–417)	NS
Months HAART	44 (29–50)	41 (31–44)	NS
VL < 400 copies/ml (mo.)	38 (22–44)	35 (21–39)	NS
previous OIs (n (%))	5 (28)	5 (24)	NS
CD4+ (cells × 10^6/l)	744 (637–940)	705 (590–803)	NS
CD4+ CD45RA+CD62L+ (cells × 10^6/l)	262 (200–369)	298 (215–404)	NS
CD4+ CD45RA-RO+ (cells × 10^6/l)	417 (328–529)	358 (288–488)	NS
CD4+ CD28+ (cells × 10^6/l)	689 (602–781)	657 (567–724)	NS
CD8+ (cells × 10^6/l)	963 (751–1209)	964 (608–1230)	NS
CD8+ HLADR CD38+ (cells × 10^6/l)	93 (58–121)	114 (71–256)	NS

Numbers are median values with inter-quartile ranges in parenthesis. SI, stimulation index; HAART, highly active antiretroviral therapy; VL, viral load; OI, opportunistic infection.

Table 2. Detailed phenotypic analysis of CD8+ T lymphocytes and HIV-1 peptide/tetramer binding CD8+ T lymphocytes of HIV-1 infected patients with long-term suppression of viral replication and absent or strong lymphoproliferative responses to HIV p24 antigen.

	Total CD8+ T cells			HIV tet+ T cells		
	HIVp24 SI < 3, n = 3	HIVp24 SI > 10, n = 4	*P*	HIVp24 SI < 3, n = 3	HIVp24 SI > 10, n = 4	*P*
CD 28	34 (7–69)	33 (25–58)	NS	43 (25–65)	19 (0–42)	NS
CD 27	58 (11–95)	74 (44–93)	NS	77 (68–82)	86 (73–93)	NS
CCR7	8 (0–40)	9 (0–29)	NS	20 (0–40)	20 (6–35)	NS
CD 62L	12 (10–43)	25 (5–31)	NS	26 (0–40)	27 (22–32)	NS

Numbers are median percentages with ranges in parenthesis. SI, stimulation index.

markers CD28, CCR7 and CD62L and did express granzyme A, a cytolytic enzyme not found in naive cells [11]. Unlike cytolytic cells, most CD8+ T cells, as well as tetramer-staining CD8+ T cells, were CD27+ and did not stain for perforin, which is needed to deliver granzymes into target cells for lysis (Fig. 1). Expression of granzyme A without perforin is a common feature of circulating CD8+ T cells reactive with antigens of viruses that cause chronic infection such as EBV, CMV and HIV in both HIV-seronegative and seropositive donors [11,14,32]. A discordance between granzyme A and perforin expression in total CD8+ T cells and HIV-specific CD8+ T cells was found for both groups in the current study. Granzyme A and perforin were detected in 80 and 5% (respectively) of tetramer+ cells and 64 and 14% of all CD8+ T cells from group A patients and in 78 and 13% of tetramer+ cells and in 60 and 16% of total CD8- T cells from group B of patients. None of these intergroup differences were statistically significant.

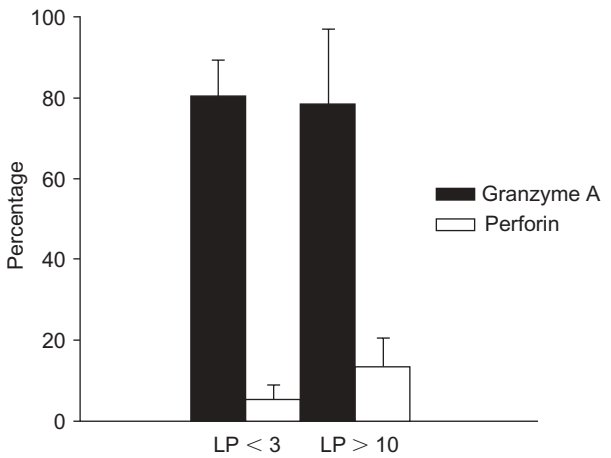

Fig. 1. A lymphoproliferative response to HIV p24 does not correlate with changes in HIV-specific CD8+ T-cell expression of cytolytic molecules. Percentages of HIV-1 specific CD8+ T cells expressing granzyme A and perforin in patients with absent (group A; SI < 3; n = 3) or strong (group B; SI > 10; n = 4) lymphoproliferation (LP) responses to HIV-*gag* antigen are shown. Differences between the groups are not significant.

Because HIV replication was well controlled pharmacologically in these donors, only a minority had sufficient numbers of HIV tetramer binding cells to meet criteria for accurate phenotypic characterization. When the analysis was expanded to include donors who had EBV or CMV tetramer-staining cells above 0.05% (17 in Group A, 13 in Group B), there were no significant differences in perforin or granzyme expression either in all of CD8+ T cells or in the EBV, CMV or HIV tetramer+ CD8+ T cells. Additionally, there was no evidence that the maturation phenotype of these antiviral CTLs was any better in persons with HIV-*gag*-specific LP responses than in persons without these responses (data not shown).

In vivo responses to vaccination are not enhanced in subjects with strong LP responses to HIV p24

Although phenotypic indices of CD8+ T-cell function were not significantly different in persons with and without HIV-*gag*-specific LP responses, we asked if CD4+ T-cell recognition of HIV-*gag*-antigen predicted the ability to mount better responses to immunization with three CD4+ T-cell dependent subunit vaccines. The two groups of patients were immunized with recall antigens (diptheria/tetanus toxoid) and with a neoantigen (KLH). Their immune responses to vaccination were assayed by measuring serum antibody, LP and DTH skin reactions. An immune response score (IRS) that gave equal weight to each of the responses to any of the antigens was generated for each subject. The immune responses after immunization to both neo- and recall antigens were similar in both groups, with total IRS 0.46 in group A and 0.47 in group B (Fig. 2). We also assayed LP in response to prevalent antigens, including *Candida albicans*, *Cytomegalovirus, Mycobacterium avium* complex, streptokinase and to pokeweed mitogen and found no differences in SIs when both groups were compared (data not shown).

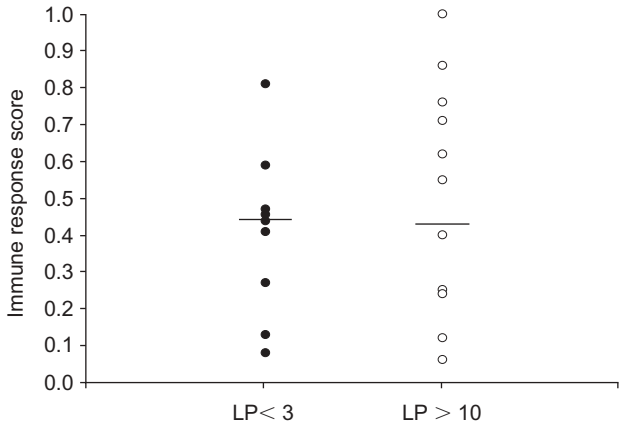

Fig. 2. Immune response scores to vaccination with recall and neo-antigens do not correlate with lymphoproliferation (LP) responses to p24. The scores represent the fraction of responses to a panel [LP, delayed type hypersensitivity (DTH), antibody] of functional immune assays that were analyzed following vaccination with tetanus-toxoid, diphtheria-toxoid and keyhole limpet haemocyanin. Bars represent median values.

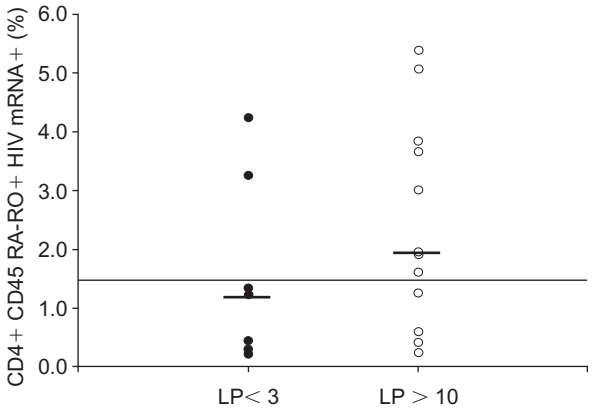

Fig. 3. A higher frequency of persistent intracellular viral replication was present in patients with lymphoproliferation (LP) in response to HIVp24 ($P < 0.05$). Bars represent median values of the percentage of CD4+ CD45+ RO+ memory cells that scored positive by *in situ* hybridization for HIV-1 *gag-pol* RNA (horizontal line at the 1.5% cutoff).

On the whole, LP responses to vaccine antigens increased after immunizations whereas responses to non-vaccine antigens remained stable. These data have been reported recently [27].

LP responses and cell-associated HIV-RNA

Because immune responsiveness was not different in the two groups of well-controlled subjects, we next asked whether differences in viral expression might underlie the differences in LP response between the groups, even though all subjects had achieved undetectable plasma virus (< 400 copies/ml) for at least 1 year. When HIV-RNA was assayed by *in situ* hybridization in cryopreserved CD45RO+ memory CD4+ T cells that had been obtained at the study initiation and prior to vaccination, more patients with strong LP responses to HIVp24 antigen had at least 1.5% of cells expressing HIV-RNA (nine of 13 patients in group B versus two of eight patients in group A) (Fig. 3). Thus the presence of a measurable LP response to HIV-1 *gag* antigen may reflect ongoing production of viral antigen within cells.

In patients with absent LP responses to HIVp24 the mean IRS was 0.44 in patients with HIV-RNA in < 1.5% of memory CD4+ T cells and 0.5 in patients with HIV-RNA in > 1.5% of memory CD4+ T cells. In patients with strong LP responses to HIVp24 the mean IRS was 0.41 in patients with HIV-RNA in < 1.5% of memory CD4+ T cells) and 0.54 in patients with HIV-RNA in > 1.5% of memory CD4+ T cells. These differences were not significant.

Discussion

While most untreated chronically HIV-infected patients lack LP responses to HIV-core antigen, many LTNP and patients treated with antiretroviral therapies early after acute infection exhibit strong T-cell proliferative responses to HIVp24 [8,17,33–35]. Although there is reason to suspect that CD4+ T-cell helper responses to HIV may help to maintain effective CD8+ T-cell-mediated protective immunity [8,18] this causal relationship has not yet been established.

Although earlier studies of ART had failed to show restoration of HIV-1-specific proliferation responses with therapy [22,36] more recently, as many has half of subjects who have had suppression of HIV-1 replication with ART demonstrate proliferation responses to HIV-1 core antigen [20,21,37,38]. In this setting, the significance of developing LP to HIV-core antigen is unclear and has not been investigated carefully.

We found no immunologic distinction between persons with strong and absent proliferation responses to HIVp24. Specifically, there was no difference in the immune phenotype of CD4+ or CD8+ T cells or in functional *in vivo* immune responses to vaccination with a panel of non-HIV neo- and recall-antigens in patients who exhibited weak or strong LP responses to HIV-core antigen following longstanding suppression of viral replication on ART. We also looked at markers that have been associated with CD8+ T-cell differentiation and function. Whereas most HIV-specific CD8+ T cells expressed granzyme A, very few in both of the patient groups stained for perforin, a molecule required for cytolysis of infected cells [11,14,39].

In the setting of long-standing therapeutic suppression of viral replication, few patients from either group had high enough frequencies of HIV-specific CD8+ T cells for detailed phenotypic analysis, thus limiting our analysis. It is also possible that differences in functional CTL activity could have been missed by these phenotypical analyses since cytolytic assays were not performed. In addition, unmeasured differences in subpopulations of T cells could have influenced the LP response to HIVp24 as recent evidence suggests that regulatory T cells may impair antigen responsiveness in HIV-infection [40,41].

Nevertheless, none of the phenotypic changes associated with CTL differentiation, distinguished the patients with HIV-specific LP responses from those without these responses. These results suggest that developing LP responses to HIV-core antigen after application of ART in chronic infection may not have the same prognostic significance as has been suggested for LTNPs, where p24 LP responses correlate with the viral setpoint [35] and also may predict the frequency of virus-specific CTL precursors [17].

There is strong evidence that ongoing viral replication directly and selectively suppresses CD4+ T-cell proliferation responses to HIV-1 antigens *in vitro* and *in vivo* [6,21]. In early combination ART studies when not all patients achieved 'complete' control of viral replication, HIV-specific LP responses were either not seen [42–44] or were infrequent [45–48]. In contrast, LP responses to HIVp24 antigen were found in a proportion (range, 28–70%) of patients when plasma HIV-RNA levels were more reliably suppressed [20,21, 37,38] .

Data from two therapeutic vaccine studies indicate that the induction of p24 proliferation responses in persons with ART-induced control of HIV replication does not appear to enhance HIV-specific CD8+ T-cell responses. In these studies, immunization with an inactivated envelope depleted HIV-1 vaccine that increased LP responses to *gag* antigen did not increase the number of HIV-reactive CD8+ T cells or the breadth of peptides they recognized [49,50] .

The preservation of HIV-1-specific LP may therefore be the result rather than the cause of control of viremia in chronic HIV-1 infection. This stated, the relationship between HIV-replication and CD4+ T-cell responses to HIV antigens may not be unimodal. Specifically, some degree of antigenic stimulation may be necessary to sustain detectable immune responses to *gag*-peptides [51–55]. In earlier studies, persistence of intracellular HIV-1 RNA in CD4+ CD45RO+ memory cells correlated with HIV-*gag*-specific LP in patients who suppressed viral replication with ART [56]. In this study, patients with strong LP responses to HIVp24 antigen were also more likely to have circulating CD4+ T-memory cells with detectable intracellular HIV-1 RNA than were patients without these LP responses. These results taken together are compatible with a model in which higher levels of viral replication decrease HIV-specific LP, whereas some lower level viral replication is needed to maintain sufficient numbers of functional HIV-specific T-helper cells to detect LP responses.

In summary, the development of HIV *gag*-specific lymphoproliferation in chronically HIV-1-infected patients who control viremia with ART does not seem to reflect an improvement in immune phenotype or function, but rather may reflect instead low levels of HIV antigen production. At this time, there is no clear evidence that the presence of these responses confers any clinical or functional advantage. As the possibility of eradication of HIV-1 infection with ART is unlikely [57], these results underscore the need for a better understanding of the correlates of immune protection to serve as a guide for attempts to augment HIV-specific immunity. Our results suggest caution in using induction of CD4+ T-cell lymphoproliferation responses as a goal for therapeutic interventions.

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