Journal of Clinical Immunology, Vol. 22, No. 6, November 2002 (©2002)

Functional Discrepancies in HIV-Specific $CD8^+$ T-Lymphocyte Populations Are Related to Plasma Virus Load

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Accepted: July 15, 2002

The potent ability of current antiretroviral drug regimens to control human immunodeficiency Virus-1 (HIV-1) replication, in conjunction with the clinical practice of structured therapeutic interruptions, provides a system in which virus levels are manipulated during a persistent infection in humans. Here, we exploit this system to examine the impact of variable plasma virus load (pVL) on the functionality of HIV-specific $CD8^+$ T-lymphocyte populations. Using both ELISpot methodology and intracellular cytokine staining for interferon (IFN)-γ to assess functional status, together with fluorochrome-labeled peptide–major histocompatibility complex (pMHC) class I tetramer analysis to detect the physical presence of $CD8^+$ T lymphocytes expressing cognate T-cell receptors (TCRs), we observed that the proportion of HIV-specific $CD8^+$ T lymphocytes capable of mounting an effector response to antigen challenge directly *ex vivo* is related to the kinetics of virus exposure. Specifically, (a) after prolonged suppression of pVL with antiretroviral therapy (ART), physical and functional measures of HIV-specific $CD8^+$ T-lymphocyte frequencies approximated; and (b) the percentage of functionally responsive cells in the HIV-specific $CD8^+$ T lymphocyte populations declined substantially when therapy was discontinued and pVL recrudesced in the same patients. These results corroborate and extend observations in animal models that describe nonresponsive $CD8^+$ T lymphocytes in the presence of high levels of antigen load and have implications for the interpretation of quantitative data generated by methods that rely on functional readouts.

KEY WORDS: $CD8^+$ T lymphocyte; HIV-1; peptide-MHC class I tetramer; intracellular cytokine staining.

INTRODUCTION

$CD8^+$ T lymphocytes play an important role in antitumor immunity and the control of infections with intracellular parasites through multiple effector mechanisms that are triggered by T-cell receptor (TCR)-mediated recognition of cognate peptide–major histocompatibility complex (pMHC) class I ligands on the target cell surface. The development of sensitive techniques to measure physical and functional aspects of antigen-specific $CD8^+$ T-lymphocyte populations at the single-cell level has led to substantial advances in our understanding of the role of these cells in disease states. Multimeric fluorochrome-labeled pMHC class I complexes allow the physical detection of $CD8^+$ T lymphocytes bearing TCRs specific for individual pMHC class I antigens (1). This technique can be combined with functional assays at the single cell level, such as flow cytometric analysis of antigen-induced intracellular cytokine production (2, 3), to assess the effector phenotype of antigen-specific $CD8^+$ T lymphocytes. This combined approach has revealed that there is marked phenotypic heterogeneity within $CD8^+$ T-

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0271-9142/02/1100-0363/0 © 2002 Plenum Publishing Corporation

Table I. Swiss HIV Cohort Study: Patient Details

Patient	On ART			Off ART		
	pVL[a]	Duration[b]	CD4 count[d]	pVL[a]	Duration[c]	CD4 count[d]
1	14	1015	669	31,656	112	523
2	<5	49	842	32,185	70	439
3	<14	98	916	13,788	336	914
4	<5	178	284	102,953	43	258
5	<5	858	749	35,354	246	705
6	<5	49	504	<5	14	471
7	<5	49	727	127,000	28	551
8	<5	709	591	171,000	224	262
9	<5	460	1450	817	225	664
10	<5	290	722	6,000	168	454
11	29	1273	666	nd	nd	nd
12	10	1337	852	789	84	542
13	31	611	582	18	14	nd
14	5	1175	366	3030	14	nd

[a]Plasma virus load (copies/ml).
[b]Duration of therapy (days).
[c]Duration of cessation of therapy (days).
[d]Total CD4 count (cells/mm^3).

lymphocyte populations specific for individual viral and tumor-derived pMHC class I antigens. In particular, it is clear that there can be discrepancies between functional and physical measurements of specific $CD8^+$ T lymphocytes. Many studies now indicate that a substantial proportion of cells within an antigen-specific $CD8^+$ T-lymphocyte population can appear functionally compromised, although the degree of dysfunction varies considerably both quantitatively and qualitatively (3–25). The factors that determine these differences, however, are poorly understood.

In this study, we use peptide–HLA class I tetramers to detect virus-specific $CD8^+$ T lymphocytes bearing appropriate TCRs, together with intracellular interferon (IFN)-γ staining and IFN-γ ELISpot analysis as readouts of functional response, to assess the effects of variable virus load on the level of responsiveness in HIV-specific $CD8^+$ T-lymphocyte populations in chronically infected patients undergoing interruptions of potent combination antiretroviral therapy (ART).

MATERIALS AND METHODS

Patients

All patients involved in this analysis are participants in the Swiss HIV Cohort Study and were recruited for the Swiss Spanish Intermittent Therapy Trial (SSITT). Patient details are summarized in Table I. In all cases, ART was initiated during the established chronic phase of HIV-1 infection. Informed consent was obtained from each patient.

Genotyping for HLA Class I

The HLA class I genotype of each patient was determined by polymerase chain reaction (PCR) using sequence-specific primers (PCR-SSP) (26).

Measurement of HIV-1 Virus Load

Virus load was quantified from cryopreserved plasma using the standard Amplicor HIV-1 monitor test, version 1.5 (Roche, Rotkreuz, Switzerland) with ultrasensitive modifications resulting in a level of detection of 50 copies HIV-1 RNA/ml or lower (27).

Cell Culture

For all experimental data shown from patient samples, cryopreserved peripheral blood leukocytes (PBL) were thawed and recovered in RPMI-1640 medium containing 10% fetal calf serum and supplemented with penicillin (100 IU/ml), streptomycin (100 μg/ml), and glutamine (2 mM). Recovery times were 1 hr prior to tetramer staining or intracellular cytokine staining (ICS) for IFN-γ, and 16 hr prior to IFN-γ ELISpot analysis.

Peptide–HLA Class I Tetrameric Complexes

Phycoerythrin (PE)-labeled peptide–HLA class I tetrameric complexes were produced as described previously (1, 28). Specific tetramers comprised the following peptide-HLA class I combinations: FLKEKGGL (Nef; residues 90–97) with HLA B8; KAFSPEVIPMF (p24

Gag; residues 30–40) with HLA B57; IPRRIRQGL (gp41 Env; residues 333–341) with HLA B7; SLYNTVATL (p17 Gag; residues 77–85) with HLA A2; and ILKEPVHGV (Pol RT; residues 464–472) with HLA A2. All residues are numbered with reference to HIV-IIIB.

Physical Analysis of HIV-Specific CD8$^+$ T-Lymphocyte Populations

Cells were stained with PE-labeled peptide-HLA class I tetrameric complexes for 30 min at 37°C, washed in PBS containing 0.1% sodium azide, stained with PerCP-labeled anti-CD8 (Becton-Dickinson, England), and FITC-labeled anti-CD38 (Dako Ltd., England) monoclonal antibodies for 20 min on ice, washed again, and then fixed with PBS containing 1% paraformaldehyde (29). Stained cells were analyzed using a Becton-Dickinson Calibur flow cytometer with CellQuest software.

Functional Analysis of HIV-Specific CD8$^+$ T-Lymphocyte Populations

Functional CD8$^+$ T lymphocyte responses to specific peptide antigens were quantified directly *ex vivo* using either IFN-γ ELISpot or IFN-γ ICS as described previously (2, 30–32). For IFN-γ ELISpot analysis, synthetic peptide epitopes matched to the patients' individual HLA class I type were used at a concentration of 2 μM; PHA (5 μg/ml) was included as the positive control, with medium alone as the negative control. Spot quantification was performed using an ELISpot reader [Autoimmun Diagnostika (AID), Germany, software version 2.1]. Results are expressed as spot-forming cells (SFC) per 10^6 PBL. Background values were subtracted from the specific response before normalization. A positive response to a given peptide epitope was defined as SFC/10^6 PBL >3 SD above background and was generally >50 SFC/10^6 total PBL. All assays were performed in duplicate. In patients 1 and 5, HIV-specific CD4$^+$ T-lymphocyte helper responses were determined similarly using IFN-γ ELISpot analysis; these assays were performed with PBL depleted of CD8$^+$ T lymphocytes as previously described (32). For IFN-γ ICS, cells were stimulated with 4 μM cognate peptide in the presence of anti-CD28 and anti-CD49d monoclonal antibodies at 0.5 μg/ml (Becton-Dickinson, England) for 6 hr at 37°C. Negative controls were stimulated with anti-CD28 and anti-CD49d monoclonal antibodies alone in the absence of peptide; positive controls included PMA (50 ng/ml) and ionomycin (500 ng/ml) in place of peptide. Brefeldin A (Sigma, England) was added at 10 μg/ml for the final 5 hr. Cells were then fixed, permeabilized, and stained with PerCP-labeled anti-CD8, PE-labeled anti-CD69, and FITC-labeled anti-IFN-γ monoclonal antibodies (Becton-Dickinson, England) prior to FACS analysis as above.

RESULTS

We studied HIV-specific CD8$^+$ T-lymphocyte responses in a large cohort of patients (n = 133) undergoing structured therapeutic interruption (STI) according to the SSITT protocol (described in Fig. 1) (33). Initially, HIV-specific CD8$^+$ T lymphocyte responses were identified and quantified with IFN-γ ELISpot screening assays using a panel of previously described optimal HIV-derived peptide epitopes matched to each individual patient's HLA class I genotype (data not shown). Based on the frequency of responses detected by IFN-γ ELISpot analysis and the availability of further PBL samples, HIV-specific CD8$^+$ T-lymphocyte populations were further characterized both physically and functionally in a selected subset of individuals.

The effects of STI on HIV epitope-specific CD8$^+$ T lymphocyte frequencies measured both physically with peptide-HLA class I tetramer staining and functionally with IFN-γ ELISpot analysis, together with concomitant changes in pVL, total CD4$^+$ T-lymphocyte counts and CD38 expression on the tetramer-positive CD8$^+$ T-lymphocyte populations, are shown for six representative individuals in Fig. 1A. In patients B, C, E, and F (SLYNTVATL-HLA A2), the increase in pVL that followed therapy interruption was associated with an increase in HIV epitope-specific CD8$^+$ T-lymphocyte frequencies determined with peptide-HLA class I tetramer staining. This was not observed in patients A, D, and F (IPRRIRQGL-HLA B7); in these cases, the size of the HIV-specific tetramer-positive CD8$^+$ T-lymphocyte populations either decreased or failed to increase when ART was discontinued (Fig. 1A). In all patients, with the exception of patient E, the increases in pVL were associated with up-regulation of the activation marker CD38 on the surface of HIV-specific CD8$^+$ T lymphocytes (Fig. 1A). Interestingly, the frequencies of functional HIV epitope-specific CD8$^+$ T lymphocytes, measured by extracellular IFN-γ release in ELISpot assays after antigenic stimulation *ex vivo,* seemed to be influenced more dramatically by the level of viral replication than the corresponding tetramer-determined population. In all patients, the frequencies of IFN-γ-secreting HIV epitope-specific CD8$^+$ T lymphocytes decreased substantially when ART was stopped and pVL increased (Fig. 1A). Interestingly, in patients B, C, and D, the

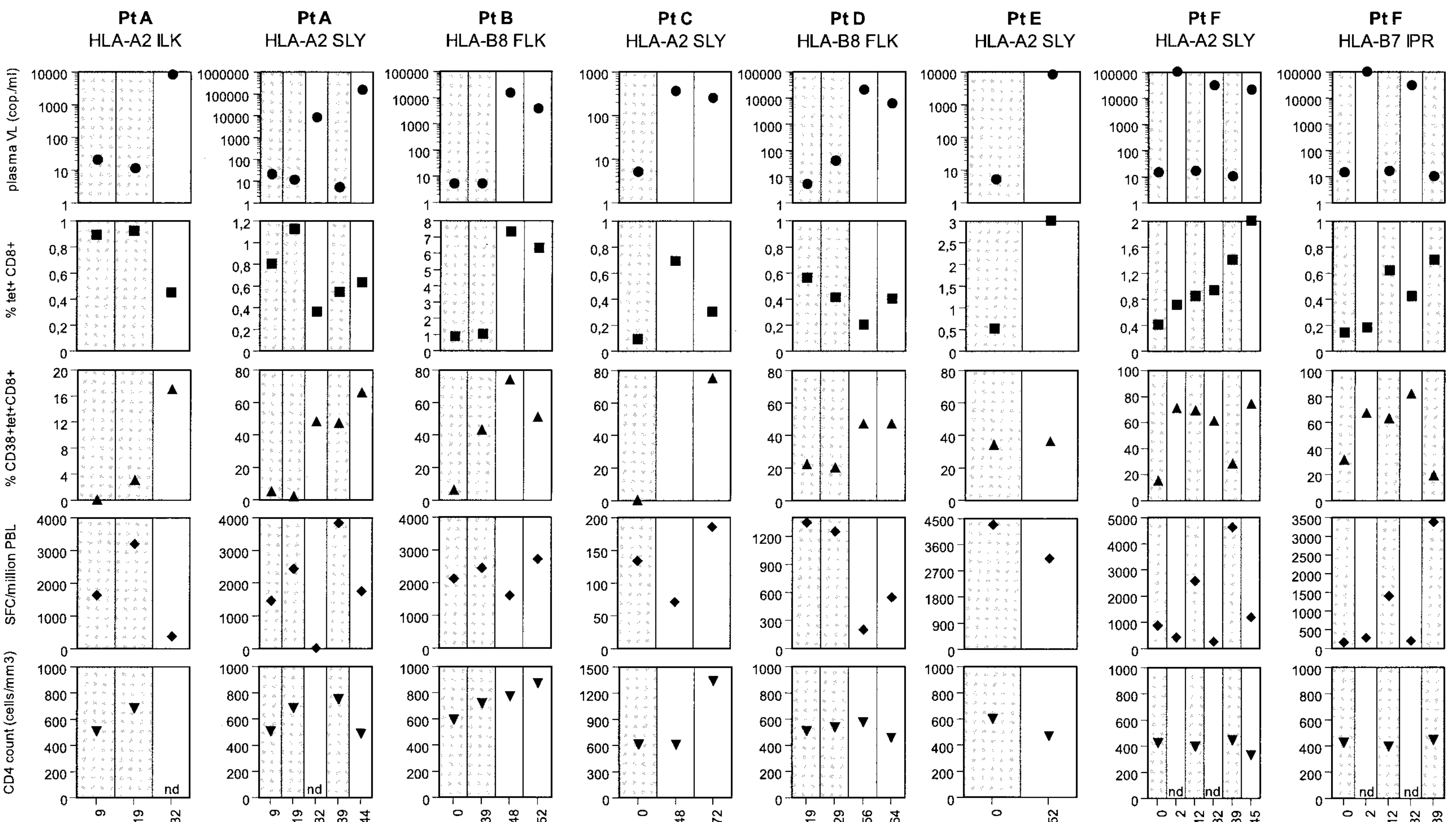

Fig. 1. Physical and functional measures of HIV-specific CD8$^+$ T lymphocyte frequencies, using peptide–HLA class I tetramer staining and IFN-γ ELISpot analysis, respectively, in patients undergoing structured therapeutic interruption (A) and in patients commencing continuous ART (B). Gray shaded areas represent time points on therapy; white shaded areas represent time points off therapy. (A) HIV-specific CD8$^+$ T-lymphocyte frequencies were analyzed in six patients (A–F) with chronic HIV-1 infection who participated in the Swiss–Spanish intermittent therapy trial (SSITT) (33). Patients enrolled in SSITT were on continuous ART, with plasma VL <50 copies/ml, for a minimum of 6 months prior to therapy interruption. The total CD4 T-cell count was >300 cells/mm^3 at enrollment. According to the SSITT treatment schedule, ART was stopped at week 0 for 2 weeks, and thereafter resumed for 8 weeks (week 2 to week 10). This cycle was repeated four times and ART was then stopped at week 40. The *x*-axis labels correspond to the number of weeks in the SSITT protocol. In the upper panels, plasma VL is shown for each patient at each time point. The frequencies of CD8$^+$ T lymphocytes staining with HIV-derived peptide–HLA class I tetramers is shown in the second row of panels; CD38 expression in these populations is shown in the third row. Corresponding functional HIV-specific CD8$^+$ T-cell frequencies, determined by IFN-γ ELISpot analysis, are shown in the fourth row. The bottom row shows total CD4 T-cell counts, where available, for the different time points. Patient identity and the HIV-derived peptide epitopes tested are shown at the top of each column. (B) Similar longitudinal analysis of HIV-specific CD8$^+$ T-lymphocyte frequencies in two patients (G and H) with chronic HIV-1 infection commencing continuous ART. Patients G and H had completed the SSITT protocol, and reinitiated treatment at week 52. The *x*-axis labels refer to week numbers according to the SSITT protocol. The data are formatted as in (A) above.

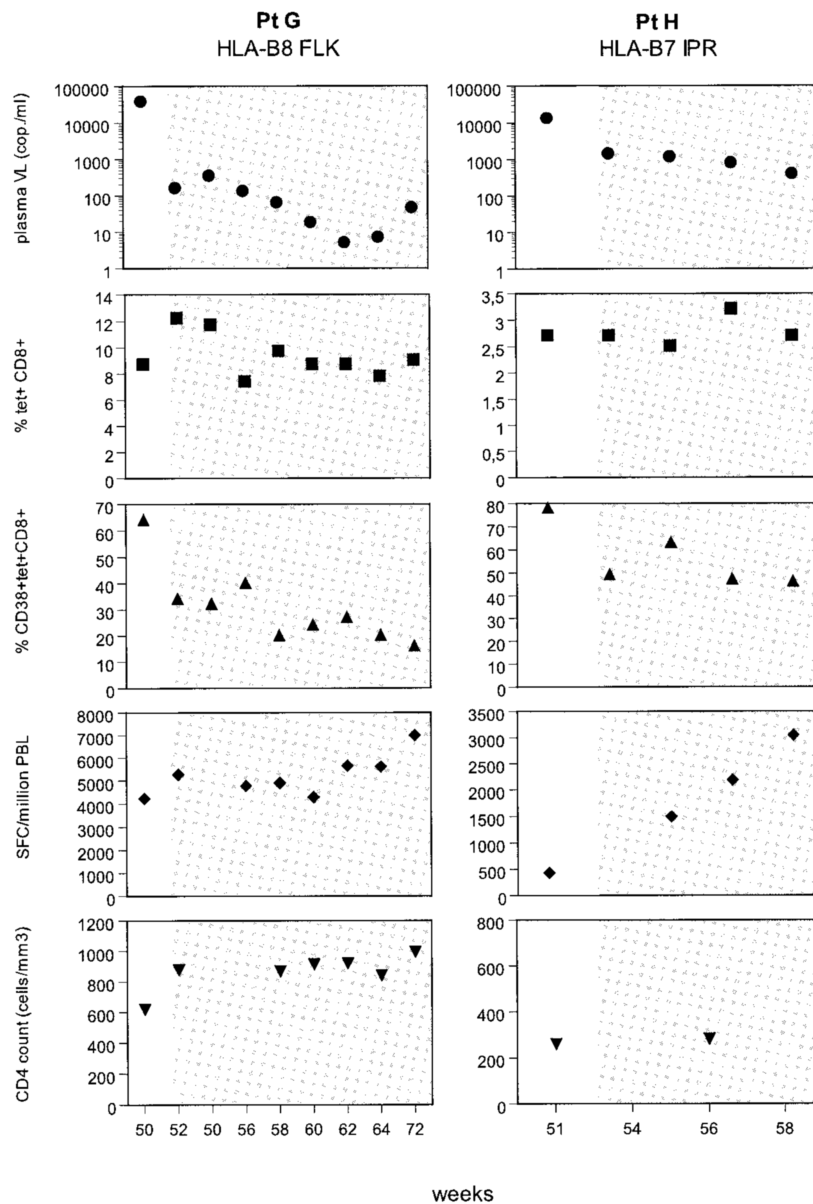

Fig. 1. (*Continued*).

functionality of HIV epitope-specific CD8^{+} T lymphocytes increased after prolonged exposure to viral replication; this functional increase was associated with a decrease in plasma VL in all three cases (Fig. 1A).

A similar analysis in two representative patients commencing continuous ART is shown in Fig. 1B. Initiation of ART resulted in a substantial reduction in pVL in both cases. During the period of observation, the frequencies of HIV epitope-specific CD8^{+} T lymphocytes that could be detected with peptide-HLA class I tetramer staining remained relatively stable, although there was a general decrease in CD38 expression on the surface of these cells over time (Fig. 1B). In contrast, the size of the functionally responsive CD8^{+} T lymphocyte populations specific for the same epitopes increased with time on ART (Fig. 1B).

These observations suggested a relationship between pVL and functionality within HIV-specific CD8^{+} T lymphocyte populations. To consolidate this observation, we studied a further 14 patients using IFN-γ ICS as an alternative measure of functionality; this experimental approach to the detection of functional responses is more

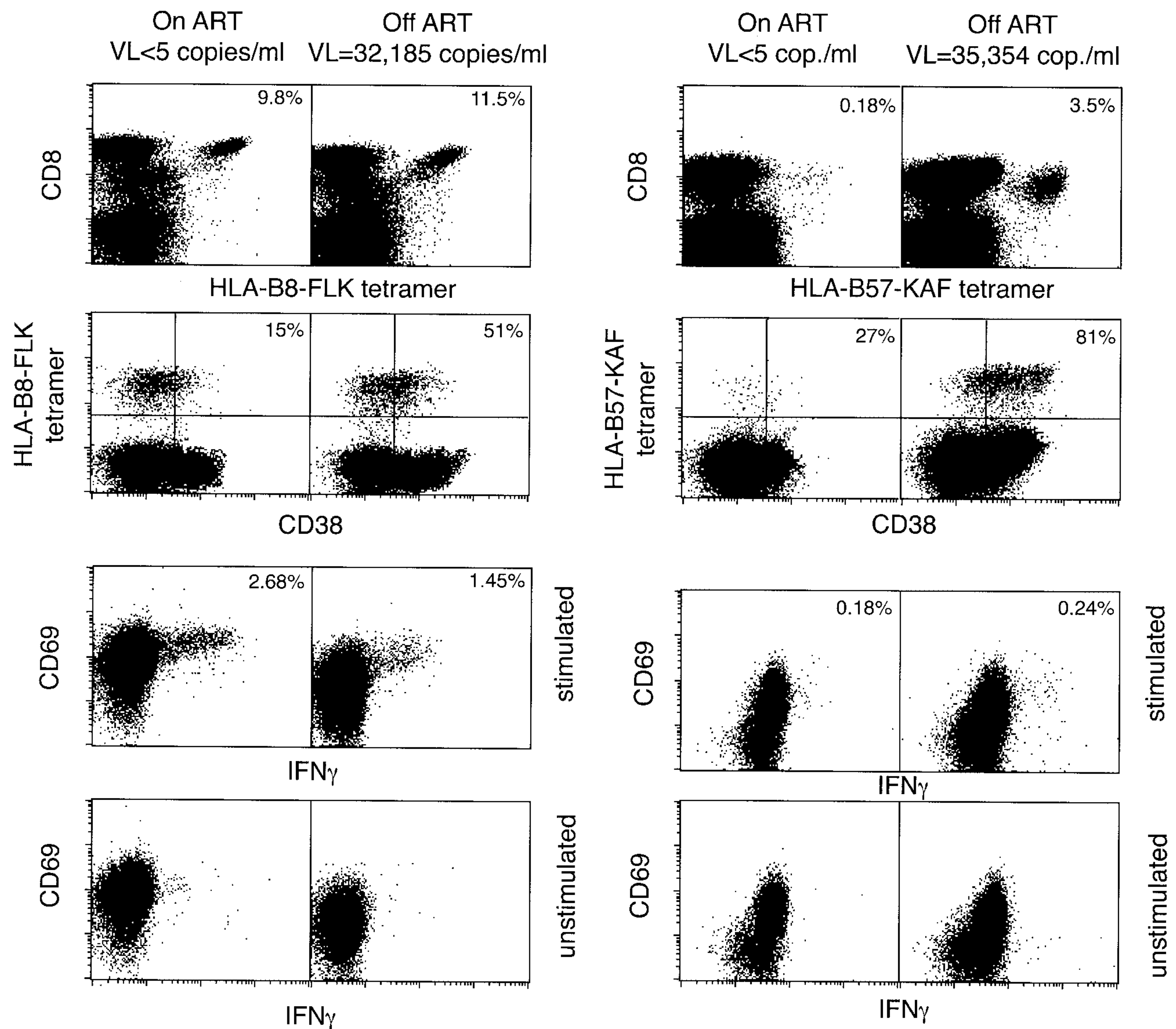

Fig. 2. *Ex vivo* phenotypic and functional characterization of HIV-specific $CD8^+$ T lymphocytes with flow cytometric methods. Patient samples were stained directly *ex vivo* with tetrameric complexes: (A) HLA B8-FLKEKGGL for patient 2; (B) HLA B57-KAFSPEVIPMF for patient 5. Staining with tetramer and anti-CD8 is shown in the upper FACS plots (inset numbers indicate the percentage of $CD8^+$ $tetramer^+$ T lymphocytes). The second row shows staining with tetramer and anti-CD38 (gated on $CD8^+$ T lymphocytes; numbers indicate the percentage of $CD8^+$ $tetramer^+$ $CD38^+$ T lymphocytes). In the lower two rows, intracellular stainings for IFN-γ, gated on $CD8^+$ T lymphocytes, are shown. The third row shows stainings for intracellular IFN-γ and CD69 after stimulation with the cognate peptide (FLKEKGGL for patient 2 and KAFSPEVIPMF for patient 5); numbers indicate the precentage of $CD8^+CD69^+IFN\text{-}\gamma^+$ T lymphocytes. The bottom row shows equivalent stainings for intracellular IFN-γ and CD69 without prior antigenic stimulation.

directly comparable to tetramer-based estimations of epitope-specific $CD8^+$ T-lymphocyte frequencies (12). Each of these 14 patients had been treated successfully with ART, and subsequently discontinued therapy for variable periods of time (Table I). We used flow cytometry to quantify the immunodominant responses in these patients both physically by direct *ex vivo* staining with the relevant peptide–HLA class I tetrameric complexes, and functionally by detection of antigen-induced intracellular IFNγ production. For each patient, we performed this analysis at two separate time points: (a) while on ART with pVL suppressed to <50 copies HIV-1 RNA/ml, and (b) after ART had been stopped (Table I).

Representative data from two individuals are shown in Fig. 2. Patient 2 was initially studied after a period of 49

days on ART. Plasma virus load (pVL) was suppressed to <5 copies HIV-1 RNA/ml at the time of analysis and the total $CD4^+$ T-lymphocyte count was 842×10^6/liter. The dominant HIV-specific $CD8^+$ T-lymphocyte response detected during IFN-γ ELISpot screening was directed against the HLA B8-restricted Nef-derived epitope FLKEKGGL (residues 90–97). Using the cognate peptide–HLA class I tetramer, 9.8% of the total $CD8^+$ T-lymphocyte population from patient 2 was found to be FLKEKGGL-specific at this time; 15% of these specific $CD8^+$ T lymphocytes expressed the activation marker CD38 (Fig. 2A). Only 2.68% of the total $CD8^+$ T-lymphocyte population mounted a detectable functional response to this epitope using IFN-γ ICS after stimulation with the FLKEKGGL peptide (Fig. 2A). Patient 2 subsequently discontinued ART. After 70 days without ART, pVL was 32,185 copies HIV-1 RNA/ml and the total $CD4^+$ T-lymphocyte count had declined to 439×10^6/liter. The frequency of the FLKEKGGL-specific response determined physically by peptide-HLA class I tetramer staining had increased to 11.5% of the total $CD8^+$ T-lymphocyte population; 51% of these specific $CD8^+$ T lymphocytes expressed CD38, consistent with increased activation in response to rising levels of antigen *in vivo* (Fig. 2A). However, a corresponding increase in the frequency of $CD8^+$ T lymphocytes capable of mounting a functional response to this epitope was not observed. In the ICS assay, only 1.45% of $CD8^+$ T lymphocytes produced IFN-γ when stimulated with the FLKEKGGL peptide (Fig. 2A). A similar increased disparity between physical and functional measures of HIV-specific $CD8^+$ T lymphocytes in response to rising pVL levels was observed in patient 5 (Fig. 2B). The dominant response in this individual was directed against the HLA B57-restricted p24 Gag-derived epitope KAFSPEVIPMF (residues 30–40). After 858 days on ART, pVL was suppressed to < 5 copies HIV-1 RNA/ml and the total $CD4^+$ T lymphocyte count was 749×10^6/liter. At this time, 0.18% of the total $CD8^+$ T-lymphocyte population stained with the specific peptide-HLA class I tetramer; 27% of the tetramer-positive $CD8^+$ T lymphocytes expressed CD38 (Fig. 2B). An identical proportion of the $CD8^+$ T-lymphocyte population responded functionally to stimulation with KAFSPEVIPMF peptide in the IFN-γ ICS assay (Fig. 2B). After 246 days without ART, pVL was 35,354 copies HIV-1 RNA/ml and the total $CD4^+$ T-lymphocyte count was 705×10^6/liter. The frequency of tetramer-positive cells measured at this time had increased to 3.5% of the total $CD8^+$ T lymphocyte population; consistent with enhanced activation, 81% of these specific $CD8^+$ T lymphocytes expressed CD38 (Fig. 2B). In contrast, there was no corresponding increase in the number of cells responding functionally to this epitope. Only 0.24% of the total $CD8^+$ T-lymphocyte population produced IFN-γ in response to *ex vivo* stimulation with the cognate peptide (Fig. 2B).

Similar analyses were performed in a further 12 patients (Table I, Fig. 3). The results indicated two consistent findings. First, physical and functional measures of HIV-specific $CD8^+$ T lymphocytes, using peptide–HLA class I tetramer staining and IFN-γ ICS, respectively, approximate after prolonged suppression of viral replication during ART. The ratio of frequencies detected by these methods expressed as IFN-γ^+/tetramer$^+$ $CD8^+$ T lymphocytes reached 100% only in those patients who had received ART for a period of 15 months or more (n = 5/6) (Fig. 3). Second, the IFN-γ^+/tetramer$^+$ ratio in HIV-specific $CD8^+$ T-lymphocyte populations decreased when ART was discontinued and virus recrudesced (Fig. 3). This decline in functional reactivity was most apparent in patients with large viral rebounds and less marked in those with smaller increases in pVL. These differences indicate that the observed cellular effects are virus-specific and not a general consequence of ART. Consistent with this, no such patterns of functional disturbance were detected in $CD8^+$ T lymphocytes stimulated with PMA/ionomycin as a positive control in each case (Fig. 3). These observations corroborate the tetramer/IFN-γ ELISpot data presented above (Fig. 1), and indicate that the degree of discrepancy between the number of $CD8^+$ T lymphocytes physically bearing antigen-specific TCRs and the number of such cells capable of responding functionally to the cognate peptide epitope directly *ex vivo* is affected by changes in pVL.

DISCUSSION

Technical advances have enabled the detailed phenotypic analysis of $CD8^+$ T lymphocytes specific for individual pMHC class I antigens directly *ex vivo*. Studies in a variety of systems indicate that not all $CD8^+$ T lymphocytes within a given antigen-specific population necessarily respond functionally to stimulation with appropriately presented cognate peptide, despite the expression of TCRs in sufficient numbers on the cell surface to engage and internalize peptide–MHC class I tetrameric complexes. However, the factors that determine the level of functional responsiveness within such antigen-specific $CD8^+$ T-lymphocyte populations are not well defined. Here, we exploited the efficient modulation of virus replication by ART in chronic HIV-1 infection to examine $CD8^+$ T-lymphocyte populations responding to rapid changes in pVL. We observed that the function-

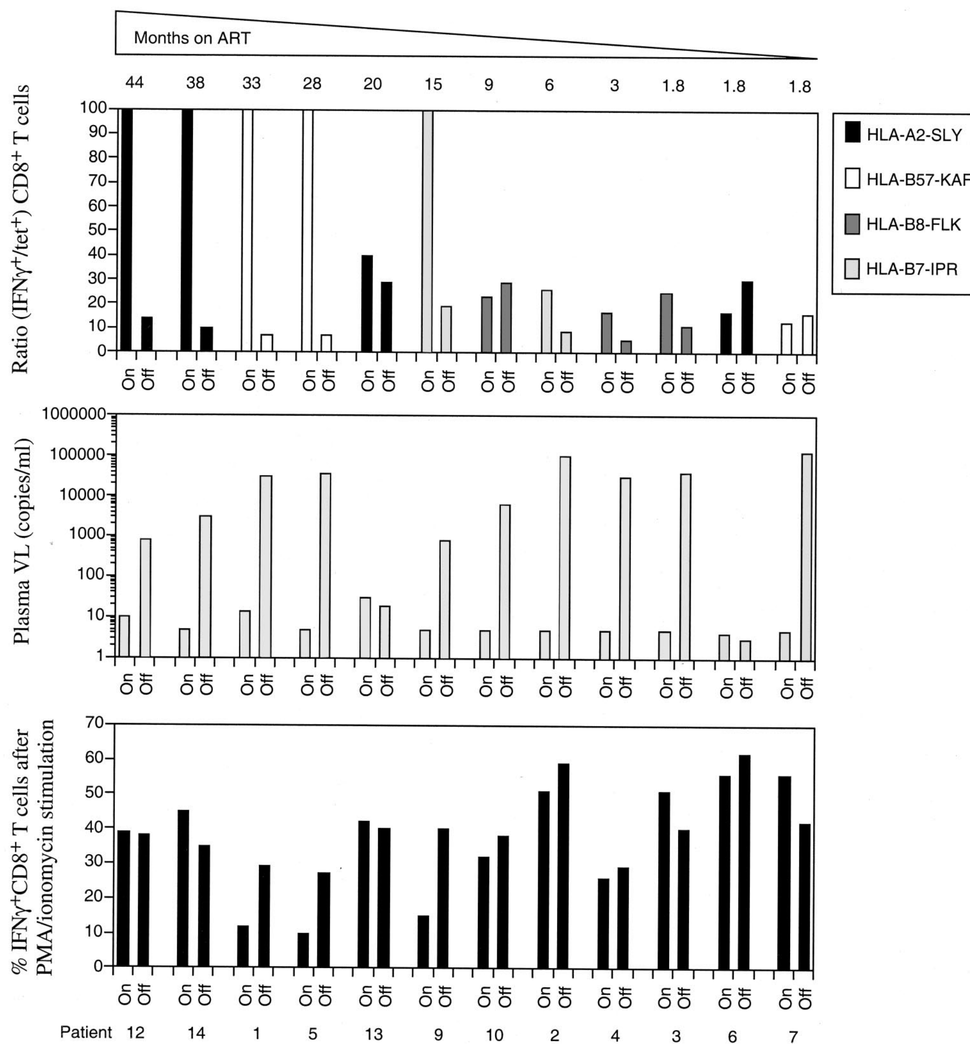

Fig. 3. Flow cytometric quantification of HIV-specific $CD8^+$ T-lymphocyte populations in consecutive samples from patients on ART who subsequently discontinue treatment. In 14 individuals, HIV epitope-specific $CD8^+$ T-lymphocyte responses were measured both physically, by staining PBL with the relevant peptide-HLA class I tetramer, and functionally, by detection of peptide antigen-induced intracellular IFN-γ production. For 12 patients, two samples were analyzed: one sample collected while on ART with pVL suppressed to below the limit of detection (On) and another sample in the absence of ART, and hence in the presence of detectable viremia (Off). For the remaining two patients, only one sample was available: patient 11, "on" ART for 42 months, pVL 29 copies/ml, ratio IFN-γ^+/tet$^+$ $CD8^+$ T lymphocytes 0.34; patient 8, "off" ART for 7 months, pVL 171,000 copies/ml, ratio IFN-γ^+/tet$^+$ $CD8^+$ T lymphocytes 0.3. The ratio of $CD8^+$IFN-γ^+ T lymphocytes and $CD8^+$tet$^+$ T lymphocytes is plotted in the upper panel for the 12 patients with two available samples (cutoff 100% as drawn). The different bar shadings represent the assayed HIV-derived $CD8^+$ T-lymphocyte epitope. Patients are ordered along the *x*-axis according to the duration of ART before assessment of the first ("on") sample. The middle panel represents the pVL of each patient at the same time points. The lowest panel shows the percentage of $CD8^+$IFN-γ^+ T lymphocytes after non-antigen-specific stimulation with PMA/ionomyin of the same samples analyzed for antigen-specific responses.

ality of HIV-specific $CD8^+$ T-lymphocyte populations, determined by either IFN-γ ELISpot or IFN-γ ICS, reflects the degree of virus exposure. The proportion of apparently inert HIV-specific $CD8^+$ T lymphocytes detected by staining with multimeric peptide–HLA class I complexes increased in relation to the level of virus rebound during therapeutic interruptions. Further, this discrepancy between physical and functional measures of HIV-specific $CD8^+$ T lymphocytes declined with prolonged suppression of viral replication on ART.

Several explanations, which are not necessarily mutually exclusive, could account for these observations.

First, HIV-specific $CD8^+$ T lymphocytes might be rendered refractory, at least in terms of IFN-γ production, to subsequent antigen recognition events following each significant encounter with cognate pMHC class I. In this model, the detection of functionally inert $CD8^+$ T lymphocytes represents a consequence of cross-sectional sampling of an antigen-specific population. At any one time, the history of antigen exposure for each $CD8^+$ T lymphocyte detected with peptide-HLA class I tetramer is unknown and a chronological spectrum exists in the visualized population. Those cells that have recently encountered antigen will be refractory to direct *ex vivo* challenge, and this proportion of such apparently dysfunctional $CD8^+$ T lymphocytes will rise in relation to antigenic load as reflected in the pVL. However, this may be an oversimplification. The number of functionally inert cells in HIV-specific $CD8^+$ T-lymphocyte populations has been shown to remain relatively low ($<$50%) at equilibrium over a range of pVL from $<$50 to $>$100,000 copies RNA/ml (12). Thus, at steady state, it appears that a more uniform level of functionality is achieved. These findings suggest that the proportion of functionally inert HIV-specific $CD8^+$ T lymphocytes is not simply a reflection of antigen load alone, and that other factors, possibly including the rate at which antigen levels increase, might play a role. Such considerations suggest a kinetic basis for the phenomenon of $CD8^+$ T-lymphocyte dysfunction and in part obviate the requirement to invoke specific mechanistic explanations for functional heterogeneity. Consistent with this, it has been shown in macaques that 100% of tetramer-positive $CD8^+$ T cells specific for a Mamu A*01-restricted simian immunodeficiency virus (SIV) epitope were functional after vaccination with a DNA prime/MVA boost approach. After challenge with pathogenic SIV and establishment of persistent plasma viremia these previously fully functional SIV-specific $CD8^+$ T lymphocytes experienced a decline in responsiveness (24). Other lines of evidence also support this hypothesis. For example, previous studies with lymphocytic choriomeningitis virus (LCMV) in mice have emphasized the role of persistently high antigen concentrations in $CD8^+$ T-lymphocyte exhaustion, a process that could be related to the antigen-induced unresponsiveness observed in this study (4, 7, 19, 34, 35). Further, we have observed antigen-induced dysfunction *in vitro* using the HIV-specific $CD8^+$ T-lymphocyte clone 003 (36); this was reversible after several days of culture with interleukin (IL)-2 in the absence of antigen (data not shown). A similar recovery of function with IL-2 exposure has been reported for freshly isolated HIV-, SIV-, and HCV-specific $CD8^+$ T lymphocytes initially lacking cytolytic or secretory functions (6, 13, 15, 25). In addition, the observation that physical and functional measures of HIV-specific $CD8^+$ T lymphocytes approximate after prolonged suppression of viral replication during ART suggests that functional recovery *in vivo* occurs in the relative absence of antigen. This is consistent with studies of persistent viral infections in mice, although it is not clear whether the dysfunctional HIV-specific $CD8^+$ T lymphocytes regain function themselves or are replaced by newly generated functional effector cells from antigen-specific precursor populations (19).

Second, when ART is discontinued, the rapidly replicating viral quasispecies that recrudesced might be antigenically distinct from that previously encountered by the HIV-specific $CD8^+$ T-lymphocyte population present in the host at that time. The variant epitopes contained within the virus population might then induce a nonresponsive state in those $CD8^+$ T lymphocytes recognizing the original antigen through phenomena such as antagonism or anergy (37). Alternatively, a new $CD8^+$ T-lymphocyte response could be mounted to the variant antigen, if binding to the relevant MHC class I molecule is retained. In this scenario, the original index peptide might be a nonagonist ligand in the context of the newly generated $CD8^+$ T-lymphocyte response; apparent dysfunction would then be due to stimulation *ex vivo* with a nonagonist ligand, while staining with pMHC class I tetramer is retained due to the incomplete specificity of these reagents (29, 38).

Third, a loss of T-cell help, either through the destruction of HIV-specific $CD4^+$ T lymphocytes or impaired dendritic cell function, could limit the capacity to mount effective functional $CD8^+$ T-lymphocyte responses to the virus (7, 22, 39, 40). We have not formally assessed HIV-specific $CD4^+$ T-lymphocyte responses in this study, although most individuals maintained total $CD4^+$ T-lymphocyte counts above 400×10^6/liter during the periods off ART. In patients 1 and 5, we measured HIV p24-specific $CD4^+$ T-cell frequencies at the time points shown in Fig. 3. On ART, HIV p24-specific $CD4^+$ T-cell frequencies were 22 and 10 SFC/10^6 CD8-depleted PBL for patients 1 and 5, respectively; off ART, the corresponding frequencies were 328 and 23 SFC/10^6 CD8-depleted PBL. Both patients showed fully functional epitope-specific $CD8^+$ T-lymphocyte responses on ART, when HIV-specific $CD4^+$ T-helper cell responses were negligible. The substantially increased HIV-specific $CD4^+$ T-helper cell response observed in patient 1 in the absence of ART was not associated with better maintenance of virus epitope-specific $CD8^+$ T-lymphocyte

functionality. These data, although anecdotal, suggest that $CD4^+$ T-cell help is not a direct determinant of functionality within $CD8^+$ T-lymphocyte populations specific for the same virus. Further, previous observations indicate that HIV-specific $CD4^+$ T-cell help declines with prolonged ART administered during the chronic phase of infection (41); functionality within our HIV-specific $CD8^+$ T-lymphocyte populations actually increased with prolonged ART.

Fourth, HIV itself might have lymphocytotoxic effects that induce HIV-specific $CD8^+$ T-lymphocyte dysfunction as part of an immune evasion strategy. However, similar findings have been reported for $CD8^+$ T-lymphocyte populations in acute hepatitis C infection, a phenomenon described as stunning (13), and in persistent LCMV infection, a process described as exhaustion (19, 34). This suggests that virus-specific $CD8^+$ T-lymphocyte dysfunction is a kinetic phenomenon and is not restricted to individual antiviral cellular immune responses.

Fifth, altered cell surface TCR expression levels due to down-regulation on antigen encounter might impact on the observed phenotype of HIV-specific $CD8^+$ T lymphocytes. The detection of antigen-specific $CD8^+$ T lymphocytes with peptide–HLA class I tetramers requires the presence of some cell surface TCR expression. However, higher TCR levels might be required to induce IFN-γ production in response to antigen. Indeed, it is known that different effector functions require different levels of TCR triggering for activation, and IFN-γ production appears to be particularly insensitive in this respect (42). However, levels of peptide–HLA class I tetramer staining, in terms of the shift in fluorescence intensity rather than absolute cell numbers, were similar in most individuals in the functional or dysfunctional states on and off ART, respectively.

Sixth, HIV-specific $CD8^+$ T lymphocyte clones with low functional avidity that stain with peptide–HLA class I tetramers might be preferentially expanded during the phase of virus rebound, while higher avidity clones could dominate the antigen-specific population under conditions of minimal antigen concentration. We have not formally assessed the clonotypic structure of the HIV-specific $CD8^+$ T-lymphocyte populations in this study, but previous studies have demonstrated discrepancies between pMHC class I tetramer staining and functional avidity (23, 43).

In summary, we have demonstrated that the proportion of $CD8^+$ T lymphocytes that exhibit an impaired functional phenotype in populations expressing TCRs specific for individual HIV-derived antigens is related to pVL. These findings have implications for the interpretation of quantitative data generated by methods that rely on functional readouts.

ACKNOWLEDGMENTS

The authors would like to thank the patients for their cooperation; Danny Douek and Mike Betts for the free exchange of ideas and protocols; Michelle LeBraz, Christine Schneider, Roland Hafner, Manuel Battegay, and Pietro Vernazza for excellent patient care; and Sabine Yerly, Friedericke Burgener, Erika Schlaepfer, and Doris Russenberger for laboratory support. This work was funded by the Schweizerische Stiftung für medizinisch-biologische Stipendien and the Novartis Foundation (AO), the Wellcome Trust (REP, AKS, SJD), the Medical Research Council (GMG, SLR-J, DAP), the Swiss HIV-Cohort Study (SHCS), supported by the Swiss National Science Foundation Grant Number 3345-062041, by the SHCS grant # 290 (HFG) and by a research grant of the Kanton of Zürich. DAP is a Medical Research Council Clinician Scientist Fellow.

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