Changes in Function of HIV-Specific T-Cell Responses with Increasing Time from Infection

Michel L. Ndongala,¹ Philomena Kamya,¹ Salix Boulet,¹ Yoav Peretz,² Danielle Rouleau,² Cécile Tremblay,² Roger Leblanc,¹ Pierre Côté,³ Jean-Guy Baril,³ RéJean Thomas,⁴ Sylvie Vézina,⁴ Mohamed R. Boulassel,⁵ Jean-Pierre Routy,¹ Rafick P. Sékaly,² and Nicole F. Bernard¹

Abstract

Recently HIV-infected individuals have virus-specific responses characterized by IFN- γ /IL-2 secretion and proliferation rarely seen in chronic infection. To investigate the timing of loss of HIV-specific T-cell function, we screened cells from 59 treatment-naïve HIV-infected individuals with known dates of infection for proteome-wide responses secreting IFN- γ /IL-2 and IFN- γ alone by ELISPOT. HIV peptide-specific proliferation was assessed by carboxyfluorescein diacetate succinimidyl ester (CFSE) dilution. The contribution of IFN- γ /IL-2 and IFN- γ -only secretion to the total HIV-specific response was compared in subjects infected <6, 6–12, and 12–36 mo earlier. The frequency of IFN- γ /IL-2-secreting cells fell, while that of IFN- γ -only secretion rose with time from infection. HIV peptide-specific proliferative responses were almost exclusively mediated by CD8⁺ T cells, and were significantly lower in cells obtained from the 12–36 mo versus <6 mo post-infection groups. By the second year of infection there was a significant difference in these functions compared to those assessed within 6 mo.

Introduction

SHORTLY AFTER HIV PRIMARY INFECTION), a spike in viremia occurs that can exceed 10⁷ copies/mL of plasma (18,19). Subsequently, viral load (VL) declines to a set point either because the appearance of HIV-specific immune responses contributes to viral suppression, or because the main HIV target population of memory CD4⁺CCR5⁺ cells is depleted, or both (11,14,34,52). HIV-specific CD8⁺ T cells are thought to play a pivotal role in viral control for several reasons, including the temporal association between the appearance of these cells and reduction in VL during acute infection (11,34), the association of HLA class I alleles such as HLA-B*27, HLA-B*57, and HLA-B*58 with slower progression to AIDS (16,31,62), and the development of HLA-allele-associated changes in viral sequences, both at the population level and in infected subjects (10,15,33,56). In addition, the positive selection of sequences within and flanking epitopes recognized by CD8⁺ T cells, which allows escape from immune pressure exerted by these cells (3,26,32,35,45,47), and the disappearance of transmitted immune escape viral variants in new hosts expressing HLA alleles that do not restrict responses to these epitopes (2,3,27,36), also support a role for

CD8⁺ T cells in the control of HIV replication. Depletion of CD8⁺ cells that may include natural killer (NK) as well as T cells in an animal model for HIV primary infection, simian immunodeficiency virus (SIV)-infected macaques, results in uncontrolled viremia until CD8⁺ T-cell numbers recover (40,57). CD4⁺ T cells are targets for HIV infection (23). In acute infection there is a precipitous loss of CCR5⁺CD4⁺ T cells, particularly in the gut-associated lymphoid tissue (14,42). Furthermore, HIV-specific CD4⁺ T cells may be preferentially activated, infected, and targeted for destruction. The remaining HIV-specific CD4⁺ cells are dysregulated and exhibit loss of functions, such as the ability to secrete perforin and IL-2, and proliferate in response to antigen stimulation in all but a subset of HIV-infected subjects (17,41,55,64). CD4⁺ T-cell dysregulation has a negative impact on the ability of these cells to provide help for HIV-specific CD8⁺ T-cell responses (37).

It has been suggested that T cells with multiple functions, including IFN- γ and IL-2 secretion, and that have the ability to proliferate, are better at controlling experimental viral infections than T cells with more limited function (58). Several studies have associated HIV-specific polyfunctional T cells with better disease control (9,30). Additionally, these

¹Research Institute of the McGill University Health Center, Montréal, Québec, Canada.

²Centre de recherche du Centre Hospitalier de l'Université de Montréal, Montréal, Québec, Canada.

³Clinique médicale Quartier Latin, Montréal, Québec, Canada.

⁴Clinique médicale L'Actuel, Montréal, Québec, Canada.

⁵Division of Hematology, Royal Victoria Hospital, McGill University Health Center, Montréal, Québec, Canada.

cells have been associated with protection from infection with pathogenic *Leishmania major*, and are induced by vaccination and yellow fever vaccine (22,53,54). HIV-specific CD8⁺ T-cell response magnitude and breadth, as detected using IFN- γ secretion assays and tetramer reagents, is compromised in acute infection (6,7,20,38,65,66). HIV-specific T cells able to proliferate and secrete both IFN- γ and IL-2 develop their full breadth and magnitude after the acute phase, and are detected in most infected individuals within the first 6 mo of infection (37,49,66). Despite the presence of these cells, most untreated HIV-infected subjects exhibit disease progression, and polyfunctional responses are rarely seen in subjects with chronic infection, except for a subset of HIV-infected individuals who spontaneously control viremia without treatment (4,9,30,37,51,55,64). In contrast, secretion of IFN- γ by HIV-specific cells, although compromised in acute infection, persists once induced well into the chronic phase of infection (1,6–8,21,50). This is analogous to what occurs in other chronic viral infections such as murine clone 13 lymphocytic choriomeningitis virus infection, in which antigen-specific IL-2 secretion is one of the first functions of memory T cells lost, whereas IFN- γ secretion is more resistant to extinction (28,63). The cause-and-effect relationship between loss of IL-2 secretion and proliferative capacity and high VL remains a topic of investigation. Although HIV-specific cells able to secrete both IFN- γ and IL-2, or IL-2 only, and to proliferate are lost as disease progresses, there is little information available addressing the timing of this loss.

In this article we sought to assess the functional evolution of HIV-specific responses in terms of the timing of loss of HIV-specific responses characterized by secretion of IFN- γ and IL-2 versus IFN- γ alone. We used a dual-color ELISPOT assay able to simultaneously detect three functional lymphocyte populations: cells secreting IFN- γ /IL-2, IFN- γ only, and IL-2 only. We screened peripheral blood mononuclear cells (PBMCs) from 59 treatment-naïve subjects infected for less than 36 mo for responses directed to the entire HIV proteome. Having previously determined that the contribution of dual IFN- γ /IL-2-secreting cells was greater, and single IFN- γ -secreting cells lower in subjects infected <6 mo versus those in the chronic phase of infection, the timing of change of HIV-specific functional responses characterized by the secretion of IFN- γ /IL-2 and IFN- γ only was ascertained by comparing the contribution of each HIV-specific functional subset to the total response in subjects sampled within 6 mo ($n = 35$), 6–12 mo ($n = 19$), and 12–36 mo ($n = 35$) of infection. Because the proliferative capacity of HIV-specific T cells is linked to their ability to secrete and maintain IL-2, we also assessed proliferation to HIV peptides of cells obtained within 6 mo and between 12 and 36 mo of infection. We found that the contribution of HIV-specific responses secreting IFN- γ and IL-2 and able to proliferate was lower in individuals tested between 12 and 36 mo of infection than in those infected <6 mo, and that this difference was even more important for peptides restricted by HLA alleles not associated with protection.

Methods

Study population

The study population included 59 treatment-naïve HIV subtype B-infected subjects. All participants provided in-

formed consent and the institutional review boards of all participating study sites approved this study. All but two study subjects were drawn from the Montreal Primary Infection (PI) cohort, which enrolls individuals within 1 y of infection and follows them for 2 y. The two individuals not enrolled in the Montreal PI cohort, MQ13 and MQ16, who were infected for 12 and 20 mo, respectively, were also treatment-naïve individuals with known dates of infection followed at the Immune Deficiency Treatment Centre clinic of the McGill University Health Centre. The date of infection was estimated for each participant using the algorithm proposed by the Acute HIV Infection Early Disease Research Program sponsored by the National Institutes of Health (5,39,49). Of the 59 individuals studied, 31 were tested more than once, such that there were samples from 35 individuals (33 males) screened within 6 mo, 19 individuals (18 males) between 6 and 12 mo, and 35 individuals (34 males) between 12 and 36 mo of infection. Ten subjects, some with time points in more than 1 study group, i.e., 6 in the <6 mo, 4 in the 6–12 mo, and 7 in the 12–36 mo post-infection groups expressed either the HLA-B27 or HLA-B57/B58 alleles previously reported to be associated with slow HIV disease progression (16,31,62).

Laboratory testing

HIV-1 enzyme immunoassay (EIA) antibody testing was performed at three university hospital sites. Western blot analysis for HIV-1 antibodies and p24 antigen capture assays were performed at the Laboratoire de Santé Publique du Québec (Ste. Anne de Bellevue, Québec, Canada). The less sensitive EIA (detuned assay), which is used to confirm timing of infection, was carried out at the University of California–San Francisco, using the Vironostica HIV-1 EIA (Organon-Teknika, Boxtel, the Netherlands). Plasma viremia was measured using the ultra-sensitive method (Ultradirect; Roche Diagnostics Corp., Indianapolis, IN), with a detection limit of 50 HIV-1 RNA copies/mL of plasma.

Cell preparation

PBMCs were obtained by venipuncture in tubes containing EDTA anticoagulant or by leukapheresis (12). PBMCs were isolated by density gradient centrifugation (Ficoll-Paque; Pharmacia, Uppsala, Sweden) and cryopreserved in 10% dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO) and 90% fetal bovine serum (Medicorp, Montréal, Québec, Canada).

Stimulatory peptides

Peptides (15-mer with 11-aa overlaps from the National Institutes of Health AIDS Research and Reference Reagent Program, Rockville, MD) corresponding to the HIV consensus clade B sequence spanning the entire HIV proteome were used to stimulate the PBMCs. Pools containing 2–15 peptides were prepared and organized into matrices of Gag, Pol, Nef, Env, and accessory gene peptide pools such that each peptide was present at a final concentration of 4 μ g/mL in two pools within each peptide matrix. An initial peptide pool matrix strategy was used to identify candidate stimulatory peptides, followed by a verification assay in which candidate peptides were tested individually at 4 μ g/mL to confirm their stimulatory capacity.

ELISPOT assay for dual IFN- γ /IL-2, single IFN- γ , and single IL-2 cytokine release

IFN- γ and IL-2 secretion by HIV-specific cells was quantified using a dual-color ELISPOT assay as described elsewhere (13). Briefly, the wells of a polyvinylidene difluoride-backed microtiter plate (Millipore, Billerica, MA) were coated with optimized concentrations of capture antibodies for both IFN- γ and IL-2 (Becton Dickinson [BD] Pharmingen, San Diego, CA). The PBMCs were thawed and plated at 2×10^5 cells per well and stimulated with peptide pools for 28 h. Cytokine secretion from individual cells was detected by sequential addition, followed by washing with a detection antibody cocktail containing a fluorescein isothiocyanate (FITC)-conjugated anti-IFN- γ antibody (Ab) (1-D1K; Mabtech, Nacka Strand, Sweden), and a biotin-conjugated anti-IL-2 Ab (BD), followed by a cocktail of horseradish peroxidase-conjugated anti-FITC monoclonal Ab (Jackson ImmunoResearch Laboratories, West Grove, PA) and streptavidin alkaline phosphatase (Jackson ImmunoResearch Laboratories). Spots corresponding to the footprint of IFN- γ /IL-2, IFN- γ , and IL-2 cytokine-secreting cells were developed by the addition of Vector Blue (Vector Laboratories, Burlingame, CA), and amino ethyl carbazole (Sigma-Aldrich) substrates. Purple (IFN- γ and IL-2 cytokine secretion), red (IFN- γ secretion), and blue (IL-2 secretion) spots in each well were counted using a CTL series 3B Immunospot analyzer (CTL Analyzers LLC, Cleveland, OH) with software version 4.0 supplied by the manufacturer. Results are expressed as spot-forming cells per million PBMCs (SFC/ 10^6 PBMCs) following subtraction of negative controls. To establish the threshold of detection of positive responses for the single and dual cytokine-secreting responses, PBMCs from 8 HIV-seronegative low-risk individuals were stimulated with the same set of stimuli as the test subjects. Uninfected individuals produced a mean of 4.2 ± 10.6 IFN- γ SFC/ 10^6 PBMCs to this peptide platform. A positive response was defined as 3 standard deviations above the mean of that seen for uninfected subjects (i.e., >36 SFC/ 10^6 PBMCs), and at least threefold greater than the autologous negative control wells. Using the same rationale, the thresholds for IL-2 and dual-cytokine secretion were set at >32 SFC/ 10^6 PBMCs and >21 SFC/ 10^6 PBMCs, respectively, and at least threefold greater than the autologous negative control wells.

5,6-Carboxyfluorescein diacetate succinimidyl ester (CFSE) dilution assay

PBMCs were thawed, washed, resuspended in cRPMI supplemented with 2% human AB serum (Wisent Inc., St-Bruno, Quebec, Canada), and labeled with $5 \mu\text{M}$ CFSE (Molecular Probes, Eugene, OR). CFSE-labeled PBMCs were stimulated in cRPMI containing 15% human AB serum with $2 \mu\text{g/mL}$ of HIV consensus B peptides identified during the screening process using the ELISPOT assay. Stimulation with media alone served as a negative control, whereas stimulation with $1 \mu\text{g/mL}$ of staphylococcal enterotoxin B (SEB) and $1 \mu\text{g/mL}$ of human cytomegalovirus lysate (Advanced Biotechnologies Inc., Columbia, MD) were used as positive control stimuli. Following 6 d of incubation at 37°C and 5% CO_2 , PBMCs were stained for cell surface markers using anti-CD3-peridinin chlorophyll protein, anti-CD8-

allophycocyanin, and anti-CD4-phycoerythrin mAbs (BD). One hundred thousand lymphocyte-gated events were acquired per sample using a FACSCalibur flow cytometer, and analyzed with FlowJo software version 8.7.1 (Tree Star, Inc., Ashland, OR). Information on the percentage and number of CFSE^{lo} cells in the CD4 and CD8 compartments was collected. Responses were defined as positive if after subtracting background they were above 0.1%.

Statistical analysis

Statistical analysis and graphical presentation were performed using InStat 3.05 and GraphPad Prism 4.01, respectively (GraphPad Software, San Diego, CA). The Kruskal-Wallis test with Dunn's post-test for multiple comparisons was used to assess between-group differences in age, CD4 counts, CD8 counts, VL, response magnitude, and contribution of each functional subset to the total HIV-specific response. The significance of between-group differences in peptide-specific proliferation of samples from two time points obtained within 6 mo and later in infection (between 8 and 17 mo of infection) was assessed using a Mann-Whitney *U* test for non-matched samples, and a Wilcoxon matched-pairs signed-rank test for paired samples. A Fisher's exact test was used to assess proportional between-group differences. Statistical significance was set at $p < 0.05$.

Results

Study population

Table 1 shows for each group the median and interquartile range (IQR) of time from infection at which samples were obtained. Also shown in Table 1 are the median (IQR) age, absolute CD4 and CD8 counts, and HIV log₁₀ VL measures of the subjects included in each time-interval-from-infection group. There were no between-group differences in age, absolute CD8 counts, and VL ($p > 0.05$ by Kruskal-Wallis test). Compared to subjects who were infected <6 mo, those infected 12–36 mo had lower CD4 counts ($p < 0.001$ by Dunn multiple comparisons test).

Changes in the percentage contribution of HIV-specific IFN- γ /IL-2- and IFN- γ -only-secreting cells with increasing time from infection

Results generated using a dual-color ELISPOT assay screening for proteome-wide HIV-specific responses provided information on the specificity, the breadth as defined by the number of individual stimulatory peptides recognized, and the magnitude of HIV-specific immune responses as defined by the sum of SFC/ 10^6 PBMCs induced by each stimulatory peptide for each of the cytokine secretion profiles detected by the assay. In previous work we showed that HIV-specific cells secreting IL-2 were only present at low frequencies and targeted few epitopes, even in individuals infected <6 mo, and were rarely detected in individuals during chronic infection (49). For this reason, in this article we focused on dual IFN- γ /IL-2 and single IFN- γ responses, and sought to assess their functional evolution during the first 3 y of infection. The magnitude of HIV-specific responses characterized by IFN- γ /IL-2 secretion and single IFN- γ secretion did not differ significantly between the three groups defined by time from infection (Fig. 1A and B;

TABLE 1. SUMMARY OF THE CLINICAL CHARACTERISTICS OF THE STUDY PARTICIPANTS INCLUDED IN THE CROSS-SECTIONAL ANALYSIS

	Gender		Age (y) ^a	CD4/ μ L blood ^a	CD8/ μ L blood ^a	Log ₁₀ RNA VL copies/mL ^a	Days from infection ^a
	M	F					
<6 months	33	2	41 (30,44)	570 (437,626)	950 (681,1190)	4.70 (4.14,5.26)	121 (82,144)
6–12 months	18	1	35 (30,39)	420 (385,463)	689 (615,854)	4.40 (4.05,4.65)	247 (230,276)
12–36 months	34	1	40 (36,43)	359 (291,449)	750 (482,1126)	4.50 (4.16,4.89)	739 (429,797)

^aMedian (interquartile range).
M, male; F, female.

$p > 0.05$ by Kruskal-Wallis test). However, the proportion of HIV-specific cells secreting IFN- γ and IL-2 decreased with time, from a median (IQR) of 21.5% (12,29) to 11% (2,20.5), and 6% (0,22) for the groups infected <6 mo, 6–12 mo, and 12–36 mo, respectively (Fig. 1C; $p = 0.019$ by Kruskal-Wallis test). The proportion of HIV-specific cells secreting IFN- γ alone increased with time from infection from 78% (71,88) to 88% (78.5,98), and 94% (78,100) in the groups infected <6 mo, 6–12 mo, and 12–36 mo, respectively (Fig. 1D; $p = 0.01$ by Kruskal-Wallis test). The contribution of HIV-specific responses to the total response for IFN- γ /IL-2-secreting cells was significantly greater, and for IFN- γ only responses signifi-

cantly lower, for comparisons between the groups infected <6 mo versus 12–36 months (Fig. 1C and D; $p < 0.05$ for both comparisons by Dunn's multiple comparisons test).

The study population included 10 individuals expressing either the HLA-B*27, HLA-B*57, or HLA-B*58 alleles, which are associated with slow HIV disease progression (16,31, 44,62). These alleles are often present in individuals who exhibit effective VL control, and who are able to maintain HIV-specific proliferation and responses characterized by the secretion of multiple cytokines, including IL-2 (4,9,25,43,46). We reasoned that inclusion of subjects with these HLA alleles may introduce a bias towards the maintenance of

FIG. 1. HIV-specific functional subsets in subjects categorized by duration of infection. Thirty-five subjects infected <6 mo, 19 infected between 6 and 12 mo, and 35 infected between 12 and 36 mo were screened for immune responses to the entire HIV proteome using a dual-color ELISPOT assay. The magnitude of proteome-wide HIV-specific responses characterized by secretion of IFN- γ /IL-2 is shown in panel A, and IFN- γ only in panel B, for the three groups categorized by time post-infection. The y-axis shows for each individual tested the magnitude of the HIV-specific response in spot-forming cells per million peripheral blood mononuclear cells (SFC/10⁶ PBMCs). The percentage contribution of HIV-specific responses characterized by IFN- γ /IL-2 secretion and IFN- γ -only secretion to the total response is shown in panels C and D, respectively. Panels E and F show the same analyses as in panels C and D, but with individuals carrying protective HLA alleles excluded from the analysis. The line through each scatterplot indicates the median response for the groups.

HIV-specific cells able to secrete IFN- γ and IL-2. To address this, we re-analyzed the data, excluding the subjects expressing these MHC class I alleles. Fig. 1E shows that the contribution of HIV-specific cells secreting IFN- γ /IL-2 to the total responses fell from 22% (11.75,30) to 11% (0,20.5) and 3% (0,22), for subjects infected for 6–12 and 12–36 mo, respectively ($p=0.02$ by Kruskal-Wallis test), while for IFN- γ only responses it rose from 78% (70,88.25) to 89% (78.5,100) and 97% (78,100) in the three study groups defined by increasing time from infection ($p=0.006$ by Kruskal-Wallis test; Fig. 1F). Between-group differences were statistically significant for those infected <6 mo versus 12–36 mo for both cytokine secretion patterns (Fig. 1E and F; $p<0.01$ and $p<0.05$ for HIV-specific cells secreting both IFN- γ /IL-2 and IFN- γ alone, respectively, by Dunn's multiple comparisons test). In contrast, for the 6, 4, and 8 subjects infected for <6, 6–12, and 12–36 mo expressing one of the protective HLA alleles, the contribution of IFN- γ /IL-2-secreting cells to the HIV-specific response was 19% (13,25), 12% (9,25), and 12% (6,21), changes that were not significantly different ($p=0.79$ by Kruskal-Wallis test).

HIV peptide-specific proliferation of PBMCs from subjects infected for defined time intervals

We used a CFSE dilution assay to assess the proliferative capacity of PBMCs specific for HIV peptides able to induce secretion of IFN- γ /IL-2 or IFN- γ only in the dual-color ELISPOT assay, to establish whether HIV-specific proliferative capacity differed in cells obtained from time points collected within 6 mo versus those taken at later times post-infection.

Flow cytometry plots showing the proliferative capacity of CD4⁺ and CD8⁺ T cells following no stimulation as a negative control, stimulation with SEB and cytomegalovirus lysate (CMV) as positive controls, and the HIV peptide pol 5543 for a study subject infected <6 mo is shown in Fig. 2A. A panel of 103 HIV peptides recognized by 17 different subjects tested within 6 mo of infection was used to induce proliferation of cells from the same study subject time point. Eighty-one HIV peptides stimulated IFN- γ /IL-2 secretion, while 22 peptides stimulated IFN- γ -only secretion in the dual-color ELISPOT assay. Of the 81 peptides stimulating IFN- γ /IL-2 secretion, 19 (23.5%) and 61 (75.3%) induced proliferation in the CD4⁺ and CD8⁺ T-cell compartments, respectively (Fig. 2B). Of the 22 peptides inducing single IFN- γ secretion, 3 (13.6%) and 11 (50%) stimulated proliferation in the CD4⁺ and CD8⁺ T-cell compartments, respectively. A larger proportion of peptides stimulating secretion of both IFN- γ /IL-2 than IFN- γ only induced CD8⁺ T-cell proliferation ($p=0.03$ by Fisher's exact test). This comparison was not significantly different for CD4⁺ T-cell proliferation, possibly due to the smaller number of responses mediated by CD4⁺ T cells. If only the peptides inducing above-background proliferation of CD8⁺ T cells were considered, the median (IQR) percentage of CFSE^{lo} cells was 1.85% (0.73,5.16) and 1.09% (0.52,2.58) for peptides inducing IFN- γ /IL-2 and IFN- γ alone, respectively, a difference that was not statistically significant ($p=0.21$ by Mann-Whitney *U* test). Therefore, HIV-specific proliferative responses detected within 6 mo of infection were generally mediated by CD8⁺ T cells, although some peptides induced proliferation

of both CD8⁺ and CD4⁺ T cells, or of only CD4⁺ T cells. A larger proportion of peptides able to stimulate IFN- γ /IL-2 than IFN- γ alone were also able to induce above-background levels of CD8⁺ T-cell proliferation.

Thirty-eight peptides recognized by six subjects tested after 6 mo of infection by dual-color ELISPOT were screened for their ability to stimulate proliferation of cells from the same study subject time point. These six subjects were infected 241, 356, 368, 372, 448, and 501 d at the time of testing. Twenty-eight and 10 peptides induced IFN- γ /IL-2- and IFN- γ -only secretion, respectively, in the dual-color ELISPOT assay. Of the 28 peptides inducing IFN- γ /IL-2 secretion 15 (53.6%) and 24 (85.7%) stimulated proliferation in the CD4⁺ and CD8⁺ T-cell compartments (Fig. 2C). Of the 10 peptides inducing IFN- γ -secretion only, 5 (50%) and 9 (90%) induced proliferation in the CD4⁺ and CD8⁺ T-cell compartments, respectively. There was a non-significant trend toward the percentage of CD8⁺ CFSE^{lo} cells being higher for responses to peptides stimulating IFN- γ /IL-2- than IFN- γ -only secretion (0.94% [0.30,2.72] and 0.29% [0.18,1.35] for peptides inducing IFN- γ /IL-2 and IFN- γ alone, respectively; $p=0.13$ by Mann-Whitney *U* test). This was also the case for proliferation of CD4⁺ T cells (0.46% [0.22,0.67] and 0.19% [0.13,0.26] CFSE^{lo} cells induced by peptides stimulating IFN- γ /IL-2 and IFN- γ only, respectively; $p=0.07$ by Mann-Whitney *U* test).

CD8⁺ T cells from subjects within 6 mo of infection had a greater capacity to proliferate than CD8⁺ T cells from individuals at later times post-infection to peptides inducing IFN- γ /IL-2 secretion ($p=0.03$ by Mann-Whitney *U* test). This was not the case for the peptide panel inducing IFN- γ -secretion only, which stimulated similar levels of CD8⁺ T-cell proliferation from subjects at both time intervals post-infection ($p=0.42$ by Mann-Whitney *U* test). As seen in Fig. 2B and C, proliferation of CD4⁺ T cells was of a lower magnitude than that of CD8⁺ T cells for both peptide panels and time-interval-from-infection groups. Comparison of the percentage of CFSE^{lo} CD4⁺ T cells induced by peptides stimulating IFN- γ /IL-2 revealed a non-significant trend toward greater proliferative capacity of samples collected before 6 mo versus later time points post-infection ($p=0.06$ by Mann-Whitney *U* test), which was not seen for peptides stimulating IFN- γ secretion only ($p=0.77$ by Mann-Whitney *U* test). In summary, this cross-sectional analysis of HIV-specific proliferative capacity showed that both CD8⁺ and CD4⁺ cells responding to peptides able to stimulate IFN- γ /IL-2 secretion in a dual-color ELISPOT assay was lower in samples collected after 6 mo of infection compared to those collected within 6 mo of infection. In contrast, the proliferative capacity of CD8⁺ and CD4⁺ cells responding to peptides stimulating IFN- γ secretion only was similar at both time intervals from infection.

For five study subjects exhibiting HIV disease progression two time points were available for testing, one from within 6 mo of infection and the other from a later time, 241, 356, 372, 448, and 501 d from infection. Twenty-seven stimulatory peptides were used to induce proliferation in paired samples tested in the same experiment. This stimulatory peptide panel induced a median (IQR) percentage of CFSE^{lo} cells of 1.09% (0.30,2.82) and 0.48 (0.20,1.33) in paired samples collected at early and late time points, respectively ($p=0.05$ by Wilcoxon matched pairs signed-ranks test) (Fig. 2D). These results confirm in paired samples collected within 6 mo and

later in infection that proliferative capacity to the same epitope declines with time from infection.

Discussion

In this report we assessed the contribution of proteome-wide HIV-specific responses characterized by secretion of

both IFN- γ and IL-2 and IFN- γ only at three time intervals post-infection in order to address the timing of loss of HIV-specific responses able to secrete IFN- γ /IL-2. Thirty-one of the 35 (88.6%) subjects tested within 6 mo of infection had HIV-specific cells capable of secreting IFN- γ and IL-2 simultaneously. While the absolute magnitude of HIV-specific responses secreting both IFN- γ and IL-2 decreased, and that

secreting IFN- γ alone increased with time from infection, this change did not achieve statistical significance when a Kruskal-Wallis test was used to assess the significance of differences in subjects tested within 6 mo versus those tested 6–12 or 12–36 mo post-infection. However, the relative contribution of responses characterized by IFN- γ /IL-2 secretion fell, while that for IFN- γ -only secretion rose with increasing time intervals post-infection. Using panels of HIV peptides able to stimulate either IFN- γ /IL-2 or IFN- γ alone to induce proliferation of cells from the same time point used for the ELISPOT assay, over 75.3% of peptides stimulating IFN- γ /IL-2 secretion and over 50% of peptides stimulating IFN- γ -only secretion also induced proliferation of CD8 $^{+}$ T cells. A lower proportion of these peptides stimulated proliferation of both CD8 $^{+}$ and CD4 $^{+}$ T cells, or stimulated only CD4 $^{+}$ T-cell proliferation. Proliferative capacity was greater for CD8 $^{+}$ than CD4 $^{+}$ T cells, and was generally higher in cells from time points collected within 6 mo of infection than from times spanning 241–501 days from infection, particularly for peptides able to induce IFN- γ /IL-2 secretion. The observation that proliferation was greater for CD8 $^{+}$ than for CD4 $^{+}$ T cells could be due to the use of 15-mers as a stimulus. Although 15-mers are known to induce CD4 $^{+}$ T-cell responses, the use of 20-mers may be more appropriate to accurately measure their immunological response profile (24). An alternative possibility is that the low level of proliferation to HIV peptides in the CD4 compartment may reflect a defect in CD4 $^{+}$ T-cell proliferative capacity.

The rationale for choosing to examine HIV-specific IFN- γ secretion in this study population is that this T-cell function is resistant to functional exhaustion in a setting of high viremia, and it is the cytokine most frequently assessed in standard ELISPOT assays that measure HIV-specific immune responses (60,63). The decision to assess HIV-specific IL-2 secretion was based on its sensitivity to functional exhaustion in a setting of high viremia (30,63,68). HIV-specific IFN- γ /IL-2-secreting cells are present in most individuals in early infection, but are uncommon in the chronic phase of progressive infection (37,49). By monitoring HIV-specific IFN- γ /IL-2-secreting cells in samples collected at defined times from infection it is possible to address when after infection the response characterized by this functional profile begins to wane. Although we only assessed IFN- γ and IL-2 secretion and proliferation, it is likely that the IFN- γ /IL-2

functional profile is a surrogate for cells with a more multifunctional profile. Most of the HIV-specific responses detected in long-term non-progressors that were able to secrete IL-2 also mediated the secretion of other cytokines and/or chemokines (9). The functional importance of IL-2 secretion relates to its role in the maintenance of CD8 $^{+}$ T-cell responses, proliferation, and differentiation into effector T cells when CD4 $^{+}$ T-cell help is limited (29,30,37).

Our observation that cells from individuals in early infection have a higher proliferative capacity than cells from later times in infection is likely to be related to changes in the functional avidity and clonal make-up of HIV-specific responses with increasing time from infection (38). Lichterfeld *et al.* found that responses characterized by CD107a expression and secretion of IFN- γ and MIP-1 β were of a higher functional avidity in individuals in acute infection with early disease, such as the subjects tested here infected <6 mo, than those to the same peptides seen in subjects infected for more than 3 y (38). Some responses were no longer detected at later times in infection, or their contribution to the total response was reduced due to the expansion of T cells bearing T-cell receptor clonotypes having low to medium avidity for their cognate antigen (38). Alternatively, a recent study has shown that the persistent antigenic load that characterizes chronic infections drives T-cell functional exhaustion through sustained antigen presentation (48).

An important proportion of the peptides inducing single IFN- γ secretion in the dual-color ELISPOT assay were also able to induce proliferation. In general, responses that include the ability to secrete IL-2 rather than IFN- γ only are correlated with proliferative capacity (37,67). The reason underlying the finding that many peptides stimulating secretion of IFN- γ only also stimulated proliferation may be related to the sensitivity of the dual-color ELISPOT assay. In other words, these responses may include cells able to secrete enough IL-2 to support proliferation, but at a frequency or in a quantity insufficient to produce a response above the cut-off for background in the dual-color ELISPOT assay. This potential limitation of the dual-color ELISPOT assay may also apply to detection of cytokine-secreting cells by intracellular cytokine staining, which has a lower threshold of detection of 0.02% specific T cells (61).

Lichterfeld *et al.* reported that the magnitude of responses characterized by the secretion of not only IFN- γ , but also

FIG. 2. HIV-specific proliferation of CD4 $^{+}$ and CD8 $^{+}$ T cells in samples obtained at times within 6 mo and after 6 mo of infection to peptides stimulating either IFN- γ /IL-2- or IFN- γ -only secretion. Panel A shows an example of a CFSE dilution experiment. Peripheral blood mononuclear cells were cultured with the stimuli shown on the right for 6d. The boxes show the percentage of CFSE 10 cells in the CD4 (left) and CD8 (right) T cell compartments. The scatterplots in panel B show the percentage of CD3 $^{+}$ CFSE 10 cells detected following a 6-day stimulation with HIV peptides. The left-hand plots show the percentage of CFSE 10 T cells in the CD4 $^{+}$, while the right-hand plots show the percentage of CFSE 10 T cells in the CD8 $^{+}$ compartments following stimulation of cells from an individual infected <6 mo. Panel C shows the percentage of CFSE 10 CD4 $^{+}$ and CD8 $^{+}$ T cells generated following the stimulation of PBMCs from 17 individuals with a panel of 81 peptides that stimulated IFN- γ /IL-2 (right-hand scatterplots), or 22 peptides that induced IFN- γ -only secretion (left-hand scatterplots) in these subjects. Panel C shows the percentage of CFSE 10 CD4 $^{+}$ and CD8 $^{+}$ T cells generated following stimulation of cells from six individuals infected more than 6 mo with a panel of 28 peptides that stimulated IFN- γ /IL-2 (right-hand scatterplots), or 10 peptides that induced IFN- γ -only secretion (left-hand scatterplots). Panel D shows changes in HIV-peptide-specific proliferation as measured by the percentage CFSE 10 cells. Paired samples from two time points from five study subjects were stimulated with a panel of 27 HIV peptides that were stimulatory at both time points in a dual-color ELISPOT assay. One sample was from a time point within 6 mo of infection, the other from a later time point in infection. A Wilcoxon matched pairs signed-ranks test was used to assess the significance of between time-point changes in the percentage of CFSE 10 cells as a measure of HIV-peptide-specific proliferative capacity.

MIP-1 β and CD107a/b expression, was of a lower magnitude in subjects infected <6 mo compared to those infected for more than 3 years (38). We did not observe differences in the magnitude of either IFN- γ or IFN- γ /IL-2 secretion for the three study groups categorized by time from infection studied here. This discrepancy may be explained by the early-infection group studied by Lichterfeld *et al.*, which included a larger proportion of individuals infected <3 mo than the <6-mo-infected population studied here. We and others have reported that the breadth and magnitude of responses in individuals infected <3 mo is compromised compared to that seen in individuals in later stages of primary infection (6,7,21).

By the second half of the first year of infection it is already possible to detect a decline in the contribution of HIV-specific cells secreting both IL-2 and IFN- γ . The contribution of IFN- γ /IL-2-secreting HIV-specific cells continues to fall in the second year of infection, but appears to plateau thereafter, as we did not detect significant differences in the contribution of IFN- γ /IL-2- and single IFN- γ -secreting cells between individuals tested between 12 and 24 mo and 24 and 36 mo of infection (data not shown). Secretion of IL-2 is linked with proliferative potential, which is lost in the majority of viremic individuals during chronic HIV infection (37,51,55,67). The loss of IL-2 secretion and persistence of IFN- γ secretion are a functional phenotype for an effector population having limited self-renewal potential. This likely has negative consequences for control of persistent viral replication. HIV-specific cells secreting IL-2 and able to proliferate also have enhanced cytolytic ability, adding to the protective potential ascribed to polyfunctional lymphocytes more likely to be seen in early infection (37,38,43,59,63).

In summary, we show that most individuals infected <6 mo develop HIV-specific IFN- γ /IL-2 responses in addition to single IFN- γ responses. These bifunctional responses peak within the first 6 mo of infection and begin to wane soon thereafter.

Acknowledgments

We wish to acknowledge Ms. Chantal Grignon and Ms. Melanie Lemay for nursing support for the leukapheresis infrastructure, Mr. Mario Legault, coordinator of the Montreal Primary Infection cohort, and Ms. Nancy Simic and Ms. Marie-Pierre Boisvert for expert technical assistance. We acknowledge Dr. Bernard Lessard, Dr. Anne Talbot, Dr. Marc-André Charon, Dr. Martin Potter, Dr. Marina Klein, Dr. Norbert Gilmore, Dr. John MacLeod, Dr. Graham Smith, Dr. Benoit Trottier, Dr. Louise Charest, Dr. Danielle Legault, Dr. Michel Boissonnault, Dr. Claude Fortin, Dr. Julian Falutz, and Dr. Julie Bruneau for study subject referral, enrollment, and clinical follow-up. We are also grateful to participants in the Montreal PI cohort. This work was supported by grants from the National Institutes of Health #AI043261-04, and the Réseau du Sida et Maladies Infectieuses du Fonds de la Recherche en Santé du Québec (FRSQ). Y.P. and S.B. were the recipient of a scholarship from the FRSQ, and J.-P.R. and was the recipient of a Chercheur Boursier scholarship from the FRSQ.

Author Disclosure Statement

No competing financial interests exist.

References

1. Addo MM, Yu XG, Rathod A, *et al.*: Comprehensive epitope analysis of human immunodeficiency virus type 1 (HIV-1)-specific T-cell responses directed against the entire expressed HIV-1 genome demonstrate broadly directed responses, but no correlation to viral load. *J Virol* 2003;77:2081–2092.
2. Allen TM, Altfeld M, Geer SC, *et al.*: Selective escape from CD8 + T-cell responses represents a major driving force of human immunodeficiency virus type 1 (HIV-1) sequence diversity and reveals constraints on HIV-1 evolution. *J Virol* 2005;79:13239–13249.
3. Allen TM, Altfeld M, Yu XG, *et al.*: Selection, transmission, and reversion of an antigen-processing cytotoxic T-lymphocyte escape mutation in human immunodeficiency virus type 1 infection. *J Virol* 2004;78:7069–7078.
4. Almeida JR, Price DA, Papagno L, *et al.*: Superior control of HIV-1 replication by CD8 + T cells is reflected by their avidity, polyfunctionality, and clonal turnover. *J Exp Med* 2007;204:2473–2485.
5. Alter G, Hatzakis G, Tsoukas CM, *et al.*: Longitudinal assessment of changes in HIV-specific effector activity in HIV-infected patients starting highly active antiretroviral therapy in primary infection. *J Immunol* 2003;171:477–488.
6. Alter G, Merchant A, Tsoukas CM, Rouleau D, *et al.*: Human immunodeficiency virus (HIV)-specific effector CD8 T cell activity in patients with primary HIV infection. *J Infect Dis* 2002;185:755–765.
7. Altfeld M, Rosenberg ES, Shankarappa R, *et al.*: Cellular immune responses and viral diversity in individuals treated during acute and early HIV-1 infection. *J Exp Med* 2001;193:169–180.
8. Betts MR, Ambrozak DR, Douek DC, *et al.*: Analysis of total human immunodeficiency virus (HIV)-specific CD4(+) and CD8(+) T-cell responses: relationship to viral load in untreated HIV infection. *J Virol* 2001;75:11983–11991.
9. Betts MR, Nason MC, West SM, *et al.*: HIV nonprogressors preferentially maintain highly functional HIV-specific CD8 + T cells. *Blood* 2006;107:4781–4789.
10. Bhattacharya T, Daniels M, Heckerman D, *et al.*: Founder effects in the assessment of HIV polymorphisms and HLA allele associations. *Science* 2007;315:1583–1586.
11. Borrow P, Lewicki H, Hahn BH, Shaw GM, and Oldstone MB: Virus-specific CD8 + cytotoxic T-lymphocyte activity associated with control of viremia in primary human immunodeficiency virus type 1 infection. *J Virol* 1994;68:6103–6110.
12. Boulassel MR, Spurr G, Rouleau D, *et al.*: Changes in immunological and virological parameters in HIV-1 infected subjects following leukapheresis. *J Clin Apher* 2003;18:55–60.
13. Boulet S, Ndongala ML, Peretz Y, *et al.*: A dual color ELISPOT method for the simultaneous detection of IL-2 and IFN-gamma HIV-specific immune responses. *J Immunol Methods* 2007;320:18–29.
14. Brenchley JM, Schacker TW, Ruff LE, *et al.*: CD4 + T cell depletion during all stages of HIV disease occurs predominantly in the gastrointestinal tract. *J Exp Med* 2004;200:749–759.
15. Brumme ZL, Tao I, Szeto S, *et al.*: Human leukocyte antigen-specific polymorphisms in HIV-1 Gag and their association with viral load in chronic untreated infection. *AIDS* 2008;22:1277–1286.
16. Carrington M, and O'Brien SJ: The influence of HLA genotype on AIDS. *Annu Rev Med* 2003;54:535–551.

17. Champagne P, Ogg GS, King AS, *et al.*: Skewed maturation of memory HIV-specific CD8 T lymphocytes. *Nature* 2001; 410:106–111.
18. Clark SJ, Saag MS, Decker WD, *et al.*: High titers of cytopathic virus in plasma of patients with symptomatic primary HIV-1 infection. *N Engl J Med* 1991;324:954–960.
19. Daar ES, Moudgil T, Meyer RD, and Ho DD: Transient high levels of viremia in patients with primary human immunodeficiency virus type 1 infection. *N Engl J Med* 1991;324: 961–964.
20. Dalod M, Dupuis M, Deschemin JC, *et al.*: Weak anti-HIV CD8(+) T-cell effector activity in HIV primary infection. *J Clin Invest* 1999;104:1431–1439.
21. Dalod M, Fiorentino S, Delamare C, Rouzioux C, Sicard D, Guillet JG, and Gomard E: Delayed virus-specific CD8 + cytotoxic T lymphocyte activity in an HIV-infected individual with high CD4 + cell counts: correlations with various parameters of disease progression. *AIDS Res Hum Retroviruses* 1996;12:497–506.
22. Darrah PA, Patel DT, De Luca PM, *et al.*: Multifunctional Th1 cells define a correlate of vaccine-mediated protection against *Leishmania major*. *Nat Med* 2007;13:843–850.
23. Douek DC, Brenchley JM, Betts MR, *et al.*: HIV preferentially infects HIV-specific CD4 + T cells. *Nature* 2002;417:95–98.
24. Draenert R, Altfeld M, Brander C, *et al.*: Comparison of overlapping peptide sets for detection of antiviral CD8 and CD4 T cell responses. *J Immunol Methods* 2003;275:19–29.
25. Emu B, Sinclair E, Favre D, *et al.*: Phenotypic, functional, and kinetic parameters associated with apparent T-cell control of human immunodeficiency virus replication in individuals with and without antiretroviral treatment. *J Virol* 2005;79: 14169–14178.
26. Feeney ME, Tang Y, Roosevelt KA, *et al.*: Immune escape precedes breakthrough human immunodeficiency virus type 1 viremia and broadening of the cytotoxic T-lymphocyte response in an HLA-B27-positive long-term-nonprogressing child. *J Virol* 2004;78:8927–8930.
27. Goepfert PA, Lumm W, Farmer P, *et al.*: Transmission of HIV-1 Gag immune escape mutations is associated with reduced viral load in linked recipients. *J Exp Med* 2008;205: 1009–1017.
28. Harari A, Dutoit V, Cellerai C, Bart PA, Du Pasquier RA, and Pantaleo G: Functional signatures of protective antiviral T-cell immunity in human virus infections. *Immunol Rev* 2006;211:236–254.
29. Harari A, Petitpierre S, Vallerian F, and Pantaleo G: Skewed representation of functionally distinct populations of virus-specific CD4 T cells in HIV-1-infected subjects with progressive disease: changes after antiretroviral therapy. *Blood* 2004;103:966–972.
30. Harari A, Vallerian F, Meylan PR, and Pantaleo G: Functional heterogeneity of memory CD4 T cell responses in different conditions of antigen exposure and persistence. *J Immunol* 2005;174:1037–1045.
31. Kaslow RA, Carrington M, Apple R, *et al.*: Influence of combinations of human major histocompatibility complex genes on the course of HIV-1 infection. *Nat Med* 1996;2:405–411.
32. Kelleher AD, Long C, Holmes EC, *et al.*: Clustered mutations in HIV-1 gag are consistently required for escape from HLA-B27-restricted cytotoxic T lymphocyte responses. *J Exp Med* 2001;193:375–386.
33. Kiepiela P, Leslie AJ, Honeyborne I, *et al.*: Dominant influence of HLA-B in mediating the potential co-evolution of HIV and HLA. *Nature* 2004;432:769–775.
34. Koup RA, Safrit JT, Cao Y, *et al.*: Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. *J Virol* 1994;68:4650–4655.
35. Leslie A, Price DA, Mkhize P, *et al.*: Differential selection pressure exerted on HIV by CTL targeting identical epitopes but restricted by distinct HLA alleles from the same HLA supertype. *J Immunol* 2006;177:4699–4708.
36. Leslie AJ, Pfafferoth KJ, Chetty P, *et al.*: HIV evolution: CTL escape mutation and reversion after transmission. *Nat Med* 2004;10:282–289.
37. Lichterfeld M, Kaufmann DE, Yu XG, *et al.*: Loss of HIV-1-specific CD8 + T cell proliferation after acute HIV-1 infection and restoration by vaccine-induced HIV-1-specific CD4 + T cells. *J Exp Med* 2004;200:701–712.
38. Lichterfeld M, Yu XG, Mui SK, *et al.*: Selective depletion of high-avidity human immunodeficiency virus type 1 (HIV-1)-specific CD8 + T cells after early HIV-1 infection. *J Virol* 2007;81:4199–4214.
39. Martro E, Suligoi B, Gonzalez V, Bossi V, Esteve A, Mei J, and Ausina V: Comparison of the avidity index method and the serologic testing algorithm for recent human immunodeficiency virus (HIV) seroconversion, two methods using a single serum sample for identification of recent HIV infections. *J Clin Microbiol* 2005;43:6197–6199.
40. Matano T, Shibata R, Siemon C, Connors M, Lane HC, and Martin MA: Administration of an anti-CD8 monoclonal antibody interferes with the clearance of chimeric simian/human immunodeficiency virus during primary infections of rhesus macaques. *J Virol* 1998;72:164–169.
41. McNeil AC, Shupert WL, Iyasere CA, Hallahan CW, Mican JA, Davey RT Jr, and Connors M: High-level HIV-1 viremia suppresses viral antigen-specific CD4(+) T cell proliferation. *Proc Natl Acad Sci USA* 2001;98:13878–13883.
42. Mehndru S, Poles MA, Tenner-Racz K, *et al.*: Primary HIV-1 infection is associated with preferential depletion of CD4 + T lymphocytes from effector sites in the gastrointestinal tract. *J Exp Med* 2004;200:761–770.
43. Migueles SA, Laborico AC, Shupert WL, *et al.*: HIV-specific CD8 + T cell proliferation is coupled to perforin expression and is maintained in nonprogressors. *Nat Immunol* 2002; 3:1061–1068.
44. Migueles SA, Sabbaghian MS, Shupert WL, *et al.*: HLA B*5701 is highly associated with restriction of virus replication in a subgroup of HIV-infected long term nonprogressors. *Proc Natl Acad Sci USA* 2000;97:2709–2714.
45. Miura T, Brockman MA, Brumme ZL, *et al.*: HLA-associated alterations in replication capacity of chimeric NL4-3 viruses carrying gag-protease from elite controllers of human immunodeficiency virus type 1. *J Virol* 2009;83:140–149.
46. Miura T, Brockman MA, Schneidewind A, *et al.*: HLA-B57/B*5801 human immunodeficiency virus type 1 elite controllers select for rare gag variants associated with reduced viral replication capacity and strong cytotoxic T-lymphocyte recognition. *J Virol* 2009;83:2743–2755.
47. Miura T, Brumme CJ, Brockman MA, *et al.*: HLA-associated viral mutations are common in human immunodeficiency virus type 1 elite controllers. *J Virol* 2009;83:3407–3412.
48. Mueller SN, and Ahmed R: High antigen levels are the cause of T cell exhaustion during chronic viral infection. *Proc Natl Acad Sci USA* 2009;106:8623–8628.
49. Ndongala ML, Peretz Y, Boulet S, *et al.*: HIV Gag p24 specific responses secreting IFN-gamma and/or IL-2 in treatment-naïve individuals in acute infection early disease (AIED) are

- associated with low viral load. *Clin Immunol* 2009;131:277–287.
50. Peretz Y, Alter G, Boisvert MP, Hatzakis G, Tsoukas CM, and Bernard NF: Human immunodeficiency virus (HIV)-specific gamma interferon secretion directed against all expressed HIV genes: relationship to rate of CD4 decline. *J Virol* 2005;79:4908–4917.
 51. Peretz Y, Ndongala ML, Boulet S, *et al.*: Functional T cell subsets contribute differentially to HIV peptide-specific responses within infected individuals: correlation of these functional T cell subsets with markers of disease progression. *Clin Immunol* 2007;124:57–68.
 52. Phillips AN: Reduction of HIV concentration during acute infection: independence from a specific immune response. *Science* 1996;271:497–499.
 53. Pulendran B, and Ahmed R: Translating innate immunity into immunological memory: implications for vaccine development. *Cell* 2006;124:849–863.
 54. Querec T, Bennouna S, Alkan S, *et al.*: Yellow fever vaccine YF-17D activates multiple dendritic cell subsets via TLR2, 7, 8, and 9 to stimulate polyvalent immunity. *J Exp Med* 2006;203:413–424.
 55. Rosenberg ES, Billingsley JM, Caliendo AM, Boswell SL, Sax PE, Kalams SA, and Walker BD: Vigorous HIV-1-specific CD4+ T cell responses associated with control of viremia. *Science* 1997;278:1447–1450.
 56. Rousseau CM, Daniels MG, Carlson JM, *et al.*: HLA class I-driven evolution of human immunodeficiency virus type 1 subtype c proteome: immune escape and viral load. *J Virol* 2008;82:6434–6446.
 57. Schmitz JE, Kuroda MJ, Santra S, *et al.*: Control of viremia in simian immunodeficiency virus infection by CD8+ lymphocytes. *Science* 1999;283:857–860.
 58. Seder RA, Darrah PA, and Roederer M: T-cell quality in memory and protection: implications for vaccine design. *Nat Rev Immunol* 2008;8:247–258.
 59. Shankar P, Russo M, Harnisch B, Patterson M, Skolnik P, and Lieberman J: Impaired function of circulating HIV-specific CD8(+) T cells in chronic human immunodeficiency virus infection. *Blood* 2000;96:3094–3101.
 60. Shiver JW, Fu TM, Chen L, *et al.*: Replication-incompetent adenoviral vaccine vector elicits effective anti-immunodeficiency-virus immunity. *Nature* 2002;415:331–335.
 61. Sun Y, Iglesias E, Samri A, Kamkamidze G, Decoville T, Carcelain G, and Autran B: A systematic comparison of methods to measure HIV-1 specific CD8 T cells. *J Immunol Methods* 2003;272:23–34.
 62. Trachtenberg E, Korber B, Sollars C, *et al.*: Advantage of rare HLA supertype in HIV disease progression. *Nat Med* 2003;9:928–935.
 63. Wherry EJ, Blattman JN, Murali-Krishna K, van der MR, and Ahmed R: Viral persistence alters CD8 T-cell immunodominance and tissue distribution and results in distinct stages of functional impairment. *J Virol* 2003;77:4911–4927.
 64. Wilson JD, Imami N, Watkins A, *et al.*: Loss of CD4+ T cell proliferative ability but not loss of human immunodeficiency virus type 1 specificity equates with progression to disease. *J Infect Dis* 2000;182:792–798.
 65. Wilson JD, Ogg GS, Allen RL, *et al.*: Direct visualization of HIV-1-specific cytotoxic T lymphocytes during primary infection. *AIDS* 2000;14:225–233.
 66. Younes SA, Trautmann L, Yassine-Diab B, *et al.*: The duration of exposure to HIV modulates the breadth and the magnitude of HIV-specific memory CD4+ T cells. *J Immunol* 2007;178:788–797.
 67. Younes SA, Yassine-Diab B, Dumont AR, Boulassel MR, Grossman Z, Routy JP, and Sekaly RP: HIV-1 viremia prevents the establishment of interleukin 2-producing HIV-specific memory CD4+ T cells endowed with proliferative capacity. *J Exp Med* 2003;198:1909–1922.
 68. Zimmerli SC, Harari A, Cellerai C, Vellelian F, Bart PA, and Pantaleo G: HIV-1-specific IFN-gamma/IL-2-secreting CD8 T cells support CD4-independent proliferation of HIV-1-specific CD8 T cells. *Proc Natl Acad Sci USA* 2005;102:7239–7244.

Address correspondence to:

Dr. Nicole F. Bernard

Research Institute of the McGill University Health Center

Montreal General Hospital

1650 Cedar Ave. Rm. C10-160

Montreal, Quebec, Canada, H3G 1A4

E-mail: nicole.bernard@mcgill.ca

Received September 24, 2009; accepted December 12, 2009.