Journal of Clinical Immunology, Vol. 26, No. 1, January 2006 (© 2006)
DOI: 10.1007/s10875-006-7518-8

HIV-1-Specific CD4$^+$ T Cell Responses in Chronically HIV-1 Infected Blippers on Antiretroviral Therapy in Relation to Viral Replication Following Treatment Interruption

EMMANOUIL PAPASAVVAS,[1] JAY R. KOSTMAN,[2,3] BRIAN THIEL,[1] MAXWELL PISTILLI,[1] AGNIESZKA MACKIEWICZ,[1] ANDREA FOULKES,[4] ROBERT GROSS,[3,4] KIMBERLY A. JORDAN,[5] DOUGLAS F. NIXON,[5,6] ROBERT GRANT,[5,6] JEAN-FRANCOIS POULIN,[5] JOSEPH M. MCCUNE,[5,6] KARAM MOUNZER,[2] and LUIS J. MONTANER[2,7]

Received: May 13, 2005; accepted: July 19, 2005

The impact of transient viral load blips on anti-HIV-1 immune responses and on HIV-1 rebound following treatment interruption (TI) is not known. Clinical and immunological parameters were measured during 40 weeks of antiretroviral therapy (ART) and following TI in an observational cohort of 16 chronically HIV-1-infected subjects with or without observed viral load blips during ART. During therapy, blips in seven subjects were associated with higher anti-HIV-1 (p24) CD4$^+$ T cell lymphoproliferative responses ($p = 0.04$), without a significant difference in T cell activation or total anti-HIV-1 CD8$^+$ T cell interferon-γ (IFN-γ) responses when compared to nine matched non-blippers. Therapy interruption resulted in a significantly higher viral rebound in blippers by 8 week despite retention of higher lymphoproliferative p24 responses ($p = 0.01$) and a rise in CD3$^+$ T cell activation ($p = 0.04$) and anti-HIV-1 CD8$^+$ T cell responses in blippers by week 4 when compared to non-blippers. Past week 4 of interruption, therapy re-initiation criteria were also met by a higher frequency in blippers by week 14 ($p < 0.04$) with no difference between groups by week 24. These data support that blippers have higher anti-HIV lymphoproliferative responses while on ART but experience equal to higher viral rebound as compared to matched non-blippers upon TI.

KEY WORDS: Blips; HIV; treatment interruption; CD4$^+$ T cell responses.

INTRODUCTION

Introduction of antiretroviral therapy (ART) has resulted in significant advancement in the treatment of human immunodeficiency virus type 1 (HIV-1) infection (1–6). In the majority of patients, treatment with ART results in a sustained suppression of plasma HIV-1 RNA levels to <50 copies/mL and restoration of immune function (7–17). However, transient increases of low level plasma HIV-1 RNA between 50 and 1000 copies/mL (blips) (18) are not uncommonly found in otherwise chronically suppressed subjects on ART (19–25) and may reflect inadequate drug levels (26), drug resistance (27), immune activation following vaccination or concomitant co-infections (28), or an inherent technical variability of the viral load assays (29). Blips and their associated HIV-1 antigenimia could be expected to drive antiviral T cell responses, which have been associated with a lower viral load or lack of disease progression as described in acute and chronic infection, respectively (30–36). An increase in antigen-specific lymphoproliferative CD4$^+$ T cell responses in these patient cohorts is commonly interpreted as affecting disease progression by supporting proliferation of antiviral and other recall CD8$^+$ T cell responses (37).

While time-defined episodes of viral replication following therapy interruption are not associated with a higher degree of viral control when compared to continuous

[1]The Wistar Institute, Philadelphia, Pennsylvania.
[2]Philadelphia Field Initiating Group for HIV-1 Trials, Philadelphia, Pennsylvania.
[3]Division of Infectious Diseases, University of Pennsylvania, Philadelphia, Pennsylvania.
[4]Center for Clinical Epidemiology and Biostatistics, University of Pennsylvania, Philadelphia, Pennsylvania.
[5]The Gladstone Institute of Virology and Immunology, University of California, San Francisco, California.
[6]Department of Medicine, University of California, San Francisco, California.
[7]To whom correspondence should be addressed at the Wistar Institute, 3601 Spruce Street, Philadelphia, Pennsylvania 19104; e-mail: montaner@wistar.org.

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therapy (38), the immune relevance of transient blips in the presence of continually suppressive therapy with regards to immunologic parameters and viral load rebound following treatment interruption (TI) is not known. We examined whether observed low level intermittent viremia between 50 and 1000 copies/mL (preceded and followed by plasma HIV-1 RNA <50 copies/mL) was associated with a change in HIV-specific responses and a differential viral rebound upon therapy interruption when compared to matched control subjects that interrupted therapy without observed blips (both groups followed equally prior to therapy interruption). For this purpose, clinical, virologic, and immunologic parameters before and after TI were followed in chronically infected patients who either did or did not have transient viremia while on continuous ART. The association between transient plasma HIV-1 RNA blips and (a) T cell markers of memory and activation and anti-HIV-1 cellular immune responses during continuous ART, and (b) levels of plasma HIV-1 RNA rebound after TI were examined.

MATERIALS AND METHODS

Patients and Study Design

As an observational study, data are presented following post hoc analysis for 16 patients with or without transient viremia (blips) while participating in the continuous therapy arm of a larger randomized controlled study investigating the effects of repeated therapy interruption in chronic HIV-1 infection (38). Only subjects with a minimum of 40 weeks of follow-up on ART before TI were included. Weeks of follow-up between on ART periods and TI are distinguished by identifying the latter period as week C0 (wC0), week C4 (wC4), etc. Demographic and clinical characteristics of the cohort are shown in Table I.

Briefly, in the larger randomized trial, 42 chronically HIV-1-infected patients on suppressive ART were enrolled. Seventy-five percent of patients were on their second to fourth regimen, while 25% were on their first regimen. Informed consent was obtained from all patients in this study and human experimentation guidelines of the US Department of Health and Human Services and of the authors' institutions were followed. Study entry criteria included being on ART with $CD4^+$ T cell count >400 cells/mm^3 and HIV-1 RNA <500 copies/mL for >6 months and <50 copies/mL at entry. Subjects described on this report were randomized in the larger trial to a first phase (phase I) of 40 weeks of continuous therapy with a final open-ended TI (phase II) subject to therapy re-initiation criteria (viral load >30,000 over three consecutive visits 2 weeks apart or a loss of >45% baseline CD4 count at any time). Study visits were every 4 weeks while on continuous ART during phase I and every 2 weeks during phase II that lasted up to 46 weeks.

Throughout follow-up on and off ART, clinical ($CD4^+$ and $CD8^+$ T cell count and percent, plasma HIV-1 RNA), immunological (T cell distribution and activation, anti-HIV-1 $CD4^+$ and $CD8^+$ T cell activity), and virological [reverse transcriptase (RT) and protease mutations] parameters were measured as described (39, 40).

Assessment of Clinical Parameters and T Cell Phenotypes

Clinical parameters ($CD4^+$ and $CD8^+$ T cell count and percent, plasma HIV-1 RNA) were measured every 4 weeks on ART and every 2 weeks off ART while whole blood was used every 4 weeks to analyze by flow cytometry T cell subset distribution (CD45RA, CD45RO, CD62L) and T cell activation (HLA-DR, CD95, CD28, CD38, TNFRII] with the following directly conjugated anti-cell surface antigen antibodies: 1) IgG1 CD3-phycoerythrin (PE), IgG1 CD38-PE, IgG1 CD62L-PE, IgG1 CD28-fluorescein isothiocyanate (FITC), IgG2b CD45RA-FITC, IgG1 CD4-allophycocyanin (APC), IgG1 CD8-APC, isotypes: mouse IgG1-PE, mouse IgG2a-PE, mouse IgG1-FITC, mouse IgG2a-FITC, mouse IgG2b-FITC, mouse IgG2b-APC, mouse IgG2a-TriColor (TC) (Pharmingen, San Diego, CA, USA); 2) IgG1 CD95-FITC, IgG2a CD4-FITC, IgG2b HLA-DR-APC, IgG2a CD4-TC, IgG2a CD8-TC, IgG2a CD45RO-TC, isotypes: mouse IgG1-APC (CalTag, Burlingame, CA, USA); and 3) IgG2a tumor necrosis factor II (TNFRII)-PE (R&D Systems, Minneapolis, MN). Briefly, 100 μL of whole blood were incubated with 10 μL of 10% mouse serum (Sigma-Aldrich, St. Louis, MO) for 10 min at room temperature and stained with the appropriate monoclonal antibody for 20 min at room temperature. The cells were then lysed with lysis buffer (Becton Dickinson FACS Lyse, Becton Dickinson Immunocytometry Systems, San Jose, CA) for 10 min at room temperature, washed twice with FACS washing buffer (1× PBS, 2.5% heat inactivated FBS, 0.1% BSA, 0.02% NaN_3) and re-suspended in FACS washing buffer. Samples were analyzed on a Becton Dickinson FACScalibur flow cytometer using the CellQuest software package for acquisition and analysis. Live cell gates were set manually during acquisition of 10,000 events for each staining. Detection thresholds were set according to isotype-matched negative controls. Results were expressed as mean fluorescent intensity (MFI) and % positive.

Table I. Demographic and Clinical Information of 16 Chronically HIV-1-Infected Patients Undergoing a Single Interruption of Therapy

Patient	Group	Sex	Ethnic group	Therapy history	Total follow-up on ART (weeks)	CD4 count at Bsl	CD8 count at Bsl	Peak plasma HIV-1 RNA during 40 weeks follow-up	Plasma HIV-1 RNA at wC0	Plasma HIV-1 RNA at wC4
S5	B	M	C	Third regimen	41	457	526	328	<50	4503
S13	B	F	AA	First regimen	40	526	536	133	<50	1156
S14	B	M	C	First regimen	41	777	611	110	<50	18,911
S21	B	M	C	Second regimen	40	444	644	174	<50	57,476
S29	B	M	AA	Third–fourth regimen	41	520	2701	241	<50	70,962
S33	B	F	AA	N/A	40	906	624	128	<50	73,066
S36	B	M	C	Second–third regimen	40	675	622	125	<50	363,774
S20	NB	M	AA	First regimen	41	497	843	<50	<50	289
S25	NB	F	AA	N/A	40	628	712	<50	<50	<50
S28	NB	M	H	First regimen	40	450	169	<50	<50	2645
S32	NB	M	C	Second–third regimen	40	818	1044	<50	<50	2354
S40	NB	M	C	Third regimen	40	502	568	<50	<50	94,489
S53	NB	M	AA	First regimen	40	826	486	<50	<50	81,957
S60	NB	M	C	First regimen	40	809	1097	<50	<50	95,361
S61	NB	M	AA	First regimen	40	645	604	<50	<50	3252
S62	NB	M	AA	Second regimen	40	736	738	<50	<50	<50

Note. ART, antiretroviral therapy; Bsl, baseline of the study on ART; wC0, wC4, week 0 and week 4 of therapy interruption, respectively; B, blippers; NB, non-blippers; M, male; F, female; C, Caucasian; AA, African American; H, Hispanic; N/A, not available.

Anti-HIV-1 T Cell Responses

HIV-1-specific lymphoproliferative responses were measured every 4 weeks on and off ART by use of fresh peripheral blood mononuclear cells (PBMCs, 250,000 cells/well, isolated by standard Ficoll-hypaque density gradient centrifugation as previously described) (41). Lymphoproliferative (LPA) responses to whole antigen associated with activation of CD4 T cell responses (7, 42–45) were tested in six replicates for the following conditions: (a) insect cell/baculovirus recombinant HIV-1 antigen [HIV-1 p24 core protein including additional amino acids of the C-terminus of p17 and N-terminus of p15 with molecular weight of 35,000 Da on sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE) (5 μg/mL, Protein Sciences, Meriden, CT)], (b) protein control provided by the manufacturers of recombinant viral antigens (5 μg/mL, Protein Sciences, USA), (c) *Candida albicans* (Greer Laboratories, Lenoir, NC), and (d) phytohemagglutinin (PHA, 5 μg/mL, Sigma-Aldrich, USA). Results were expressed as Δcounts per minute (Δcpm) scores and stimulation index (SI) scores calculated respectively as:

Δcpm = antigen stimulated mean cpm$-$internal control stimulated mean cpm.
SI = antigen stimulated mean cpm/internal control stimulated mean cpm.

HIV-1-specific interferon-γ (IFN-γ) secreting CD8$^+$ T cell responses were measured in cryopreserved PBMCs by enzyme-linked immunospot (ELISPOT) as described (40). Briefly, 96-well filtration plates (Millipore, France) were coated overnight at 40°C with 5 μg/mL of the primary anti-IFN-γ mab (Mabtech, Stockholm, Sweden), washed four times with 1× PBS and blocked with RPMI containing 5% PHS for 1 h. Uninfected and infected (recombinant vaccinia virus expressing either Gag, Pol, Env, Nef, Rev, or Tat or no HIV-1 antigen as control) PBMC were added to the wells and incubated overnight. Plates were washed four times with 1× PBS prior addition of the secondary antibody at 1 μg/mL for 2 h. Plates were washed four times in PBS with 0.1% Tween 20 and avidin-bound horseradish peroxidase H was added to the wells for 1 h at room temperature. Plates were washed four times with 0.1% Tween 20 and diaminobenzidene tetrahydrochloride was added. The spots were counted with a stereomicroscope and vaccinia control signals for each donor were substracted from each corresponding HIV-specific response. Only spots with a fuzzy border and a brown color were counted. Results were expressed as spot forming cells (SFC) per 10^6 PBMC. From our previous studies of HIV-1-infected subjects we have established this response to be CD8$^+$ T cell mediated and have proposed a descriptive range for the strength of CTL responses: Low (SFC 10–200 per 10^6 PBMC), moderate (SFC 201–500 per 10^6 PBMC), high (SFC >501 per 10^6 PBMC) (46, 47). For data analysis, responses per protein were added to measure total CD8$^+$ T cell responses against HIV-1.

Thymic Activity

T cell receptor (TCR) rearrangement excision circles (sjTREC) were measured in cryopreserved PBMC at entry and at baseline of TI. A two-steps quantitative PCR (nested) was used as described (48) [except that β-globin was used as the reference gene instead of a portion of the CD3γ chain gene].

In summary, larger fragment of the sjTREC and the β-globin were sequentially cloned into the same Bluescript^{TM} vector (Stratagene, La Jolla, CA). This plasmid, containing both amplicons, was used to generate standard curves for real-time quantification of TREC. Parallel quantification of the sjTREC, together with the β-globin amplicon was performed for each sample using the TaqMan^{TM} technology (ABI, Perkin-Elmer, Boston, MA). This protocol allowed us to precisely normalize for the input DNA in each quantification, thus providing an accurate TREC frequency.

Briefly, cells (approximately 2×10^5 PBMCs) were lysed in Tween 20 (0.1%), NP-40 (0.1%), and Proteinase K (100 μg/mL) for 30 min at 56°C and then 15 min at 98°C. Multiplex PCR amplification was performed for sjTREC together with the β-globin gene in a final volume of 50 μL (10 min initial denaturation at 95°C, 30 s at 95°C, 30 s at 60°C, 2 min at 72°C for 22 cycles) using the outer 3′/5′ primer pairs. The linearity of this first round multiplex assay was demonstrated in triplicate experiments up to 24 cycles when using a maximum of 2×10^5 PBMCs. These PCR conditions were used for all subsequent experiments. Following the first round of amplification, the PCR products were diluted 10-fold prior to simplex, real-time amplification using the TaqMan^{TM} technology. PCR conditions in the TaqMan^{TM} experiments were: 10 min initial denaturation at 95°C, 30 s at 95°C, 2 min at 60°C for 40 cycles. Fluorescence measurements were performed at the end of each of the annealing/extension phase steps. For each PCR product, the TREC and β-globin second round amplification were performed in different plate wells but in the same run and quantified using a serially dilute standard curve. This highly sensitive nested quantitative PCR assay has a detection threshold of one copy out of 10^5 cells. The results were expressed as absolute number of TRECs per 10^5 cells. Quantification of

sjTREC frequencies was performed in duplicate. Primers and probes used are shown in Table II.

Viral Mutations

Viral mutations in the protease and the RT genes were analyzed by retrospective genotyping of cryopreserved plasma samples with a viral load >50 copies/mL on and off ART, using the TruGene™ Assay as described (39). The genotyping TrueGene™ assay accurately identified 97.6% of codons and codon mixtures at 54 sites associated with drug resistance in blinded repeated testing of blood plasma specimens from HIV-1-infected adults (49). Resistant genotypes were defined by the presence of viral sequences associated with impaired drug susceptibility or virologic suppression as specified by the International AIDS Society-USA mutations panel (50). Genotypic resistance to a protease inhibitor (PI) was defined by the presence of at least one primary mutation in the protease gene (D30N, M46I, G48V, V82A, I84V, or L90M), while genotyping resistance to nucleoside reverse transcriptase inhibitors (NRTIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs) was defined by the presence of any mutation in the RT gene (M41L, E44D, K65R, D67N, any insertion at T69, K70R, L74V, V75T, V118I, Q151M, M184I/V, L210W, T215Y/F, K219Q/E for the NRTIs and L100I, K103N, V106A, V108I, Y181C/I, Y188C/L/H, G190A for the NNRTIs) (51).

Statistics

Analysis of data was performed after variables were summarized with means, medians, standard deviations (SDs), standard errors (SEs), and ranges for both groups. Unless specified otherwise, results are expressed as medians, while 25 and 75% interquartiles ranges (IQR) are shown in parenthesis. For analysis and graphing purposes plasma HIV-1 RNA <50 copies/mL was considered as equal to 50 copies/mL (threshold of detection). Each group of data was analyzed for normal distribution by the Shapiro–Wilk W-test ($p > 0.05$) and all subsequent comparisons between groups were two-tailed. Differences amongst groups were tested using One-way Anova t test for normally distributed data or the Wilcoxon/Kruskal–Wallis Tests (rank sums) for non-normally distributed data. Linear regression analysis was used to estimate associations between the different sets of data (all independent values were included as continuous data points). Pairwise correlations using the Pearson correlation coefficient or the Spearman's Rho test were performed accordingly. Linear regression and correlation analysis results were considered significant if $r > 0.3$ and $p < 0.05$ (52). Chi-square test was employed to determine frequency differences between groups with regards to re-initiation of therapy following TI. A two-sided alpha level of 0.05 ($p < 0.05$) was considered significant. All descriptive analysis and statistical tests were performed using JMP 4.0 (SAS Institute, Cary, NC, USA).

RESULTS

Increased Anti-HIV-1 Lymphoproliferative T Cell Responses in Blippers Together with Lack of Difference in Other Variables During Antiretroviral Therapy

During a period of observation of 40 weeks on therapy (characteristics for cohort listed in Table I), 7 of 16 subjects showed one or more viral load blip episodes (50–999 copies/mL), while the remaining 9 subjects sustained plasma HIV-1 RNA of <50 copies/mL at all time-points tested (Fig. 1). Treatment history was confirmed for 14/16 subjects before start of follow-up with half (7/14) on their first regimen and the rest on their second to fourth regimen. Patients had no clinical history to document previous treatment failures in association with a resistance profile. No change in ART was observed in any of the subjects under follow-up.

No significant differences ($p > 0.05$) were noted between the two groups regarding variables measuring clinical parameters, whole blood T cell distribution, or activation changes or anti-HIV-1 $CD8^+$ T cell ELISPOT, except for a significant difference in anti-HIV LPA responses against HIV-1 p24 antigen. More precisely, the blipping group showed significantly higher anti-HIV-1 p24 response at baseline and at time of TI (also referred to as week 0 of therapy interruption wC0, $p = 0.04$ for Δcpm) as compared to the non-blipping group (Table III and IV Fig. 2, top left). Consistent with this difference, 6/7 of the blippers versus 3/9 of the non-blippers showed a higher frequency of $SI > 3$ against HIV-1 p24 antigen as defined by >30% of time-points tested while on ART ($SI \leq 3$ in the remaining time-points). By contrast, no difference in the frequency of positive LPA responses against *C. albicans* or PHA (Table III and IV) or their absolute amount at any time-point were observed between the two groups. Importantly, no difference was observed in total anti-HIV $CD8^+$ T cell responses by ELISPOT between the two groups at the time of TI suggesting that patients with a blipping history had higher HIV-specific $CD4^+$ rather than $CD8^+$ T cell responses. In addition, no correlation was found between the frequency of the blips and the level of anti-HIV LPA or total anti-HIV $CD8^+$ T cell responses at the time of TI. While a significant difference in TREC was observed at the last observed

Table II. Oligonucleotides and Probes Used for sjTREC Quantification

Conventional PCR (first amplification using *outer* 5′/3′ primers)	TaqMan PCR (second amplification using "nested" primers: "*inner*" 5′/3′)	TaqMan probes
sjTREC forward 5′-CAGCCCTCTCCAAGGCAAAAT-3′	sjTREC forward 5′-CCCTTTCAACCATGCTGACAC-3′	sjTREC 5′-*FAM*-TCTGGTTTTTGTAAAGGTGCCCACTCCTG-*TAMRA*-3′
sjTREC reverse 5′-ACATTTGCTCCGTGGTCTGTG-3′	sjTREC reverse 5′-GGGTGCAGGTGCCTATGC-3′	
β-globin forward 5′-GAGGGCTGAGGGTTTGAAGT-3′	β-globin forward 5′-GAAGAGCCAAGGACAGGTACG-3′	β-globin 5′-*FAM*-CACAGGGTGAGGTCTAAGTGATGACAG-*TAMRA*-3′
β-globin reverse 5′-TTGCCCCACAGGGCAGTAACGGCAGA-3′	β-globin reverse 5′-CCAACCCTAGGGTGTGGCT-3′	

Note. sjTREC, T cell receptor rearrangement excision circles; FAM/TAMRA, fluorochromes used (FAM in 5′ excites, while TAMRA in 3′ quenches).

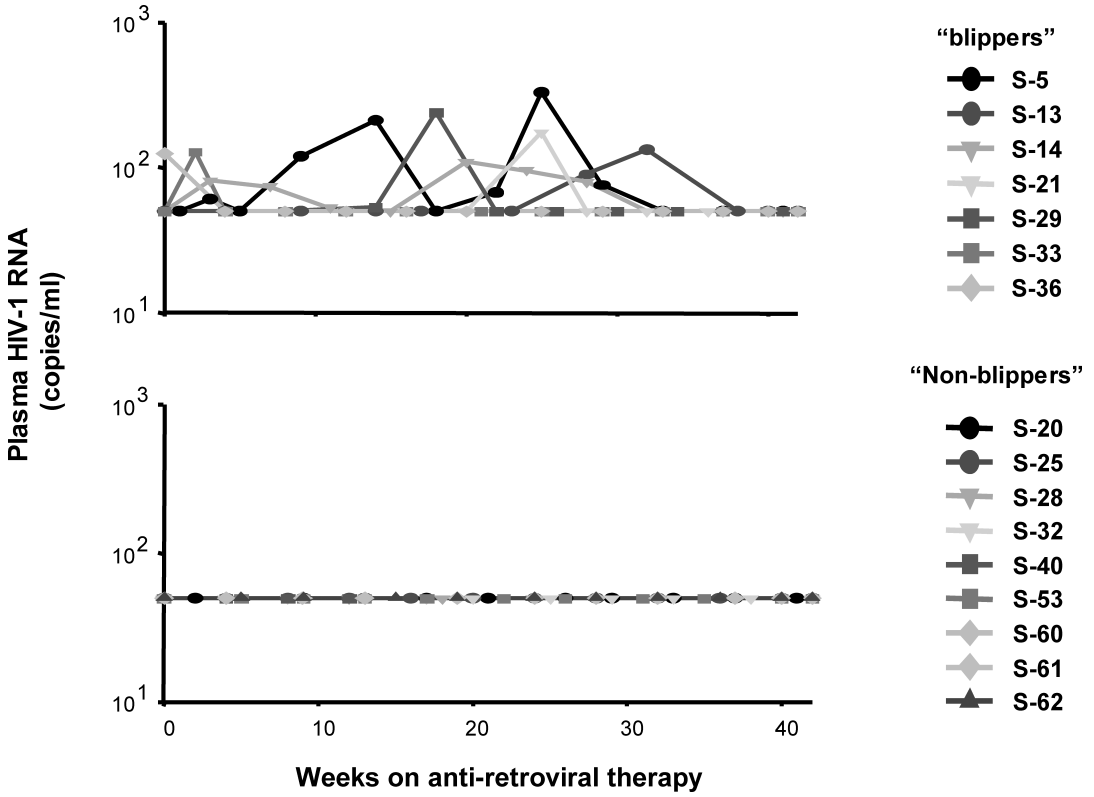

Fig. 1. Plasma HIV-1 RNA measurements in chronically HIV-1-infected patients under ART determine presence or absence of blips. Graph shows viral load in patients with blips (>50 and <1000 copies/mL, *top*) and patients with no blips (<50 copies/mL, *bottom*) during a median of 40 weeks of follow-up under continuous "suppressive" ART. For analysis and graphing purposes plasma HIV-1 RNA <50 copies/mL was considered as equal to 50 copies/mL (threshold of detection). Patients in each group are shown at the *right side* of the graphs.

point on therapy between groups, the lack of a difference at baseline, the small magnitude of change noted for this variable, and the lack of associated changes within the naïve T cell subsets reduces the potential that this change represents a differential thymic activity between groups.

Finally, viral blips measured in this study were predominantly amplified as wild-type for protease and RT regions except in two of seven blipping subjects. More precisely in patient S13, the RT mutation M184V was observed in presence of 3TC- (EPIVIR, GlaxoSmithKline, Research Triangle park, NC) containing regimen, while in patient S21 the RT mutations M184V, A62V, and V75T were observed in presence of 3TC- and d4T- (ZERIT, Bristol-Myers Squibb, New York, NY) containing regimens.

Lack of Difference in Viral Rebound Amongst Groups During Therapy Interruption Despite Increased Anti-HIV Lymphoproliferative Responses in Blippers

Following therapy interruption, both groups showed viral rebound as early as 2 weeks [wC2: mean plasma HIV-1 RNA in blippers = 67841.571 copies/mL (STE: 67474.419), mean plasma HIV-1 RNA in non-blippers = 25639.222 (STE: 22986.791)] (Fig. 3). The blipping group showed higher viral rebound with a significant difference observed at 8 weeks of therapy interruption [wC8: mean plasma HIV-1 RNA in blippers = 87419.8 copies/mL (STE: 47701.75), mean plasma HIV-1 RNA in non-blippers = 12252.5 (STE: 6191.543)] ($p = 0.04$). In support of a greater impact of viral load on the blipping arm, a significant increase in the frequency of $CD3^+/HLA\text{-}DR^+$ ($p = 0.01$) and of memory and activated $CD8^+$ T cells ($CD8^+/CD45RO^+/CD45RA^-$, $p = 0.04$; $CD8^+/HLA\text{-}DR^+$, $p = 0.04$) was observed in this group at 4 weeks of TI (no difference observed in non-blipping group at this time-point). Furthermore, a significant decrease in naïve $CD8^+$ T cells and on the expression of CD28 ($CD8^+/CD62L^+/CD45RA^+$, $p = 0.01$; $CD8^+/CD28^+$, $p = 0.01$) was also present after 4 weeks of TI in the blipping group. Total anti-HIV $CD8^+$ T cell responses following TI showed a four-fold increase in the blipping group ($p = 0.06$) and a two-fold increase in the non-blipping group ($p = 0.03$) from values at start

Table III. Median Data of the Changes in Immune Parameters During Therapy and Therapy Interruption in "Blippers" and "Non-Blippers"

	Data median (25%, 75% IQR)					
	"Blippers" ($n = 7$)			"Non-Blippers" ($n = 9$)		
Parameters	Bsl	wC0	wC4	Bsl	wC0	wC4
Clinical data						
$CD4^+$ (cells/mm^3)	526 (457–777)	517 (509–808)	439 (421–657)	645 (499.5–813.5)	720 (631.5–875)	559.5 (505.7–629.7)
$CD8^+$ (cells/mm^3)	622 (536–644)	584 (484–890)	679 (501–864)	712 (527–943)	734 (636–848)	675 (439–947)
T cell distribution						
$CD4^+/CD45RA^+/CD62L^+$ (%)	48.2 (19–59.5)	41.8 (18.2–51.1)	45.8 (15.3–52.6)	40.4 (32.3–57.8)	40 (26.5–49.8)	35.1 (28.5–47.6)
$CD4^+/CD45RA^-/CD45RO^+$ (%)	40.4 (35.6–74)	50.5 (45.3–65.2)	49.7 (42.2–63.3)	49.2 (38.7–59.7)	55.4 (43.9–63.1)	58.8 (48.8–61.6)
$CD8^+/CD45RA^+/CD62L^+$ (%)	43 (22.8–60.8)	37.5 (24.6–50.2)	29.8 (15.1–42.3)	45.5 (24.4–56.6)	40.9 (22.6–52.5)	35.6 (18.9–51.4)
$CD8^+/CD45RA^-/CD45RO^+$ (%)	34.6 (16.1–39.9)	39.2 (26.4–44.1)	47.3 (30.9–59.4)	21.4 (14.4–29.3)	27.5 (16.1–38)	28.4 (19.3–46.8)
T cell activation						
$CD3^+/CD95^+$ (%)	53.9 (38.4–62.9)	54.2 (42.4–66.4)	54.9 (40.8–69.3)	60.7 (57.2–64.5)	60.8 (39.4–69.4)	67.2 (44–76)
$CD3^+$/HLA-DR^+ (%)	6 (4.3–8.2)	6.2 (5.3–9.5)	14.9 (8.9–29.1)	5.7 (3.5–11.6)	4.9 (1.6–10.8)	11.3 (4.5–20)
$CD4^+$/HLA-DR^+ (%)	3.6 (0.6–6.1)	2.2 (2–3.2)	2.5 (2.3–4.5)	5.1 (2.4–7.2)	2.9 (1.8–8.6)	4.4 (1.6–6.1)
$CD4^+/TNFRII^+$ (%)	8.1 (5.8–11.5)	9.5 (8.9–19.4)	10 (8.1–16.5)	14 (10.5–19.5)	10.7 (9.4–19.7)	14.5 (8.8–21.5)
$CD4^+/CD28^+$ (%)	95.5 (94.3–98)	96.7 (94.9–97.3)	94.5 (89.4–95.8)	94.6 (92.1–99)	94.9 (92.4–98.6)	93.7 (74.5–98.7)
$CD4^+/CD38^+$ (%)	60.4 (45.4–64.9)	59.4 (52.2–70.8)	63 (59–71.9)	58.2 (49.9–70)	61.5 (49.8–73.4)	60.9 (49.7–68.3)
$CD8^+$/HLA-DR^+ (%)	0.4 (0.2–1)	0.5 (0.4–2.1)	7 (1.3–12.5)	2 (0.5–4.7)	6.2 (1.4–27.3)	7 (3.7–10.6)
$CD8^+/TNFRII^+$ (%)	19.9 (12.5–28.7)	12.7 (8.4–31.6)	19.4 (17.5–33.9)	17.6 (13.6–25)	13.4 (8–19.2)	11.5 (6.1–24.8)
$CD8^+/CD28^+$ (%)	65.3 (39.5–65.7)	58.5 (39.1–71.5)	39.3 (30.6–57.3)	64.1 (33.5–69.3)	57.7 (29.9–69.3)	49.6 (23.7–73.3)
$CD8^+/CD38^+$ (%)	39.7 (27.9–58.6)	41.6 (26.8–61.1)	62.9 (51–81.3)	57 (30.2–64.7)	53.8 (30.6–57.8)	42.8 (33.7–63.2)
Thymic activity						
sjTREC/10^5 $CD3^+$ cells	718 (82–3322)	988 (193.2–2069.4)	N/A	402.2 (101.2–723.9)	187.04 (131.53–431.18)	N/A
T cell responses						
LPA against p24 (Δcpm)	28,836 (7443–70,838)	49,879 (11,757–93,097)	30,351 (14,097–85,454)	7748 (1099–24,847)	2735 (0–23,911)	473 (0–20,786)
LPA against *Candida* (Δcpm)	78,409 (56,791–85,203)	96,636 (83,775–112,907)	65,679 (42,135–98,664)	113,669 (58,533–140,395)	75,370 (63,325–116,489)	88,429 (28,131–105,437)
LPA against PHA (Δcpm)	30,339 (16,415–48,796)	32,837 (21,526–34,898)	33,719 (20,543–53,828)	43,596 (37,570–56,248)	48,785 (27,009–66,389)	77,379 (51,172–100,965)
ELISPOT (SFC per 10^6 PBMC)	408 (137–2599)	273 (0–983)	1099 (81–1813)	507 (278.3–1093)	355 (106.5–728.5)	732 (524.8–1088)

Note. IQR, interquartile; Bsl, baseline of the study on ART; wC0, wC4, week 0 and week 4 of therapy interruption, respectively; N/A, not applicable; ELISPOT results expressed as SFC (spot forming cells) per 10^6 PBMC are against total HIV as described in 'Material and methods' Section.

Table IV. Analysis of the Changes in Immune Parameters During Therapy and Therapy Interruption in "Blippers" and "Non-Blippers"

	Analysis (p values)						
	Within group						
	"Blippers" ($n = 7$)		"Non-blippers" ($n = 9$)		Between group		
Parameters	Bsl vs. wC0	wC0 vs. wC4	Bsl vs. C0	wC0 vs. wC4	Bsl	wC0	wC4
Clinical data							
$CD4^+$ (cells/mm^3)	0.93	0.1	0.07	**0.02**	0.61	0.18	0.41
$CD8^+$ (cells/mm^3)	0.57	0.81	0.2	0.76	0.83	0.67	0.86
T cell distribution							
$CD4^+/CD45RA^+/CD62L^+$ (%)	0.55	0.93	0.19	0.24	0.88	0.93	0.59
$CD4^+/CD45RA^-/CD45RO^+$ (%)	0.19	0.59	0.13	0.12	0.96	0.86	0.59
$CD8^+/CD45RA^+/CD62L^+$ (%)	0.7	**0.01**	0.37	0.32	0.95	0.96	0.45
$CD8^+/CD45RA^-/CD45RO^+$ (%)	0.17	**0.04**	**0.03**	0.19	0.22	0.28	0.11
T cell activation							
$CD3^+/CD95^+$ (%)	0.54	0.97	0.31	0.31	0.34	0.92	0.63
$CD3^+$/HLA-DR^+ (%)	0.8	**0.01**	0.05	0.07	0.68	0.7	0.28
$CD4^+$/HLA-DR^+ (%)	0.28	0.62	0.91	0.73	0.59	0.22	0.36
$CD4^+/TNFRII^+$ (%)	0.46	0.93	0.54	0.68	0.05	0.45	0.44
$CD4^+/CD28^+$ (%)	0.29	**0.03**	0.57	0.09	0.95	0.87	0.87
$CD4^+/CD38^+$ (%)	0.34	0.17	0.93	0.45	0.56	0.82	0.54
$CD8^+$/HLA-DR^+ (%)	0.57	**0.04**	0.1	0.46	0.03	0.05	0.96
$CD8^+/TNFRII^+$ (%)	0.93	0.15	0.12	0.73	0.75	0.59	0.16
$CD8^+/CD28^+$ (%)	0.72	**0.01**	0.94	0.46	0.75	0.52	0.67
$CD8^+/CD38^+$ (%)	0.72	**0.04**	0.13	0.98	0.33	0.86	0.06
Thymic activity							
sjTREC/10^5 $CD3^+$ cells	0.68	N/A	0.33	N/A	0.49	**0.03**	N/A
T cell responses							
LPA against p24 (Δcpm)	0.24	0.17	0.84	0.74	**0.04**	**0.04**	**0.01**
LPA against *Candida* (Δcpm)	0.09	0.05	0.26	0.47	0.16	0.48	0.84
LPA against PHA (Δcpm)	0.74	0.61	0.93	**0.03**	0.07	0.08	**0.005**
ELISPOT (SFC per 10^6 (Δcpm)	0.18	0.06	0.84	**0.03**	0.9	0.67	0.84

Note. Bsl, baseline of the study on ART; wC0, wC4, week 0 and week 4 of therapy interruption, respectively; N/A, not applicable; ELISPOT results expressed as SFC (spot forming cells) per 10^6 PBMC are against total HIV as described in 'Material and methods' Section.

of TI. As observed while on therapy, LPA responses against HIV-1 p24 antigen remained higher in the blipping arm ($p = 0.01$ for Δcpm) (Fig. 2, bottom left). A lack of correlation between cellular anti-HIV responses (LPA or ELISPOT data) at time of interruption and viral rebound at weeks 4, 6, or 8 of TI was present in either group as shown in Table V (analysis was not performed for further time-points on TI due to sample number limitations). Following week 4, subjects in each group started to meet re-treatment criteria due to sustained viral loads >30,000 copies/mL with the blipping arm having a significantly higher frequency of subjects back on therapy by week 14 (4/7) as compared to the non-blipping group (1/9, $p = 0.04$). This difference between groups was sustained up to week 24 suggesting that although the blipping arm may have reached higher levels of viral rebound after 12 weeks off therapy, both groups had the potential to experience similar viral rebounds if followed to 24 weeks off therapy.

Analysis of rebounding viral sequences in all subjects showed no difference in the genotypic profile of resistant viruses between groups. Briefly, in the blipping group, only the M184V mutation was still detected in one (patient S13) out of the two patients with RT mutations detected during viral blips on ART, while in the non-blipping group the mutations K101K/Q and K70K/R were detected in 2/9 patients (patients S32 and S40, respectively, mutations were not associated with patients regimen at time of TI).

DISCUSSION

We conducted an observational study in order to examine the effect of viral load blips observed during antiretroviral therapy in chronically HIV-1-infected patients on the virologic and immune responses during therapy and its interruption. We observed higher anti-HIV $CD4^+$ T cell responses before and after therapy interruption in blippers when compared to matched non-blippers (Fig. 2). Surprisingly, the presence of significantly higher lymphoproliferative responses in blipping subjects was associated with a higher rather than lower viral rebound upon therapy

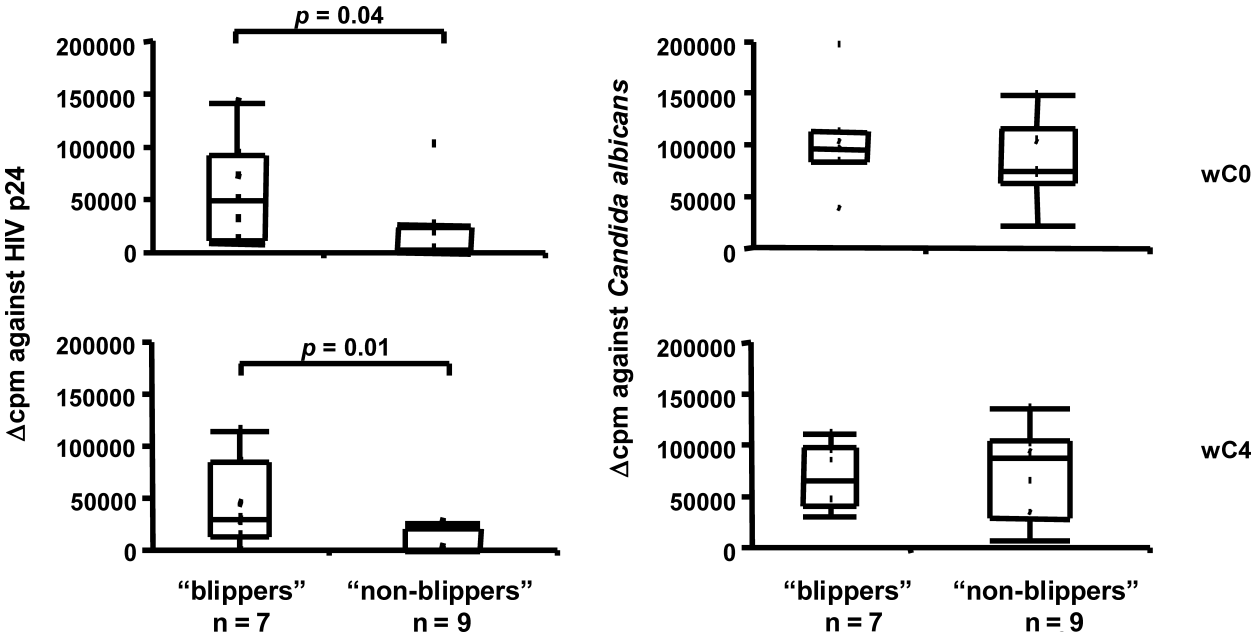

Fig. 2. Significantly higher anti-HIV-1 CD4$^+$ T cell responses in blippers compared to non-blippers at time of treatment interruption and at week 4 of treatment interruption. CD4$^+$ T cell lymphoproliferative responses against HIV-1 p24 (*left panels*) and *C. albicans* (*right panels*), expressed as Δcounts per minute (Δcpm), in patients with viral load blips (>50 and <1000 copies/mL) and patients with no blips (<50 copies/mL) at week 0 of therapy interruption (wC0, *top panels*) and week 4 of treatment interruption (wC4, *bottom panels*) are shown. Non-parametric Wilcoxon/Kruskal–Wallis tests (rank sums) were performed for comparisons between the groups. Data shown as interquartile box plots (median and 25th–75th interquartiles), with significant *p* values on the *top* of each graph.

interruption (Fig. 3). The latter was an unexpected finding that suggests that viral blips in patients on ART can be indicative of a higher viral rebound upon therapy interruption. While viral rebound during ART has been reported before (20–23), this is the first study that compares viral rebound from matched subjects with the presence or absence of viral blips in defining correlates of viral control upon therapy interruption.

Although the presence of anti-HIV-1 lymphoproliferative responses in chronically infected subjects on ART in conjunction with low T cell activation profiles has been reported by us and others (40, 53), our data supports that the LPA anti-HIV response is significantly associated with a history of intermittent viral blips. Our data challenges the assumption that the presence of lymphoproliferative responses in long-term non-progressors would suggest

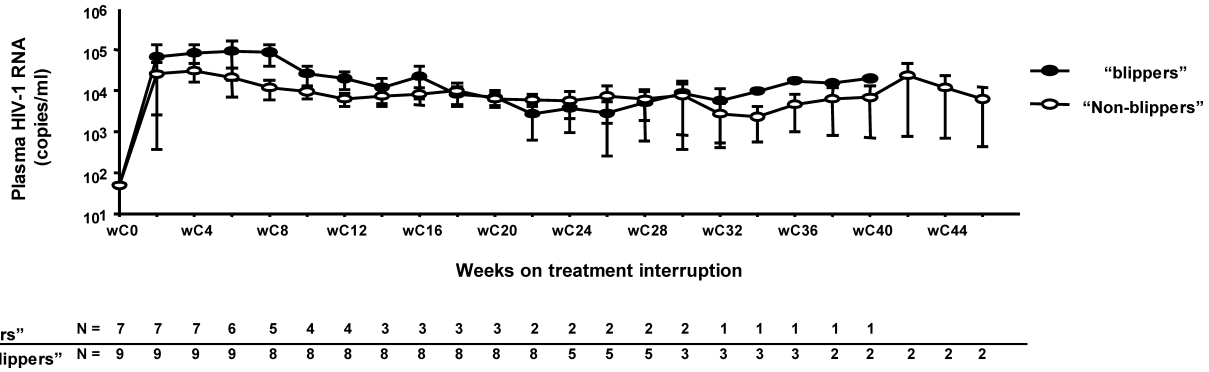

Fig. 3. Viral rebound during treatment interruption between blippers and non-blippers. Plasma HIV-1 RNA levels (mean ± SE) per group during a period of follow-up of 46 week after treatment interruption. *Bottom* table shows number of patients at time-points shown for viral load in the second panel. The decrease in viral load over time is due to the re-initiation of therapy in patients with higher viral load.

Table V. Correlations Amongst Anti-HIV T Cell Responses at Week 0 of Treatment Interruption and Plasma HIV-1 RNA During Treatment Interruption in "Blippers" and "Non-Blippers"

	"Blippers"			"Non-Blippers"		
	Log HIV-1 RNA (copies/mL)			Log HIV-1 RNA (copies/mL)		
Parameters	wC4	wC6	wC8	wC4	wC6	wC8
$CD4^+$ T cell responses against HIV p24 at wC0 (Δcpm)						
r	−0.7	−0.46	−0.34	+0.3	−0.14	−0.18
p	0.06	0.35	0.57	0.43	0.72	0.67
N	7	6	5	9	9	8
Total anti-HIV-1 $CD8^+$ T cell responses at wC0 (IFN-γ, SFC/10^6 PBMC)						
r	+0.01	+0.79	+0.91	−0.24	−0.02	+0.24
p	0.97	0.05	0.03	0.52	0.95	0.57
N	7	6	5	9	9	8

Note. wC0, wC4, wC6, or wC8, weeks 0, 4, 6, or 8 of therapy interruption, respectively; SFC, spot forming cells.

that these responses are a direct correlate of viral control if present in chronic infected subjects on ART. Our observation that viral replication is associated with an increase in lymphoproliferative responses, as suggested by the blipping history, is supported by previous longitudinal data from studies characterizing the relation between lymphoproliferative responses and IL-2-induced viral blips on ART (54) and from therapy interruption studies (41) even if progressively lost upon repeated (55) or extended (56) therapy interruptions. Our observations documenting higher anti-HIV-1 lymphoproliferative responses by week 4 of therapy interruption at the same time that viral replication is observed is consistent with our prior observations (41). The latter may reflect a temporal association between antigen and T cell function upon viremia that later progresses to a loss of this response upon continual viral replication as supported by data from McNeil *et al*. (56) and others showing an inverse relationship between these variables (43, 57–63).

While pathogenesis studies have associated the CD4 lymphoproliferative response with low viral loads supporting a mechanistic role for these responses in achieving viral control (17, 42, 64), our study strongly suggests that additional factors may need to be characterized in defining correlates to control of viral replication. From the host side, factors such as a dysfunction of the innate immune system (65, 66), clonal exhaustion (67), maturation and functional impediments of the $CD8^+$ T cells (68, 69) may provide barriers to viral control in chronic infection in spite of higher lymphoproliferative responses. From a virological perspective, *in vivo* viral diversity with regards to the antigen used *in vitro* or relative to different reservoirs *in vivo* (70–73) also may need to be established before characterizing the $CD4^+$ T cell response measured in relation to viral control *in vivo*. Taken together, our data does not exclude the lymphoproliferative response as a component of viral control but suggests that its presence in chronically infected persons on ART is more likely to be indicative of viral antigenimia than a strong correlate to viral control upon therapy interruption.

Our observations showing that therapy interruption and viral rebound will increase T cell activation and anti-HIV $CD8^+$ T cell responses are also consistent with previous data (74). We interpret that the significantly higher levels of activation observed in the blipping as compared to the non-blipping reflect the lower number of subjects with high viral load rebound (>5000 copies/mL) in the latter group (3/9) when compared to the former group (5/7) at 4 weeks of TI.

In contrast to previous empirical data suggesting that multiple blips are predictive of virologic failure (75), our study did not observe a relation between blips and lack of viral suppression (22). In spite of our limited sample size, resistance data collected during the study did not support a difference in the viral populations between subjects as a dominant factor associated with the presence of lymphoproliferative responses in the blipping group. In contrast to the findings of Cohen *et al*. (20), showing an association of viral blips with selection of drug-resistant virus, viral blips measured in this study were predominantly amplified as wild-type.

Our current data indicate that although viral blips during ART may provide sufficient antigen to activate higher levels of $CD4^+$ HIV-specific responses against HIV-1 p24, these responses are not a significant correlate to viral control upon therapy interruption but instead were indicative of a higher viral rebound. It remains to be determined whether correlates for viral control outcomes in subjects receiving ART may be addressed by other quantitative or qualitative criteria such as measures of HIV-specific

$CD8^+$ T cells proliferation or the frequency of IL-2 secreting, $CD4^+$ central memory T cells as suggested by recent data (76).

ACKNOWLEDGMENTS

We would like to thank the HIV-1 patients who participated in this study (presented at the 11th Conference on Retroviruses and Opportunistic Infections, February 8–11, 2004, San Francisco, USA, Abstract A-93.) and their providers; C. Gallo, E. C. Moore, T. Liegler, J. Javier, L. Chow, and K. Plamenco for laboratory support; and the Philadelphia FIGHT Board and Staff for study support. No commercial affiliations were present. This work was primarily supported by a grant to L. J. Montaner from the National Institute of Allergy and Infectious Disease NIH AI48398. Additional support was provided by The Philadelphia Foundation (Robert I. Jacobs Fund), The Stengel-Miller family, AIDS funds from the Commonwealth of Pennsylvania, and from the Commonwealth Universal Research Enhancement Program, Pennsylvania Department of Health. Resistance data was also supported by the California University wide AIDS Research Program and the Gladstone Institute of Virology and Immunology. Dr. Gross effort was supported by K08 MH01584 (RG) and the PENN Center for AIDS Research (P30 AI060352). Douglas F. Nixon and Joseph M. McCune are Elizabeth Glaser Scientists of the Elizabeth Glaser Pediatric AIDS Foundation. Joseph M. McCune is a recipient of the Burroughs Wellcome Fund Clinical Scientist Award in Translational Research.

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