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BRIEF REPORT

Impact of Treatment Interruption on HIV Reservoirs and Lymphocyte Subsets in Individuals Who Initiated Antiretroviral Therapy During the Early Phase of Infection

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Therapeutic strategies for achieving sustained virologic remission are being explored in human immunodeficiency virus (HIV)–infected individuals who began antiretroviral therapy (ART) during the early phase of infection. In the evaluation of such therapies, clinical protocols should include analytical treatment interruption (ATI); however, the immunologic and virologic impact of ATI in individuals who initiated ART early has not been fully delineated. We demonstrate that ATI causes neither expansion of HIV reservoirs nor immunologic abnormalities following reinitiation of ART. Our findings support the use of ATI to determine whether sustained virologic remission has been achieved in clinical trials of individuals who initiated ART early during HIV infection.

Keywords. HIV reservoirs; acute/early HIV infection; analytical treatment interruption; antiretroviral therapy.

Current antiretroviral therapy (ART) has proven to be unsuccessful at eradicating human immunodeficiency virus (HIV) from an infected individual because the vast majority of individuals experience plasma viral rebound upon treatment interruption [1]. Given that lifelong ART is currently required to maintain suppression of plasma viremia, novel therapeutic strategies aimed at achieving sustained virologic remission in the absence of ART are currently being explored [2]. HIV-infected individuals who began ART during the acute/early phase of infection are more likely to respond to therapeutic interventions aimed at inducing ART-free virologic remissions, possibly as a result of smaller HIV reservoirs, more-homogeneous viral quasispecies, and relatively intact immune systems, compared with individuals who initiated ART during the chronic phase of infection [3]. Therefore, these early treated individuals have been the focus of intense study. In this regard, the efficacy of any therapeutic strategy designed to achieve ART-free virologic remission can only be adequately assessed with analytical treatment interruption (ATI) [4]. Yet, there has been concern that plasma viral rebound resulting from ATI may have deleterious immunologic and virologic consequences. Previous studies have demonstrated that short-term ATI did not lead to permanent expansion of the HIV reservoir or irreversible damages to the immune system of infected individuals who initiated ART during the chronic phase of infection [5–8]. Furthermore, it has been shown that a brief course of ATI followed by immediate reinitiation of ART after the plasma viremia level rebounded to >1000 copies/mL did not lead to higher levels of HIV DNA in $CD4^+$ T cells of individuals who initiated ART during the acute phase (Fiebig stage I) of infection [9]. Given the greater chance of achieving ART-free remission in early treated individuals, comprehensive assessment of curative interventions in such individuals may require extended periods of ATI despite high levels of plasma viral rebound [10]. Therefore, it is important to investigate the potential consequences of ATI in patients with significant levels of plasma viremia occur before reinitiation of ART. We conducted the present study to address the effect of ATI on immunologic and virologic parameters in early treated individuals.

MATERIALS AND METHODS

Study Participants

Twenty-two individuals with HIV infection who initiated ART and who had previously participated in a therapeutic vaccine trial [10] were included in this study (Table 1). Following a phase in which they received vaccine or placebo, all study subjects underwent ATI and resumed ART if they met any of the following criteria: a decrease of >30% in baseline $CD4^+$ T-cell count or a decrease in the absolute $CD4^+$ T-cell count to <350 cells/mm^3, a sustained (for ≥ 4 weeks) plasma viremia level of >50 000 copies/mL [10]. Blood and leukapheresed products were collected in accordance with protocols approved by the Institutional Review Board of the National Institute of Allergy and Infectious Diseases, National Institutes of Health. All subjects provided written informed consent.

Longitudinal Measurements of HIV Reservoirs

The dynamics of HIV reservoirs were examined in all participants before ATI (referred to as the "pre-ATI" period), during ATI (the "ATI" period), and following reinitiation of ART (the "post-ATI" period). The frequency of cells carrying HIV DNA was determined by droplet digital polymerase chain reaction

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Table 1. Profiles of Human Immunodeficiency Virus (HIV)–Infected Study Subjects

Subject Identifier	Viral Suppression Duration, y	$CD4^+$ T-Cell Parameter Before ATI		$CD8^+$ T-Cell Parameter Before ATI		Peak Plasma Viremia Level During ATI, Copies/mL	ATI Duration, d	ART Duration After ART Reinitiation, d
		Count, Cells/mm^3	Percentage	Count, Cells/mm^3	Percentage			
1	4.4	855	47	309	17	164 792	62	890
2	5.8	511	39	301	23	105 918	126	1071
5	7.0	559	43	468	36	8 405 097	47	947
6	2.7	1443	51	679	24	21 997	127	910
7	3.2	495	34	466	32	86 049	121	894
10	7.2	582	27	798	37	1605	139	766
12	8.2	1628	59	441	16	118 209	140	850
13	12.1	1540	54	428	15	113 416	144	879
14	14.0	679	39	470	27	24 667	117	888
15	4.0	932	45	725	35	416	114	413
17	3.8	584	42	348	25	5248	319	640
19	3.5	545	34	400	25	25 358	207	697
20	1.9	508	33	523	34	401 130	139	690
21	1.8	524	33	715	45	3223	117	738
22	5.6	1025	50	492	24	162 388	120	706
23	2.8	603	37	505	31	3262	126	645
24	2.6	484	37	536	41	1935	217	557
25	11.7	514	34	454	30	72 026	86	632
26	2.7	647	45	475	33	15 247	112	596
28	2.4	649	34	458	24	362 467	56	580
29	2.4	721	34	912	43	32 046	115	511
31	4.3	465	26	1091	61	2180	242	360
Median value	3.9	594	38	473	31	28 702	124	702

Abbreviations: ATI, analytical treatment interruption; ART, antiretroviral therapy.

(PCR) analysis (Bio-Rad Laboratories), using restriction endonuclease (MscI; New England BioLabs)–digested genomic DNA isolated from $CD4^+$ T cells as previously described [5]. The level of cell-associated HIV RNA was determined by reverse transcription PCR (RT-PCR). Total RNA was isolated (by the RNeasy Mini kit; Qiagen) from $CD4^+$ T cells, followed by synthesis of complementary DNA (qScript XLT cDNA Master Mix, Quanta Biosciences) and droplet digital PCR analysis (Bio-Rad Laboratories) as previously described [5]. HIV RNA copy numbers were normalized per 1×10^6 copies of the housekeeping gene *TBP*, which encodes TATA-box binding protein. The level of inducible HIV was determined by stimulating 10^6 $CD4^+$ T cells with a phorbol ester (50 nM of prostratin analog 11c) [11] in triplicate for 48 hours, followed by quantitation of cell-free HIV RNA, using the Cobas Ampliprep/Cobas Taqman HIV-1 Test, version 2.0 (Roche Diagnostics). The frequency of cells carrying replication-competent HIV was determined by quantitative coculture assays, using serially diluted (1×10^6, 200 000, 40 000, 8000, 1600, and 320) and replicates of 5×10^6 $CD4^+$ T cells as described elsewhere [5].

Longitudinal Measurements of Immune Parameters

The levels of lymphocyte subsets were determined by staining peripheral blood mononuclear cells with the following fluorophore-conjugated antibodies: CD3 (clone SK7), CD4 (clone SK3), CD8 (clone SK1), CD19 (clone SJ25C1), CD16 (clone B73.1), CD56 (clone NCAM16.2), CD38 (clone HB7), and HLA-DR (clone L243; BD Biosciences). Flow cytometry data were acquired on a BD FACS Canto II flow cytometer with FACSDiva software and analyzed using FlowJo.

Statistical Analysis

Three-way comparisons were performed using the Friedman test, followed by pair-wise comparisons with the Wilcoxon signed rank test if findings were statistically significant.

RESULTS

The study cohort comprised 22 HIV-infected individuals who initiated ART during the acute/early phase of infection and subsequently participated in a therapeutic vaccine trial [10]. The median duration of the ATI phase was 124 days (range, 56–242 days), and the plasma viremia level rebounded in all participants, with a median peak level of 28 702 copies/mL (range, 416–8 405 097 copies/mL). All study subjects resumed ART, per the predefined criteria [10], and received antiretroviral drugs for a median of 702 days (range, 360–1071 days) at the time of analysis. During ATI, the vast majority of study participants experienced significant

increases in the frequency of CD4$^+$ T cells carrying HIV DNA and cell-associated HIV RNA (Figure 1A). However, following reinitiation of ART, the frequency of CD4$^+$ T cells carrying HIV DNA and cell-associated HIV RNA returned to baseline levels, and there were no significant differences for either virologic marker between the pre-ATI and post-ATI time points (Figure 1A). Given that a large proportion of HIV present in infected CD4$^+$ T cells is replication defective, we conducted 2 additional assays designed to address the level of infectious HIV in CD4$^+$ T cells. First, the frequency of cells carrying inducible HIV was assessed before ATI and following reinitiation of ART, by stimulating CD4$^+$ T cells for 48 hours with a phorbol ester (prostratin analog). Second, we conducted quantitative coculture assays at the pre-ATI and post-ATI time points to assess the change in the size of the HIV reservoir carrying replication-competent virus. As shown in Figure 1A, there were no significant differences in frequencies of CD4$^+$ T cells carrying either inducible or replication-competent HIV between the pre-ATI and post-ATI periods. Of note, when the data were analyzed after excluding the study subjects who had received the therapeutic vaccine regimens [10], there was no impact on the changes in virologic markers shown in Figure 1A. Collectively, these data indicate that ATI and subsequent reinitiation of ART does not alter the size of HIV reservoirs in early treated individuals.

Having established that ATI leading to plasma viral rebound followed by reinitiation of ART had no measurable impact on

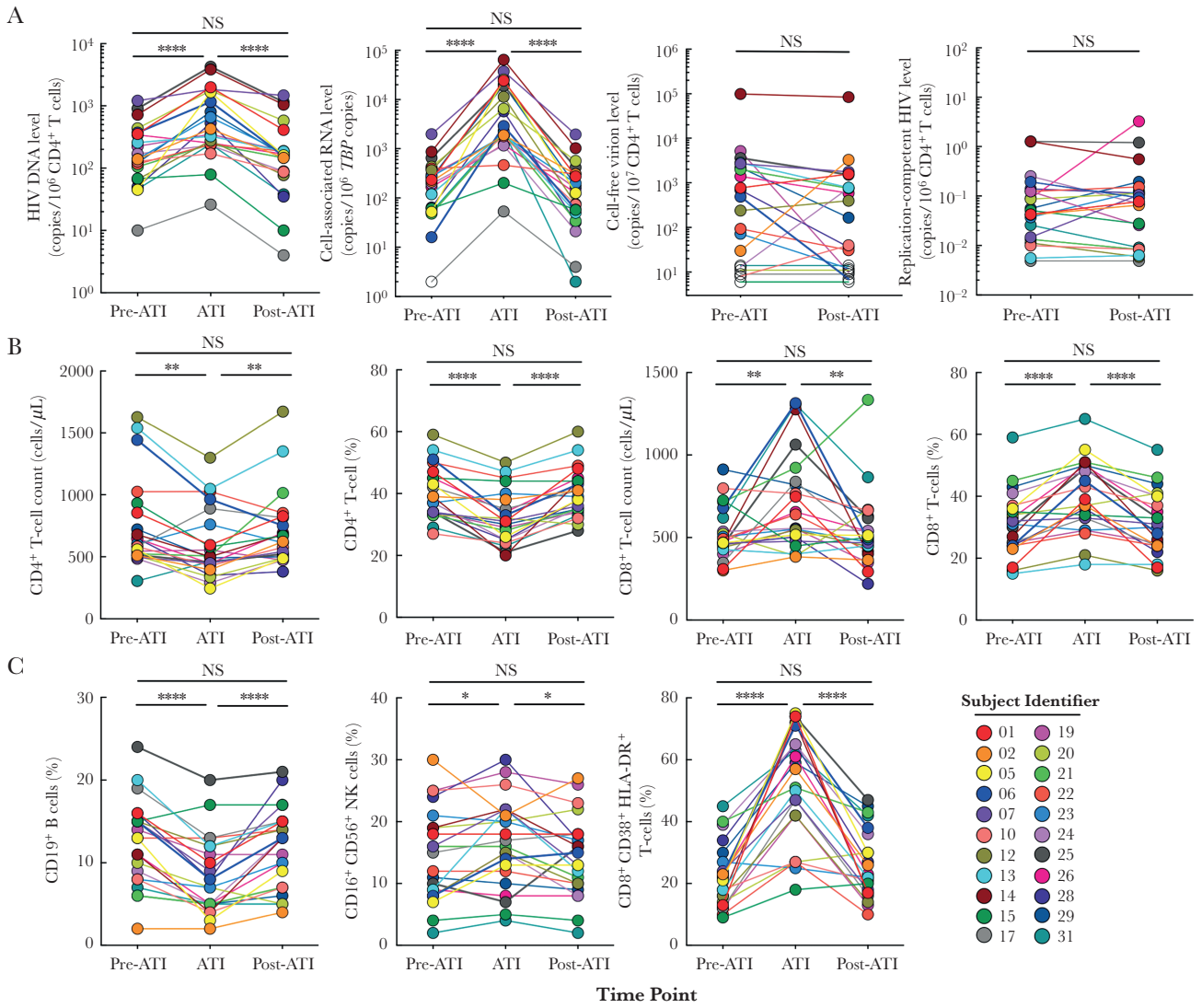

Figure 1. Kinetics of human immunodeficiency virus (HIV) reservoirs and immunologic parameters before and following analytical treatment interruption (ATI) and reinitiation of antiretroviral therapy (ART). *A*, The frequency of CD4$^+$ T cells carrying HIV DNA and cell-associated viral RNA was measured before ATI (the "pre-ATI" period), following ATI (the "ATI" period), and upon reinitiation of ART (the "post-ATI" period). The level of inducible cell-free virions and replication-competent HIV was measured before ATI and following reinitiation of ART. Open symbols indicate values under limits of detection. *B* and *C*, The frequency and cell count of CD4$^+$ and CD8$^+$ T cells (*B*) and the frequency of B (CD19$^+$), natural killer (CD16$^+$CD56$^+$), and CD8$^+$CD38$^+$HLA-DR$^+$ T cells (*C*) are shown at the pre-ATI, ATI, and post-ATI time points. NK, natural killer; NS, not significant. **P* < .05, ***P* < .01, and *****P* < .0001, by the Wilcoxon signed rank test.

the size of the persistent HIV reservoir, we examined several immune parameters longitudinally. During ATI, all subjects experienced a transient decrease in the numbers and frequencies of $CD4^+$ T and B ($CD19^+$) cells, as well as increases in $CD8^+$ T, natural killer ($CD16^+CD56^+$), and $CD8^+CD38^+HLA\text{-}DR^+$ T cells that coincided with plasma viral rebound (Figure 1B and 1C). Despite these changes, levels of all immune parameters examined returned to baseline, with no significant difference between pre-ATI and post-ATI time points (Figure 1B and 1C). Taken together, these data demonstrate that active HIV replication/rebounding plasma viremia following ATI contributes to transient perturbations in the frequency of circulating lymphocyte populations that return to pre-ATI frequencies following reinitiation of ART.

DISCUSSION

The development of therapeutic strategies aimed at achieving sustained virologic remission following discontinuation of ART has received considerable attention in light of overwhelming evidence that HIV reservoirs persist during clinically effective ART [12, 13] and that rapid plasma viral rebound occurs upon cessation of therapy in the vast majority of infected individuals [2]. Efforts to determine the efficacy of therapeutic interventions in clinical trials in which ATI was not used have included a variety of qualitative and quantitative laboratory-based assays designed to measure the size of persistent viral reservoirs [14], as well as immune responses against HIV [15]. However, most of these assays are unable to predict the likelihood of achieving durable control of HIV in infected individuals following ATI and as such are of limited clinical relevance [2]. Given that the plasma viremia level remains the only clinically relevant virologic marker available, clinical trials that evaluate therapeutic agents aimed at achieving ART-free virologic remission should include treatment interruption with intensive and extended monitoring of relevant immunologic and virologic parameters. In this regard, several studies have addressed the impact of ATI and reinitiation of ART on the dynamics of HIV reservoirs and immune markers in cohorts of infected individuals who initiated ART during the chronic phase of infection [5–8]. These studies have shown that a short course of ATI does not lead to irreversible expansion of the HIV reservoir or damage to the immune system [5–8]. However, there is a growing interest in exploring novel therapeutic interventions in HIV-infected individuals who have not thus far been extensively studied for the effects of ATI and reinitiation of ART, namely those who began ART during the acute/early phase of infection. Previous studies have shown that, compared with initiation of ART during the chronic phase of infection, early intervention is associated with smaller viral reservoir size, homogeneous viral quasispecies, and preservation of immune function, factors that might predict a better likelihood of controlling HIV replication following discontinuation of ART [3]. Given the reported differences in immunologic and virologic parameters associated with early versus delayed initiation of ART [3], the evaluation of such parameters following ATI and subsequent reinitiation of ART in early treated individuals is warranted. In this regard, a recent study involving a small number of HIV-infected individuals who initiated ART during the earliest possible stage of infection (ie, Fiebig stage I) revealed that the plasma viremia level rebounded with a median of 26 days following treatment interruption [9], similar to the time to rebound reported in cohorts of individuals whose therapy was initiated during the chronic stage of infection [1, 6]. Although the ATI study involving early treated patients did not find evidence of changes in the frequency of $CD4^+$ T cells carrying HIV DNA between the pre-ATI period and 6 months after reinitiation of ART, the findings were confounded by a prompt restart of ART, defined per protocol, as soon as the plasma viremia level reached 1000 copies/mL. It is possible that a longer period of treatment interruption may have provided more valuable information without compromising the clinical status of the patients. In this regard, we previously demonstrated that a substantial proportion of HIV-infected individuals who initiated ART early in the course of infection spontaneously controlled their plasma viremia for extended periods following high levels of initial plasma viral rebound [10]. Had we reinitiated ART immediately when the patients reached 1000 copies/mL, the subsequent prolonged control of plasma viremia observed in the absence of ART would have been missed. Based on these observations, we feel that it is justified and safe for clinical trials of potentially curative interventions in early treated HIV patients to include a period of ATI that could potentially lead to transiently high levels of plasma viral rebound. The study subjects in the present study underwent ATI that lasted a median of 124 days, with a median peak plasma viremia of 28 702 copies/mL followed by a median ART duration of 702 days after ATI. Despite the relatively high peak plasma viremia level and longer duration of ATI as compared to the previous study [9], we did not find evidence of irreversible expansion of the measurable HIV reservoir or alterations in subsets of immune cells in the peripheral blood. Future experiments involving phylogenetic analyses of rebounding virus and persistent HIV reservoirs, as well as in-depth examination of immune responses to the virus, would be necessary to support our findings. One caveat of our study was the inclusion of study subjects who received therapeutic vaccines before the ATI phase [10]. However, it is unlikely that the vaccine regimen had an impact on the parameters examined in this study, given the lack of effect of the vaccine regimens on the size of HIV reservoirs and HIV-specific immune responses [10]. Indeed, exclusion of the vaccine group from our current analyses and inclusion of only the placebo group had no impact on the overall conclusions. However, further investigations are warranted, including analyses of tissue compartments where a higher viral burden than that in the peripheral blood is expected. Collectively, our data

indicate that ATI with close monitoring of plasma viremia and inclusion of strict ART restart guidelines is safe. Furthermore, we propose that the importance of including ATI in clinical trials designed to examine the efficacy of therapeutic interventions aimed at achieving ART-free HIV remissions in infected individuals who initiated ART early in the course of infection outweighs the potential risks of ATI.

Notes

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