Expanded Cytotoxic T-cell Lymphocytes Target the Latent HIV Reservoir

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Enhanced human immunodeficiency virus (HIV)-specific immunity may be required for HIV eradication. Administration of autologous, ex vivo expanded, virus-specific, cytotoxic T-lymphocytes derived from HIV-infected patients on suppressive antiretroviral therapy (HXTCs) are a powerful tool for proof-of-concept studies. Broadly specific, polyclonal HXTCs resulting from ex vivo expansion demonstrated improved control of autologous reservoir virus compared to bulk CD8+ T cells in viral inhibition assays. Furthermore, patient-derived HXTCs were able to clear latently infected autologous resting CD4+ T cells following exposure to the latency-reversing agent, vorinostat. HXTCs will be ideal reagents to administer with precise control in future in vivo studies in combination with latency-reversing agents.

Keywords. adoptive T-cell therapy; ex vivo expanded T cells; HIV cure; HIV eradication; HIV immunology; HIV T cells; latent reservoir; vorinostat.

The latent human immunodeficiency virus (HIV) reservoir remains a major obstacle to eradication of persistent infection [1]. Therapies that clear latent HIV infection may require both a means to reactivate virus protein expression, and an immune response that recognizes and kills infected cells as they leave the latent state [2]. The extent antiviral immune response may be inadequate to clear such latently infected cells [3].

Augmented, polyclonal, functional antiviral responses have been created by ex vivo expansion of virus-specific T cells. These cells have successfully treated viral infections after hematopoietic stem cell transplantation and Epstein-Barr virus–associated malignancies [4, 5]. The use of ex vivo expanded T cells from HIV-infected individuals in place of potent antiretroviral therapy (ART) was first tested over a decade ago (reviewed in [6]). These studies established the safety of adoptive transfer of HIV-specific T cells, but cellular therapy by itself had only transient antiviral efficacy, due in part to the reliance on monoclonal T cells, extensive in vitro expansion, and their use in actively viremic patients. In a more recent study, HIV-specific CD8+ T cells derived from ART-suppressed patients were found to be persistent in the blood and capable of homing to rectal tissue following reinfusion, but virologic outcomes were not assessed [7].

In this study, we examine the ability of functional, broadly specific cytotoxic T cells (HXTCs) derived from 6 HIV+ individuals, clinically diverse but aviremic on durable ART, to target the latent reservoir ex vivo. We find that ex vivo expanded, polyclonal T cells broadly specific for multiple HIV epitopes [8] in Gag, Pol, and Nef (HXTCs) can be produced in clinically relevant numbers. These studies set the stage for testing of HXTCs in ART-suppressed patients. This combination approach may allow clearance of residual low-level viremia and if coadministered with latency reversing agents, might clear the latent reservoir itself.

MATERIALS AND METHODS

Patient Population
HXTCs were derived from HIV-infected donors with undetectable plasma viremia (<50 copies/mL) on stable ART. Participants were drawn from cohorts who began treatment within 45 days of diagnosis of acute HIV (AHI) or during chronic HIV infection (CHI). For in vivo vorinostat (VOR) exposure, patients received 3 doses of 400 mg VOR, with a leukapheresis performed at baseline and 6 hours following the third dose. Written informed consent was obtained from each patient and the study approved by the University of North Carolina, Baylor College of Medicine, and Children’s National Medical Center Biomedical Institutional Review boards.

Generation of HXTCs
HXTCs were generated as previously described (Supplemental Methods and [8]).
Viral Inhibition Assay
CD8+ T cells were isolated from peripheral blood mononuclear cells (PBMCs) by positive selection (EasySep human CD8+ Selection Kit, Stem Cell). CD8-depleted PBMCs were preactivated with 2 µg/mL phytohemagglutinin (PHA; Remel, Lenexa, KS) and 60 U/mL interleukin 2 (IL-2) and spinoculated at 2500 rpm for 90 minutes with either JR-CSR or autologous reservoir virus (AR) (obtained from pooled supernatant from outgrowth assays of resting CD4+ T cells for each patient) at a multiplicity of infection of 0.01. 5 x 10^4 targets/well were cocultured with HXTCs, autologous unexpanded CD8+ T cells, or no effectors, in 0.2 mL of Roswell Park Memorial Institute 1640 media supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, and 5 U/mL IL-2. Supernatant was assayed on day 7 by p24 enzyme-linked immunosorbent assay (AB, Rockville, Maryland). For major histocompatibility complex class I (MHC-I)–blocking experiments, targets were incubated with 10 µg/mL of clone w6/32 antibody (Pierce) for 1 hour prior to coculture.

Latency Clearance Assay
To measure the reduction in viral recovery in a viral outgrowth assay following the addition of autologous effector cells, resting CD4+ T cells were isolated from a leukeapheresis product as previously described [9] and exposed to PHA (4 µg/mL) and IL-2 (60 U/mL) for 24 hours or VOR (335 nM, 6 hours) (Merck Research Laboratories), and plated in a limiting dilution manner at 5 × 10^4 targets/well for 24 hours or VOR (335 nM, 6 hours) (Merck Research Laboratories), and plated in a limiting dilution manner at 0.5 to 2.5 × 10^6 cells/well in replicate wells. Drug was then washed off and effectors added (E:T 1:10). Cells were cocultured for 24 hours, then cultured with allogeneic CD8-depleted PBMCs from an HIV-negative donor to amplify virus. Antigen p24 was measured on day 15 for each well.

Statistical Analysis
Statistical comparisons between groups were analyzed using the Wilcoxon signed rank test for latency clearance assays (LCAs) when results were taken in aggregate across patients (n = 6). Significance for each individual patient was analyzed using a 2-tailed Student t test for viral inhibition assays (n = 3 replicates) and based on the variance of the outgrowth assay for LCAs. P < .05 was considered significant.

RESULTS AND DISCUSSION
HXTCs were derived from HIV-infected individuals on ART (Supplementary Table 1). Patients 231 and 250 initiated ART during AHI; suppressive ART was maintained for 6 years. Patients 423, 425, 492, and 532 initiated ART during CHI and suppressed for 3 to 11 years. Nadir CD4+ T-cell counts ranged from 16 to 516. Human leukocyte antigen (HLA) markers associated with either rapid disease progression (patient 532, HLA-B35*03; patient 423, HLA-B07*02) or immune control (HLA-B27*05, patient 250) were found.

HXTCs were predominantly CD8+ T cells (median, 89.1%), with a smaller population of CD4+ T cells (median, 7.4%). Most HXTCs were effector memory T cells (median, 87.2%) with a small subpopulation (median, 4.9%) of central memory T cells (Supplementary Figure 1A). HXTC generation was optimized to allow for production of T cells that are capable of further expansion upon restimulation, and HXTCs proliferate normally in response to PHA stimulation (Supplementary Figure 1B). Clinically relevant numbers of Nef-, Gag-, and Pol-specific HXTCs are recovered after expansion by this method [8]. In these studies, expansions ranging from 40-fold (patient 250) to >200-fold expansion (patients 423 and 492) were obtained.

Using a modification of an in vitro viral inhibition assay that has been associated with viral control in vivo [10], we tested the antiviral capacity of HXTCs against productively infected cells. As viral genomes with mutations that might lead to cytotoxic T-lymphocyte (CTL) escape can be found in the latent reservoir [11], we examined whether polyclonal HXTCs could recognize and inhibit epitopes derived from the latent reservoir by using AR, harvested from outgrowth assays of autologous resting CD4+ T cells.

In this assay, coculture with HXTCs at an effector to target ratio of 1:1 resulted in a significantly greater reduction of AR production than did unexpanded CD8+ T cells in patient 532 (13.2% of maximal AR production with HXTCs versus no reduction with CD8+ T cells; P < .01), and a trend toward greater reduction of AR production in patient 250 (2% of maximal AR production with HXTCs versus 20.8% with unexpanded CD8+ T cells; P = .3; Figure 1A, see Supplementary Figure 2A for absolute p24 values). MHc class I blockade significantly decreased the observed inhibition (P < .01) (Figure 1A, patient 532). Even at a lower E:T (1:10), superior antiviral effect was conferred by HXTCs compared to CD8+ T cells in most cases (Figure 1A). Notably, at E:T 1:10, the unexpanded CD8+ T cells (Figure 1A) performed no better than did CD8+ T cells from HIV-seronegative donors (Supplementary Figure 2B). Similar overall results with HXTCs were obtained against the HIV-1 clade B clone JR-CSR, with a trend toward a superior reduction in p24 production with HXTCs compared to unexpanded CD8+ T cells in all patients, although not significantly so in patient 425 due to the moderate restriction exerted by unexpanded CD8+ T cells (Supplementary Figure 2C). HXTCs are capable of detecting and responding to autologous virus derived from resting CD4+ T cells with greater efficiency than unexpanded CD8+ T cells in an MHC class I-restricted manner.
Viral epitope expression by productively infected cells may not mirror the rare antigen presented by latently infected resting cells following exposure to a reactivating agent. Hence, we developed a novel LCA to measure the reduction in viral recovery...
from cultures of resting CD4+ T cells mediated by the addition of effector cells following effective reversal of latency. Using this assay, we queried the ability of HXTCs to clear HIV from cultures of resting CD4+ T cells from aviremic, ART-treated, HIV-infected individuals, as these cells are induced to express latent virus. This is in contrast to previous studies that have relied on primary cell models to evaluate the impact of CD8 T cells in the context of latency [3]. Using the LCA, coculture of HXTCs with autologous PHA/IL-2-stimulated resting CD4+ T cells from 6 patients resulted in a median 50% reduction in recovery of virus \( (P < .05; \text{Figure 1D}) \) in a reproducible manner. Interestingly, HXTCs from 4 of the 6 patients respond to all 3

Figure 2. Impact of VOR on antiviral capacity of CD8+ T cells. A, CD8+ T cells from a representative patient (532) were pretreated with escalating doses of VOR for 24 hours prior to coculture with autologous CD8-depleted PBMCs superinfected with JR-CSF at an E:T of 1:10 or 1:1. B, The impact of increasing doses of VOR on proliferation of CFSE-labeled CD8+ T cells following stimulation with PHA/IL-2 is shown (C). Antiviral capacity of ex vivo CD8s isolated either before (“Pre-VOR”) or 6 hours following a third in vivo dose of VOR given 72 hours apart (“Post-VOR”) was compared in a viral inhibition assay. D, “Pre-” and “Post-VOR” CD8s were compared in a latent virus inhibition assays. Error bars represent SEM of \( n = 3 \) replicates, \( p \) calculated by Student \( t \) test. *Denotes \( P < .01 \) for CD8s compared to control with no CD8s (except for (A) exposure to 500 nM VOR, \( P = .03 \); (C) pre-VOR CD8 at E:T 1:10, \( P < .05 \)). Abbreviations: CFSE, carboxyfluorescein diacetate succinimidyl diester; IL-2, interleukin 2; PBMCs, peripheral blood mononuclear cells; PHA, phytohemagglutinin; SEM, standard error of the mean; VOR, vorinostat.
cognate antigens, gag, nef, and pol, in in vitro Elispot assays, while HXTCs from patient 423 responded to only nef and those from patient 250 to gag only [8], perhaps explaining the weaker antiviral activity of HXTCs from patients 250 and 423.

HXTCs led to a reduction in viral recovery in all 6 patients beyond that achievable with unexpanded CD8+ T cells. Unexpanded CD8+ cells exhibited little ability to blunt viral recovery following reactivation of CD4+ T cells by PHA (median 90%, $P = \text{NS}$), consistent with findings in primary cell models of latency [3, 12] and recent studies in which unexpanded T cells from aviremic patients showed no measurable HIV-specific response to cognate peptides in Elispot assays [8]. Strongly enhanced activity was even seen in a CHI patient with a history of a low nadir CD4 count and the HLA-B35*03 allele (patient 532) associated with rapid disease progression.

Due to the need to use allogeneic HIV-seronegative cells to efficiently recover latent HIV, we cannot exclude a contribution of immune response to allogeneic stimuli. However, HXTCs possess specific, enhanced antiviral effect compared to unexpanded CD8+ T cells, consistent with results obtained in the viral inhibition assays performed without allogeneic cells (Figure 1A), and strongly correlate with the results of the LCA ($r = 0.67; \quad P = .01$; Supplementary Figure 2D).

Responding effector cells must recognize HIV antigens induced by latency-reversing agents. We therefore confirmed our findings using a physiologically relevant exposure to the histone deacetylase (HDAC) inhibitor VOR [13]. HXTCs increased viral clearance substantially in all 3 patients tested (Figure 1C).

HDAC inhibition used to induce the expression of latent HIV might have deleterious immunomodulatory effects [14]. We tested the impact of physiologically relevant exposures of VOR on the antiviral capacity of CD8+ T cells. VOR concentrations peak near 335 nM at 4 hours, with rapid clearance of drug within 8 hours [13]. Pretreatment with VOR for up to 24 hours at concentrations as high as 1000 nM did not significantly impair CD8+ T-cell antiviral activity at an E:T of 1:1 or 1:10 (Figure 2A); neither did VOR impair the ability of the CD8+ T cells to proliferate in response to PHA stimulation (Figure 2B). These results are consistent with those from a recent study, which did not find any impairment of CTL activity at physiologically relevant exposures to VOR [14]. However, it is possible that HDAC inhibitors with longer half-lives, which we did not study, may exert a negative effect on CTL function.

The impact of in vivo VOR exposure on the immune response will reflect a more complex interplay of factors than can be fully modeled ex vivo. Using samples from an ongoing clinical trial (NCT01319383), we compared the antiviral capacity of CD8+ T cells obtained either before (“pre-VOR”) or 6 hours following a third in vivo dose of 400 mg VOR (“post-VOR”). We found no reduction in the ability of post-VOR CD8s to inhibit productive viral infection (Figure 2C) or to eliminate infected cells as they emerged from latency (Figure 2D). In fact, at higher E:T ratios, post-VOR CD8s exerted an unexpectedly higher degree of viral inhibition than did pre-VOR CD8s (Figure 2C), possibly reflecting antigenic stimulation resulting from transient in vivo reversal of latency, although this finding merits further evaluation.

In summary, we show that HXTCs are able to inhibit productive viral infection with autologous reservoir virus and reduce viral recovery following reactivation of resting CD4+ T cells from latency with VOR. It will be important to bear in mind that an impact on the latent reservoir may not be seen in the absence of reactivation of latency, and thus combination studies of immune therapeutics with latency-reactivating agents may be needed. Therefore, it is encouraging that we did not observe any deleterious effects of in vivo exposure of VOR on the antiviral capacity of HXTCs, providing further data to support the combination of HXTCs with VOR in future studies.

**Supplementary Data**

Supplementary materials are available at The Journal of Infectious Diseases online (http://jid.oxfordjournals.org). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

**Notes**

**Acknowledgments.** We thank R. Bateson, N. Dahl, and B. Allard for technical support, J. Kuruc for study management, A. Crooks for study coordination, and M. Hudgens for statistical analysis. We thank the HIV-positive volunteers involved in this project for their donations to this research.

**Financial support.** This work was supported by National Institutes of Health (NIH) U19-AI096113 to D. M. for the Collaboratory of AIDS Researchers for Eradication, T32-AI07001 to J. S., AI136211 to C. B., P30-A1504100 to R. I. S. for the University of North Carolina Center for AIDS Research, a Bristol Myers Squibb Virology Fellowship to J. S., and by NIH contract PACT NIH-NHLBI-NO1 HB37163.

**Potential conflicts of interest.** All authors: No reported conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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