

Initial HIV-1 Antigen-Specific CD8⁺ T Cells in Acute HIV-1 Infection Inhibit Transmitted/Founder Virus Replication

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CD8-mediated virus inhibition can be detected in HIV-1-positive subjects who naturally control virus replication. Characterizing the inhibitory function of CD8⁺ T cells during acute HIV-1 infection (AHI) can elucidate the nature of the CD8⁺ responses that can be rapidly elicited and that contribute to virus control. We examined the timing and HIV-1 antigen specificity of antiviral CD8⁺ T cells during AHI. Autologous and heterologous CD8⁺ T cell antiviral functions were assessed longitudinally during AHI in five donors from the CHAVI 001 cohort using a CD8⁺ T cell-mediated virus inhibition assay (CD8 VIA) and transmitted/founder (T/F) viruses. Potent CD8⁺ antiviral responses against heterologous T/F viruses appeared during AHI at the first time point sampled in each of the 5 donors (Fiebig stages 1/2 to 5). Inhibition of an autologous T/F virus was durable to 48 weeks; however, inhibition of heterologous responses declined concurrent with the resolution of viremia. HIV-1 viruses from 6 months postinfection were more resistant to CD8⁺-mediated virus inhibition than cognate T/F viruses, demonstrating that the virus escapes early from CD8⁺ T cell-mediated inhibition of virus replication. CD8⁺ T cell antigen-specific subsets mediated inhibition of T/F virus replication via soluble components, and these soluble responses were stimulated by peptide pools that include epitopes that were shown to drive HIV-1 escape during AHI. These data provide insights into the mechanisms of CD8-mediated virus inhibition and suggest that functional analyses will be important for determining whether similar antigen-specific virus inhibition can be induced by T cell-directed vaccine strategies.

During acute human immunodeficiency virus type 1 (HIV-1) infection (AHI), virus replicates rapidly and is typically shed at high levels in mucosal fluids and circulating blood plasma. HIV-1-specific CD8⁺ T cells are among the earliest immune responses that arise before peak viremia and contribute to the initial viral decline (3, 32), resulting in virus escape mutations within the first 50 days of infection (22). These cytotoxic T lymphocyte (CTL)-driven escape mutations that appear rapidly in the transmitted/founder (T/F) virus during acute infection demonstrate that the initial CD8⁺ T cell response applies pressure on early virus replication. Vaccine strategies that can elicit rapid and durable HIV-1-specific CD8⁺ T cell responses from which the T/F virus cannot escape will probably be critical for inhibiting virus replication *in vivo* and for controlling viral load (VL) in the absence of sterilizing immunity. Recent proof-of-concept vaccine studies in nonhuman primates (24) have demonstrated that vaccine-elicited CD8⁺ T cell responses can substantially control virus replication and contribute to protection. Identifying key attributes of CD8⁺ T cells that associate with virus control and determining whether CD8⁺ T cells exert antiviral pressure is important for understanding the quality of CD8⁺ T cell responses that vaccine strategies should target. A detailed understanding of the functional properties of CD8⁺ T cells that arise during the time of initial viremic control in acute infection will inform vaccine design by focusing on strategies that can decrease viremia.

The phenotypic and functional properties of CD8⁺ T cells that can mediate natural control of virus replication in an exclusive group of individuals, virus controllers (VCs), have been characterized (18, 46, 47). Additionally, T cell polyfunctionality (1, 2)

and Gag specificity (6, 28, 48) are associated with viremic control and a slower disease course in HIV-1-positive individuals. Reduction in viremia by CD8⁺ T lymphocytes without the lysis of infected cells was demonstrated in simian immunodeficiency virus (SIV) infection of nonhuman primates (NHP) (27, 31, 58), demonstrating that the antiviral activity of CD8⁺ T lymphocytes includes soluble components. A recent paper has given evidence that immune pressure is not limited to cytotoxicity of infected cells (16), as the total CD8⁺ T cell response to acute infection was shown to possess a high contribution of MIP-1β-producing CD8⁺ T cells. We also previously demonstrated that the percentage of MIP-1β-positive CD8⁺ T cells correlated positively with virus inhibition in virus controllers and vaccinees (18).

Here, we have utilized a novel combination of four assays used in concert to assess CD8⁺ T cell function during acute HIV-1 infection. The ability of primary CD8⁺ T lymphocytes to inhibit HIV-1 virus replication (full-length HIV-1 T/F infectious molecular clones) via cell-cell contact and also through soluble mechanisms was determined. Antigen-specific cytokine responses of these same CD8⁺ T cells were determined both through multiparameter intracellular cytokine-staining assays and antigen-spe-

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cific cytokine secretion in cell culture supernatants. We found that, following HIV-1 transmission, there was rapid and durable elicitation of autologous HIV-1-specific CD8⁺ T cell-mediated inhibition of the T/F virus along with rapid elicitation but less durable breadth for heterologous transmitted viral species. Similar to HIV-1 controllers, the functional HIV-1 antigen-specific CD8⁺ T cells studied here can mediate inhibition of virus by soluble components. In one patient with virus escape mutations from defined CTL function during acute infection (22), we demonstrated functional escape from CD8⁺ T cell-driven virus inhibition. Moreover, this is the first report that CD8⁺ T cells mediating soluble inhibition were of the same antigen specificities as the CD8⁺ T cells that had previously been determined to drive virus escape. Altogether, these data demonstrate that multifunctional HIV-1-inhibitory CD8⁺ T cell populations against both autologous and heterologous circulating HIV-1 strains arise rapidly following infection.

MATERIALS AND METHODS

Patient cohorts. HIV-1-positive patients were enrolled through the Center for HIV/AIDS Vaccine Immunology HIV-1 infection prospective cohort study. CHAVI 001 acute patients were identified as positive for HIV-1 viral RNA in plasma but having been negative or discordant for HIV-1 serology at a screening visit. Blood samples were obtained from patients at multiple time points over several years, and samples taken during the acute phase of infection were classified according to the criteria of Fiebig et al. (17). HIV-1 clade C-positive elite controllers were enrolled through the chronic arm of the CHAVI 001 cohort. HIV-1 clade B elite virus controllers were enrolled through the Duke University Medical Center adult infectious diseases clinic. Elite controllers included in this study were antiretroviral therapy naïve, had CD4⁺ lymphocyte counts of more than 600 per ml of blood, and had viral loads that remained below the level of detection (48 copies/ml blood) or, in the case of one subject, demonstrated infrequent, isolated viral RNA blips of <2,500 copies/ml blood. One additional VC did not control virus to an elite (undetectable) level but maintained a VL of <2,000 RNA copies/ml. HIV-1-negative control CD8⁺ T cells were obtained as leukopaks, either from the American Red Cross or from healthy uninfected donors through the Duke IQAC program. The CHAVI Acute and Chronic Cohorts study and the Duke virus controller study were reviewed and approved by the institutional review boards of Duke University Medical Center. All participants provided written informed consent for study participation.

PBMC isolation and cell subset preparation. Peripheral blood mononuclear cells (PBMC) were isolated using Ficoll-Paque Plus (GE Healthcare) density gradient centrifugation. PBMC were frozen (90% fetal bovine serum [FBS]–10% dimethyl sulfoxide) and stored at –180°C until analyzed. On the day of analysis, cells were thawed in a 37°C water bath, transferred to prewarmed RPMI 1640 (Gibco/Invitrogen) supplemented with 10% heat-inactivated FBS, and examined for recovery and viability. Cells were activated by OKT3 and anti-CD28 antibodies for 3 to 6 days and cultured in RPMI 1640 supplemented with 20% FBS, penicillin-streptomycin, and 20 U/ml recombinant human interleukin-2 (IL-2) (PeproTech). Activated T cells were separated into CD8⁺ and CD8[–] subsets using a CD8⁺ T cell isolation kit and CD8⁺ depletion beads, respectively (Miltenyi Biotech).

Transmitted founder viruses/infectious molecular clones. This study utilized full-length infectious molecular clones (IMC) (40) for expressing the T/F virus sequences inferred from CHAVI 001 subjects infected, respectively, with clade B (40, 49) and clade C HIV-1 (H. Ding and C. Ochsenauber, unpublished data). The T/F sequence derivation for and construction of the clade C IMC were conducted essentially as previously described (40). Virus nomenclature is as follows. Clade B T/F viruses are CH040.c, CH058.c, CH077.t, and CH106.c. Clade C T/F viruses are CH042.c, CH162.c, and CH185.c. For subjects 700010077 (CH077) and

700010058 (CH058), escape mutations in CTL epitopes have been demonstrated at 6 months postinfection (22). IMC representing the consensus sequence of full-length genomes of circulating viruses at the 6-month sampling time point were constructed using methods involving chemical synthesis of overlapping subgenomic fragments followed by ligation and cloning, essentially as described previously (34, 49), and are referred to CH058.mo6 and CH077.mo6, respectively.

Replication-competent virus stocks of the IMC were generated as described previously (15, 18, 29, 40). Briefly, proviral DNA was transfected into 293T cells by using Fugene HD (Roche). Working stocks were amplified by passaging virus in human PBMC (American Red Cross). PBMC-derived virus supernatants were collected every 2 to 3 days and filtered through a 0.45- μ m syringe filter, and titers were determined on TZM-bl cells (obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, from John C. Kappes, Xiaoyun Wu, and Tranzyme, Inc.) (12, 43, 56).

CD8 VIA. The ability of primary CD8⁺ T cells to inhibit virus replication in autologous CD4⁺ T cell-enriched targets was measured using an adaptation of a previously described CD8 virus inhibition assay (CD8 VIA) (18). In brief, PBMC targets were depleted of CD8⁺ T cells by magnetic bead separation as described above. CD8⁺ T cell effectors were isolated by negative selection using the CD8⁺ T cell isolation kit (Miltenyi). Target PBMC were infected by spinoculation at $1,200 \times g$ for 2 h at a multiplicity of infection (MOI) of 0.1. Following spinoculation, infected targets were plated at 2.25×10^4 cells per well in a flat-bottom 96-well tissue culture plate. CD8⁺ effectors were serially diluted 2-fold from 4.5×10^5 to 5.6×10^4 cells/ml and added to autologous infected targets at corresponding effector-to-target (E/T) ratios of 2:1, 1:1, 1:2, and 1:4. The infectivity controls consisted of infected targets without added CD8⁺ effectors. Cultures were incubated at 37°C and 5% CO₂ for 6 to 7 days with a change of 50% fresh medium at day 3. Virus production was measured at day 6 or 7 by transfer of supernatants onto TZM-bl cells. A single dose of supernatant was transferred for all wells, and in addition, supernatants from control wells and 2:1 E/T wells were titrated on TZM-bl cells by serial dilution. Following 2 days of incubation, the TZM-bl cells were lysed and firefly luciferase measured using the BriteLite Plus reagent (Perkin Elmer) on a Berthold EG&G luminometer. Virus inhibition was calculated as either the log reduction in relative light units (RLU) from wells containing CD8⁺ T cells compared with that in control wells lacking CD8⁺ T cells or the log reduction in virus titer (50% tissue culture infective dose [TCID₅₀]) at a 2:1 E/T ratio compared with the reduction in the infectivity control virus titer.

HIV-1 peptide stimulation and soluble-factor virus inhibition assay (HIV-1-specific CD8 transwell VIA). Twelve pools of overlapping 15-amino-acid peptides that represent potential T cell epitopes (PTE) (33, 37) (obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH) were created to represent the following HIV-1 regions based on HXB2 position: Env1 4 to 296, Env2 297 to 488, Env3 489 to 602, Env4 603 to 840, Gag1 1 to 128, Gag2 131 to 361, Gag3 362 to 486, Pol1 1 to 152, Pol2 156 to 447, Pol3 452 to 709, Pol4 711 to 988, and Nef 1 to 193. Positively selected CD8⁺ T cells (following 2 days of rest after separation) were stimulated with peptide pools at 2 μ g/ml with anti-CD28 and anti-CD49d (1 μ g/ml each; Becton Dickinson) in PBMC medium (as described above) for 5.5 h. Following stimulation, CD8⁺ cells were diluted in PBMC medium to 3.2×10^5 cells/ml and aliquots placed in the upper chambers of a 96-well transwell plate (0.4- μ m polycarbonate membrane; Corning, Millipore) at 3.2×10^4 cells per well (2:1 E/T). Trypsinized TZM-bl cells were added to the bottom chambers of the transwell plate with DEAE-dextran (15 μ g/ml) and virus at an MOI of 0.2. Following 48 h of incubation at 37°C and 5% CO₂, virus replication was measured by quantification of cell-derived luciferase using BrightLite reagent (Perkin Elmer) as described above. Virus suppression was determined as the log reduction in RLU in wells containing CD8⁺ T cells compared with that in control wells lacking CD8⁺ T cells, and significant virus inhibition was calculated as >0.2-log reduction in RLU. Virus sup-

TABLE 1 Demographics and clinical data

Patient	Age	Ethnicity	Gender	Cohort ^a	Clade	HLA			CD4 range	VL range	ART
						A	B	C			
700010077 (CH077)	23	AA	M	Acute	B	02/02	53/57	04/18	664–914	1.8e5–1.7e3	Naïve
700010106 (CH106)	56	W	M	Acute	B	11/24	35/35	04/04	277–869	1.5e7–<50	Week 1
701010199 (CH199)	19	AA	M	Acute	B	66/30	58/42	03/17	485–546	1.2e5–3.5e3	Naïve
702010157 (CH157)	21	African	M	Acute	C ^b	24/34	44/–	04/04	256–382	>7.5e5–7.6e3	Naïve
702010736 (CH736)	20	African	M	Acute	C ^b	29/29	42/45	04/06	213–367	>7.5e5–1.6e5	Naïve
707010616 (CH616)	62	African	F	EC	C ^b	02/68	07/57	07/07	889–2397	<40	Naïve ^c
707010668 (CH668)	42	African	F	EC	C ^b	01/30	42/81	17/18	610–743	<48	Naïve ^c
707010735 (CH735)	38	African	F	EC	C ^b	29/30	14/42	08/05	439–715	<40	Naïve ^c
707010774 (CH774)	24	African	F	EC	C ^b	30/68	53/57	04/18	1134–1151	57	Naïve ^c
VC18 0873–5017	48	W	M	EC	B	02/03	27/35	01/04	1222	209	Naïve ^c
VC9 0873–5014	58	W	M	VC	B	03	07/40	03/07	848	1,020	Naïve

^a EC, elite controllers; VC, virus controllers.

^b Presumed virus clade based on geographic location.

^c ART status based on clinical data.

pression from stimulated CD8⁺ cells from an HIV-1-seronegative donor was subtracted as background for each peptide pool.

Cytokine measurement. Supernatants were collected from the bottom chambers of the transwells (HIV-1-specific CD8 transwell VIA, described above) following the 2-day incubation and prior to TZM-bl cell lysis and were stored at –80°C. Cytokine levels (e.g., macrophage inflammatory protein 1α [MIP-1α], MIP-1β, gamma interferon [IFN-γ], interferon-inducible protein of 10 kDa [IP-10], IL-4, and IL-1α) were measured using a Milliplex Map cytokine/chemokine immunoassay Luminex kit (Millipore) according to the manufacturer's instructions and as previously described (51).

Multiparameter intracellular cytokine-staining assay. PBMC were stimulated with the PTE peptide pools described above. Stimulation with 1 μg/ml anti-CD3 (OKT3) and 1 μg/ml anti-CD28 was used as positive controls, as was stimulation with a peptide pool representing the cytomegalovirus (CMV) pp65 protein (JPT, Germany). The titer of each antibody was determined to obtain the saturating concentration used for the final staining. The stimulations were conducted in the presence of 1 μg/ml anti-CD49d monoclonal antibody (MAB) (clone L25; BD), anti-CD107a phycoerythrin (PE)-Cy5 (clone H4A3; eBioscience), 5 μg/ml brefeldin A (Sigma-Aldrich), and 1 μg/ml Golgi Stop (BD) for 5.5 h at 37°C in 5% CO₂. After washing, the cells were stained with aqua blue viability indicator (LIVE/DEAD fixable dead cell stain kit; Invitrogen) in phosphate-buffered saline for 20 min at room temperature. Cells were then washed and stained for 20 min at room temperature with a surface stain cocktail containing anti-CD4-Cy5.5-PE (clone M-T477; Biolegend), anti-CD8-QD605 (clone RPA-T8; Invitrogen), anti-CD27-Cy7-PE (clone M-T271; BD Bioscience), anti-CD57-QD565 (clone NK-1; AbD, Serotec; conjugated in G. Ferrari's laboratory), and anti-CD45RO-PE-Texas red (clone UCHL1; Beckman Coulter). PBMC were subsequently fixed and permeabilized with Cytofix/Cytoperm and Perm/Wash buffer (Pharmingen, San Diego, CA) for 20 min, washed twice, and stained with anti-CD3-Cy7-antigen-presenting cell (APC) (clone SK7; BD Bioscience), anti-IFN-γ-Alexa Fluor 700 (B27; BD Bioscience), anti-IL-2-APC (clone MQ1-17H12), anti-MIP-1b-fluorescein isothiocyanate (FITC) (clone 24006; R&D Systems), and perforin PE (clone B-D48; Cell Sciences) for 1 h at 4°C. After washing and fixation, all samples were acquired on a custom-made LSRII (BD Bioscience, San Jose, CA) within the next 24 h. Gates were set to include singlet events, live CD3⁺ cells, lymphocytes, and CD4⁺ and CD8⁺ subsets. From the total CD4⁺ and CD8⁺ populations, the naïve subset was identified as CD45RO[–] CD27⁺. This subset was excluded from the subsequent analysis, and only the memory population was included. Within the memory population, the central memory (CM) CD45RO⁺ CD27⁺ (RO⁺ 27⁺), effector (E) CD45RO⁺ CD57⁺ (RO⁺ 57⁺), and terminal effector (TE) CD45RO[–] CD57⁺ (RO[–] 57⁺) popula-

tions were identified. Antigen-specific populations were identified within the memory population as single-function cells shown in the sequential single-cytokine/-chemokine/-degranulation gates. Responses were considered positive if the percentage of antigen-specific cells was 3-fold above the background and greater than 0.05% after background subtraction. Data analysis was performed using FlowJo 8.8.4 software (TreeStar).

Statistical analyses. Statistical analyses were performed using Prism 5 software (GraphPad). Correlations between cytokine expression level and virus inhibition were calculated using Pearson's correlations.

RESULTS

Breadth of inhibition of transmitted/founder viruses by acute CD8⁺ T cells. We examined the breadth of CD8⁺ T cell virus inhibition during acute infection using primary CD8⁺ T lymphocytes from CHAVI 001 acute-infection subjects (Table 1). CD8⁺ T cells isolated from the peripheral blood during the acute phase of infection (Fiebig stages 1 through 5) were tested for the ability to inhibit both autologous and heterologous T/F virus replication in primary CD4⁺ T lymphocytes. To test the breadth of CD8⁺ T cell-mediated virus inhibition across relevant circulating clade B and clade C viruses, a panel of seven full-length T/F IMC and two 6-month full-length IMC were examined in a VIA assay. CD8⁺ T cells isolated during acute infection inhibited both autologous and heterologous viruses at the earliest time points examined with a trend toward a higher magnitude of inhibition of autologous viruses. Acute CD8⁺ T cells reduced T/F virus titers by a mean of 1.5 logs more than seronegative controls at an E/T ratio of 2:1 (Fig. 1). We previously demonstrated that virus controllers have potent CD8 antiviral responses against IMC that contain T/F envelope sequences (18). Here, we refined our studies by utilizing recently constructed IMC of both clade B (40) and C HIV-1 that represent the complete HIV-1 T/F genomes inferred from subjects during acute infection. Additionally, we tested CD8⁺ T cells from four elite controllers from the CHAVI 001 established-infection cohort (presumed clade C infection, based on clade C accounting for 100% of HIV-1 sequences analyzed from these clinical sites) and one elite controller from the Duke infectious diseases clinic to compare to the breadth of acute CD8⁺ T cell responses. Similar to CD8⁺ T cells from acute infection, elite controllers exhibited a breadth of inhibition of the panel of T/F viruses with a magnitude that was 1.8 logs greater than that of seronegative controls. While the mean potency exerted by acute CD8⁺ T cells against T/F vi-

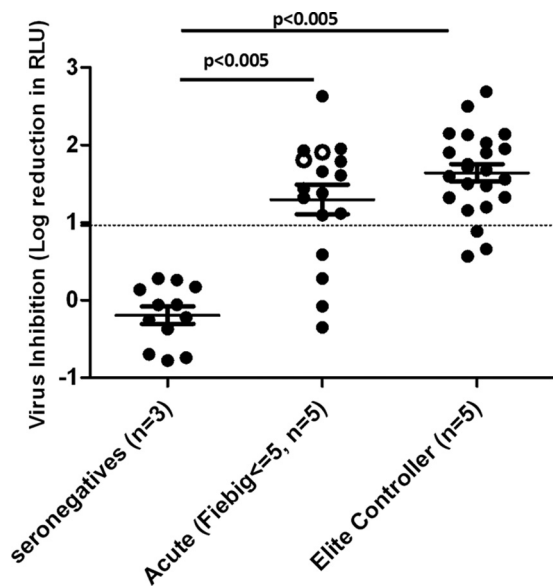


FIG 1 CD8⁺ T cells from HIV-1 donors before or during the time of seroconversion suppress T/F viruses at levels similar to suppression in elite controllers. CD8⁺ T cells isolated from 3 seronegative donors, 5 HIV-1-positive donors (Fiebig score of ≤ 5), and 5 HIV-1-positive elite controllers were tested for antiviral activity against a panel of 7 T/F IMC in the CD8 VIA. Dots represent the log reduction in virus replication (RLU) for each CD8 donor against a single virus at an E/T ratio of 2:1 compared to the results from a CD8-depleted infection control. Statistical significance was determined by using the mean virus inhibition across T/F viruses for each donor (Student's *t* test). The dotted line indicates a log inhibition cutoff for positivity of 0.96 (3 standard deviations above the mean seronegative donor T/F virus inhibition).

rus was lower than that exerted by elite controllers, this difference was not found to be significant.

Ontogeny and durability of inhibition of heterologous and autologous T/F viruses by acute CD8⁺ T cells. To examine the ontogeny of CD8⁺ T cell virus inhibition, we examined CD8⁺ T cells isolated longitudinally over the course of the earliest stages of acute infection from four HIV-1-infected donors. We first measured the ontogeny and durability of the acute CD8⁺ T cell-mediated response with a panel of heterologous T/F viruses using the CD8 VIA (Fig. 2). CD8⁺ T cells from both presumed clade B infections (subjects CH199 and CH106) and presumed clade C infections (subjects CH157 and CH736) and isolated from Fiebig stages 1 to 5 of acute infection broadly mediated the inhibition of several heterologous viruses. Inhibition of virus from both matched and mismatched clades was observed, with 91% of heterologous viruses inhibited by >1 log at the earliest time point tested (Fig. 2 and Table 2). As shown in Fig. 2, the potency of mean virus inhibition of heterologous T/F viruses mirrors the dynamics of virus load over time, suggesting that the level of inhibitory activity might be dependent on constant antigen stimulation. We then measured the ontogeny and durability of autologous CD8⁺ T cell-mediated virus inhibition using longitudinal acute samples and T/F virus from the same subject, CH106 (Fig. 3). While CD8 inhibition of heterologous virus in this subject rapidly declined 1 to 2 logs concurrent with the resolution of viremia (after antiretroviral therapy [ART]) (Table 1 and Fig. 2 and 3), CD8-mediated inhibition of autologous T/F virus was maintained at peak levels 48 weeks following enrollment (Fig. 3). These data demonstrate

the rapid and durable development of the virus-specific inhibitory CD8⁺ T cell memory response despite reduced viremia, and yet they also highlight the limitation of the CD8⁺ T cell response to novel (heterologous) viruses in the requirement for continuous antigen stimulation.

Impaired CD8⁺ T cell inhibition of early T cell escape viruses compared to inhibition of T/F viruses. To determine whether CD8-mediated virus inhibition could have a role in driving virus escape, we examined cells obtained at three consecutive early time points from an acute HIV-1-positive donor beginning at Fiebig stage 4 (subject CH077). Epitopic changes in the virus consistent with virus escape from CD8⁺ T cell IFN- γ responses in this HLA-B57 donor were characterized in previous studies (16, 22). Using the CD8 VIA, we examined the ability of CD8⁺ cells isolated during acute infection to inhibit replication of the autologous T/F virus and a later autologous IMC which represents the consensus sequence of circulating viruses at 6 months following enrollment and contains identified T cell escape mutations. In addition, we examined virus inhibition of paired heterologous T/F and 6-month viruses derived from another HLA-B57 donor (subject CH058). We found that both autologous and heterologous T/F viruses were inhibited by 2 logs during a span of 3 weeks during acute infection (Fig. 4A). However, CD8⁺ T cells obtained during acute infection were impaired in their ability to inhibit the 6-month autologous and heterologous viruses. These 6-month viruses were not significantly resistant to inhibition by heterologous (not HLA-matched) acute-infection CD8⁺ T cells (Fig. 4B), suggesting a common mechanism of escape, possibly to HLA-B57-restricted epitopes, exerted by autologous donor CD8⁺ T cells. These data are congruent with previous findings that virus rapidly escapes from CD8⁺ T cell responses in this donor. Taken together, these data demonstrate that virus can rapidly escape from specific CD8⁺ T cell responses during acute infection, diminishing the ability of CD8⁺ T cells to control virus replication in early infection.

Acute-infection CD8⁺ T cells can inhibit transmitted/founder viruses via soluble-factor virus inhibition. We previously demonstrated that virus inhibition correlates with MIP-1 β expression (18) and that strongly suppressive CD8⁺ T cells up-regulate the expression of inflammatory cytokines and chemokines, including MIP-1 α , MIP-1 β , IFN- γ , IP-10, and IL-1 α (51). To evaluate whether antigen-specific acute-infection CD8⁺ T cells with inhibitory capacity against T/F viruses can also mediate soluble-factor virus inhibition, we employed a novel assay using a transwell system to measure the inhibition of T/F virus infection of target cells in one chamber following HIV-1 peptide stimulation of CD8⁺ T cells present in the other chamber. First, CD8⁺ T cells from a virus controller (VC9) were examined for the ability to mediate soluble HIV-1 inhibition following HIV-1 antigen stimulation. Aliquots of CD8⁺ T cells were stimulated with pools of HIV-1 peptides representing potential T cell epitopes (PTE) and cultured with HIV-1-infected TZM-bl cells separated from the effector cells by a semipermeable membrane. As shown in Fig. 5A, soluble inhibitory activity was stimulated by multiple HIV-1 peptide pools, with the most potent responses resulting from Gag2, Pol2, and Nef peptide pool stimulation. Both R5-tropic T/F viruses and an X4-tropic laboratory-adapted virus (IIB) were inhibited via soluble CD8⁺ T cell responses. Virus inhibition correlated with the percentage of HIV-1-specific CD8⁺ cells expressing CD107a, MIP-1 β , and IFN- γ , as determined by flow cytometric

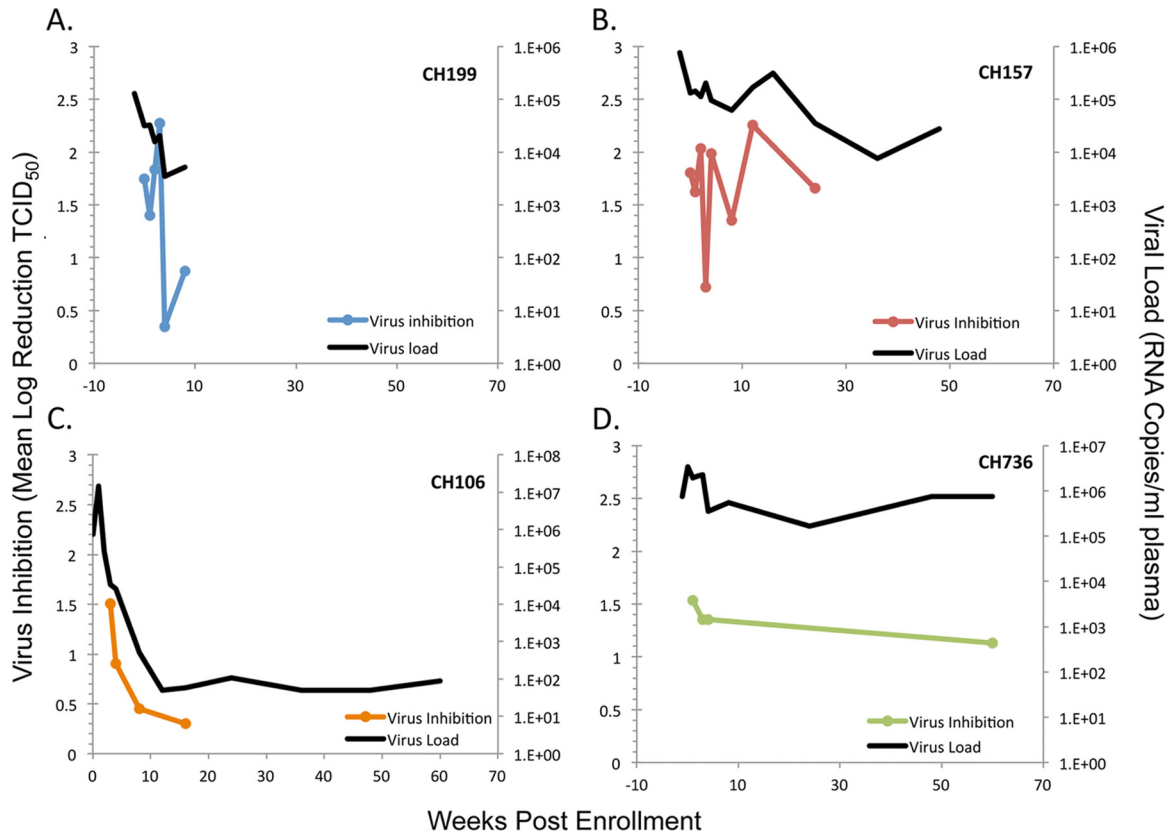


FIG 2 CD8⁺ T cell-mediated virus inhibition of heterologous T/F viruses is dynamic during the acute phase of infection. Antiviral activity was measured from CD8⁺ T cells isolated longitudinally from 4 HIV-1-positive patients (3 ART-naïve and CH106 who began ART at study week 1) during acute infection (shown as log reduction in virus titer [TCID₅₀] from the results for a CD8-depleted control). Mean inhibition across T/F viruses is shown for 4 patients: (A) 700010199; (B) 702010157; (C) 702010736; (D) 700010106.

analysis (Fig. 5B). Following the 2-day inhibition assay, cytokine levels in supernatants drawn from the bottom chamber of the transwell system were measured. Antigen-specific virus inhibition significantly correlated with secretion of cytokines/chemokines (MIP-1 α , MIP-1 β , IFN- γ IP-10, and IL-1 α), as shown in Fig. 5C. Next, we determined whether CD8⁺ T cells from acute HIV-1 infection could mediate antigen-specific soluble-factor inhibition similar to that observed in a virus controller. CD8⁺ T cells isolated longitudinally from five subjects with acute HIV-1, CH106, CH199, CH157, CH736 (Fig. 6), and CH077 (Fig. 7), were examined for the ability to inhibit a clade-matched T/F virus (CH040.c,

clade B, or CH042.c, clade C, respectively). HIV-1 antigen-specific CD8⁺ T cells from the earliest acute-infection time point examined were capable of mediating soluble inhibition of T/F virus replication. In each of the four subjects followed longitudinally (Fig. 6), who were studied for ≥ 8 weeks, the earliest dominant responses diminished over time, and less potent responses against a wider range of epitopes began to emerge. The antigen-specific CD8⁺ T cell responses varied among the acute-infection subjects. However, in all five subjects, Nef-specific CD8-mediated virus inhibition was observed, with Gag 2 CD8⁺ T cell responses being the next most frequent in two of five responders (Fig. 6E). These data demonstrate, for the first time, that antigen-specific CD8⁺ T cells from acute HIV-1 infection can mediate soluble-component inhibition of T/F viruses.

The subsets of antigen-specific CD8⁺ T cells that mediate inhibition by soluble factors are the same as those that drive virus escape. Since we demonstrated that virus inhibition by CD8⁺ T cells from subject CH077 (Fig. 4A) was impaired against autologous virus carrying T cell escape mutations (CH077.mo6) but not the T/F virus (CH077.t), we sought to evaluate whether CD8⁺ T cells from acute infection could mediate antigen-specific soluble-factor virus inhibition corresponding to the same antigen-specific CD8⁺ T cells that have been shown to drive virus escape in this subject. Thus, we evaluated the ability of CD8⁺ T cells from CH077 to inhibit autologous T/F through soluble

TABLE 2 CD8⁺ T cell-mediated virus inhibition

Inhibition of viruses examined in patient:							
CH106		CH199		CH157		CH736	
Wk	No. of viruses	Range (log virus inhibition)	No. of viruses	Range (log virus inhibition)	No. of viruses	Range (log virus inhibition)	No. of viruses
0			2	1.8–1.8	3	0.9–2.8	
1			2	1.1–1.8	5	0.5–2.3	5
2			2	1.6–2.1	4	0.9–3.2	
3	5	0.9–1.8	2	2.1–2.5	5	0.5–0.9	4
4	5	0–1.4	2	0.4–0.4	5	1.5–2.7	4
8	5	0–1.8	2	0.7–1.1	4	0.5–1.8	
12					3	0.9–3.6	
16	5	0.5–1.5					
24					3	0.5–2.7	
60							4

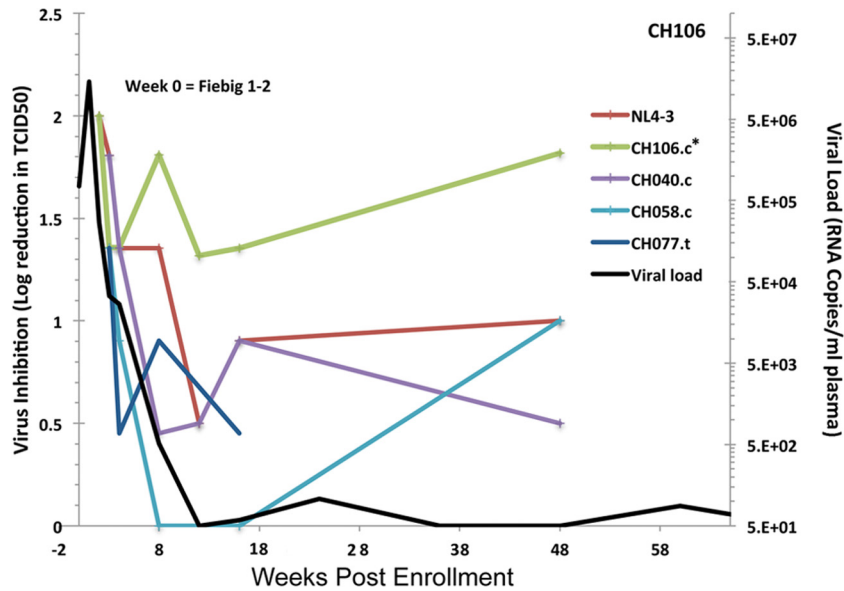


FIG 3 CD8⁺ T cell-mediated inhibition of autologous virus is maintained even in the absence of high viral loads. Antiviral activities exerted by CD8⁺ T cells that were isolated longitudinally from a clade B-infected HIV-1-positive patient during acute infection are shown as log reductions in titer (TCID₅₀) compared to the results for the CD8 T cell-depleted control sample. CD8⁺ T cell-mediated inhibition of 3 heterologous T/F viruses (CH040.c, CH058.c, and CH077.t), one autologous T/F virus (CH106.c), and NL4-3 is shown over 48 weeks for patient 7000010106.

means and in an antigen-specific manner. Using the HIV-1-specific CD8-transwell VIA assay described above, we found that acute CD8⁺ T cells from CH077 produced soluble factors capable of inhibiting autologous T/F viruses, specifically after recognizing epitopes found within the Env2, Gag2, and Nef regions (Fig. 7A). These antigen-specific CD8⁺ T cell data add to the previous work that demonstrated rapid virus escape mutations in these regions

during this same time frame (16, 22). Elevated levels of the cytokines MIP-1α, MIP-1β, IFN-γ, IP-10, and IL-1α were measured in cell culture supernatants following Env2, Gag2, and Nef peptide pool stimulation. The concentrations of these cytokines correlated positively with the levels of virus suppression (Fig. 7B and Table 3). These data further suggest that inhibitory CD8⁺ T cell populations arise rapidly following infection and are multifunc-

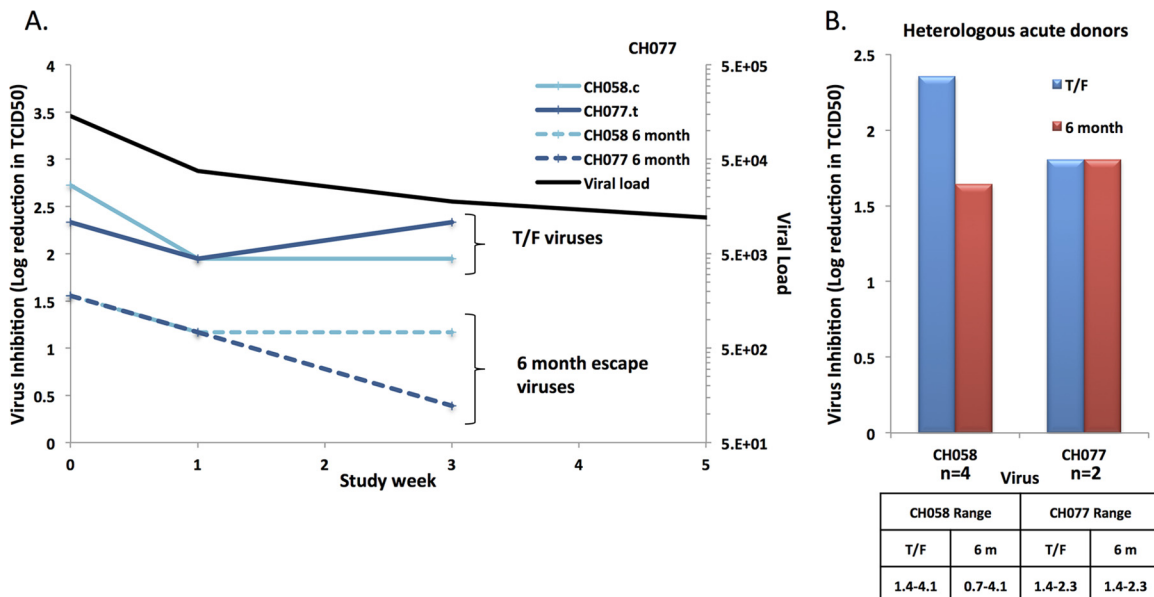


FIG 4 Virus rapidly escapes from CD8-driven pressure exerted early after infection. (A) CD8⁺ T cells isolated from 3 early time points (enrollment to week 3) were tested in the CD8 VIA for inhibitory capacity against autologous (CH077.t) and heterologous (CH058.c) T/F and 6-month viruses. Inhibition of virus replication (TCID₅₀) relative to the results for a CD8-depleted control is shown. (B) Inhibition of T/F and paired 6-month virus by CD8⁺ T cells from heterologous acute patients is shown as log reduction in virus titer (TCID₅₀) relative to the results for a CD8-depleted control. The range of inhibition for heterologous donors is shown as log reduction in virus titer at 2:1 E/T.

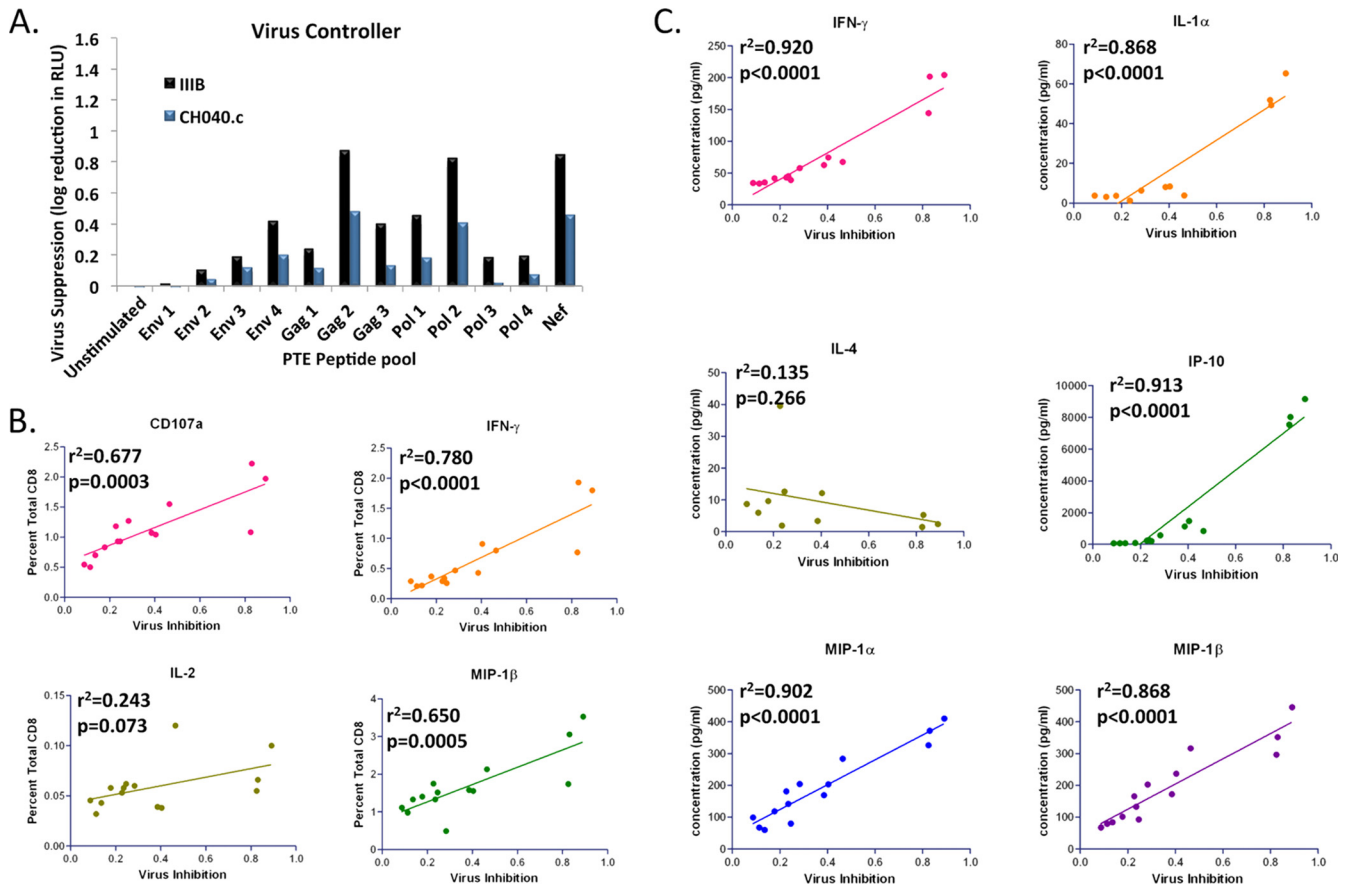


FIG 5 Soluble inhibition of HIV-1 correlates with cytokine expression in CD8⁺ T cells from a virus controller. Transwell CD8 VIA was used to assess the soluble-factor inhibitory capacity of PTE peptide-stimulated CD8⁺ T cells from a virus controller. Suppression of virus replication is shown as log reduction in RLU from the results for an infected TZM-bl control. Background is determined using peptide pool-stimulated CD8⁺ T cells from a seronegative donor and has been subtracted. (A) Suppression of CH040.c T/F and IIIB viruses by peptide pool-stimulated VC9 CD8⁺ T cells is shown. (B) Each panel shows the percentage of total CD8⁺ T cells expressing the indicated cytokine of CD107a as determined by flow cytometric analysis. (C) Each panel shows supernatant cytokine levels as measured by Luminex following peptide stimulation of CD8⁺ T cells and coculture with infected CD4⁺ T cell targets. Lines indicate best fit, by least squares method. Pearson's correlations and *P* values are shown (Prism software).

tional and that soluble inhibition of HIV-1 replication mediated by CD8⁺ T cells during acute infection may play a role in driving virus escape.

DISCUSSION

Identification of the functional properties of CD8⁺ T cells responsible for effective virus control in HIV-1-infected humans will have a profound impact on vaccine design. In working toward this goal, it is important to delineate the functional antiviral capacity and the antigen specificity of HIV-1-specific CD8⁺ T cells that are elicited during acute infection and are responsible for decreasing the initial high level of viremia. CD8⁺ T cells are quite heterogeneous and are characterized by their capacity to mediate lysis of HIV-1-specific target cells, secrete cytokine, and proliferate (52). Virus controllers have potent suppression of viremia due to the contribution of specific CD8⁺ T cell effector functions (11, 25, 39), and these attributes of effector CD8⁺ T cells are being studied intensively. Cell surface expression of CD107a is associated with the capacity of antigen-specific CD8⁺ T cells to eliminate infected cells and to inhibit HIV-1 replication. Phenotypic markers denoting transitional memory cells are also associated with the ability to inhibit HIV-1 replication (18, 30). Furthermore, the characteriza-

tion of the cytokine profile of CD8⁺ T cells has identified MIP-1 β as a correlate of virus control (16) and virus inhibition (18). In total, CD8⁺ T cells can inhibit virus replication through either lysis of infected CD4⁺ T cells or inhibition of virus replication (7, 13, 41, 54, 55, 59). This inhibition may involve suppression through the release of soluble β -chemokines that can directly block the entry of CCR5-using viruses (8, 14). In this study, we examined the early ontogeny, magnitude, and virus specificity of CD8⁺ T cell-mediated virus inhibition. This comprehensive investigation of acute CD8⁺ T cell-mediated anti-HIV-1 responses, utilizing complementary assays (Fig. 8), includes characterization of soluble-factor virus inhibition against autologous and heterologous full-length T/F infectious molecular clones from clades B and C during the well-defined early stages of AHI according to Fiebig et al. (17).

An important indicator of the quality of vaccine-elicited CD8⁺ T cell responses will be the ability of CD8⁺ T cells to inhibit circulating strains of HIV-1. CD8⁺ T cells from acute infection of all five patients that we examined were able to inhibit multiple T/F viruses representing current circulating strains. However, this ability declined over the course of AHI. In CH106, we found that

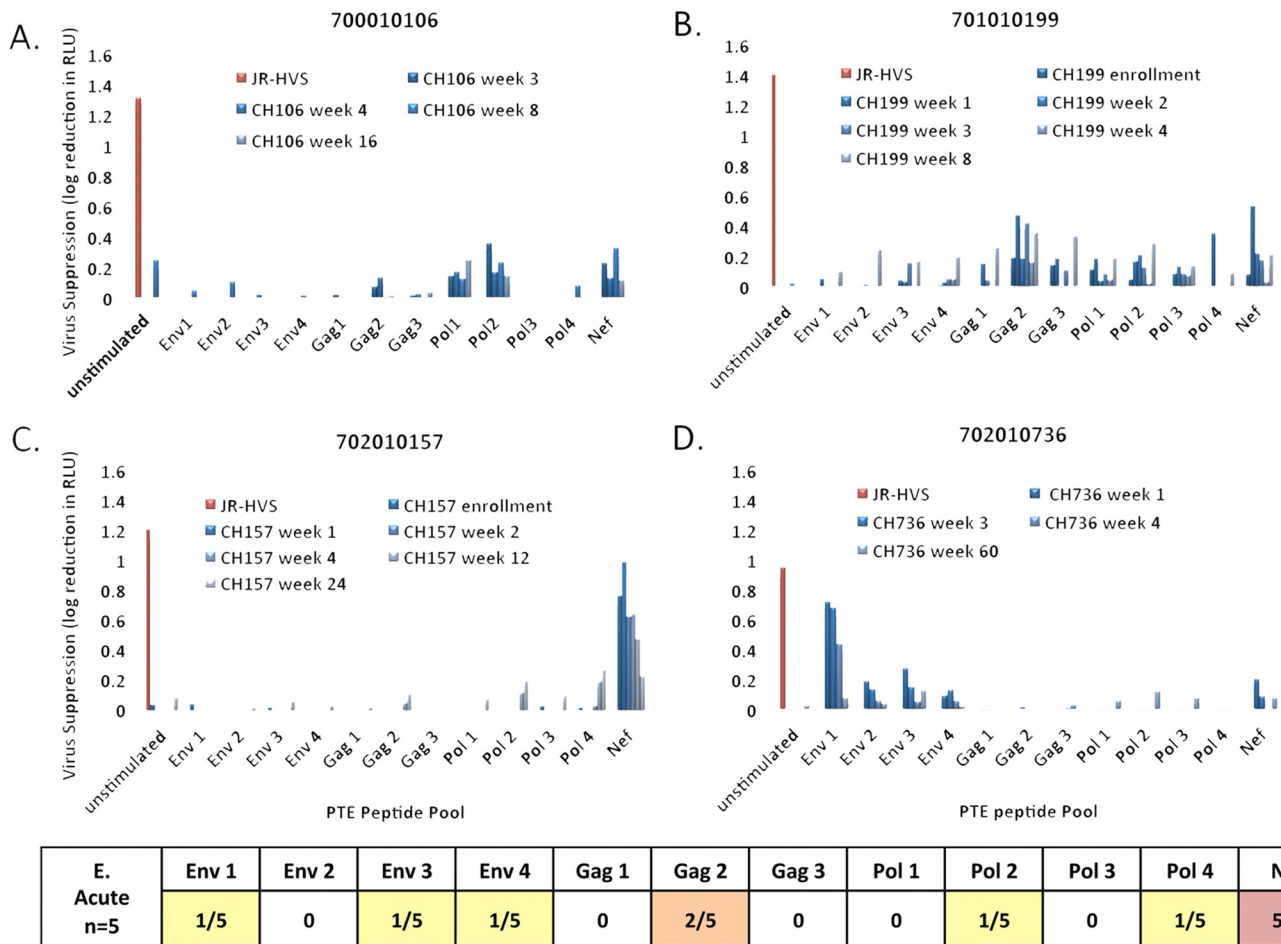


FIG 6 CD8⁺ T cells inhibit replication of T/F viruses through antigen-specific release of soluble inhibitors. Transwell CD8 VIA assay was used for longitudinal assessment of the soluble-factor inhibitory capacity of HIV-1 peptide-stimulated CD8⁺ T cells from 4 acute donors. Suppression of virus replication is shown as log reduction in RLU from the results for an infected TZM-bl control. Results for the JR-HVS CD8⁺ T cell line positive control are shown in red. Background is determined using peptide pool-stimulated CD8⁺ T cells from a seronegative donor and has been subtracted. (A) Soluble-factor suppression of CH040.c virus by 700010106 CD8⁺ T cells. (B) Soluble-factor suppression of CH040.c virus by 701010199 CD8⁺ T cells. (C) Soluble-factor suppression of CH042.c virus by 702010157 CD8⁺ T cells. (D) Soluble-factor suppression of CH042.c virus by 702010736 CD8⁺ T cells. (E) A summary of positive responses seen at enrollment or week 1 (cutoff of 2.5 standard deviations above the seronegative mean after background subtraction).

inhibition of autologous virus was more durable than heterologous virus inhibition. This was in contrast to virus controllers, who demonstrated breadth of virus inhibition well into the chronic stage of infection. There are many potential explanations for this difference that warrant further investigation. CD8⁺ T cell responses that have maintained specificity for autologous epitopes may have been selectively expanded during acute infection. However, CD8⁺ T cell responses to more conserved regions across the virus isolates measured here might not be as readily amplified and so may represent a decreasing fraction of the total CD8⁺ T cell response over time. Future studies, where the primary CD8⁺ T cells from acute infection are not limiting, could test a range of CD8⁺ effector cells to evaluate the quantitative differences in heterologous and autologous CD8⁺ T cell responses. CD8⁺ T cell inhibition against heterologous T/F viruses may also be more dependent on the level of virus replication (Fig. 2). Further studies with larger cohorts are needed to determine if the levels of antigen stimulation and heterologous activity are linked. Alternatively, in CH106, the preference for the durability of autologous virus inhi-

tion could be due to early ART that results in better preservation of the immune response, thus maintaining the initial antiviral response against the T/F virus. Cao et al. (4) studied two acutely infected subjects using IFN-γ enzyme-linked immunosorbent spot (ELISPOT) and ⁵¹Cr release assays and concluded that the quality of the earliest CD8⁺ T cell response in HIV-1 infection may be the best target for HIV-1 vaccine strategies, because these earliest responses probably develop in the presence of CD4⁺ T cell help and are able to significantly decrease initial viremia. Similarly, we find that the early CD8⁺ T cell response may provide insight into the quality of the CD8⁺ T cell responses required for an effective HIV-1 vaccine.

HIV-1-specific CD8⁺ T cells from acute infection consistently mediated soluble inhibition of T/F viruses. Although there were differences among the patients in terms of which HIV-1 peptides elicited soluble-factor virus inhibition, the ability of Nef-stimulated T cells to mediate soluble inhibition was common to all acute subjects studied here (Fig. 6). In contrast to virus controllers, we found that the memory T cell response mediating soluble-factor

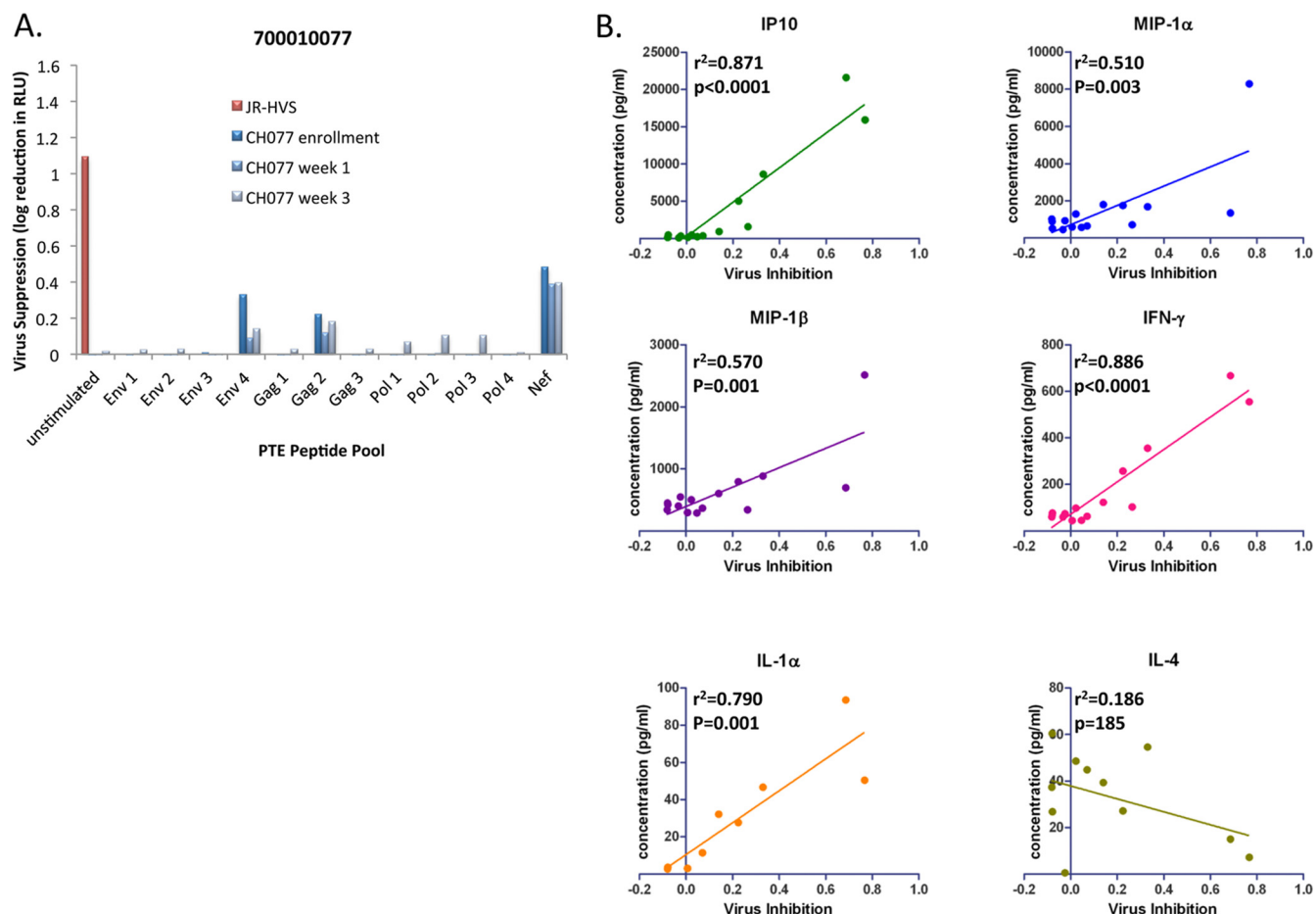


FIG 7 Soluble-factor inhibition of autologous T/F virus corresponds with rapid virus escape and correlates with cytokine release. Transwell CD8 VIA was used for longitudinal assessment of the soluble-factor inhibitory capacity of PTE peptide-stimulated CD8⁺ T cells from an acute patient. Suppression of virus replication is shown as log reduction in RLU from the results for an infected TZM-bl control. Results for the JR-HVS CD8⁺ T cell line control are shown in red. Background is determined using peptide pool-stimulated CD8⁺ T cells from a seronegative donor and was subtracted. (A) Suppression of CH077.t T/F virus by peptide pool-stimulated 700010077 acute CD8⁺ T cells is shown. (B) Each panel shows supernatant cytokine levels as measured by Luminex following peptide stimulation of CD8⁺ T cells and coculture with infected CD4⁺ T cell targets. Pearson's correlations and P values are shown (Prism software).

virus inhibition was limited to selected specificities of HIV-1 antigen-stimulated CD8⁺ T cells. Our evaluation of the CD8⁺ antigen-specific soluble HIV-1 inhibitory response is complementary to the findings of Radebe et al., who recently demonstrated restricted antigen specificity of CD8⁺ T cells and a common Nef

response, as measured by IFN-γ secretion, in a clade C cohort (45).

In this study, we found that acute CD8⁺ T cell-mediated virus inhibition was composed of soluble virus-inhibitory factors secreted from epitope-specific (Env, Gag, and Nef) CD8⁺ T cells.

TABLE 3 Correlation of soluble factors and CD8⁺ T cell virus inhibition during acute HIV-1 infection and for an HIV-1-positive virus controller^a

Patient, time of sampling	Statistic	IFN-γ	IL-1α	IL-4	IP-10	MIP-1α	MIP-1β
700010077, enrollment	r ²	0.886	0.790	0.186	0.871	0.510	0.570
	P value	<0.0001	0.001	0.185	<0.0001	0.003	0.001
701010199, week 1	r ²	0.520	0.382	0.348	0.469	0.513	0.669
	P value	0.002	0.139	0.026	0.005	0.003	0.0002
702010157, enrollment	r ²	0.585	0.544	0.0004	0.926	0.402	0.479
	P value	<0.0001	0.155	0.910	<0.0001	<0.0001	<0.0001
702010736, week 1	r ²	0.658	0.768	0.053	0.739	0.950	0.887
	P value	0.0008	<0.0001	0.471	0.028	<0.0001	<0.0001
VC9 0873-5014	r ²	0.920	0.868	0.135	0.913	0.902	0.868
	P value	<0.0001	<0.0001	0.266	<0.0001	<0.0001	<0.0001

^a Supernatant cytokine levels as measured by Luminex following peptide stimulation of CD8⁺ T cells and coculture with infected CD4⁺ T cell targets. Pearson's correlation coefficients and P values are shown (Prism).

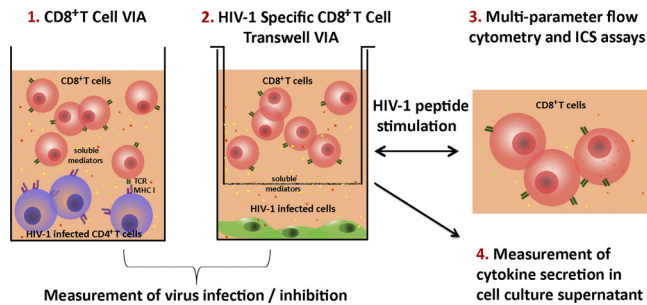


FIG 8 Schematic of methods for assessing CD8⁺ T cell antiviral function in acute HIV-1 infection. Four assays were utilized in concert to assess CD8⁺ T cell function. Two assays measure the ability of primary CD8⁺ T lymphocytes to inhibit HIV-1 virus replication (using full-length HIV-1 transmitted/founder infectious molecular clones) via coculture and through soluble mechanisms: a CD8⁺ virus inhibition assay (CD8 VIA) (1) and an HIV-1 peptide stimulation and soluble-factor inhibition assay (HIV-1-specific CD8 transwell VIA) (2). The other two assays are for cell surface marker phenotype and intracellular cytokines from HIV-1 peptide-stimulated primary CD8⁺ T lymphocytes using multiparameter intracellular cytokine staining assays (3) and antigen-specific cytokine secretion in cell culture supernatants using Luminex multiplex cytokine measurements (4). The combination of the four assays using the same PBMC specimen provides a comprehensive measurement of CD8⁺ T cell antiviral function against both autologous and circulating HIV-1 isolates.

β -Chemokines are important soluble mediators of control in HIV-1 infection (13) and have been shown to mediate CD8⁺ T cell antiviral activity (8). Importantly, CD8⁺ T lymphocytes secrete high levels of MIP-1 α and MIP-1 β that correlate with asymptomatic HIV-1 infection (9). We have reported that MIP-1 β and CD107a were markers strongly associated with virus inhibition by virus controllers and HIV-1 vaccinees (18). Furthermore, recent work indicates that MIP-1 β -expressing CD8⁺ T lymphocytes exert immune pressure early in HIV-1 infection (16). Analysis of the cytokine profile released by CD8⁺ T lymphocytes may aid in determining the CD8⁺ T cell functions that should be elicited by therapeutics or a protective HIV-1 vaccine. We previously analyzed the mRNA levels and cytokine secretion from HIV-1+ virus controllers (50) to identify the cytokine profile of CD8⁺ T lymphocytes that exhibit soluble-factor virus inhibition (51). Our profile includes several genes studied previously in the context of CD8⁺ T lymphocyte-mediated virus inhibition (MIP-1 α , MIP-1 β , IL-13, I-309, IP-10, and granulocyte-macrophage colony-stimulating factor [GM-CSF] [8, 20, 21, 26, 36, 57]). These results underscore the multifactorial nature of the CD8⁺ T cell effector response and highlight the need for a better understanding of how these responses are elicited and maintained.

The ability of T/F viruses to escape from CD8⁺ T cell-mediated virus inhibition had not been previously reported. Notably, we examined patient CH077, who has documented virus escape from CTL, and measured both total and soluble-factor virus inhibition of the autologous T/F virus from this subject. We found that that HIV-1 CD8⁺ T cells that mediated inhibition of autologous T/F virus (Fig. 4) were of the same antigen specificity as had been previously shown to be responsible for virus escape (22). Specifically, the Gag2 responses include cells responsive to the IW9 and TW10 epitopes present in this individual, from which escape was documented between 102 and 159 days following screening. Furthermore, rapid virus escape from regions represented in the Env4 and Nef pools was documented. Together, these data suggest that

MHC peptide-induced stimulation produces factors that decrease virus replication. These factors may be commonly stimulated in memory cells, are broadly active, and are mediated from the same antigen-specific CD8⁺ T cell subsets that are capable of driving virus escape. Further analysis of HIV-1 peptide stimulation in a virus controller demonstrated significant increases in specific soluble antiviral activity, and this activity correlated positively with the levels of cells expressing CD107a, IFN- γ , and MIP-1 β ($P < 0.001$, Pearson's correlation) but not IL-2. We previously identified that this CD8⁺ T cell phenotype in virus controllers and vaccinees correlated with the capacity to inhibit virus replication (18). This study was uniquely suited to address the question of whether virus escape from known epitope-specific CD8⁺ T cell responses (IFN- γ ELISPOT) extends to escape from CD8-mediated virus-inhibitory function. As more infectious molecular clones are generated that represent CD8⁺ T cell escape variants in other subjects, the frequency of escape from CD8⁺ T cell virus inhibition during acute infection can be better evaluated.

Transmission risk has been positively associated with viral load in peripheral blood and mucosal secretions (5, 23, 44). Individuals in the acute stages of HIV-1 infection, prior to viral decline and set point, have a greater probability of passing their HIV-1 infection to partners (42). Recently, a landmark study demonstrated that dramatic reduction in HIV-1 viral loads by the early introduction of antiretroviral therapy reduced the risk of transmission to seronegative sexual partners by 96% (10). While effective vaccine designs aim to prevent HIV-1 acquisition, the likelihood of breakthrough infections must be considered. Vaccine strategies that can also elicit cellular immune responses capable of decreasing viremia could dramatically reduce the transmission risk in the absence of sterilizing immunity. The resultant effects at the population level could serve to significantly reduce the incidence of HIV-1 globally. In addition to effects at the population level, vaccines that can elicit CD8⁺ T cell populations with effective, durable, and rapid antiviral responses may reduce the cell-associated viral load (19) and preserve CD4⁺ T cell immunity despite infection, thus providing an overall health benefit to the individual.

Some HIV-1 vaccine strategies have been shown to elicit CD8⁺ T cells that can inhibit virus replication (18, 53). A comparison in future vaccine studies of the quality of the vaccine-elicited cellular response to that of the initial inhibitory CD8⁺ T cell response in acute infection will be an important component in evaluating the quality of the HIV-1 vaccine response. Proof-of-concept studies in nonhuman primates provide evidence that effector memory CD8⁺ T cells at mucosal sites (24) and antigen-specific CD8⁺ T cell responses (38) could play a major role in protection. Thus, the generation of CD8⁺ T cells that can inhibit a diverse panel of HIV-1 isolates is an important goal for vaccine design. The strategy reported here of multiple CD8⁺ T cell function assays with T/F viruses, akin to the way neutralizing antibodies are systematically studied (35), can provide a comprehensive platform for evaluating the quality of vaccine-elicited HIV-1-specific T cells.

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