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HIV-Specific CD8+ Lymphocytes in Semen Are Not Associated with Reduced HIV Shedding1,2

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Sexual contact with HIV-infected semen is a major driving force behind the global HIV pandemic. Little is known regarding the immune correlates of virus shedding in this compartment, although HIV-1-specific CD8+ T cells are present in semen. We collected blood and semen from 27 chronically HIV-infected, therapy-naïve men without common sexually transmitted infections or urethral inflammation and measured HIV-1 RNA viral load and cytokine/chemokine levels in both compartments. HIV-1 RNA levels were 10-fold higher in blood than semen, but discordantly high semen shedding was associated with higher semen levels of the proinflammatory cytokines IL-6, IL-8, IL-12, and IFN-γ. Virus-specific CD8+ T cell epitopes were mapped in blood by IFN-γ ELISPOT, using an overlapping HIV-1 clade B peptide matrix, and blood and semen CD8+ T cell responses were then assayed ex vivo using intracellular IFN-γ staining. HIV-specific CD8+ responses were detected in 70% of semen samples, and their frequency was similar to or higher than blood. There was no correlation between the presence of virus-specific CD8+ T cells in semen and levels of HIV-1 RNA shedding. Among participants with detectable CD8+ IFN-γ semen responses, their relative frequency was not associated with reduced HIV-1 RNA shedding, and their absolute number was correlated with higher levels of HIV-1 RNA semen shedding (r = 0.6; p = 0.03) and of several proinflammatory cytokines. Neither the presence nor the frequency of semen HIV-specific CD8+ T cell IFN-γ responses in semen correlated with reduced levels of HIV RNA in semen. The Journal of Immunology, 2005, 175: 4789–4796.

The HIV-1 pandemic has claimed over 20 million lives, and 42 million people are currently infected (www.who.int/hiv/pub/epidemiology/epi2003/en/). Given the scale of the global pandemic and the fact that most transmission is sexual, HIV-1 mucosal transmission is surprisingly inefficient, with a transmission risk of 0.1-1% per sexual contact (1). When sexual transmission of HIV-1 does occur, it takes place in two broad steps. First, the virus must be shed in the genital fluids (semen or cervicovaginal secretions) of the infected partner. Second, the virus must cross the mucosal epithelium of the uninfected partner and establish persistent infection. Previous investigations have found a strong association between levels of virus in blood and the probability of transmitting HIV-1 to a monogamous sex partner (2), and this likely relates to the fact that the amount of virus in the genital tract tends to correlate with levels in the blood plasma (3–6). Despite the correlation between levels of virus in the blood and genital tract, some individuals may shed disproportionately high or low levels of HIV-1 in the semen in comparison to blood, a phenomenon that may have profound public health implications (4, 7). The immune control of HIV-1 shedding in semen, particularly in such “discordant” shedders, is poorly understood.

Systemic HIV-specific CD8+ T cell responses are clearly important in host HIV-1 immune control, as evidenced by their temporal association with viral control in humans and animal models, and by the strong immune selection pressure that they place on the virus (8–10). However, the association between the frequency of systemic HIV-specific CD8+ T cells and levels of plasma virus is less clear, with different groups reporting an inverse correlation (11, 12), a positive correlation (13), or no correlation at all (7, 14). HIV-specific CD8+ responses are present in the genital tract of both HIV-infected and exposed uninfected subjects (15–20), but their role in controlling levels of viral shedding at mucosal surfaces, or in mediating protection against infection, has not been elucidated. Nonetheless, in animal models it is mucosal, not systemic, CD8+ lymphocytes that are most critical in mediating protection against mucosal viral challenge (21, 22). Furthermore, after vaginal SIV infection the earliest virus-specific CD8+ T cells detected are in the vaginal mucosa (23), and HIV-1 is known to replicate preferentially within mucosal lymphoid tissue, particularly that of the gut (24, 25). These observations suggest that mucosal CD8+ T cell responses may be important in controlling mucosally acquired HIV-1 infection and therefore perhaps in controlling levels of viral shedding.

Although CD8+ T cell responses place a strong immune pressure on HIV-1, excessive systemic immune activation is associated with higher levels of plasma viremia and with more rapid disease progression. Negative prognostic markers of immune activation

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include elevated expression of CD38 and increased levels of inflammatory cytokines such as IL-6 and TNF-α (26–28). In addition, high levels of HIV-1 RNA in the gut mucosa of HIV-1-infected men taking antiretroviral therapy are associated with gastrointestinal mucosal cytokine activation (29). However, the impact of the systemic or genital tract cytokine milieu on levels of HIV-1 in semen is not known.

To study the role of host immune factors in HIV-1 semen shedding, we have assayed ex vivo CD8\(^+\) T cell responses in semen for the first time and have examined the association between CD8\(^+\) responses, the local and systemic cytokine milieu, and levels of HIV-1 RNA in the semen of HIV-1-infected, therapy-naïve men.

Materials and Methods

Study population

Antiretroviral therapy-naïve gay men with chronic HIV-1 infection were recruited through the Canadian ImmuneDeficiency Research Collaborative. Clinicians were asked to enroll participants not expected to require antiretroviral therapy within the next 2 years. All subjects provided informed, written consent. The study protocol was approved by Research Ethics Boards at the Mount Sinai Hospital, the University Health Network, Toronto, and at the University of Toronto.

Sample processing

At the recruitment visit (visit 1), 24 ml of venous blood were collected into acid citrate dextran for CD8\(^+\) epitope mapping. At two subsequent visits, paired blood and semen specimens were collected within an hour of each other. Semen samples were collected by masturbation into a dry sterile container at visit 2 and into 10 ml of sterile RPMI 1640 containing antibiotic and antimitotic at visit 3. Samples were processed within 2 h of collection. A first-void urine sample was screened for the presence of leukocytes using a standard urine dipstick (Bayer Diagnostics) and for infection by either Neisseria gonorrhoeae or Chlamydia trachomatis using the Amplicor CT/NG assay (Roche Diagnostic Systems). Any participant with urethral leukocytes, gonorrhea, or chlamydia was excluded from analysis. Seminal plasma was isolated by centrifugation at 850 \(\times\) g for 10 min, and the semen cell pellet was then resuspended in 10 ml of sterile R10 medium. PBMCs and seminal fluid mononuclear cells were then isolated by Ficoll-Hypaque density centrifugation. Blood and seminal plasma were immediately frozen at 

\(-86^\circ C\).

HIV-1 shedding in semen

Blood and semen RNA viral load (VL)\(^a\) was measured using the Versant HIV-1 RNA 3.0 assay (bDNA; Bayer Diagnostics) because RT-PCR-based assays have been found to be inhibited by semen constituents in other studies (30). VLs were either measured directly in blood and seminal plasma or in the supernatant of semen samples collected into RPMI 1640. In the latter case, semen VL was appropriately corrected for the dilution factor.

Epitope mapping using the IFN-γ ELISPOT

HIV-1-specific CD8\(^+\) T cell responses were mapped in blood using an IFN-γ ELISPOT assay, as described previously (31, 32). PBMCs were incubated at 1 \(\times\) 10\(^5\)/well with a matrix of 756 15-mer peptides, overlapping by 11 amino acids, spanning the entire clade B HIV-1 genome (obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health). Each peptide appeared uniquely in two separate matrix pools at a final working concentration of 2 \(\mu\)M. All responses detected using the matrix pools were confirmed using individual 15-mer peptides. Response frequencies were calculated using an automated ELISPOT counter (Cellular Technology), and a positive response was defined as an HIV peptide-specific response 1) at least 2-fold higher than background (PBMC + 2 \(\mu\)M of DMSO) and 2) \(\geq 100\) spot-forming units (SFU)/million cells. All responses were confirmed to be CD8\(^+\) mediated using IFN-γ intracellular cytokine staining.

\(^a\) Abbreviations used in this paper: VL, viral load; SFU, spot-forming unit; CBA, cytokine bead array; SLPI, secretory leukocyte protease inhibitor.

Ex vivo stimulation and intracellular IFN-γ staining

A pool of all responding epitopes mapped in blood was used to examine HIV-specific IFN-γ responses in blood and semen (19). Briefly, 1 \(\times\) 10\(^5\) blood mononuclear cells were incubated for 1 h at 37°C in 5% CO\(_2\) with medium alone, staphylococcus enterotoxin B (3 \(\mu\)g/ml), or the HIV epitope pool (each peptide at 10 \(\mu\)g/ml), and semen mononuclear cells were split in two vials and incubated with medium alone or the HIV epitope pool. Brefeldin A (BD Biosciences Immunocytometry Systems) was then added (1 \(\mu\)g/ml), and samples were incubated for 5 h, permeabilized, and stained with combinations of CD3-FITC, CD3-PerCP, CD8-PE, CD8-PerCP, CD4-PE, CD69-FITC, CD103-PerCP, CD3-APC, and IFN-γ-APC (BD Biosciences Immunocytometry Systems). Samples were acquired using a FACS caliber flow cytometer (BD Systems), and data analysis was performed using FlowJo analytical software (Tree Star). A positive response was defined as an HIV-specific response 1) at least 2-fold higher than the unstimulated control and 2) a CD8\(^+\) HIV-1-specific response frequency of \(\geq 0.05\%\).

Cytokine bead array (CBA)

Cytokine levels in blood and semen plasma were measured using CBA (BD Biosciences Immunocytometry Systems), according to the manufacturer’s instructions (33). Cytokines/chemokines assayed were IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, TNF-α, IFN-γ, IL-12p70, RANTES, monokine induced by IFN-γ (MIG/CXCL9), macrophage chemotactic protein (MCP1/CLL2), and IFN-inducible protein-10 (IP-10/CXCL10). Blood and semen plasma samples were incubated for 3 h at room temperature with a mixture of Ab-coupled beads (50 \(\mu\)l/sample) and PE-conjugated secondary Abs (50 \(\mu\)l/sample) against each cytokine. Samples were then washed with 2% paraformaldehyde, resuspended in 150 \(\mu\)l of PBS wash buffer, and analyzed using flow cytometry.

Measurement of innate immune factors

Levels of secretory leukocyte protease inhibitor (SLPI) and lactoferrin were measured in seminal plasma by ELISA (Quantikine Human SLPI kit, R&D Systems; Enzyme Immunoassay for Human Lactoferrin, Oxford Bio-medical Research), according to the manufacturers’ instructions, after dilution of 1/6,400 and 1/3,000 in diluent buffer. ELISA plates were read in a standard 96-well Thermomax reader (Molecular Devices) at 450 nm using 570 nm as a reference.

Statistical analysis

SPSS 11 for the Macintosh OS X (SPSS) was used for statistical analysis. Comparisons between the blood and semen of study subjects were performed using the paired samples t test. The Mantel-Haenszel \(\chi^2\) test with calculation of likelihood ratios and confidence intervals was used to compare dichotomous variables between study groups, and comparison of means between groups was performed by one-way ANOVA. Linear association of continuous variables was assessed using the Pearson correlation coefficient. Independent associations of HIV-1 RNA shedding were examined in a multivariable linear regression model.

Results

Participant demographics

Twenty-seven chronically HIV-1-infected, therapy-naïve gay men consented to take part in the study. Their median CD4\(^+\) T cell count was 550/mm\(^3\) (range, 120–1260/mm\(^3\)). All participants had been HIV-1 infected for at least 6 mo. No participant had clinical urethritis or genital ulcer disease or laboratory evidence of infection by Treponema pallidum, C. trachomatis, or N. gonorrhoeae. A first-void urine dipstick for leukocytes (a screen for urethral inflammation) was negative for all participants.

HIV-1 RNA VL in blood and semen

The median blood plasma VL was 20,302 copies/ml plasma (range, <50–401,448 copies). HIV RNA was detected in the semen of 21 of 27 participants (78%), and there was no evidence of PCR inhibition in any assay. Levels of HIV-1 RNA in semen were highly variable, with a median value of 758 copies/ml semen plasma (range, <50–210,350 copies). Only one participant had an undetectable VL in blood, and this individual also had no virus detected in semen. Six participants (22%) had an undetectable semen VL, and their blood VL was quite variable, with a median of
9,893 copies/ml plasma (range, 50–76,014 copies). As reported in a preliminary analysis (7), semen VL was positively correlated with HIV-1 levels in blood plasma (Pearson correlation coefficient, \( r = 0.4; p = 0.02 \)), although semen levels of virus were >10-fold lower than in blood (3.1 vs 4.3 \( \log_{10} \) copies/ml; \( p < 0.001, \) paired samples \( t \) test). There was an inverse correlation between blood CD4+ T cell counts and the RNA VL in blood (\( r = -0.5; p = 0.02 \)) but not semen (\( r = -0.3; p = 0.2 \)).

Although HIV-1 RNA levels in blood and semen were correlated overall, some participants clearly demonstrated disproportionate shedding of HIV-1 RNA in semen. To study the role of semen immune factors on discordant HIV-1 shedding, participants were therefore divided into three groups (7): group 1 had an undetectable semen VL (nonshedders; \( n = 6 \)); group 2 had a semen VL < 60% that of blood plasma (discordant shedders; \( n = 17 \)); and group 3 had a semen viral load \( \geq 60\% \) that in blood plasma (discordant shedders; \( n = 4 \)).

**Systemic HIV-specific CD8+ IFN-γ responses and semen HIV RNA shedding**

Peripheral blood HIV-specific CD8+ responses were screened using the IFN-γ ELISPOT, and HIV-1-specific responses were detected in 27 of 27 participants. Most epitopes fell within HIV-1 Gag (33%), Pol (26%), Env (17%), or Nef (6.7%). Individuals responded to a mean of 8.6 epitopes (range, 3–20 epitopes), and the mean total magnitude (sum) of HIV-1-specific CD8+ responses was 6,833 SFU/million cells (range, 770–25,955 SFU). All IFN-γ responses were confirmed to be CD8+ T cell mediated using intracellular cytokine staining and flow cytometric analysis. As noted in a preliminary report (7), there was no association between the frequency of systemic (blood) HIV-1-specific CD8+ IFN-γ responses and the RNA VL in either blood (\( r = 0.3; p = 0.1 \)) or semen (\( r = 0.2; p = 0.4 \)). In addition, there was no association between the breadth or specificity of blood CD8+ T cells responses and VL at either site. The weak inverse association previously described between the magnitude and breadth of HIV-1 Tat-specific responses and semen HIV-1 shedding was no longer apparent with the increased sample size (\( p = 0.8 \) and \( p = 0.9 \), respectively).

**Semen proinflammatory cytokines and HIV-1 RNA shedding**

Cytokine/chemokine levels were assayed directly in semen and blood plasma using the CBA. Semen levels of several proinflammatory cytokines were associated with discordant shedding of HIV-1. In particular, disproportionate semen HIV-1 RNA shedding was associated with increased levels of IL-6 (\( p = 0.008 \); Fig. 1a), IFN-γ (\( p = 0.04 \)), IL-12 (\( p = 0.008 \)), and IL-8 (\( p = 0.02 \); Fig. 1b). No association was seen between HIV-1 RNA shedding in semen and the other cytokines or chemokines assayed in semen, and there was no correlation between levels of cytokines/chemokines blood plasma and the HIV-1 RNA VL in blood or semen.

**Ex vivo measurement of HIV-specific CD8+ T lymphocytes**

Based on the association of local inflammatory cytokines, including IFN-γ, with HIV RNA shedding, we went on to examine the association of HIV-specific, IFN-γ-producing CD8+ T cells in semen with HIV shedding. At a follow-up clinic visit, semen samples were collected from 20 participants by masturbation into 10 ml of sterile RPMI 1640 to preserve lymphocyte viability (with correction for the resulting dilution factor when assaying semen RNA VL and cytokine levels).

Semen mononuclear cells were split into two equal volumes, one used as a negative control and the second sample to evaluate semen HIV-specific responses assayed by IFN-γ intracellular staining after short-term ex vivo stimulation with a pool of HIV-1 epitopes previously mapped in blood. Cells were permeabilized and stained, and the median number of gated CD3+ T lymphocytes acquired per preparation was 2,862 cells (range, 476–70,030 T cells). Expression of CD103 is a marker for mucosal lymphocytes in both the male and female genital tracts and the gastrointestinal tract (19, 34, 35). CD103+ expression was measured for 12 subjects and was more common on semen than blood CD3+ T cells (4.4 vs 0.1%; \( p = 0.003, \) paired samples \( t \) test; Fig. 2). CD8+ T cells constituted a higher proportion of T cells in the semen than in blood (69.9 vs 52.9%; \( p < 0.001, \) paired samples \( t \) test), as has been described in the semen of HIV-uninfected men (36, 37). Seminal CD8+ T cells were also more activated than those in blood, with much higher levels of spontaneous IFN-γ release (1.3% of

![FIGURE 1. Semen proinflammatory cytokines and discordant HIV shedding. Participants (n = 27) were divided into three shedding groups: nonshedders (group 1; n = 6) had an undetectable semen VL; discordant shedders (group 2; n = 17) had a semen VL < 60% that in blood plasma; and discordant shedders (group 3; n = 4) had a semen viral load >60% that in blood plasma. There was a stepwise association between discordant HIV-1 RNA shedding and semen levels of several proinflammatory cytokines, including IL-6 (p = 0.008; a) and IL-8 (p = 0.02; b).](http://www.jimmunol.org)
HIV-1-specific CD8\(^+\) T cell responses were detected in the peripheral blood of all participants (20 of 20), and a response to the same pool of HIV-1 epitopes was detected in most semen samples (14 of 20; 70%; Fig. 3 for representative example and summary data). After correction for background IFN-γ release, HIV-1-specific CD8\(^+\) responses were present in semen at a significantly higher frequency than blood (2.6 vs 0.7% of CD8\(^+\) T cells; \(p = 0.04\)). There were no detectable HIV-1-specific CD8\(^+\) responses in the semen of 6 of 20 participants (30%) despite a strong CD8\(^+\) response in blood (Fig. 3a, lower panel: representative example). In four of six cases, this may have been due to a low numbers of gated T cell events (<1000 gated cells), but in two of six cases, there was no semen response seen despite relatively high numbers of gated CD3\(^+\) T cells (mean, 5.5 \times 10^7 gated T cells). Lack of response was unlikely to be due to anergy of semen T cells because staphylococcus enterotoxin B stimulation induced a robust IFN-γ response in the semen of both a representative HIV responder (4% of CD3/CD8\(^+\) T cells) and a nonresponder (11.5% of CD3/CD8\(^+\) T cells). The frequency of HIV-1-specific IFN-γ CD8\(^+\) T cells in blood was similar in participants with and without CD8\(^+\) responses detected in semen (0.7 vs 1.3% of CD8\(^+\) T cells, respectively; \(p = 0.3\)). No HIV-specific responses were seen in the blood or semen of five HIV-uninfected controls after stimulation with a pool of the eight HIV epitopes most commonly recognized by infected participants.

**Semen HIV-1-specific CD8\(^+\) T cell responses and HIV-1 RNA levels**

Semen levels of HIV-1 RNA at the time of the semen CD8\(^+\) assay, a mean of 177 days after the baseline assessment (range, 73–313 days), were highly correlated with HIV-1 RNA levels measured at enrollment (\(r = 0.7; \ p = 0.001\); data not shown), confirming the robust nature of our semen VL assay. There was no association between the presence/absence of detectable HIV-1-specific CD8\(^+\) T cell IFN-γ responses in semen and the level of HIV-1 RNA: the mean semen VL was 4.0 log_{10} RNA copies/ml in nonresponders (\(n = 6\)) and 3.7 log_{10} copies/ml in responders (\(n = 14\); \(p = 0.6\)). Among participants with a detectable semen CD8\(^+\) response (\(n = 14\)), no association was seen between the relative frequency of semen HIV-specific CD8\(^+\) T cells and the semen VL when the frequency of HIV-1-specific CD8\(^+\) T cells was expressed as a percentage of all semen CD8\(^+\) T cells (\(r = -0.2; \ p = 0.5\); Fig. 4a).

Because the total number of T cells in semen varied widely between study participants, the absolute number of HIV-1-specific CD8\(^+\) T cells in a given semen sample was also calculated for each participant, and this absolute number was then transformed into a logarithmic scale. When analyzed in this fashion, there was a positive correlation between the semen CD8\(^+\) T cell response and levels of HIV-1 RNA so that a higher absolute number of HIV-1-specific, IFN-γ-producing CD8\(^+\) T cells in semen was associated with higher semen HIV-1 RNA shedding (\(r = 0.6; \ p = 0.03\); Fig. 4b). When the total number of CD3\(^+\) T cells in each semen sample was calculated and then transformed into a logarithmic scale, there was also a trend to a positive correlation with the semen HIV-1 RNA load (\(r = 0.5; \ p = 0.07\)), and the absolute number of HIV-1-specific CD8\(^+\) T cells was closely related to the total number of CD3\(^+\) T cells in a given sample (\(r = 0.8; \ p = 0.001\)).

Participants were again divided into three groups based on the concordance of HIV-1 RNA levels in blood and semen, as described earlier: nonshedders (\(n = 5\)), discordant shedders (\(n = 8\)), and discordant shedders (\(n = 7\)). There was no significant association between discordant HIV-1 semen shedding and the frequency of semen CD8\(^+\) T cell responses (percentage of semen CD8\(^+\) T cells 2.5, 2.0, and 1.8, respectively; \(p = 0.8\)) or the absolute number of virus-specific CD8\(^+\) T cells (log_{10} absolute number of responding cells 1.6, 1.8, and 2.0, respectively; \(p = 0.6\)).

**HIV-1-specific CD8\(^+\) T cell responses in semen and local inflammation**

Because our baseline analysis had found that discordant HIV-1 semen shedding was associated with higher local levels of the proinflammatory cytokines, we examined the relationship among semen CD8\(^+\) T cell responses, proinflammatory cytokines, and semen HIV-1 RNA shedding. Levels of several proinflammatory cytokines in semen were again positively correlated with higher HIV-1 shedding, including IFN-γ (\(r = 0.5; \ p = 0.02\)), TNF-α (\(r = 0.4; \ p = 0.08\)), IL-6 (\(r = 0.4; \ p = 0.05\)), and IL-8 (\(r = 0.5; \ p = 0.04\); Fig. 5a). In addition, there was a positive correlation between the log_{10} absolute number of HIV-1-specific semen CD8\(^+\) T cell IFN-γ responses and local levels of the proinflammatory cytokines.
TNF-α (r = 0.5; p = 0.02), IL-6 (r = 0.6; p = 0.006), and IL-8 (r = 0.5; p = 0.02; Fig. 5b). Therefore, the absolute number of HIV-1-specific, IFN-γ-producing CD8+ T cells in semen was associated with both increased semen HIV-1 RNA shedding and increased levels of proinflammatory cytokines in semen.

**Soluble innate factors in semen and HIV-1 RNA levels**

Both SLPI and lactoferrin were detected in the semen of all participants. The mean level of SLPI was 29.0 μg/ml (median, 27.3 μg/ml; range, 1.1–72.5 μg/ml) and of lactoferrin was 168.4 ng/ml (median, 169.2 ng/ml; range, 5.1–566.4 ng/ml). There was no association between SLPI levels and semen HIV-1 RNA detection (33.1 μg/ml in HIV-1 shedders vs 22.3 μg/ml in nonshedders; p = 0.3), semen HIV-1 RNA levels (r = 0.24; p = 0.3), or any of the proinflammatory cytokines (data not shown). Although lactoferrin levels did not vary based on the detection of HIV-1 RNA (195.1 ng/ml in HIV-1 shedders vs 115.3 in nonshedders; p = 0.3), there was a positive correlation between lactoferrin and levels of HIV-1 RNA (r = 0.54; p = 0.01), and lactoferrin also tended to be higher in discordant shedders (115.3 ng/ml in nonshedders, 145.0 ng/ml in concordant shedders, 259.6 ng/ml in discordant shedders; p = 0.07). No association was found between levels of lactoferrin and semen cytokine/chemokines.

**Discussion**

Although contact during sex with semen containing HIV-1 is a major driving force in the global pandemic, little is known about the immune correlates of HIV-1 shedding in semen. There is no question that systemic levels of virus are an important predictor of semen virus load (4, 5, 38–41), but our study confirms the observation that discordance between levels of virus in the blood and semen is relatively common (4). Therefore, understanding the immune basis of HIV-1 semen shedding, and particularly of discordant shedding, is important for the development of both immunotherapeutics and rational public health policy. Virus-specific CD8+ T cell responses are critical in the control of HIV-1 by an infected person (9, 10), but there is controversy as to whether there is a direct correlation between the frequency of systemic virus-specific CD8+ T cells and the blood VL (12, 13, 42, 43), and the optimal assay technique to measure these responses is also unclear.
HIV-1-specific CD8$^+$ T cells are present in the mucosa of the female cervix (15, 19), in semen (20), and in the gastrointestinal mucosa (45, 46). However, previous assay techniques have not allowed precise quantitation of responses in semen and have not attempted to correlate mucosal response levels with shedding of HIV-1.

For the first time, we have been able to assay functional CD8$^+$ T cell responses directly ex vivo in the semen of HIV-1-infected men and to examine their association with semen VL. HIV-1-specific, IFN-γ-producing CD8$^+$ T cells were present in the semen of most participants, and their frequency in semen exceeded that measured in blood. However, no association was seen between the detection of semen CD8$^+$ responses and HIV-1 RNA shedding in semen. Indeed, among those with a detectable CD8$^+$ semen response, higher absolute numbers of virus-specific CD8$^+$ T cells in semen correlated with increased levels of HIV-1 RNA. The fact that CD8$^+$ responses were also associated with higher semen proinflammatory cytokines, including IL-6, IFN-γ, and IL-8, suggests a possible mechanism for this observation. Although limited semen lymphocyte numbers necessitated screening semen responses using epitopes that had been mapped in blood, this should not affect our results because the ontogeny and Ag specificity of mucosal HIV-specific CD8$^+$ T cells closely mirror that of blood (16, 17, 20, 45).

These observations do not prove that HIV-1-specific CD8$^+$ T cells are themselves inducing inflammation and increased viral shedding in semen. Our study shows a clear association between semen inflammation, HIV-1 RNA shedding, and CD8$^+$ T cell responses, but we cannot demonstrate causation. Increased levels of inflammatory cytokines in the blood have been associated with higher VL in blood plasma and in the gastrointestinal mucosa with higher HIV-1 levels in the mucosal tissues of the gut (29). Therefore, it is possible, and perhaps more intuitive, that inflammatory cytokines in semen might induce viral shedding and that the increased virus-specific CD8$^+$ T cell numbers are secondary to inflammation and/or the resultant higher levels of virus. The absolute number of HIV-1-specific CD8$^+$ T cells in semen tended to mirror the total number of CD3$^+$ T cells so that any inflammatory process
recruiting CD3+ T cells might increase both HIV-1 shedding and local numbers of CD3+ T cells, including HIV-1-specific CD8+ T cells. If both HIV-1 semen shedding and CD8+ T cell responses were secondary to increased levels of proinflammatory cytokines, then it will be important to identify the cause of local semen inflammation. The semen cytokine milieu was not simply a reflection of systemic cytokine levels because there was no significant correlation of proinflammatory cytokine levels in semen and blood. Men were screened for gonorrhea and chlamydia, as well as for urethral inflammation due to other factors, so classical sexually transmitted infections should not have been responsible.

Several innate mucosal factors, including SLPI and lactoferrin, have anti-HIV activity and are found at high levels at many mucosal surfaces. Lactoferrin can block HIV-1 replication in T cells, block dendritic cell uptake of HIV-1 by binding to DC-SIGN, and may reduce breast milk transmission of HIV-1 (47, 48). SLPI has direct anti-HIV activity and is induced in the oral mucosa by HIV-1, and levels of SLPI in the female genital tract and breast milk have been correlated with reduced mother-child transmission of HIV-1 (49–51). We did not find that either factor was associated with reduced HIV-1 semen RNA: indeed, lactoferrin was associated with increased HIV shedding. This may relate to different levels or effects of these innate factors at different mucosal sites or could perhaps reflect induction of lactoferrin as an antiviral defense in response to higher local levels of HIV-1.

Our CBA analyses involved the measurement of levels of 14 different cytokines/chemokines in semen, and so without correction for multiple comparisons, one might expect at least one significant association with HIV shedding, using a two-sided α error of 0.05. However, the hypothesis of an association between HIV-1 RNA levels and semen inflammation was generated at the first study visit and was then confirmed at a second separate visit, making it extremely unlikely that this represents a chance finding. In addition, an association arising through chance would be unlikely to result in the repeated clustering of associations with only the proinflammatory cytokines and not with the other cytokines/chemokines screened.

Although this cross-sectional study cannot prove that HIV-1-specific CD8+ T cell responses in semen increase semen HIV-1 RNA load, they were clearly not associated with reduced virus shedding in chronic HIV infection. Whether the induction or boosting of semen CD8+ T cell responses by therapeutic vaccines or other immunotherapeutics would affect semen shedding or transmission cannot be addressed by our study. Although the association of semen CD8+ T cell responses with higher HIV shedding might imply that this will not be a useful therapeutic strategy, our use of IFN-γ production as a means of identifying HIV-1-specific CD8+ T cells might bias to finding an association between inflammatory cytokines and CD8+ T cell responses. Future studies might consider alternate means to measure semen responses, including MHC class I peptide tetramers, Ag-specific proliferation, expression of the lytic marker CD107Δ, or the production of alternate cytokines/chemokines, particularly IL-2 or MIP-1β. The ability of blood CD8+ T cells to proliferate in response to cognate epitope, rather than IFN-γ production, has been linked to enhanced viral control in HIV-1-infected long-term nonprogressors (52). Similar studies may also be useful in elucidating the role of semen CD8+ T cells in control of HIV-1 shedding and transmission. Nonetheless, our results do imply that when therapeutic HIV vaccines are developed that boost HIV-1-specific CD8+ T cell immunity, the impact of such vaccines on semen CD8+ T cell responses and levels of HIV shedding should be carefully monitored.

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Disclosures
The authors have no financial conflict of interest.

References