Modulation of the CCR5 Receptor/Ligand Axis by Seminal Plasma and the Utility of In Vitro versus In Vivo Models

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ABSTRACT Sexual HIV-1 transmission occurs primarily in the presence of semen. Although data from macaque studies suggest that CCR5+/CD4+ T cells are initial targets for HIV-1 infection, the impact of semen on T cell CCR5 expression and ligand production remains inconclusive. To determine if semen modulates the lymphocyte CCR5 receptor/ligand axis, primary human T cell CCR5 expression and natural killer (NK) cell anti-HIV-1 antibody-dependent beta chemokine production was assessed following seminal plasma (SP) exposure. Purified T cells produce sufficient quantities of RANTES to result in a significant decline in CCR5bright T cell frequency following 16 h of SP exposure (P = 0.03). Meanwhile, NK cells retain the capacity to produce limited amounts of MIP-1α/MIP-1β in response to anti-HIV-1 antibody-dependent stimulation (median, 9.5% MIP-1α+ and/or MIP-1β+), despite the immunosuppressive nature of SP. Although these in vitro experiments suggest that SP-induced CCR5 ligand production results in the loss of surface CCR5 expression on CD4+ T cells, the in vivo implications are unclear. We therefore vaginally exposed five pigtail macaques to SP and found that such exposure resulted in an increase in CCR5+ HIV-1 target cells in three of the animals. The in vivo data support a growing body of evidence suggesting that semen exposure recruits target cells to the vagina that are highly susceptible to HIV-1 infection, which has important implications for HIV-1 transmission and vaccine design.

IMPORTANCE The majority of HIV-1 vaccine studies do not take into consideration the impact that semen exposure might have on the mucosal immune system. In this study, we demonstrate that seminal plasma (SP) exposure can alter CCR5 expression on T cells. Importantly, in vitro studies of T cells in culture cannot replicate the conditions under which immune cells might be recruited to the genital mucosa in vivo, leading to potentially erroneous conclusions about the impact of semen on mucosal HIV-1 susceptibility.

KEYWORDS CCR5, HIV, RANTES, T cells, chemokines, nonhuman primate, semen, seminal plasma

Successful establishment of human immunodeficiency virus type 1 (HIV-1) infection at the genital mucosa involves the presence of CD4+ CCR5+ T cells, which are the primary target of transmitted/founder viruses (1, 2). Fluctuations in mucosal target cell frequency are now understood to have an important impact on the risk of HIV-1 acquisition (3). Notably, certain contraceptives or HIV-1 vaccine candidates may alter...
the availability of HIV-1 target cells (4–8). Importantly, HIV-1 transmission predominantly occurs across mucosal surfaces and frequently in the context of semen, which has potent immunomodulatory activity (9). Seminal plasma (SP) contains both pro- and antiviral factors and initiates a proinflammatory response in the genital tract that induces the recruitment of immune cells following intercourse (10).

Multiple studies have shown that SP exposure results in the induction of CCL20/MIP-3α expression in the genital mucosa (10–12). In animal models, CCL20 appears to play a substantial role in the recruitment of HIV-1 target cells (CD4+ CCR6+ T cells [13]) to the genital mucosa (14), suggesting a mechanism by which SP may preferentially recruit T cells that are highly susceptible to HIV-1 infection to the vaginal mucosa. While CCR6 expression does define a subset of HIV-1 target cells, modulation of the HIV-1 coreceptor CCR5 would likely have a substantial, and perhaps more critical, role in determining the frequency of targets cells at the genital mucosa. In contrast to CCR6/MIP-3α, there is less consensus around the impact of SP exposure on lymphocyte production of the CCR5 ligands (RANTES/CCL5, MIP-1α, or MIP-1β), expression of CCR5 on CD4+ T cells, and alterations in CD4+ CCR5+ T cell frequency in the vaginal mucosa.

While in vitro studies of ectocervical explants exposed to SP have demonstrated SP to induce RANTES secretion (15), the contribution of lymphoid cells versus epithelial cells to CCR5 ligand accumulation in this context has not been assessed. The capacity for activated T cells to secrete RANTES and MIP1α/β is well described, and this secretion is an important mechanism by which HIV-1 infection can be suppressed (via CCR5 ligation and internalization) (16). Recently, however, it has become clear that natural killer (NK) cells are also important producers of CCR5 ligands in response to direct and CD16-mediated stimulation (17, 18). Indeed, NK cell β-chemokine secretion occurs in response to autologous HIV-1-infected CD4+ T cells and may represent a mechanism to block viral entry at initial foci of infection. We have previously shown that SP inhibits gamma interferon (IFN-γ) production by NK cells and conventional T cells (9), raising questions about whether these cells can produce β-chemokines in the presence of semen.

In addition to the induction of CCR5 ligand secretion, it remains possible that SP could also alter T cell CCR5 expression directly. Published studies to date have reported contrasting effects of SP exposure on T cells; one study reported that SP induces CCR5 expression on primary T cells after 8 h of exposure (19), while another reported a transient loss of CCR5 expression after 6 h of SP exposure but increased CCR5 after 24 h of exposure (20). Consensus is further complicated by the use of phytohemagglutinin (PHA)-stimulated T cells in some assays but not in others and by the use of cell lines rather than primary cells (21). An underlying weakness of all these studies is the use of closed in vitro culture systems that cannot recapitulate any recruitment of HIV-1 target cells from the circulation into the genital mucosa.

In this study, we sought to characterize the mechanisms by which SP might interact with the lymphocyte CCR5 receptor/ligand axis using in vitro assays to assess the impact of SP on primary human peripheral blood mononuclear cells (PBMC) and an in vivo system to evaluate the impact of mucosal exposure of pigtail macaques (PTM; Macaca nemestrina) to SP. We quantified the impact of SP on CCR5 expression on both high-density CCR5-expressing nonconventional T cells and low-density CCR5+ conventional T cells and determined the capacity of bulk PBMC, isolated T cells, and anti-HIV-1 antibody-stimulated NK cells to produce CCR5 ligands following SP exposure. Finally, we demonstrate that SP-induced CCR5 ligand production in closed in vitro systems results in the downregulation of CCR5 on T cells, while SP exposure in vivo fails to downregulate CCR5 and, in some cases, results in increased frequencies of CD4+ CCR5+ T cells at the vaginal mucosa.

RESULTS

Impact of pooled SP on lymphocyte viability in vitro. The known cytopathic impact of SP on lymphocytes during in vitro cell culture is an important consideration for studies of cellular phenotype and function (20). We exposed cells to SP at a final
concentration of 1%, which aims to reflect a balance between the likely physiological concentration of SP in the vaginal tract following sexual intercourse (~10%, according to Sharkey et al. [11]) and the issue of in vitro cytotoxicity. To confirm that this approach does not result in substantial cell death, we assessed the viability of bulk PBMC following exposure to 1% SP for 5 or 16 h (Fig. 1A). In five different PBMC donors, there was no change in T cell viability after 5 h of culture (median of 99.9% viable for both untreated and SP exposed) and only a marginal drop in viability after 16 h (medians of 99.7% viable for untreated and 99.3% viable for SP exposed [Fig. 1B]). There was no appreciable change in the frequency of any T cell subset (CD4+, CD8+, Vγ6+ gamma
delta, or mucosa-associated invariant T cells (MAIT cells; CD3\(^+\) Va7.2\(^+\) CD161\(^+\))} after 16 h of SP exposure (Fig. 1C). Interestingly, when T cells were isolated from fresh PBMC and then exposed to SP, they were more susceptible to SP-induced cell death after 16 h of culture (Fig. 1D). However, across six PBMC donors, the median proportion of viable T cells remained high in SP-exposed T cell cultures (median of 94.5% viable, compared to 99.7% viable in untreated controls), and all donors exhibited cell viability of >85% (Fig. 1E).

**Loss of CCR5 expression on high-density CCR5\(^+\) nonconventional T cells following SP exposure.** To assess the impact of SP exposure on T cell CCR5 expression, we first monitored CCR5 expression levels on MAIT cells and V\(\delta\)2\(^+\) gamma delta T cells (Fig. 2A). These nonconventional T cells express substantially higher levels of surface CCR5 protein than conventional CD4\(^+\) and CD8\(^+\) T cells (Fig. 2B), thereby providing a sensitive readout of CCR5 internalization due to ligand binding by flow cytometry. Exposure of cultured whole PBMC to a 1% SP pool for 5 h did not affect either V\(\delta\)2\(^+\) or MAIT cell CCR5 expression, but by 16 h, CCR5 median fluorescent intensity (MFI) was significantly decreased on both T cell subsets (medians of 32.5% of untreated control for V\(\delta\)2\(^+\) cells and 40.3% for MAIT cells; \(P = 0.014\) and 0.009, respectively) (Fig. 2C and D). Despite documented differences in the cytokine profiles of individual SP samples (22), five HIV-1-uninfected SP samples induced levels of CCR5 downregulation remarkably similar to those of the SP pool (Fig. 2E). Similarly, when the proportion of CCR5\(^+\) cells was quantified, there was found to be a significant reduction in CCR5\(^+\) V\(\delta\)2\(^+\) T cells (but not MAIT cells) after 16 h of SP exposure (\(P = 0.009\)) (Fig. 2F).

**Modulation of CD4\(^+\) and CD8\(^+\) T cell CCR5 expression by SP.** Similar to the SP-induced downregulation of CCR5 on nonconventional T cells, both CD4\(^+\) and CD8\(^+\) T cells exhibited lower CCR5 surface density after 16 h, but not 5 h, of exposure to the SP pool in bulk PBMC (61.3% of untreated control MFI for CD4\(^+\) cells [\(P = 0.009\)] and 50.2% of control for CD8\(^+\) cells [\(P = 0.014\)]) (Fig. 3A and B). Once again, individual SP samples caused a consistent loss of CCR5 expression on T cells, regardless of the donor (Fig. 3C). Unlike for V\(\delta\)2\(^+\) and MAIT cells, however, the reduction in CCR5 surface density on CD4\(^+\) T cells occurred through two mechanisms: a loss of CCR5\(^{bright}\) cells and an accumulation of a population of CCR5\(^{low}\) cells (\(P < 0.02\) for both) (Fig. 3D and E). Importantly, the increased frequency of CCR5\(^{low}\) cells was substantially greater than the loss of CCR5\(^{bright}\) cells, suggesting de novo CCR5 expression. The appearance of this CCR5\(^{low}\) subset was dependent on both PBMC donor and SP donor and was more pronounced in some of the individual SP samples than in the pool (Fig. 3D).

The concurrent decrease in CCR5\(^{bright}\) cells and the expansion of CCR5\(^{low}\) CD4\(^+\) T cells following SP exposure may explain current discrepancies in the literature regarding the impact of SP on CD4\(^+\) T cell CCR5 expression (19, 20). To assess whether SP-induced CCR5\(^{low}\) CD4\(^+\) T cells represent an expansion of putative HIV-1 target cells, we assessed the phenotype of this population. Th17 cells are preferential target cells for HIV-1 infection and can be identified by expression of CCR6 (23). The CCR5\(^{low}\) population expressed significantly less CCR6 following SP exposure (\(P = 0.016\)) (Fig. 3F), suggesting that low-level CCR5 expression was not induced on Th17 cells. Indeed, the frequency of CCR5\(^+\) CCR6\(^+\) cells declined overall as a proportion of the bulk CD4\(^+\) T cell subset following SP exposure (\(P = 0.031\)) (Fig. 3G).

**T cell production of RANTES following SP exposure results in CCR5 downregulation.** The consistency of CCR5 downregulation on T cells exposed to SP samples collected from independent donors, together with the time delay observed prior to loss of CCR5 expression, led us to hypothesize that prolonged exposure to SP caused one or more lymphocyte subsets to produce RANTES or other CCR5 ligands that resulted in CCR5 internalization. We therefore isolated CD3\(^+\) T cells from bulk PBMC prior to SP exposure. Consistent with the effects of SP on bulk PBMC, exposure of isolated T cells to 1% SP resulted in downregulation of CCR5 expression after 16 h on all T cell subsets examined (median CCR5 expressions of 49.8% of untreated control for V\(\delta\)2\(^+\) cells, 57.4% for MAIT cells, 56.2% for CD4\(^+\) T cells, and 48.0% for CD8\(^+\) T cells; \(P < 0.05\) for
all), confirming that the impact of SP on T cell CCR5 expression does not require the presence of other lymphocyte subsets (Fig. 4A and B). Despite the loss of CCR5 MFI, there was no change in the proportion of CCR5 cells within the Vδ2 or MAIT cell populations (data not shown). In contrast, there was a significant decrease in the frequency of CCR5bright cells and a significant increase in the frequency of CCR5low
FIG 3 Modulation of CCR5 expression on conventional T cells by seminal plasma. (A) Representative plots demonstrating a reduction in CCR5 MFI on CCR5^+ cells (indicated in the upper right of the CCR5 gate) among both CD4^+ and CD8^+ T cells following 16 h of SP pool exposure. (B) Exposure of whole PBMC to a 1% SP pool for 16 h results in a significant decrease in CCR5 MFI on CCR5^+ CD4^+ and CD8^+ T cells (n = 5). Differences from the untreated control were assessed by Friedman test with Dunn’s posttest. *, P < 0.05; **, P < 0.01. (C) CCR5 MFI following 16 h of exposure to either the SP pool or individual HIV-1-uninfected SP samples (SP 1 to SP 5) in a representative PBMC donor. (D) Representative plots of CCR5 expression on CD4^+ T cells following 16 h of exposure to a 1% SP pool or an individual SP sample. Arrows indicate population of expanded CCR5^low cells compared to that in an untreated control. (E) Quantification of CCR5^high and CCR5^low cells among CD4^+ T cells after 16 h of culture (n = 7). Statistics were assessed by Wilcoxon test. (F) Quantification of CCR6 expression on CCR5^low CD4^+ T cells in the presence or absence of a 1% SP pool (n = 7). Statistics were assessed by Wilcoxon test. (G) CCR5 and CCR6 coexpression on CD4^+ T cells following 1% SP exposure and quantification of the proportion of CCR5^-CCR6^- cells within the bulk CD4^+ T cell population in the presence or absence of SP (n = 7). Statistics were assessed by Wilcoxon test.
CD4$^+$ and CD8$^+$ T cells ($P = 0.031$ for all) (Fig. 4C), similar to our observations in the bulk PBMC cultures.

Analysis of chemokines present in the supernatant of SP-exposed PBMC cultures revealed a 6.6-fold median increase in RANTES concentration after 16 h (median of 410 pg/ml in RF10 control versus 2,698 pg/ml in 1% SP culture) (Fig. 5A). The concentration of RANTES in RF10 with the 1% SP pool was below the limit of detection (<31.5pg/ml), confirming that de novo RANTES expression from PBMC was induced by SP exposure. Addition of 3 ng/ml of recombinant RANTES to PBMC culture resulted in the rapid downregulation of CCR5 on unconventional T cells and a loss of CCR5$^{bright}$ CD4$^+$ and CD8$^+$ T cells ($P < 0.05$ for all) (Fig. 5B), suggesting that RANTES might be responsible for the observed changes in CCR5 following SP exposure.

To determine if RANTES was responsible for the observed loss of surface CCR5 protein, and whether T cells were the source of the RANTES, we exposed isolated T cells to SP in the presence of a neutralizing antibody against RANTES or an isotype control. SP-treated T cells cultured with anti-RANTES antibody exhibited significantly increased CCR5 MFI on both V$\gamma$2$^+$ T cells (from a median 50.3% of untreated control to 77.7%) ($P = 0.031$) and MAIT cells (from 54.6% of untreated control to 71.1%) ($P = 0.031$) compared to an isotype control, confirming the contribution of RANTES in CCR5 downregulation after SP exposure (Fig. 5C and D). The inclusion of additional neutralizing antibodies against MIP-1$\alpha$ and MIP-1$\beta$ did not result in any further increase in CCR5 surface density (data not shown), pointing to RANTES as the primary driver of CCR5 downregulation. Interestingly, the addition of the neutralizing RANTES antibody to the SP-treated T cell culture partially restored the population of CCR5$^{bright}$ CD4$^+$ T cells ($P = 0.031$) but did not abrogate the expansion of the CCR5$^{low}$ cells ($P > 0.05$) (Fig. 5E and F). Similar results were obtained for CD8$^+$ T cells ($P = 0.031$ for CCR5$^{bright}$ and $P > 0.05$ for CCR5$^{low}$) (Fig. 5G).

To confirm that the increased frequency of CCR5$^{low}$ CD4$^+$ and CD8$^+$ T cells was not due to SP-induced preferential cell death of the CCR5$^-$ population, we sorted freshly isolated CCR5$^-$ CD4$^+$ and CD8$^+$ T cells from two donors in the cohort. These cells were cultured for 16 h in either RF10 or 1% SP 3, the SP sample which induced the highest
FIG 5 Role of SP-induced RANTES in mediating CCR5 downregulation. (A) PBMC were exposed to a 1% SP pool for 16 h
(n = 3 PBMC donors). Cell culture supernatants were collected and RANTES expression was quantified by ELISA. Each
sample was run in duplicate. (B) PBMC were cultured for 30 or 60 min with 3 ng/ml of recombinant RANTES. Plots show
the change in CCR5 MFI for Vδ2+ and MAIT cells or the change in proportion of CCR5bright cells for CD4+ and CD8+ T cell
populations (n = 6 PBMC donors). Statistics were assessed by Friedman test with Dunn’s posttest. *, P < 0.05; **, P < 0.01.
(C and D) Representative histograms (C) and quantification (D) of CCR5 MFI on Vδ2+ and MAIT cells in isolated T cell
cultures treated with SP and either a neutralizing anti-RANTES antibody (2 μg/ml) or an IgG isotype control (n = 6 PBMC
donors). Differences were assessed by Wilcoxon test. (E) Representative plots of CD4+ T cell populations segregated based
(Continued on next page)
frequency of CCR5<sup>low</sup> cells (Fig. 3D). Following culture, 24.6 to 44.7% of CD4<sup>+</sup> and CD8<sup>+</sup> T cells from both donors expressed low levels of CCR5, compared to 0.9 to 4.2% of cells in the RF10 controls, demonstrating the capacity of SP to induce low-level de novo CCR5 expression on CCR5<sup>−</sup> T cells (Fig. 5H). In all cases, cell viability of the sorted and cultured cells remained at >87%.

Inhibition of CD16-mediated Ca<sup>2+</sup> mobilization in NK cells by short-term SP exposure. T cells are unlikely to be the only lymphocyte subset capable of producing β-chemokines following SP exposure. We and others have previously demonstrated that SP inhibits NK cell-mediated cytolyis and IFN-γ production following both direct and antibody-dependent stimulations (9, 24–26). Distinct from NK cell IFN-γ production, NK cell production of β-chemokines is thought to require less stimulation and occur in a shorter period (27). Therefore, we determined the temporal dynamics of NK cell inhibition by SP by first monitoring the capacity of NK cells to respond to CD16 cross-linking by mobilization of intracellular calcium stores (Fig. 6A). PBMC exposed to a 1% SP pool for 3 h had a nonsignificant inhibition of calcium flux (10.7% inhibition of untreated control; P > 0.05); however, 5 h of SP exposure resulted in significant inhibition (19.1%; P = 0.027) of CD16-induced calcium mobilization (Fig. 6B and C). Even after 5 h of SP exposure, NK cells still exhibited normal responses to the calcium ionophore ionomycin, suggesting that inhibition of CD16-induced signaling by SP is not due to cytotoxicity (data not shown).

Relative inhibition of anti-HIV-1 antibody-dependent NK cell β-chemokine production and IFN-γ production by SP. Given that inhibition of NK cell functions mediated through CD16 appears to occur following a 3- to 5-h delay after SP exposure, we hypothesized that NK cell functions requiring shorter stimulation periods and/or less cumulative stimulation, such as β-chemokine production, would be less inhibited than IFN-γ (27). To assess this possibility in the setting of HIV-1-specific antibody-mediated NK cell activation, we exposed NK cells to HIV-1 gp120-coated target cells in the presence of a 1:1,000 dilution of HIV-1 immunoglobulin (HIVIG), with or without 1% SP present. Following stimulation, NK cells were stained to detect intracellular expression of IFN-γ, MIP-1α, and MIP-1β and surface expression of the degranulation marker CD107a. Anti-HIV-1 antibody-dependent stimulation triggered NK cell IFN-γ production (median of 2% (interquartile range [IQR], 1.2 to 4.5%)) and CD107a expression (6.08% [13.6 to 28.5%]) (Fig. 6D). Addition of 1% SP to these conditions resulted in statistically significant decreases in IFN-γ production (0.27% [0.13 to 0.62%]; P = 0.002), CD107a mobilization (0.93% [0.50% to 1.4%]; P = 0.002), and the percentage of cells producing MIP-1α and/or MIP-1β (9.5% [7.1 to 12.5%]; P = 0.002) (Fig. 6E). Although SP reduced the ability of stimulated NK cells to produce MIP-1α and/or MIP-1β, the percent inhibition of MIP-1α and/or MIP-1β production by SP was significantly lower than the SP-mediated inhibition of IFN-γ production or CD107a expression (54.4% versus 88.8% and 85.4%, respectively; P = 0.0004 versus IFN-γ and P = 0.01 versus CD107a) (Fig. 6F).

Vaginal exposure to SP in a nonhuman primate model highlights discrepancies between in vitro and in vivo observations. Our in vitro data suggest that SP induces the secretion of β-chemokines over the course of 16 h after SP exposure. Combined with data from tissue explants demonstrating production of RANTES in response to SP (15), it seems clear that SP exposure likely results in elevated CCR5 ligand concentrations in the genital tract. In vitro, this manifests as reductions in T cell surface CCR5

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**FIG 5 Legend (Continued)**

on surface density of CCR5 following 16 h of exposure to 1% SP pool, with anti-RANTES neutralizing antibody (2 μg/ml) or an isotype control. (F and G) Analysis of the proportion of CCR5<sup>high</sup> or CCR5<sup>low</sup> CD4<sup>+</sup> (F) and CD8<sup>+</sup> (G) T cells cultured in the presence of SP and a neutralizing anti-RANTES antibody, expressed as a percentage of the untreated IgG isotype control (n = 6 PBMC donors). Bars indicate medians, with IQR. Differences were assessed by Wilcoxon test. (H) CCR5<sup>−</sup>CD4<sup>+</sup> and CD8<sup>+</sup> T cells were sorted from freshly isolated PBMC and cultured in the presence or absence of 1% SP 3 for 16 h. Plots indicate de novo CCR5 expression following cell culture. Data are representative of those from two PBMC donors.
FIG 6 Inhibition of NK cell function following seminal plasma treatment. (A) Representative plot of intracellular calcium flux in response to CD16 triggering. Cells were gated on live lymphocytes and then CD3⁺/CD56dim cells. Red arrows indicate the time of 3G8 or cross-linking F(ab')₂ additions (at 30 and 60 s). (B) Representative plot of Fluo4-AM MFI over time in CD3⁺/CD19⁻/CD56dim NK cells. Dashed lines indicate the time of 3G8 or cross-linking F(ab')₂ additions (at 30 and 60 s). Calcium flux in cells that were cultured for 5 h in the absence of SP (untreated) was compared to the flux elicited in cells that were cultured for 5 h but exposed to an individual SP sample for 3 or 5 h. The plot is representative of cells exposed to either individual SP samples or the SP pool. (C) The (Continued on next page)
expression, but in vivo, it is unclear whether CCR5 might be similarly downregulated or whether increased frequencies of CCR5+ T cells might traffick to the vaginal mucosa. To address this issue, we studied the in vivo response to SP using pigtail macaques (PTM). We first confirmed that in vitro culture of PTM PBMC with SP induced the same CCR5 expression patterns as observed for human PBMC. Indeed, 16 h of SP exposure on PTM PBMC results in an accumulation of CCR5dim CD4+ T cells that do not coexpress CCR6 (Fig. 7A and B) and an overall reduction of CCR5 MFI (Fig. 7B), which was highly consistent with the observed response of human PBMC (Fig. 3).

We therefore collected baseline vaginal biopsy specimens from five female PTM. Two weeks later, the same animals were exposed anogenitally to a pool of SP from aviremic HIV-1-infected donors, and a second vaginal biopsy specimen was collected 24 h after SP exposure. While not statistically significant, three out of the five macaques exhibited an increase in the frequency of CCR5+ cells within the CD4+ T cell population, with a particularly notable increase in CCR5 frequency among the two animals with low baseline CCR5+ CD4+ T cell frequencies (Fig. 7C). In contrast to the in vitro data, there was no consistent impact of SP exposure on CCR5 surface density (Fig. 7C). Overall, these data are consistent with elevated recruitment of CCR5-expressing T cells to the vaginal mucosa following CCR5 ligand production, rather than β-chemokine-induced CCR5 downregulation.

**DISCUSSION**

Despite the highly immunosuppressive nature of SP, these results indicate that not all immune cell functions are equally inhibited by SP exposure. We have demonstrated that NK cells retain the capacity to produce MIP-1α/MIP-1β in the presence of SP and that isolated T cells can secrete sufficient quantities of CCR5 ligand to result in CCR5 internalization following SP exposure in vitro. The contrasting impacts of SP-induced T cell-mediated RANTES production (resulting in CCR5 downregulation on CCR5hi T cells) and SP-induced accumulation of low levels of CCR5 expression on previously CCR5- CD4+ T cells may provide some clarity to the inconsistent conclusions of previous studies in this field (19–21). Depending on the resolution of CCR5 surface expression by flow cytometry and how CCR5 expression is measured (MFI versus proportion of positive cells), SP could be concluded to either increase or decrease cell surface CCR5. Combined with the artificially elevated levels of CCR5 expressed on PHA-activated primary T cells or immortalized cell lines, it has been difficult to understand the relationship between SP exposure and CCR5+ CD4+ T cell frequency in a more physiological setting. Our results clearly demonstrate the loss of CCR5hi T cells, mostly attributable to RANTES secretion into the culture supernatant, and an increase in CD4+ T cells expressing low levels of surface CCR5 following SP exposure.

Consistent with previous studies (9, 26), SP robustly inhibited NK cell degranulation and IFN-γ secretion in response to HIV-1 antibody-mediated activation (by 85 to 88.9%). It was therefore somewhat surprising that secretion of MIP-1α and MIP-1β was impaired by only approximately 50% despite SP exposure. The time-dependent decrease in CD16-triggered calcium flux following SP exposure may partially explain this observation, as NK cells are reported to have a lower threshold and more rapid capacity to

**FIG 6 Legend (Continued)**

stimulation-induced AUC of the SP-treated samples is expressed as a percentage of the untreated control for four PBMC donors (median with IQR). Differences were assessed by Friedman test with Dunn’s multiple-comparison posttest. (D) Gating strategy to assess NK cell function following stimulation with gp120-coated CEM.NKr cells and HIVIG. Lymphocytes were gated on FSC-A versus FSC-H to identify singlets, and NK cells were defined as CD3- CD16+CD56dim cells. Incubation of PBMC with gp120-coated CEM.NKr cells resulted in minimal background expression of IFN-γ, CD107a, and MIP-1α/MIP-1β. Addition of HIVIG resulted in NK cell recognition of CEM.NKr target cells and induced expression of IFN-γ, CD107a, and MIP-1α/MIP-1β, which is inhibited in the presence of seminal plasma (SP). (E) Addition of 1% SP to the PBMC/CEM.NKr/antibody coculture results in a significant inhibition of IFN-γ, CD107a, and MIP-1α/MIP-1β expression by NK cells (n = 10 PBMC donors). (F) The SP-induced percent inhibition of IFN-γ and CD107a expression is significantly greater than the inhibition observed for MIP-1α/MIP-1β (n = 10 PBMC donors). Differences were assessed by Wilcoxon test or Friedman test with Dunn’s multiple-comparison posttest.
FIG 7 Response of macaque T cells to SP exposure in vitro and in vivo. (A) Representative staining of CD4+ T cell CCR5 expression and CCR5/CCR6 coexpression following exposure of macaque PBMC to 1% SP 3 for 16 h. (B) Quantification of CCR5 MFI on CCR5+ CD4+ T cells and proportion of CCR5dim CD4+ T cells following 16 h of SP exposure (n = 5). (C) Vaginal biopsy specimens were collected from five female macaques at baseline. Two weeks later, animals were vaginally exposed to 2.5 ml of seminal plasma, and a second biopsy specimen was collected 24 h later. CD4+ T cells (identified as live, CD45+ EpCam- CD20- CD14- CD3+ CD4+ lymphocytes) were assessed for expression of CCR5 and CCR5 surface density (MFI). Plots are representative of results from two animals with low baseline CCR5 expression.
produce β-chemokines than other antiviral cytokines (27). Overall these data, combined with observations from ectocervical explants exposed to SP (15), begin to portray a more comprehensive description of the impact of SP on mucosal immunity to HIV-1. In addition to possible epithelial cell production of RANTES (15), SP is capable of eliciting RANTES secretion by T cells. Meanwhile, antibody-mediated activation of NK cells in the presence of SP also results in the secretion of MIP-1α and MIP-1β. What has remained unclear from in vitro experiments, however, is what impact this β-chemokine production may have on CCR5⁺ CD4⁺ T cell frequencies in vivo, as the concentrations of any CCR5 ligands in vitro likely have little relationship to the accumulation of these proteins in the genital tract.

The use of PTM exposed vaginally to SP provides important evidence regarding this issue and overcomes the limitations of the in vitro model, which includes potentially non-physiologically relevant concentrations of chemokines as well as an inability to recapitulate any aspects of T cell recruitment to the mucosa via chemotaxis. In vitro, PTM and human PBMC respond similarly to SP exposure. It is extremely interesting, therefore, that vaginal SP exposure resulted in an increase in CCR5⁺ CD4⁺ T cells in the vagina among some animals without affecting CCR5 surface density. It is highly probable that this difference reflects the ability of SP to recruit additional immune cells from the circulation in vivo, as well as reflecting SP-induced contributions from the vaginal epithelium and other cell types not reflected in PBMC samples.

Together, the weight of evidence from a variety of PBMC, tissue explant, and nonhuman primate models appears to favor a model in which SP promotes an HIV-1-susceptible genital mucosa. Chemotaxis experiments have suggested that mucosal RANTES accumulation results in the recruitment of monocytes (15), which likely contribute to a proinflammatory immune environment that promotes viral infection. We have shown that SP promotes the accumulation of CCR5⁻ T cells in vivo, which is similar to observations of CCL20/MIP-3α-mediated recruitment of HIV-1 target cells (14). Simultaneously, we have shown that antiviral immune responses at the foci of infection (including NK cell production of IFN-γ and degranulation) might be inhibited as early as 3 to 5 h following SP exposure through a mechanism involving the suppression of intracellular calcium signaling. The balance of evidence therefore seems to support the view that exposure to SP promotes HIV-1 transmission and infection while impairing antiviral immune responses.

These data are relevant to both general studies of HIV-1 transmission and the development of a successful HIV-1 vaccine, which will need to achieve a balance between inducing protective antibody and/or CD8⁺ T cell-mediated responses in the genital mucosa without arousing additional CD4⁺ T helper cell activation/CCR5 expression and thus increasing the availability of HIV-1 target cells (5, 28). We speculate that simian immunodeficiency virus (SIV) challenge studies that include SP exposure would be more relevant than current models in order to account for the possibility of increased target cell availability that may compromise vaccine efficacy. Indeed, results from multiple human and macaque trials have suggested that the recruitment of activated CD4⁺ CCR5⁺ T cells to the mucosa underlies the lack of protection observed in several vaccine candidates (5–8). When CCR5⁺ CD4⁺ T cells become productively infected, resident antiviral immune responses will be critical to control the early spread of infection. If the cytolytic capacity of NK cells is inhibited in the presence of SP, while the expression of chemotactic proteins such as CCL20, RANTES, and MIP-1α/β is induced from T cells, novel vaccine candidates will need to be formulated to function in this altered immune environment. Future studies involving T cell samples derived from the vaginal or rectal mucosa, in combination with mucosal tissue explant models, will therefore be particularly informative in further dissecting the impact of SP on HIV-1 target cell availability and mucosal vaccine responses. Additionally, future in vivo studies will be informative in determining whether the elevated frequency of CCR5⁺ CD4⁺ T cells represents chemotactic recruitment of new target cells to the mucosa, or simply CCR5 upregulation on existing mucosa-resident T cells.
MATERIALS AND METHODS

Human cohort participants. Whole blood was collected from 13 HIV-1-uninfected donors. SP samples from five individual HIV-1-uninfected donors and an SP pool from multiple HIV-1-uninfected human donors were purchased from BioIVT (Westbury, NY). Pooling SP from 70 aviremic, HIV-1-infected human donors used for pigtail macaque experiments was obtained from the Opposites Attract cohort study (22). Informed consent was obtained before collection and storage of all biological samples, and ethics approval was granted by all participating institutions.

Animals. Female pigtail macaques aged 2 to 3 years were purchased from the Australian National Breeding Colony. A total of 2.5 ml of an SP pool (derived from 70 HIV-1-infected donors with plasma viral loads of <40 copies/ml) was inserted atraumatically into both the vagina and rectum of the animals using a transfer pipette. Vaginal biopsy specimens (~3 mm) were collected using pinch biopsy forceps placed 5 cm into the vagina. Biopsy samples were taken 2 weeks prior to SP exposure and 24 h postexposure. Samples were transported on ice. Biopsy tissues were washed in RPMI medium and incubated in digestion buffer (0.1 mg/ml of collagenase and 1.5 U/ml of DNase in RF10 [see below]) at 37°C for 2 h. The buffer and remaining tissue samples were passed through a 70-μm filter and washed in RPMI 1640 medium (Life Technologies, Grand Island, NY) supplemented with 10% fetal calf serum (FCS), penicillin, streptomycin, and L-glutamine (RF10). The resulting cell pellet was resuspended in RF10, passed through a 30-μm cell filter, and then processed immediately for antibody staining and flow cytometry.

Cell culture and stimulation for T cell immunology. PBMC were isolated by Ficoll gradient separation (GE Healthcare, Madison, WI) and cultured in RF10. Where described, SP (either pooled or individual) was added to cell culture at a final concentration of 1% for 3, 5, or 16 h. Identical SP exposure protocols were used for human and macaque PBMC experiments. In some experiments, neutralizing antibodies against RANTES (MAB678), MIP-1α (MAB270), and MIP-1β (MAB271) (R&D Systems, Minneapolis, MN) or an IgG isotype control (MOPC-21) (BioLegend, San Diego, CA) were added to cell culture at a concentration of 2 μg/ml. In one experiment, recombinant human RANTES protein (Peprotech) was added to cell culture at 3 ng/ml.

T cell isolations. CD3⁺ T cells were isolated from whole PBMC by negative selection using an EasySep human T cell isolation kit according to the manufacturer’s protocol (StemCell Technologies, Vancouver, BC, Canada). Isolation purity was confirmed by flow cytometry and ranged from 94.0% to 98.5%.

RANTES ELISA. To determine the concentration of RANTES in cell culture, the human CCL5/RANTES Quantikine enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems) was used according to the manufacturer’s instructions. Briefly, 1.0 × 10⁵ PBMC were stimulated for 16 h at 37°C with 1% (vol/vol) pooled seminal plasma and supernatants were collected by centrifugation at 500 × g for 5 min. Supernatants were diluted 1:10 in calibrator diluent (or used neat [unstimulated control]) and added to the anti-human RANTES coated microplate for 2 h. Plates were then washed three times in wash buffer and incubated with horseradish peroxidase (HRP)-conjugated anti-human RANTES antibody for 1 h. Following three further wash steps, plates were developed for 20 min with tetramethylbenzidine substrate solution, reactions were stopped with sulfuric acid, and absorbance was measured at 450 nm.

Absorbance readings were averaged for each sample, the background was subtracted, and a five-parameter logistic (5-PL) curve was fitted to the recombinant RANTES standards using Prism 7. Concentrations were determined by interpolation of the standard curve.

Detection of CCR5 by flow cytometry. For quantification of CCR5 expression levels, human PBMC were washed and incubated with the following antibodies/dyes for 30 min at 4°C: anti-CD3 BV786 or BV515 (clone SK7), BD Biosciences, San Jose, CA), anti-CD4 BV605 (clone RPA-T4), anti-CD8 BV505 (clone RPA-T8), anti-CD161 allophycocyanin (APC) (clone 7D12; BD Biosciences, San Diego, CA), anti-NKG2A antibodies (clone RPA-T4), anti-CD8 BV650 (clone RPA-T8), anti-CD161 allophycocyanin (APC) (clone 7D12; BD Biosciences, San Diego, CA), anti-Vβ7.2 BV510 (clone 3C10), anti-CCR5 BV421 (clone J418F1), and anti-CCR6 BV786 (clone G034E3) (all from BioLegend, San Diego, CA), and fixable LIVE/DEAD blue (Life Technologies, Grand Island, NY). For PTM samples, single cell suspensions from vaginal biopsy specimens were stained with CD45 BV395 (clone DO58-1283; BD Biosciences), CD3 Alexa Fluor 488 (clone SP3-2; BD Biosciences), CD4 BV605 (clone L200; BD Biosciences), and CCR5 BV421 (clone J418F1; BioLegend) as well as exclusion markers for neutrophils, monocytes, B cells, and NK cells (using anti-EpCam, CD66abce, CD14, CD20, and CD56 antibodies). In vitro PTM PBMC/SP exposure experiments were assessed using the same CD3, CD4, and CCR5 antibody clones.

Samples were acquired on a BD LSRFortessa with FACS Diva. Flow cytometry data were analyzed in FlowJo (TreeStar, Inc., Ashland, OR). Calculations of median fluorescent intensity (MFI) were performed for cells gated to express the marker of interest only.

Cell sorting. To sort CCR5⁺ CD4⁺ and CD8⁺ T cells, freshly isolated PBMC were stained with CD3 BV786, CD4 BV605, CD8 BV505, CCR5 BV421, and viability dye. Cells were sorted on a BD FACS Aria III using CCR5 fluorescence minus one (FMO) to set the CCR5 gate. Cells were cultured at 2 × 10⁶/ml for 16 h in the presence or absence of 1% SP. After culture, cells were stained with CCR5 APC and a different viability dye to measure de novo CCR5 expression.

Intracellular Ca²⁺ release. PBMC were cultured for 3 or 5 h with (or 5 h without) 1% pooled SP. Cells were then washed twice with phosphate-buffered saline (PBS) and loaded with 3 μM Fluor-AM plus 0.02% Pluronic F-127 (Life Technologies, Grand Island, NY) for 45 min at room temperature. Cells were washed twice with PBS and then stained with the following antibodies: anti-CD3 peridinin chlorophyll protein (PerCP)-Cy5.5 (clone SK7), anti-CD19 PE-CF594 (clone HIB19), and anti-CD56 PE-Cy7 (clone NCA116.2) (all from BD Biosciences, San Jose, CA). Cells were washed and incubated at 37°C until the time of assay. A baseline fluorescent reading was collected on a BD LSRFortessa for 30 s, at which time...
anti-CD16 monoclonal antibody (mAb) (clone 3G8) was added to the cells at 5 μg/ml. A second baseline reading was collected for 30 s, at which time goat anti-mouse IgG F(ab)\(^{-}\) (Sigma) was added to the cells at 10 μg/ml to stimulate calcium flux through CD16. Stimulation with ionomycin was used as a positive control. Fluorescent readings were taken for a total of 4 min. Calcium mobilization in the CD3–CD19–CD56\(^{dim}\) NK cell population was analyzed using the Program (1 10 \(^{6}\) at a 1:10 ratio in the presence or absence of a 1:1,000 dilution of HIV-1 (NIH AIDS Reagent Program). Some renditions of the assay included a 1:100 dilution of SP to assess the effects of SP on NK cell activation. Incubations were conducted for 5 h at 37°C in the presence of 5 μg/ml of brefeldin A (Sigma) and APC-H7-conjugated anti-CD107a antibody (clone H4A3; BD Biosciences). Following stimulation, cells were surface stained with BV786-conjugated anti-CD3 (clone SK7; BioLegend) and PE-Cy7-conjugated anti-CD56 antibodies (clone NCAM16.2; BD Biosciences). Next, cells were fixed with formaldehde, washed, and incubated in permeabilization buffer (BD). Cells were then stained with Alexa Fluor 700-conjugated anti-IFN-γ (clone 4S.B11; BioLegend) and fluorescein isothiocyanate (FITC)-conjugated anti-MIP-1α (clone D21-1351; BD Biosciences) antibodies. Samples were then washed, fixed, and acquired with a BD LSRFortessa flow cytometer and analyzed with FlowJo software (Tree Star Inc.).

**Statistical analysis.** Data were analyzed in GraphPad Prism, version 7 (GraphPad Software, La Jolla, CA). Paired data were analyzed using two-tailed nonparametric Wilcoxon tests or a Friedman test with FlowJo v10 Kinetics function. To compare the calcium flux induced by 3G8 cross-linking in the presence or absence of SP, the area under the curve (AUC) was utilized to assess NK cell activation (9). Briefly, PBMC (1 10 \(^{6}\)) were incubated with HIV-1TL gp120 coated (3 μg/ml) or uncoated CEM.NK-CCR5 cells (both from the NIH AIDS Reagent Program) (1 10 \(^{6}\)) at a 1:1 ratio in the presence or absence of a 1:1,000 dilution of HIV-1 (NIH AIDS Reagent Program). Some renditions of the assay included a 1:100 dilution of SP to assess the effects of SP on NK cell activation. Incubations were conducted for 5 h at 37°C in the presence of 5 μg/ml of brefeldin A (Sigma) and APC-H7-conjugated anti-CD107a antibody (clone H4A3; BD Biosciences). Following stimulation, cells were surface stained with BV786-conjugated anti-CD3 (clone SK7; BioLegend) and PE-Cy7-conjugated anti-CD56 antibodies (clone NCAM16.2; BD Biosciences). Next, cells were fixed with formaldehde, washed, and incubated in permeabilization buffer (BD). Cells were then stained with Alexa Fluor 700-conjugated anti-IFN-γ (clone B27; BD Biosciences), APC-conjugated anti-CD154 (clone RPA-T4; BD Biosciences), and fluorescein isothiocyanate (FITC)-conjugated anti-MIP-1α (clone D21-1351; BD Biosciences) antibodies. Samples were then washed, fixed, and acquired with a BD LSRFortessa flow cytometer and analyzed with FlowJo software (Tree Star Inc.).

**Statistical analysis.** Data were analyzed in GraphPad Prism, version 7 (GraphPad Software, La Jolla, CA). Paired data were analyzed using two-tailed nonparametric Wilcoxon tests or a Friedman test with Dunn’s multiple-comparison posttest. P values of less than 0.05 were considered statistically significant.

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**REFERENCES**


