Modulation of the CCR5 receptor/ligand axis by seminal plasma and the utility of *in vitro* versus *in vivo* models

Running head: Chemokine regulation by semen

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Abstract

Sexual HIV-1 transmission occurs primarily in the presence of semen. Although data from macaque studies suggests $CCR5^{+}CD4^{+}$ 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 $CCR5^{bright}$ T cell frequency following 16 hours 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-$1\alpha^{+}$MIP-$1\beta^{+}$), 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 out of five 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.

53 **Introduction**

54 Successful establishment of HIV-1 infection at the genital mucosa involves the presence of
55 $CD4^+CCR5^+$ T cells, which are the primary target of transmitted/founder viruses(1, 2).
56 Fluctuations in mucosal target cell frequency are now understood to have an important
57 impact on the risk of HIV-1 acquisition(3). Notably, certain contraceptives or HIV-1 vaccine
58 candidates may alter the availability of HIV-1 target cells(4-8). Importantly, HIV-1
59 transmission predominantly occurs across mucosal surfaces and frequently in the context of
60 semen, which has potent immunomodulatory activity(9). Seminal plasma (SP) contains both
61 pro- and anti-viral factors, and initiates a pro-inflammatory response in the genital tract that
62 induces the recruitment of immune cells following intercourse(10).

63

64 Multiple studies have shown that SP exposure results in the induction of CCL20/MIP-3α
65 expression at the genital mucosa(10-12). In animal models, CCL20 appears to play a
66 substantial role in the recruitment of HIV-1 target cells ($CD4^+CCR6^+$ T cells(13)) to the genital
67 mucosa(14), suggesting a mechanism by which SP may preferentially recruit T cells that are
68 highly susceptible to HIV-1 infection to the vaginal mucosa. While CCR6 expression does
69 define a subset of HIV-1 target cells, modulation of the HIV-1 co-receptor CCR5 would likely
70 have a substantial, and perhaps more critical, role in determining the frequency of targets
71 cells at the genital mucosa. In contrast to CCR6/MIP-3α, there is less consensus around the
72 impact of SP exposure on lymphocyte production of the CCR5 ligands (RANTES/CCL5, MIP-
73 1α or MIP-1β), expression of CCR5 on $CD4^+$ T cells and alterations in $CD4^+CCR5^+$ T cell
74 frequency at the vaginal mucosa.

75

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 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 IFNγ producion 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 hours of exposure(19), while another reported a transient loss of CCR5 expression after six hours of SP exposure, but increased CCR5 after 24 hours of exposure(20). Consensus is further complicated by the use of PHA-stimulated T cells in some assays, but not in others, or 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 (*Macaca nemestrina,* PTM) to SP. We quantified the impact of SP on CCR5 expression on both high-density CCR5-expressing non-conventional T cells as well as 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* failed to downregulate CCR5 and, in some cases, resulted 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 five or 16 hours (Fig 1A). In five different PBMC donors, there was no change in T cell viability after five hours of culture (median 99.9% viable for both untreated and SP exposed), and only a marginal drop in viability after 16 hours (median 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+, $V\delta2^+$ gamma delta or mucosal associated invariant T cells (MAIT cells, $CD3^+V\alpha7.2^+CD161^{++}$)) after 16 hours 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 hours 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 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 $CCR5^+$ non-conventional 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 $V\delta2^+$ gamma delta T cells (Fig 2A). These non-

conventional 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 1% SP pool for five hours did not affect either $V\delta2^+$ or MAIT cell CCR5 expression, but by 16 hours, CCR5 MFI was significantly decreased on both T cell subsets (median 32.5% of untreated control for $V\delta2^+$ cells, and 40.3% for MAIT cells, p=0.014 and 0.009, respectively, Fig 2C, D). Despite documented differences in the cytokine profile of individual SP samples(22), five HIV-1-uninfected SP samples induced remarkably similar levels of CCR5 downregulation as the SP pool (Fig 2E). Similarly, when the proportion of $CCR5^+$ cells was quantified, there was a significant reduction in $CCR5^+$ $V\delta2^+$ T cells (but not MAIT cells) after 16 hours of SP exposure (Fig 2F, p=0.009).

Modulation of $CD4^+$ and $CD8^+$ T cell CCR5 expression by SP

Similar to the SP-induced downregulation of CCR5 on non-conventional T cells, both $CD4^+$ and $CD8^+$ T cells exhibited lower CCR5 surface density after 16, but not five, hours 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, B). Once again, individual SP samples caused a consistent loss of CCR5 expression on T cells, regardless of the donor (Fig 3C). Unlike $V\delta2^+$ 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-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

158 donor and SP donor, and was more pronounced in some of the individual SP samples than in
159 the pool (Fig 3D).
160
161 The concurrent decrease in CCR5^{bright} cells and the expansion of CCR5^{low} CD4^{+} T cells
162 following SP exposure may explain current discrepancies in the literature regarding the
163 impact of SP on CD4^{+} T cell CCR5 expression(19, 20). To assess whether SP-induced CCR5^{low}
164 CD4^{+} T cells represent an expansion of putative HIV-1 target cells, we assessed the
165 phenotype of this population. Th17 cells are preferential target cells for HIV-1 infection, and
166 can be identified by expression of CCR6(23). The CCR5^{low} population expressed significantly
167 less CCR6 following SP exposure (p=0.016, Fig 3F), suggesting that low-level CCR5 expression
168 was not induced on Th17 cells. Indeed, the frequency of CCR5^{+}CCR6^{+} cells declined overall
169 as a proportion of the bulk CD4^{+} T cell subset following SP exposure (p=0.031, Fig 3G).
170
171 *T cell production of RANTES following SP exposure results in CCR5 downregulation*
172 The consistency of CCR5 downregulation on T cells exposed to SP samples collected from
173 independent donors, together with the time delay observed prior to loss of CCR5
174 expression, led us to hypothesize that prolonged exposure to SP caused one or more
175 lymphocyte subsets to produce RANTES or other CCR5 ligands that resulted in CCR5
176 internalization. We therefore isolated CD3^{+} T cells from bulk PBMC prior to SP exposure.
177 Consistent with the effects of SP on bulk PBMC, exposure of isolated T cells to 1% SP
178 resulted in downregulation of CCR5 expression after 16 hours on all T cell subsets examined
179 (median CCR5 expression of 49.8% of untreated control for Vδ2^{+} cells, 57.4% for MAIT cells,
180 56.2% for CD4^{+} T cells, and 48.0% for CD8^{+} T cells; p<0.05 for all), confirming that the impact
181 of SP on T cell CCR5 expression does not require the presence of other lymphocyte subsets

(Fig 4A, B). Despite the loss of CCR5 MFI, there was no change in the proportion of $CCR5^+$ cells within the $V\delta2^+$ or MAIT cell populations (not shown). In contrast, there was a significant decrease in the frequency of $CCR5^{bright}$ cells and a significant increase in the frequency of $CCR5^{low}$ $CD4^+$ 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 16hrs (median 410pg/mL in RF10 control versus 2698pg/mL in 1% SP culture; Fig 5A). The concentration of RANTES in RF10 with 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 3ng/mL 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 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 $V\delta2^+$ (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, D). The inclusion of additional neutralizing antibodies against MIP-1α and MIP-1β did not result in any further

206 increase in CCR5 surface density (not shown), pointing to RANTES as the primary driver of
207 CCR5 downregulation. Interestingly, the addition of the neutralizing RANTES antibody to
208 the SP-treated T cell culture partially restored the population of $CCR5^{bright}$ $CD4^{+}$ T cells
209 ($p=0.031$), but did not abrogate the expansion of the $CCR5^{low}$ cells ($p>0.05$, Fig 5E-F). Similar
210 results were observed for $CD8^{+}$ T cells ($p=0.031$ for $CCR5^{bright}$, $p>0.05$ for $CCR5^{low}$; Fig 5G).
211
212 To confirm that the increased frequency of $CCR5^{low}$ $CD4^{+}$ and $CD8^{+}$ T cells was not due to SP-
213 induced preferential cell death of the $CCR5^{-}$ population, we sorted freshly isolated $CCR5^{-}$
214 $CD4^{+}$ and $CD8^{+}$ T cells from two donors in the cohort. These cells were cultured for 16hrs in
215 either RF10 or 1% SP #3, the SP sample which induced the highest frequency of $CCR5^{low}$ cells
216 (Fig 3D). Following culture, 24.6-44.7% of $CD4^{+}$ and $CD8^{+}$ T cells from both donors expressed
217 low levels of CCR5, compared to 0.9-4.2% of cells in the RF10 controls, demonstrating the
218 capacity of SP to induce low-level de novo CCR5 expression on $CCR5^{-}$ T cells (Fig 5H). In all
219 cases, cell viability of the sorted and cultured cells remained >87%.
220
221 *Inhibition of CD16-mediated Ca^{2+} mobilization in NK cells by short-term SP exposure*
222 T cells are unlikely to be the only lymphocyte subset capable of producing β-chemokines
223 following SP exposure. We and others have previously demonstrated that SP inhibits NK
224 cell-mediated cytolysis and IFNγ production following both direct and antibody-dependent
225 stimulations(9, 24-26). Distinct from NK cell IFNγ production, NK cell production of β-
226 chemokines is thought to require lesser stimulation and occur in a shorter time period(27).
227 As such, we determined the temporal dynamics of NK cell inhibition by SP, by first
228 monitoring the capacity of NK cells to respond to CD16 cross-linking by mobilization of
229 intracellular calcium stores (Fig 6A). PBMC exposed to a 1% SP pool for three hours had a

non-significant inhibition of calcium flux (10.7% inhibition of untreated control, p>0.05), however, five hours of SP exposure resulted in significant inhibition (19.1%, p=0.027) of CD16-induced calcium mobilization (Fig 6B, C). Even after five hours of SP exposure, NK cells still exhibited normal responses to the calcium ionophore ionomycin, suggesting that inhibition of CD16-induced signalling by SP is not due to cytotoxicity (not shown).

Relative inhibition of anti-HIV-1 antibody-dependent NK cell β-chemokine production and IFNγ production by SP

Given that inhibition of NK cell functions mediated through CD16 appears to occur following a three-to-five hour 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 IFNγ(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 1:1000 dilution of HIV-1 immunoglobulin (HIVIG), with or without 1% SP present. Following stimulation, NK cells were stained to detect intracellular expression of IFNγ, MIP-1α and MIP-1β, and surface expression of the degranulation marker CD107a. Anti-HIV-1 antibody-dependent stimulation triggered NK cell IFNγ production (median 2% [1.2-4.5% IQR]), CD107a expression (6.08% [3.1-10.0%]) as well as high percentages of NK cells producing MIP-1α and/or MIP-1β (20.5% [13.6-28.5%]) (Fig 6D). Addition of 1% SP to these conditions resulted in statistically significant decreases in IFNγ production (0.27% [0.13-0.62%], p=0.002), CD107a mobilization (0.93% [0.50%-1.4%], p=0.002) and the percentage of cells producing MIP-1α and/or MIP-1β (9.5% [7.1-12.5%], 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

254 significantly lower than the SP-mediated inhibition of IFNγ production or CD107a expression
255 (54.4% vs. 88.8% and 85.4%, respectively, p=0.0004 vs IFNγ and p=0.01 vs CD107a) (Fig 6F).
256
257 *Vaginal exposure to SP in a non-human primate model highlights discrepancies between in*
258 *vitro and in vivo observations*
259 Our *in vitro* data suggests that SP induces the secretion of β-chemokines over the course of
260 16 hours after SP exposure. Combined with data from tissue explants demonstrating
261 production of RANTES in response to SP(15), it seems clear that SP exposure likely results in
262 elevated CCR5 ligand concentrations at the genital tract. *In vitro*, this manifests as
263 reductions in T cell surface CCR5 expression, but *in vivo,* it is unclear whether CCR5 might be
264 similarly downregulated, or whether increased frequencies of $CCR5^{+}$ T cells might traffick to
265 the vaginal mucosa. To address this issue, we studied the *in vivo* response to SP using pigtail
266 macaques (PTM). We first confirmed that *in vitro* culture of PTM PBMC with SP induced the
267 same CCR5 expression patterns as observed for human PBMC. Indeed, 16 hours of SP
268 exposure on PTM PBMC results in an accumulation of $CCR5^{dim}$ $CD4^{+}$ T cells that do not co-
269 express CCR6 (Fig 7A, B), and an overall reduction of CCR5 MFI (Fig 7B), which was highly
270 consistent with the observed response of human PBMC (Fig 3).
271
272 We therefore collected baseline vaginal biopsies from five female PTM. Two weeks later,
273 the same animals were exposed anogenitally to a pool of SP from aviremic HIV-1-infected
274 donors, and a second vaginal biopsy was collected 24 hours after SP exposure. While not
275 statistically significant, three out of five macaques exhibited an increase in the frequency of
276 $CCR5^{+}$ cells within the $CD4^{+}$ T cell population, with a particularly notable increase in CCR5
277 frequency among the two animals with low baseline $CCR5^{+}CD4^{+}$ T cell frequencies (Fig 7C).

278 In contrast to the *in vitro* data, there was no consistent impact of SP exposure on CCR5
279 surface density (Fig 7C). Overall, this data is consistent with elevated recruitment of CCR5-
280 expressing T cells to the vaginal mucosa following CCR5 ligand production, rather than β-
281 chemokine-induced CCR5 downregulation.
282

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283 **Discussion**

284

285 Despite the highly immunosuppressive nature of SP, these results indicate that not all
286 immune cell functions are equally inhibited by SP exposure. We have demonstrated that NK
287 cells retain the capacity to produce MIP-1α/MIP-1β in the presence of SP, and that isolated
288 T cells can secrete sufficient quantities of CCR5 ligand to result in CCR5 internalization
289 following SP exposure in vitro. The contrasting impact of SP-induced T cell-mediated
290 RANTES production (resulting in CCR5 downregulation on $CCR5^{hi}$ T cells) and SP-induced
291 accumulation of low levels of CCR5 expression on previously $CCR5^{-}CD4^{+}$ T cells may provide
292 some clarity to the inconsistent conclusions of previous studies in this field(19-21).
293 Depending on the resolution of CCR5 surface expression by flow cytometry and how CCR5
294 expression is measured (MFI vs proportion of positive cells), SP could be concluded to either
295 increase or decrease cell surface CCR5. Combined with the artificially elevated levels of
296 CCR5 expressed on PHA-activated primary T cells or immortalized cell lines, it has been
297 difficult to understand the relationship between SP exposure and $CCR5^{+}CD4^{+}$ T cell
298 frequency in a more physiological setting. Our results clearly demonstrate the loss of
299 $CCR5^{bright}$ T cells, mostly attributable to RANTES secretion into the culture supernatant, and
300 an increase in $CD4^{+}$ T cells expressing low levels of surface CCR5 following SP exposure.

301

302 Consistent with previous studies(9, 26), SP robustly inhibited NK cell degranulation and IFNγ
303 secretion in response to HIV-1 antibody-mediated activation (by 85-88%). It was therefore
304 somewhat surprising that secretion of MIP-1α and MIP-1β was only impaired by 50%
305 despite SP exposure. The time-dependent decrease in CD16-triggered calcium flux following
306 SP exposure may partially explain this observation, as NK cells are reported to have a lower
307 threshold and more rapid capacity to produce beta 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 at 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*, both 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 non-human primate models appears to favour a model where 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 pro-inflammatory 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 IFNγ and degranulation) might be inhibited as early as three-to-five hours following SP exposure through a mechanism involving the suppression of intracellular calcium signalling. The balance of evidence therefore seems to support the view that exposure to SP promotes HIV-1 transmission and infection, while impairing anti-viral immune responses.

This data is relevant to both general studies of HIV-1 transmission as well 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 at 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 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 anti-viral immune responses will be critical to control the early spread of infection. If the cytolytic capacity of NK cells are 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.

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364 **Methods**

365 *Human cohort participants:* Whole blood was collected from 13 HIV-1-uninfected donors. SP
366 samples from five individual HIV-1-uninfected donors and a SP pool from multiple HIV-1-
367 uninfected human donors were purchased from BioIVT (Westbury, New York, USA). Pooled
368 SP from 70 aviremic, HIV-1-infected human donors used for pigtail macaque experiments
369 was obtained from the Opposites Attract cohort study(29). Informed consent was obtained
370 before collection and storage of all biological samples, and ethics approval were granted by
371 all participating institutions.

372

373 *Animals:* Female pigtail macaques aged 2-3 years were purchased from the Australian
374 National Breeding Colony. 2.5mL of a SP pool (derived from 70 HIV-1-infected donors with
375 plasma viral loads <40 copies/mL) was inserted atraumatically into both the vagina and
376 rectum of the animals using a transfer pipette. Vaginal biopsies (~3mm) were collected
377 using pinch biopsy forceps placed 5cm into the vagina. Biopsy samples were taken 2 weeks
378 prior to SP exposure and 24 hours post-exposure. Samples were transported on ice. Biopsy
379 tissues were washed in RPMI and incubated in digestion buffer (0.1mg/mL collagenase and
380 1.5U/mL DNase in RF10) at 37°C for two hours. The buffer and remaining tissue samples
381 were passed through a 70μm filter and washed in RPMI 1640 media (Life Technologies,
382 Grand Island, New York, USA) supplemented with 10% FCS, penicillin, streptomycin, and l-
383 glutamine (RF10). The resulting cell pellet was resuspended in RF10 and passed through a
384 30μm cell filter, then processed immediately for antibody staining and flow cytometry.

385

386 *Cell culture and stimulation for T cell immunology:* PBMC were isolated by Ficoll gradient
387 separation (GE Healthcare, Madison, Wisconsin, USA) and cultured in RF10. Where

388 described, SP (either pooled or individual) was added to cell culture at a final concentration
389 of 1% for three, five or 16 hours. Identical SP exposure protocols were used for human and
390 macaque PBMC experiments. In some experiments, neutralizing antibodies against RANTES
391 (MAB678), MIP-1α (MAB270), MIP-1β (MAB271) (R&D Systems, Minneapolis, Minnesota,
392 USA) or an IgG isotype control (MOPC-21) (BioLegend, San Diego, California, USA) were
393 added to cell culture at a concentration of 2μg/mL. In one experiment, recombinant human
394 RANTES protein (Peprotech) was added to cell culture at 3ng/mL.
395
396 *T cell isolations:* $CD3^+$ T cells were isolated from whole PBMC by negative selection using an
397 EasySep Human T cell Isolation kit according to the manufacturer's protocol (StemCell
398 Technologies, Vancouver, British Columbia, Canada). Isolation purity was confirmed by flow
399 cytometry and ranged from 94.0% to 98.5%.
400
401 *RANTES ELISA:* To determine the concentration of RANTES in cell culture, the Human
402 CCL5/RANTES Quantikine® ELISA kit (R&D Systems) was used according to the
403 manufacturer's instructions. Briefly, 1.0 x 10^6 PBMC were stimulated for 16 h at 37°C with
404 1% v/v pooled seminal plasma and supernatants were collected by centrifugation at 500 *g*, 5
405 minutes. Supernatants were diluted 1:10 in calibrator diluent (or used neat; unstimulated
406 control) and added to the anti-human RANTES coated microplate for two hours. Plates were
407 then washed three times in wash buffer and incubated with the HRP-conjugated anti-human
408 RANTES antibody for one hour. Following three further wash steps, plates were developed
409 for 20 minutes with tetramethylbenzidine substrate solution, stopped with sulfuric acid and
410 absorbance measured at 450nm. Absorbance readings were averaged for each sample,
411 background subtracted and a five-parameter logistic (5-PL) curve fitted to the recombinant

412 RANTES standards using Prism 7. Concentrations were determined by interpolation of the
413 standard curve.
414
415 *Detection of CCR5 by flow cytometry:* For quantification of CCR5 expression levels, human
416 PBMC were washed and incubated with the following antibodies/dyes for 30 minutes at 4°C:
417 anti-CD3 BV786 or BV510 (clone SK7, BD Biosciences, San Jose, California, USA), anti-Vδ2 PE
418 (clone B6), anti-CD4 BV605 (clone RPA-T4), anti-CD8 BV650 (clone RPA-T8), anti-CD161 APC-
419 Fire750 (clone HP-3G10), anti-Vα7.2 BV510 (clone 3C10), anti-CCR5 BV421 (clone J418F1),
420 anti-CCR6 BV786 (clone G034E3) (all from BioLegend, San Diego, California, USA), and
421 fixable live/dead blue (Life Technologies, Grand Island, New York, USA). For PTM samples,
422 single cell suspensions from vaginal biopsies were stained with CD45 BUV395 (clone D058-
423 1283, BD Biosciences), CD3 Alexa488 (clone SP34-2, BD Biosciences), CD4 BV605 (clone
424 L200, BD Biosciences) and CCR5 BV421 (clone J418F1, Biolegend) as well as exclusion
425 markers for neutrophils, monocytes, B cells and NK cells (using anti-EpCam, CD66abce,
426 CD14, CD20 and NKG2A antibodies). In vitro PTM PBMC/SP exposure experiments were
427 assessed using the same CD3, CD4 and CCR5 antibody clones.
428 Samples were acquired on a BD LSRFortessa with FACS Diva. Flow cytometry data were
429 analysed in FlowJo (TreeStar, Inc., Ashland, Oregon, USA). Calculations of median
430 fluorescent intensity (MFI) were performed for cells gated to express the marker of interest
431 only.
432
433 *Cell sorting:* To sort $CCR5^-$ $CD4^+$ and $CD8^+$ T cells, freshly isolated PBMC were stained with
434 CD3 BV786, CD4 BV605, CD8 BV650, CCR5 BV421 and viability dye. Cells were sorted on a
435 BD FACS Aria III using a CCR5 FMO to set the CCR5 gate. Cells were cultured at $2x10^6$/mL for

16hrs 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 Ca^{2+} release: PBMC were cultured for three or five hours with (or five hours without) 1% pooled SP. Cells were then washed twice with PBS and loaded with 3µM Fluo4-AM + 0.02% Pluronic F-127 (Life Technologies, Grand Island, New York, USA) for 45 minutes at room temperature. Cells were washed twice with PBS and then stained with the following antibodies: anti-CD3 PerCP-Cy5.5 (clone SK7), anti-CD19 PE-CF594 (clone HIB19), and anti-CD56 PE-Cy7 (clone NCAM16.2) (all from BD Biosciences, San Jose, California, USA). 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 seconds, at which time anti-CD16 mAb (clone 3G8) was added to the cells at 5µg/mL. A second baseline reading was collected for 30 seconds, at which time goat anti-mouse IgG F(ab')2 (Sigma) was added to the cells at 10µg/mL to stimulate calcium flux through CD16. Stimulation with ionomycin was used as a positive control. Fluorescent readings were taken for a total of four minutes. Calcium mobilization in the $CD3^{-}CD19^{-}CD56^{dim}$ NK cell population was analysed using the 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 calculated for the initial baseline reading (30 seconds) and subtracted from the AUC for the length of the stimulation after addition of goat anti-mouse IgG (to give a "stimulation-induced AUC"). The stimulation-induced AUC value for the SP-treated samples was then expressed as a percentage of the stimulation-induced AUC for the untreated sample.

NK cell activation assays: A previously reported protocol, modified to incorporate staining for MIP-1α and MIP-1β production, was utilized to assess NK cell activation(9). Briefly, PBMC ($1x10^6$) were incubated with 3μg/mL HIV-1_{bal} gp120-coated or uncoated CEM.NKr-CCR5 cells (both from NIH AIDS Reagent Program) ($1x10^5$) at a 10:1 ratio in the presence or absence of a 1:1000 dilution of HIVIG (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 five hours at 37°C in the presence of 5μg/mL 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 formaldehyde, washed and incubated in permeabilization buffer (BD). Cells were then stained with Alexa Fluor 700-conjugated anti-IFNγ (clone B27, BD Biosciences), APC-conjugated anti-MIP-1α (clone REA257, Miltenyi Biotech) and FITC-conjugated anti-MIP-1β (clone D21-1351, BD Biosciences) antibodies. Samples were then washed, fixed and acquired with a BD LSRFortessa flow cytometer and analysed with FlowJo software (Tree Star Inc.).

Statistical analysis: Data were analysed in GraphPad Prism, version 7 (GraphPad Software, La Jolla, California, USA). Paired data was analysed using two-tailed non-parametric Wilcoxon tests or a Friedman test with Dunn's multiple comparisons post-tests. P values of less than 0.05 were considered statistically significant.

Acknowledgements:

The authors would like to thank the PBMC and semen donors for their involvement in the study. The authors would like to acknowledge the Opposites Attract Study site investigators and sites participating in the Semen Sub-Study: Nittaya Phanuphak (Thai Red Cross AIDS Research Centre, Bangkok, Thailand); Beatriz Grinsztejn (Evandro Chagas Institute of Clinical Research, Rio de Janeiro, Brazil); Catherine Pell (Taylor Square Private Clinic, Sydney, Australia); Mark Bloch (Holdsworth House Medical Practice, Sydney, Australia); David Baker (East Sydney Doctors, Sydney, Australia); David J Templeton (RPA Sexual Health, Sydney, Australia); Anna M McNulty (Sydney Sexual Health Centre, Sydney, Australia); and David A Cooper (St Vincent's Hospital, Sydney, Australia). The work was funded by a project grant from the National Health and Medical Research Council (NHMRC) of Australia to MSP. Funding for the Opposites Attract Study was received from the National Health and Medical Research Council, Australia; amfAR, Foundation for AIDS Research, USA; ViiV Healthcare, UK; and Gilead Sciences, USA. JAJ and SJK are funded by NHMRC fellowships. SRL is an NHMRC practitioner fellow.

Figure legends

Figure 1. Impact of SP on lymphocyte viability during cell culture. Cells were cultured with 1% final concentration of SP for five or 16 hours in RF10 media. (A) Representative plots of bulk PBMC forward scatter (FSC) /side scatter (SSC) characteristics and viability after 16 hours of culture in either RF10 alone or RF10 + 1% SP. Cells were gated based on FSC-area versus SSC-area to identify lymphocytes. Singlets were identified by FSC-area versus height. $CD3^+$ viability dye^- (live) cells were gated for further downstream analysis. (B) Quantification of T cell viability in bulk PBMC cultures after five or 16 hours. After gating on lymphocytes and singlets, total $CD3^+$ were gated, and the proportion of viability dye^- (live) cells was quantified. (C) PBMC were cultured for 16 hours in the presence or absence of SP pool and the frequency of $CD3^+V\delta2\ TCR^+$, $CD3^+V\delta2\ TCR^-Va7.2^+CD161^{++}$ (MAIT), $CD4^+$ and $CD8^+$ T cells were quantified. (D) Representative viability dye staining of isolated T cell cultures. $CD3^+$ cells were gated among the lymphocyte population, and subsequently gated as viability dye^+ (dead) or viability dye^- (live). (E) Quantification of the proportion of live cells after 16 hours of culture of isolated T cells in RF10 media alone or RF10 + 1% SP.

Figure 2. Reduction of CCR5 expression on non-conventional T cells following seminal plasma exposure. (A) Gating strategy used to identify and compare CCR5 expression on conventional and non-conventional T cell populations. Lymphocytes were gated based on FSC-A and FSC-H to identify single cells, followed by gating on viable $CD3^+$ T cells. $V\delta2^+$ gamma delta T cells were identified based on Vδ2 TCR expression. MAIT cells were identified as $V\delta2^-$ cells with a $V\alpha7.2^+CD161^{++}$ phenotype. $V\delta2^-$ non-MAIT conventional T cells were gated for CD4 and CD8 co-receptor expression. (B) Comparison of CCR5 expression levels among $V\delta2^+$, MAIT, CD4 and CD8 T cell populations. Gates for CCR5 expression were

520 determined based on fluorescence minus one (FMO) controls. The proportion of CCR5$^+$ cells
521 within each population and the median fluorescent intensity (MFI) of CCR5$^+$ cells is
522 expressed in the upper left corner of each plot. (C) Representative histograms of Vδ2$^+$ and
523 MAIT cell CCR5 expression following five hour or 16 hour exposure to 1% pooled seminal
524 plasma (SP) in whole PBMC culture compared to an untreated control. (D) The MFI of CCR5
525 on CCR5$^+$ Vδ2$^+$cells (black) or MAIT cells (red) following 1% SP treatment was determined as
526 a percentage of the MFI of the untreated control at each timepoint (n=5). Differences
527 compared to the untreated control assessed by Friedman test with Dunn's post-test,
528 *p<0.05, **p<0.01. (E) Histograms of Vδ2$^+$ and MAIT cell CCR5 expression following 16
529 hours of exposure to either the SP pool (SP pool) or individual HIV-1-uninfected SP samples
530 (SP1-5). (F) Quantification of the change in proportion of CCR5$^+$ Vδ2$^+$cells (black) or MAIT
531 cells (red) following 1% SP treatment compared to untreated controls at each timepoint
532 (n=5). Differences compared to the untreated control assessed by Friedman test with
533 Dunn's post-test, **p<0.01.
534
535 **Figure 3. Modulation of CCR5 expression on conventional T cells by seminal plasma** (A)
536 Representative plots demonstrating a reduction in CCR5 MFI on CCR5$^+$ cells (indicated in the
537 upper right hand of the CCR5 gate) among both CD4$^+$ and CD8$^+$ T cells following 16 hours of
538 seminal plasma (SP) pool exposure. (B) Exposure of whole PBMC to 1% SP pool for 16 hours
539 results in a significant decrease in CCR5 MFI on CCR5$^+$ CD4$^+$ (black) and CD8$^+$ (blue) T cells
540 (n=5). Differences compared to the untreated control assessed by Friedman test with
541 Dunn's post-test, *p<0.05, **p<0.01. (C) CCR5 MFI following 16 hours of exposure to either
542 the SP pool or individual HIV-1-uninfected SP samples (SP1-5) in a representative PBMC
543 donor. (D) Representative plots of CCR5 expression on CD4$^+$ T cells following 16 hours of

544 exposure to 1% SP pool or an individual SP sample. Arrows indicate population of expanded
545 CCR5^{low} cells compared to untreated control. (E) Quantification of CCR5^{bright} and CCR5^{low}
546 cells among CD4^{+} T cells after 16 hours of culture (n=7). Statistics assessed by Wilcoxon test.
547 (F) Quantification of CCR6 expression on CCR5^{low} CD4^{+} T cells in the presence or absence of
548 1% SP pool (n=7). Statistics assessed by Wilcoxon test. (G) CCR5 and CCR6 co-expression on
549 CD4^{+} T cells following 1% SP exposure, and quantification of the proportion of CCR5^{+}CCR6^{+}
550 cells within the bulk CD4^{+} T cell population in the presence or absence of SP (n=7). Statistics
551 assessed by Wilcoxon test.
552
553 **Figure 4. Impact of SP on isolated T cell CCR5 expression.** Bulk T cells were isolated from
554 PBMC and exposed to 1% SP pool for five or 16 hours. (A) Quantification of CCR5 MFI on
555 CCR5^{+} Vδ2^{+} (black) and MAIT (red), or (B) CD4^{+} (black) and CD8^{+} (blue) T cells following SP
556 exposure (n=6). Differences assessed by Friedman test with Dunn's post-test, $p<0.05$. (C)
557 Quantification of the proportion of CCR5^{bright} and CCR5^{low} cells among the CD4^{+} and CD8^{+} T
558 cell populations after 16 hours of SP exposure (n=6). Statistics assessed by Wilcoxon test.
559
560 **Figure 5. Role of SP-induced RANTES in mediating CCR5 downregulation.** (A) PBMC were
561 exposed to 1% SP pool for 16 hours (n=3 PBMC donors). Cell culture supernatants were
562 collected and RANTES expression was quantified by ELISA. Each sample was run in duplicate.
563 (B) PBMC were cultured for 30 or 60min with 3ng/mL recombinant RANTES. Plots show the
564 change in CCR5 MFI for Vδ2^{+} and MAIT cells or the change in proportion of CCR5^{bright} cells
565 for CD4^{+} and CD8^{+} T cell populations n=6 PBMC donors. Statistics assessed by Friedman test
566 with Dunn's post-test. *$p<0.05$, **$p<0.01$. (C) Representative histograms and (D)
567 quantification of CCR5 MFI on Vδ2^{+} and MAIT cells in isolated T cell cultures treated with SP

and either a neutralizing anti-RANTES antibody (2µg/mL) or an IgG isotype control (n=6 PBMC donors). Differences assessed by Wilcoxon test. (E) Representative plots of $CD4^+$ T cell populations segregated based on surface density of CCR5 following 16 hours of exposure to 1% SP pool, with anti-RANTES neutralizing antibody (2µg/mL) or isotype control. (F) Analysis of the proportion of $CCR5^{bright}$ or $CCR5^{low}$ $CD4^+$ and (G) $CD8^+$ T cells cultured in the presence of SP and a neutralizing anti-RANTES antibody, expressed as a percentage of the IgG isotype control (n=6 PBMC donors). Bars indicate median, with IQR. Differences assessed by Wilcoxon test. (H) $CCR5^-$ $CD4^+$ and $CD8^+$ T cells were sorted from freshly isolated PBMC and cultured in the presence or absence of 1% SP 3 for 16hrs. Plots indicate *de novo* CCR5 expression following cell culture. Data is representative of two PBMC donors.

Figure 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, then $CD3^-CD56^{dim}$ cells. Red arrows indicate the time of 3G8 or cross-linking $F(ab')_2$ additions (at 30 and 60 seconds). (B) Representative plot of Fluo4-AM MFI over time in $CD3^-CD19^-CD56^{dim}$ NK cells. Dashed lines indicate the time of 3G8 or cross-linking $F(ab')_2$ additions (at 30 and 60 seconds). Calcium flux in cells that were cultured for five hours in the absence of seminal plasma (SP) (black line) was compared to the flux elicited in cells that were cultured for five hours but exposed to an individual SP sample for three (blue line) or five (red line) hours. The plot is representative of cells exposed to either individual SP samples or the SP pool. (C) The 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 assessed by Friedman test with Dunn's multiple comparison post-test. (D) Gating strategy to assess NK cell function following stimulation with gp120-

592 coated CEM.NKr cells and HIVIG. Lymphocytes were gated on FSC-A vs FSC-H to identify
593 singlets, and NK cells were defined as $CD3^{-}CD56^{dim}$ cells. Incubation of PBMC with gp120-
594 coated CEM.NKr cells results in minimal background expression of IFNγ, CD107a and MIP-
595 1α/MIP-1β. Addition of HIVIG results in NK cell recognition of CEM.NKr target cells and
596 induces expression of IFNγ, CD107a and MIP-1α/MIP-1β, which is inhibited in the presence
597 of seminal plasma (SP). (E) Addition of 1% SP to the PBMC/CEM.NKr/antibody co-culture
598 results in a significant inhibition of IFNγ, CD107a and MIP-1α/MIP-1β expression by NK cells
599 (n=10 PBMC donors). (F) The SP-induced percent inhibition of IFNγ and CD107a expression is
600 significantly greater than the inhibition observed for MIP-1α/MIP-1β (n=10 PBMC donors).
601 Differences assessed by Wilcoxon test or Friedman test with Dunn's multiple comparisons
602 post-test.
603
604 **Figure 7. Response of macaque T cells to SP exposure *in vitro* and *in vivo*.** (A)
605 Representative staining of $CD4^{+}$ T cell CCR5 expression and CCR5/CCR6 co-expression
606 following exposure of macaque PBMC to 1% SP #3 for 16 hours. (B) Quantification of CCR5
607 MFI on $CCR5^{+}$ $CD4^{+}$ T cells and proportion of $CCR5^{dim}$ $CD4^{+}$ T cells following 16 hour SP
608 exposure (n=5). (C) Vaginal biopsies were collected from five female macaques at baseline.
609 Two weeks later, animals were vaginally exposed to 2.5mL of seminal plasma, and a second
610 biopsy was collected 24 hours later. $CD4^{+}$ T cells (identified as live, $CD45^{+}EpCam^{-}CD20^{-}CD14^{-}$
611 $CD3^{+}CD4^{+}$ lymphocytes) were assessed for expression of CCR5 and CCR5 surface density
612 (MFI). Plots are representative of two animals with low baseline CCR5 expression.
613
614

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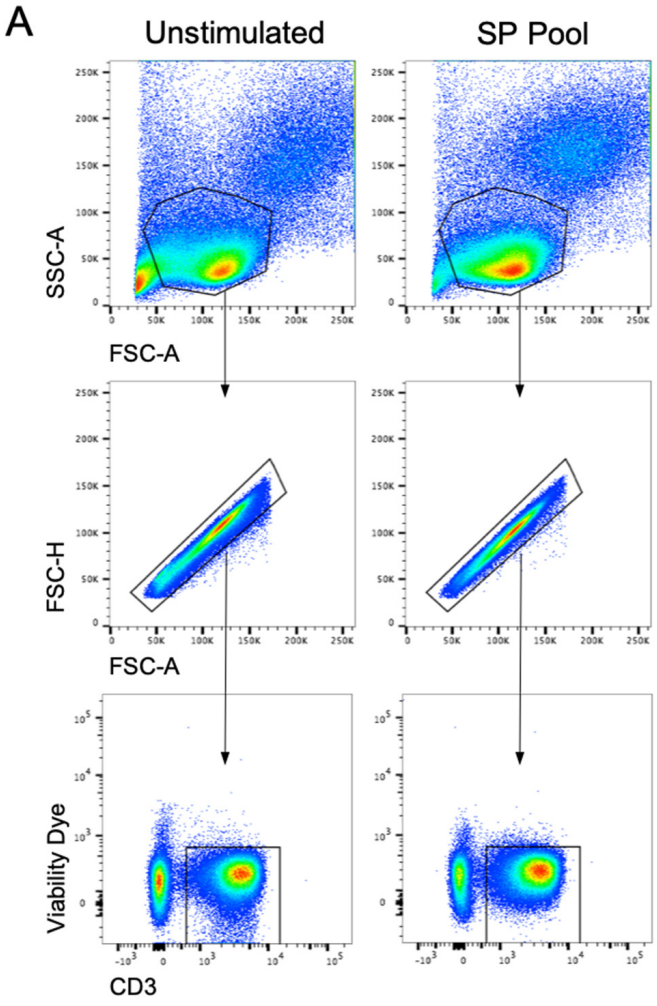
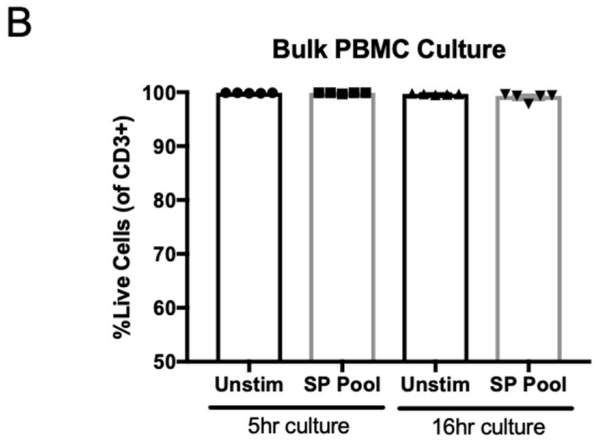
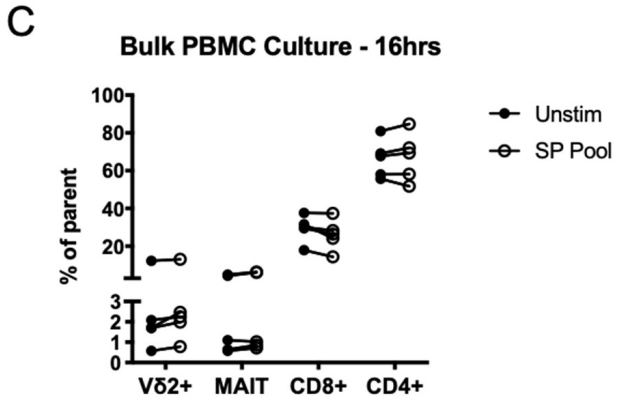
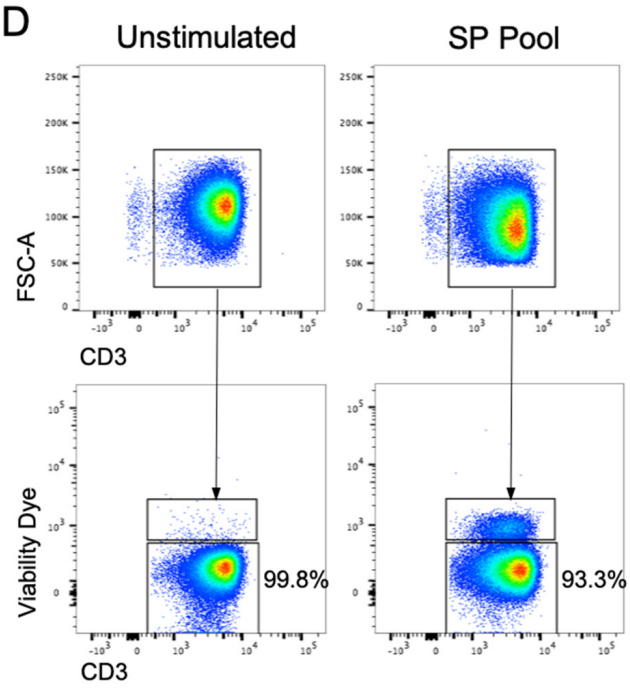
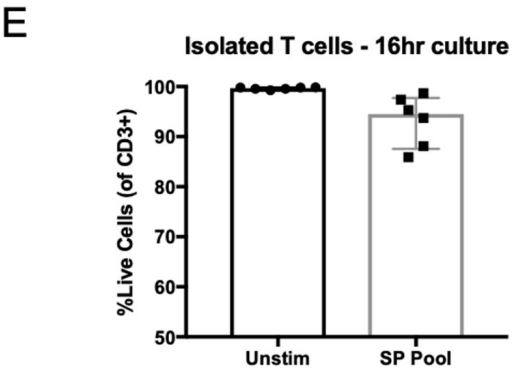










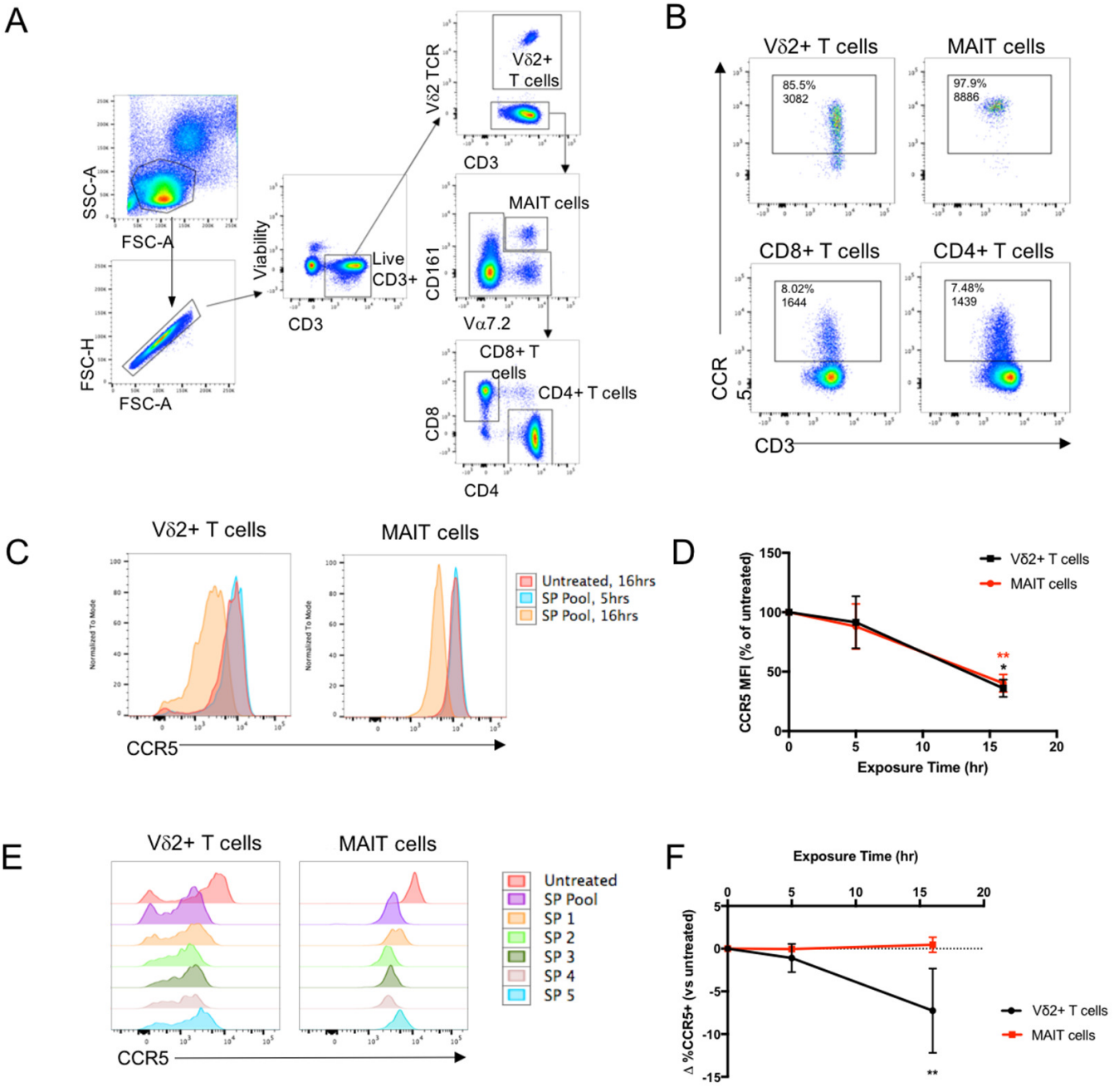


Figure 2

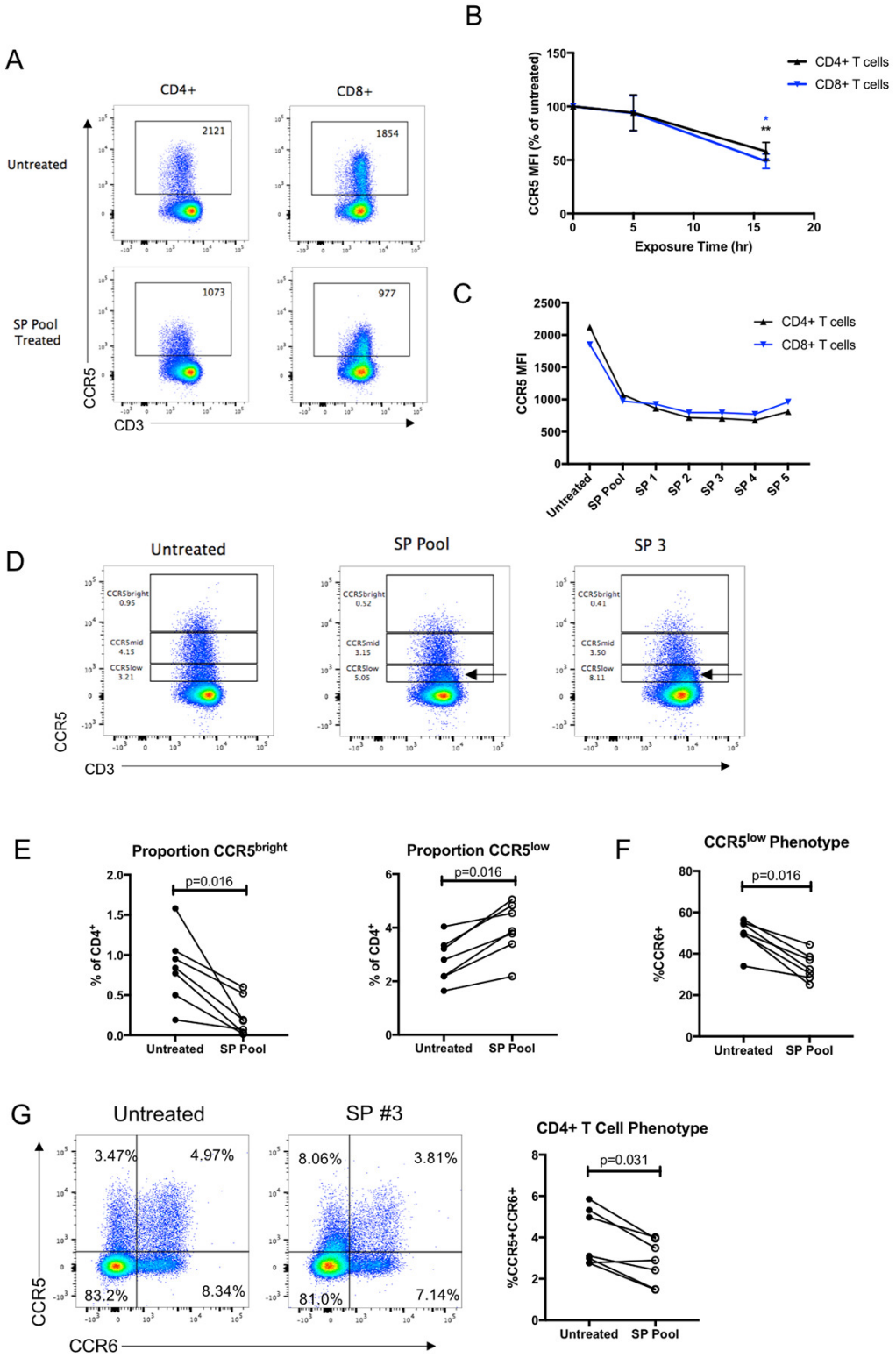


Figure 3

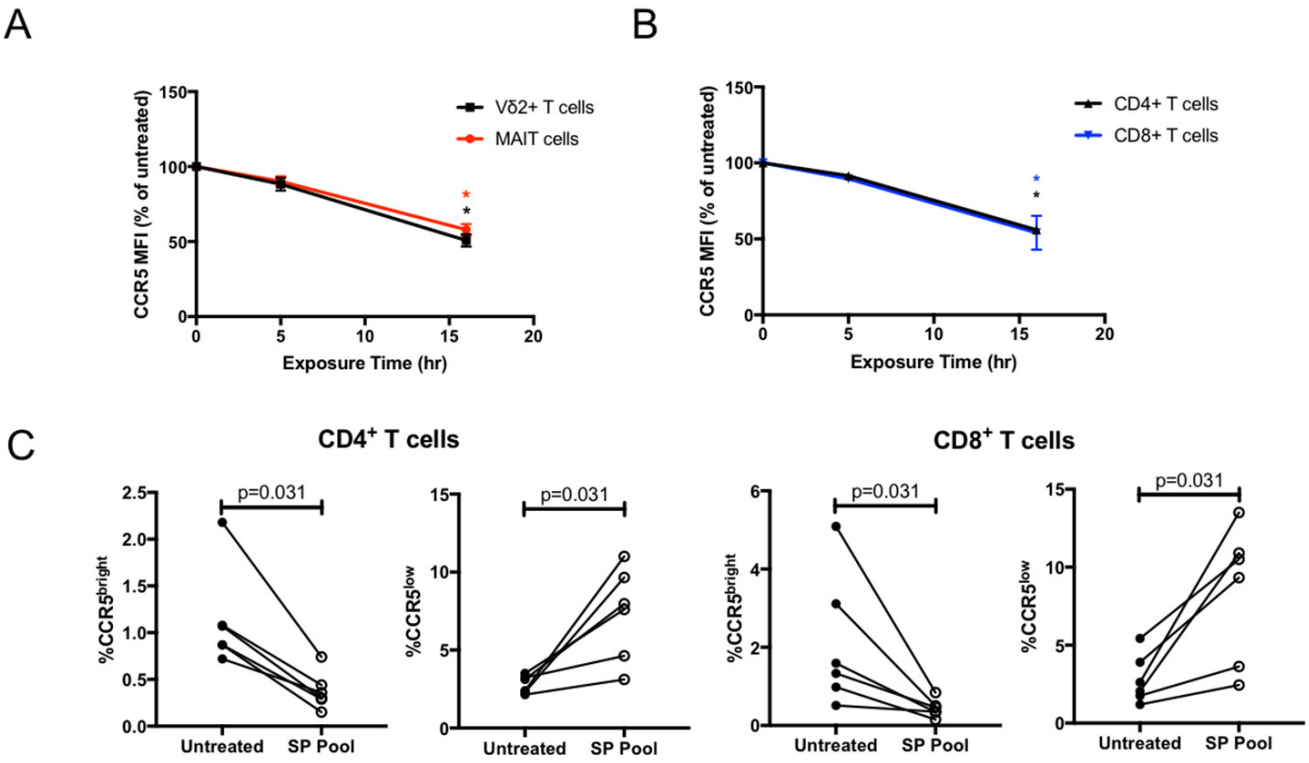


Figure 4

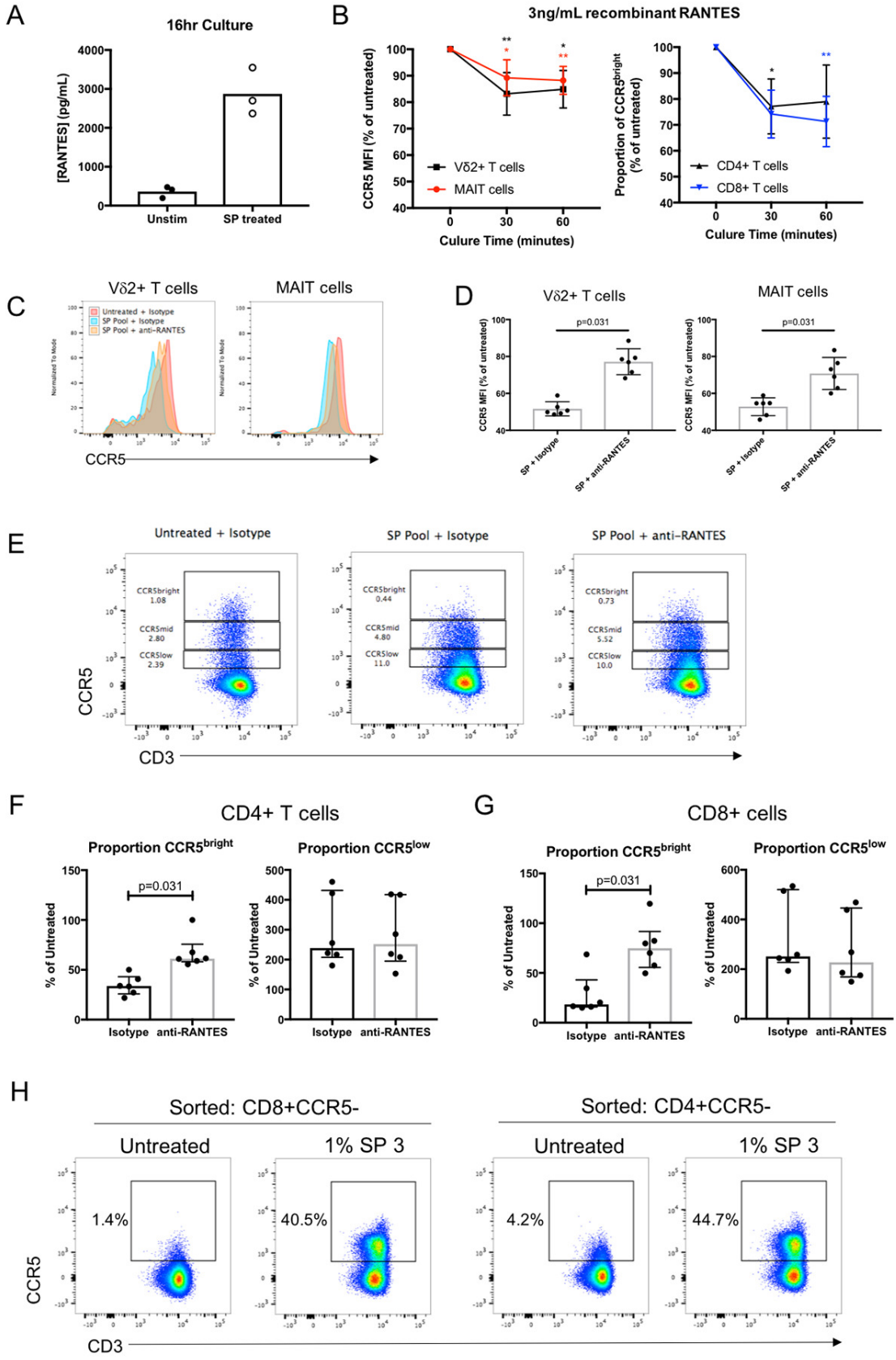
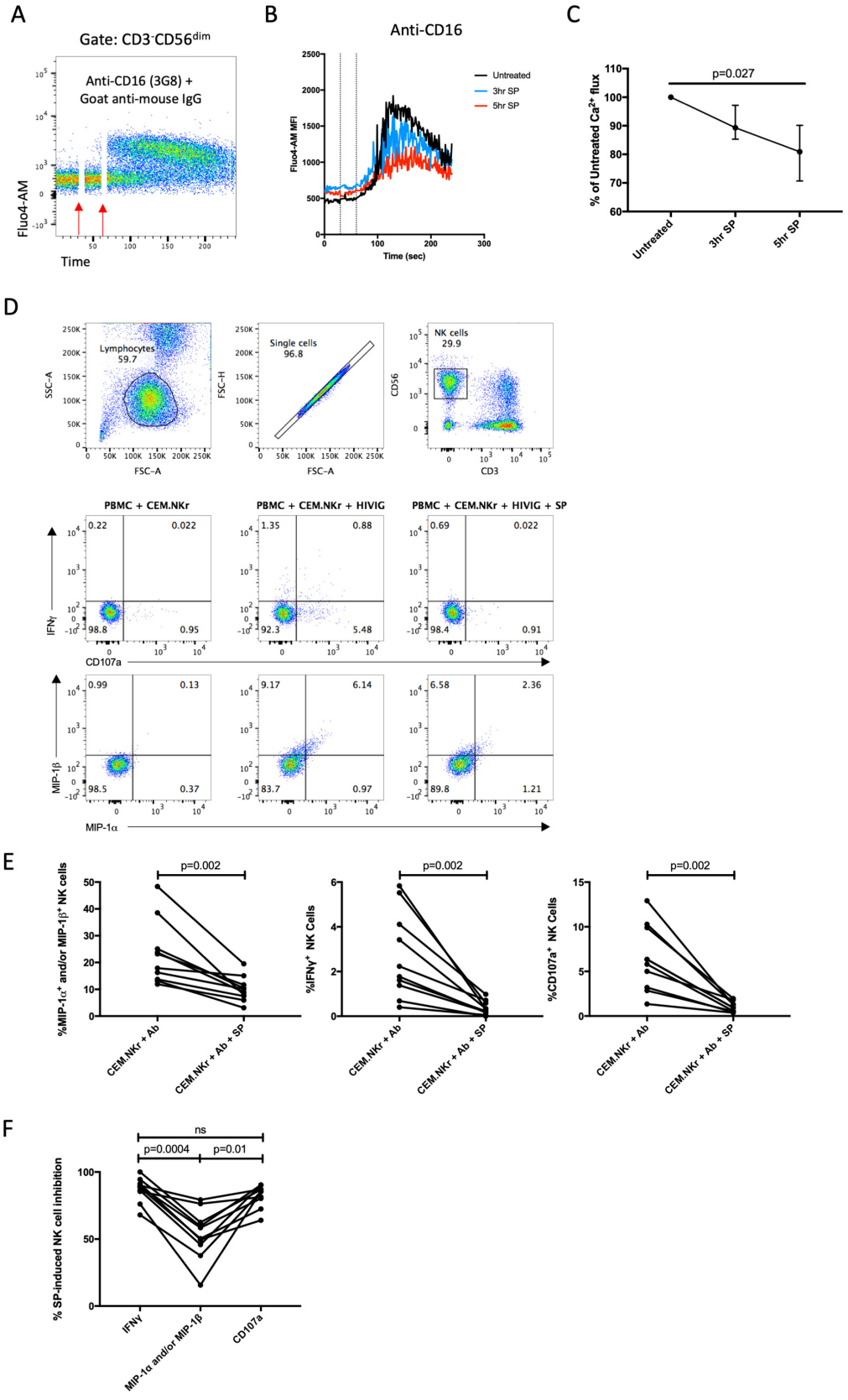


Figure 5



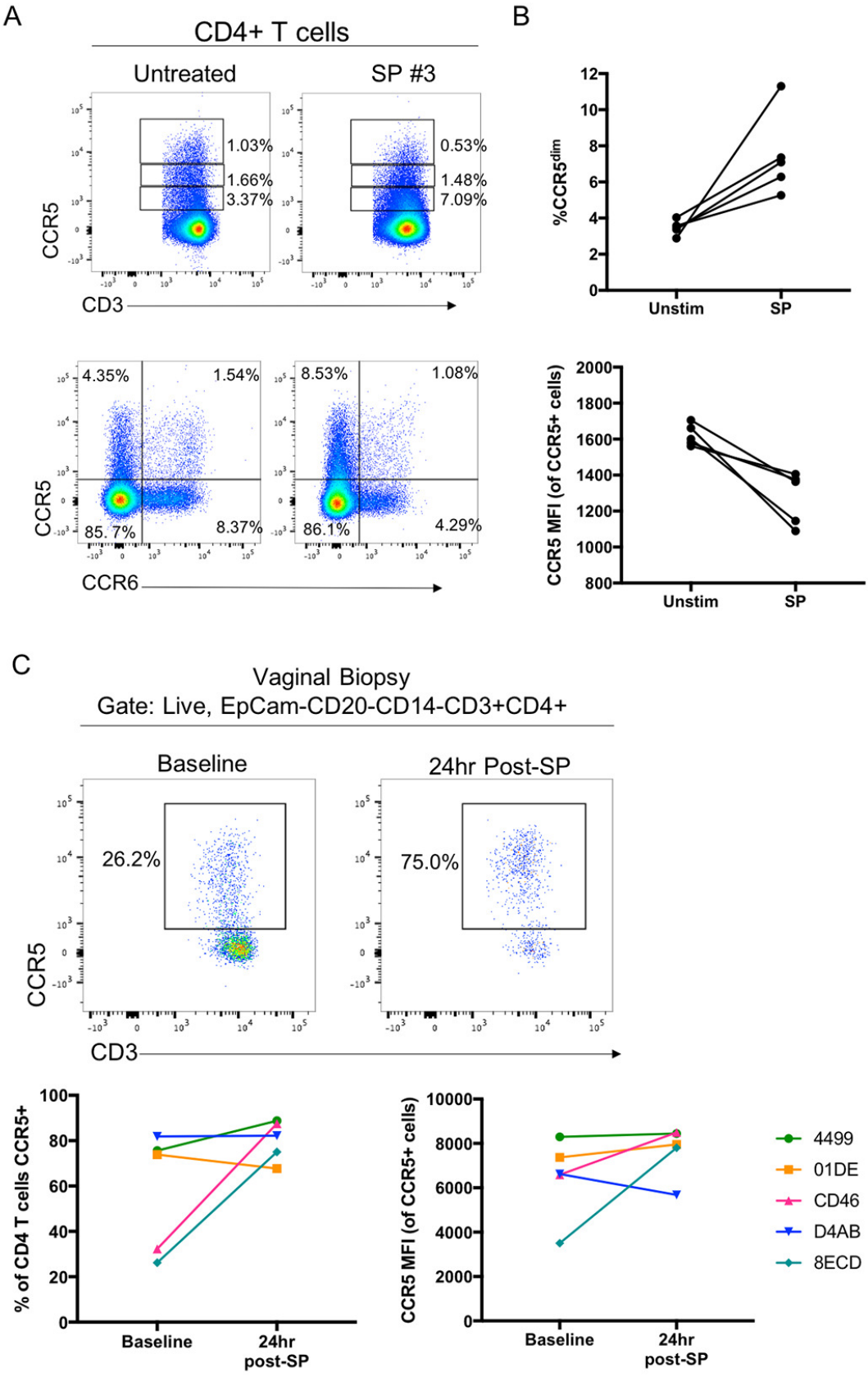


Figure 7