Modulation of innate and adaptive cellular immunity relevant to HIV-1 vaccine design by seminal plasma

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Objectives: Mucosal exposure to HIV-1 infection generally occurs in the presence of semen. Immunomodulation by seminal plasma is well described in the reproductive biology literature. Little is known, however, about the impact of seminal plasma on innate and adaptive anti-HIV-1 cellular immunity.

Design: The study investigated the effects of seminal plasma on immune responses considered important for prophylactic HIV-1 vaccine development, namely innate and adaptive cellular immunity mediated by natural killer (NK) cells and T cells, respectively.

Methods: The ability of seminal plasma to modulate direct, antibody-dependent and cytokine-stimulated NK cell activation was assessed utilizing intracellular cytokine staining. Direct and antibody-dependent cellular cytotoxicity was assessed using lactate dehydrogenase release assays. The effects of seminal plasma on T-cell activation upon stimulation with staphylococcus enterotoxin B or HIV-1 Gag peptides were assessed by intracellular cytokine staining. The impact of seminal plasma on redirected cytolysis mediated by T cells was measured using lactate dehydrogenase release assays.

Results: Both direct and antibody-dependent NK cell activation were dramatically impaired by the presence of either HIV-1-uninfected or HIV-1-infected seminal plasma in a dose-dependent manner. Additionally, seminal plasma suppressed both direct and antibody-dependent NK cell-mediated cytolysis, including anti-HIV-1 antibody-dependent cytolysis of gp120-pulsed CEM.NKr-CCR5 cells. Finally, seminal plasma attenuated both HIV-1 Gag-specific and staphylococcus enterotoxin B-induced CTL activation.

Conclusions: Semen contains potent immunosuppressors of both NK cell and $CD8^+$ T-cell-mediated anti-HIV-1 immune responses. This could impede attempts to provide vaccine-induced immunity to HIV-1.

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Introduction

A prophylactic vaccine is needed to curb the ongoing HIV-1 epidemic. Most HIV-1 transmissions occur via sexual exposure at the mucosa, where it is thought combinations of humoral and cellular immune responses could provide sterilizing immunity. Indeed, in nonhuman primate (NHP) models, passive delivery of broadly neutralizing antibodies or induction of $CD8^+$ T lymphocytes (CTLs) via vaccination confers protection against chimeric simian human immunodeficiency virus challenges or can control simian immunodeficiency virus infections, respectively [1–3]. Additional evidence from live-attenuated vaccine trials in NHP suggests induction

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of non-neutralizing antibodies can provide protection from challenge with pathogenic viruses through the ability of such antibodies to trigger cellular functions via interactions with constant region receptors (FcRs) [4,5]. Despite the generation of promising data in NHP models, success in human HIV-1 vaccine clinical trials has been limited [6–10]. Most notable is the modest efficacy of the RV144 trial, which provided partial protection from infection and highlighted a potential role for non-neutralizing antibodies targeting the V1V2 region as correlates of protection [11].

Mucosal exposure to HIV-1 almost always occurs in the presence of semen. Seminal plasma has been known for decades to be a potent immunosuppressant, impairing a wide array of primary human immune cells including T-lymphocytes, natural killer (NK) cells, and macrophages [12]. This has been well described in the reproductive biology literature, which has highlighted seminal plasma-induced immunomodulation and its importance for male fertility and promoting impregnation [13–15]. By extension, immunomodulation by seminal plasma could theoretically alter anti-HIV-1 immune responses within mucosal sites of exposure [16–18]. Interestingly, we have previously observed that anti-HIV-1 antibody-dependent NK cell activation induced by HIV-1-positive whole seminal plasma was weaker than that induced by dose-equivalent IgG purified from the same seminal plasma, suggesting seminal plasma may inhibit antibody-dependent NK cell activation [19].

Given the potential for seminal plasma to modulate immune responses, we investigated the ability of seminal plasma to restrain an array of anti-HIV-1 cellular immune responses. We utilized assays measuring direct or antibody-dependent NK cell activation or cytolysis, as well as assays to measure the activation and cytolytic functions of CTL. We provide robust evidence of the ability of seminal plasma to suppress key anti-HIV-1 immune responses.

Materials and methods

Participants

Whole blood was collected from six HIV-1-infected study participants recruited from the Melbourne Sexual Health Centre. Five of these patients were on antiretroviral therapy and exhibited suppressed virus (<20 RNA copies/ml), whereas one patient was untreated at the time of sampling and had a viral load of 184 000 RNA copies/ml. The six study participants had a mean $CD4^+$ T-cell count of 493 cells/μl, ranging from 160–893 cells/μl. Whole blood was also collected from 14 HIV-1-uninfected controls, and peripheral blood mononuclear cells (PBMCs) were subsequently isolated by Ficoll gradient separation (GE Healthcare, Madison, Wisconsin, USA). Isolated PBMCs were then washed and resuspended in RF10 media (RPMI 1640, supplemented with 10% FCS, penicillin, streptomycin, and l-glutamine; Life Technologies, Grand Island, New York, USA). Four HIV-1-uninfected seminal plasma samples were purchased from BioreclamationIVT (Westbury, New York, USA), whereas three HIV-1-infected seminal plasma samples were obtained from the HIV STAR trial in Thailand [20]. Lastly, blood plasma from a single HIV-1-infected donor was obtained from a client of the Melbourne Sexual Health Centre to serve as a source of anti-HIV-1 antibodies for anti-HIV-1 NK cell activation assays. Informed consent was obtained before collection of all biological samples, and the ethics committees of all participating institutions approved the described studies.

Pulsing of CEM.NKr-CCR5 with gp120

CEM.NKr-CCR5 cells were labelled with 3 ug/ml HIV-1_{Bal} gp120 (Both obtained from the NIH AIDS Reagent Program) and incubated for 90 min at 4°C, as previously described [21]. Uncoated cells, used as controls, were also incubated for 90 min at 4°C. Subsequently, the cells were washed and resuspended in RF10.

Coating of 721.221 target cells with Rituximab

Major histocompatibility complex class I (MHC-I or HLA-I) deficient 721.221 B cells, a known target for anti-CD20-mediated NK cell activation and killing (a kind gift from Dr Andrew Brooks, Department of Microbiology and Immunology, University of Melbourne), were suspended at 1×10^6 cells/ml in RF10 and coated with 5 μg/ml of the anti-CD20 monoclonal antibody, Rituximab (RTX) (Roche, Dee Why, New South Wales, Australia) [22]. The cells were then incubated for 1 h at 4°C. Uncoated cells, used as controls or for direct activation of NK cells, were also incubated 1 h at 4°C in the absence of antibody. After incubation, the cells were washed and resuspended in RF10.

NK cell activation assays

To study NK cell activation through either direct or anti-CD20 antibody-dependent stimulation, 1×10^5 RTX-coated or uncoated 721.221 target cells were coincubated with 1×10^6 isolated PBMCs. HIV-1-infected or HIV-1-uninfected seminal plasma diluted in RF10 were added to the cells as required. Brefeldin A (5 μg/ml; Sigma-Aldrich, St. Louis, Missouri, USA) and 5 μg/ml monensin (BD Biosciences, East Rutherford, New Jersey, USA) were added to the cell suspension for a final volume of 100 μl. After a 5 h incubation at 37°C with 5% CO_2, cells were harvested then stained with anti-human CD3 PerCP (clone SK7; BD Biosciences, San Jose, California, USA) and anti-human CD56 PE Cy7 (clone NCAM16.2; BD Biosciences) for 30 min at room temperature in the dark. Cells were next fixed with 1% formaldehyde (Sigma-Aldrich) for 10 min before being permeabilized by 1 × FACS permeabilizing solution 2 for 10 min (BD Biosciences). Next, cells were stained for 1 h at room temperature with IFN-γ AF700 (clone B27; BD

Biosciences). Finally, cells were again fixed with 1% formaldehyde, and acquired by flow cytometry.

Similarly, to measure anti-HIV-1 antibody-dependent NK cell activation 1×10^5 gp120-coated CEM.NKr-CCR5 cells were incubated with 1×10^6 PBMC in the presence or absence of a 1 : 1000 dilution of HIV-1-infected plasma. To assess the ability of seminal plasma to modulate anti-HIV-1 antibody-dependent NK cell activation a range of dilutions of HIV-1-uninfected seminal plasma was added as required, with RF10 added to control wells instead. The remainder of the assay set up, incubation, processing and acquisition were completed as described above.

To study NK cell activation through cytokine stimulation, 1×10^6 isolated PBMCs were incubated with 10 ng/ml of IL-12 and 5 ng/ml of IL-15, in the presence or absence of dilutions of seminal plasma, at a final volume of 100 μl for 18 h at 37°C with 5% CO_2. Next, 5 μg/ml brefeldin A was added to the cells, which was followed by another 6 h incubation at 37°C with 5% CO_2. The cells were then harvested and stained for IFNγ expression as described above.

Cytotoxic T-lymphocyte activation assay

Briefly, 150 μl of fresh whole blood from six HIV-1-infected study participants attending the Melbourne Sexual Health Centre was incubated with 5 μg/ml brefeldin A and 5 μg/ml monensin in the presence of either the 5 μg/ml of a pool of overlapping 15mer HIV-1 consensus subtype B gag peptides(NIH AIDS Reagent Program), 5 μg/ml staphylococcal enterotoxin B (SEB; Sigma-Aldrich) or an equivalent volume of DMSO. Additionally, to assess the ability of seminal plasma to modulate CTL responses a 1 : 100 dilution of seminal plasma, or an equivalent volume of RF10, were added to arrive at a final volume of 200 μl. After a 5 h incubation at 37°C with 5% CO_2, cells were stained with anti-human CD3 PerCP (clone SP34–4; BD Biosciences) and anti-human CD8 PE-Cy7 (clone SK1; BD Bioscience) for 30 min at room temperature in the dark. Subsequently, cells were treated with 1 × lysing solution (BD Bioscience) for 10 min at room temperature, before being permeabilized by 1 × FACS permeabilizing solution 2 (BD Biosciences) for another 10 min. Lastly, cells were stained for 1 h at room temperature with IFN-γ AF700 (clone: b27; BD Biosciences), before being fixed with 1% formaldehyde, and acquired by flow cytometry.

Lactate dehydrogenase release assay

The LDH assay was conducted with the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega, Madison, Wisconsin, USA), as previously described [21]. To measure NK cell-mediated antibody-dependent and direct cytotoxicity, 1×10^4 Ab-coated or uncoated target cells (721.221 cells or gp120-labelled CEM.NKr-CCR5 cells) were co-incubated with 1×10^5 isolated PBMCs. To study CTL-mediated cytotoxicity, 1×10^4 P815 cells (a kind gift from Dr Andrew Brooks, Department of Microbiology and Immunology, University of Melbourne)were used as target cells in the presence of anti-CD3 antibody (clone OKT3; eBioscience), and coincubated with 1×10^5 isolated PBMCs. Background spontaneous LDH release of target and effector cells was assessed by incubation of each cell population alone, while maximum LDH release from target cells was assessed through the addition of lysis solution. Dilutions of seminal plasma were added to the respective wells as required, with RF10 added to the control wells instead, for a final volume of 100 μl. Plates were spun at 250*g* for 4 min then incubated for 4 h at 37°C. Following incubation, plates were spun at 250 g for 4 min, before 50 μl of supernatant was removed from each well and transferred to an ELISA plate (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Subsequently, 50 μl of substrate was added to each well, and the plate was incubated for 30 min in the dark at room temperature. Finally, 50 μl of stop solution was added to each well, and the absorbance was read at 492 nm. Observed cytotoxicity was calculated as such: % cytotoxicity = [(experimental−effector spontaneous−target spontaneous)/(target maximum−target spontaneous)] × 100.

Statistical analysis

All data analyses were conducted using Graphpad Prism version 6.0 (GraphPad Software Inc., La Jolla, California, USA). Paired data sets were compared with Wilcoxon matched pairs tests. Differences between the groups were considered significant when $P < 0.05$. Data throughout the article are presented in the [median (range)] format.

Results

Modulation of direct and antibody-dependent natural killer cell activation by seminal plasma

To ascertain the immunomodulatory impact of seminal plasma, we investigated the effect of seminal plasma on the ability of NK cells, a key lymphocyte of the innate immune system, to become activated either directly in response to foreign cells or via stimulation through the CD16 Fc receptor. We first studied NK cell responses to a HLA-I deficient B cell line (i.e., 721.221 cells), with or without coating of the target cells with RTX, an anti-CD20 antibody capable of triggering NK cell-mediated antibody-dependent cellular cytotoxicity (ADCC) responses. Seminal plasma collected from a HIV-1-uninfected donor was added at a final dilution of 1 : 100 to freshly isolated PBMCs from a single HIV-1-uninfected donor. This dilution has been previously used to simulate the concentration of seminal plasma present in the uterus following coitus [23]. The presence of seminal plasma reduced direct activation of NK cells against the uncoated 721.221 cells as quantified by intracellular staining for IFN-γ (i.e., from 2.04 to 0.37%, Fig. 1a). Seminal plasma

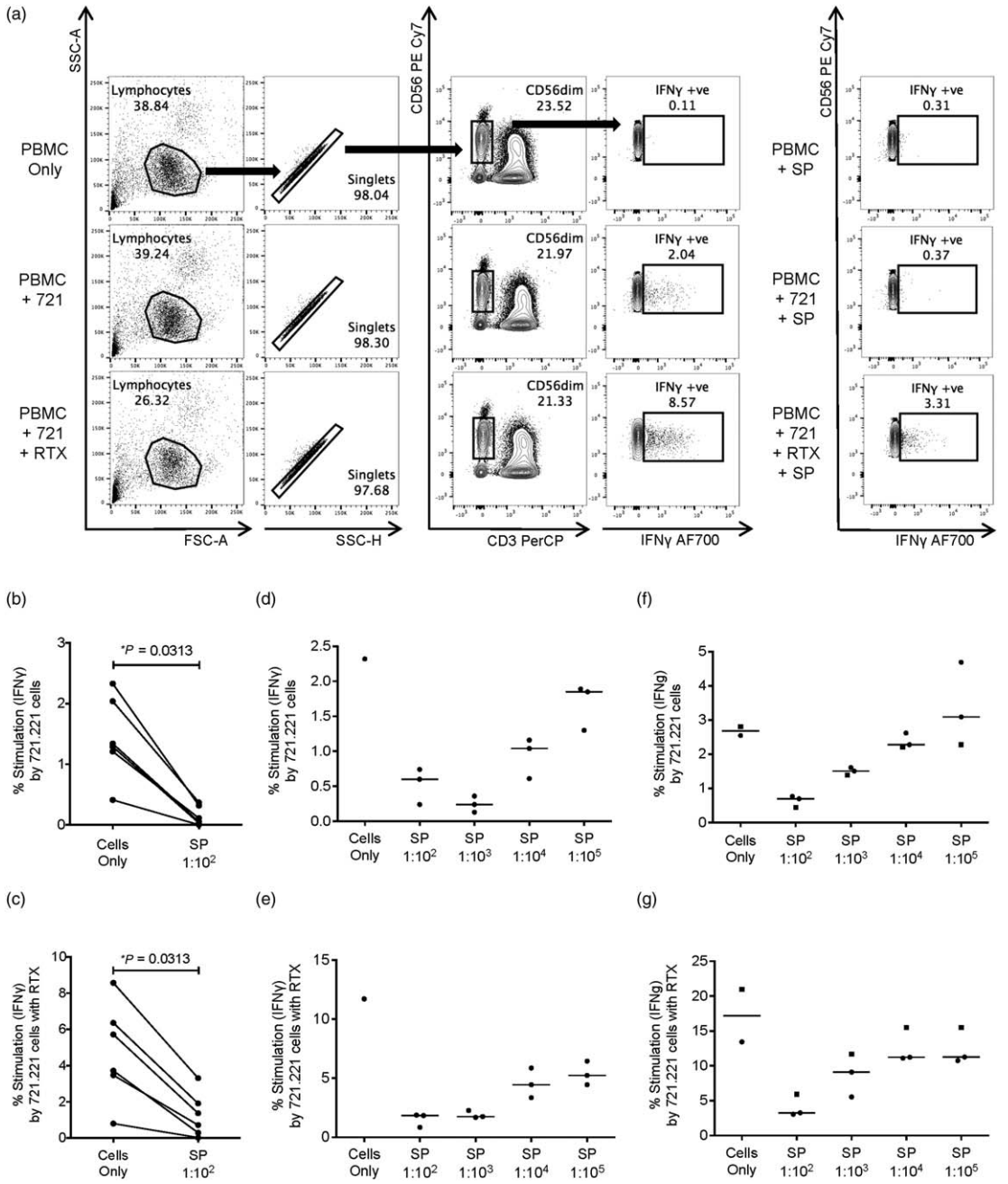

Fig. 1. Effects of seminal plasma on direct and antibody-dependent activation of NK cells. FACs, fluorescence-activated cell sorting; RTX, , Rituximab; SP, seminal plasma. (a) Freshly isolated PBMCs from an HIV-1-uninfected donor were incubated with either uncoated 721.221 cells or RTX-coated 721.221 cells in the presence or absence of a 1 : 100 final dilution of SP from an HIV-1-uninfected donor to assess its immunomodulatory effects. (a) FACs plots depict the gating strategy used to define $CD3^{-}CD56^{dim}$ NK cells, as well as to assess their activation (i.e., IFN-γ production). (b,c) Next, assays were expanded to include six HIV-1-uninfected PBMC donors. (b) The graph depicts the relative IFN-γ production by NK cells upon stimulation with 721.221 target cells in the presence and absence of SP. (c) The graph depicts the relative IFN-γ production by NK cells upon stimulation with RTX-coated 721.221 target cells in the presence and absence of SP. (d,e) Next, PBMC from a single HIV-1-uninfected donor were stimulated with uncoated or RTX-coated 721.221 target cells in the presence of a series of dilutions of SP from three HIV-uninfected donors. (d) The graph depicts the dose-dependent effects of SP upon direct NK cell activation. (e) The graph depicts the dose-dependent effects of SP upon antibody-dependent NK cell activation. (f,g) Similarly, PBMC from a two HIV-1-uninfected donors were stimulated with uncoated or RTX-coated 721.221 target cells in the presence of a series of dilutions of SP from three HIV-1-infected donors. (f) The graph depicts the dose-dependent effects of HIV-1-infected SP upon direct NK cell activation. (g) The graph depicts the dose-dependent effects of HIV-1-infected SP upon antibody-dependent NK cell activation. Statistical significance values were obtained using a nonparametric Wilcoxon matched pairs test.

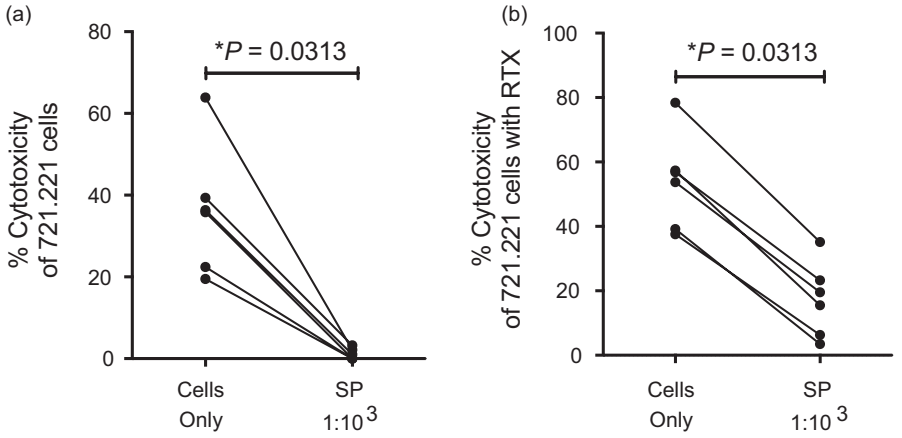

Fig. 2. Effect of seminal plasma on NK cell-mediated cytolysis. RTX, Rituximab; SP, seminal plasma. (a,b). Freshly isolated PBMCs from six HIV-1-uninfected donors were assessed for their ability to kill either uncoated 721.221 cells or RTX-coated 721.221 cells, in the presence or absence of a 1 : 1000 final dilution of SP from a single HIV-1-uninfected donor, using the LDH-release assay. (a) The graph depicts the relative cytolysis of uncoated 721.221 target cells in the presence and absence of SP. (b) The graph depicts the relative cytolysis of RTX-coated 721.221 target cells in the presence or absence of SP. Statistical significance levels were assessed using a nonparametric Wilcoxon matched pairs test.

also abrogated NK cell production of IFN-γ upon stimulation with RTX-coated 721.221 cells (i.e., from 8.57 to 3.31%, Fig. 1a). To verify that the observed inhibitions were not restricted to specific host–donor interactions, we screened multiple HIV-1-uninfected PBMC donors. Seminal plasma decreased IFN-γ production across six PBMC donors upon both direct stimulation with 721.221 target cells [1.32% (0.41–2.33%) vs. 0.08% (0.00–0.37%), $P = 0.031$; Fig. 1b] and RTX-coated 721.221 target cells [4.72% (0.80–8.57%) vs. 1.05% (0.03–3.31%), $P = 0.031$; Fig. 1c].

We next screened seminal plasma samples from three distinct HIV-1-uninfected donors to confirm that the ability of seminal plasma to attenuate NK cell activation is common across individuals. Notably, all three seminal plasmas inhibited both direct NK cell activation (Fig. 1d) and antibody-dependent NK cell activation (Fig. 1e) in a dose-dependent manner. To establish if HIV-1-positive seminal plasma would behave similarly, we repeated the assay with seminal plasma collected from three HIV-1 infected individuals against the PBMCs isolated from two healthy donors. Seminal plasma from all three HIV-1-infected donors inhibited direct activation of NK cells (Fig. 1f) and antibody-dependent NK cell activation (Fig. 1g) in a dose-dependent manner.

Modulation of direct and antibody-dependent cytolysis by seminal plasma

We next assessed if the presence of seminal plasma decreased NK cell cytolytic activity. Using a killing assay based on the release of LDH, we were able to quantify the levels of direct and antibody-dependent cytolysis of 721.221 target cells in the presence and absence of seminal plasma. We observed dramatic and statistically significant reductions in direct cytolysis when PBMCs from six donors were incubated with 721.221 target cells in the presence of seminal plasma (1 : 1000 final dilution) [36.07% (19.50–63.88%) vs. 0.50% (0.00–3.26%), $P = 0.031$; Fig. 2a]. The addition of seminal plasma (1 : 1000 final dilution) also triggered a statistically significant drop in ADCC activity against RTX-coated 721.221 cells [55.29% (37.57–78.39%) vs. 17.54 (3.48–35.16%), $P = 0.031$; Fig. 2b].

Modulation of cytokine-induced NK cell activation by seminal plasma

We then questioned if the observed suppression of NK cell activation by seminal plasma was restricted to stimulations requiring effector–target interactions. Freshly isolated PBMCs samples from six donors were stimulated with both IL-12 and IL-15 overnight in the presence or absence of seminal plasma. NK cells exposed to seminal plasma exhibited decreased IFN-γ production as detected by intracellular staining [39.41% (24.08–50.27%) vs. 11.72% (8.58–17.82%), $P = 0.031$; Fig. 3a]. This inhibitory effect was observed in a dose-dependent manner, as shown with three PBMC samples (Fig. 3b). These findings suggest that the inhibition by seminal factors of NK cell activation is not limited to that of effector–target interactions.

Modulation of anti-HIV-1 antibody-dependent natural killer cell activation by seminal plasma

The RV144 trial and live attenuated vaccine trials in primates suggest that ADCC is potentially important for HIV-1 vaccine design [4,11]. Thus, we sought to examine if the immunosuppressive properties of seminal plasma would influence the anti-HIV-1 immune response mounted by NK cells. HIV-1 negative seminal plasma

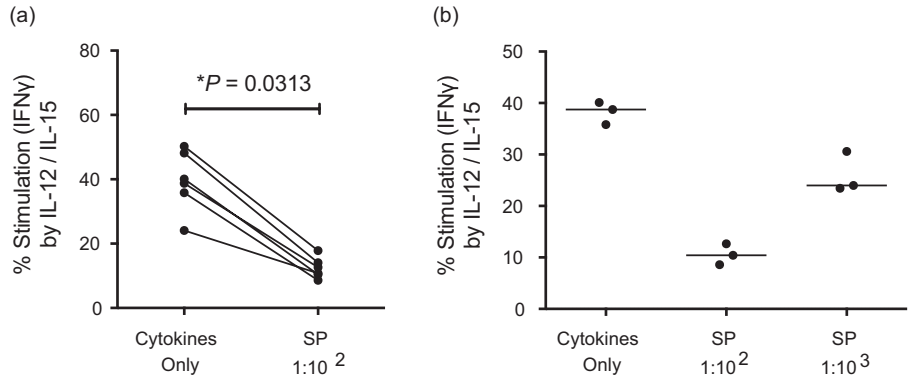

Fig. 3. Effects of seminal plasma on cytokine-induced activation of NK cells. SP, seminal plasma. (a) Freshly isolated PBMCs from six HIV-1-uninfected donors were stimulated overnight with a combination of IL-12 and IL-15 in the presence or absence of a 1 : 100 final dilution of SP from an HIV-1-uninfected donor. The graph depicts the relative IFN-γ production by NK cells, as measured by intracellular cytokine staining, upon stimulation with IL-12 and IL-15 in the presence and absence of seminal plasma. (b) Freshly isolated PBMCs from three HIV-1-uninfected donors were stimulated overnight with a combination of IL-12 and IL-15 alone and in the presence of two dilutions of SP (i.e. 1 : 100, 1 : 1000) from a single HIV-1-uninfected donor. The graph depicts the dose-dependent effects of seminal plasma upon cytokine-induced NK cell activation. Statistical significance levels were assessed using a nonparametric Wilcoxon matched pairs test.

was added to the coculture of freshly isolated PBMCs and gp120-labelled CEM.NKr-CCR5 cells in the presence of HIV-1-infected plasma. The presence of seminal plasma inhibited NK cell activation across multiple donors, as measured by levels of intracellular IFN-γ [5.35% (0.70–19.10%) vs 1.00% (0.03–2.60%), $P = 0.0313$; Fig. 4a]. This suppression of anti-HIV-1 antibody-dependent NK cell activation occurred in a dose-dependent fashion (Fig. 4b). Similarly, experiments assessing the ability of seminal plasma to inhibit anti-HIV-1 antibody-dependent activation of purified NK cells revealed robust inhibition (Data not shown). These results were corroborated by the attenuated ADCC of gp120-labelled CEM.NKr-CCR5 cells by seven PBMC samples in the presence of HIVIg and HIV-1 negative seminal plasma, as detected by released LDH [13.90% (8.18–35.75%) vs. 2.02% (0.00–6.77%), $P = 0.031$; Fig. 4c]. Taken together, our observations highlight the potency of

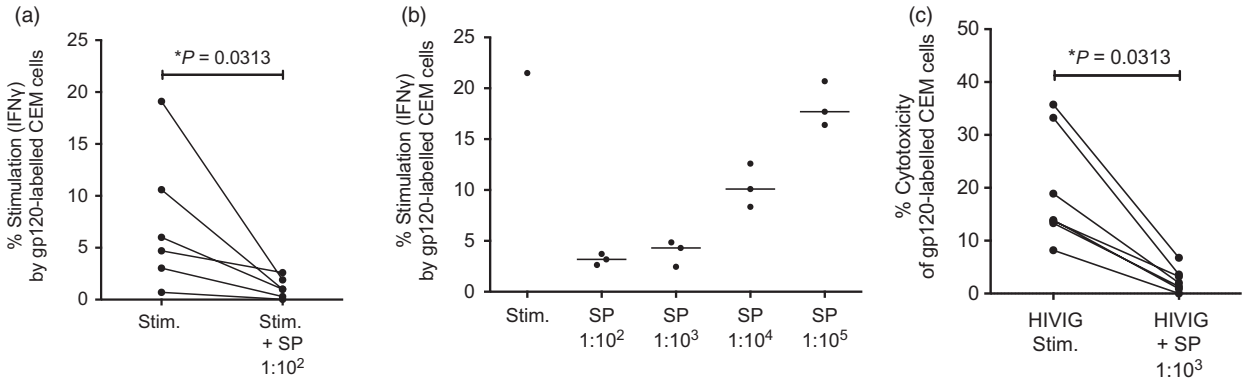

Fig. 4. Effects of seminal plasma on anti-HIV-1 antibody-dependent NK cell responses. SP, seminal plasma. (a) Freshly isolated PBMCs from six HIV-1-uninfected donors were incubated with gp120-coated CEM.NKr-CCR5 cells in the presence of a 1 : 1000 dilution of plasma from an HIV-1-infected donor. To assess its immunomodulatory effects, SP from an HIV-1-uninfected donor was added to the assay at a 1 : 100 final dilution. The graph depicts the relative activation of NK cells within PBMC upon stimulation in the presence and absence of SP. (b) PBMCs from a single HIV-1-uninfected donor were stimulated with gp120-coated CEM.NKr-CCR5 cells in the presence of a 1 : 1000 dilution of plasma from an HIV-1-infected donor and a series of dilutions of SP from three HIV-1-uninfected donors. The graph depicts the dose-dependent effects of SP upon anti-HIV-1 antibody-dependent activation of NK cells. (c) Anti-HIV-1 ADCC activity of PBMCs from seven HIV-1-uninfected donors, against gp120-coated CEM.NKr-CCR5 target cells in the presence of a 1 : 1000 dilution of HIVIG, was assessed in the presence and absence of a 1 : 1000 dilution of SP from an HIV-1-uninfected donor using the LDH-release assay. The graph depicts the relative anti-HIV-1 ADCC observed in the presence and absence of SP. Statistical significance values were obtained using a nonparametric Wilcoxon matched pairs test.

seminal plasma in hindering NK cell participation in the immune response against HIV-1 infection.

Modulation of anti-HIV-1 $CD8^+$ T lymphocytes responses by seminal plasma

Beyond NK cells, the reproductive biology literature shows seminal plasma to impair an array of primary human immune cells, including T lymphocytes [12]. To determine if seminal plasma suppresses anti-HIV-1 cellular immune responses other than those mediated by NK cells, we studied the effects of seminal plasma on CTLs. First, we investigated the effects of seminal plasma on $CD8^+$ CTL activation with the superantigen SEB. SEB was added to freshly isolated whole blood samples from six HIV-1-infected patients in the presence or absence of seminal plasma from a HIV-1-uninfected donor. The addition of seminal plasma resulted in a statistically significant reduction in CTL responses as measured by IFN-γ [5.08% (0.08–9.81%) vs. 1.53% (0.03–3.90%), $P=0.031$; Fig. 5a,b]. Eliciting broad, robust local HIV-1-specific CTL responses has been the strategy for several vaccines against HIV-1 infection. We next stimulated six HIV-1-positive whole blood samples with a set of overlapping 15-mer Gag peptides in the presence and absence of seminal plasma from a HIV-1-uninfected donor. All six study participants had detectable Gag-specific CTLs and a statistically significant reduction in IFN-γ producing $CD8^+$ T cells was detected following stimulation in the presence of seminal plasma [0.25% (0.07–0.69%) vs. 0.01% (0.01–0.11%), $P=0.031$; Fig. 5a and 5c]. We next ascertained if the presence of seminal plasma had an effect on T-cell-mediated cytolysis. PBMCs from six HIV-1-uninfected donors were coincubated with the murine P815 mast cell line and purified anti-CD3 antibody to induce redirected cytolysis [24]. These incubations were done in the presence or absence of seminal plasma from a HIV-1-uninfected donor. The presence of seminal plasma (final dilution 1 : 1000) significantly reduced T-cell-mediated cytolysis [12.63% (7.46–60.36%) vs. 0.08% (0.00–16.66%), $P=0.031$; Fig. 5d]. These results suggest that the immunomodulatory effects of seminal plasma also extend to T cells.

Discussion

We have previously shown that HIV-1-positive whole seminal plasma induced weaker anti-HIV-1 antibody-dependent NK cell activation than did dose-equivalent IgG purified from the same seminal plasma [19]. Taken together with the vast literature on reproductive biology highlighting the potency of seminal plasma-induced immunomodulation [12,15,16], we hypothesized that the presence of seminal plasma could impair an array of anti-HIV-1 cellular immune responses. In the current manuscript, we provide robust data implicating seminal plasma in the suppression of anti-HIV-1 cellular immune responses, namely NK cells and T lymphocytes. These data have important ramifications for vaccine design and highlight a crucial element lacking from most discussions of preventive HIV-1 interventions – the presence of seminal plasma as a viral delivery vehicle [25].

The exact factor(s) conferring seminal plasma with immunomodulatory properties has been extensively discussed in the reproductive biology literature. That we observed potent inhibition after serial dilutions and blanket suppression of both NK cells and T cells suggests that the factors involved are present in high concentrations in semen and wide acting. Active transforming growth factor beta has been shown to be a potent immunomodulator of the female reproductive tract and is present at elevated levels in semen [26]. Similarly, seminal CD52 has been shown to suppress the classical complement pathway [27]. The increased prostaglandins present in seminal plasma have also been previously suggested to hinder viral clearance during infections of the reproductive tract [16]. In addition, Alexander *et al.* (1987) [28] showed in rhesus macaques, vaginal and rectal infusions of human semen resulted in the elevation of blood prostaglandins. Prostasomes have also been previous implicated in the immunosuppression of NK cells by seminal plasma [29]. The interpretation of experiments on the effects of seminal plasma on NK cells, however, are limited as they were performed with purified/fractionated constituents from seminal plasma, and did not accurately represent the multifactorial immunomodulatory effects at play [29,30]. In addition, these assays were conducted over 12–24 h, and extended exposure to seminal plasma could induce cytotoxicity and influence results, as suggested by Kim *et al.* (2010) [31].

The hours immediately following HIV-1 exposure are a crucial window for preventing infection. Indeed, it has been shown in macaques that cell-free simian immunodeficiency virus rapidly disseminates from the mucosa to the lymph nodes within the first 4 h following rectal inoculation [32]. There, the virus establishes infection, and eventually latent reservoirs. Given the urgency of curtailing the spread of infectious virus, it is paramount to mount an effective early immune response at the site of entry. Here, we have demonstrated that within 5 h of exposure, the addition of diluted seminal plasma significantly hampered anti-HIV-1 immune responses by both NK cells and T lymphocytes. This hindrance could provide a window of opportunity for HIV-1 dissemination and subsequent infection.

Although the prospect of semen interfering with immune responses to HIV-1 and allowing viral dissemination to occur is intriguing, many questions remain about the in-vivo relevance of immune suppression by seminal plasma. Indeed, the interaction of seminal plasma with factors present within the mucosal sites of HIV-1 transmission could determine if immune suppression observed *in vitro* translates to *in vivo.* Future experiments should attempt to

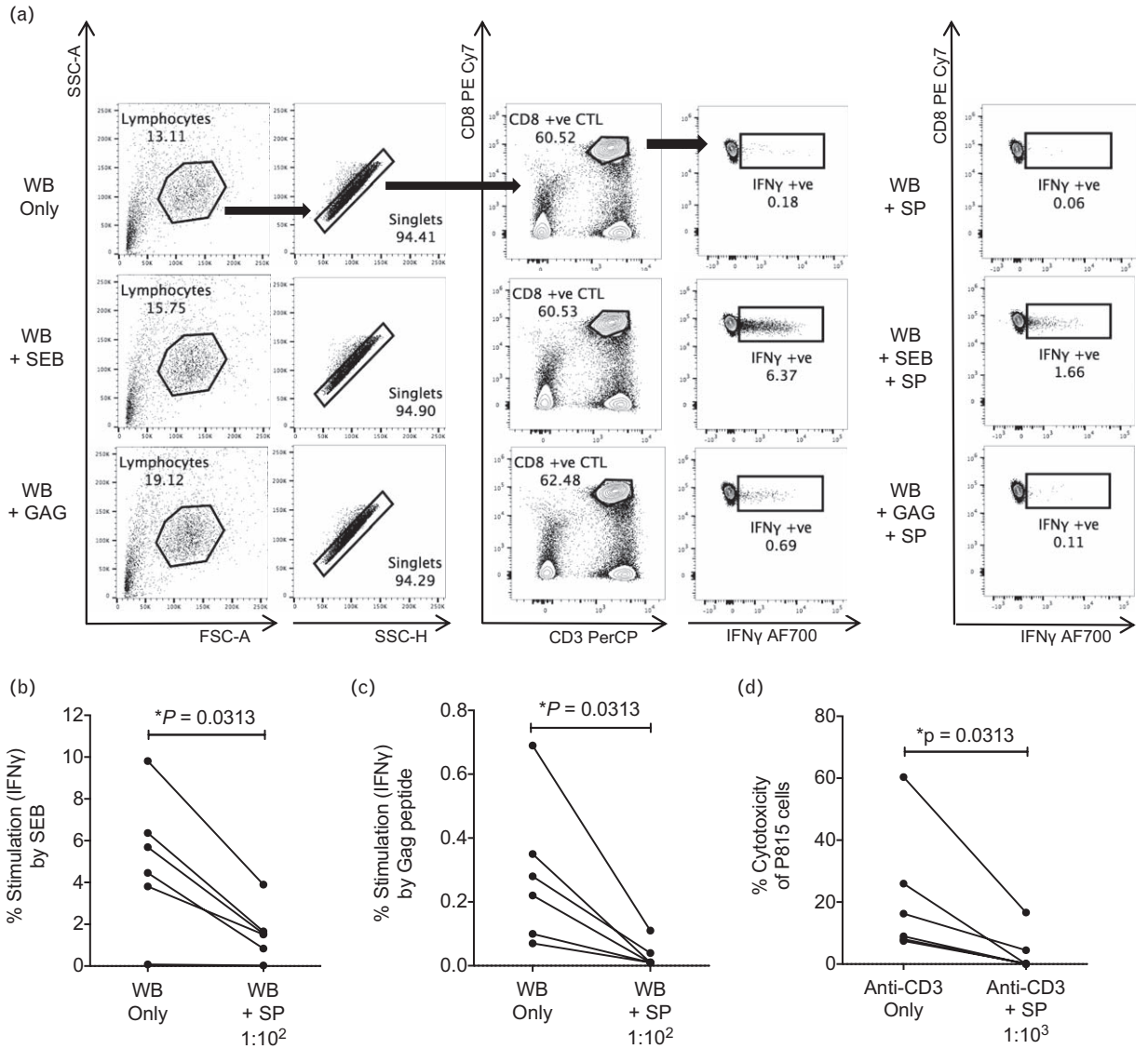

Fig. 5. Effects of seminal plasma on T-lymphocyte responses. CTL; $CD8^+$ T lymphocytes; FACs, fluorescence-activated cell sorting; SEB, staphylococcal enterotoxin B; SP, seminal plasma. (a–c) Whole blood was collected from six HIV-1-infected donors and T-lymphocyte responses were assessed by the addition of SEB or 15-mer Gag peptide pool. To assess the impacts of SP on T-lymphocyte responses stimulations were conducted in the presence or absence of a 1 : 100 final dilution of SP from a single HIV-1-uninfected donor. T-lymphocyte responses were assessed through intracellular cytokine staining to detect IFN-γ production by $CD3^+CD8^+$ lymphocytes. (a) FACs plots depict the gating strategy used to identify $CD3^+CD8^+$ T cells, as well as assess their activation (i.e., IFNγ production). (b) The graph depicts the relative IFN-γ production by $CD3^+CD8^+$ T lymphocytes upon SEB stimulation in the presence and absence of SP. (c) The graph depicts the relative IFN-γ production by $CD3^+CD8^+$ T lymphocytes upon Gag peptide pool stimulation in the presence and absence of SP. (d) The impact of SP on T-lymphocyte-mediated cytolysis was assessed using the LDH-release assay. Freshly isolated PBMCs from six HIV-1-uninfected donors were incubated with P815 target cells in the presence of anti-CD3 antibody to elicit redirected cytolysis. Incubations were conducted in the presence or absence of a 1 : 1000 final dilution of SP from single HIV-1-uninfected donor. The graph depicts the relative redirected cytolysis of P815 target cells in the presence and absence of SP. Statistical significance values were obtained using a nonparametric Wilcoxon matched pairs test.

address this possibility by diluting seminal plasma within rectal or vaginal fluids prior to addition to assays assessing cellular immunity. In addition to the possibility of factors within mucosal fluids attenuating immune suppression mediated by seminal plasma, there are questions about the location of effector cells at mucosal sites and the ability of seminal plasma to influence the functionality of these cells. Recently, NK cells were identified within both the colorectal and female genital mucosal tissues of humans [33]. The majority of these cells were localized within the subepithelial lamina propria. Additionally, it has recently been shown that anti-HIV-1 CTL can be established

within the vaginal mucosa of mice after an intranasal prime/intravaginal boost vaccination protocol with an influenza construct modified to express HIV-1 p24 [34]. Whether or not immunosuppressive factors within seminal plasma can access potential effector cells within mucosal tissues and alter their behavior is an important question to be addressed by future research.

In conclusion, our findings show that seminal plasma is a potent suppressor of key NK cell and T-lymphocyte anti-HIV-1 immune responses, and may attenuate vaccine-induced responses upon HIV-1 exposure. Our results highlight seminal plasma as a potentially under-recognized impediment to HIV-1 vaccine efficacy.

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Conflicts of interest

There are no conflicts of interest.

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