Exogenous and endogenous hyaluronic acid reduces HIV infection of CD4\(^+\) T cells

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Preventing mucosal transmission of HIV is critical to halting the HIV epidemic. Novel approaches to preventing mucosal transmission are needed. Hyaluronic acid (HA) is a major extracellular component of mucosa and the primary ligand for the cell surface receptor CD44. CD44 enhances HIV infection of CD4\(^+\) T cells, but the role of HA in this process is not clear. To study this, virions were generated with CD44 (HIV\(_{CD44}\)) or without CD44 (HIV\(_{mock}\)). Exogenous HA reduced HIV infection of unstimulated CD4\(^+\) T cells in a CD44-dependent manner. Conversely, hyaluronidase-mediated reduction of endogenous HA on the cell surface enhanced HIV binding to and infection of unstimulated CD4\(^+\) T cells. Exogenous HA treatment reduced activation of protein kinase C alpha via CD44 on CD4\(^+\) T cells during infection with HIV\(_{CD44}\). These results reveal new roles for HA during the interaction of HIV with CD4\(^+\) T cells that may be relevant to mucosal HIV transmission and could be exploitable as a future strategy to prevent HIV infection.

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Prevention of HIV transmission is still the most direct way to stem the HIV/AIDS epidemic.\(^1\) However, to date, large-scale clinical trials of vaccines to produce an HIV-specific antibody or a T-cell response to prevent HIV infection have been disappointing.\(^2,3\) As 80% of HIV infection occurs through sexual contact,\(^4\) there is intense interest in the prevention of HIV mucosal transmission. To design a better strategy to prevent mucosal transmission of HIV, we need to more fully understand the mechanism of HIV mucosal transmission.\(^5\)

Mucosal tissues are the front-line defense against pathogen invasion and greatly impede HIV transmission. Studies using the simian immunodeficiency virus (SIV) rhesus macaque model demonstrate that the genital tract mucosal barrier limits exposure of CD4\(^+\) T cells, dendritic cells and macrophages to the majority of the viral inoculum, and only a small number of infectious virions pass through the mucosal barrier to establish the infected founder population.\(^6,7\) These findings are confirmed by clinical studies showing that a small number of infectious virions breach the mucosal barrier to infect resting CD4\(^+\) T cells, generating a clonal or oligoclonal founder population.\(^5,8,9\)

Mucosal integrity has an important role in HIV transmission, and mucosal inflammation can increase HIV transmission.\(^10–12\) The mucosal tissues are composed of epithelial cells, extracellular matrix, interstitial cells and surface mucus. In addition to providing a full complement of host immune cells that variably facilitate or impede HIV infection, the mucosal surface also serves as a physical barrier to mucosal HIV invasion. Mucosal mucus can trap HIV virions\(^13\) and reduce virion movement.\(^14\) An acidic vaginal mucosal environment can decrease the rate of HIV sexual transmission.\(^15\) How these effects on mucosal HIV transmission are mediated remains largely unknown.\(^5,9\)

The surface of the mucosal layer is a scaffold with extracellular matrix; a major component of the extracellular matrix is hyaluronic acid (HA, or hyaluronan). HA is a large glycosaminoglycan that can be remodeled and degraded by hyaluronidase. On the surface of the cells, HA polymers extend up to 25 \(\mu\)m in length, forming pericellular coats. HA interaction with its receptors can induce cellular signaling and is involved in mucosal tissue homeostasis and maintenance of tissue integrity.\(^16–18\) HA is also a regulator of immunity. HA interaction with its main receptor, CD44, regulates recruitment and extravasation of T cells into sites of inflammation\(^19,20\) and participates in the inflammatory process.\(^16,21\) HA interaction with CD44 can reduce cytokine production from macrophages in the setting of inflammation\(^22\) and lowers protein kinase C alpha (PKCa) activity to decrease histamine release from leukemic cell lines.\(^23\)

There are reasons to believe that HA–CD44 receptor interactions may influence mucosal transmission of HIV. Clinical studies have found that mucosal integrity, activation of T cells and secretion of cytokines are each involved in mucosal HIV transmission,\(^5,9\) and each is modulated by HA–CD44 receptor binding. Studies have also reported that the primary HA receptor, CD44, is incorporated into HIV-1 virions\(^24,25\) and that CD44 on the HIV virion surface maintains its biological function, such as binding to HA.\(^26\) Moreover, CD44 on HIV virions enhances HIV-1 infectivity for
primary CD4+ T cells. However, the effect of HA on HIV-1 infectivity remains poorly understood. The main aim of this study was to assess the role of HA in HIV infection. We observed that exogenous HA reduced HIV infectivity when both virions and CD4+ T cells expressed CD44. Effects were seen on both early infection events like viral binding and probably later events through reduction of PKCa activation, whereas treatment with hyaluronidase reduced endogenous HA thickness and enhanced susceptibility of CD4+ T cells to infection.

RESULTS
Exogenous HA reduces HIV infectivity on unstimulated peripheral blood mononuclear cells, but only for virus bearing CD44

CD44 is found on HIV virions from either peripheral blood mononuclear cell (PBMC) cultures or directly in patient plasma. In contrast, 293T cells do not express appreciable levels of endogenous CD44. Transfection of an expression vector containing CD44 complementary DNA (cDNA) from donor CD4 T cells resulted in CD44 protein expression in 293T cells (Supplementary Figure 1), and

Figure 1 Exogenous HA reduced infectivity of HIV virions containing CD44. (a and b) M7-Lue cell assays. Relative light units is a measurement of luciferase activity. (a) Exogenous HA (Sigma) reduced infectivity of HIVCD44 (filled bar), but not HIVmock (open bar). (b) Exogenous HA decreased HIVPBMC infectivity in a dose-dependent fashion. (c and d) Primary healthy donor unstimulated PBMCs' assays. (c) Exogenous HA reduced HIV-1 p24 production from HIVCD44 (filled bar) infection, but failed to reduce HIVmock (open bar) infection. (d) Exogenous HA lowered HIV RNA in T-20-treated cells infected with HIVCD44 (filled bar), but not HIVmock (open bar). (c and d) data are mean ± s.e.m. of triplicate samples and represent three independent experiments. (a and b): data are mean ± s.e.m. of triplicate samples and represent three independent experiments. (c and d): data are mean ± s.e.m. of triplicate samples and represent three independent experiments. (e and f): data are mean ± s.e.m. of triplicate samples and represent three independent experiments. (g and h): data are mean ± s.e.m. of triplicate samples and represent three independent experiments. (i and j): data are mean ± s.e.m. of triplicate samples and represent three independent experiments.
Figure 2 Exogenous HA-mediated reduction of HIV infection required CD44 on target cells. (a) Exogenous HA (Sigma) had no impact on either HIVCD44 (filled bar) or HIVmock (open bar) infection of Jurkat-E6.1 cells, which lack CD44. (b) Jurkat-E6.1 cells expressed CD44 after transfection with a CD44 expression vector, but not with an empty vector (pcDNA3.1). Intracellular staining was performed using isotype control (R&D systems; open histogram) or anti-human CD44-APC (R&D systems; closed histogram). (c) On top is percent of intracellular staining of HIV-p24+ cells (KC-57RD1-PE; Beckman Coulter) and bottom is the amount of HIV-p24 in the supernatant. Exogenous HA reduced HIV-p24 staining in CD44-transfected Jurkat cells infected with HIVCD44, but not HIVmock. Exogenous HA had no effect on HIV-p24 staining when either HIVCD44 or HIVmock were used to infect Jurkat-E6.1 cells transfected with empty vector. Viral production was assessed by measurement of p24 in the supernatant. The effects of HA were analogous to those seen with intracellular staining for p24 in c. (Data in a are mean±s.e.m. of triplicate samples and representative of three independent experiments, whereas data in b and c are from duplicate samples and representative of two independent experiments.)
this expressed CD44 protein was able to bind to HA (Supplementary Figure 2). Simultaneous transfection of the cloned CD44 expression vector with pNL-4-3 into 293T cells was used to generate HIV virions containing CD44 (HIV\textsubscript{CD44}), whereas cotransfection of pNL-4-3 along with an empty vector (pcDNA3.1) produced virions without CD44 (HIV\textsubscript{mock}). CD44 is a transmembrane protein whose extracellular domain can be cleaved by membrane proteases.\textsuperscript{28,29} To test whether virions produced by CD44\textsuperscript{+} cells contain CD44, we utilized a centrifugation-based method\textsuperscript{30} for concentrating virions that should exclude free protein present in the supernatant. Supernatants were obtained from cells transfected with HIV plus empty vector (HIV\textsubscript{mock}), CD44 plus empty vector (CD44 alone) or CD44 plus HIV (HIV\textsubscript{CD44}), and aliquots of supernatant and virions (resuspended from centrifuged supernatant) were tested for CD44 by enzyme-linked immunosorbent assay (ELISA). No CD44 was detected in supernatant or virions from cells transfected with HIV plus empty vector. CD44 was present in similar concentrations in supernatants from cells transfected with CD44 plus empty vector and CD44 plus HIV, but was enriched only in the virions from cells transfected with CD44 plus HIV (Supplementary Figure 3). Both viral stocks (HIV\textsubscript{CD44} and HIV\textsubscript{mock}) were tested on the virus sf rom cell transfected with CD44 plus HIV (Supplementary similar concentrations in supernatant sf rom cell transfected with CD44, and aliquots of supernatant and virions (resuspended from centrifuged supernatant) were tested for CD44 by enzyme-linked immunosorbent assay (ELISA). No CD44 was detected in supernatant or virions from cells transfected with HIV plus empty vector. CD44 was present in similar concentrations in supernatants from cells transfected with CD44 plus empty vector and CD44 plus HIV, but was enriched only in the virions from cells transfected with CD44 plus HIV (Supplementary Figure 3). Both viral stocks (HIV\textsubscript{CD44} and HIV\textsubscript{mock}) were tested on the reporter T cell line M7-Lue, which contains the lucerase gene under the control of the HIV long terminal repeat (LTR), providing a measure of infectivity.\textsuperscript{31} In the presence of 10% fetal calf serum (FCS), viral stock containing CD44 (HIV\textsubscript{CD44}) demonstrated higher infectivity than HIV without CD44 (HIV\textsubscript{mock}) in both M7-lue cell line (Supplementary Figure 4) and primary CD4 T cells (Supplementary Figure 5). HIV produced from 293T cells cotransfected with CD44 (HIV\textsubscript{CD44}) has a similar infectivity compared with HIV produced by PBMCs (HIV\textsubscript{PBMC}). This was not true for HIV generated from 293T cells without CD44 (HIV\textsubscript{mock}) (Supplementary Figure 6). Reports have indicated that FCS not only contains HA but it can also influence HA synthesis\textsuperscript{32} and hyaluronidase activity.\textsuperscript{33} To limit the confounding effects of FCS during the study of HA effects on HIV infection, we removed FCS when the cells inoculated with HIV. This resulted in reduced infectivity of viral stocks irrespective of virion CD44 expression (Supplementary Figure 7).

Earlier studies reported that exogenous HA could interrupt T-cell rolling and impede extravasation of T cells into an inflamed site through interactions with CD44.\textsuperscript{34} To study whether exogenous HA can impact HIV infectivity, M7-Lue cells were treated with exogenous HA for 1 h, then inoculated with HIV\textsubscript{CD44} or HIV\textsubscript{mock} produced from 293T cells and normalized by p24. HIV infectivity was measured at day 3 post infection by luciferase activity as relative light units. Exogenous HA reduced infectivity of HIV\textsubscript{CD44} significantly but had no effect on the infectivity of HIV\textsubscript{mock} (Figure 1a). This inhibitory effect of exogenous HA for HIV containing CD44 was confirmed in experiments with HIV produced from PBMCs (HIV\textsubscript{PBMC}), which contain CD44.\textsuperscript{25,26} The reduction of infectivity of HIV by HA was dosage dependent (Figure 1b).

To further confirm that exogenous HA treatment can reduce infectivity of HIV containing CD44, unstimulated, primary PBMCs from healthy donors were treated with exogenous HA, and then inoculated with viral stocks of HIV\textsubscript{CD44} or HIV\textsubscript{mock} normalized by p24, then stimulated with phytohemagglutinin (PHA) and IL-2 for 24 h. HIV infectivity was assessed by measurement of supernatant HIV-1 p24 7 days post infection. Exogenous HA treatment significantly reduced HIV-1 p24 production during HIV\textsubscript{CD44} infection, but had no impact on p24 production during HIV\textsubscript{mock} infection (Figure 1c).

Because exogenous HA was added before HIV infection, we attempted to distinguish effects of HA on binding vs entry by using the entry inhibitor enfuvirtide (T-20).\textsuperscript{34} Unstimulated, primary PBMCs were treated with exogenous HA for 1 h, then exposed to an equivalent inoculum by p24 of HIV\textsubscript{CD44} or HIV\textsubscript{mock} for 5 h in the presence of 10\,µg\,ml\textsuperscript{-1} of enfuvirtide (T-20). Afterwards, the cells were extensively washed, and the cellular HIV RNA was measured. Exogenous HA treatment significantly reduced cell surface HIV RNA

![Figure 3](https://example.com/figure3.png) Hyaluronidase treatment enhanced HIV infection of unstimulated CD4\textsuperscript{+} T cells. Hase (hyaluronidase treatment; Sigma); medium (medium treatment); Hase + HA (hyaluronidase treatment with 100\,µg of exogenous HA (Sigma)); and medium + HA (medium treatment with 100\,µg of exogenous HA). (a) Hyaluronidase (Hase) treatment enhanced HIV\textsubscript{CD44} (filled bar) infection of M7-lue cells, but exogenous HA reversed the enhancement (Hase + HA). However, hyaluronidase (Hase) treatment failed to impact HIV\textsubscript{mock} (open bar) infectivity. (All data are mean ± s.e.m. of triplicate samples and representative of three independent experiments). (b) Treatment of healthy donor unstimulated CD4\textsuperscript{+} T cells by hyaluronidase (Hase) boosted HIV\textsubscript{CD44} (filled bar) infection, but not HIV\textsubscript{mock} (open bar) infection, and exogenous HA reduced this enhancement (Hase + HA). (All data are mean ± s.e.m. from duplicate samples and representative of at least three donors.)
in unstimulated PBMC infected with HIV_{CD44}, but not cell surface HIV RNA in HIV_{mock} infection (Figure 1d). These results indicate that exogenous HA may interfere with HIV binding, but only for HIV bearing CD44.

**CD44 on recipient cells is involved in exogenous HA’s reduction of HIV infection**

Although CD44 is the principal receptor for HA, HA also binds to other receptors on the cell surface, such as the receptor for...
PKCa is involved in exogenous HA-mediated reduction of HIV infectivity. G66976 treatment reduced HIVCD44 infection (filled bar) to a degree similar to exogenous HA in M7-Lue cells (a) and primary healthy donor unstimulated CD4⁺ T cells (b). However, G66976 treatment had no impact on infection of HIVmock (open bar). DMSO (Sigma) is a solvent for G66976 (Sigma). (All data are mean ± s.d. of triplicate samples and representative of three independent experiments). (c) CD44 co-immunoprecipitation western blot. Unstimulated CD4⁺ T cells were treated with 0 μg or 100 μg of exogenous HA (Sigma), then infected with either HIVCD44 or HIVmock for 5 h. Cellular protein was extracted and immunoprecipitated with anti-CD44 antibody. The western blots were done using three antibodies: (1) anti-human CD44 (CD44); (2) anti-phosphorylated PKCa (p-PKCa); and (3) anti-PKCa (PKCa). (d) The ratio of p-PKCa and PKCa from the mean intensity of each band shown in c. (All data are mean ± s.e.m. from duplicate experiments representing at least three donors.)

Hyaluronidase reduced the thickness of surface HA on unstimulated CD4⁺ T cells and enhanced HIV binding. (a–c) The images are Z-stack projections from laser scanning microscopy analysis of cell surface HA on CD4 T cells. Images are 40 ×; HA-staining with Alexa488 (blue); cell membrane staining with Texas Red (Life Technologies); and nuclear staining with DAPI (Life Technologies). (a) CD4 T cells without hyaluronidase treatment, and (b) CD4 T cells with hyaluronidase treatment. (c) The thickness of HA on the surface of the cell, from an average of 20 cells after hyaluronidase treatment (Hase) or no treatment (Medium). (d and e) A total of 50 000 cells of each staining were measured by flow cytometry (fluorescence-activated cell sorting (FACS)); healthy primary unstimulated CD4 T cells were treated with or without hyaluronidase (Hase; Sigma) for 1 h, then stained with Alexa488-labeled HA-binding protein. (d) FACS result showing cells without HA staining (dash line), cells treated with hyaluronidase (Hase; Sigma) and cells treated with control medium (open histogram) followed by HA staining. (e) The mean fluorescence after treatment with hyaluronidase (Hase) or medium (Medium). (f and g) HIV-1 p24 staining on unstimulated CD4⁺ T cells. A total of 100 000 cells of each unstimulated CD4⁺ T cells were treated with or without hyaluronidase for 1 h, incubated with HIVPMBCs for 5 h, then stained mouse anti-HIV-1p24 (Santa Cruz Biotech) and Donkey anti-mouse IgG antibody with AlexaFluor@680 (Life Technologies). (f) FACS result showing cells treated with hyaluronidase (Hase) or medium (Medium) and stained with isotype control (closed histogram) or HIV-1p24-Alexa-680 (open histogram). (g) Percent of HIV-1-p24-positive cells treated with hyaluronidase (Hase) or with medium (Medium). (All data are mean ± s.e.m. from duplicate experiments and representative of at least three donors.)
Hyaluronan-mediated motility (RHAMM), tumor necrosis factor (TNF)-stimulated gene-6/TNF-α-induced protein six (TSG-6), lymphatic vessel endothelial HA receptor (LYVE-1), hyaluronan receptor for endocytosis (HARE), liver endothelial cell clearance receptor (LEC receptor) and TLR-4. To assess whether CD44 on the target cell is required to reveal the inhibitory effect of exogenous HA on HIV infectivity, a cell line lacking expression of the CD44, the Jurkat-E6.1 cell line was studied. Jurkat-E6.1 cells were treated with exogenous HA before exposure to either HIVCD44 or HIVmock normalized by p24. HIV infectivity was assessed by measuring HIV-1 p24 production on day 7 post infection. Exogenous HA reduced HIV-p24 intracellular staining, and HIV-1 p24 on day 3 post infection. Exogenous HA reduced HIV-p24 intracellular staining and HIV-p24 production of HIVCD44 but not HIVmock in CD44-transfected E6.1 cells, whereas HA did not affect the ability of either virus to infect empty vector-transfected Jurkat-E6.1 cells (Figure 2c). These data indicate that CD44 expression on target cells is also required for exogenous HA to reduce infection by HIVCD44.

**Hyaluronidase treatment can enhance HIVCD44 infectivity on unstimulated CD4+ T cells**

Endogenous HA has been observed to form a cushion on the cell surface, to have an anti-adhesive role and to impede ligand access to cell surface receptors. Hyaluronidase treatment can reduce endogenous HA from the cell surface, thus promoting cell–cell contact. To determine whether endogenous HA on cell surfaces has an effect on HIV infection, M7-Lue cells were treated with hyaluronidase and then exposed to p24-equivalent inocula of HIVCD44 or HIVmock. HIV infectivity was assessed on day 3 post infection by measuring luciferase activity. Hyaluronidase treatment significantly enhanced infectivity of HIVCD44; however, this enhancement was reduced when exogenous HA was added. In contrast, hyaluronidase treatment had no effect on infectivity of HIVmock (Figure 3a). This latter observation indicates that the inhibitory effect of HA for HIV infectivity is not simply through non-specific steric hindrance.

**PKCa signaling is important for reduction of HIVCD44 infection of unstimulated CD4+ T cells by exogenous HA**

Others have reported that interaction of exogenous HA with CD44 can reduce PKCa activation, that PKCa has a critical role in HIV infectivity and that the selective PKCa inhibitor ‘G69676’ can inhibit HIV infection. To further investigate the role of PKCa in HA–CD44 modulation of HIV infectivity, M7-Lue cells were treated with G69676, dimethylsulphoxide (DMSO) or exogenous HA, then incubated with either HIVCD44 or HIVmock normalized by p24. HIV infectivity was assessed on day 3 post infection by measurement of luciferase activity. G69676 significantly reduced infectivity of HIVCD44 compared with the DMSO control, and the magnitude of this reduction was comparable to exogenous HA treatment. However, G69676 had no impact on the infectivity of HIVmock (Figure 5a). Next, unstimulated, primary CD4+ T cells from healthy controls were first treated with either G69676 or exogenous HA, then exposed for 5 h to either HIVCD44 or HIVmock normalized by p24. The toxicity of these treatments on the primary unstimulated CD4+ T cells was evaluated using the ViaCount assay (Guava, Millipore, Billerica, MA, USA), and neither cell number nor viability was affected (Supplementary Figure 10). The infectivity of HIV was assessed 7 days post infection by measurement of HIV-1 p24 in the supernatant. As expected, both G69676 and exogenous HA significantly reduced infectivity of HIVCD44 but had no effect on infectivity of HIVmock (Figure 5b). These results suggest that CD44 is involved in reduction of HIV infectivity.
of HIV infection by both Gø6976 and exogenous HA and that both treatments have a similar effect on HIV infection.

From these results, we hypothesize that reduction of infectivity of HIV by exogenous HA may involve the PKCa signaling pathways distal to CD44. To test this hypothesis, we evaluated PKCa phosphorylation, which corresponds to activation of PKCa, in unstimulated CD4+ T cells treated with exogenous HA and exposed to HIV. Following treatment with exogenous HA and exposure to HIV, CD4+ T cells were lysed and cellular proteins were extracted, immunoprecipitated with anti-CD44 antibody and analyzed using western blot. Treatment with exogenous HA had no effect on the amount of total PKCa protein in any condition (Figure 5c), but phosphorylated PKCa was reduced following exogenous HA treatment, and the ratio of phosphorylated PKCa/PKCa was significantly reduced in exogenous HA-treated unstimulated CD4+ T cells infected with HIVCD44 (Figure 5d). These results suggest that reduction of HIVCD44 infectivity of unstimulated CD4 T cells by exogenous HA is mediated by inhibition of PKCa activation.

**DISCUSSION**

Devising more effective interventions to prevent mucosal HIV transmission will require a deeper understanding of the interplay between virus and target cells in the mucosal environment. Studies of both human and rhesus macaque models indicate that mucosal tissues provide multiple mechanisms to prevent HIV and SIV infection, including physical barriers and innate and adaptive immune responses. However, a small amount of HIV-1 and SIV can penetrate the mucosal barrier, gaining access to the submucosa (or other CD44 ligands) and CD44 on unstimulated CD4 T cells, thus reducing targets that are needed for efficient transmission.

HA is the main component of the extra cellular matrix in the mucosal tissue. The main receptor for HA, CD44, is highly expressed in all cells, including CD4+ T cells, and in mucosal tissues, especially those along the reproductive tract. HA interactions with CD44 are involved in mucosal immunity and participate in mucosal inflammation. It has been reported that exogenous HA can interfere with interaction of endogenous HA with CD44, limiting recruitment of T cells by reducing T-cell adhesion, preventing T-cell rolling and inhibiting T-cell extravasation into an inflamed site. Other studies identify the recruitment of T cells into submucosa as important and necessary for mucosal HIV transmission.

Our study demonstrates that exogenous HA reduces HIV infection of unstimulated PBMCs and unstimulated CD4+ T cells and that this reduction of HIV infectivity is CD44 dependent. This inhibitory effect of HA was only observed when both HIV virions (HIVCD44 and HIVPBMC) and recipient cells (Figure 2c) were CD44+. These results indicate that exogenous HA may reduce HIV infectivity by interfering with interactions between CD44 on HIV virions, endogenous HA (or other CD44 ligands) and CD44 on unstimulated CD4+ T cells. Others have reported that the endogenous HA coat on the surface of cells can prevent extraneous or indiscriminant ligand binding and can prevent infection with Newcastle disease virus, vesicular stomatitis virus and rubella virus. Reducing the thickness of HA on the cell surface by hyaluronidase treatment can make target cells more susceptible to Newcastle disease virus, vesicular stomatitis virus and rubella virus infection. We also observed that treatment of unstimulated CD4+ T cells with hyaluronidase reduced the thickness of endogenous HA on the cell surface, allowing more HIV particles to bind (Figures 4c and d), an effect that could be reversed by the addition of exogenous HA (Figure 3b). Although these experimental results suggest that the inhibitory effects of HA on HIV infection occur at least in part at an early stage of the viral life cycle, including effects localizable to viral binding, further work is needed.

Interactions of HA with CD44 induce cellular signaling and regulate immune responses. Other studies have observed that HA can reduce PKCa activity through CD44 to decrease expression of inflammatory regulators such as TNFa, IL-6, IL-18 and MMP-7. PKCa is a classic isoform of PKC that is highly expressed in CD4 T cells. PKCa and CD44 are localized in lipid microdomains (lipid rafts), and HIV engagement with CD4 in lipid rafts is required for HIV infection. In turn, PKCa has been implicated as important for HIV infection, and Gø6976, a PKCa selective inhibitor, has been observed to reduce HIV infection. This study is the first to show that virion CD44 is necessary for the reduction of HIV infectivity by Gø6976 and that Gø6976 treatment has an effect analogous to treatment with exogenous HA (Figures 5a and b). This finding is compatible with a model where PKCa signaling is triggered by bridging of virion and cell surface CD44 by multivalent ligands. Our findings are reminiscent of the report by Chang et al. that alpha-defensin-1 reduces HIV infectivity through decreased cellular phosphorylation of PKC, but not the amount of PKC protein. These investigators further determined that blocking phosphorylation of PKC resulted in inhibitory effects at later stages of the viral life cycle, including nuclear import and viral transcription. Such effects therefore represent at least a second, later stage in the viral life cycle that can be modulated by manipulating HA–CD44 interactions.

These findings are also similar to other recent data showing that anti-CD44 antibody beads enhance HIV infection only for HIV produced by PBMC, but not for HIV produced by 293T cells. Under some conditions, different HA fragments are known to mediate CD44 interactions that augment T-cell activation. In the current work, a commercial source of HA was used, as has been reported by many other investigators. Although several experiments were performed with different sizes of HA (both high and low molecular weight), we did not observe consistent differences, but rather found that all sizes of exogenous HA inhibited HIV infection under the conditions tested (Supplementary Figure 11). One possible effect of exogenous HA regardless of size may be its ability to compete with ligands (perhaps including some natural species of HA) that bind to CD44. We also observed that exogenous HA reduced HIV infectivity in unstimulated CD4+ T cells to a degree greater than that observed in stimulated CD4+ T cells (Supplementary Figures 12 and 13). In such a model, excess exogenous HA may disrupt the optimal stoichiometry necessary for endogenous ligands to bind and bridge CD44, an effect that may be more important for the infection of resting CD4 T cells.

It is notable that HA, hyaluronidase and CD44 are all highly expressed and have important roles in maintaining the integrity and function of the mucosal tissue along the reproductive tract.
Hyaluronidase is also a major constituent of semen. Both HA and hyaluronidase are highly upregulated during sexual intercourse as well as inflammation. The processes of reproductive fertilization and HIV transmission intersect at many levels anatomically and functionally. Both involve the reproductive tract and permissiveness of mucosal tissue and surface barriers. Clinical studies have demonstrated that inflammation in the mucosal tissue can facilitate HIV transmission, and that transmission rates are higher for HIV in semen compared with blood. The identification of topical agents that can be applied to mucosal surfaces to prevent mucosal HIV transmission has been identified as a priority area for research and development in the effort to control the HIV/AIDS epidemic.

In summary, HA present on the surface of unstimulated CD4+ T cells can influence HIV infection. Both HA and hyaluronidase have well-described roles in reproductive and inflammatory processes. Our data now show that the status of the HA coat may have an important role in the initial interaction of HIV virions with resting CD4+ T cells, a feature that may be particularly relevant for mucosal HIV transmission. Intriguingly, exogenous HA appears to block HIV engagement and infection of target cells. Furthermore, HA interactions with virion and cell surface CD44 modulate PKCα activation and may have additional inhibitory effects on later stages of the viral life cycle. HA is a non-immunogenic natural biopolymer that has been used in a variety of clinical applications. These findings should be further explored in vivo and, if confirmed, could lead to the development of novel interventions to reduce HIV mucosal transmission.

METHODS

CD44 gene construction

Cloning of CD44 gene. A cDNA of the standard isoform of human CD44 was generated from RNA extracted from healthy donor CD4+ T cells using SuperScript III Reverse Transcribease (Life Technologies, Carlsbad, CA, USA) according to the manufacturer’s instructions and amplified using Platinum TaqDNA High Fidelity Polymerase (Life Technologies). Oligonucleotides used in reverse-transcription PCR were 5’-CACGCTCTGGCAAGTTTCGTCGCCGATCTCGT-3’ and 3’-CD44 (5’-TGAATGATGCAATGGACGAAGAAGTACAGATA TTATATGATG-3’). The CD44 gene was cloned into pcDNA3.1/V5-His TOPO TA Expression vector (Life Technologies). The CD44 expression plasmid was purified and sequenced, and the CD44 clone was verified to be a perfect match to the standard isoform of human CD44 (GenBank AJY11921).

Isolation of PBMCs and CD4+ T cells from healthy donors

PBMCs were isolated from healthy donors as previously published, and CD4+ T cells were isolated from PBMCs using the EasySep Human CD4+ T cell Enrichment kit (STEMCell, Vancouver, BC, Canada) according to the manufacturer’s instructions.

Generation of HIV virus stock with or without CD44 from 293T cells and from PBMCs

Viral stock generated from 293T cells. 293T cells (ATCC, Manassas, VA, USA) were cultured in complete Dulbecco’s modified Eagle’s medium (Life Technologies) supplemented with penicillin (50 U ml−1), streptomycin (50 μg ml−1), 2 μM of L-glutamine (Life Technologies) and 10% heat-inactivated FCS (Sigma, St Louis, MO, USA), then cotransfected with 10 μg of pNL4-3 (NIH AIDS Research and Reference Reagent Program) and 5 μg CD4 plasmid (to generate HIVΔGlu), or with empty pcDNA3.1/V5-His TOPO TA Expression vector (to make HIVMock) using FuGENE HD Transfection Reagent (Roche, Indianapolis, IN, USA) according to the manufacturer’s instructions. Three days later, the supernatant was collected and HIV production was assessed using an HIV-1 p24 ELISA assay (PerkinElmer, Waltham, MA, USA).

HIV viral stock generated from PBMCs. Isolated PBMCs were stimulated with 3 μg ml−1 of PHA-M (Roche) and 20 μM of human-IL-2 (HIL-2; Roche) for 3 days; viral supernatant from transfection of 293T cells was used to infect pooled mixed stimulated PBMCs prepared from two healthy donors. The supernatant from HIV-infected PBMCs was collected at day 7 and day 11. Viral titers were determined by HIV-1 p24 ELISA (PerkinElmer).

Reduction of HA from cell surface

A total of 1 x 10^6 of unstimulated CD4+ T cells or M7-lue cells were washed three times in RPMI without FCS and incubated in RPMI with 12 U ml−1 of hyaluronidase (Sigma) for 60 min, followed by removal of supernatant and three washes with RPMI without FCS.

Co-immunoprecipitation with CD44 western blotting

A total of 2 x 10^6 unstimulated healthy donor CD4+ T cells were washed twice with cold phosphate-buffered saline and lysed using the Mammalian Cell Lysis kit (Sigma). The lysates were centrifuged at 4°C for 10 min at 12 000 x g to pellet cellular debris, and the clarified lysate was transferred to a fresh pre-chilled tube. Lysates were precleared by incubation with protein A-Sepharose (Sigma) for 1 h at 4°C, subjected to immunoprecipitation with anti-CD44 (Santa Cruz Biotech, Santa Cruz, CA, USA) or antibody to phosphorylated-PKA (Santa Cruz Biotech). Detection was performed using goat anti-mouse immunoglobulin G (IgG)- conjugated horseradish peroxidase (Sigma) and ECL Advance tablets (Sigma). The bands were analyzed using Quantity One 1-D analysis Software (Bio-Rad, Hercules, CA, USA).

Staining HA and HIV (p24) on unstimulated CD4+ T cells

HA staining. We adapted a method from Lin37 to stain HA on the unstimulated CD4+ T cell surface. Unstimulated CD4+ T cells were fixed by acid-formaldehyde and stained with biotinylated hyaluronan-binding protein (Calbiochem, Gibbstown, NJ, USA) overnight, then stained with Alexa488-streptavidin (Life Technologies) for 1 h at 4°C. The Eluate was neutralized by 2 ml of cold wash buffer (lysis buffer plus 0.5 M NaCl), then eluted from the protein G-Sepharose using 50 μl of elution buffer (1 ml glycine, 0.25 M NaCl, pH 2.8). The Eluate was neutralized by 2 ml of 1 M Tris–HCl (pH 9.5), then subjected to 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. The membrane was treated with blocking buffer (5% fat-free milk, 50 μg/ml Tris–HCl (pH 7.5), 0.2 M NaCl and 0.2% volume Tween-20), then incubated overnight at 4°C in blocking buffer containing 1:250 dilution of antibody to PKCa (Santa Cruz Biotech, Santa Cruz, CA, USA) or antibody to phosphorylated-PKA (Santa Cruz Biotech). Detection was performed using goat anti-mouse immunoglobulin G (IgG)- conjugated horseradish peroxidase (Sigma) and ECL Advance tablets (Sigma). The bands were analyzed using Quantity One 1-D analysis Software (Bio-Rad, Hercules, CA, USA).

HIV staining. Unstimulated CD4+ T cells were incubated with HIV for 5 h, fixed with 2% formaldehyde and incubated with mouse anti-HIV-1 p24 antibody at 4°C overnight, then stained with donkey anti-mouse IgG antibody conjugated with Alexa Fluor647 (Life Technologies) for 2 h. The HIV-1-p24-positive cells were measured by flow cytometry (fluorescence-activated cell sorting). For laser scanning microscopy analysis, the cells were further stained, cell membranes were stained with Texas Red (Life Technologies) and nuclei stained with DAPI (4,6-diamidino-2-phenylindole; Life Technologies). LSM510 META laser scanning microscope (Carl Zeiss Microimaging Inc., Thornwood, NY, USA) was used to scan the cells with Z-stack projections. For fluorescence-activated cell sorting analysis, 50 000 HA-stained cells were analyzed by EasyCyte6HT-2L (Millipore, Billerica, MA, USA).

Assays for HIV infection

M7-lue cell (5.25.EGFP.Lue.M7 cell, a gift from Dr David Montefiori (Duke University) and Dr Ned Landsdau (NYU)). A total of 1 x 10^6 cells were washed with complete RPMI without FCS, then treated with study reagents, incubated with 1000 pg HIV-p24 of HIV for 5 h, washed three times with complete RPMI and resuspended in complete RPMI. HIV infectivity was measured by using Bright-Glo Luciferase assay system (Promega, Madison, WI, USA) and...
measured with a plate reader (VICTOR3; PerkinElmer) at 3 days post infection. The luciferase activity is presented as relative light units.

**Jurkat (Ek1) cell (NIH AIDS Research and Reference Reagent Program).** For untransfected Jurkat cells, HIV infection conditions were the same as for the M7-Luc cell line, but HIV infectivity was measured by HIV-1 p24 production at 7 days post infection using the HIV-1p24 ELISA kit (PerkinElmer). For CD44 +/− empty vector-transfected Jurkat cells, after 3 days of transfection, the cells were infected with 5 ng of HIV-p24 for 5 h, then washed three times with complete RPMI, resuspended in complete RPMI and cultured for 3 days. These cells were analyzed for HIV infection by two methods: first, by intracellular staining for HIV-p24 (KC-57RD1-PE; Beckman Coulter, Fullerton, CA, USA) and CD44 (anti-huCD44-APC; R&D system); and second, measuring HIV-1 p24 in the supernatant by p24 ELISA (PerkinElmer).

**Unstimulated CD4 +/− T cells and unstimulated PBMCs.** A total of 1 x 10⁶ healthy donor unstimulated cells were washed with RPMI without FCS, treated with different reagents, inoculated with 1000 pg HIV-p24 of HIV-5 for 5 h, washed three times with complete RPMI, resuspended in complete RPMI with 5 μg of PHA-M and 20 U of hIL-2 for 24 h, washed three times with completed RPMI and finally resuspended in completed RPMI with 20 U of hIL-2. HIV infectivity was measured by HIV-1 p24 production in supernatant at day 7 post infection by using the HIV-1p24 ELISA kit (PerkinElmer).

**Statistical analyses**

All M7-Luc and Jurkat cell assays were run in triplicate and repeated at least three times. Experiments with primary PBMCs and CD4 +/− T cells were run in duplicate and repeated with at least three different donors. Data averages and s.d. were calculated in Microsoft Excel 2008 and presented as means ± s.d. Significance was assessed by a t-test, with P < 0.05 considered significant.

**CONFLICT OF INTEREST**

The authors declare no conflict of interest.

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