

## Syndecan-Fc Hybrid Molecule as a Potent *In Vitro* Microbicidal Anti-HIV-1 Agent<sup>∇</sup>

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**In the absence of a vaccine, there is an urgent need for the development of safe and effective topical microbicides to prevent the sexual transmission of human immunodeficiency virus type 1 (HIV-1). In this study, we proposed to develop a novel class of microbicides using syndecan as the antiviral agent. Specifically, we generated a soluble syndecan-Fc hybrid molecule by fusing the ectodomain of syndecan-1 to the Fc domain of a human IgG. We then tested the syndecan-Fc hybrid molecule for various *in vitro* microbicidal anti-HIV-1 properties. Remarkably, the syndecan-Fc hybrid molecule possesses multiple attractive microbicidal properties: (i) it blocks HIV-1 infection of primary targets including T cells, macrophages, and dendritic cells (DC); (ii) it exhibits a broad range of antiviral activity against primary HIV-1 isolates, multidrug resistant HIV-1 isolates, HIV-2, and simian immunodeficiency virus (SIV); (iii) it prevents transmigration of HIV-1 through human primary genital epithelial cells; (iv) it prevents HIV-1 transfer from dendritic cells to CD4<sup>+</sup> T cells; (v) it is potent when added 2 h prior to addition of HIV-1 to target cells; (vi) it is potent at a low pH; (vii) it blocks HIV-1 infectivity when diluted in genital fluids; and (viii) it prevents herpes simplex virus infection. The heparan sulfate chains of the syndecan-Fc hybrid molecule are absolutely required for HIV-1 neutralization. Several lines of evidence suggest that the highly conserved Arg298 in the V3 region of gp120 serves as the locus for the syndecan-Fc hybrid molecule neutralization. In conclusion, this study suggests that the syndecan-Fc hybrid molecule represents the prototype of a new generation of microbicidal agents that may have promise for HIV-1 prevention.**

The dominant cell surface heparan sulfate proteoglycans (HSPG) (25, 29, 30, 33) are syndecans, which are transmembrane receptors highly expressed on adherent cells (macrophages and epithelial and endothelial cells) but poorly expressed on suspension cells (T cells) (2, 3, 4, 10, 35). Their ectodomain bears three linear heparan sulfate (HS) chains, which are composed of a repetition of a sulfated disaccharide motif (1). The sulfation pattern of HSs dictates the ligand specificity of syndecans (1). HSPG, including syndecans, serve as receptors for human deficiency virus type-1 (HIV-1) (16), herpes simplex virus (HSV) (7), human papillomavirus (HPV) (13, 37), and human T-lymphotropic virus type 1 (HTLV-1) (19, 20). Pretreatment of target cells such as macrophages with heparinase, an enzyme that removes HS moieties from syndecans, significantly reduces HIV-1 infectivity (35). Although syndecans do not alleviate the requirement for CD4 and chemokine receptors for viral entry (35), these *in cis* attachment receptors amplify HIV-1 infection by promoting viral adsorption to the surface of permissive cells. Syndecans also serve as *in trans* receptors for HIV-1 (2, 16). HIV-1 binds syndecans richly expressed on the endothelium and remains infectious for a week, whereas cell-free virus loses its infectivity after a single day (2). Moreover, HIV-1 attached onto the endothelium via

syndecans represents an *in trans* source of infection for circulating T cells (2). Primary HIV-1, HIV-2, and simian immunodeficiency virus (SIV) isolates produced from peripheral blood mononuclear cells (PBMCs) exploit syndecans (2). Furthermore, syndecans on microvascular endothelial cells play a significant role in cell-free HIV-1 transmigration through the blood-brain barrier (3). Thus, HIV-1 has maximized its utilization of syndecans in the body.

A single conserved arginine (Arg298) in the V3 region of gp120 governs HIV-1 binding to syndecans (11). An amine group on the side chain of this residue is absolutely required for syndecan utilization by HIV-1 (11). HIV-1 binds syndecans via a 6-O sulfation (11) within the HS chains, demonstrating that this binding is not the result of random interactions between basic residues and negative charges but the result of specific contacts between gp120 and a well-defined sulfation in syndecans. Surprisingly, the Arg298 in gp120 that mediates HIV-1 binding to syndecans also mediates HIV-1 binding to CCR5 (42), suggesting that HIV-1 recognizes similar motifs on syndecans and CCR5 (11). Supporting this hypothesis, the 6-O sulfation recognized by HIV-1 on syndecans mimics the sulfated tyrosines recognized by HIV-1 in the N terminus of CCR5 (11). The finding that CCR5 and syndecans are exploited by HIV-1 via a single determinant echoes the mechanisms by which chemokines utilize these two disparate receptors and suggests that the gp120/chemokine mimicry may represent a common strategy in microbial pathogenesis.

More recent work suggests that syndecans play a critical role in HIV-1 transmission (4). HIV-1 transmission includes trans-

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migration of HIV-1 through the genital epithelium and subsequent capture and transfer of infectious particles from dendritic cells (DC) and/or Langerhans cells (LC) to T cells (31, 34, 38). Importantly, human cervical and vaginal mucosal epithelia richly express syndecans *in vivo* (4). HS chain removal by heparinase treatment prevents HIV-1 transcytosis through primary human cervical and vaginal mucosal epithelia (4). The Arg298 V3 HIV-1 mutant, which cannot interact with syndecans (11), fails to transcytose (4). Thus, syndecans facilitate HIV-1 epithelial transmigration *in vitro*. More recently, we identified syndecan-3 as an HIV-1 receptor on DC (12). Syndecan-3 stabilizes the captured virus, enhances DC infection *in cis*, and promotes transfer to T cells (12). Removal of the HS from the cell surface by heparinase or by silencing syndecan-3 by small interfering RNA (siRNA) partially inhibited HIV-1 transmission by immature DC (12), whereas neutralizing both syndecan-3 and DC-SIGN abrogated HIV-1 capture and subsequent transmission (12). Our findings that syndecans modulate both HIV-1 epithelial transmigration and HIV-1 transfer from DC to CD4<sup>+</sup> T cells suggest that syndecans represent new targets for the development of topical microbicides.

Nearly 40 million people currently live with HIV-1/AIDS; the majority of them reside in developing countries. It has been estimated that there are over 14,000 new HIV-1 infections per day in adults aged 15 to 50 years, with 50% of the newly infected being women aged 15 to 24 years (21, 22). In the absence of a vaccine, there is thus an urgent need for the development of safe and effective topical microbicides to prevent the sexual transmission of HIV-1 (14, 26). Topical microbicides are currently defined as vaginally or rectally applied products that prevent male-to-female or female-to-male transmission of HIV-1 infectious particles (39).

In this study, we proposed to develop a novel class of microbicides using syndecan as the antiviral agent. Specifically, we generated a soluble syndecan-Fc hybrid molecule by fusing the ectodomain of syndecan-1 to the Fc domain of a human IgG. We then tested this syndecan-Fc hybrid molecule for its *in vitro* microbicidal properties. Remarkably, the syndecan-Fc hybrid molecule possesses multiple attractive microbicidal properties: (i) it blocks HIV-1 infection of primary targets including T cells, macrophages, and DC; (ii) it exhibits a broad range of antiviral activity against primary HIV-1 isolates, multidrug-resistant HIV-1 isolates, HIV-1, and SIV; (iii) it prevents transmigration of HIV-1 through human primary genital epithelial cells (PGEC); (iv) it prevents HIV-1 transfer from DC to CD4<sup>+</sup> T cells; (v) it is potent when added 2 h prior to addition of HIV-1 to target cells; (vi) it is potent at a low pH; (vii) it blocks HIV-1 infectivity when diluted in genital fluids; and (viii) it inhibits HSV infection. Thus, this syndecan-Fc hybrid molecule represents the prototype of a new generation of microbicidal agents that may have promise for HIV-1 prevention.

#### MATERIALS AND METHODS

**Viruses.** 293T cells were transfected with proviral plasmid (9 μg) and vesicular stomatitis virus G (VSV-G) envelope plasmid (1 μg). At day 2, VSV-G pseudotyped viruses (5 μg of p24) were harvested and used to acutely infect Jurkat cells (2 × 10<sup>6</sup> cells). Two days after infection, viruses were harvested, and p24 content was measured by enzyme-linked immunosorbent assay (ELISA; PerkinElmer, Waltham, MA). The following proviral constructs were used: wild-type pNL4.3 (X4); pNL4.3-BaL (R5), in which the wild-type NL4.3 envelope was switched for the R5 BaL envelope; pNL4.3ΔEnv, which lacks gp160; and pNL4.3-

eGFP (X4) and pNL4.3-BaL-eGFP (R5), which encode the enhanced green fluorescent protein (eGFP) gene instead of the *nef* gene (6). Primary HIV-1, drug-resistant HIV-1, HIV-2, and SIV were obtained through the National Institutes of Health (NIH) AIDS Research and Reference Reagent Program and amplified in activated human PBMCs (5 × 10<sup>6</sup> cells) as described previously (5). The following HIV-1 group M viruses were used: (i) 92RW021, a subtype A Env, subtype A, R5 virus (contributed by WHO Network for HIV Isolation and Characterization); (ii) 92UG029, a subtype A Gag, subtype A Env, X4 (syncytium-inducing [SI]) virus (WHO Network for HIV Isolation and Characterization); (iii) 92TH026, a subtype B Gag, subtype B Env, R5 (non-syncytium-inducing [NSI]) virus (the UNAIDS Network for HIV Isolation and Characterization); (iv) 92HT599, a subtype B Env, X4 (SI) virus (Neal Halsey and the Division of AIDS [DAIDS], NIAID); (v) 93IN101, a subtype C Env, R5 (NSI) virus (Robert Bollinger and the UNAIDS Network for HIV Isolation and Characterization and the DAIDS, NIAID); (vi) 98IN017, a subtype C Env, X4 virus (Robert Bollinger and the UNAIDS Network for HIV Isolation and Characterization and the DAIDS, NIAID); courtesy of the NIBSC Center for AIDS Reagents, United Kingdom); (vii) 92UG005, a subtype D Gag, subtype D Env, R5 virus (UNAIDS Network for HIV Isolation and Characterization and the DAIDS, NIAID); (viii) 92UG024, a subtype D Gag, subtype D Env, X4 (SI) virus (UNAIDS Network for HIV Isolation and Characterization and the DAIDS, NIAID); (ix) 92TH006, a subtype E Env, R5 (NSI) virus (UNAIDS Network for HIV Isolation and Characterization and the DAIDS, NIAID); (x) 93TH053, a subtype E Env, X4 virus (UNAIDS Network for HIV Isolation and Characterization and the DAIDS, NIAID); (xi) 93BR029, a subtype F Gag, subtype F Env, R5 (NSI) virus (UNAIDS Network for HIV Isolation and Characterization and the DAIDS, NIAID); (xii) 93BR020, a subtype F Gag, subtype F Env, R5 and X4 (SI) virus (UNAIDS Network for HIV Isolation and Characterization and the DAIDS, NIAID); (xiii) RU132, a subtype G Env, R5 virus (A. Bobkov, D. I. Ivanovsky Institute of Virology, Moscow, Russia; supplied by Jonathon Weber, Imperial College, London, United Kingdom); (xiv) Jv1083, a subtype G Env, R5 virus (A. Abimiku); and (xv) JR-CSF, a laboratory-adapted R5 (NSI) virus (I. Chen). The following drug-resistant viruses were used: HIV-1<sub>74V</sub>/MT-2, a reverse transcriptase (RT) inhibitor-resistant X4 virus (HXB2; contributed by B. Larder, courtesy of the MRC AIDS Directed Program) (abbreviated RT<sub>74V</sub>); HIV-1<sub>LAI-M184V</sub> (lamivudine [3TC]-resistant), an RT inhibitor-resistant X4 virus (LAI; J. Mellors and R. Schinazi) (RT<sub>M184V</sub>); HIV-1 M46I/L63P/V82T/I84V, a protease (PR) inhibitor-resistant R5X4 virus (E. Emimi) (PR<sub>M46I/L63P/V82T/I84V</sub>); and pNL4.3 gp41 (36G) V38A/N42D, a fusion inhibitor (T20)-resistant X4 virus (Trimeris, Inc.) (T20<sub>V38A/N42D</sub>). The following additional retroviruses were used: SIV<sub>mac251/43H</sub> (contributed by R. Desrosiers), SIV<sub>syk</sub> 1.2 (V. Hirsch and P. Johnson), HIV-2<sub>CDC310342</sub> (M. Rayfield for S. Wiktor), and HIV-2<sub>7312A</sub> (F. Gao and B. Hahn).

**Cells.** TZM-bl cells were obtained through the NIH AIDS Research and Reference Reagent Program. TZM-bl cells express CD4, CXCR, and CCR5, which render them susceptible to infection, and contain an integrated *Escherichia coli lacZ* gene driven by the HIV-1 long terminal repeat (LTR) (44). Upon infection, Tat production from the integrated provirus leads to activation of the *lacZ* reporter, resulting in synthesis of β-galactosidase in these cells. Infected cells are identified by enzymatic activity measurement at 48 h postinfection, allowing quantitation after a single round of infection, as described previously (44). Blood-derived CD4<sup>+</sup> T lymphocytes, macrophages, and DC were isolated as described previously (5). Briefly, human PBMCs were purified from fresh blood by banding on Ficoll-Hypaque (30 min at 800 × g at 25°C) (GE Healthcare Bio-Sciences Corp., Piscataway, NJ). Primary human CD4<sup>+</sup> T cells were purified from PBMCs by positive selection with anti-CD4 Dynabeads and subsequent release using Detachabead (Invitrogen Corp., Carlsbad, CA). Cells were cultured in RPMI medium 1640 (Invitrogen Corp., Carlsbad, CA) supplemented with 10% fetal calf serum (FCS; Thermo Fisher Scientific Inc., Waltham, MA), minimal essential medium (MEM) amino acids, L-glutamine, MEM vitamins, sodium pyruvate (Invitrogen Corp., Carlsbad, CA), and penicillin plus streptomycin (Mediatech, Inc., Manassas, VA) and were subsequently activated with bacterial superantigen staphylococcal enterotoxin B (SEB; 100 ng/ml) and mitomycin C-killed PBMCs from another donor (cell ratio of 10:1 PBMCs to CD4<sup>+</sup> cells). Three days after stimulation, cells were split 1:2 in medium containing interleukin-2 ([IL-2] final concentration, 200 units/ml; NIH AIDS Research and Reference Reagent Program). Cultures were then split 1:2 every 2 days in IL-2 medium and infected with HIV-1 at 7 days after stimulation. For generating primary human macrophages, monocytes were purified from human PBMCs by negative selection (Invitrogen Corp., Carlsbad, CA) and activated and cultured at a cell concentration of 10<sup>6</sup> cells/ml in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS (Thermo Fisher Scientific Inc., Waltham, MA), MEM amino acids, L-glutamine, MEM vitamins, sodium pyruvate (Invitro-



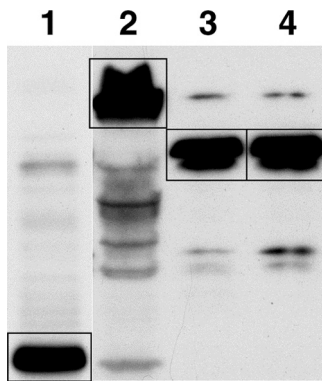


FIG. 1. Western blot analysis of recombinant syndecan-Fc hybrid molecules. Protein A-purified Fc (1  $\mu$ g) (lane 1), syndecan-1-Fc (1  $\mu$ g) (lane 2), syndecan-1-Fc (1  $\mu$ g) pretreated for 2 h at 37°C with a mixture of heparinases I, II and III (10 U) (lane 3), and the heparan sulfate chainless syndecan-1-Fc mutant called  $\Delta$ 3HS-syndecan-1-Fc (lane 4) (1  $\mu$ g) were analyzed by Western blotting using anti-human Fc IgG (1/1,000 dilution). Fc and syndecan-1-Fc proteins (bands) are boxed.

**Syndecan-1-Fc ELISA.** Nunc MaxiSorb eight-well strip plates were coated with anti-syndecan-1 IgG (clone ID4) or control isotype IgG (10  $\mu$ g/ml) for 16 h at 4°C. Wells were washed extensively and blocked in PBS-BSA (1 mg/ml) for 4 h at room temperature. Medium containing (or not) syndecan-1-Fc was added to wells overnight at 4°C. Wells were washed, and captured syndecan-1-Fc molecules were revealed using anti-human Fc-horseradish peroxidase-IgG (1  $\mu$ g/ml). Wells were washed, and *o*-phenylenediamine dihydrochloride substrate was added. After color development, the reaction was stopped with 4N sulfuric acid, and wells (triplicate) were read on a plate reader at 490 nm.

**Cytotoxicity.** Cells were plated in clear-bottom 96-well plates (BD Biosciences, San Jose, CA). Cells were serially diluted from 55,000 cells to 25 cells in 100  $\mu$ l of complete DMEM or RPMI medium. Fifteen microliters of the CellQuant-MTTM reagent (Gentaur, Kampenhout, Belgium) was added, and cells were incubated for 4 h at 37°C. Then, 100  $\mu$ l of the solubilization solution was added, and the plate was shaken for 1 h at room temperature. The  $A_{570}$  was measured on a SpectraMax 384 Plus reader (Molecular Devices, Sunnyvale, CA). A linear relationship was observed between the  $A_{570}$  value and the cell number. The detection limit was estimated to be 950 cells from the blank control. To determine the cytotoxicity of Fc proteins on cells, 55,000 cells were plated per 80- $\mu$ l well in clear-bottom 96-well plates. Cells were treated twice daily with 200  $\mu$ g/ml of syndecan-1-Fc, Fc, or 0.01% saponin (Sigma-Aldrich, St. Louis, MO) for a week. No washes were performed to maintain a continuous exposure of cells to Fc proteins.

## RESULTS

**Construction, expression, amplification, and purification of the syndecan-Fc hybrid molecule.** The ectodomain of human syndecan-1 was subcloned in frame with the Fc domain of human IgG1. For amplification of the expression of Fc and syndecan-1-Fc, a GS amplification system was used that allows selection and amplification in CHO cells in a glutamine-free environment in the presence of methionine sulfoximine, an inhibitor of the GS encoded by the vector (10). For production of stable cell lines expressing Fc and syndecan-1-Fc, CHO cells were transfected with pRSC-GS-Fc and pRSC-GS-syndecan-1-Fc and grown in G-DMEM containing methionine sulfoximine. After 2 to 3 weeks of selection, clone supernatants were analyzed for Fc content by Western blotting using anti-Fc antibodies (Fig. 1; Fc and wild-type syndecan-1-Fc, lanes 1 and 2, respectively). Positive clones were then amplified for 2 to 3 additional weeks, and Fc and syndecan-1-Fc proteins were

batch purified from 1 to 2 liters of cell supernatant by protein A affinity. The rationale for linking syndecan-1 to the Fc of human IgG1 was that the Fc moiety allowed efficient secretion of a syndecan molecule, which harbored heparan sulfate chains, into the extracellular medium and allowed subsequent affinity purification on a protein A column. Moreover, the Fc moiety was extremely useful to conduct ELISA, FACS analysis, and pulldown studies. Syndecan-1 was chosen because it is the most studied syndecan member and because it is highly expressed on genital epithelial cells (4).

**Anti-HIV-1 spectrum of the syndecan-Fc hybrid molecule.** We first tested the ability of the syndecan-Fc hybrid molecule to block the infection of CD4<sup>+</sup> CXCR4<sup>+</sup> CCR5<sup>+</sup> HeLa TZM-bl reporter ( $\beta$ -galactosidase) cells by three distinct categories of viruses. The first category includes HIV-1 isolates representative of various HIV-1 subtypes with different coreceptor usage, either CCR5 (R5 viruses) or CXCR4 (X4 viruses). The second category includes HIV-1 isolates that are resistant to reverse transcriptase (RT), protease (PR), or fusion (T20) inhibitors. The third category is other retroviruses such as HIV-2 and SIV isolates. Viruses were added to TZM-bl cells together with Fc or syndecan-1-Fc for 4 h; cells were washed, and infection was measured 48 h after infection as  $\beta$ -galactosidase activity. Importantly, we found that syndecan-1-Fc, but not Fc, efficiently blocks infection of all HIV-1, HIV-2, and SIV isolates (Table 1).

**The syndecan-Fc hybrid molecule blocks HIV-1 infection of CD4<sup>+</sup> T lymphocytes, macrophages, and DC.** An ideal microbicide should inhibit HIV-1 infection of primary *in vivo* targets of HIV-1. We thus asked whether syndecan-1-Fc could block HIV-1 infection in human primary CD4<sup>+</sup> T lymphocytes, macrophages, and DC. Cells were exposed to HIV-1 (R5 JR-CSF) together with syndecan-1-Fc or Fc control. Twenty-four hours postinfection, cells were washed, and viral replication was monitored by measuring the amount of HIV-1 capsid protein present in the cell culture supernatant by p24 ELISA every 2 days for up to 10 to 14 days thereafter. Remarkably, syndecan-1-Fc, but not Fc, blocked HIV-1 infection of all three *in vivo* cell targets of HIV-1 (Fig. 2A). We conducted similar inhibitory experiments using increasing concentrations of Fc or syndecan-1-Fc rather than a fixed concentration of Fc proteins. IC<sub>50</sub> and IC<sub>90</sub> values are presented in Table 2. Importantly, syndecan-1-Fc exhibited no cytotoxic effect on target cells (Fig. 2B). We used the natural detergent saponin as positive control for cytotoxicity (Fig. 2B). These results suggest that the syndecan-Fc hybrid molecule has the potential to specifically prevent infection of T cells, macrophages, and DC during the early steps of HIV-1 transmission.

**Antiviral mechanism of action of the syndecan-Fc hybrid molecule.** After demonstrating the broad potency of the syndecan-Fc hybrid molecule at blocking HIV-1 infection of its primary *in vivo* targets, we examined its mechanisms of antiviral action. Since we along with others previously reported that cell surface HSPG including syndecans facilitate the initial adsorption of the virus onto target cells (16, 25, 29, 30, 33), we asked whether syndecan-1-Fc interferes with HIV-1 adsorption onto CD4<sup>+</sup> T lymphocytes and macrophages. To address this issue, HIV-1 (JR-CSF) together with Fc or syndecan-1-Fc was added to CD4<sup>+</sup> T lymphocytes and macrophages. After 2 h at 4°C, cells were washed and lysed, and amounts of attached



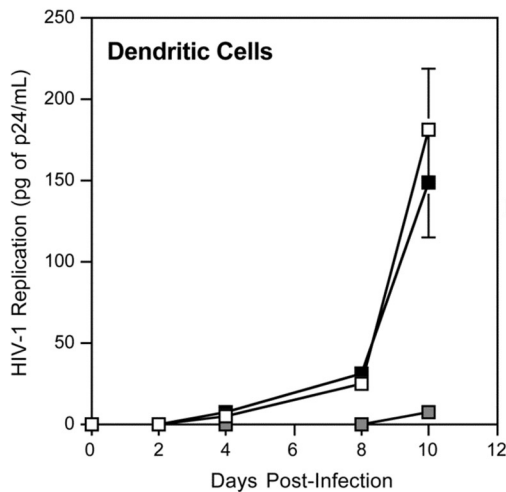
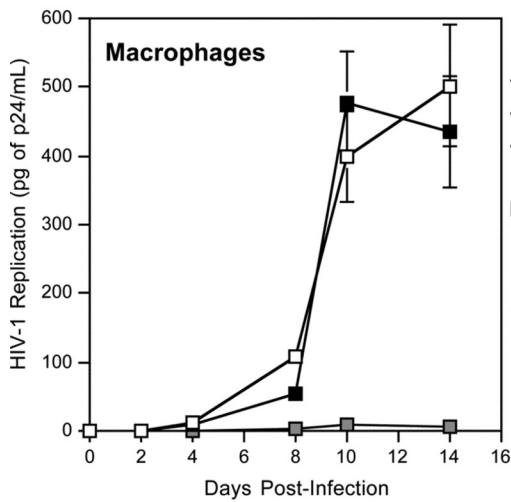
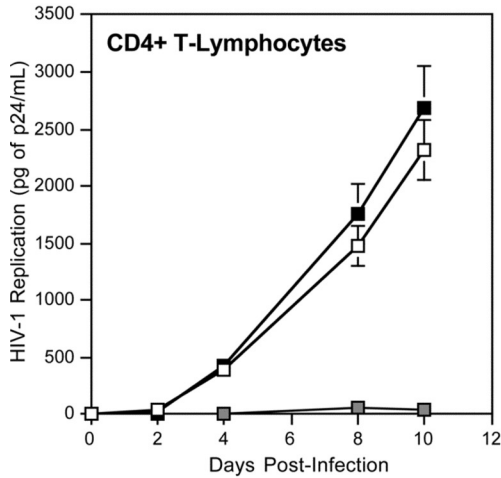
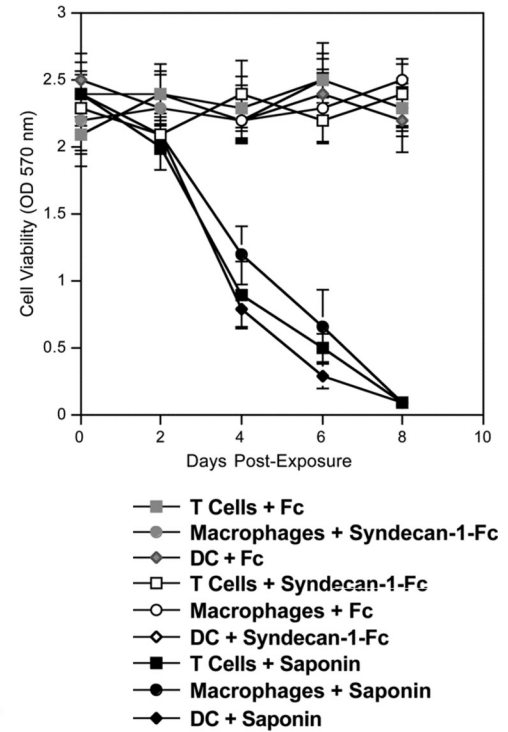
**A.****B.**

FIG. 2. The syndecan-Fc hybrid molecule blocks HIV-1 infection of primary *in vivo* cell targets. (A) Blood-derived CD4<sup>+</sup> T lymphocytes, macrophages, or DC ( $0.5 \times 10^6$  cells) were exposed to JR-CSF (1 ng of p24) and syndecan-1-Fc or control Fc (5  $\mu$ g/ml) for 1 day at 37°C and washed. The amount of virus released was monitored by measuring amounts of capsid protein in cell supernatants by p24 ELISA. The points are median values, and error bars represent standard errors of triplicates. The *P* values were calculated using a one-sided *t* test assuming equal variances. d, day. (B) Cells were serially diluted from 55,000 cells to 25 cells in 100  $\mu$ l of complete medium. Fifteen microliters of the CellQuant-MTTM reagent was added, and cells were incubated for 4 h at 37°C. Then, 100  $\mu$ l of the solubilization solution was added, and the plate was shaken for 1 h at room temperature. The  $A_{570}$  was measured on a SpectraMax 384 Plus reader. To determine the cytotoxicity of Fc proteins, cells (55,000) were treated twice daily with 200  $\mu$ g/ml of syndecan-1-Fc, Fc, or 0.01% saponin for a week. No washes were performed to maintain a continuous exposure of cells to Fc proteins. OD, optical density.



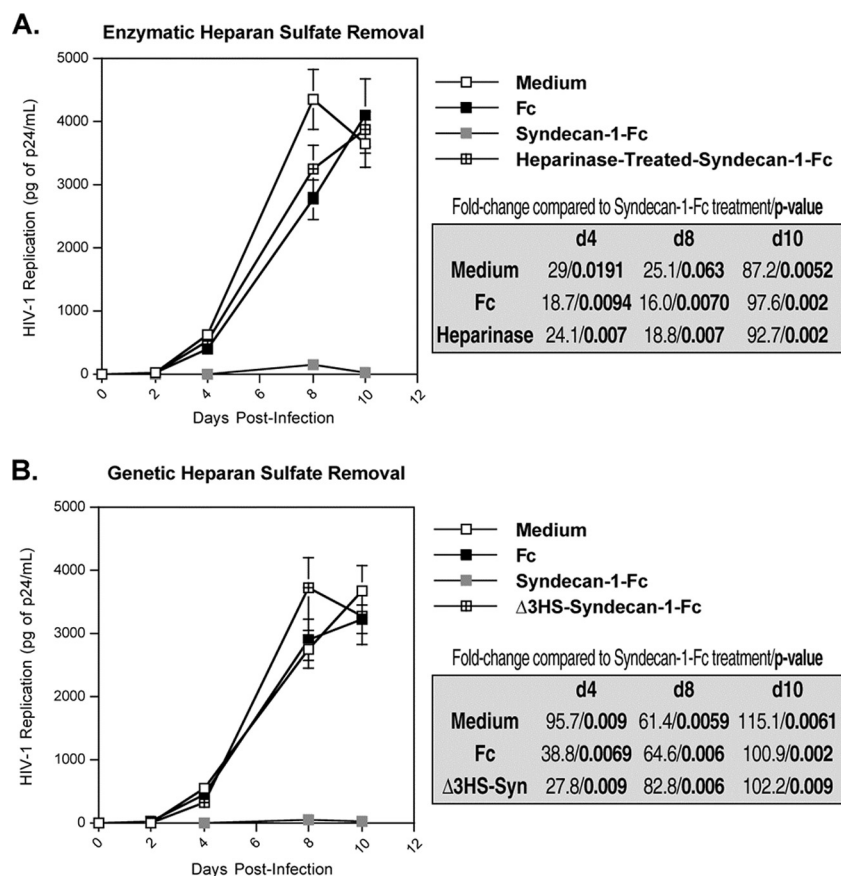


FIG. 4. HS chain removal abrogates the anti-HIV activity of the syndecan-Fc hybrid molecule. (A) Blood-derived  $CD4^+$  T lymphocytes ( $0.5 \times 10^6$  cells) were exposed to JR-CSF (1 ng of p24) and untreated syndecan-1-Fc, heparinase-treated syndecan-1-Fc, or control Fc (5  $\mu\text{g}/\text{ml}$ ) for 1 day at  $37^\circ\text{C}$  and washed. The amount of virus released was monitored by measuring amounts of p24 capsid protein in cell supernatants by ELISA. (B) The same experiment as in panel A was performed except that the  $\Delta 3HS$ -syndecan-1-Fc, which is devoid of its HS chains after mutagenesis of its three glycosaminoglycan attachment sites, was tested for anti-HIV-1 activity. The points are median values, and error bars represent standard errors of triplicates. The  $P$  values were calculated using a one-sided  $t$  test assuming equal variances.

the experiment since we found that adding the fusion protein 1 h before adding the virus to cells or at the same time similarly inhibited HIV-1 infection (Fig. 5). In the absence of Fc proteins, HIV-1 efficiently transmigrated through PGEC, in contrast to a virus lacking gp160 (Fig. 6A), suggesting that the viral glycoprotein is absolutely required for transcytosis and that the PGEC layer does not allow nonspecific transmigration of viruses. Amounts of transcytosed viruses were quantified by measuring amounts of particulate capsid in the basal chamber. Remarkably, syndecan-1-Fc, but not Fc, prevents HIV-1 transmigration through PGEC (Fig. 6A). We obtained similar results when we applied syndecan-1-Fc together with virus addition (data not shown). Importantly, syndecan-1-Fc exhibited no cytotoxic effect on PGEC (Fig. 6C).

We then examined the capacity of the syndecan-Fc hybrid molecule to prevent DC-mediated transmission of HIV-1. We took advantage of a replication-defective virus (NL4.3 $\Delta$ Env-eGFP), which does not encode Env but which has been pseudotyped with HIV-1 Env (6). Pseudotyped viruses can infect cells because they contain Env. However, cells that have been infected by pseudotyped viruses cannot produce infectious viruses because *de novo* viruses do not encode Env. Thus, the use of pseudotyped viruses allowed the analysis of the

effect of the syndecan-Fc hybrid molecule on the transmission of infectious particles from DC to T cells independently of DC infection. DC were incubated with wild-type NL4.3-eGFP and NL4.3-BaL-eGFP or pseudotyped NL4.3 $\Delta$ Env-eGFP/gp160 Env viruses. Two hours later, at which time the entry of the virus into DC is completed (18), syndecan-1-Fc or control Fc was added. After 2 h, DC were washed to remove both free virus and Fc proteins. To measure DC-T-cell transmission, Jurkat T cells were added for 3 days, and the percentage of infected T cells (gated with an anti-CD3 antibody) was analyzed by FACS. Only pseudotyped viruses that have been rapidly transferred from DC to T cells through the virological synapse (independently of DC infection) can infect T cells. Indeed, pseudotyped viruses that infect DC can no longer infect T cells because they do not encode Env. Because DC were washed before T cells were added, the T-cell infection by the pseudotyped virus observed in Fig. 6B could arise only from pseudotyped particles that were transferred from DC to T cells. Importantly, syndecan-1-Fc added to DC prevents subsequent T-cell infection with the pseudotyped virus (Fig. 6B). We obtained similar results when syndecan-1-Fc was added to DC 1 h before virus was added (data not shown). This finding suggests that syndecan-1-Fc neutralizes HIV-1 either before



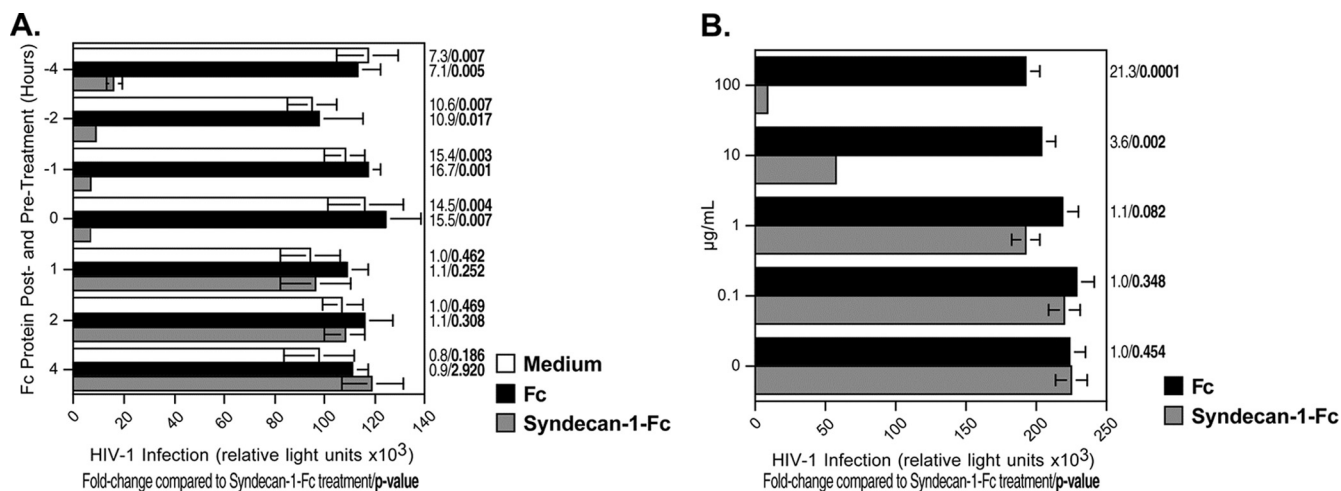


FIG. 5. Antiviral effect of the syndecan-Fc hybrid molecule before, during, or after virus exposure. (A) Syndecan-1-Fc or control Fc (5 µg/ml) was added to TZM-bl cells 1, 2, or 4 h before (negative values on the y axis) or after (positive values on the y axis) the addition of HIV-1 (R5 JR-CSF) (1 ng of p24) or together (time zero) with the virus. Infection was measured 48 h after infection as β-galactosidase activity. (B) Activated PBMCs (200,000 cells) were infected with JR-CSF (500 pg of p24) for 3 days, washed three times to remove cell-free virus, and added to TZM-bl cells in the presence of increasing concentrations of syndecan-1-Fc or control Fc (0.1 to 100 µg/ml) diluted in a pool ( $n = 6$ ) of seminal plasma. Infection was scored as above. The points are median values, and error bars represent standard errors of triplicates. The  $P$  values were calculated using a one-sided  $t$  test assuming equal variances.

DC infection or during DC transfer to T cells. Thus, syndecan-1-Fc interferes with HIV-1 transmission at multiple levels: genital epithelial transmigration, DC infection or transfer to T cells, and T cell infection.

**Antiviral activities of the syndecan-Fc hybrid molecule are preserved after low pH and genital fluid exposure.** An ideal microbicide should maintain its potency in the genital environment. We thus examined the inhibitory effect of the syndecan-Fc hybrid molecule under conditions that mimic the genital environment, such as exposure to low pH and genital fluids. We first examined the antiviral effect of the syndecan-Fc hybrid molecule at various pHs. Syndecan-1-Fc and Fc were incubated at pH 2 to 9 and tested for their antiviral activities. As expected, syndecan-1-Fc treated at a neutral pH (pH 7) neutralizes HIV-1. Importantly, syndecan-1-Fc maintained its potency in a basic (pH 8) or acidic (pH 4) environment (Fig. 7A), suggesting that antiviral activities of the syndecan-Fc hybrid molecule are preserved at low pH. We then examined the antiviral effect of the syndecan-Fc hybrid molecule in seminal plasma, cervical fluid, and vaginal mucus. Seminal plasma was separated from sperm by centrifugation at  $500 \times g$  for 10 min. The pH was determined before and after the seminal plasma was mixed with syndecan-1-Fc or Fc diluted in PBS. We found that the original pH of seminal plasma is approximately 8.0 to 8.05 ( $n = 8$ ). Efficacy of syndecan-1-Fc against HIV-1 in the presence of seminal plasma was conducted as follows. Fifty microliters of serially 2-fold diluted syndecan-1-Fc in DMEM was mixed with 50 µl of JR-CSF and 50 µl of either seminal plasma or DMEM. The mixtures were added to TZM-bl indicator cells, and infection was scored. Similar studies were conducted with cervical fluid and vaginal mucus. Importantly, syndecan-1-Fc maintains its potent antiviral effect when diluted in each of these genital fluids (Fig. 7B). Together, these features are important with regard to the future use of the syndecan-Fc hybrid molecule as a microbicide.

**The syndecan-Fc hybrid molecule blocks HSV infection.** Since genital herpes increases transmission of HIV-1 (17), an ideal microbicide should inhibit HIV-1 as well as HSV infection. We thus investigated whether the syndecan-Fc hybrid molecule could also block HSV infection. To address this issue, Vero cells were exposed to HSV-1-GFP or HSV-2-GFP (multiplicity of infection [MOI] of 0.1) in the presence of syndecan-1-Fc or Fc. After 2 days, HSV infection was scored by FACS by measuring the percentage of GFP-positive (GFP<sup>+</sup>) Vero cells. Importantly, we found that syndecan-1-Fc, but not Fc, inhibits HSV-1 and HSV-2 infection (Fig. 8A). It is likely that syndecan-1-Fc blocks HSV infection by interfering with the interaction between HSV glycoproteins and entry receptors on Vero cells. Since HSV exploits HS proteoglycans to enhance its infection (7), it is likely that syndecan-1-Fc blocks HSV infection by interfering with the attachment of HSV to cell surface HS expressed on Vero cells (i.e., syndecans). Supporting this hypothesis, HS removal by enzymatic (heparinase treatment) or genetic ( $\Delta 3$ HS-syndecan-1-Fc) manipulation abrogates the anti-HSV effect of the syndecan-Fc hybrid molecule (Fig. 8A). As above (Fig. 2B, 5B and 6B), syndecan-1-Fc exhibited no cytotoxic effect on Vero cells (Fig. 8B). Dual neutralization of HIV-1 and HSV infection represents an additional microbicidal property for the syndecan-Fc hybrid molecule.

## DISCUSSION

In this study, we report that a syndecan-Fc hybrid molecule has potent antiviral activity against HIV-1. Interestingly, many antiviral properties of syndecan-1-Fc satisfy important preconditions for the use of syndecan-1-Fc as a potential microbicide. Syndecan-1-Fc (i) blocks HIV-1 infection of primary targets including T cells, macrophages and DC; (ii) exhibits a broad range of antiviral activity against primary HIV-1 isolates, multidrug resistant HIV-1 isolates, HIV-2, and SIV; (iii) prevents

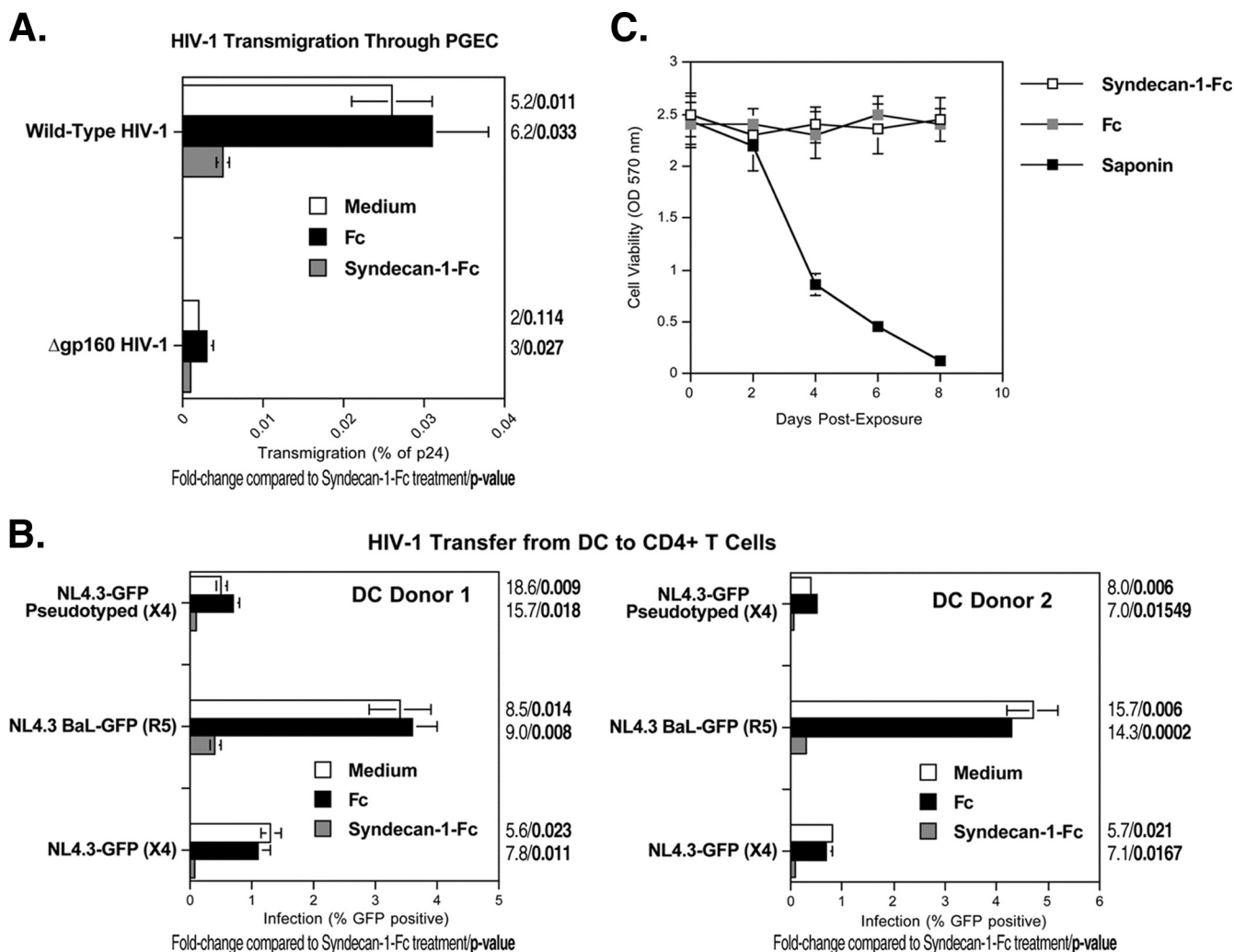


FIG. 6. The syndecan-Fc hybrid molecule inhibits both HIV-1 transmigration and HIV-1 transfer from DC to T cells. (A) Pseudotyped NL4.3ΔEnv/gp160 R5 Env virus (25 ng of p24) or Gp160-deficient NL4.3ΔEnv virus (25 ng of p24) was added to the apical surface of PGEC. Syndecan-1-Fc or control Fc (5 μg/ml) was added to PGEC 30 min before the virus was added. Amounts of transcytosed viruses in the basal chamber were quantified by p24 ELISA. Results are expressed as a percentage of p24 of the original inoculum. The points are median values, and error bars represent standard errors of triplicates. (B) DC (100,000 cells) were incubated for 2 h at 37°C with wild-type NL4.3-eGFP (X4 virus) and NL4.3-BaL-eGFP (R5 virus) or with the pseudotyped NL4.3ΔEnv-eGFP/gp160 X4 Env virus (25 ng of p24). Syndecan-1-Fc or control Fc (5 μg/ml) was added 2 h later. DC were washed 2 h after the Fc proteins were added, Jurkat T cells (100,000 cells) were added for 3 days, and the percentage of infected Jurkat T cells was analyzed. The percentage of HIV-infected Jurkat T cells (gated by using an anti-CD3 antibody) in DC-T-cell coculture was analyzed by GFP expression on day 5. Error bars represent standard errors of duplicates. (C) The experiment is as described in the legend of Fig. 2B.

transmigration of HIV-1 through primary human genital epithelial cells; (iv) prevents HIV-1 transfer from DC to T cells; (v) is potent when added 2 h prior to addition of HIV-1 to cells; (vi) is potent at a low pH; (vii) efficiently blocks HIV-1 infectivity when diluted in genital fluids; and (viii) blocks HSV infection. Thus, this syndecan-Fc hybrid molecule represents the prototype of a new generation of microbicidal agents that may have promise for HIV-1 prevention.

We previously showed that HIV-1 binds to cell surface syndecans via a highly conserved residue (Arg298) within the V3 region of gp120 (11). Interestingly, the same residue is critical for gp120 binding to the CCR5 chemokine receptor (42). It is thus likely that the syndecan-Fc hybrid molecule blocks HIV-1 infection by interacting with Arg298 and, therefore, by inter-

fering with both viral adsorption and coreceptor-mediated entry. Since compounds that interrupt gp120-syndecan interactions have never been exploited as microbicides, they represent a novel class of microbicides and thus differ from existing tools. One advantage of using compounds that target gp120-syndecan interactions as microbicides is that all primary R5, X4, and R5X4 HIV-1 as well as HIV-2 isolates bind syndecans. Thus, these compounds will have a broader impact than, for example, RANTES derivatives, which neutralize R5 viruses only (24). Since we found that compounds that block gp120-syndecan interactions also block gp120-CCR5 interactions (11), their dual inhibitory effects represent another advantage of using them as microbicides. Supporting this notion, we found that the syndecan-Fc hybrid molecule blocks a broad spectrum of

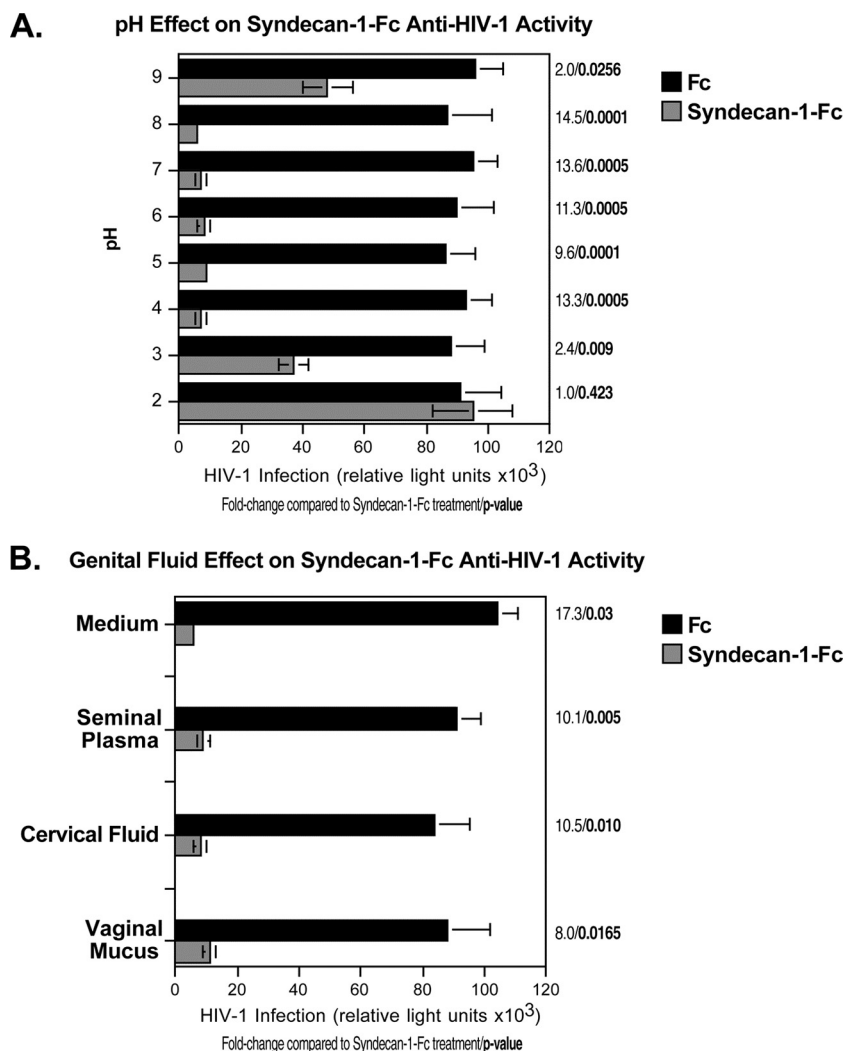


FIG. 7. The syndecan-Fc hybrid molecule is still active after low pH and genital fluid exposure. (A) TZM-bl cells (20,000 cells) were exposed to JR-CSF (100 pg of p24). Syndecan-1-Fc or control Fc was treated for 8 h at various pHs and added to cells (final concentration of 5  $\mu\text{g/ml}$ ) together with virus. Infection was measured 48 h postinfection by  $\beta$ -galactosidase activity. (B) Syndecan-1-Fc or control Fc was diluted in medium, a pool ( $n = 6$ ) of seminal plasma, a pool of cervical fluid ( $n = 8$ ), and a pool ( $n = 4$ ) of vaginal mucus. The dilutions were then added to TZM-bl cells together with HIV-1 (100 pg of p24). Infection was measured 48 h postinfection by  $\beta$ -galactosidase activity. Results are expressed as relative light units. The points are median values, and error bars represent standard errors of triplicates. The  $P$  values were calculated using a one-sided  $t$  test assuming equal variances.

X4, R5, and R5X4 viruses. Although it is likely that *in vivo* syndecan-1-Fc-resistant HIV-1 variants should arise, we were unsuccessful at developing such resistant variants *in vitro*. Nevertheless, it is important to emphasize that the “perfect” microbicide will likely be composed of several anti-HIV-1 agents that target viral proteins other than gp120 (i.e., RT) and therefore should neutralize emerging syndecan-1-Fc-resistant HIV-1 variants. Another advantage of using a syndecan-Fc hybrid molecule as a microbicide is that many sexually transmitted pathogens also exploit syndecans for host colonization, such as HSV (7), HPV (13, 37), and *Neisseria gonorrhoeae* (15, 41). Supporting this hypothesis, we found that the syndecan-Fc hybrid molecule efficiently blocks HSV infection (Fig. 8).

One cannot exclude the possibility that syndecan-1-Fc has adverse effects in the vaginal environment after its topical administration. One can envision that the hybrid molecule, by

capturing specific cytokines or chemokines via its HS chains, enhances the suppression of immune activation and increases the inflammation state mediated by HIV-1 (9). Similarly, syndecan-1-Fc may play a detrimental role during HIV-1 transmission by capturing and neutralizing cationic antimicrobial polypeptides (i.e., defensins), which are the principal effector molecules of mucosal innate immunity against microbes and viruses such as HIV-1 (8). However, we do not anticipate that syndecan-Fc (60 to 90 kDa), which is extremely soluble and does not make aggregates even at high concentrations, would cause discomfort to humans or animals. Indeed, the ectodomain of syndecans, which we fused to an IgG1 Fc portion, is constitutively and constantly shed by human cells without affecting cell viability (1). The extracellular domain of syndecan-1 is proteolytically cleaved at a juxtamembrane site by a tissue inhibitor of metalloprotease-3-sensitive metalloprotein-

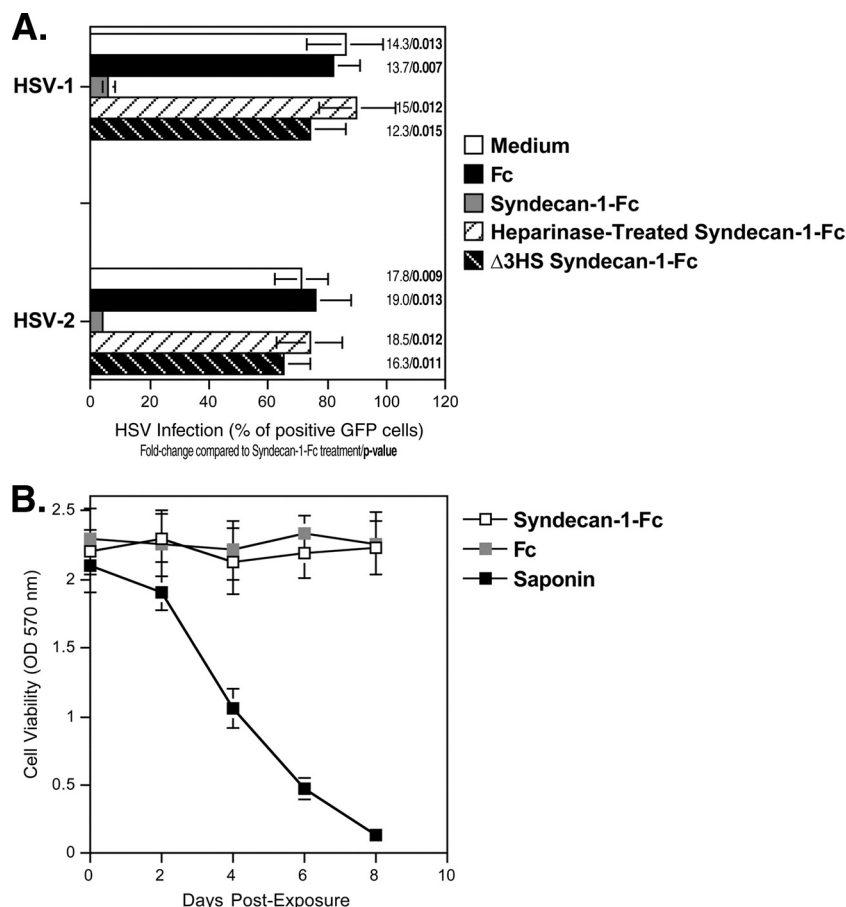


FIG. 8. The syndecan-Fc hybrid molecule blocks HSV infection. Vero cells (250,000 cells) were exposed to HSV-1-GFP or HSV-2-GFP (MOI of 0.1) together with control Fc, syndecan-1-Fc, heparinase-treated syndecan-1-Fc, or  $\Delta$ 3HS syndecan-1-Fc lacking HS chains (5  $\mu$ g/ml). After 2 days, Vero cell infection was scored by FACS by quantifying the percentage of GFP<sup>+</sup> cells. Data are expressed in percentage of GFP<sup>+</sup> cells. The points are median values, and error bars represent standard errors of triplicates. The *P* values were calculated using a one-sided *t* test assuming equal variances. (B) The experiment is as described in the legend of Fig. 2B.

ases in response to a variety of physiological stimulators in a process known as shedding (43). Shedding converts syndecan-1 from a membrane-bound coreceptor into a soluble effector capable of binding the same ligands. Thus, the nature of syndecan-1-Fc (ectodomain of existing human receptors fused to human antibody fragments) significantly diminishes the risk of adverse effects.

Another disadvantage of using syndecan-1-Fc as a topical microbicide is that its antiviral effect wanes in 4 h (Fig. 5). Interestingly, we found by FACS analysis that syndecan-Fc binds efficiently to HeLa cells (data not shown). It is likely that syndecan-Fc binds to HeLa cells by binding either to Fc receptors expressed on HeLa cells or to cytokines, chemokines, or growth factors bound onto the surface of HeLa cells. An adequate formulation of syndecan-1-Fc allowing a slow release of the fusion protein should, hopefully, partially alleviate this obstacle. Since syndecan-1-Fc loses its antiviral effect when added to cells 1 h after virus exposure, it would therefore need to be applied shortly before coitus and would not have long-lasting effects like other potential microbicides.

A series of microbicides has been developed (i.e., sulfated and sulfonated polymers) that most likely work similarly to

syndecan-1-Fc. Importantly, a majority of these microbicidal candidates (carrageenan [the active ingredient in Carraguard], cellulose sulfate, PRO-2000, and dextran sulfate) failed to provide protection in humans (27, 40). It has been proposed that this failure arises from a limited *in vitro* potency of these sulfated compounds against R5 viruses, the most commonly transmitted strains of HIV-1 (27, 40). Moreover, evidence emerged that cellulose sulfate enhances HIV-1 infection *in vitro*, particularly of R5 viruses (27, 40). Thus, although syndecan-1-Fc efficiently blocks both X4 and R5 viruses and does not promote HIV-1 infection, it is still possible that the topical administration of syndecan-1-Fc *in vivo* would not provide any protection. Importantly, syndecan-1-Fc is currently being tested in a vaginal HIV-1 transmission model in macaque. Indeed, we believe that any microbicide candidate should demonstrate proof of both safety and efficacy in a macaque model before being considered for human trials. Evidently, success in a macaque model would not guarantee that syndecan-1-Fc will protect women, but a failure to protect monkeys would be considered a serious sign that it lacks potency.

Another concern with regard to the future development of syndecan-1-Fc as a microbicide is that it exhibits a potent

antiviral activity only when used at a  $\mu\text{g/ml}$  range (Tables 1 and 2). To avoid having to use too high a concentration of syndecan-1-Fc *in vivo*, we propose to optimize the potency of the Fc hybrid molecule. We previously reported that the degree of sulfation of heparan sulfates directly correlates with their potential to neutralize HIV-1 (3). Specifically, we showed that the removal of the 6-O sulfation dramatically decreases the anti-HIV-1 effect of heparan sulfate analogs, whereas oversulfation significantly increases their inhibitory effect (3). Thus, with regard to future optimization of syndecan-1-Fc, we will examine whether manipulating the degree of sulfation of syndecan-1-Fc would improve its potency and therefore would diminish the amounts of syndecan-1-Fc needed for protection.

Protein microbicides such as syndecan-1-Fc could represent promising alternatives to the current generation of small-molecule drugs. However, producing and distributing such proteins in key areas of HIV endemicity are difficult and expensive because of the lack of a fermentor and a cold chain. Interestingly, many therapeutic proteins have been expressed in plants, including a large number of vaccine candidates and various recombinant antibody formats (32). The most important benefits of using plants such as maize seeds over fermentor-based systems include the enhanced stability of recombinant proteins accumulating in the endosperm, which means that a cold chain is not necessary for product distribution (32), as well as the economy of large-scale production, which will make the product more affordable. In other words, if syndecan-1-Fc provides protection in a vaginal SHIV transmission model in macaques, the production of syndecan-1-Fc in plants should be considered.

In conclusion, this study demonstrates that syndecan-1-Fc possesses numerous microbicidal *in vitro* properties against HIV-1. Specifically, syndecan-1-Fc can block HIV-1 transmission at multiple levels: *trans*-epithelial migration, DC-mediated transfer to T cells, and *cis* infection of CD4<sup>+</sup> T lymphocytes, macrophages, and DC. Future safety and efficacy *in vivo* studies using humanized mice and macaque vaginal simian-human immunodeficiency virus (SHIV) transmission models will determine whether syndecan-Fc hybrid molecules truly represent prototypes of a new generation of microbicidal agents against HIV-1.

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