Spermatozoa capture HIV-1 through heparan sulfate and efficiently transmit the virus to dendritic cells

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Semen is the main vector for HIV-1 dissemination worldwide. It contains three major sources of infectious virus: free virions, infected leukocytes, and spermatozoa-associated virions. We focused on the interaction of HIV-1 with human spermatozoa and dendritic cells (DCs). We report that heparan sulfate is expressed in spermatozoa and plays an important role in the capture of HIV-1. Spermatozoa-attached virus is efficiently transmitted to DCs, macrophages, and T cells. Interaction of spermatozoa with DCs not only leads to the transmission of HIV-1 and the internalization of the spermatozoa but also results in the phenotypic maturation of DCs and the production of IL-10 but not IL-12p70. At low values of extracellular pH (~6.5 pH units), similar to those found in the vaginal mucosa after sexual intercourse, the binding of HIV-1 to the spermatozoa and the consequent transmission of HIV-1 to DCs were strongly enhanced. Our observations support the notion that far from being a passive carrier, spermatozoa acting in concert with DCs might affect the early course of sexual transmission of HIV-1 infection.

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Abbreviations used: BMA, biotin-labeled mannose coupled to albumin; DC-SIGN, DC-specific intercellular adhesion molecule 3-grabbing nonintegrin; HS, heparan sulfate; MFI, mean fluorescence intensity; MR, mannose receptor; PLC, phospholipase C.

The UNAIDS/World Health Organization AIDS epidemic update estimated that 33 million people were living with HIV at the end of 2007. New infections have been occurring in the past few years at a rate of 3.2 million per year, and almost 2 million individuals succumbed to AIDS-related diseases in 2007 (UNAIDS, 2007). Most infections are acquired through sexual transmission during vaginal or anal intercourse, with semen being the major transmission vector for HIV-1 (Miller and Shattock, 2003; Pope and Haase, 2003; Haase, 2005; Lederman et al., 2006).

Semen contains three major sources of infectious virus: free virions, spermatozoa-associated virions, and infected leukocytes (Miller and Shattock, 2003; Gupta and Klasse, 2006; Lederman et al., 2006; Hladik and McElrath, 2008). The role of each of these sources in sexual transmission of HIV-1 is not well defined. Few studies have addressed this important question. Free virus and seminal infected leukocytes appear to play an important role in sexual transmission of HIV-1 (Miller and Shattock, 2003; Gupta and Klasse, 2006; Lederman et al., 2006; Hladik and McElrath, 2008). The role of spermatozoa, however, has been a matter of debate (Mermin et al., 1991; Dussaix et al., 1993; Quayle et al., 1997; Pudney et al., 1998), in spite of the fact that the presence of viral particles and/or nucleic acids in spermatozoa from HIV-1–infected men has been largely demonstrated using a variety of techniques (Baccetti et al., 1994, 1998; Bagasra et al., 1994; Nuovo et al., 1994; Dulioust et al., 1998; Muciaccia et al., 1998, 2007; Barboza et al., 2004).

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The identity of the receptor for HIV-1 expressed in the spermatozoa remains unclear. On the basis of its ability to recognize the HIV gp120, it has been proposed that mannose receptors (MRs) might function as HIV-1 receptors on the spermatozoa (Bandivdekar et al., 2003; Cardona-Maya et al., 2006; Fanibunda et al., 2008). It has also been shown that the gp120 can be recognized by galactosyl-alkyl-acylglycerol, a glycolipid expressed on the spermatozoa, suggesting that, as described for keratinocytes and epithelial cells, this molecule also contributes to the attachment of HIV-1 to the spermatozoa (Brogi et al., 1996, 1998).

After deposition of HIV-1 on the mucosa, the virus must cross the mucosal epithelium to interact with T $CD4^+$ lymphocytes, macrophages, and DCs, which are the most important targets of infection (Mermin et al., 1991; Miller and Shattock, 2003; Haase, 2005; Gupta and Klasse, 2006; Lederman et al., 2006; Hladik and McElrath, 2008). These cells express the HIV-1 receptors CD4 and coreceptors CCR5 or CXCR4 which are required for infection (Haase, 2005; Gupta and Klasse, 2006; Lederman et al., 2006; Hladik and McElrath, 2008). It is now clear that DCs are able to capture HIV-1 at entry sites and transport the virus to draining lymph nodes, where HIV-1 is efficiently transmitted to T $CD4^+$ cells, which become the center of viral replication (Geijtenbeek et al., 2000; Gurney et al., 2005; Wilkinson and Cunningham, 2006; Wu and KewalRamani, 2006).

The pathways used by HIV-1 to cross the mucosal epithelium are not well defined. The virions may transcytose through the genital epithelium (Gupta and Klasse, 2006; Hladik and McElrath, 2008) or may pass the barrier through genital lesions, either as cell-free or cell-associated virus (Piot and Laga, 1989; Serwadda et al., 2003; Galvin and Cohen, 2004). The latter possibility could account for the association of HIV infections with sexually transmitted diseases (Miller and Shattock, 2003; Haase, 2005; Lederman et al., 2006). It is of note that epithelial microabrasions in the vagina are detected in 60% of healthy women after consensual intercourse (Norvell et al., 1984; Guimarães et al., 1997), suggesting that the presence of genital microlesions represent a frequent scenario for the transmission of HIV-1. Anal intercourse is also often associated with mucosal trauma and, because the rectal epithelium is only one cell layer thick, it provides a low degree of protection against trauma, favoring the access of virus to the underlying target cells (Shattock and Moore, 2003). Moreover, the access of HIV-1 to target cells may be facilitated by other mechanisms such as the binding of HIV-1 to DC projections that extend to the luminal surface (Pope and Haase, 2003; Haase, 2005; Wu and KewalRamani, 2006; Sharkey et al., 2007). Transmission may also be favored by the induction of a local inflammatory response triggered by semen (Pandya and Cohen 1985; Thompson et al., 1992; Berlier et al., 2006; Sharkey et al., 2007).

In this paper, we analyze the interaction of HIV-1 with human spermatozoa and the ability of spermatozoa to transmit the virus to immature DCs. We found that spermatozoa capture HIV-1 and efficiently transmit the virus to DCs through a mechanism that requires cell-to-cell contacts. DC–spermatozoa contacts lead to the phenotypic maturation of the DCs and the production of IL-10 (but not IL-12). Acidic values of extracellular pH similar to those found in the vaginal mucosa after sexual intercourse markedly increased both the attachment of HIV-1 to the spermatozoa and the consequent transmission of HIV-1 to DCs. Our results suggest that spermatozoa-associated virus may play a central role in the sexual transmission of HIV-1.

RESULTS

Spermatozoa capture HIV-1 through heparan sulfate (HS)

In a first set of experiments, we analyzed the capture of HIV-1 BAL (R5 tropic) and HIV-1 IIIB (X4 tropic) by human spermatozoa. Fig. 1 (A and B) shows that spermatozoa capture both strains in a similar fashion. Fig. 1 A also shows that

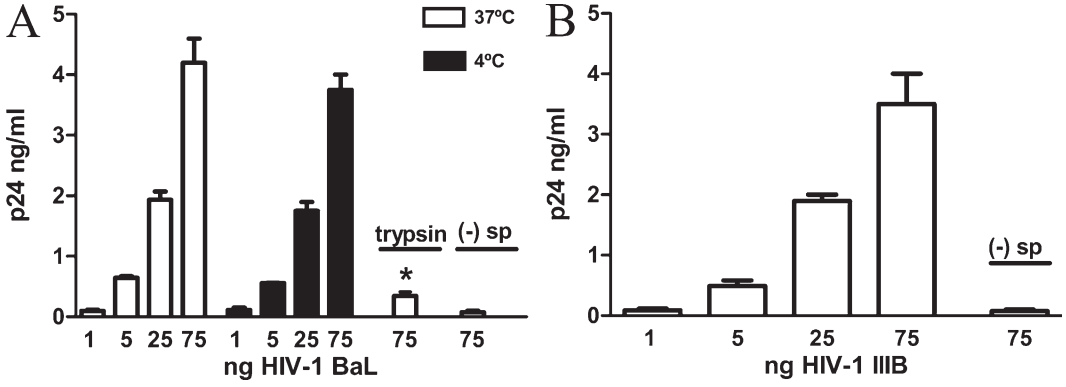

Figure 1. Capture of HIV-1 by human spermatozoa. Spermatozoa (1.5×10^6/200 µl) were incubated with different amounts of HIV-1 BAL (R5-tropic; A) or HIV-1 IIIB (X4-tropic; B) for 60 min at 37 or 4°C. Cells were then washed thoroughly, lysed, and assayed for p24 antigen by ELISA. Results are the mean ± SEM of six to eight experiments performed in triplicate. In A, spermatozoa was cultured with HIV-1 BAL containing 75 ng p24 for 60 min at 37°C, washed thoroughly, treated with 1,000 U/ml trypsin for 15 min at 37°C, lysed, and assayed for p24 antigen by ELISA. (−) sp represents the values of p24 found in wells in which spermatozoa were omitted (nonspecific attachment of HIV-1 to the wells). The asterisk represents statistical significance ($P < 0.05$ vs. untreated spermatozoa incubated with 75 ng HIV-1 BAL).

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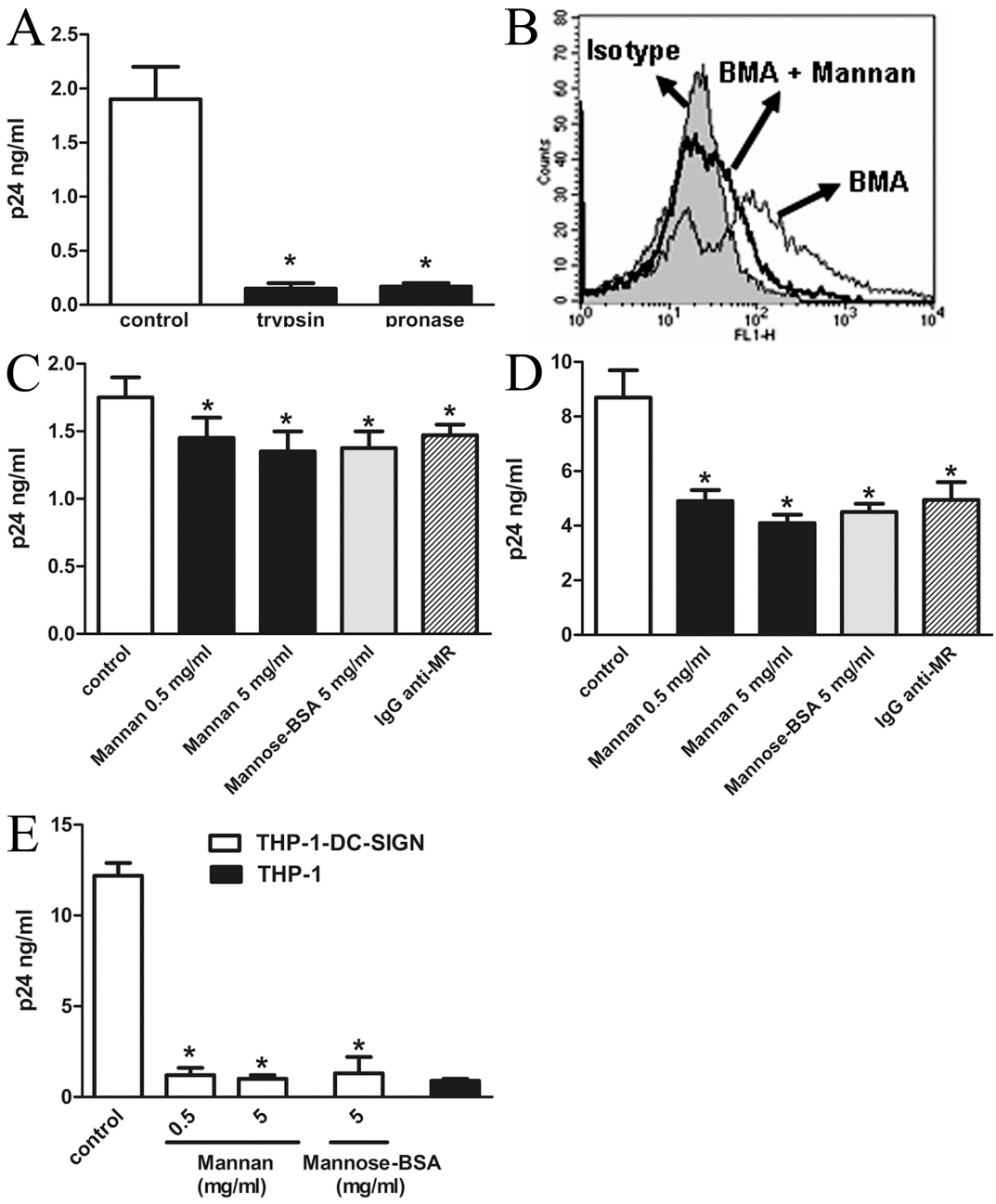

Figure 2. MRs do not play a major role in the capture of HIV-1 by spermatozoa. (A) Spermatozoa were treated with 1,000 U/ml trypsin or 20 µg/ml pronase for 15 min at 37°C, washed thoroughly, and 1.5 × 10^6/200 µl were incubated with HIV-1 BAL containing 25 ng p24 for 60 min at 37°C. Cells were then washed, lysed, and assayed for p24 antigen by ELISA. Results are the mean ± SEM of five experiments performed in triplicate. Asterisks represent statistical significance ($P < 0.05$ vs. controls). (B) The expression of MRs in the spermatozoa was analyzed by measuring the binding of albumin bovine-α-D-mannopyranosylphenyl isothiocyanate-BMA revealed by streptavidin-FITC. Assays were performed by incubating spermatozoa for 30 min at 37°C with 100 µg/ml BMA in the absence or presence of 5 mg/ml mannan. The gray histogram represents spermatozoa incubated only with streptavidin-FITC (isotype). A representative experiment ($n = 4$) is shown. (C) Spermatozoa (1.5 × 10^6/200 µl) were incubated with HIV-1 BAL containing 25 ng p24 for 60 min at 37°C in the absence or presence of mannan, mannose-BSA, or blocking antibodies directed to MR. Cells were washed thoroughly, lysed, and assayed for p24 antigen by ELISA. Results are the mean ± SEM of four to eight experiments performed in duplicate. (D) Human macrophages (10^5/100 µl), obtained from monocytes cultured with GM-CSF for 5 d, were incubated with HIV-1 BAL containing 25 ng p24 for 60 min at 37°C in the absence or presence of mannan, mannose-BSA, or blocking antibodies directed to MR. Cells were washed thoroughly, lysed, and assayed for p24 antigen by ELISA. Results are the mean ± SEM of three to four experiments performed in duplicate. (E) B-THP-1-DC-SIGN$^+$ cells (2.5 × 10^5/200 µl) were incubated with HIV-1 BAL containing 25 ng p24 for 60 min at 37°C in the absence or presence of mannan or mannose-BSA, washed thoroughly, lysed, and assayed for p24 antigen by ELISA. Results are the mean ± SEM of three to five experiments performed in duplicate. Asterisks in C–E represent statistical significance ($P < 0.05$ vs. controls). The capture mediated by THP-1 cells, which do not express DC-SIGN, is also shown (black bar).

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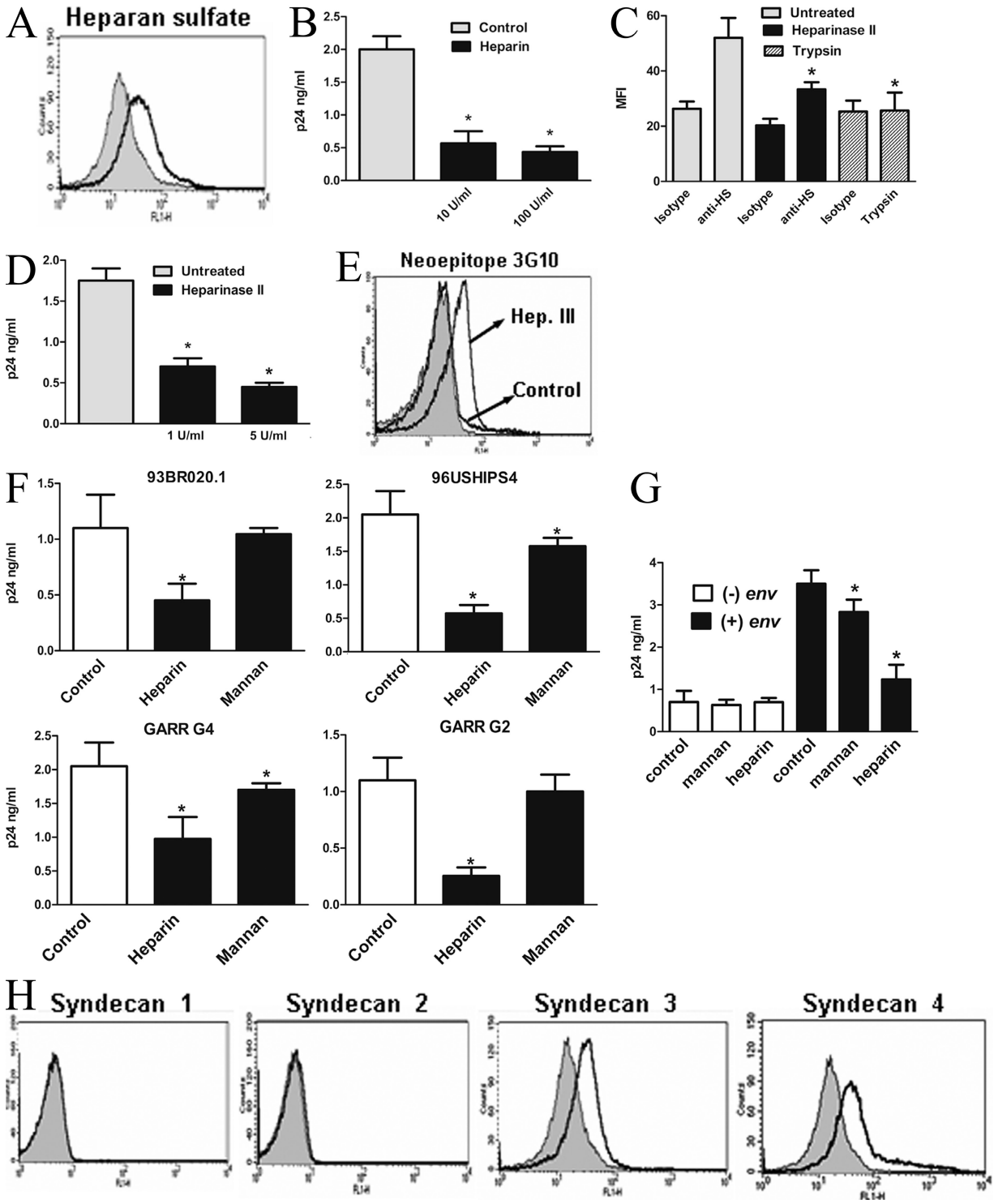

Figure 3. Capture of HIV-1 by spermatozoa is mainly mediated through HS. (A) The expression of HS on spermatozoa was analyzed by flow cytometry using anti–HS-specific antibodies (clone 10E4). Isotype control (gray histogram) and 10E4 labeled (open histogram) are depicted. A representative experiment ($n = 7$) is shown. (B) Spermatozoa (1.5×10^6/200 µl) were incubated with HIV-1 BAL containing 25 ng of p24 for 60 min at 37°C in the absence or presence of 10 and 100 U/ml heparin, washed thoroughly, lysed, and assayed for p24 antigen by ELISA. Results are the mean ± SEM of six experiments performed in triplicate. Asterisks represent statistical significance ($P < 0.05$ vs. controls). (C) Spermatozoa were treated with 5 U/ml heparinase II for 60 min at 25°C or 1,000 U/ml trypsin for 15 min at 37°C. Then the expression of HS was analyzed by flow cytometry. The results are expressed as the MFI ± SEM of five experiments performed in duplicate. Asterisks represent statistical significance ($P < 0.05$ vs. the expression of HS in controls). (D) Spermatozoa were treated with 1 and 5 U/ml heparinase II for 60 min at 25°C. Then their ability to capture HIV-1 was assayed as described for Fig. 2 A.

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spermatozoa capture similar amounts of HIV-1 at 37°C and 4°C and that treatment with trypsin effectively removed bound HIV-1, indicating that the majority of the spermatozoa-attached virus remains at the cell surface.

Fig. 2 A shows that pretreatment of spermatozoa with either trypsin or pronase almost completely prevented the binding of HIV-1, suggesting that a protein receptor mediates HIV binding. We then analyzed the expression of the different known receptors for HIV-1 in human spermatozoa. Consistent with previously published data (el-Demiry et al., 1986; Wolff and Anderson, 1988; Kim et al., 1999), CD4 expression was not detected on the spermatozoa (unpublished data). Other published studies have shown that spermatozoa express MR (Benoff et al., 1993; Chen et al., 1995). Consistent with these studies, we found that spermatozoa bind biotin-labeled mannose coupled to albumin (BMA) and that the binding of BMA to spermatozoa was almost completely inhibited by mannan, an inhibitor of the family of C-type lectin receptors, including MR and DC-specific intercellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN; Hong et al., 2002; Nguyen and Hildreth, 2003; Fig. 2 B). Treatment of spermatozoa with 1,000 U/ml trypsin for 15 min at 37°C resulted in a diminished ability to bind BMA, with a 79 ± 18% decrease in the mean fluorescence intensity (MFI; mean ± SEM; $n = 4$; $P < 0.01$, untreated vs. trypsin-treated spermatozoa).

It has been proposed that MR is an attachment receptor for HIV-1 on spermatozoa (Bandivdekar et al., 2003; Cardona-Maya et al., 2006; Fanibunda et al., 2008). Using three MR inhibitors, mannan, mannose-BSA, and blocking antibodies directed to MR, we observed only a slight inhibitory effect on the binding of HIV-1 to spermatozoa (Fig. 2 C). The efficiency of these agents to block MR was tested using macrophages differentiated from peripheral blood monocytes. It has been shown that ∼60% of the capture of HIV by macrophages is MR dependent (Nguyen and Hildreth, 2003). Consistent with these data, we observed ∼50% inhibition of HIV-1 capture using either mannan, BSA-mannose, and blocking antibodies directed to MR (Fig. 2 D). Moreover, both mannan and mannose-BSA almost completely abrogate the binding of HIV-1 to B-THP-1–DC-SIGN–positive cells (Fig. 2 E), thus confirming the efficiency of the inhibitors used. We conclude that MR does not contribute critically to the attachment of HIV-1 to spermatozoa.

Previous studies have shown that HS interacts with gp120 (de Parseval et al., 2005; Crublet et al., 2008), allowing the attachment of HIV-1 to macrophages (Saphire et al., 2001), DCs (de Witte et al., 2007), epithelial cells (Wu et al., 2003), and endothelial cells (Argyris et al., 2003). Studies performed by flow cytometry showed that spermatozoa express HS (Fig. 3 A). Heparin, an agent able to inhibit the attachment of HIV-1 to HS (Saphire et al., 2001; de Parseval et al., 2005; Parish, 2006; Crublet et al., 2008), prevented the capture of HIV-1 by spermatozoa (Fig. 3 B) without affecting the binding of BMA (MFI values of 269 ± 64 and 281 ± 43 for binding assays performed in the absence or presence of 100 U/ml heparin, respectively; mean ± SEM; $n = 4$). Heparinase II, which removes HS from heparin-like glycosaminoglycans (Parish, 2006; Bishop et al., 2007), reduced the expression of HS and the capture of HIV-1 (Fig. 3, C and D). As expected, treatment with trypsin, which almost completely prevented HIV capture (Fig. 2 A), also resulted in the abrogation of HS expression (Fig. 3 C). Because the expression of HS detected in the spermatozoa surface is relatively low, we then analyzed whether this result could be related to the antibody used to detect HS (mAb 10E4). Ruling out this possibility, Fig. S1 shows high levels of HS revealed by the mAb 10E4 in the human intestinal epithelial cell line HT-29 and a marked reduction in cell staining after heparinase II treatment. Cleavage of HS by heparinase III (heparitinase) results not only in the destruction of the epitope recognized by the anti-HS mAb 10E4 but also in the expression of a neoepitope recognized by the mAb 3G10 (Jones et al., 2005; Kureishy et al., 2006). Consistent with these studies, we found that treatment of spermatozoa with heparinase III resulted in a diminished expression of the HS epitope 10E4 (69 ± 13% decrease in the MFI; mean ± SEM; $n = 4$; $P < 0.05$) and the neoexpression of the epitope 3G10 (Fig. 3 E).

Similar to the observations made with HIV-1 BAL and HIV-1 IIIB, experiments performed with primary HIV-1 isolates (Table S1) indicated that heparin strongly inhibited the capture of HIV-1 by spermatozoa, whereas mannan exerted only a weak inhibitory effect (Fig. 3 F). Moreover, Fig. 3 G shows that HIV-1 *env*$^-$ virus binds poorly to the spermatozoa surface, supporting a critical role for gp160 in the binding of HIV-1 to spermatozoa.

Two classes of HS are found in mammalian cells; syndecans and glypicans (Kreuger et al., 2006; Parish, 2006; Bishop et al., 2007). All syndecans are able to mediate the attachment

Results are the mean ± SEM of five experiments performed in triplicate. Asterisks represent statistical significance ($P < 0.05$ vs. controls). (E) Spermatozoa were treated with 5 U/ml heparinase III for 60 min at 25°C. Then the expression of the neoepitope 3G10 was analyzed by flow cytometry. Isotype (gray histogram) and 3G10-labeled untreated (control) or heparinase III–treated spermatozoa (open histograms) are shown. Isotype controls were similar for untreated and heparinase III–treated spermatozoa. A representative experiment ($n = 4$) is shown. (F) Spermatozoa (1.5×10^6/200 µl) were incubated with different primary HIV-1 isolates containing 25 ng of p24 for 60 min at 37°C in the absence or presence of 100 U/ml heparin or 5 mg/ml mannan, washed thoroughly, lysed, and assayed for p24 antigen by ELISA. Results are the mean ± SEM of three to five experiments performed in triplicate. Asterisks represent statistical significance ($P < 0.05$ vs. controls). (G) HIV-1 pseudotypes were produced as described in Materials and methods. Spermatozoa (1.5×10^6/200 µl) were incubated with HIV-1 env$^-$ or HIV-1 env$^+$ pseudotypes containing 40 ng of p24 for 60 min at 37°C in the absence or presence of 5 mg/ml mannan or 100 U/ml heparin, washed thoroughly, lysed, and assayed for p24 antigen by ELISA. Results are the mean ± SEM of three to four experiments performed in triplicate. Asterisks represent statistical significance ($P < 0.05$ vs. controls). (H) The expression of syndecans 1–4 was analyzed by flow cytometry. Gray histograms correspond to isotype controls. In each case, a representative experiment ($n = 3$–6) is shown.

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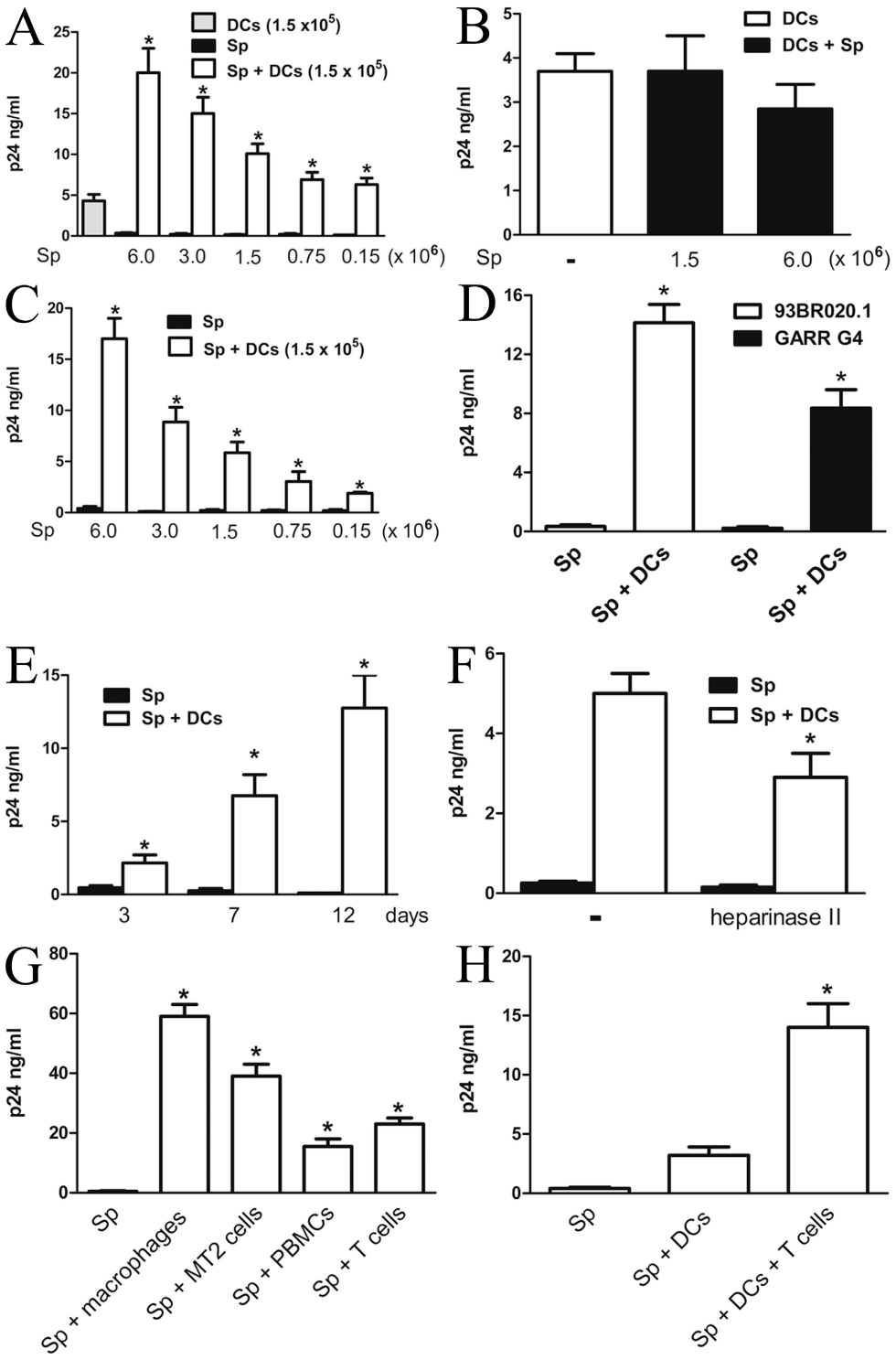

Figure 4. Spermatozoa efficiently transmit HIV-1 to DCs, macrophages, and CD4⁺ T cells. (A) DCs were incubated for 60 min at 37°C with HIV-1 containing 25 ng of p24, in the absence (gray bar) or presence of different numbers of spermatozoa (Sp; open bars). Cells were then washed four times to remove free virus, and the infection of DCs was revealed after 7 d by measuring the amount of p24 in cell supernatants. Black bars represent spermatozoa incubated alone with HIV-1 containing 25 ng p24. Results are the mean ± SEM of four experiments performed in duplicate. Asterisk represents statistical significance ($P < 0.05$ vs. DCs). (B) DCs (10^5/200 µl) were incubated with HIV-1 BAL containing 25 ng p24 for 60 min at 37°C. Cells were then washed thoroughly to remove free virus and then DCs were cultured with or without 1.5 or 6.0 × 10^6 spermatozoa. Infection of DCs was revealed after 7 d. The results are expressed as the mean ± SEM of four experiments performed in duplicate. (C) Spermatozoa suspended at different concentrations were incubated with HIV-1 BAL containing 25 ng p24/200 µl of spermatozoa suspension for 60 min at 37°C. Cells were then washed thoroughly, different numbers

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of HIV-1 to nonpermissive cells via HS chains (Gallay, 2004; de Parseval et al., 2005). Fig. 3 H shows that spermatozoa do not express syndecans 1 and 2 but express syndecans 3 and 4. The second class of cellular HS is glypicans. There are six family members of glypicans in mammals. All of them are attached to the outer cell surface of the plasma membrane by a glycosylphosphatidylinositol anchor (Kreuger et al., 2006; Bishop et al., 2007). Thus, they can be released from the cell surface by phospholipase C (PLC). To determine the possible involvement of glypicans in the attachment of HIV-1, we determined whether treatment of spermatozoa with PLC resulted in the inhibition of HIV-1 capture. Fig. S2 shows that treatment with 2 U/ml PLC did not prevent the binding of HIV-1 to the spermatozoa, suggesting that it does not involve glypicans. The efficacy of PLC treatment was tested in neutrophils. As expected, treatment with 2 U/ml PLC almost completely removed CD16, a molecule attached by a glycosylphosphatidylinositol anchor, as detected by flow cytometry (unpublished data).

Spermatozoa efficiently transmit HIV-1 to DCs, macrophages, and CD4+ T cells

DCs are able to capture HIV-1 at entry sites and transport the virus to draining lymph nodes where HIV-1 is transmitted to T $CD4^+$ cells (Geijtenbeek et al., 2000; Gurney et al., 2005; Wilkinson and Cunningham, 2006; Wu and KewalRamani, 2006). We analyzed whether spermatozoa were able to modulate the course of DC infection by HIV-1, using DCs obtained from monocytes cultured for 5 d with GM-CSF plus IL-4 (Sallusto and Lanzavecchia, 1994). DCs were incubated for 60 min at 37°C with HIV-1 BAL, in the absence or presence of different numbers of spermatozoa. Cells were then washed four times to remove free virus, and the infection of DCs was revealed after 7 d of culture. Controls included spermatozoa incubated with HIV-1 in the absence of DCs (Fig. 4 A, black bar). As shown in Fig. 4 A, the presence of spermatozoa markedly enhanced the infection of DCs at all ratios. No enhancing effect on DC infection was observed when spermatozoa were added to DCs previously incubated with HIV-1 (Fig. 4 B), suggesting that spermatozoa might increase the attachment and/or entry of the virus into DCs.

We then analyzed whether the facilitation of DC infection involves the transfer of HIV-1 from the spermatozoa to DCs. Spermatozoa were incubated first with HIV-1 BAL, washed four times to remove free virus, and cultured for 7 d with DCs at different spermatozoa/DC ratios. As shown in Fig. 4 C, spermatozoa bound HIV-1–infected DCs efficiently (effective infection was observed even at spermatozoa/DC ratios as low as 1:1). Similar results were observed using primary HIV-1 isolates instead of HIV-1 BAL (Fig. 4 D). A time course of infection of DCs after exposure to spermatozoa-bound HIV-1 is shown in Fig. 4 E. Supporting a role for HS in the transmission of HIV-1 from the spermatozoa to DCs, we found that treatment with heparinase II significantly impaired the ability of spermatozoa to transmit the infection to DCs (Fig. 4 F). In all cases, studies of DC viability performed by fluorescence microscopy after 7 d of culture revealed levels of viability >80%.

To gain insight into the capacity of spermatozoa to act as HIV carriers, we analyzed their ability to transmit HIV-1 to macrophages, PBMCs, and T cells. Spermatozoa were first incubated with HIV-1 for 60 min at 37°C, washed four times to remove free virus, and cultured for 7 d with macrophages, the $CD4^+$ T cell line MT2, activated PBMCs, and activated T cells purified from peripheral blood of normal donors. Our results showed that spermatozoa efficiently transmit HIV-1 to macrophages, MT2 cells, PBMCs, and normal T cells (Fig. 4 G). Lastly, we analyzed whether the capture of spermatozoa-bound

of HIV-1–treated spermatozoa were incubated with (open bars) or without DCs (black bars) over 7 d, and the infection of DCs was then analyzed. Results are the mean ± SEM of four to five experiments performed in duplicate. Asterisks represent statistical significance ($P < 0.05$ vs. spermatozoa cultured without DCs). (D) Spermatozoa (1.5×10^6/200 µl) were incubated with the primary HIV-1 isolates 93BR020.1 or GARR-G4 containing 25 ng p24 for 60 min at 37°C, and then cells were washed thoroughly. Spermatozoa and DCs were co-cultured during 7 d at a spermatozoa/DC ratio of 10:1, and the infection of DCs was then analyzed. Results are the mean ± SEM of three experiments performed in duplicate. Asterisks represent statistical significance ($P < 0.05$ vs. controls). (E) Spermatozoa (1.5×10^6/200 µl) were incubated with HIV-1 BAL containing 25 ng p24 for 60 min at 37°C, washed thoroughly, and co-cultured with 10^5 DCs during 3, 7, and 12 d at a spermatozoa/DC ratio of 10:1. Infection of DCs was then analyzed. Black bars represent the amount of p24 found in the supernatants of HIV-1–treated spermatozoa cultured without DCs. Results are the mean ± SEM of four experiments performed in duplicate. Asterisks represent statistical significance ($P < 0.05$ vs. controls). (F) Spermatozoa (1.5×10^6 /200 µl) were treated with 5 U/ml heparinase II for 60 min at 25°C, incubated with HIV-1 BAL containing 25 ng p24 for 60 min at 37°C, washed thoroughly, and co-cultured during 7 d with DCs at a spermatozoa/DC ratio of 10:1. Infection of DCs was then revealed as described in A. Black bars represent the levels of p24 antigen in the supernatants of HIV-1–treated spermatozoa cultured alone. Results are the mean ± SEM of four experiments performed in duplicate. The asterisk represents statistical significance ($P < 0.05$ vs. controls). (G) Spermatozoa (1.5×10^6/200 µl) were incubated with 25 ng HIV-1 BAL or HIV-1 IIIB for 60 min at 37°C. Cells were then washed thoroughly and incubated with macrophages, the T cell line MT2, PHA plus IL-2–activated PBMCs, or purified $CD3^+$ T cells activated by PHA plus IL-2 (1.5×10^5 cells/200 µl). Spermatozoa treated with HIV-1 BAL were used in macrophage cultures, whereas spermatozoa treated with HIV-1 IIIB were used for MT2 cells, PBMCs, and purified T cells. Cellular infection was analyzed after 7 d of culture. Results are the mean ± SEM of three to four experiments performed in duplicate. Asterisks represent statistical significance ($P < 0.05$ vs. spermatozoa cultured alone). (H) Spermatozoa (1.5×10^6/200 µl) were incubated with HIV-1 IIIB containing 25 ng of p24 for 60 min at 37°C, and then cells were washed thoroughly. Spermatozoa and DCs were co-cultured during 60 min at 37°C at a spermatozoa/DC ratio of 10:1. Cells were then treated with 1,000 U/ml trypsin for 15 min at 37°C, washed, and cultured with or without 3.0×10^5 T cells purified from peripheral blood and activated with PHA plus IL-2. The amount of p24 antigen in cell supernatants was analyzed after 7 d of culture. Results are the mean ± SEM of three experiments performed in duplicate. The asterisk represents statistical significance ($P < 0.05$ vs. Sp + DCs).

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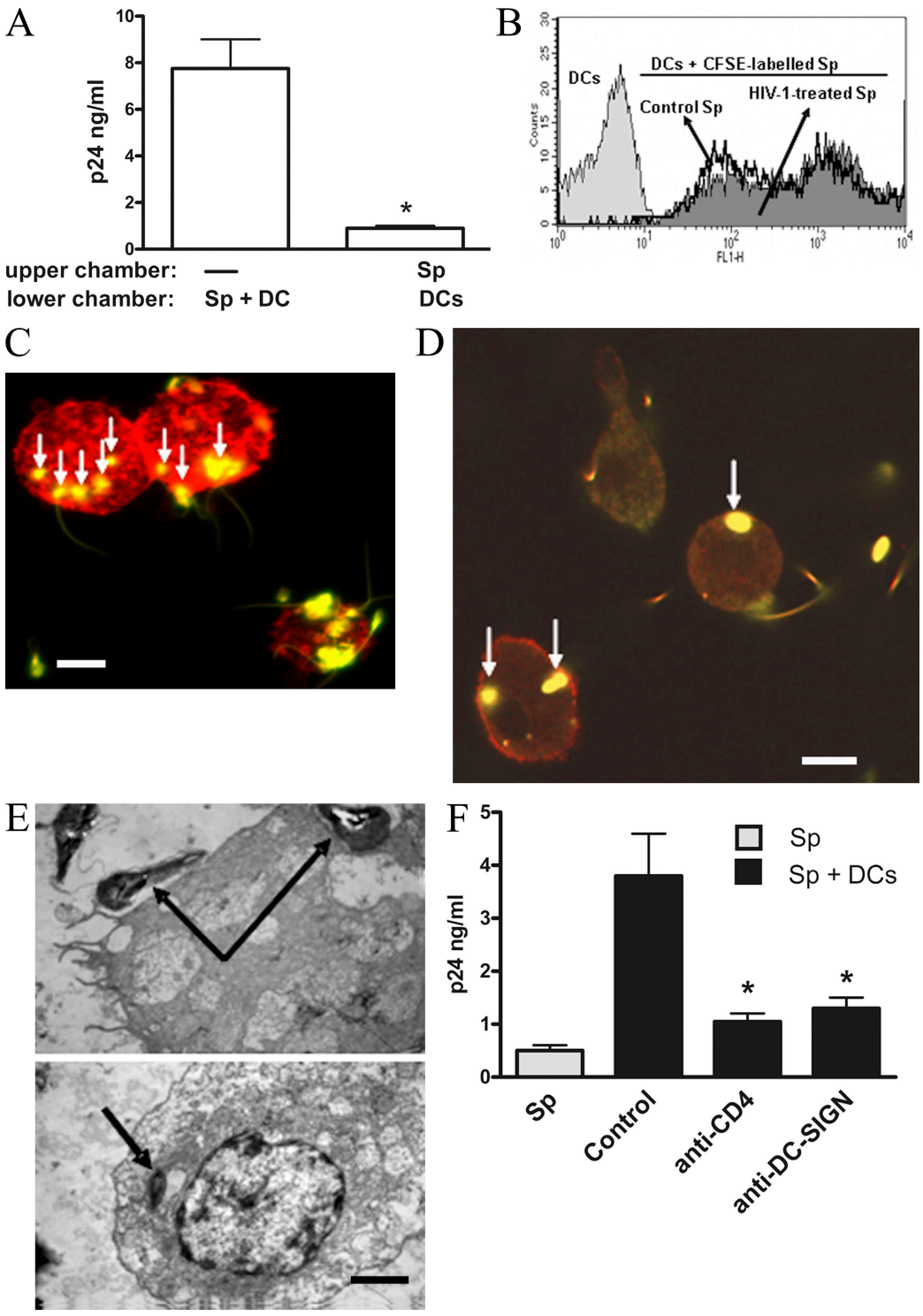

Figure 5. Spermatozoa strongly interact with DCs. (A) Spermatozoa (5×10^6/400 µl) were incubated with 50 ng HIV-1 BAL for 60 min at 37°C. Cells were then washed thoroughly. Experiments were performed in 24-transwell plates with a polycarbonate filter (0.2-µm pore size). 5×10^6 HIV-1–treated spermatozoa were in the upper compartment and 5×10^5 DCs were seeded in the lower compartment. Controls were performed by incubating together HIV-1–treated spermatozoa and DCs in the lower compartment. Cells were cultured for 7 d and the infection of DCs was evaluated by measuring the amount of p24 in the supernatants of DC cultures. Results are the mean ± SEM of five experiments performed in triplicate. The asterisk represents statistical significance ($P < 0.05$ vs. controls). (B) CFSE-labeled spermatozoa (1.5×10^6/200 µl) were incubated with or without HIV-1 BAL containing 25 ng p24 for 60 min at 37°C. Cells were then washed thoroughly and were incubated with unlabeled DCs at a spermatozoa/DC ratio of 10:1 during 1 h at 37°C. The interaction of spermatozoa and DCs were then analyzed by flow cytometry in the gate of DCs, which could be easily distinguished from spermatozoa because

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HIV-1 enables DCs to transmit the virus to T cells. HIV-1–treated spermatozoa and DCs were incubated for 60 min at 37°C. To remove spermatozoa-bound HIV-1, cells were then treated with trypsin to minimize the transmission of HIV-1 from spermatozoa directly to T cells. Cells were then washed thoroughly and cultured with or without activated T cells for 7 d. Fig. 4 H shows that the addition of T cells resulted in a marked increase in p24 levels in culture supernatants, whereas flow cytometry revealed T cell infection by p24 intracellular immunostaining (not depicted).

Interaction of spermatozoa with DCs leads to the internalization of the spermatozoa and the phenotypic maturation of DCs

To examine whether transmission of HIV-1 required the contact between spermatozoa and DCs, experiments were performed using 24-transwell chambers with a polycarbonate filter (0.2-µm pore size). Spermatozoa were incubated with HIV-1 containing 50 ng p24 for 60 min at 37°C, washed four times to remove free virus, and included in the upper chamber of the transwell system (5×10^6 spermatozoa/well). The lower chamber included 5×10^5 DCs. Controls were performed by incubating together HIV-treated spermatozoa and DCs in the lower compartment. Infection of DCs was evaluated after 7 d of co-culture. Our results shown that DCs remain uninfected when spermatozoa and DCs were placed in different chambers of the transwell system (Fig. 5 A), suggesting that the transmission of HIV-1 from the spermatozoa to DCs actually requires cell-to-cell contact.

The requirement for direct cell–cell contact for HIV transmission suggests that the DCs may actually phagocytose the spermatozoa. To evaluate internalization, we labeled the spermatozoa with CFSE. Binding of the CFSE-labeled spermatozoa to the DCs was quantified using flow cytometry. As shown in Fig. 5 B, virtually all DCs bind the green spermatozoa, regardless of the presence of HIV on the spermatozoa. The interaction of DCs with spermatozoa was also analyzed by fluorescence microscopy, using CFSE-labeled spermatozoa and DCs labeled with PE-labeled IgG anti–HLA-DR antibodies (ratio of 10:1). A representative image is shown in Fig. 5 C, with arrows indicating spermatozoa attached and/or ingested by DCs. Consistent with flow cytometer data, microscopic analysis revealed that >90% of DCs bind or ingest at least one spermatozoa with a mean of 2.7 ± 1.1 (mean ± SE; $n = 6$) per DC. To determine whether spermatozoa were phagocytosed by DCs, additional studies were performed by laser confocal microscopy and electron microscopy. These studies showed that a significant fraction of the spermatozoa was actually taken up by DCs (Fig. 5, D and E). We then performed a new set of experiments to define the fraction of ingested and attached spermatozoa. As expected, when spermatozoa and DCs were incubated at 4°C, no ingestion of spermatozoa was observed and bound spermatozoa were released from DCs by trypsin treatment (unpublished data). We then used trypsin to distinguish internalized versus attached spermatozoa. Spermatozoa and DCs were incubated together for 60 min at 37°C. After washing, cells were treated or not with 1,000 U/ml trypsin for 15 min at 37°C, and the number of spermatozoa associated with DCs was determined by fluorescence microscopy. Treatment with trypsin resulted in a marked reduction in the number of spermatozoa associated with DCs ($47 \pm 13\%$ reduction; $n = 4$; mean ± SEM), suggesting that almost half of the spermatozoa associated to DCs are not ingested and remain attached to the DC surface.

Although the relative contribution of attached versus ingested spermatozoa in the infection of DCs remains to be defined, we speculated that ingestion of spermatozoa might provide an alternative pathway for the infection of DCs, enabling HIV-1 to infect DCs without the participation of receptors classically involved in HIV-1 attachment or infection, such as DC-SIGN and CD4 (Turville et al., 2003; Piguet and Steinman, 2007; Sabatté et al., 2007). Transmission of HIV-1 from the spermatozoa to DCs was markedly impaired by blocking antibodies directed to either DC-SIGN or CD4 (Fig. 5 F), suggesting that phagocytosis of spermatozoa does not provide an alternative pathway for the infection of DCs.

The capacity of HIV-1 to hijack DCs for viral dissemination depends not only on the capture of HIV-1 by DCs but also on the migration of DCs to lymph nodes, a response associated with the phenotypic maturation of DCs (Turville et al., 2003; Piguet and Steinman, 2007). We therefore investigated the effect of spermatozoa on the maturation of DCs. Spermatozoa and DCs were cultured for 24 h and the phenotype of DCs was then analyzed by flow cytometry. Fig. 6 A shows the phenotype of immature DCs used in these experiments. As expected, cells were CD1a positive, CD14 negative, and CD83 and CCR7 negative and expressed low to intermediate levels of HLA-DR, CD86, and CD40. Spermatozoa effectively trigger the phenotypic maturation of DCs, as indicated by the

of their higher values of forward light scatter. Histograms from a representative experiment ($n = 7$) are shown. (C and D) CFSE-labeled spermatozoa (1.5×10^6/200 µl) were incubated during 60 min at 37°C with DCs, previously labeled with PE–anti–HLA-DR antibodies, at a spermatozoa/DC ratio of 10:1. The interaction of spermatozoa and DCs were then analyzed by fluorescence microscopy (C) or laser confocal microscopy (D). Bars, 10 µm. (E) Spermatozoa (1.5×10^6/200 µl) were incubated during 60 min at 37°C with DCs at a spermatozoa/DC ratio of 10:1. The interaction of spermatozoa and DCs were then analyzed by electron microscopy. Representative images are shown. Arrows in C–E indicate spermatozoa attached to or ingested by DCs. Bars: (top) 0.5 µm; (bottom) 1.5 µm. (F) Spermatozoa (1.5×10^6/200 µl) were incubated with HIV-1 BAL containing 25 ng p24 for 60 min at 37°C and washed thoroughly. DCs were pretreated, or not, with blocking antibodies directed to either CD4 or DC-SIGN for 15 min at 4°C. Blocking antibodies were used at a concentration three- to fivefold higher than those needed to saturate all binding sites, as determined by FACS analysis. Spermatozoa and DCs were co-cultured during 7 d at a spermatozoa/DC ratio of 10:1, and the infection of DCs was then analyzed by measuring the levels of p24 antigen in cell supernatants. Also shown in the figure is the amount of p24 found in the supernatants of HIV-1–treated spermatozoa cultured for 7 d without DCs (gray bar). The results are expressed as the mean ± SEM of four experiments performed in duplicate. Asterisks represent statistical significance ($P < 0.05$ vs. controls).

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marked increase in the expression of HLA-DR, CD86, CD40, CD83, and CCR7, the chemokine receptor responsible for the homing of DCs to lymph nodes (Fig. 6 B). As expected, LPS also induced the phenotypic maturation of DCs, as indicated by the increase in the expression of all the markers analyzed. The maturation of DCs induced by spermatozoa cannot be attributed to contaminating LPS in the spermatozoa suspensions because supernatants collected from spermatozoa suspensions were completely unable to change the phenotype of DCs (unpublished data). Moreover, spermatozoa lysates and spermatozoa supernatants were shown to be free of endotoxin (2×10^7 spermatozoa/100 µl; <0.5 endotoxin U/ml) as determined using a Limulus amebocyte lysate test. Fig. 6 (C and D) shows that the interaction with spermatozoa not only induced the phenotypic maturation of DCs but also the production of high levels of IL-10 but not IL-12 p70 (in contrast with LPS which induced both cytokines). We conclude that the spermatozoa induce DCs to mature phenotypically and to produce IL-10.

Extracellular acidosis markedly enhances the attachment of HIV-1 to spermatozoa and the consequent transmission of HIV-1 to DCs

The healthy vaginal environment is markedly acidic with pH values between 4.0 and 6.0 (García-Closas et al., 1999; Thinkhamrop et al., 1999). In contrast, normal values of semen pH vary between 7.2 and 8.0 (Harraway et al., 2000), and it is well known that deposition of semen in the vaginal mucosa raises the pH of vaginal secretion to slightly acidic values ranging from 6.0 to 7.0 (Masters and Johnson, 1961; Bouvet et al., 1997). We therefore analyzed whether these pH values modulated the capture of HIV-1 by spermatozoa. Spermatozoa were cultured with HIV-1 during 60 min at 37°C at extracellular pH values of 7.3 (controls), 6.8, 6.5, and 6.0, and the capture of HIV-1 was then analyzed. Fig. 7 A shows that the capture of HIV-1 by spermatozoa was markedly increased (over fourfold) at pH 6.5 or 6.0 compared with neutral pH. Similar results were observed using primary HIV-1 isolates (Fig. 7 B). No changes in sperm viability were observed at pH 6.8 and 6.5, whereas a slight decrease was found at pH 6.0 (unpublished data). In contrast, Fig. 7 C shows that the capture of the virus by the spermatozoa at pH 6.5 was markedly inhibited by heparin, suggesting that, as at neutral pH, the binding of HIV-1 to the spermatozoa at pH 6.5 is dependent on the expression of HS by the spermatozoa. Moreover, Fig. 7 D shows that the expression of HS did not increase in spermatozoa incubated at pH 6.5, supporting the idea that the enhanced attachment of HIV-1 to spermatozoa observed under acidic conditions is not related to changes in the amount of HS expressed at the spermatozoa surface.

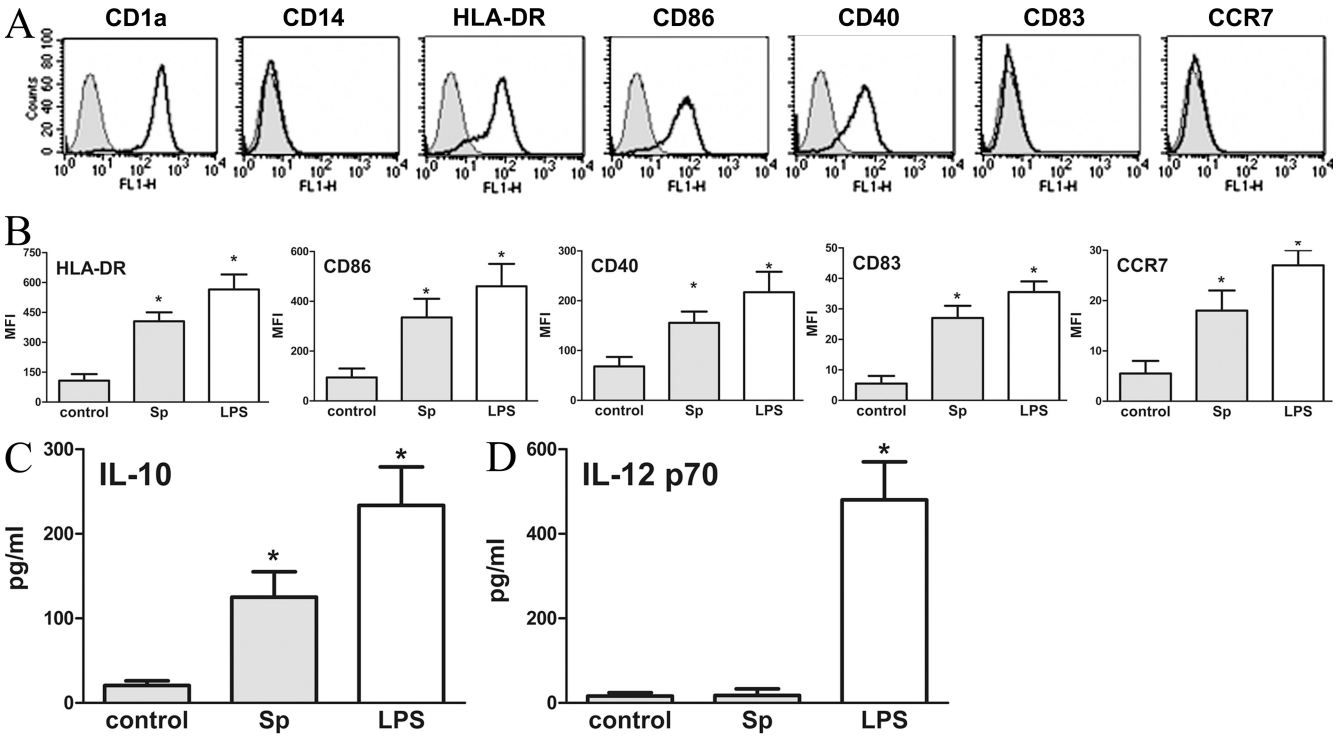

Figure 6. Interaction with spermatozoa leads to the phenotypic maturation of DCs and the production of IL-10. (A) Representative histograms of the phenotype of immature DCs. Gray histograms represent isotype controls. (B) Spermatozoa (Sp; 1.5×10^6/200 µl) were incubated for 24 h at 37°C with DCs at a spermatozoa/DC ratio of 10:1, and the phenotype of DCs was then analyzed by flow cytometry. The phenotype of DCs incubated alone or in the presence of spermatozoa or 100 ng/ml LPS during 24 h at 37°C is also shown. Results are expressed as MFI values and represent the arithmetic mean ± SEM of 11 experiments. Asterisks represent statistical significance ($P < 0.05$ vs. controls). (C and D) The production of IL-10 and IL-12p70 was evaluated in cell supernatants of DCs cultured alone or in the presence of spermatozoa or 100 ng/ml LPS during 24 h at 37°C. Results are expressed in picograms per milliliter and represent the arithmetic mean ± SEM of 10 experiments. Asterisks represent statistical significance ($P < 0.05$ vs. controls).

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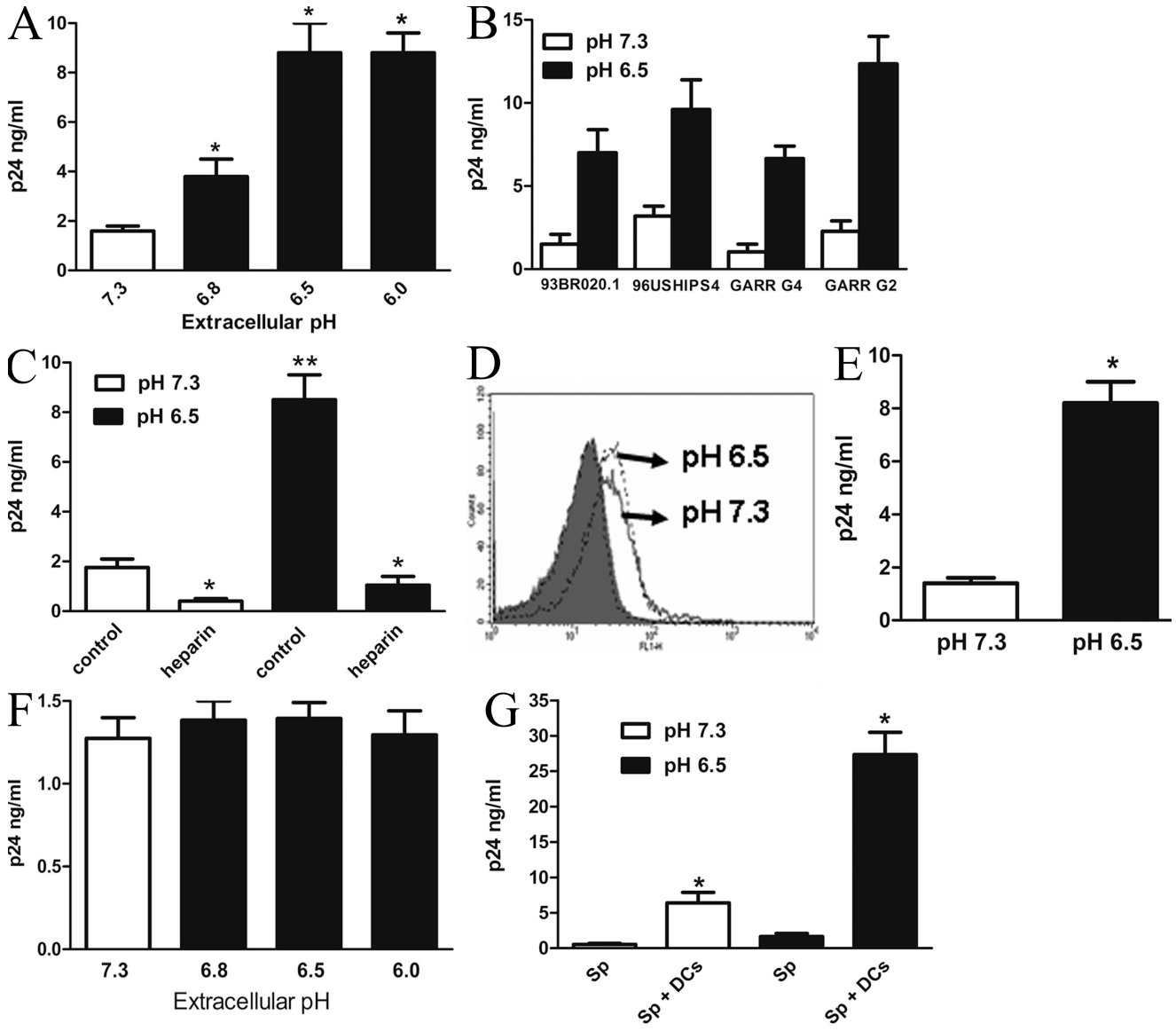

Figure 7. Acidic values of extracellular pH increase HIV-1 binding to spermatozoa and the subsequent transmission of HIV-1 to DCs. (A) Spermatozoa (Sp; 1.5×10^6/200 µl) were incubated with HIV-1 BAL containing 25 ng of p24 for 60 min at 37°C at pH 7.3 (controls), 6.8, 6.5, or 6.0. Cells were then washed thoroughly, lysed, and assayed for p24 antigen by ELISA. The results are expressed as the mean ± SEM of five experiments performed in triplicate. Asterisks represent statistical significance ($P < 0.05$ vs. pH 7.3). (B) Spermatozoa (1.5×10^6/200 µl) were incubated with different primary HIV-1 isolates containing 25 ng p24 for 60 min at 37°C at pH 7.3 or 6.5. Cells were then washed thoroughly, lysed, and assayed for p24 antigen by ELISA. A representative experiment performed in duplicate ($n = 2–3$) is shown. (C) Spermatozoa (1.5×10^6/200 µl) were incubated with HIV-1 BAL containing 25 ng p24 for 60 min at 37°C at pH 7.3 (controls) or 6.5, in the absence or presence of 10 U/ml heparin. Cells were then washed thoroughly, lysed, and assayed for p24 antigen by ELISA. The results are expressed as the mean ± SEM of four experiments performed in triplicate. *, $P < 0.05$, heparin vs. controls, at either pH 7.3 or 6.5; **, $P < 0.05$, controls at pH 6.5 vs. controls at 7.3. (D) Spermatozoa (1.5×10^6/200 µl) were incubated for 60 min at 37°C at pH 7.3 or 6.5, and the expression of HS was then analyzed by flow cytometry. The gray histogram represents isotype control. A representative experiment ($n = 3$) is shown. (E) 100 µl of whole semen were diluted 1:1 with culture medium, and the pH was adjusted to 7.3 (control) or 6.5. HIV-1 BAL was added containing 25 ng p24, and the samples were incubated for 60 min at 37°C. After centrifugation, cells pellets were washed thoroughly, lysed, and assayed for p24 antigen by ELISA. The results are expressed as the mean ± SEM of four experiments performed in triplicate. The asterisk represents statistical significance ($P < 0.05$ vs. pH 7.3). (F) DCs (1.5×10^5/200 µl) were incubated with HIV-1 BAL containing 25 ng p24 for 60 min at 37°C at pH 7.3 (controls), 6.8, 6.5, or 6.0. Cells were then washed thoroughly, lysed, and assayed for p24 antigen by ELISA. The results are expressed as the mean ± SEM of three experiments performed in triplicate. (G) Spermatozoa (1.5×10^6/200 µl) were incubated with HIV-1 BAL containing 25 ng p24 for 60 min at 37°C, at pH 7.3 or 6.5. Cells were then washed thoroughly and incubated with or without DCs for 7 d at a spermatozoa/DC ratio of 10:1. The infection of DCs was then analyzed by measuring the levels of p24 antigen by ELISA in cell supernatants. The results are expressed as the mean ± SEM of four experiments performed in duplicate. Asterisks represent statistical significance ($P < 0.05$ vs. spermatozoa cultured without DCs).

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To analyze the influence of extracellular pH on the capture of HIV-1 in a more physiological setting, experiments were done using whole semen instead of isolated spermatozoa. 100 µl of semen were diluted 1:1 with culture medium, the pH was adjusted to values of 7.3 (control) and 6.5, and capture assays were performed by incubating semen and HIV-1 containing 25 ng p24 for 60 min at 37°C. The cell pellet was then washed four times and the attachment of HIV-1 was evaluated. As shown in Fig. 7 E, and as observed for isolated spermatozoa, incubation of HIV-1 with whole semen at pH 6.5 also resulted in a marked increase in cellular attachment of HIV-1. To confirm that the virus was actually attached to spermatozoa and not to seminal leukocytes, spermatozoa were first purified from the semen by the technique of swim-up and were then resuspended in autologous seminal plasma diluted 1:1 with culture medium. The pH was adjusted to values of 7.3 and 6.5, and capture assays were performed as described in the Fig. 7 A legend. A marked increase in the attachment of HIV-1 at pH 6.5 was also observed under these experimental conditions, with a 585 ± 132% increase in the attachment of HIV-1 at pH 6.5 versus 7.3 (mean ± SEM; $n = 4$; $P < 0.01$). This result suggest that the enhancement of HIV-1 capture observed at pH 6.5 in assays performed in whole semen actually reflects an increased interaction between virus and spermatozoa. Interestingly, the enhancing effect of acidic values of pH on the cellular binding of HIV-1 does not appear to represent a general phenomenon. In fact, no enhancing effect was observed when the attachment of HIV-1 to DCs was analyzed (Fig. 7 F).

Because previous studies have shown that acidic values of pH are able to induce the inactivation of HIV-1 (Kempf et al., 1991; Connor, 2006), we investigated if the HIV-1 captured by the spermatozoa at pH 6.5 retained its ability to infect DCs. As shown in Fig. 7 G the increased capture of HIV-1 by spermatozoa at pH 6.5, as compared with pH 7.3, resulted in a marked increase (more than fourfold) in the transmission of HIV-1 from the spermatozoa to DCs. Together, these results suggest that, after sexual intercourse, the mildly acidic pH of the vagina might favor the dissemination of HIV-1 infection via the concerted action of spermatozoa and DCs.

DISCUSSION

In the present study, we show that human spermatozoa capture HIV-1 and efficiently transmit the virus to DCs in a process that requires cell-to-cell contact. The interaction of spermatozoa and DCs not only leads to the uptake of the spermatozoa but also results in the phenotypic maturation of DCs. In addition, we show that acidic values of extracellular pH, similar to those found in the vaginal mucosa after sexual intercourse, markedly increase the attachment of HIV-1 to the spermatozoa and the consequent transmission of HIV-1 to DCs. Interestingly, transmission of spermatozoa-bound HIV-1 was not restricted to DCs. It was also observed for macrophages, PBMCs, and normal T cells.

How could spermatozoa gain access to DCs at the female genital tract? This might occur under two different scenarios. Microabrasions of the mucosal surface induced either by mechanical stress during intercourse (Norvell et al., 1984; Guimarães et al., 1997) or by genital ulcer diseases (Piot and Laga, 1989; Serwadda et al., 2003; Galvin and Cohen, 2004) may allow spermatozoa to directly access DCs as well as macrophages and $CD4^+$ T cells, the three major targets of HIV-1 infection. It is of note that this is not an unusual scenario; in fact, epithelial microabrasions in the vagina are usually detected in 60% of healthy women after consensual intercourse (Norvell et al., 1984). The access of spermatozoa to DCs may also be facilitated by an alternative mechanism: the interaction of spermatozoa with DC projections that extend to, or near, the luminal surface of the mucosa (Shattock and Moore, 2003; Chieppa et al., 2006).

HS is a ubiquitous linear glycosaminoglycan composed of 30–400 repeats of a sulfated disaccharide motif with distinct sugar sequences and sulfation patterns (Parish, 2006; Bishop et al., 2007). Previous studies have shown that HS functions as an ancillary attachment factor for HIV-1 in DCs, macrophages, and epithelial and endothelial cells (Saphire et al., 2001; Wu et al., 2003; Argyris et al., 2003; Gallay, 2004; de Witte et al., 2007). The ability of HS to recognize HIV-1 appears to depend on four HS binding domains identified in the V2 and V3 loops, in the C-terminal domain, and within the CD4-induced bridging sheet of the gp120 (de Parseval et al., 2005; Crublet et al., 2008).

We found that human spermatozoa express HS. Moreover, we observed that treatment of spermatozoa with heparinase II reduced the attachment of HIV-1 supporting the involvement of HS. Interestingly, it has been shown recently that human papillomavirus 16 efficiently binds to the spermatozoa surface and also that the binding is inhibited by heparin and carrageenan, supporting the participation of HS-like molecules in the mechanisms responsible for virus attachment (Pérez Andino et al., 2009).

Our results support the notion that HS might enable spermatozoa to capture HIV-1 and to transmit the virus to $CD4^+$ target cells promoting the spreading of the infection. A similar role has been proposed for HS expressed by epithelial (Wu et al., 2003) and endothelial (Argyris et al., 2003) cells. Interestingly, although previous studies have shown that the avidity of gp120 for HS is usually higher for X4 virus than R5 virus (Moulard et al., 2000; Fletcher et al., 2006), our observations indicated that both HIV-1 BAL (R5) and HIV-1 IIIB (X4) interact with spermatozoa with similar efficiency. This suggests that, besides HS, other attachment factors might contribute to the binding of HIV-1 to the spermatozoa surface.

MRs expressed by leukocytes belong to the family of C-type lectin receptors, which recognize mannose-type carbohydrates found on virus, bacteria, and fungi (Figdor et al., 2002; Cambi and Figdor, 2003). Theses receptors play an important role in the capture of HIV-1 by macrophages and DCs (Figdor et al., 2002; Cambi and Figdor, 2003; Trujillo et al., 2007). Our results support that MR plays a complementary role in the attachment of HIV-1 to spermatozoa because mannan, mannose-BSA, and blocking antibodies directed to

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MR modestly inhibited the binding of HIV-1 to spermatozoa. A recent study (Fanibunda et al., 2008) has shown that MR expressed by the spermatozoa bind the HIV gp120, suggesting that these receptors might play an important role in the binding of HIV-1. Our results do not support this view and reinforce the notion that the ability of a given receptor to bind soluble recombinant gp120 is not necessarily predictive of its ability to capture HIV-1. In fact, elegant studies performed in cell lines transfected with the C-type lectin receptors langerin, DC-SIGN, and LSECTin (lymph node sinusoidal endothelial cell C-type lectin) have clearly shown that all of these receptors effectively recognize recombinant soluble gp120, whereas only DC-SIGN demonstrated capacity to attach the virus (Gramberg et al., 2008). Differences in the content or the surface exposure of gp120 glycans and/or in the multimerization status of the gp120 between soluble recombinant gp120 and the gp120 expressed on the virus surface may explain these apparently contrasting results.

DCs play a crucial role in the induction of adaptive immunity (Guermonprez et al., 2002). Owing to their unique localization at mucosal surfaces and their extraordinary ability to capture antigens, DCs are among the first potential targets of HIV infection during sexual transmission (Wilkinson and Cunningham, 2006; Wu and KewalRamani, 2006). Thus, after vaginal inoculation with simian immunodeficiency virus in macaques, DCs appear to be the predominant cell type infected during the early phase of the infection (Hu et al., 2000). Moreover, studies performed in infected men showed that DCs account for >90% of the virus bound to total mononuclear cells from the rectal mucosa, in spite of the fact that DCs represent <5% of these mononuclear cells (Gurney et al., 2005). A large body of evidence supports the notion that HIV-1 hijacks DC function. Instead of, or at the same time as, stimulating the adaptive immune response against HIV-1, DCs mediate HIV-1 transmission to T $CD4^+$ cells in the lymphoid tissues, contributing to the spreading of infection (Wilkinson and Cunningham, 2006; Wu and KewalRamani, 2006). We found that spermatozoa efficiently transfer the virus to immature DCs, which transmit the virus to T cells. Additional experiments performed in transwell chambers showed that transmission of HIV-1 from the spermatozoa to DCs requires cell-to-cell contact, whereas studies performed by flow cytometry, fluorescence microscopy, confocal microscopy, and electron microscopy revealed that spermatozoa strongly interact with DCs. Interestingly, this interaction leads to both the endocytosis of the spermatozoa (almost 50% of the attached spermatozoa were internalized) and the phenotypic maturation of DCs. These processes were not related to the presence of HIV-1 on the spermatozoa because they were similarly observed using either untreated or HIV-treated spermatozoa. Importantly, as reported for DCs challenged with free HIV-1 (Turville et al., 2003; Piguet and Steinman, 2007; Sabatté et al., 2007), we found that trans-infection of DCs mediated by spermatozoa was markedly prevented by blocking antibodies directed to either DC-SIGN or CD4, supporting the notion that this mechanism do not provide an alternative route for the infection of DCs by HIV-1. Interestingly, our results support the notion that DC-SIGN expressed by DCs interacts with HIV-1 on the spermatozoa surface. The mechanisms through which DC-SIGN promotes the infection of DCs by spermatozoa-bound HIV-1 are not yet defined. DC-SIGN might promote the attachment of HIV-1 to DCs (Geijtenbeek and van Kooyk, 2003). Moreover, it might protect the virus from degradation (Geijtenbeek and van Kooyk, 2003) and might also activate signaling pathways in DCs able to promote HIV spreading (Hodges et al., 2007).

Our results are consistent with previous studies (Scofield et al., 1992; Scofield, 1998) showing that spermatozoa interact with B lymphocytes, activated T cells, and monocytes. The authors showed that the binding of leukocytes and spermatozoa appeared to be dependent on the interaction of HLA-DR molecules expressed by leukocytes and a CD4-like structure expressed by the spermatozoa because it was impaired by antibodies directed to either HLA-DR or CD4. We analyzed whether a similar mechanism was involved in the interaction between spermatozoa and DCs. Consistent with previous published data (el-Demiry et al., 1986; Wolff and Anderson, 1988; Kim et al., 1999), we found no expression of CD4 in the spermatozoa. However, because we cannot rule out that spermatozoa express very low amounts of CD4, undetectable by flow cytometry, we performed a set of experiments using blocking antibodies directed to either CD4 (clone SIM.4) or HLA-DR (clone L243). Only a low inhibition of the interaction of spermatozoa and DCs (< 30%) was observed when it was performed in the presence of both blocking antibodies (unpublished data), raising the question of the existence of HLA-DR–independent mechanisms responsible for the interaction between spermatozoa and DCs. Supporting this possibility, we observed that HLA-DR–negative cells, such as resting neutrophils, were also able to interact with viable spermatozoa (unpublished data).

It is usually assumed that the acidic environment of the vagina provides a protective mechanism against sexual transmission of HIV-1. In fact, the pH of vaginal secretions usually ranged from 4.0 to 6.0 (García-Closas et al., 1999; Thinkhamrop et al., 1999), and early studies showed that the exposure of free HIV-1 to pH values lower than 5.0 results in virus inactivation (Kempf et al., 1991). More recent studies performed with primary HIV-1 isolates instead of laboratory strains showed considerable variability to inactivation by acidic pH, and for some isolates it was observed that the infectivity of HIV-1 was actually enhanced after exposure at pH 4.0–5.0 (Connor, 2006). Regarding the influence of vaginal pH on the infectivity of HIV-1, it is very important to consider that semen does not only carry the virus but also acts as a buffer of vaginal secretions, leading the pH of the vaginal mucosa to values usually ranged from 6.0 to 7.0 for several hours after sexual intercourse (Masters and Johnson, 1961; Bouvet et al., 1997). We found that the attachment of HIV-1 to spermatozoa was markedly increased when it was assayed at pH 6.0–7.0 instead of neutral pH (pH 7.3). Importantly, these results challenge the view that acidic environment of

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the vagina provides a protective mechanism against sexual transmission of HIV-1 and support the notion that after sexual intercourse a fraction of free HIV-1 found in semen becomes attached to the spermatozoa, thus enhancing its intrinsic ability to infect $CD4^+$ cells.

We speculate that spermatozoa, in addition to their ability to transmit the virus to DCs, might influence the course of the immune response against HIV by modulating the function of mucosal DCs in the receptive partner. Although this hypothesis remains to be tested, our results showing that spermatozoa not only induce the phenotypic maturation of DCs but also the production of IL-10, but not IL-12 p70, and the expansion of Foxp3-expressing $CD4^+CD25^+$ regulatory T cells (unpublished data) support the notion that the interaction with spermatozoa might drive mucosal DCs into a tolerogenic profile.

There are no candidate vaccines for HIV that could induce sterilizing immunity and protect against HIV infection. The best opportunity to prevent sexual transmission of HIV-1 clearly lies at the sites of mucosal entry. However, a better understanding of the earliest events governing the mucosal transmission of HIV is needed to develop rational strategies that prevent HIV infection. Vasectomized men are still able to transmit HIV-1 (Coombs et al., 2003), indicating that sexual transmission of the virus can proceed without spermatozoa. However, spermatozoa might be able to modulate the sexual transmission of HIV-1. Our observations support that far from being a passive carrier, spermatozoa acting in concert with DCs might affect the course of sexual transmission of HIV-1 infection by influencing both viral spreading and, perhaps, the profile of the adaptive immune response directed to HIV-1.

MATERIALS AND METHODS

Reagents. LPS from *Escherichia coli*, recombinant human IL-4, recombinant human GM-CSF, trypsin, pronase, PHA, heparin, heparinase II, heparinase III, PLC, mannan, and mannose-BSA were obtained from Sigma-Aldrich. Ficoll-Hypaque and Percoll were obtained from GE Healthcare. Blocking IgG monoclonal antibodies directed to either DC-SIGN (clone 120526) or CD4 (SIM.4) were obtained from the AIDS Research and Reference Reagent Program (AIDS Division, National Institute of Allergy and Infectious Disease, National Institutes of Health). Blocking IgG monoclonal antibody to MR (LS-C16312) was obtained from Lifespan Biosciences.

Semen samples and spermatozoa isolation. All protocols using human cells were approved by the Ethical Committee of the National Academy of Medicine (Buenos Aires, Argentina). Fresh semen samples were obtained from healthy fertile volunteer donors (aged 25–45 yr) after 3–5 d of abstinence. Informed consent was obtained from each patient before spermatozoa collection. Semen samples were obtained by masturbation from normozoospermic donors, according to World Health Organization standards (WHO, 1999). After complete liquefaction, semen samples were subjected to spermatozoa selection using the swim-up procedure (WHO, 1999). Motile cells were resuspended in RPMI 1640 medium supplemented with 0.3% BSA. Only ejaculates determined to have normal semen parameters (WHO, 1999) were used.

Preparation of human DCs and macrophages. PBMCs were isolated from healthy volunteers by standard density gradient centrifugation on Ficoll-Hypaque. Monocytes were purified by centrifugation on a discontinuous Percoll gradient. In brief, PBMCs were suspended in $Ca2^+/Mg2^+$-free Tyrode solution supplemented with 0.2% EDTA and incubated during 30 min at 37°C. During this incubation, the osmolarity of the medium was gradually increased from 290 to 360 osmol/liter by the addition of 9% NaCl. Three different Percoll fractions were layered in polypropylene tubes: 50% at the bottom, followed by 46 and 40%. PBMCs (5×10^6/ml) were layered at the top, and they were centrifuged at 400 *g* for 20 min at 4°C. Monocytes were recovered at the 50/46% interface. The purity was checked by FACS analysis using an anti-CD14 mAb and was found to be >85%. To obtain DCs, monocytes were cultured in RPMI 1640 medium (Life Technologies) supplemented with 10% fetal calf serum, 50 U/ml penicillin, 50 μg/ml streptomycin, and 0.1 mM nonessential amino acids (complete culture medium; all from Life Technologies) at 10^6 cells/ml with 10 ng/ml IL-4 and 10 ng/ml GM-CSF, as previously described (Sallusto and Lanzavecchia, 1994). On day 6, the cells were analyzed by FACS. To obtain macrophages, monocytes were cultured in complete culture medium with 50 ng/ml GM-CSF for 5 d.

Cell lines and virus. The HIV-1 BAL using CCR5, the HIV-1 IIIB using CXCR4, the primary HIV-1 isolates HIV-1 93BR020.1 and HIV-1 96USHIPS4, and the cell lines MT2, B-THP-1, and B-THP-1-DC-$SIGN^+$ were obtained from the AIDS Research and Reference Reagent program. The cell line HT29 (American Tissue Culture Collection) derived from human colon adenocarcinoma was maintained as a subconfluent monolayer in DMEM/F12 nutrient mixture, supplemented with 2 mmol/liter glutamine, 10% fetal bovine serum, 50 U/ml penicillin, and 50 μg/ml streptomycin (Thermo Fisher Scientific). The primary HIV-1 isolates GARR G4 and GARR G2 were obtained from the Laboratory of Cellular Biology and Retrovirus (Hospital de Pediatría Juan P. Garrahan, Buenos Aires, Argentine). The HIV isolates BAL, 93BR020.1, 96USHIPS4, GARR G4, and GARR G2 were grown on 10 U/ml IL-2 (R&D Systems) plus PBMC stimulated with 10 μg/ml PHA. The HIV-1 IIIB isolate was obtained from H9HTLV-IIIB supernatants. The viruses were concentrated by ultracentrifugation at 28,000 rpm for 90 min at 4°C (L2-65B ultracentrifuge; Beckman Coulter), and the virus pellet was suspended in RPMI 1640 medium. p24 antigen levels were determined by ELISA (Abbott Diagnostics), and virus input into assays was a function of p24 antigen concentration. Pseudotypes were produced, as previously described, by transiently cotransfecting (Lipofectamine 2000; Invitrogen) human embryonic kidney 293T cells with the proviral pNL-Luc-E-R^+ vector (Connor et al., 1995), which lacks the *env* gene, and the expression vector pCMV harboring the gene coding for the HIV-1 R5 (BAL) envelope protein. 293T cells were also transfected only with pNL-Luc-E-R^+. Supernatants from 293T cells were harvested 72 h after transfection or cotransfection and p24 levels were measured by ELISA.

Quantitation of cellular apoptosis and viability by fluorescence microscopy. Quantitation was performed using 100 μg/ml of the fluorescent DNA-binding dye acridine orange to determine the percentage of cells that had undergone apoptosis and 100 μg/ml ethidium bromide to differentiate between viable and nonviable cells. With this method, nonapoptotic cell nuclei show structure or variations in fluorescence intensity that reflect the distribution of euchromatin and heterochromatin. In contrast, apoptotic nuclei exhibit highly condensed chromatin that is uniformly stained by acridine orange. In fact, the entire apoptotic nucleus is present as bright spherical beads. To assess the percentage of cells showing morphological features of apoptosis, at least 200 cells were scored in each experiment.

Flow cytometry. Fluorescein isothiocyanate– or phycoerythrin-conjugated mAbs directed to CD1a, CD14, CD4, CD80, CD86, HLA-DR, CD83, and CCR7 were obtained from BD. mAb 10E4 (mouse IgM) directed to HS chains and mAb 3G10 (mouse IgG) directed to a neoepitope expressed by heparinase III–treated HS were obtained from Seikagaku Corporation. Rat IgG monoclonal antibodies directed to human syndecan-1 (clone 359103) and syndecan-2 (clone 305515), as well as goat polyclonal antibodies directed to human syndecan-3 and syndecan-4, were obtained from R&D Systems. FITC-conjugated IgG antibodies directed to rat IgG or goat IgG were obtained from Sigma-Aldrich. Analysis was performed by using a FACS flow cytometer and CellQuest software (BD).

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Enzymatic treatments. Spermatozoa were treated with the following: 1,000 U/ml trypsin for 15 min at 37°C, 20 μg/ml pronase for 15 min at 37°C, 1 and 5 U/ml heparinase II for 1 h at 25°C, 5 U/ml heparinase III for 1 h at 25°C, or 2 U/ml PLC for 90 min at 37°C. Cells were then washed and used in subsequent experiments.

HIV-1 capture assays. Spermatozoa were suspended in culture complete medium at a concentration of 1.5×10^6/200 μl and were incubated with HIV-1 stocks containing the indicated amounts of p24 antigen for 60 min at 37°C. Cells were then washed thoroughly, pelleted, lysed, and assayed for HIV p24 antigen by ELISA. Controls of nonspecific attachment of HIV-1 were performed by incubating HIV-1 in the absence of spermatozoa. When indicated, capture assays were performed at acidic values of extracellular pH. In these experiments, 1.5×10^6 spermatozoa were suspended in 200 μl of culture complete medium previously adjusted to pH values of 6.8, 6.5, and 6.0 by the addition of a precalculated volume of isotonic HCl solution. HIV-1 stocks containing the indicated amounts of p24 antigen were then added, and capture assays were performed as described in this section. Capture assays were also performed using whole semen instead of isolated spermatozoa. In these experiments, 100 μl of whole semen were diluted 1:1 with culture medium, and the pH was adjusted to 7.3 (control) or 6.5 by the addition of a precalculated volume of isotonic HCl solution. HIV-1 was then added and the samples were incubated for 60 min at 37°C. After centrifugation, cells pellets were washed thoroughly, lysed, and assayed for p24 antigen by ELISA.

HIV-1 trans-infection assays. To analyze whether spermatozoa were able to transmit HIV-1 to DCs, 1.5×10^6 spermatozoa cells in 200 μl of complete medium were first incubated with HIV-1 for 60 min at 37°C. Cells were then washed thoroughly and coincubated with DCs suspended in culture complete medium supplemented with 10 ng/ml IL-4 and 10 ng/ml GM-CSF at different spermatozoa/DC ratios in a final volume of 200 μl in 96-well U-bottom plates. Supernatants, harvested at 7 d of culture, were assayed for p24 antigen by ELISA. Trans-infection of macrophages and MT2 cells was performed by incubating 1.5×10^6 spermatozoa cells in 200 μl of complete medium with HIV-1 for 60 min at 37°C. Cells were then washed thoroughly and were coincubated with macrophages or MT2 cells at a spermatozoa/macrophage MT2 ratio of 10:1 in a final volume of 200 μl. Supernatants, harvested at 7 d of culture, were assayed for p24 antigen by ELISA. Trans-infection of PBMCs and T cells purified from PBMCs by positive magnetic selection (purity >95%; CD3 MicroBeads; Miltenyi Biotec) was performed as described in this section, using cells activated by 10 U/ml IL-2 plus 10 μg/ml PHA for 72 h.

When indicated, trans-infection assays were performed in 24-transwell chambers with a polycarbonate filter (0.2-μm pore size). In these experiments spermatozoa were incubated with HIV-1 containing 50 ng/ml of p24 for 60 min at 37°C, washed four times to remove free virus, and included in the upper chamber of the transwell system (5×10^6 spermatozoa/well). 5×10^5 DCs were seeded in the lower chamber, and infection was evaluated after 7 d of culture by measuring the amount of p24 in the supernatants of the DC chamber.

Analysis of the interaction between spermatozoa and DCs. Analysis was performed by flow cytometry, fluorescence microscopy, confocal microscopy, and electronic microscopy. Analysis by flow cytometry was performed using spermatozoa labeled with 5 μM of CFSE for 15 min at 37°C. CFSE-labeled spermatozoa (1.5×10^5/200 μl) were incubated in the absence or presence of HIV-1 BAL containing 25 ng of p24 for 60 min at 37°C. Cells were then washed four times and were cultured for 60 min at 37°C with unlabeled DCs, using a spermatozoa/DC ratio of 10:1. DCs display values of forward light scatter that are markedly higher than those of spermatozoa, enabling us to define a gate for DCs which exclude free spermatozoa. Interaction of spermatozoa and DCs was also analyzed by fluorescence microscopy (Carl Zeiss, Inc.) and confocal microscopy (Eclipse E800 laser confocal microscope; Nikon), using CFSE-labeled spermatozoa and DCs labeled with PE–anti–HLA-DR antibodies, using a spermatozoa/DC ratio of 10:1. Studies using electron microscopy were performed with a transmission electron microscope (1011; JEOL) at 60 kV. Images were recorded with a cooled charge-coupled device digital camera (MegaView III; Olympus).

Production of IL-10 and IL-12p70 by DCs. Spermatozoa (1.5×10^6/200 μl) were incubated for 24 h at 37°C with DCs, at a spermatozoa/DC ratio of 10:1, and the production of IL-10 and IL-12p70 was evaluated by ELISA (R&D Systems), according to the manufacturer's recommendations.

Statistics. All statistical comparisons were performed by using analysis of variance. P-values of <0.01 and <0.05 were considered statistically significant.

Online supplemental material. Fig. S1 shows the expression of HS in the human intestinal epithelial cell line HT-29. Fig. S2 shows that spermatozoa treatment with PLC did not prevent HIV-1 capture. Table S1 illustrates the properties of primary HIV-1 isolates. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20091579/DC1.

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