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Human beta-defensins 2 and -3 cointernalize with human immunodeficiency virus via heparan sulfate proteoglycans and reduce infectivity of intracellular virions in tonsil epithelial cells

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Abstract

We previously showed that expression of the anti-HIV innate proteins human beta-defensin 2 (hBD2) and hBD3 in adult oral epithelial cells reduces HIV transepithelial transmission by inactivation of virus. However, fetal/infant oral epithelia lack beta-defensin expression, leading to transmission of HIV. The mechanisms of hBD2- and hBD3-mediated HIV inactivation in adult oral epithelial cells are poorly understood. Here we found that heparan sulfate proteoglycans (HSPGs) on the apical surfaces of epithelial cells facilitate simultaneous binding of hBDs and HIV gp120 to the cell surface. HSPG-facilitated binding of hBDs and HIV gp120 to the cell surface did not affect viral attachment. HBD2 or -3 cointernalized with virions in endosomes, formed oligomers, and reduced infectivity of HIV. The anti-HIV effect of combining hBD2 and hBD3 was substantially higher than that of the individual peptides. These findings advance our understanding of the mechanisms of anti-HIV resistance in adult oral epithelium.

INTRODUCTION

The mucosal epithelia of the oropharyngeal, gastrointestinal and anogenital tracts play a critical role in initial HIV transmission; however, viral transmigration via epithelium varies

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considerably at different epithelial sites. For example, oral transmission of HIV through adult oropharyngeal mucosal epithelium is rare: The infection rate per oral sexual exposure is estimated to be 0.00–0.04% (Page-Shafer et al., 2006; Tudor-Williams and Lyall, 1999; UNAIDS, 2011). In contrast, mother-to-child transmission (MTCT) of HIV through fetal/neonatal oral and gastrointestinal epithelia is much more common. Without intervention, the rate of MTCT can reach 30–45% (Boyle et al., 2013; Lehman and Farquhar, 2007; UNAIDS, 2013; Wood et al., 2013). Likewise, HIV transmission is about 10 times more frequent through cervicovaginal epithelium than through adult oral epithelium (Anderson et al.; Baggaley et al., 2013; Baggaley et al., 2010; Campo et al., 2006; Pope and Haase, 2003; Rothenberg et al., 1998; Scully and Porter, 2000; Younai, 2001), and HIV transmission through anal sex is estimated to be 18 times higher than the risk through vaginal sex (Baggaley et al., 2013; Baggaley et al., 2010; Boily et al., 2009a; Boily et al., 2009b). Although the variability of HIV transmission through different mucosal epithelial sites is well documented, very little is known about the role of epithelial-specific biological factors, including that of innate immune proteins, in the modulation of transepithelial transmission of virus.

Mucosal epithelia express multiple epithelial-specific anti-HIV innate immune proteins, including human beta-defensins 2 (hBD2), hBD3, and secretory leukocyte protease inhibitor (SLPI), which may reduce viral mucosal transmission (Borrow et al., 2010; Jana et al., 2005; Ma et al., 2004; McNeely et al., 1997; Sun et al., 2005; Wahl et al., 1997; Wang et al., 2003; Wang et al., 2004; Weinberg et al., 2011; Weinberg et al., 2006). However, expression of anti-viral innate proteins may vary in mucosal epithelia at different geographic sites and could depend on age. For example, adult oral mucosa constitutively expresses high levels of hBD2, hBD3, and SLPI, but expression of these innate proteins in fetal and infant oral epithelium is very low (Dale et al., 2001; Dunsche et al., 2002; Jana et al., 2005; Moutsopoulos et al., 2007; Quinones-Mateu et al., 2003; Sun et al., 2005; Tugizov et al., 2011). Expression of hBD2, hBD3, and SLPI in the genital mucosa is not stable and depends on the menstrual cycle; i.e., hBD3 and SLPI are expressed during the secretory phase, and hBD2 has been detected only during menstruation (King et al., 2003; Moriyama et al., 1999). Expression of hBD2, hBD3 and SLPI in established polarized cervical epithelial cells *in vitro* is barely detectable (Tugizov et al., 2011). There are higher levels of innate protein expression in saliva than in rectal fluid, as shown by comparative proteomic analysis (Romas et al., 2014).

Defensins are small, 3- to 5-kDa cysteine-rich cationic innate proteins with a broad spectrum of antimicrobial and antiviral properties (Dhople et al., 2006; Schroder and Harder, 1999). The anti-HIV functions of hBD2 and hBD3 have been investigated in both X4- and R5-tropic viruses (Sun et al., 2005; Wang et al., 2003; Wang et al., 2004; Weinberg et al., 2006). These defensins bind to the HIV envelope and inactivate both X4- and R5-tropic viruses (Quinones-Mateu et al., 2003; Sun et al., 2005; Wang et al., 2003; Wang et al., 2004; Weinberg et al., 2006). hBD2 and hBD3 downregulate C-X-C chemokine receptor type 4 and inhibit entry of X4-tropic HIV-1 (Quinones-Mateu et al., 2003). hBD2 and hBD3 also inhibit HIV replication at an early stage by reducing reverse transcription activity of the virus (Sun et al., 2005).

Although the anti-HIV activity of hBD2 and hBD3 has been investigated in HIV-susceptible $CD4^+$ T lymphocytes and peripheral blood mononuclear cells (PBMC), the molecular mechanisms of antiviral functions of defensins in mucosal epithelial cells, the first target for viral transmission, have been poorly investigated. HIV mucosal transmission is initiated by transcytosis of virions through epithelial cells or by paracellular passage of virus between cells if tight junctions are disrupted (Mesquita et al., 2009; Tugizov et al., 2012). HIV transepithelial transcytosis has been shown by using polarized epithelial cells of oral, intestinal, vaginal, and endometrial origin with intact tight junctions (Bobardt et al., 2007; Bomsel, 1997; Gupta et al., 2013; Kinlock et al., 2014; Kohli et al., 2014; Meng et al., 2002; Saidi et al., 2007; Tugizov et al., 2011).

HIV transcytosis through fetal and infant oral mucosal epithelium (Tugizov et al., 2011, 2012) may play a critical role in mother to child transmission (MTCT), which is an important pathway for the spread of HIV (UNAIDS, 2013). MTCT of HIV via oral mucosal epithelia may originate from amniotic fluid *in utero* (Jaspan et al., 2004; Maiques et al., 2003; Mundy et al., 1987), from amniotic and cervicovaginal fluids at birth, and from breast milk postnatally (Nussenblatt et al., 2005; Semba, 2000; Semba and Neville, 1999; Willumsen et al., 2000).

HIV transmitted through adult oral epithelial cells is rendered noninfectious, whereas virions that pass through fetal oral epithelial cells remain highly infectious (Tugizov et al., 2011, 2012). Inactivation of HIV by adult oral epithelial cells is mediated by high-level expression of hBD2 and hBD3, and their lack of expression in fetal and infant oral epithelium leads to the passage of infectious virions. The mechanisms of hBD-associated protection of HIV transmission through adult oral epithelium are poorly understood. We hypothesized that since heparan sulfate proteoglycans (HSPGs) bind to HIV gp120 and hBD3 (Bobardt et al., 2007; Bobardt et al., 2004b; Bobardt et al., 2003; Hazrati et al., 2006), this interaction may play a role in enhancing innate anti-viral functions of hBDs in oral mucosal epithelium. HSPGs are membrane glycoproteins composed of a core protein and one or more heparan sulfate glycosaminoglycan chains — linear polysaccharides composed of alternating N-acetylated or N-sulfated glucosamine units and uronic acids (Sarrazin et al., 2011). Here we investigated the possible interactions of HSPGs with hBDs and HIV in oral epithelium and their role in hBD2- and hBD3-mediated inactivation of HIV in oral epithelial cells. We used polarized infant tonsil epithelial cells as a model system for HSPG-hBD-HIV interactions since these cells may serve as a better model system for MTCT of HIV. Herein, we report that HSPGs on the apical surfaces of tonsil epithelial cells facilitate simultaneous binding of hBDs and HIV to the cell surface. Subsequent cointernalization of hBDs with HIV into vesicular compartments of oral epithelial cells leads to a reduction in infectious activity of intracellular virus.

RESULTS

Expression of hBD2 and hBD3 in polarized adult and infant tonsil epithelial cells

We previously showed that the high-level expression of hBD2 and hBD3 in adult oral mucosal epithelium is in contrast to the low level or lack of expression of these proteins in the infant oral epithelium (Tugizov et al., 2011). Here we established polarized epithelial

cells from primary tonsil keratinocytes of 3 independent adult and 3 independent infant donors. Cells were grown in Transwell filter inserts for 10 to 14 days. All cells expressed tight junction protein zonula occludens (ZO-1) in a ring shape (Fig. 1A), and transepithelial resistance was 600 to 700 Ω/cm^2 indicating development of epithelial polarity. Immunostaining of adult AT#1 and infant IT#1 polarized epithelial cells for hBD2 and hBD3 showed strong expression of both proteins in the adult tonsil epithelial cells (Fig. 1B). About 95% of the adult cells were strongly positive for both hBD2 and hBD3. In contrast, hBD2 and hBD3 expression in infant tonsil epithelial cells was greatly reduced (Fig. 1B). These findings were corroborated by two other tonsil keratinocyte cultures that were isolated from independent donors of adult and infant origin (data not shown). Furthermore, Western blot analysis confirmed the immunostaining data showing that hBD2 and hBD3 were detected in all 3 adult epithelial cell lines (Fig. 1C). In contrast, a Western blot of infant tonsil cultures isolated from 3 independent donors showed only a trace amount of hBD2 (Fig. 1B). hBD3 was not detected in these infant cells.

HSPGs facilitate hBD2 and hBD3 binding to the apical surfaces of polarized oral epithelial cells

We have shown that adult oral epithelial cells express and secrete hBD2 and hBD3 and inactivate transcytosed HIV (Tugizov et al., 2011). However, it was not clear whether HIV is inactivated upon binding of the virus to the cell surface by secreted hBDs, or during viral transcytosis in the vesicular/endosomal compartments, or both. To determine if hBDs inactivate HIV before transcytosis upon virion attachment to the cell surface, we first examined hBD binding to the apical cell surface. Hazrati showed that hBD3 binds to HSPG, but the study was done using a cell-free system (Hazrati et al., 2006). Here, we examined hBD2 and hBD3 binding to HSPG in polarized infant epithelial cells. A domain-specific assay of HSPG in polarized adult and infant epithelial cells showed that HSPG was expressed in both apical and basolateral membranes of polarized cells of adult and infant origins (Fig. 2A). Heparinase treatment of infant oral epithelial cells removed the majority of HSPGs; i.e., approximately 70% of cells stained negatively after heparinase treatment (Fig. 2B).

To determine the binding of hBD2 and hBD3 to apical HSPG, the apical membrane of polarized infant epithelial cells was treated or not treated with heparinase, and recombinant hBD2 and hBD3 binding to HSPG was examined by surface biotinylation assay. The data showed that both hBDs bound to the cell surface. Quantitative analysis of protein bands that bound to the cell surface showed that treatment with heparinase substantially (i.e., ≈80–90%) reduced hBD binding (Fig. 2C). To confirm equal protein loading in heparinase-treated and -untreated samples, we examined apically expressed T-cell immunoglobulin and mucin domain 1 (TIM-1) protein (Kondratowicz et al., 2011) in the surface biotinylated samples. Similar levels of TIM-1 detection indicated that protein loading was equal in heparinase-treated and -untreated samples. Reduction of hBD binding by heparinase treatment indicates that HSPG facilitate the binding of both hBD2 and hBD3 to the apical surface of polarized oral epithelial cells.

Previously, we showed that polarized adult and fetal oral epithelial cells express HIV coreceptors CXCR4, CCR5 and galactosylceramide (GalCer) on the apical surfaces of epithelial cells (Tugizov et al., 2011). Immunostaining of infant IT#1 cells for CXCR4, CCR5 and GalCer showed that infant oral epithelial cells were also positive for these proteins (Fig. 2D). Approximately 30% of cells were positive for CXCR4 and CCR5, and 50% were positive for GalCer. To determine if these HIV coreceptors play a role in hBD binding to the apical cell surface, we preincubated polarized tonsil epithelial cells with antibodies against CXCR4, CCR5 and GalCer. HBD2 and hBD3 binding was then examined in a domain-specific surface labeling assay. The data showed that none of these antibodies inhibit or reduce hBD binding to infant tonsil epithelial cells (Fig. 2E), indicating that these coreceptors do not play a role in hBD interaction with tonsil epithelial cells.

HSPGs facilitate simultaneous binding of hBD2 and hBD3, and HIV gp120 to the apical surfaces of polarized oral epithelial cells

Above, we showed that HSPGs facilitate hBD2 and hBD3 binding to the apical surfaces of infant oral epithelial cells. It has been shown that HSPG also bind to HIV gp120 (Bobardt et al., 2007; Bobardt et al., 2004b; Bobardt et al., 2003), suggesting that HSPG may simultaneously interact with hBDs and HIV gp120 on the apical surface of polarized oral epithelial cells. To study this possibility, polarized infant cells were treated or not treated with heparinase and incubated with hBD2 and hBD3 for 30 min at 4°C. One set of cells was not incubated with hBDs. After extensive washing, cells were incubated with HIV-1_{SF33} for 1 h at 4°C, and gp120 was immunoprecipitated. Western blot analysis of hBD2 and hBD3 in the gp120 precipitates showed that gp120 coprecipitated with both hBD2 and hBD3 (Fig. 3A, left upper panels). In cells without hBD treatment, coprecipitation of gp120 with hBD2 and hBD3 was not detected (Fig. 3A, right panel). Heparinase treatment substantially reduced gp120 coprecipitation with hBD2 and hBD3. To determine if heparinase treatment affects HIV binding to the cell surface, we examined the presence of gp120 in the immunoprecipitated samples of the hBD2- or hBD3-treated cells (Fig. 3A, left middle panels). Quantitative analysis of the Western blot data showed that heparinase treatment reduced the amount of gp120 in the immunoprecipitated samples by approximately 30% and 60% in hBD2 and hBD3-treated cells, respectively, compared to untreated cells (Fig. 3A, left lower panels). The reduction of gp120, i.e., HIV binding to the cell surface, correlated with the reduction of gp120 coprecipitation with hBD2 and hBD3. Thus, the data in Fig. 2C and Fig. 3A show that HSPGs bind simultaneously to hBD2 and hBD3 and to HIV gp120 on the apical cell surfaces of oral epithelial cells.

To confirm this finding, we examined hBD2 and hBD3 binding to HIV gp120 in the presence or absence of HSPGs using purified heparan sulfate proteoglycan. Incubation of HIV-1_{SF33} with hBD2 or hBD3 in the presence of purified HSPGs in cell culture media for 1 h at 37°C led to binding of both hBDs to gp120 (Fig. 3B, left upper panels). In contrast, in the absence of HSPGs, hBD2 and hBD3 binding to HIV gp120 was not detected, thus showing that the simultaneous binding of HSPGs to hBD2 and hBD3 and to HIV gp120 is critical for gp120 coprecipitation with hBDs. Presence of gp120 in these samples was approximately similar (Fig. 3B, left middle and lower panels). Chondroitin sulfate did not

facilitate hBD binding to HIV gp120 (data not shown), indicating the specific role of HSPGs in binding of hBD2 and hBD3 to HIV gp120.

To verify the antibody specificity in the immunoprecipitation experiments, gp120-immunoprecipitated samples of hBD2- (Fig. 3B, right panel) and hBD3 (data not shown)-treated and HIV bound cells were blotted with normal mouse IgG. Lack of higher bands similar with gp120 and lower bands similar with hBD2 and hBD3 indicate that the anti-gp120, and hBD2 and hBD3 antibodies specifically recognized these proteins.

To determine whether cell-bound hBDs form oligomers, gp120-coprecipitated hBD2 and hBD3 was examined by Western blot under nonreducing conditions. The results show that hBD2 and hBD3 were detected at ~5 kDa (Fig. 3C), which was consistent with their monomers, indicating that hBD binding to the cell surface and HIV gp120 did not lead to their oligomer formations. These data indicate that HIV gp120 binding to hBD2 and hBD3 is indirect and is facilitated by HSPG, which binds to both HIV gp120 and hBDs.

HSPG-mediated hBD2 and hBD3 binding to the apical surfaces of polarized cells did not affect HIV attachment and did not alter infectivity of cell-bound virus

To study the role of hBD2 and hBD3 in HIV's binding to the apical surface of polarized cells and the infectivity of cell-bound virus, we performed an HIV binding and infectivity experiment in the presence or absence of hBD2 and hBD3 in the infant cells. One set of cells was preincubated with antibodies against HSPG, and another set was pretreated with heparinase. HBD2 or hBD3 (1, 10, or 100 μg/ml each) was added to the apical surfaces for 30 min at 4°C. The highest concentrations of hBD2 and hBD3 in this study were established on the basis of their higher level of local expression in oral epithelium, which may reach 100 μg in 100-μm-thick epithelial tissue (Shi et al., 1999). Relevant concentrations of hBDs (from 0.8 μg/ml to 100 μg/ml) were used in other studies (Quinones-Mateu et al., 2003; Sun et al., 2005; Wang et al., 2003; Wang et al., 2004; Weinberg et al., 2006). Cells without hBDs served as a control. Analysis of the cytotoxicity assay and measurement of transepithelial resistance of polarized cells treated with 100 μg/ml hBD2 and/or hBD3 for 24 h showed that hBDs were not cytotoxic to the cells (Fig. 4A upper panel) and did not reduce transepithelial resistance (TER) of polarized cells (Fig. 4A lower panel).

Previously, we showed that hBD2 and hBD3 may play a role in inactivation of dual-tropic, R5 and X4-tropic HIV-1, which were transcytosed through polarized oral epithelial cells. Therefore, to determine the role of hBDs in HIV attachment, we used dual-tropic HIV-1_{SF33}, R5-tropic HIV-1_{SF170} and X4-tropic HIV-$1_{92UG029}$ viruses. After washing of cells from unbound virions, attachment of HIV-1_{SF33}, HIV-1_{SF170} and HIV-$1_{92UG029}$ viruses was examined by p24 ELISA assay. Antibodies against HSPG and heparinase treatment reduced HIV attachment to the apical surface of polarized cells, indicating a role for HSPG in viral binding. However, increasing concentrations of hBD2 and hBD3 did not reduce HIV attachment (Fig. 4B, upper panel) to the apical membranes, indicating that HSPG-facilitated binding of hBDs to gp120 may not be critical for viral attachment to the cell surface.

We next examined the infectivity of apically-bound HIV in the presence or absence of hBD2 and hBD3 in concentrations similar to those in the attachment assay. Infection of TZM-bl

cells with apically-bound HIV-1_{SF33}, HIV-1_{SF170} and HIV-$1_{92UG029}$ virions showed that hBD2 and hBD3 did not reduce the infectious activity of HIV attached to the cell surface (Fig. 4B, lower panel), indicating that hBDs do not play a role in viral inactivation before its internalization and transcytosis.

Comparative analysis of HSPG expression in TZM-bl and polarized infant tonsil epithelial IT#1 cells by Western blot showed that TZM-bl cells also express HSPGs; however, their expression is substantially lower than that in tonsil cells (Fig. 4C). Expression of HSPG in TZM-bl cells is involved in HIV binding (Bobardt et al., 2004a; Mondor et al., 1998). TZM-bl cells also express the main viral receptor CD4 and coreceptors CCR5 and CXCR4, which mediate viral attachment and entry (Platt et al., 1998; Wei et al., 2002).

HSPGs facilitate cointernalization of hBD2, hBD3, and HIV into polarized infant tonsil epithelial cells

To study internalization of hBDs into polarized tonsil epithelial cells, we added recombinant hBD2 or hBD3 (100 μg/ml of each) to the apical surfaces of hBD-negative polarized infant cells. Cells without hBD treatment served as a control. After 30 min incubation at 37°C, cells were fixed, permeabilized and immunostained for hBD2 or hBD3. Confocal immunofluorescence analysis showed that approximately 50 and 80% of cells became strongly positive for hBD2 and hBD3, respectively (Fig. 5A, upper panels). The vesicular pattern of the hBDs suggested their penetration into endosomal compartments. Internalization of hBD2 and hBD3 was also detected with lower concentration (40 μg/ml) of these proteins (data not shown). HBD expression in untreated cells was sparse; i.e., <1% of untreated (endogenous) cells were positive for hBDs (Fig. 5A, lower panels). To study the internalization of hBDs into polarized epithelial cells by Western blot assay, hBD2 or hBD3 was added to the apical surface as described above. After 30 min incubation at 37°C, the cells were washed, treated with heparinase to remove uninternalized HSPG-bound hBDs and dissociated with 0.25% trypsin. Intracellular hBD2 and hBD3 were detected by Western blot, which showed that recombinant hBD2 and hBD3 rapidly penetrate into polarized infant tonsil epithelial cells (Fig. 5B). Quantitative analysis of protein bands showed that penetration of hBD3 was approximately 2 fold higher than penetration of hBD2.

In the next experiments we compared hBD internalization by incubating hBD-exposed cells at 4°C and 37°C for 30 min. Cells were then washed, treated with heparinase and dissociated with 0.25% trypsin. Quantitative analysis of Western blot data of internalized hBDs showed that incubation of cells at 4°C substantially (80–90%) inhibited the penetration of hBD2 and hBD3 into cells (Fig. 5C). These findings suggest that hBD internalization occurred by an endocytotic mechanism.

To study the role of HSPGs in cointernalization of hBD2 and hBD3 with HIV, we incubated heparinase-treated and untreated cells with hBDs at 4°C for 30 min. As we showed above, incubation of hBDs at 4°C should allow them to bind to the cell surface but prevent their internalization into cells. Thus, this approach was used to determine hBDs and HIV cointernalization. HBD-bound cells at 4°C were washed and incubated with HIV-1_{SF33} for 1 h at 37°C. Detection of intracellular hBD2, hBD3 and HIV p24 by Western blot showed that only trace amounts of internalized hBDs were seen in heparinase-treated cells. In contrast, in

untreated cells approximately 7- and 5-fold more intracellular hBD2 and hBD2, respectively, were detected than in heparinase-treated cells (Fig. 5D). Detection of HIV p24 in heparinase-treated cells was also substantially (4- to 7-fold) lower than that in untreated cells, indicating that HSPGs play a role in cointernalization of hBDs and HIV into oral epithelial cells.

To detect cointernalization of HBDs with HIV by confocal microscopy, we first incubated cells with hBD2 and hBD3 for 30 min at 4°C. Cells were then washed and incubated with HIV-1_{SF33} for 1 h at 37°C. Confocal microscopy showed that both hBD2 and hBD3 were cointernalized with HIV into polarized tonsil epithelial cells (Fig. 5E).

Internalized hBD2 and hBD3 reduce the infectious activity of intracellular HIV

To determine if internalized hBDs inactivate intracellular virus, hBD2 and/or hBD3 was added to the apical surfaces of polarized infant tonsil epithelial cells for 30 min at 4°C. Cells were then washed, and HIV-$1_{SF33,}$ HIV-1_{SF170} or HIV-$1_{92UG029}$ was added to the apical surfaces of polarized infant tonsil epithelial cells for 2 h at 37°C. Cells were trypsinized using 0.25% trypsin, which removes attached virions from the cell surface. To confirm this, we examined trypsin-mediated removal of attached virions from the cell surface. HIV-1_{SF33} was added to the apical surface of polarized cells, which were incubated at 4°C for 1 h. Cells were then washed and either trypsinized or not. Next, cells were washed and lysed, and HIV p24 Western blot assay was used to detect attached virions. No virions were detected after trypsin treatment (Fig. 6A), indicating that our experimental conditions are optimized for study of the infectious activity of only the internalized virions.

Following trypsin treatment, the hBDs- and HIV-incubated cells were examined for viral entry and infectious activity of intracellular virions. One set of epithelial cells was lysed and internalized HIV was measured by ELISA. These data showed that hBD2 and hBD3, individually or in combination, did not affect viral entry (Fig. 6B, upper panel). The other set of epithelial cells was disrupted and used for infection of TZM-bl cells. Results showed that the infectivity of intracellular virus was reduced by both hBD2 and hBD3, alone and in combination (Fig. 6B, lower panel). Inactivation of dual-tropic HIV-1_{SF33} virus by hBD2 and hBD3 was approximately 40% and 70%, respectively (Fig. 6B, lower panel). HBD2 and hBD3 reduced infectivity of R5-tropic HIV-1_{SF170} by 20% and 40%, respectively. Reduction of X4-tropic HIV-$1_{92UG029}$ virus infectivity by both hBD2 and hBD3 was substantial, i.e. approximately 80%. In general, hBD2- or hBD3-induced inactivation of R5-tropic virus was lower than that of dual- and X4-tropic viruses. HIV inactivation by a combination of hBD2 and hBD3 led to a substantial reduction (about 80–95%) in viral infectivity of all three viruses (Fig. 6B, lower panel). These results indicate that cointernalization of hBD2 and/or hBD3 with HIV in infant tonsillar epithelial cells reduces considerably the infectivity of intracellular virions. Anti-HIV effects were also detected in HIV-1_{SF33} virus at a lower concentration of hBD2 and/or hBD3 (40 ng/ml) (Fig. 6C). HIV infectivity in the experiments above (Fig. 6B and C) was also tested in PBMCs, and similar data were obtained (data not shown).

To determine whether hBD2 and/or hBD3 maintain anti-HIV activity in the presence of human breast milk, we collected and pooled breast milk from 5 donors. Polarized cells were

preincubated with hBDs and HIV-1_{SF33} in the presence or absence of breast milk, and viral infectivity was examined in TZM-bl cells. The data showed (i) that breast milk alone, in the absence of hBDs, reduced HIV infectivity by approximately 15% (Fig. 6D), (ii) that the hBDs, individually, retained their ability to inhibit HIV infectivity in the presence of breast milk and (iii) that the combination of hBD2 and −3, in the presence of breast milk, was best at inhibiting HIV infectivity. A lower concentration of hBD2 and/or hBD3 (40 μg/ml of each) in the presence of breast milk also showed an anti-HIV effect in polarized infant tonsil cells (data not shown).

hBD2 and hBD3 reduce HIV infectivity in the vesicular/endosomal compartments of polarized infant tonsil epithelial cells

To determine whether virus is inactivated within the vesicular/endosomal compartments, we incubated polarized cells with hBD2 and hBD3 in combination for 30 min at 4°C. One set of cells was not treated with hBDs. Cells were incubated with HIV-1_{SF33} for 2 h at 37°C and vesicular/endosomal compartments were isolated by cell fractionation. To identify the early and late endosomes in a total of 13 fractions, each fraction was examined for EEA1 and LAMP-1, respectively, by Western blot assay. Fractions 4, 5 and 6, representing late endosomes, were positive for LAMP-1 (Fig. 7A). EEA-1 was detected in fractions 7, 8, 9 and 10, which represent early vesicles. In hBD-treated cells, both hBD2 and hBD3 were detected in early and late endosomes. Endogenous hBDs were not detected in cells not treated with hBD. HIV p24 Western blotting also showed that HIV and both hBD2 and hBD3 were localized in the early and late endosomal compartments (Fig. 7A).

To determine the role of hBDs in HIV infection, we combined HIV containing late and early endosomal fractions from hBD-treated and untreated cells into two independent pools. HIV p24 ELISA of these vesicular pools showed that approximately equal numbers of virions were present in vesicular/endosomal compartments of hBD-treated and untreated polarized cells (Fig. 7B, upper panel), indicating that hBD2 and hBD3 did not affect HIV entry into vesicular/endosomal compartments. To determine the infectivity of intravesicular HIV, TZM-bl cells were infected with equal amounts of vesicular pool 1 and pool 2. Luciferase assay showed that TZM-bl cells infected with vesicular fractions isolated from hBD2- and hBD3-treated cells was reduced to 80–90% compared to hBD-untreated cells (Fig. 7B, lower panel). These findings indicate that hBD2-and hBD3-mediated HIV inactivation occurs within both the early and late vesicular/endosomal compartments.

hBD2–HIV and hBD3-HIV complexes internalized into vesicular/endosomal compartments of infant oral epithelial cells, and hBD2 and hBD3 form oligomers

To determine whether hBD2 and hBD3 bind to virions in the vesicular compartments, we examined their binding to intracellular HIV gp120 by immunoprecipitation and Western blot assays. HIV-1_{SF33} at 200 ng/ml p24 was added to the apical surfaces of polarized hBD-positive adult and hBD-negative infant tonsil epithelial cells. Upon internalization of virions, hBD2 and hBD3 were detected in gp120-immunoprecipitated samples from hBD-positive adult tonsil cells but not from infant cells (Fig. 8A). These results indicate that both hBDs bind to HIV in the vesicular compartments of adult oral epithelial cells, which express high levels of hBDs.

To determine if HSPG-facilitates hBD–HIV complex cointernalization into endosomal compartments, we treated the apical surfaces of polarized infant oral epithelial cells with or without heparinase in prior hBD/HIV binding and entry procedures. Western blotting of gp120 precipitates for hBD2 or hBD3 showed that gp120-hBD binding was detected only in heparinase nontreated cells (Fig. 8B, upper panels). The lack of gp120 binding to hBDs in heparinase-treated cells indicates that the HSPG-mediated hBD–HIV gp120 complex cointernalized into vesicular compartments. Detection (Fig. 8B, middle panes) and measurement (lower panels) of gp120 in the immunoprecipitated samples showed that intracellular gp120 was lower in the heparinase treated cells than in untreated cells due to reduction of HIV binding to the cell surface (Fig. 4B). Nevertheless, these lower levels of intracellular gp120 in heparinase treated cells did not coprecipitate with hBD2 and hBD3, confirming that HSPG play a role in cointernalization of the hBD-HIV complex.

To determine if hBD2 and hBD3 form oligomers in the endosomal compartments, we examined these HIV-bound hBDs by Western blot assay under nonreducing conditions. The results showed that hBD2 and hBD3 were detected at ~5 kDa, ~10/12 kDa and ~15/17 kDa (Fig. 8C), indicating that these were monomers, dimers and trimers, respectively; however, most of the hBDs were detected as oligomers. These findings indicate that HIV-bound hBD2 and hBD3 in the vesicular/endosomal compartments were oligomerized.

DISCUSSION

To study the molecular mechanism of hBD-mediated HIV inactivation in oral epithelial cells, we established polarized infant tonsil keratinocytes, which do not express hBD2 and hBD3. Application of recombinant hBD2 and hBD3 and HIV to the apical (mucosal) surfaces of hBD-negative polarized infant cells showed that both hBDs and HIV bound to the cell surface and were internalized into vesicular/endosomal compartments. Although hBD2 and hBD3 did not play a direct role in the infectivity of HIV upon viral attachment to the cell surface, both defensins independently and, in combination, reduced the infectious activity of intracellular virus. These data are consistent with HIV inactivation in adult oral epithelial cells expressing hBD2 and hBD3, and with the preservation of viral infectivity by suppressing hBD2 and hBD3 expression using siRNA and intracellular neutralization of these hBDs by specific antibodies (Tugizov et al., 2011).

Our results show that both hBD2 and hBD3 interact with HIV envelope protein gp120 on the apical surface of polarized oral epithelial cells. This interaction is facilitated by HSPG, which are expressed on the apical surface of oral epithelial cells and simultaneously bind to hBD2, hBD3 and HIV gp120. The binding of hBD2 and hBD3 to HIV gp120 in the presence of HSPG and lack of hBD-gp120 binding in the absence of HSPG in a cell-free system indicate that HSPG is important for the interaction of hBDs with the HIV viral envelope. Furthermore, lack of hBD binding to gp120 in heparinase treated cells clearly indicates that these cell surface proteoglycans play a critical role in the interaction of hBDs to HIV gp120.

It is well documented that HIV gp120 binds to HSPG through the V3 extracellular domain of gp120 (Batinic and Robey, 1992; Harrop and Rider, 1998; Rider et al., 1994; Roderiquez

et al., 1995; Vives et al., 2005). hBD2 and hBD3's interaction with HSPG does not compete with gp120 binding to HSPG. The heparan sulfate chains express vast structural diversity on same cells, which may facilitate their simultaneous binding to different proteins (Dreyfuss et al., 2009; Sarrazin et al., 2011). Thus, it is possible that chains of HSPG in the same oral epithelial cells may independently bind to hBD2, hBD3, or HIV gp120, and that this may prevent potential competition between hBD and HIV in binding to HSPG.

The interaction of hBD2 and hBD3 with HIV gp120 on the cell surface and in the endosomal compartments indicates cointernalization and cotrafficking of hBDs and HIV into the same endosomes. Defensins are internalized into cervical epithelial cells, as shown by Hazrati et al. (Hazrati et al., 2006). Binding and internalization of hBD3 was higher than that of hBD2, suggesting that the binding affinity of hBD3 to HSPG could be higher than the binding affinity of hBD2 to HSPG. Cointernalization of hBD2 and hBD3 with HIV into oral epithelial cells was facilitated by HSPG, which are a well-known receptors for the endocytosis of multiple cellular and viral proteins and viruses, including HIV (Bobardt et al., 2007; Bobardt et al., 2004b; Christianson and Belting, 2014; Connell and Lortat-Jacob, 2013; Saidi et al., 2007).

The interaction of hBD2 and hBD3 with HIV gp120 on the cell surface did not lead to viral inactivation, in contrast to cointernalization of hBD2 and hBD3 with HIV into endosomal compartments. The intracellular antiviral function of hBD2 and hBD3 correlates with their oligomer formations. These findings suggest that the internalization of hBDs and HIV into endosomal compartments creates a favorable environment for the oligomerization of hBD2 and hBD3, thus increasing their antiviral function. It is possible that molecular interactions between the hBDs and HIV, or between hBDs and unidentified endosomal proteins in the vesicular environment, may play a critical role in the induction of hBD oligomerization. The molecular mechanisms of intravesicular interactions between hBDs, HIV, and endosomal proteins in the vesicular/endosomal compartments will require elucidation.

We have shown that hBD2 and hBD3 are colocalized with HIV in the early endosomes of polarized oral epithelial cells (Tugizov et al., 2011), which are sorting compartments with a nonacidic environment (Jovic et al., 2010). Although HIV transcytosis through these compartments should not be inactivated by neutral pH, transcytosed virions were inactivated (Tugizov et al., 2011). Our current data from purified endosomes show that hBD2 and hBD3 cointernalization with HIV into early endosomes causes inactivation of virus, indicating that viral inactivation during transcytosis occurs in the early endosomes of polarized oral epithelial cells. Notably, HIV isolated from the late endosomes in hBD-untreated cells is infectious, suggesting entry of virus into the spherical, nonacidic late endosomal compartment called a multivesicular body (MVB) (Fader and Colombo, 2009; Piper and Katzmann, 2007). However, it is also possible that hBD-mediated HIV inactivation occurs first in the early endosomes, and that these inactivated virions are later delivered into late endosomes, including MVB. hBD penetration into both early and late MVBs is critical for the inactivation of virus in these compartments, leading to intraepithelial clearance of the virus.

The molecular mechanisms of hBD2- and hBD3-mediated HIV inactivation are not well understood. Defensins bind to biological membranes and form multimeric pores, leading to the disruption of membranes (Bonucci et al., 2013; Ganz et al., 1985; Hancock and Lehrer, 1998; Lehrer and Ganz, 2002; Nizet et al., 2001; White et al., 1995; Zasloff, 2002). It is possible that intravesicular HIV inactivation in oral epithelial cells is due to hBD2- and hBD3-mediated disruption of viral membranes, which could be substantially increased by cointernalization of the hBDs in the same vesicular/endosomal compartments and by their oligomer formation. The role of oligomeric hBD2 and hBD3 in the disruption of HIV membranes in the vesicles has yet to be demonstrated.

The binding of defensins to the viral surface may cause cross-linking of viral glycoproteins, affecting their mobility (Leikina et al., 2005). Immobile glycoproteins may not expose their fusion domains and thus may not be able to induce the fusion of viral and cellular membranes; i.e., become dysfunctional for viral entry. It is possible that the interaction of hBD2 and/or hBD3 with intracellular HIV gp120 in polarized oral epithelial cells may cross-link viral envelope glycoproteins, leading to their immobilization. Oligomerization of virion-bound hBDs in the vesicles may play a critical role in the cross-linking and immobilization of HIV gp120. Although transcytosis of HIV with immobile gp120 may still allow the release of virus from epithelial cells, it may not be able to infect CD4 T lymphocytes. Binding of immobile gp120 to CD4 cannot induce the conformational changes in gp120 that are critical for the exposure of fusion peptide of gp41 (Rizzuto et al., 1998; Tamm and Han, 2000; Wilen et al., 2012). We have shown that hBD2 and hBD3 are secreted from polarized adult oral epithelial cells (Tugizov et al., 2011). HBD2 and hBD3 were detected in saliva (Ghosh et al., 2007), suggesting that they may be secreted from mucosal epithelium and/or salivary glands into saliva. HBDs secreted into the mucosal environment may bind to HSPG of the mucosal epithelium, which may facilitate hBD binding to viral gp120 and internalization of hBD-bound HIV into endosomal compartments of oral epithelial cells, where hBDs are oligomerized and inactivate the intravesicular virions.

Functionally active oligomers of hBD2 and hBD3 in endosomal compartments of polarized oral epithelial cells indicate that the oligomers of hBDs rather than their monomers have a strong antiviral effect in the inhibition of transcytotic transmission of HIV through oral mucosal epithelium. HBDs may not be highly functional in the inhibition of paracellular HIV transmission through disrupted epithelium, because extracellular hBDs may not efficiently bind to viral envelope in the absence of HSPG and may not form dimers/oligomers in the extracellular environment.

Human breast milk did not inhibit the anti-HIV activity of hBD2 and/or hBD3, suggesting that the presence of recombinant hBDs in breast milk may reduce postnatal HIV MTCT. Breast milk without hBDs reduced HIV infectivity by approximately 15%, which could have been due to endogenous breast milk associated antiretroviral proteins, including lactoferrin, mucin and tenescin-C (Fouda et al., 2013; Habte et al., 2008; Moriuchi and Moriuchi, 2001; Newburg and Walker, 2007). Breast milk contains hBD2, however, it was detectable only in the colostrum (0.5 ng/ml), but not in the mature breast milk (Savilahti et al., 2015).

In summary, our data show that recombinant hBD2 and hBD3 bind to and internalize into infant tonsil epithelial cells that express very low levels of endogenous hBDs, which could be due to developmental regulation of their expression (Meyerholz et al., 2006; Patil et al., 2005; Starner et al., 2005). Cell surface HSPGs play a critical role in simultaneous binding to hBD2 and hBD3 and to HIV gp120 upon viral attachment and in the subsequent cointernalization of HIV and hBD into vesicular/endosomal compartments of infant oral epithelial cells. hBD2- and/or hBD3-mediated inactivation of HIV occurs in the vesicular/endosomal compartments by formation of oligomer hBDs, which could be critical for inhibiting the mother-to-child transmission of HIV. It could also be a mechanism of the rare HIV oral transmission via the adult oral epithelium, which expresses high levels of hBD2 and hBD3 (Tugizov et al., 2011, 2012). The lack of hBD2 and hBD3 expression in fetal, neonatal, and infant oral epithelia could reduce the innate protective function of oral epithelia and contribute to the high rate of HIV MTCT. This idea is supported by the high level of oral transmission of simian immunodeficiency virus in infant macaques (Baba et al., 1996; Ruprecht et al., 1998). Our data suggests that approaches designed to increase the levels of expression of hBD2 and hBD3 in infant oral mucosal epithelium or the topical application of recombinant hBD2 and hBD3 could prevent or reduce postnatal HIV MTCT.

MATERIALS AND METHODS

Ethics statement

This study was conducted according to the principles expressed in the Declaration of Helsinki and was approved by the Committee on Human Research of the University of California, San Francisco (IRB approval # H8597–30664-03). All subjects provided written informed consent for the collection of tissue and breast milk samples.

Viruses and cells

Laboratory-adapted dual (X4-R5)-tropic HIV-1_{SF}33 and the primary isolates R5-tropic HIV-1_{SF170} and X4-tropic HIV-1_{92U}g029 were grown in peripheral blood mononuclear cell (PBMC), which were obtained from HIV-negative donors. Before infection, PBMC were activated with 2.5 μg/ml phytohemagglutinin (Sigma) and 1 μg/ml interleukin-2 (BD Biosciences) for 3 days.

TZM-bl cells, obtained from the NIH AIDS Research and Reference Reagent Program, were contributed by John Kappes and Xiaoyun Wu. These cells are a genetically engineered HeLa cell clone that expresses CD4, CXCR4, and CCR5 and contains Tat-responsive reporter genes for firefly luciferase and *Escherichia coli* p-galactosidase under regulatory control of an HIV-1 long terminal repeat (Platt et al., 1998; Wei et al., 2002).

Primary oral epithelial keratinocytes were expanded from tonsil tissue samples collected after routine tonsillectomies in three adults 35, 41, and 44 years of age and referred to as AT#1, AT#2 and AT#3, respectively. Furthermore, primary keratinocytes were isolated from tonsils of three infants at 3 months and 2 and 3 years of age and referred to as IT#1, IT#2 and IT#3, respectively. The keratinocytes were grown in KGM medium (Lonza), and the purity of the epithelial cells was examined by detection of pankeratin using a cocktail of

anti-keratin antibodies containing Ab-1 and Ab-2 (Thermo Fisher Scientific), and only those epithelial cell populations that were 100% positive for keratin were used. Large numbers of keratinocytes from each tissue were propagated at early passages and frozen in liquid nitrogen.

Polarized cells were established in 0.45-μm Transwell two-chamber filter inserts, as described in our previous work (Tugizov et al., 2003; Tugizov et al., 2013a; Tugizov et al., 2013b; Tugizov et al., 2011). The polarity of epithelial cells was verified by immunodetection of the tight junction protein ZO-1 and measurement of transepithelial resistance (TER) using a Millicell-ERS voltohmmeter, as described in our previous work (Tugizov et al., 2003; Tugizov et al., 2013a; Tugizov et al., 2013b; Tugizov et al., 2011).

Confocal immunofluorescence assay

For immunofluorescence assays, cells were fixed with 4% paraformaldehyde and 2% sucrose in PBS for 5 min, and then permeabilized with 0.01% Triton X-100 in 4% paraformaldehyde for 5 min. For detection of hBD2 and hBD3, goat anti-hBD2 and goat anti-hBD3 (R&D Systems) were used. HSPGs and GalCer were detected with mouse monoclonal antibodies 10E4 (US Biological) and MAB342 (Millipore), respectively. CCR5 and CXCR4 were immunostained with mouse monoclonal antibodies (both from R&D and provided by the NIH AIDS Reagent Program). For detection of HIV, mouse anti- p24 antibody was used (NIH AIDS Reagent Program). Tight junction protein ZO-1 was detected using rabbit antibody (Invitrogen). Secondary antibodies labeled with fluorescein isothiocyanate or tetramethyl rhodamine isothiocyanate were purchased from Jackson ImmunoResearch. The specificity of each antibody was confirmed by negative staining with the corresponding isotype control antibody. Cell nuclei were counterstained with TO-PRO-3 iodide (blue) or propidium iodide (red) (Molecular Probes). Cells were analyzed by using a krypton-argon laser coupled with a BioRad MRC2400 confocal head. The data were analyzed by using BioRad LaserSharp software.

Western blot assay

Cells were extracted with 1.0% Triton X-100 buffer (150 mM NaCl, 10 mM Tris/HCl, pH 8.0, and a cocktail of protease inhibitors). Proteins were separated on a 4–20% gradient SDS-polyacrylamide gel. hBD2 and hBD3 were detected by using mouse and goat antibodies from Santa Cruz Biotechnology and R&D Systems. Heparan sulfate proteoglycans (HSPG) were detected by using mouse monoclonal antibody 7E12 (Millipore). Protein bands were visualized by using ECL Western blotting detection reagents (Amersham), and an equal protein load was confirmed by detection of β-actin (Ambio). HIV gp120 and p24 were detected by using mouse monoclonal antibodies ID6 and #24-2, respectively (both from NIH AIDS Research and Reference Reagent Program).

Generation of recombinant hBD2 and hBD3

Recombinant hBD2 and hBD3 were generated as described previously (Feng et al., 2005). Briefly, hBD2 and hBD3 cDNA was cloned into pET-30c (Novagen). IPTG was used to induce expression of the recombinant hBD2/3 His-tag fusion protein. Ni-NTA affinity chromatography was used to isolate hBD2/3 fusion protein. Mature hBD2/3 was released by

enterokinase (Novagen) digestion. HBD2/3 was purified with RP-HPLC and identified by mass spectroscopy.

hBD binding and penetration assays

The apical surfaces of polarized oral epithelial cells were pretreated with 10 U/ml heparinase (Sigma) for 30 min. For the hBD binding assay, cells were washed with cold phosphate-buffered saline (PBS, pH 7.2), and recombinant hBD2 or hBD3 was added to the apical surfaces. Cells were incubated at 4°C for 30 min and washed 3 times with PBS; surface-bound hBDs were detected by domain-specific labeling assay (see below). For hBD penetration, hBD2 or hBD3 was added to the apical surfaces of polarized cells, which were incubated for 30 min at 37°C. Cells were washed and trypsinized to remove uninternalized hBDs, and intracellular hBD2 and hBD3 were detected by Western blot. hBD2 and hBD3 binding to the apical surfaces of infant oral epithelial cells in the presence of anti- CXCR4, CCR5 and GalCer antibodies was examined by using the following reagents: mouse monoclonal anti-CXCR4 (clone 12G5; 10 μg/ml), anti-CCR5 (pool of clone 2D7; 50 μg/ml), and mouse monoclonal anti-GalCer (25 μg/ml) (Millipore). Antibodies were added to the apical membranes of polarized cells, which were then incubated for 1h at 4°C before the addition of hBDs. All antibodies to CXCR4 and CCR5 were provided by the NIH AIDS Reagent Program. A parallel set of experiments was performed in which cells were treated with a pool of appropriate isotype controls for the anti-GalCer (IgG1a), CXCR4, and CCR5 (mouse IgG2a and IgG2b) antibodies. The concentrations of isotype antibodies were similar to the concentrations of specific antibodies. Cells were washed, and hBD2 and hBD3 (100 μg/ml of each) were added to cells, which were incubated at 4°C for 30 min. Cells were washed, and hBD binding was examined by domain-specific surface labeling assay.

Domain-selective surface labeling assay

Polarized cells were incubated with 200 μg/ml sulfo-NHS-LC-biotin (Thermo Fisher Scientific Inc.) from apical or basolateral membranes for 30 min as described in our previous work (Sufiawati and Tugizov, 2014; Tugizov et al., 2003; Xiao et al., 2009). Cells were washed with Tris saline (10 mM Tris-HCl, pH 7.4, 120 mM NaCl), and extracted in 1% Triton X-100 lysis buffer containing protease inhibitors. Biotinylated proteins were precipitated with streptavidin–agarose beads in lysis buffer (Thermo Fisher Scientific). Proteins were separated on a 4–20% Tris-glycine SDS-polyacrylamide gel and transferred to nitrocellulose membranes (GE Healthcare). HSPG were detected by using mouse monoclonal antibody 7E12. Bands were visualized by using the ECL detection system (GE Healthcare). To determine hBD2 and hBD3 binding to HSPG on the apical surface, one set of cells was treated with heparinase for 30 min, while a second set was not treated. Cells were then washed, and recombinant hBD2 and hBD3 were added for 1 h at 4°C. Cells were biotinylated as described above and lysed. hBD2 and hBD3 were detected by Western blot assay using mouse monoclonal antibodies. To confirm equal protein load, the samples were examined for T-cell immunoglobulin and mucin domain 1 (TIM-1) protein by Western blot using rabbit polyclonal antibody (LSBio).

HIV attachment and entry assays in the presence or absence of hBD2 and hBD3

Recombinant hBD2 and hBD3 were obtained from Santa Cruz Biotechnology, as well as from Dr. Aaron Weinberg's laboratory. All experiments with hBD2 and hBD3 were performed in keratinocyte growth medium (Lonza). The toxicity of hBDs on polarized cells was examined by using an MTT Cell Viability Assay Kit (Biotium, Inc.). One set of cells was pretreated with antibodies against HSPG (25 mg/ml) or isotype control antibodies (25 μg/ml). Another set of cells was pretreated with 10 U/ml heparinase (Sigma) for 30 min. hBD2 and hBD3 were added to the apical surfaces of polarized cells, and cells were incubated at 4°C for 30 min. Cells were washed 3 times with PBS, pH 7.0, and dual (X4-R5)-tropic HIV-1_{SF33}, and R5-tropic HIV-1_{SF170} or X4-tropic HIV-$1_{92UG029}$ virions (40 ng/ml of each) were added to the apical surfaces of the cells. For the HIV attachment assay, cells were incubated at 4°C for 1 h and then washed 3 times and lysed with Triton X-100 buffer. Surface-bound HIV was measured by ELISA. For HIV entry, cells were incubated with defensins at 4°C for 30 min and then washed and incubated with HIV at 37°C for 2 h. Uninternalized virions from the cell surface were removed with 0.25% trypsin as previously described (Tugizov et al., 2012). Cells were then lysed, and internalized virus was examined by using p24 ELISA (PerkinElmer).

HIV infectivity assay

To determine the infectivity of HIV attached to the apical surface of polarized cells, with or without hBD2 and hBD3, defensins were added to polarized cells for 30 min at 4°C, and then cells were washed three times and incubated with virus for 1 h at 4°C. Cells were washed three times, dissociated from inserts by using cell scrapers (CELLTREAT), and homogenized by using motor-driven grinders connected with disposable pellet pestles, which spin 2000–3000 round per minute (Pellet Pestle Cordless Motor, Kimble Kontes). Pestle ends are specially designed to mate with 1.5 ml Eppendorf microtubes. Homogenization of cells using spinning pellet pestles was performed for 1 min, leading to the disruption of vesicles. Protein concentrations of homogenates were measured by using the Bradford protein assay, and equal amounts of homogenate were used to infect PBMC or TZM-bl cells. PBMC infected with HIV were cultured for 1 or 2 weeks, and viral infection was measured by ELISA detection of HIV p24. HIV-infected TZM-bl cells were maintained for 2 days, and HIV infection was evaluated by detecting luciferase activity as described previously (Sun et al., 2005). Luciferase expression was measured using Bright-GloTM Luciferase Assay System (Promega) following manufacturer's instructions.

To determine the infectivity of HIV penetrating into polarized epithelial cells, cells were incubated with defensins at 4°C for 30 min and then washed and incubated with HIV for 2 h at 37°C. In some experiments, incubation of defensins and HIV was done in the presence of breast milk from HIV-uninfected, healthy, breastfeeding mothers. Breast milk was collected from healthy women using breastpumps. All breast milk samples were mature, i.e., not colostrum, and collected within 6 months of breast-feeding. A pool of whole breast milk from 5 donors was used. Defensins were resuspended in breast milk and added to the apical surfaces of cells (0.3 ml per insert), which were incubated at 4°C for 30 min. Cells were then washed and incubated with HIV resuspended in breast milk for 2 h at 37°C. Cells were

washed and trypsinized to remove uninternalized virions from the cell surface, homogenized, and used to infect PBMC or TZM-bl cells.

Isolation of vesicular/endosomal compartments containing HIV and hBDs

Polarized cells were treated with or without hBD2 and hBD3 (50 μg/ml each) at 4°C for 30 min and then washed. Cells were exposed to HIV (200 ng/ml) at 37°C for 2 h. Uninternalized virions were removed with trypsin, and cells were homogenized in a buffer containing 10 mM Tris (pH 7.5), 0.25 M sucrose, 1 mM EDTA, and protease inhibitors as described (Grigorov et al., 2006). To remove nuclei, homogenized cells were centrifuged at 1,000 rpm for 10 min at 4°C. The postnuclear supernatant was gently mixed with sucrose to a final concentration of 63%. Samples were placed at the bottom of a centrifuge tube and overlaid with 45% sucrose and then 10% sucrose. Samples were centrifuged at 35,000 rpm for 16 h at 4°C. Fractions were collected from the top, and each fraction was analyzed by Western blotting using antibodies for markers of early (early endosomal marker-1, EEA1) and late (lysosome-associated membrane protein 1, LAMP-1) endosomes (both from Abcam), HIV p24 (U.S. Biological), and hBD2 (Santa Cruz Biotechnology) and hBD3 (RayBiotech).

HIV gp120 binding to hBD2 and hBD3

To detect HIV-1 gp120 binding to cell surface hBDs, recombinant hBD2 and hBD3 (100 μg/ml each) were added to the apical surfaces of polarized infant tonsil epithelial cells. Cells were incubated at 4°C for 1 h. Before incubation with hBD, one set of cells was treated with 10 U/ml heparinase (Sigma) for 30 min, and cells were then washed and exposed to hBDs. Cells were washed 3 times with cold PBS, pH 7.0, and HIV-1_{SF33} (200 ng/ml) was added to the apical surfaces of polarized cells. Cells were incubated at 4°C for 1 h, washed and lysed, and HIV gp120 was immunoprecipitated by using a mouse monoclonal antibody to HIV-1 gp120 (ID6; NIH AIDS Research and Reference Reagent Program). The hBD2 and hBD3 in the gp120 precipitates was detected by Western blotting with mouse monoclonal antibodies. To examine HIV gp120 binding to intracellular hBDs, we exposed polarized adult and infant tonsil epithelial cells to HIV-1_{SF33} at 37°C for 2 h. Uninternalized virions were then removed with trypsin, cells were lysed, and HIV gp120 was immunoprecipitated using mouse monoclonal antibodies ID6. hBD2 and hBD3 were detected by Western blot using mouse monoclonal antibodies. Detection of HIV gp120 protein in the gp120-immunoprecipitate samples was performed by Western blot assay using the same ID6 antibodies that were used for the immunoprecipitation assays.

Proteins were separated by using Tris-glycine precast gels (Invitrogen) under reducing and nonreducing conditions. For reducing conditions, samples were denatured in Tris-glycine SDS sample buffer containing 2.5% β-mercaptoethanol and run on the gels by using Tris-glycine SDS running buffer (both from Invitrogen). For nonreducing conditions, samples were processed in Tris-glycine native sample buffer without p-mercaptoethanol and run on gels in Tris-glycine native running buffer, which does not contain SDS (both from Invitrogen). Both denatured and native samples were boiled at 80°C for 5 min.

To examine hBD2 and hBD3 binding to HIV-1 gp120 in the presence or absence of purified HSPG, HIV-1 $_{SF33}$ at 100 ng p24 /ml was incubated with hBD2 or hBD3 (100 μg/ml of each) in the presence or absence of purified HSPG (100 μg/ml) in cell culture media for 1 h at 37°C. Purified HSPGs and chondroitin sulfate were purchased from Sigma-Aldrich. gp120 was then precipitated and hBDs were detected by Western blot as described above.

Statistical analysis

Differences in p24 values of PBMC and TZM-bl cells infected with HIV in the presence or absence of hBD2 or hBD3 were compared by using Student's *t*-test; P values < 0.05 were considered significant.

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Research Highlights

- The heparan sulfate proteoglycans (HSPGs) on the apical surfaces of oral epithelial cells facilitate simultaneous binding of hBDs and HIV gp120 to the cell surface.
- HBD2 or -3 cointernalized with virions in endosomes, formed oligomers, and reduced infectivity of HIV.
- The anti-HIV effect of combining hBD2 and hBD3 was substantially higher than that of the individual peptides.
- These findings advance our understanding of the mechanisms of anti-HIV resistance in adult oral epithelium.

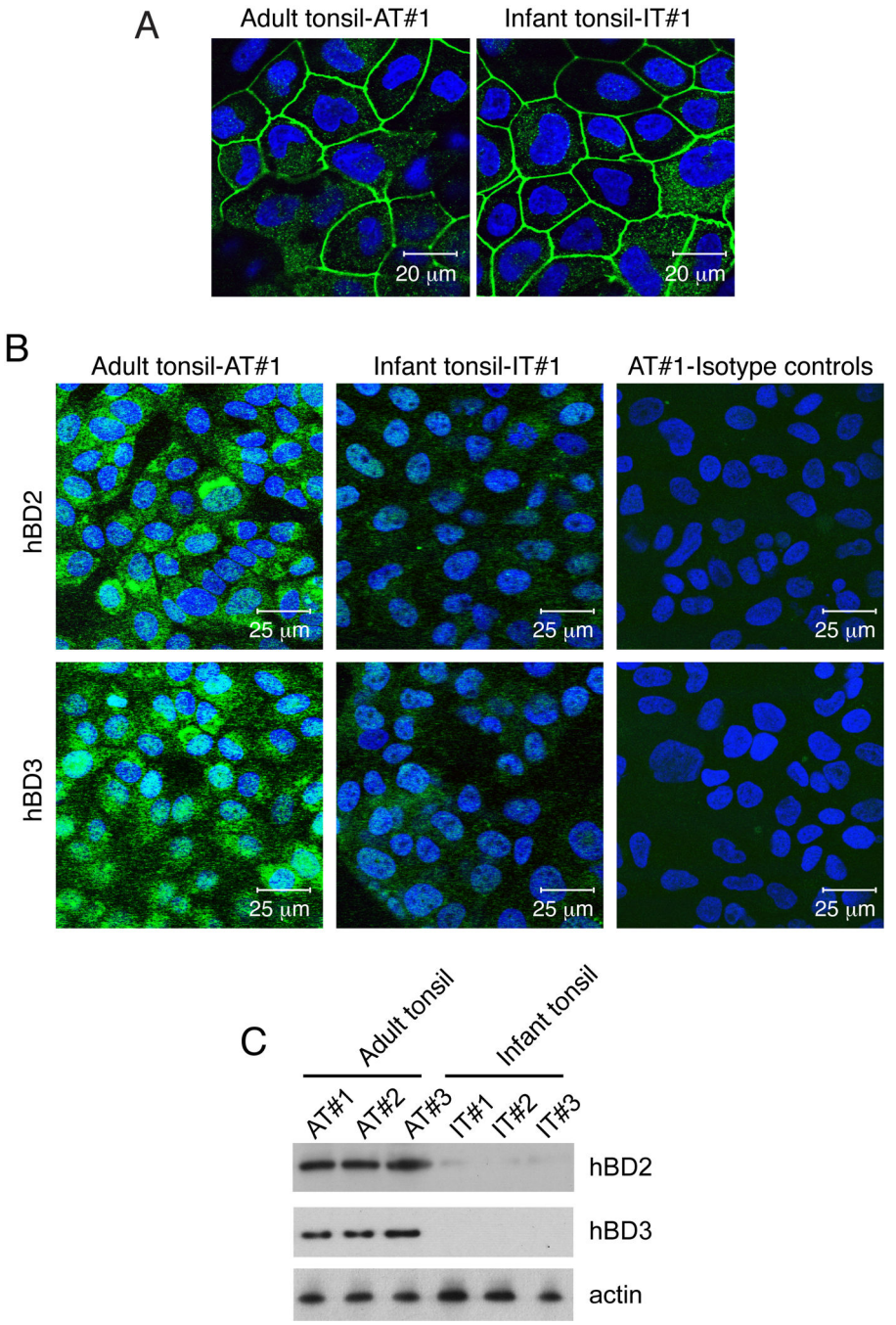

Fig. 1.

Expression of anti-HIV innate proteins hBD2 and hBD3 in adult but not infant oral epithelial cells. (A) Adult AT#1 and infant IT#1 epithelial cells were grown under polarized conditions and immunostained for ZO-1 (green). Cells were analyzed by confocal microscopy, and representative XY images in single plane are shown. Cell nuclei are counterstained in blue. (B) Polarized AT#1 and IT#1 cells were immunostained for hBD2 and hBD3 (both in green). (C) Polarized adult and infant epithelial cells isolated from independent donors were

lysed, and hBD2 and hBD3 were detected by Western blot assay. Beta-actin was used as a loading control.

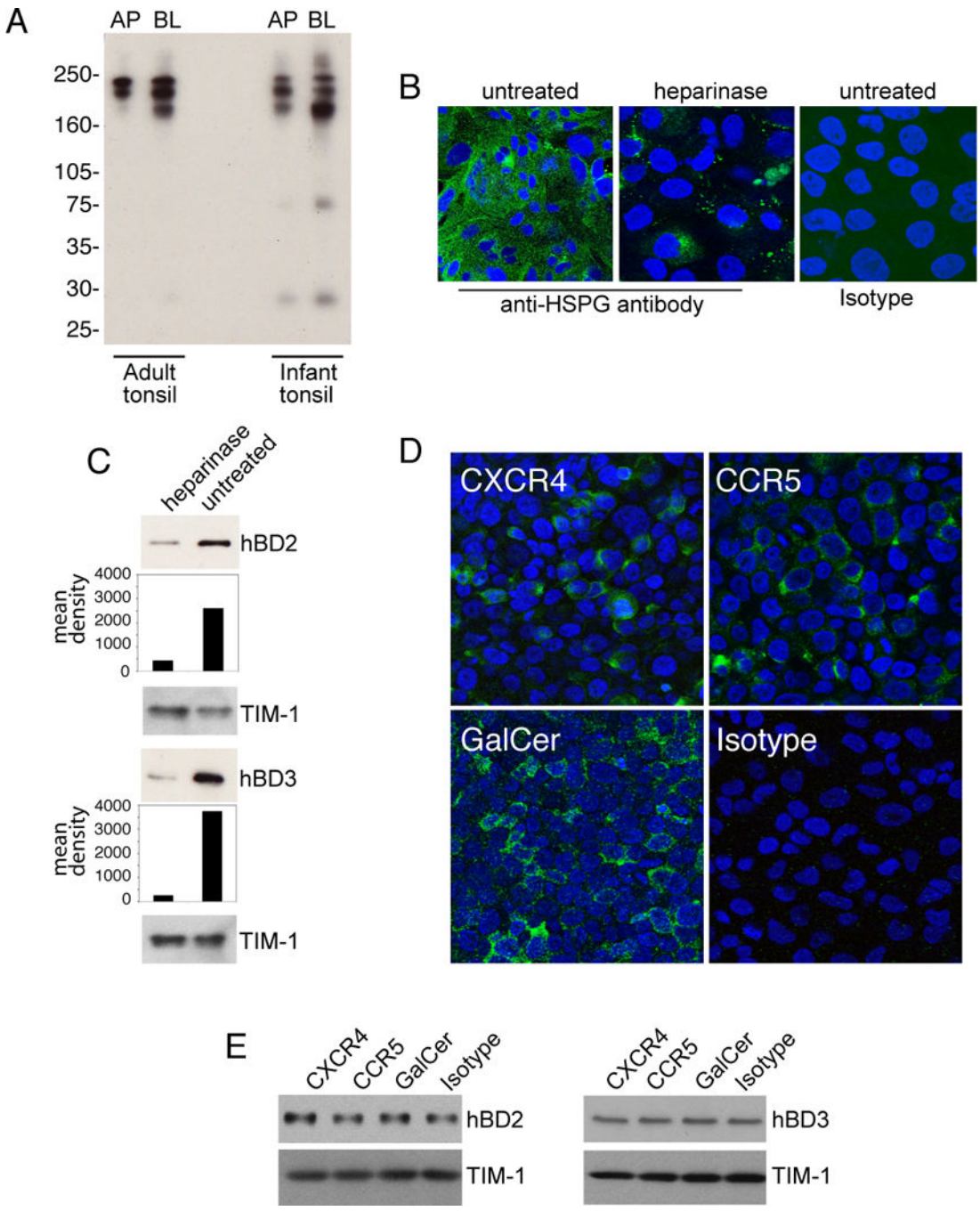

Fig. 2.

HSPGs facilitate hBD2 and hBD3 binding to the apical surfaces of infant oral epithelial cells. (A) HSPGs were detected in apical (AP) and basolateral (BL) membranes of polarized adult AT#1 and infant IT#1 tonsil epithelial cells by domain-specific biotinylation. (B) The apical and basolateral surfaces of infant IT#1 polarized epithelial cells were treated with heparinase, and untreated cells served as a control. Cells were fixed and immunostained with mouse monoclonal antibodies (10E4) against HSPG (green). (C) The apical surfaces of infant IT#1 polarized epithelial cells were treated with heparinase or untreated. Recombinant

hBD2 and hBD3 (100 μg/ml each) were added to apical membranes, and cells were incubated for 30 min at 4°C. Cells were washed, cell surfaces were biotinylated, and biotinylated proteins were precipitated with streptavidin–agarose beads. hBD2 and hBD3 were detected in the streptavidin precipitates by Western blot assay (upper panels). To verify equal protein loading in the heparinase-treated and untreated samples, these samples were examined for TIM-1 protein (lower panels). The mean densities of pixels in the hBD2 and hBD3 bands were measured by ImageJ software; the results for each gel are shown as a bar graph under each blot. (D) Polarized infant oral epithelial cells (IT#1) were immunostained for CCR5, CXCR4 and GalCer (all green). (E) Polarized IT#1 cells were preincubated with antibodies to CCR5, CXCR4 and GalCer at 4°C for 1 h and after washing were incubated with hBD2 or hBD3 (100 μg/ml of each) for 30 min at 4°C. Cells were washed and hBDs were detected by domain-specific labeling assay. All experiments presented in panels A, B and C were performed 3 times using IT#1, IT#2 and IT#3 cells, and representative data from IT#1 are presented. Experiments in panels D and E were repeated twice by using IT#1 and IT#3, and representative IT#1 data are presented.

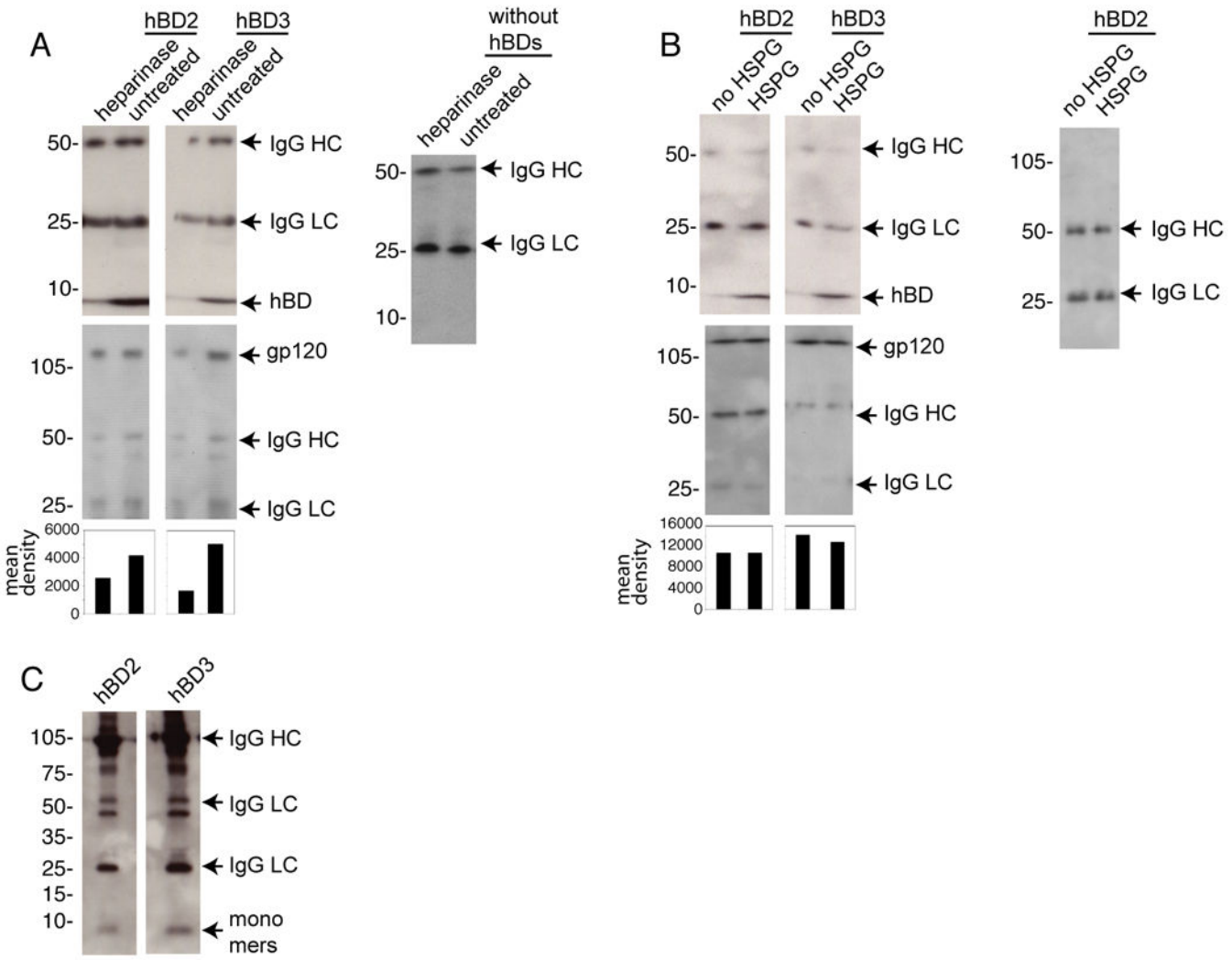

Fig. 3.

HSPGs facilitate hBD2 and hBD3 binding to HIV gp120. (A) The apical membranes of IT#1 cells were treated with heparinase or untreated. hBD2 and hBD3 (100 μg/ml each) were added to the apical surfaces, incubated at 4°C for 30 min, and, after extensive washing, HIV-1_{SF33} (200 ng/ml) was added. Cells were incubated for 1 h at 4°C and washed, and gp120 was immunoprecipitated. (Left upper panels) hBD2 and hBD3 were detected in the gp120 precipitates by Western blotting. (Left middle panels) Immunoprecipitated samples were blotted with anti-gp120 antibodies. (Left lower panels) The mean densities of pixels in the gp120 protein bands were measured by ImageJ software and shown as a bar graph under each blot. IgG LC, immunoglobulin light chain; IgG HC, immunoglobulin heavy chain. (Right panel) Cells were treated with heparinase or untreated and gp120 was precipitated in absence of hBD2 and hBD3. (B) HIV-1_{SF33} at 100 ng p24 /ml was incubated with hBD2 or hBD3 (100 μg/ml of each) in the presence or absence of purified HSPGs (100 μg/ml) in cell culture media for 1 h at 37°C. (Left upper panels) HIV-1_{SF33} gp120 was precipitated and hBDs were detected by Western blot under reducing conditions. (Left, middle panels) Immunoprecipitated samples were blotted with anti-gp120 antibodies. (Left lower panels)

The gp120 protein bands were quantified by ImageJ and mean densities of pixels are shown as a bar graph under each blot. (Right panel) HIV gp120 immunoprecipitated samples of hBD2 treated and HIV bound cells were blotted with normal mouse IgG. (C) hBD2 or hBD3 was added to the apical surfaces of polarized infant IT#1 cells for 30 min at 4°C. To remove unbound hBDs, cells were washed and HIV-1_{SF33} (200 ng/ml) was added for 1 h at 4°C. Cells were washed, lysed, and gp120 was immunoprecipitated. Proteins were separated under nonreducing conditions in 16% Tris-glycine gel. hBD2 and hBD3 were detected by Western blotting. A, B and C. Similar results were obtained in 2 independent experiments using infant IT#1 and IT#3 tonsil epithelial cells.

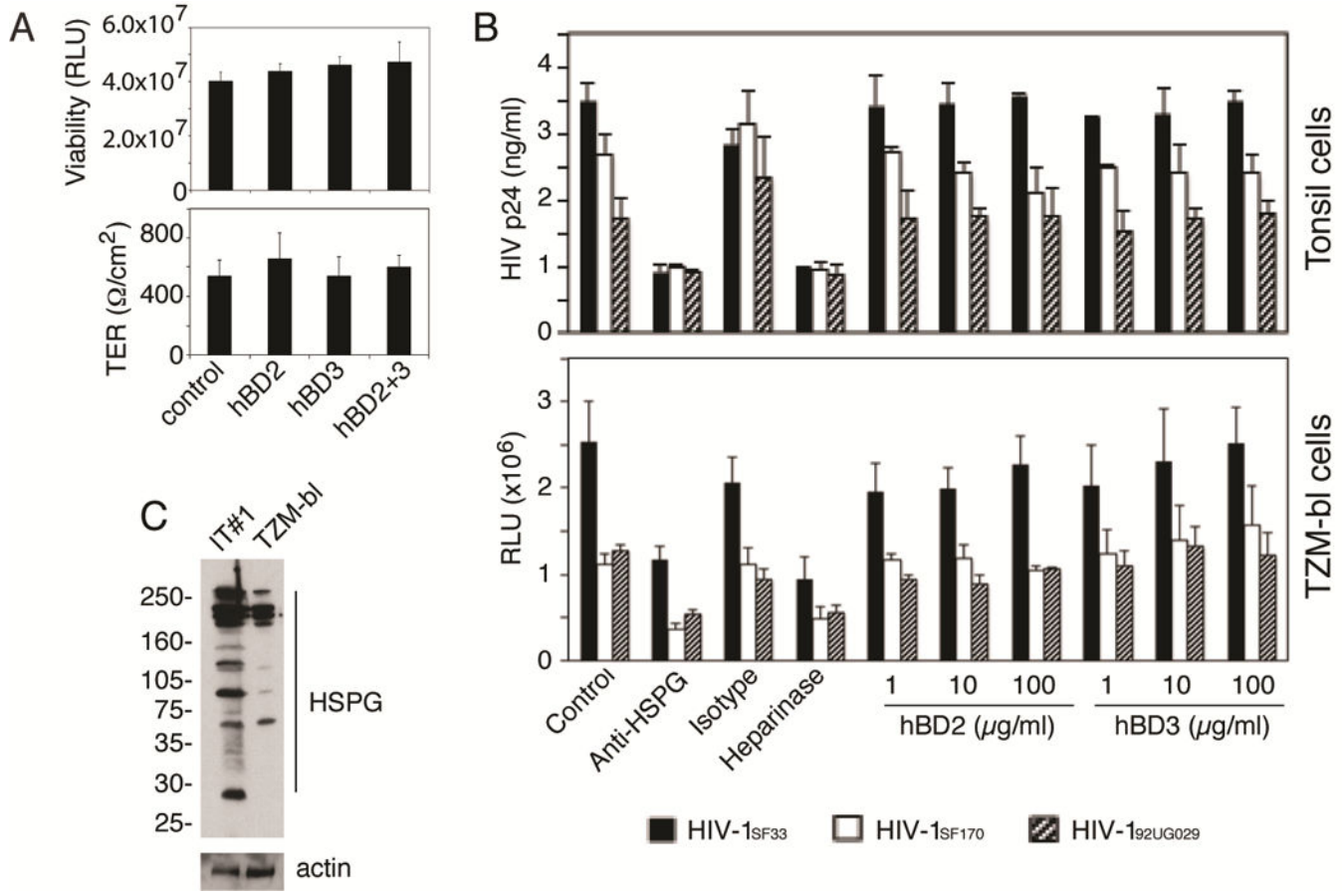

Fig. 4.

hBD and HIV interaction does not affect virus attachment to the cell surface and does not inactivate cell-bound virus. (A) Polarized infant IT#1 tonsil epithelial cells were incubated with hBD2, hBD3 and hBD2+hBD3 for 24 h and cell viability (upper panel) and TER (lower panel) were examined. RLU, relative light units. TER, transepithelial resistance. (B) Polarized cells were preincubated with antibodies to HSPG or treated with heparinase. (Upper panel) Next, sets of cells were preincubated with hBD2 and hBD3 in increasing concentrations for 1 h at 4°C. Cells were washed, and HIV-1_{SF33}, HIV-1_{SF170}, or HIV-$1_{92UG029}$ virions (40 ng/ml each) were added to the apical surfaces of polarized cells for 1 h at 4°C. Cells were washed, and HIV binding was examined by p24 ELISA. Bars indicate standard errors of the means (n=3). (Lower panel). hBDs and HIV exposed cells for attachment were washed, homogenized, and used to infect TZM-bl cells. After 48 h HIV infection was evaluated by luciferase assay. RLU, relative luciferase unit. These experiments were performed twice using IT#1 and twice in IT#3 cells, and average data of four experiments was presented. (C) Infant tonsil epithelial IT#1 and TZM-bl cells were grown under polarized and nonpolarized conditions, respectively, and HSPGs were detected by Western blot assay. Detection of beta-actin was used as a loading control.

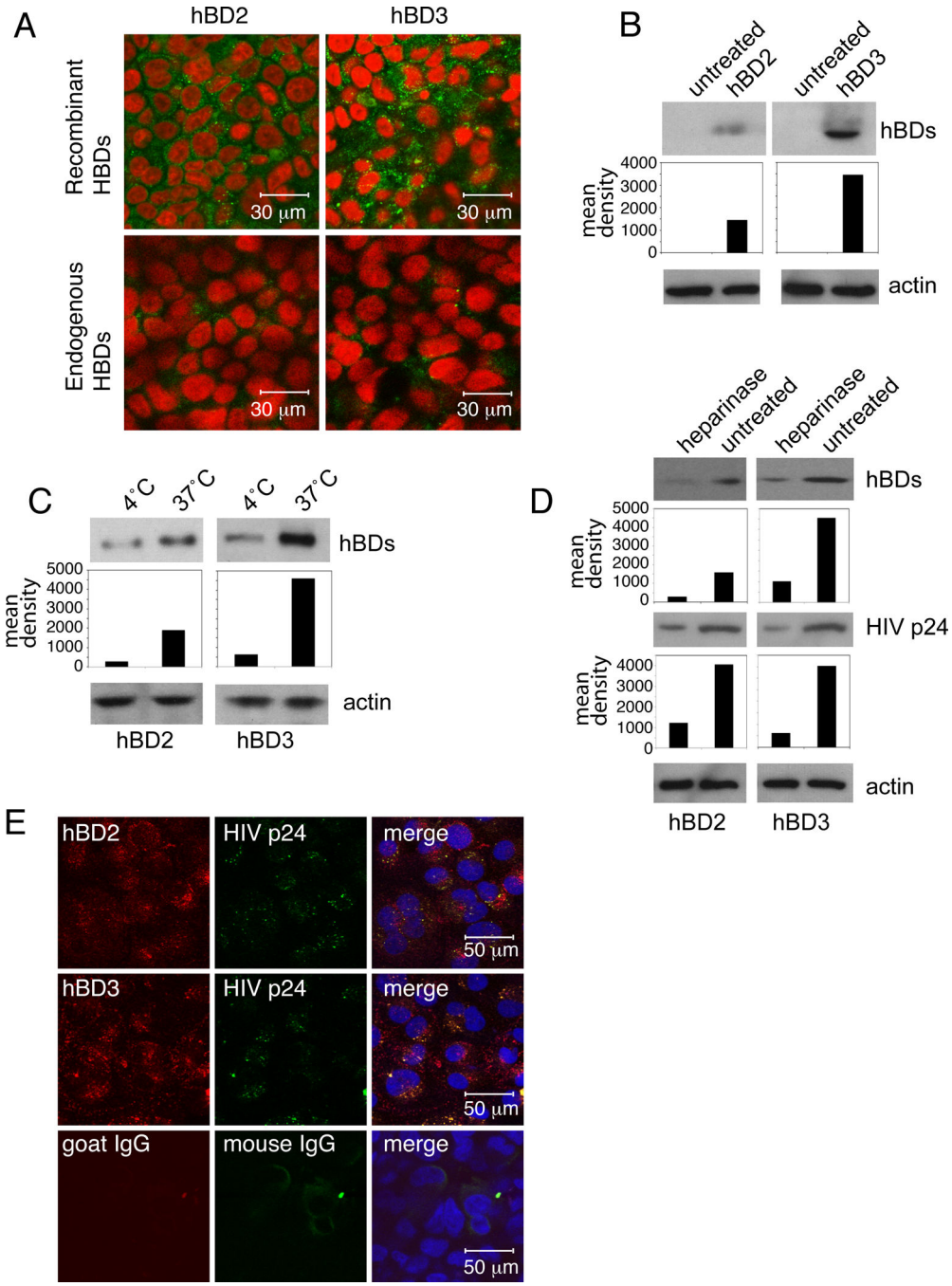

Fig. 5.

HSPG-mediated cointernalization of hBDs and HIV into polarized infant tonsil epithelial cells. (A) Recombinant hBD2 and hBD3 (100 μg/ml each) were added to the apical surfaces of polarized infant IT#2 cells and incubated at 37°C for 30 min. Untreated cells served as a control. Cells were fixed, permeabilized and immunostained for hBD2 and hBD3 (both in green) and analyzed by confocal microscopy. Cell nuclei are stained in red. Representative XY images in single plane are shown. (B) Cells were treated with heparinase and dissociated with 0.25% trypsin to remove uninternalized hBDs from the cell surface, and intracellular

hBD2 and hBD3 were detected by Western blotting. The mean densities of pixels in the protein bands were measured by ImageJ software and the results for each gel are shown as a bar graph under each blot. (C) Recombinant hBD2 or hBD3 was added to the apical surfaces of IT#2 polarized cells, which were incubated at 4°C and 37°C for 30 min. Then, uninternalized hBDs were removed by heparinase and trypsin treatments, and internalized hBD2 and hBD3 were detected by Western blot assay. (D) IT#2 cells were treated with heparinase or not treated, washed, and incubated with hBD2 and hBD3 for 30 min at 4°C. Cells were washed and incubated with HIV-1_{SF33} for 1 h at 37°C. Cells were dissociated with 0.25% trypsin, and intracellular hBD2, hBD3 and HIV p24 were detected by Western blotting. B, C and D. The mean densities of pixels in the protein bands are shown as a bar graph under each blot. (E) hBD2 and hBD3 were added to the apical surfaces of IT#2 polarized cells for 30 min at 4°C. Then, cells were washed and incubated with HIV-1_{SF33} for 30 min at 37°C. Cells were fixed, immunostained for hBDs (red) and HIV (green), and examined by confocal microscopy. Yellow in merged panels (upper and middle raw) indicates colocalization of HBDs with HIV. A-D. Results shown are from one representative experiment out of three experiments using IT#2 and IT#3 cells.

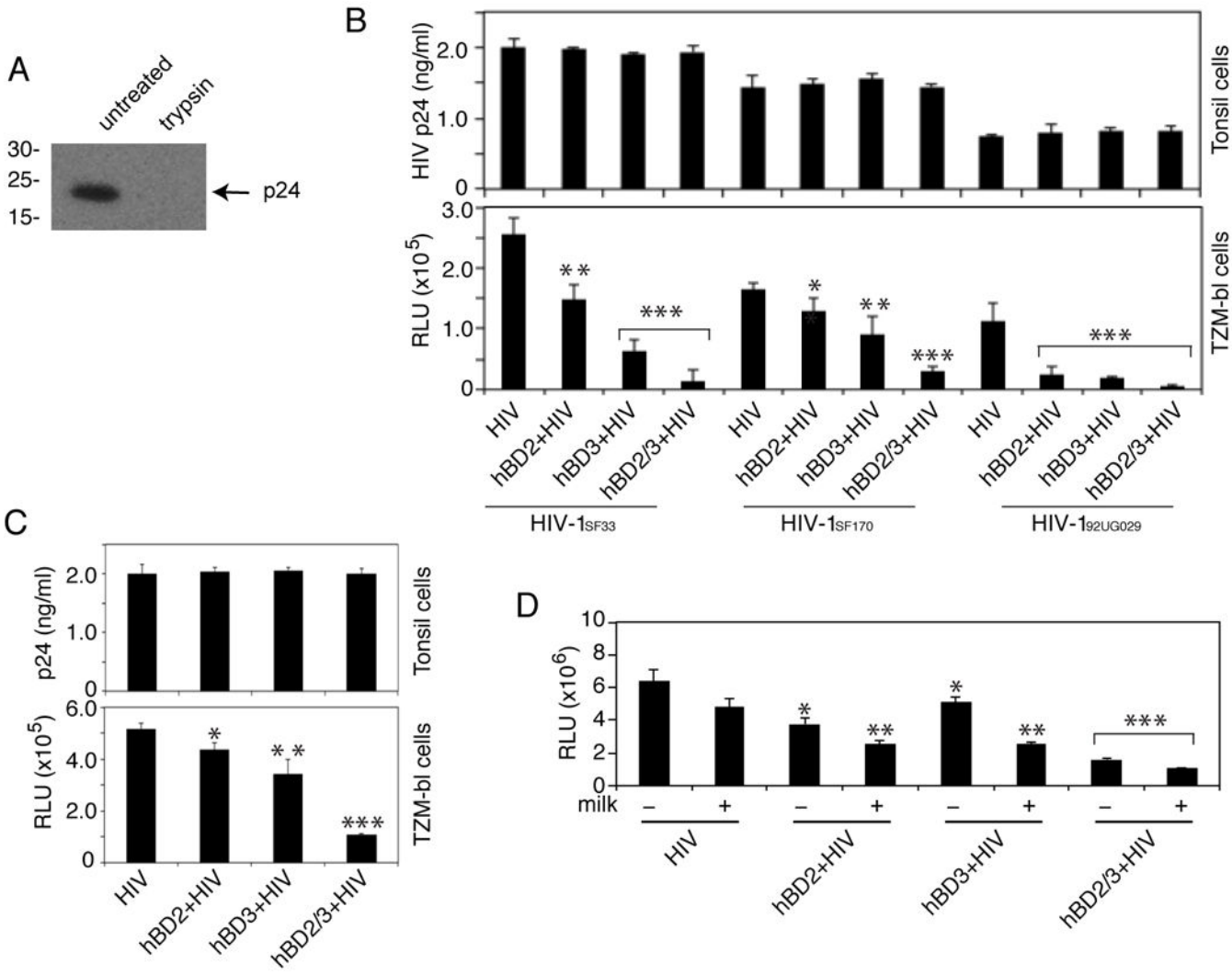

Fig. 6.

Internalization of hBD2 and/or hBD3 into infant tonsil cells inactivates intracellular HIV. (A) Polarized infant IT#2 tonsil epithelial cells were incubated with 40 ng/ml of HIV-1_{SF33} virions for 1 h at 4°C. Then, one set of cells was washed and trypsinized with 0.25% trypsin. Another set of cells was not treated with trypsin. Dissociated cells were washed and lysed, and HIV p24 was examined by Western blot. (B) IT#2 cells were incubated with hBD2 and/or hBD3 (100 μg/ml of each in individual and 50 μg/ml of each in combination) for 30 min at 4°C. Cells were washed, and HIV-1_{SF33}, HIV-1_{SF170} and HIV-$1_{92UG029}$ virions were added to the apical surfaces of polarized cells. Cells were incubated for 2 h at 37°C. Then, cells were trypsinized to remove uninternalized virions and equally divided into two tubes. Cells in one tube were lysed and used for detection of intraepithelial HIV by using p24 ELISA (upper panel). Cells in the other tube were homogenized and used for infection of TZM-bl cells (lower panel). After 48 h, HIV infection was measured by Bright-GloTM Luciferase Assay. (C) Polarized infant epithelial cells IT#3 were incubated with hBD2 and/or hBD3 (40 μg/ml of each in individual and 20 μg/ml of each in combination) for 30 min at 4°C. Cells were washed, and HIV-1_{SF33} virions were added to the apical surfaces of

polarized cells for 2 h at 37° and viral infectivity was examined in TZM-bl cells. (D) hBD2 and/or hBD3 (100 μg/ml of each in individual and 50 μg/ml of each in combination) were resuspended in pooled breast milk from 5 donors and added to the polarized infant IT#2 cells for 30 min at 4°C. HIV-1_{SF33} was resuspended in breast milk and added to the cells for the next 2 h at 37°C. Cells without breast milk served as a control. Cells were washed and trypsinized to remove uninternalized virions from the cell surface, homogenized, and used to infect TZM-bl cells. B, C and D. Bars indicate standard errors of the means (n=3). *$P <$ 0.01, **$P < 0.001$, ***$P < 0.0001$, compared with the HIV-uninfected control group. Three independent experiments using IT#2 and IT#3 cells showed similar results, and representative data from one experiment are presented.

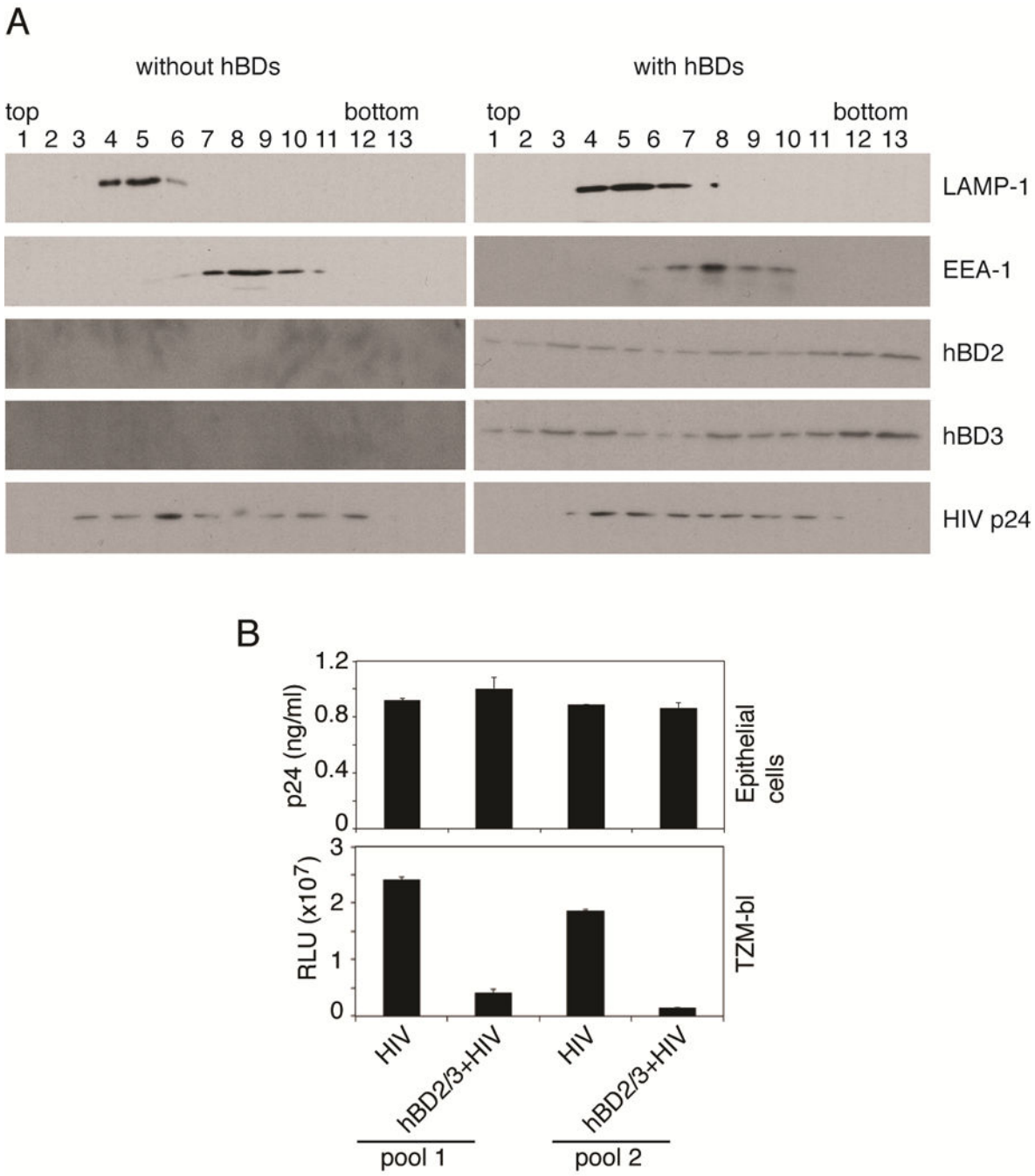

Fig. 7.

hBD2 and hBD3 inactivate HIV in early and late endosomal compartments of polarized infant tonsil epithelial cells. (A) hBD2 and hBD3 (50 μg/ml each) in combination were added to the apical surfaces of infant polarized epithelial cells IT#2 for 30 min at 4°C. One set of cells was not treated with hBDs (left panel). Cells were washed and incubated with HIV-1_{SF33} (200 ng/ml p24) for 2 h at 37°C. Cells were dissociated with trypsin, and homogenized and vesicular fractions were separated in sucrose gradients. Each fraction was examined for EEA-1, LAMP-1, hBD2, hBD3 and HIV p24 by Western blot. (B) Vesicular

fractions 4, 5 and 6 (pool 1) and 7, 8, 9, and 10 (pool 2) were combined and examined for HIV p24 by ELISA (upper panel). TZM-bl cells were infected with pool 1 and pool 2, and HIV infection was examined after 48 h (lower panel). Bars indicate standard errors of the means. Results with similar trends were obtained in 2 independent experiments using IT#2 and IT#3 infant tonsil epithelial cells.

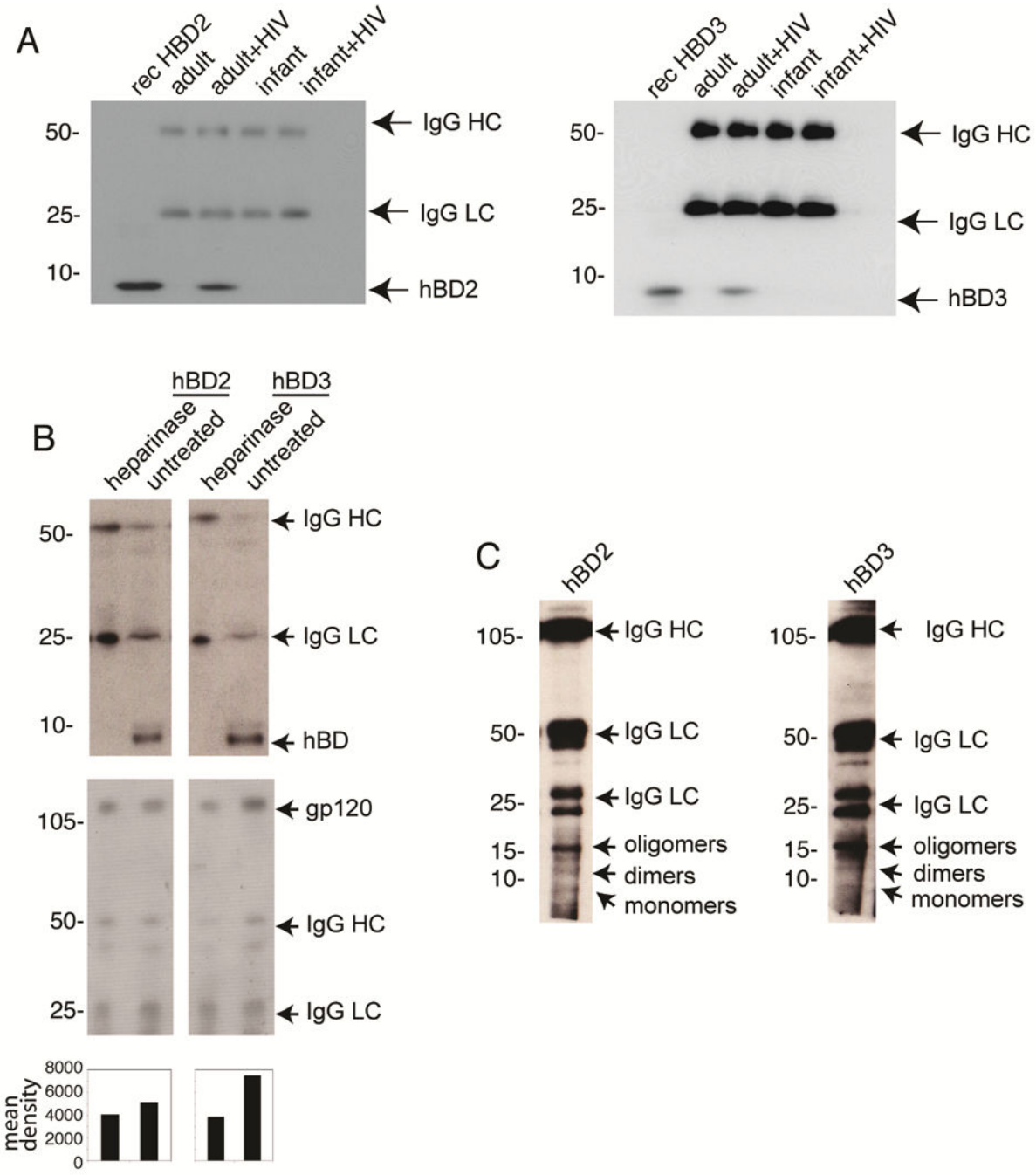

Fig. 8.

hBD2 and hBD3 bind to intracellular HIV. (A) HIV-1_{SF33} at 200 ng/ml p24 was added to the apical surfaces of polarized adult AT#1 and infant IT#1 tonsil epithelial cells, which were incubated for 2 h at 37°C. Then, uninternalized HIV was removed with trypsin, cells were homogenized, and the postnuclear supernatant was lysed and immunoprecipitated with anti-gp120 antibodies. hBD2 (left) and hBD3 (right) in gp120 precipitates were detected by Western blotting. (B) Apical membranes of infant polarized epithelial cells IT#1 were treated with heparinase or untreated. hBD2 or hBD3 (100 μg/ml each) was added to the

apical surfaces for 30 min at 4°C. Cells were washed, and HIV-1_{SF33} (200 ng/ml) was added for 2 h at 37°C. Extracellular HIV and hBDs were removed with trypsin, cells were lysed, and postnuclear supernatant was used for gp120 immunoprecipitation. (Upper panels) hBD2 and hBD3 were detected in gp120 precipitates by Western blotting. (Middle panels) Immunoprecipitated samples of the upper panels were blotted with anti-gp120 antibodies. (Lower panels) The gp120 protein bands of immunoprecipitated samples were measured by ImageJ software and mean densities of pixels for each band are shown as a bar graph under each blot. (C) Polarized infant epithelial cells IT#1 were treated with hBDs and HIV as described in panel B, and gp120 precipitates were separated under nonreducing conditions in 16% Tris-glycine gel. hBD2 and hBD3 were detected by Western blotting. Similar data were obtained from 2 independent experiments using infant IT#1 and IT#2 cells.