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Human Blood-Circulating Basophils Capture HIV-1 and Mediate Viral *trans*-Infection of CD4$^+$ T Cells

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ABSTRACT

Cell-associated HIV-1 infection has been proposed to play a pivotal role in the spread of HIV-1 infection. Granulocytes are a category of white blood cells, comprising mainly basophils, neutrophils, and eosinophils, and participate in various inflammatory reactions and defense against pathogens. Here, we investigated the role of human blood granulocytes in the dissemination of HIV-1. These cells were found to express a variety of HIV-1 attachment factors (HAFs). Basophils expressed HAFs dendritic cell (DC)-specific intercellular adhesion molecule 3 (ICAM3)-grabbing nonintegrin (DC-SIGN), DC immunoreceptor (DCIR), heparan sulfate proteoglycan (HSPG), and α4β7 integrin and mediated the most efficient capture of HIV-1 on the cell surface. Neutrophils were found to express DCIR and demonstrated limited efficiency of viral capture. Eosinophils expressed α4β7 integrin but exhibited little or no virus-binding capacity. Intriguingly, following direct contact with CD4$^+$ T cells, viruses harbored on the surface of basophils were transferred to T cells. The contact between basophils and CD4$^+$ T cells and formation of infectious synapses appeared necessary for efficient HIV-1 spread. In HIV-1-infected individuals, the frequency of basophils remained fairly stable over the course of disease, regardless of CD4$^+$ T depletion or the emergence of AIDS-associated opportunistic infections. Collectively, our results provide novel insights into the roles of granulocytes, particularly basophils, in HIV-1 dissemination. Thus, strategies designed to prevent basophil-mediated viral capture and transfer may be developed into a new form of therapy.

IMPORTANCE

Cell-associated HIV-1 infection has been proposed to play a pivotal role in the spread of HIV-1 infection. Here, we demonstrated that human blood-circulating granulocytes, particularly basophils, can capture HIV-1 and mediate viral *trans*-infection of CD4$^+$ T cells. The expression of a variety of HIV-1 attachment factors, such as the C-type lectins, etc., facilitates viral capture and transfer. Intriguingly, the frequency of basophils in patients with different levels of CD4$^+$ T counts remains fairly stable during the course of disease. Our results provide novel insights into the roles of granulocytes, particularly basophils, in HIV-1 dissemination. We suggest that strategies designed to prevent basophil-mediated viral capture and transfer may be a new direction for the development of anti-HIV therapy.

Granulocytes are a category of white blood cells (WBCs) characterized by the presence of lobulated nuclei and secretory granules in their cytoplasm. Blood-circulating granulocytes comprise mainly neutrophils, basophils, and eosinophils. Neutrophils make up the majority (50% to 60%) of circulating WBCs; basophils constitute only 0.5% to 1% and eosinophils less than 6%. Granulocytes are differentiated from bone marrow hematopoietic stem cells; they normally circulate in the bloodstream and are recruited to peripheral tissue under certain pathological conditions (1–5). Granulocytes participate in various inflammatory reactions. Basophils and eosinophils are known to modulate allergic disorders and autoimmune diseases (6–11).

Granulocytes play crucial roles in combating invading pathogens. The expression by human granulocytes of a broad range of pattern recognition receptors suggests that they play a role in various forms of host innate immunity (12–14), and evidence is mounting that granulocytes are essential to the regulation of host adaptive immunity (6, 15, 16). Activated granulocytes release various intracellular granule proteins or cytokines to suppress or directly kill invading microbes and parasites (17–24) or to recruit other host immune cells to combat pathogens. Neutrophils are "professional" phagocytes that rapidly engulf and degrade invaders or form extracellular traps to kill extracellular pathogens (23). Eosinophils have been described as capable of modulating the functions of other immune cells (16, 25).

The interplays between granulocytes and HIV-1 and their contribution to HIV-1 disease progression remain elusive. The majority of peripheral blood neutrophils do not express CD4 molecules on their surface. Previous work showed that 4 of 51 (7.8%) HIV-1-infected individuals and 3 of 25 (12%) uninfected individuals had CD4 expression on their peripheral blood neutrophils

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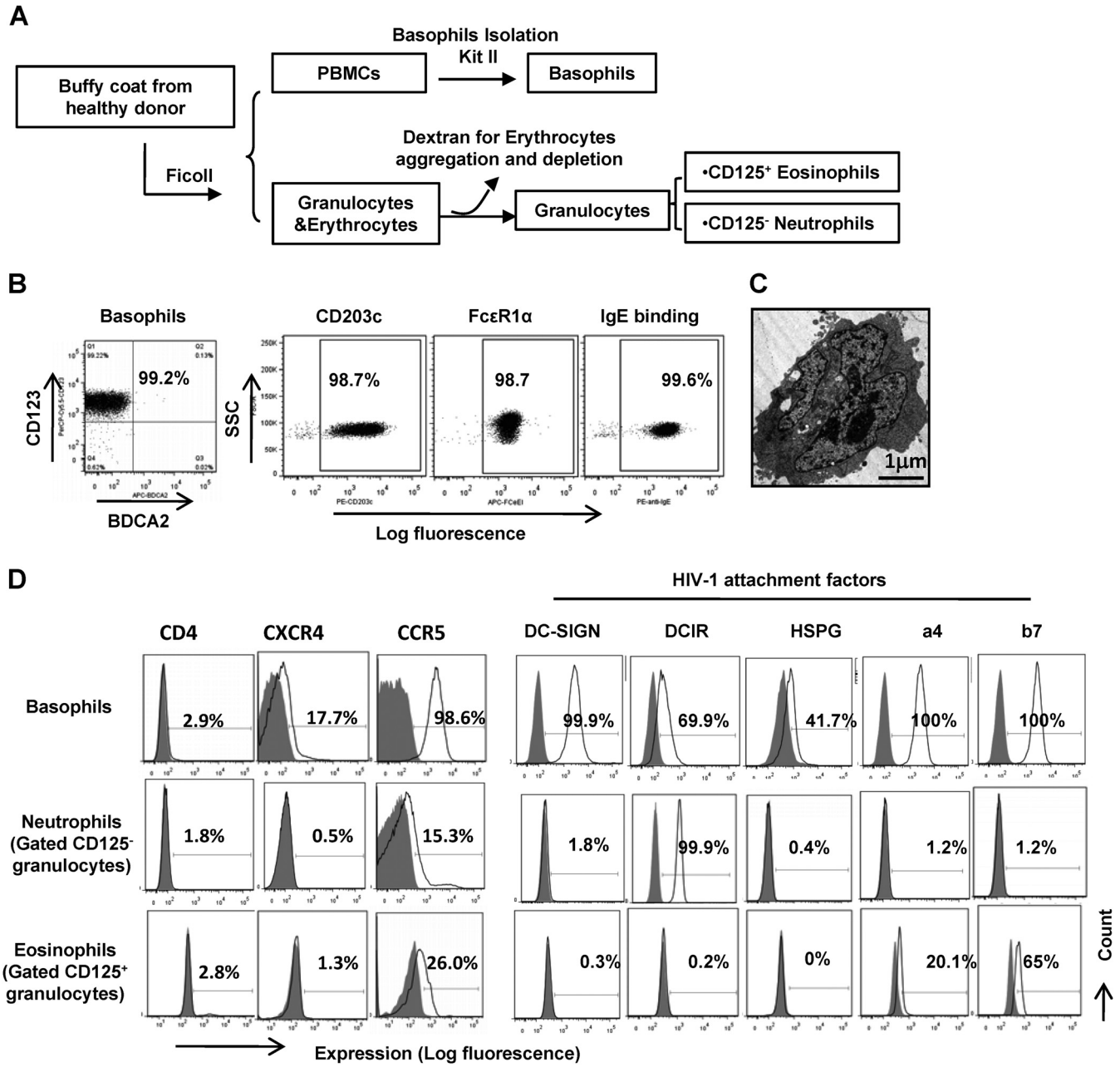

FIG 1 Expression of HIV-1 receptors and attachment factors by granulocytes. (A) Enrichment of granulocytes from peripheral blood of healthy donors. (B) Phenotype of purified basophils analyzed by immunostaining with specific antibodies and detected using flow cytometry. SSC, side-scattered light. (C) Visualization of basophils under TEM. (D) Expression of HIV-1 (co)receptors and attachment factors analyzed by immunostaining with specific antibodies and detected using flow cytometry. The positive percentage for immunostaining is noted, and results from one donor representative of six are shown.

(26); moreover, HIV-1 infection does not alter CD4 expression levels (26). Neutrophils are capable of releasing defensins and other antimicrobial peptides that suppress HIV-1 replication (27, 28). HIV-1 infection induces apoptosis and functional impairment of neutrophils, compromising their capacity for phagocytosis, oxidative burst, bacterial killing, etc. As a result, neutrophils in AIDS patients are unable to control opportunistic pathogens (29–32). HIV-1 virions can induce basophil degranulation, and HIV-1 gp120 can activate basophils isolated from healthy human donors to release histamine, cysteinyl leukotrienes, and the Th2 cytokines interleukin (IL)-4, IL-5, IL-10, and IL-13 (33). In some studies, the HIV-1 Tat accessory protein was also shown to induce the release of IL-4 and IL-13 from basophils of healthy donors (34, 35). The shift from Th1 cytokine production to Th2 cytokine production, partially caused by the HIV-1-induced degranulation of basophils, is believed to contribute to the creation of an allergy-like Th2 bias in HIV-1-infected adults and children (34, 36, 37). A recent study showed that eosinophil-secreted neurotoxin suppresses infection by HIV-1 and other single-stranded RNA viruses (38). Further elucidation of the interaction between HIV-1 and

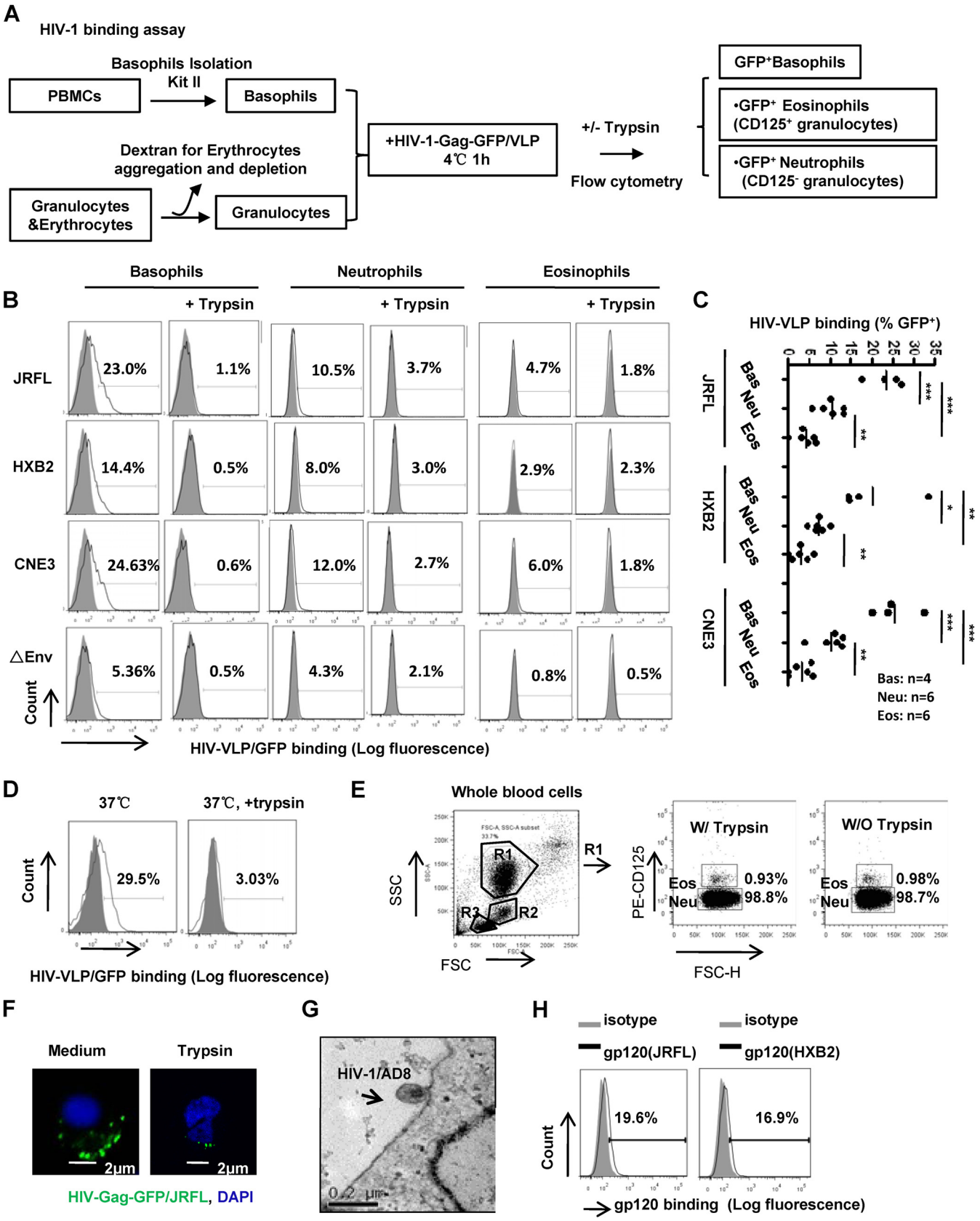

FIG 2 The binding of HIV-1 or gp120 on granulocytes. (A) Procedures for granulocyte enrichment and the HIV-1-binding assay. (B and C) Detection of HIV-1 VLP binding on granulocytes by flow cytometry. VLPs containing Gag-GFP were incubated with granulocytes at 4°C, and VLPs and ΔEnv were used as the

granulocytes may help to increase the understanding of HIV-1/AIDS pathogenesis.

Cell-associated HIV-1 infection has been proposed to play a pivotal role in HIV-1 spread (39–43). Dendritic cells (DCs) provide one of the best described cell models for understanding cell-mediated HIV-1 capture and dissemination (39, 42–45). The vaginal and rectal routes are the most frequent areas for HIV-1 mucosal or sexual infection, and these submucosa-located DCs are among the early targets for virus and subsequently contribute to the spread of HIV-1 infection to $CD4^+$ T cells (42, 43, 46–49). Both infectious particles captured during HIV-1 exposure and newly synthesized progeny virions could be transferred by DCs to T cells (39, 42, 50). Several host cellular HIV-1 attachment factors (HAFs) for mediating HIV-1 capture through interactions with glycosphingolipids in the virus lipid bilayer have been identified. These HAFs include the C-type lectin receptors DC-specific intercellular adhesion molecule 3 (ICAM3)-grabbing nonintegrin (DC-SIGN), langerin, DC immunoreceptor (DCIR; also known as CLEC4A), mannose receptor, and heparan sulfate proteoglycan (HSPG) molecules and $\alpha4\beta7$ integrins (39, 42, 45, 51–55). Viruses could be transferred via the infectious synapses formed between DC and $CD4^+$ T target cells or via the exosome secretion pathway (39, 41, 47, 56, 57). Lipopolysaccharide (LPS)-matured DCs significantly facilitated HIV-1 endocytosis and efficiently concentrated HIV-1 at the infectious synapses and thereby enhanced viral transmission (39, 41). C-type lectin receptor sialic acid-binding Ig-like lectin-1 (Siglec-1; also known as CD169) expressed on LPS-matured DCs, which could be induced by type 1 interferon for expression, was recently shown to mediate HIV-1 capture and spread (58).

In this study, we demonstrated that blood-circulating granulocytes, particularly basophils, can capture HIV-1 particles and transfer them to $CD4^+$ T cells for robust infection. Additionally, the various HAFs expressed on cell surfaces mediated viral binding and transfer. We also found that blood basophil frequencies are similar in patients at different stages of HIV infection; thus, blocking the capacity of the basophils for capturing and transferring virus to T cells may form a new therapeutic strategy.

MATERIALS AND METHODS

Ethics statement. The blood samples and clinical data were collected from HIV-1-infected individuals and healthy donors by licensed physicians at The Third People's Hospital in Kunming, Yunnan, China. The study was approved by the Medical Ethics Review Committee of The Third People's Hospital, and signed informed consent was obtained from each of the participants.

Cell culture. A Ficoll-Paque density gradient medium was used to separate peripheral blood mononuclear cells (PBMCs) from fresh buffy coats collected from healthy donors. Basophils were negatively selected from PBMCs using Basophil Isolation kit II (Miltenyi Biotec) and maintained in RPMI 1640 medium in the presence of 10 ng/ml of recombinant human IL-3 (rhIL-3). Blood constituents containing a mixture of erythrocytes and granulocytes were harvested and then added to dextran 2000 at a concentration of 6% to aggregate and deplete the erythrocytes. Neutrophils and eosinophils were further separated using anti-CD16 antibody-coated magnetic beads (Miltenyi Biotec) as described in previous studies (59, 60). Neutrophils were maintained in RPMI 1640 medium supplemented with 100 ng/ml of granulocyte-macrophage colony-stimulating factor (GM-CSF), and eosinophils were maintained in RPMI 1640 medium supplemented with 20 ng/ml of rhIL-3 and 20 ng/ml of GM-CSF. $CD14^+$ monocytes were isolated from PBMCs using anti-CD14 antibody-coated magnetic beads (Miltenyi Biotec) and treated with 50 ng/ml of GM-CSF and rhIL-4 for 5 days to generate dendritic cells, as described in previous studies (41, 61). Primary $CD4^+$ T lymphocytes were purified from PBMCs using anti-CD4 antibody-coated magnetic beads (Miltenyi Biotec). The Hut/CCR5 $CD4^+$ T-lymphocyte cell line was generously provided by Li Wu (The Ohio State University, USA) (41). All cytokines were purchased from R&D Systems.

HIV-1 stocks. Pseudotyped single-cycle-infectious HIV stocks were generated via calcium-phosphate cotransfection of HEK293T cells with pLAI-ΔEnv–Luc and plasmids expressing JRFL (CCR5-tropic) or HXB2 (CXCR4-tropic) HIV envelope proteins (Env), as described previously (41). HIV-like particles (VLPs) was generated by cotransfecting HEK293T cells with a plasmid containing Gag-green fluorescent protein (Gag-GFP) and plasmids expressing JRFL, HXB2, and CNE3 (CCR5-tropic) HIV envelope proteins as reported previously (62). The plasmid expressing CNE3 envelope protein, cloned from an HIV-1 isolate circulating in China (63), was provided by Lin-Qi Zhang (Tsinghua University, China). The replication-competent HIV-1-AD8 (CCR5-tropic) virus was produced by transfection with the pNLAD8 vector containing HIV-1 proviral DNA. Cell-free supernatant was harvested, filtered, and titrated using a $p24^{gag}$ capture enzyme-linked immunosorbent assay (ELISA).

Flow cytometry. Cells were stained with specific monoclonal antibodies (MAbs) or isotype-matched IgG controls. The staining was performed using MAbs directed against the following human molecules (clone numbers and sources are given in parentheses): peridinin chlorophyll protein (PerCP)-Cy5.5-CD123 (6H6; eBioscience); allophycocyanin (APC)-BDCA2 (AC144; MACS); phycoerythrin (PE)-CD203c (NP4D6; Bielefeld); APC-FcεR1α (AER-37; eBioscience); APC-CD15 (VIMC6; MACS); PE-CD125 (A14; BD); PE-CD4 (L3T4; eBioscience); APC-CXCR4 (12G5; BD Pharmingen); APC-Cy7-CCR5 (2D7; BD Pharmingen); PE–DC-SIGN (eB-h209; eBioscience); and fluorescein-DCIR (50586; R&D Systems). Purified unlabeled antibodies directed against human DC-SIGN (120507; Abcam), HSPG (A7L6; Abcam), α4 (EPR1355Y; Abcam), and β7 (EP5948; Abcam) were used in some of the experiments, and secondary anti-mouse IgG-fluorescein isothiocyanate (FITC) or anti-rat IgG-FITC was used for detection. In the appropriate experiments, human IgE protein (ab90392; Abcam) was used first, followed by PE-conjugated anti-human IgE antibodies (MHE-18; Biolegend). The stained cells were analyzed using a Fortessa flow cytometer (BD Pharmingen) with FlowJo 7.6.1 software.

HIV-Gag-GFP/VLP or gp120 binding assay. The granulocytes or monocyte-derived dendritic cells (MDDCs) were incubated with the VLPs (40 ng $p24^{gag}$) for 1 h at 4°C and then washed. The amount of

control to detect nonspecific binding. Trypsin was used to treat the cells for 5 min at room temperature to remove surface-bound VLPs. The percentage of GFP-positive cells is labeled, and the results for at least four donors are summarized and analyzed in panel C. *, $P < 0.05$; **, $P < 0.01$; **, $P < 0.001$ (representing statistically significant differences in a paired *t* test). Bas, basophils; Neu, neutrophils; Eos, eosinophils. (D) Association of HIV-1 VLPs with basophils at 37°C. (E) CD125 expression upon trypsin treatment. The whole-blood cells were treated with 0.25% trypsin (W/Trypsin) for 5 min at room temperature or left untreated (W/O Trypsin), and then CD125 was immunostained with specific antibodies, followed by the secondary antibodies, the population of granulocytes was gated, and the CD125 level on either eosinophils or neutrophils was analyzed using flow cytometry. FSC, forward scatter; FSC-H, forward-scattered light height. (F) Confocal microscopy was used to observe the basophil-mediated uptake of HIV-Gag-GFP/JRFL VLP. (G) The basophil-mediated uptake of HIV-1-AD8 was visualized by TEM. (H) Binding of gp120 on basophils. The recombinant gp120 of HIV-1 JRFL or HXB2 was added to purified basophils, which were then immunostained with anti-gp120 antibodies, followed by secondary antibodies. The cells were examined using flow cytometry. Positive percentages for immunostaining are noted, and results from one donor representative of four are shown.

Gag-GFP associated with the cells was quantified by flow cytometry, and the mean fluorescence intensity was calculated. The cells were treated with 0.25% trypsin (without EDTA) (Invitrogen) for 5 min at room temperature to remove VLPs bound to the cell surface. The viral particle bound to cell surface was also visualized using confocal microscopy and transmission electron microscopy (TEM) as described below. To perform the HIV-1 gp120 binding assay, the cells were incubated with 5 μg/ml gp120 (JRFL or HXB2) (eEnzyme) in adherent buffer (1 mM $CaCl_2$, 2 mM $MgCl_2$, 5% bovine serum albumin [BSA], pH 7.4) for 1 h at 4°C. The cells were then fixed with 4% paraformaldehyde (PFA) (Sigma-Aldrich) for 10 to 20 min and stained with goat anti-gp120 antibodies (SAB3500463; Sigma-Aldrich). Subsequently, the cells were stained with FITC-conjugated secondary anti-goat antibodies (sc-2356; Santa Cruz Biotechnology).

HIV-1 transmission and enhancement assay. The HIV-1 transmission assay was performed using a luciferase reporter system as previously described (41). In brief, the donor cells (2×10^5) were loaded with pseudotyped, single-cycle-infectious, luciferase-containing HIV-luc/JRFL or HIV-luc/HXB2 (10 ng $p24^{gag}$) reporter virus, separately, for 2 h at 37°C, washed thoroughly, and then cocultured with Hut/CCR5 (2×10^5) cells for 3 days or with phytohemagglutinin (PHA)-P-stimulated primary $CD4^+$ T cells (2×10^5) for 5 days. Enhancement assays were performed as previously described (56, 64); briefly, HIV-luc/JRFL (1 or 4 ng $p24^{gag}$) (i) was loaded to basophils (2×10^5) for 2 h and the pulsed basophils were cocultured with Hut/CCR5 cells for an additional 2 days or (ii) was added directly to Hut/CCR5 cells for 2 days of infection. A commercially available kit (Promega) was used to analyze viral infection by measuring luciferase activity in the cell lysate.

To perform the transmission blocking assay, some of the donor cells were treated prior to viral inoculation with 10 μg/ml of anti-HSPG MAbs (A7L6; Abcam), anti-DC-SIGN antibodies (120507; Abcam), and anti-DCIR antibodies (216110; R&D), or with 20 μg/ml of mannan, for 1 h at 4°C. The others were treated with 10 mM EGTA during viral inoculation. In some of the experiments, 0.25% trypsin was used to treat the cells for 5 min at room temperature to remove cell surface-bound viruses before coculturing. As displayed in the figures and described previously (41), transwell plates with a 0.4-μm-pore-size membrane were used to separate the donor cells from the target cells.

Confocal microscopy. The granulocytes were incubated with 40 ng $p24^{gag}$ HIV-Gag-GFP/JRFL VLP for 1 h and then seeded onto poly-L-lysine-coated microscope slides (PolyScience). The cells were fixed with 4% PFA for 10 min at room temperature and immunostained with purified MAbs (1 μg/ml) against human DC-SIGN (120507; Abcam), DCIR (216110; R&D), HSPG (A7L6; Abcam), α4 (EPR1355Y; Abcam), or β7 (EP5948; Abcam), and the secondary antibody Alexa 546-labeled goat anti-mouse IgG (Invitrogen) (1 μg/ml) was used for immunostaining. The nuclei were stained with DAPI (4′,6-diamidino-2-phenylindole).

To enable the formation of infectious synapses, the virus-loaded granulocytes were first cocultured with Hut/CCR5 cells for 30 min in polystyrene tubes at 37°C before being seeded on slides. The cells were then fixed with 4% PFA for 10 min or 2 h at room temperature. Purified MAbs against human FcεR1α (9E1; Abcam), β-tubulin (clone SAP.4G5; Sigma-Aldrich), DC-SIGN (120507; Abcam), and CD4 (clone Q4120; Sigma-Aldrich) were added first, and then the secondary antibodies Alexa 546-labeled goat anti-mouse IgG or Alexa 350-labeled goat anti-mouse IgG (Invitrogen) (1 μg/ml) were used for immunostaining. All of the slides were mounted in a fluorescent mounting medium (Dako) and observed under a laser scanning confocal microscope (Leica SP5).

TEM. The use of transmission electron microscopy (TEM) to visualize the interactions between HIV-1 and host cells has been described in a previous publication (41). Briefly, granulocytes were pulsed with replication-competent HIV-1-AD8 (5 ng $p24^{gag}$), and the virus-loaded granulocytes were cocultured with Hut/CCR5 cells for 1 h before fixation. Thin sections were examined using a Jeol JEM-1230 TEM operating at 100 kV.

Statistical analysis. SigmaStat software was used to perform paired and unpaired *t* tests to analyze statistically significant differences.

RESULTS

Blood-circulating granulocytes express HAFs. The buffy coats collected from the healthy donors were separated using a Ficoll-Paque density gradient medium, and a layer of PBMCs was harvested, along with the constituents containing a mixture of erythrocytes and granulocytes (Fig. 1A). Basophils were enriched directly from the PBMCs by negative isolation, using Basophil Isolation kit II. During this process, T cells, NK cells, B cells, monocytes, dendritic cells, erythroid cells, platelets, neutrophils, and eosinophils were depleted by a cocktail of biotin-conjugated antibodies against CD3, CD4, CD7, CD14, CD15, CD16, CD36, CD45RA, HLA-DR, and CD235a followed by anti-biotin antibody-coated magnetic beads. More than 98% of the basophils isolated presented a $CD123^+$ $CD203c^+$ $BDCA2^-$ cell phenotype and expressed the FcεR1 high-affinity IgE receptor (Fig. 1B). The basophils were also shown by TEM to exhibit phenotypes with polylobed nuclei and condensed chromatin patterns (Fig. 1C). The constituents containing erythrocytes and granulocytes were added to dextran to aggregate and deplete the erythrocytes. The remaining granulocytes were distinguished by immunostaining with anti-CD125 antibodies, as eosinophils, unlike neutrophils, express a high level of CD125 on the cell surface (65) (Fig. 1A).

First, the expression of HIV-1 (co)receptors was measured. The three types of granulocyte were immunostained with specific antibodies and detected by flow cytometry. The eosinophils were gated as $CD125^+$ granulocytes and the neutrophils as $CD125^-$ granulocytes. None of the granulocytes collected from the tested donors were observed to express the CD4 molecule. All of the granulocytes expressed CCR5, but only the basophils expressed CXCR4 (Fig. 1D).

In addition to entry receptors, viruses subvert a wide variety of molecules expressed on the cell surface as viral attachment receptors, such as HSPG, lectins, integrins, scavenger receptors, sialic acids, and glycolipids and other carbohydrate moieties (66–71). HSPG molecules, α4β7 integrins, and the C-type lectins of DC-SIGN, DCIR, and mannose receptors have been shown to bind with HIV-1 gp120 (39, 51–55). We found that the basophils expressed multiple HAFs, such as DC-SIGN, DCIR, HSPG, and α4β7 integrin. The $CD125^-$ neutrophil granulocytes expressed DCIR, and the $CD125^+$ eosinophils expressed α4β7 integrin (Fig. 1D). Together, these data demonstrate that granulocytes express a variety of HAFs on their surface.

Basophils efficiently capture HIV-1 particles on the cell surface, whereas neutrophils perform viral capture less efficiently. As multiple HAFs are expressed on granulocytes, it is important to measure which ones mediate viral binding. Purified basophils and a mixture of eosinophil and neutrophil granulocytes were incubated with VLPs containing HIV-Gag-GFP. The VLPs were pseudotyped with envelope proteins of JRFL, HXB2, or CNE3, and the VLPs/ΔEnv that did not incorporate HIV-1 envelope proteins were used to monitor nonspecific binding (Fig. 2A). At 4°C, basophils were the most efficient cells for VLP capture; neutrophils bound fewer VLPs, and eosinophils displayed little capacity for VLP binding (Fig. 2B and C). No bind-

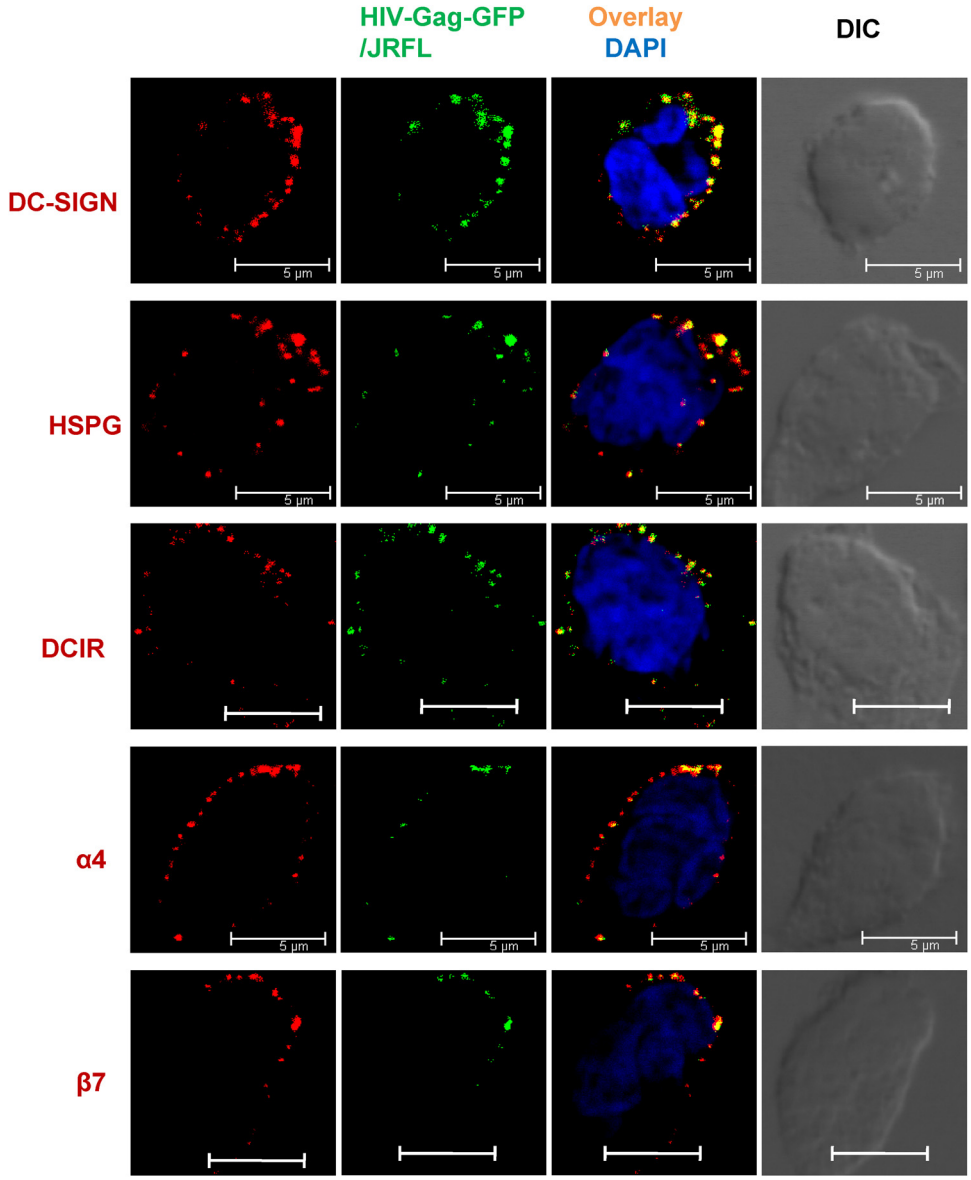

FIG 3 The colocalization of HIV-VLP with HAFs. Purified basophils were incubated with HIV-Gag-GFP/JRFL VLP (40 ng $p24^{gag}$) for 1 h at 4°C and seeded on poly-L-lysine-coated microscope slides. Cells were fixed and immunostained with specific antibodies against human DC-SIGN, HSPG, DCIR, and α4 or β7 integrin followed by secondary Alexa 546-labeled goat anti-mouse IgG antibodies. Nuclei were detected with DAPI, and cells were observed by confocal microscopy. Bar, 5 μm. DIC, differential interference contrast.

ing was observed with the VLP/ΔEnv, indicating that the binding was envelope dependent (Fig. 2B). The majority of the cell-associated VLPs could be removed by trypsin digestion at 4°C (Fig. 2B), and when the temperature was shifted to 37°C to enable endocytosis, trypsin digestion also removed the majority of the basophil-captured VLPs (Fig. 2D). These findings demonstrate that the VLPs were bound preferentially to the cell surface. Trypsin treatment could remove some molecules from the cell surface (41) but did not alter the proportions of CD125-positive and -negative cells, as shown in Fig. 2E.

Confocal microscopy was used to observe the binding of VLPs to the cell surface of basophils (Fig. 2F), and replication-competent HIV-1-AD8 was used to visualize the binding of virus to basophils under TEM (Fig. 2G). Condensed chromatin of the basophils was observed (Fig. 2G). To confirm that the basophil-mediated VLP binding was envelope dependent, we examined the binding of recombinant HIV-1 gp120 protein to basophils. Both HIV-1 JRFL-derived gp120 glycoprotein and HXB2-derived gp120 glycoprotein were found to bind to basophils (Fig. 2H).

Collectively, these data demonstrate that basophils were responsible for the most efficient capture of HIV-1 particles on the cell surface and that this process was HIV-1 envelope dependent.

The HIV-1 bound on basophils whose spread was mediated by HAFs could *trans*-infect $CD4^+$ T cells. We found that granulocytes express a variety of HAFs on the cell surface (Fig. 1D) and that basophils are responsible for the most efficient capture of

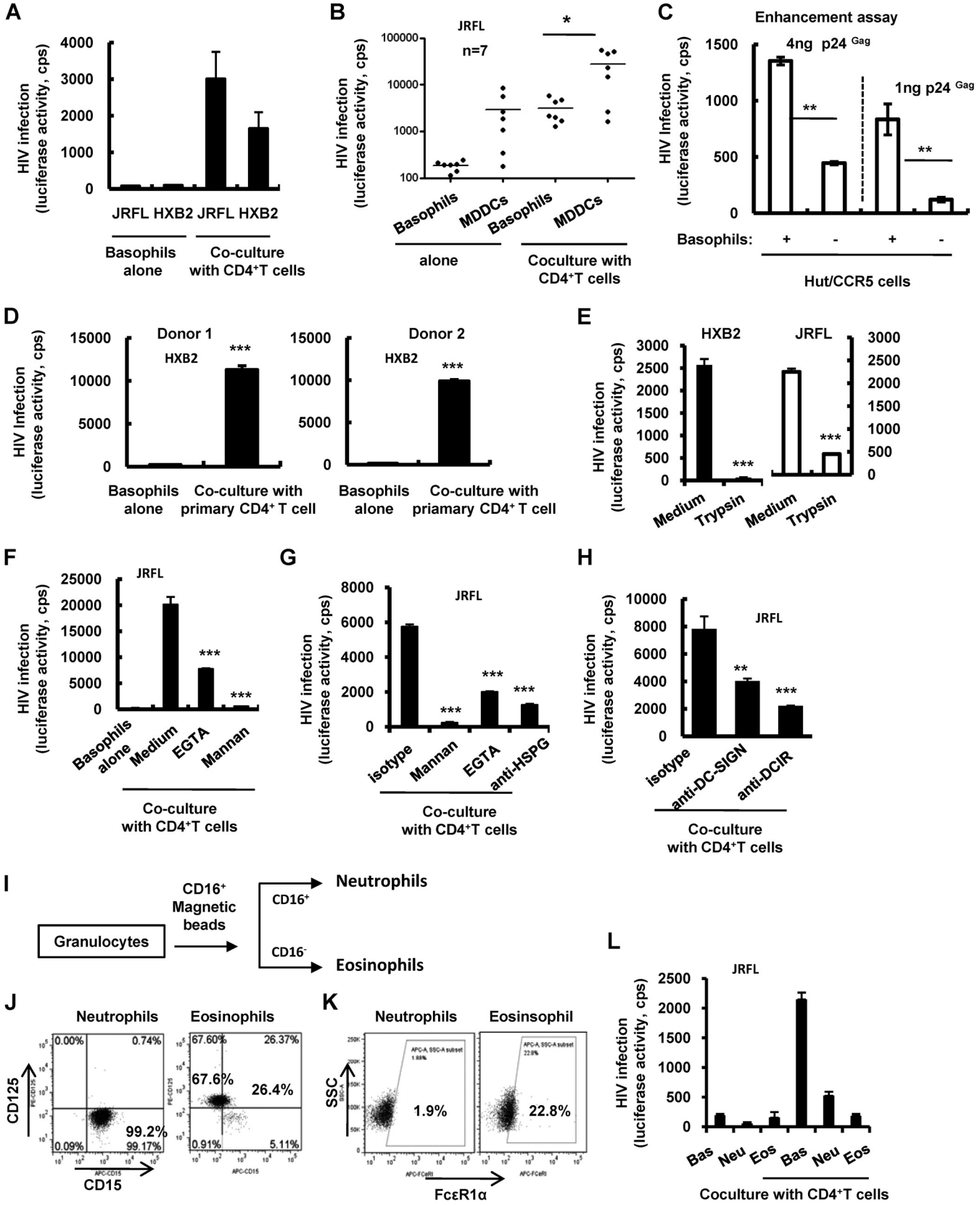

FIG 4 Granulocyte-mediated HIV-1 transmission. (A) Basophil-mediated HIV-1 transmission to CD4$^+$ T cells. The purified basophils were incubated with single-cycle-infectious HIV-luc/JRFL or HXB2 for 2 h. The virus-loaded basophils were then washed and cocultured with and without Hut/CCR5 cells for 3 days. The cells were lysed to measure luciferase activity and thereby quantify HIV-1 infection. (B) Summary of data collected on basophil-mediated HIV-1

HIV-1 particles (Fig. 2B and C). Next, we observed the overlay of HIV-VLP with HAFs under a confocal microscope. Colocalizations of HIV-VLPs with all tested HAFs were observed on basophils (Fig. 3), indicating the role of HAFs in viral capture.

To investigate whether basophils are capable of transferring surface-bound viruses to $CD4^+$ T cells, purified basophils were pulsed with pseudotyped single-cycle HIV-luc/JRFL or HIV-luc/HXB2 viruses and then cocultured with $CD4^+$ T cell Hut/CCR5 for 3 days. The infection caused by the transfer of HIV-1 to the Hut/CCR5 cells was monitored by measuring luciferase activity. Basophils were found to transfer HIV-1 to the cocultured Hut/CCR5 cells, leading to robust infection (Fig. 4A and B), although HIV-1 spread was mediated less effectively by the basophils than by the autologous MDDCs (Fig. 4B), and, as expected, no obvious viral replication was observed in the basophils (Fig. 4A and B). We also performed an enhancement assay to show significantly increased viral infection mediated by cell-associated HIV-1 compared with cell-free viruses (Fig. 4C). Additionally, the PHA-activated primary $CD4^+$ T cells were used as the target cells to demonstrate the viral transmission mediated by basophils (Fig. 4D).

Treating the virus-harbored basophils with trypsin before coculturing them with the T cells was found to significantly diminish HIV-1 transmission (Fig. 4E), suggesting that surface-bound HIV-1 particles play the greatest role in viral transfer. The incubation of basophils with mannan or antibodies against HSPG, DC-SIGN, or DCIR before viral inoculation was found to significantly diminish basophil-mediated HIV-1 transmission (Fig. 4F, G, and H), demonstrating that these HAFs mediate viral spread. The binding of glycoprotein mediated by HSPG and C-type lectin can be blocked by prior treatment with mannan (55, 72, 73). We found that prior treatment with mannan almost completely eradicated basophil-mediated HIV-1 transmission (Fig. 4F and G), indicating that HSPG, DC-SIGN, and DCIR molecules might have the most prominent roles in HIV-1 capture and spread. As glycoprotein capture by C-type lectins is Ca^{2+} dependent (45), the addition of EGTA to chelate Ca^{2+} ions during viral inoculation with basophils impaired gp120 capture, leading to diminished viral transmission (Fig. 4F and G).

The capacity of neutrophils and eosinophils to mediate HIV-1 *trans*-infection was also investigated. Neutrophils were found to be much less frequently involved than basophils in HIV-1 binding, and eosinophils showed little or no capacity to bind HIV-1 (Fig. 2B and C). $CD125^-$ neutrophils exhibited the $CD16^+$ phenotype, whereas $CD125^+$ eosinophils exhibited the $CD16^-$ phenotype. Therefore, neutrophils and eosinophils could be separated using anti-$CD16^+$ antibody-coated magnetic microbeads (Fig. 4I). The purity was confirmed by flow cytometry, as the $CD16^+$ granulocyte population showed a neutrophil phenotype of $CD125^-$ $CD15^+$ $Fc\varepsilon R1^-$ and the $CD16^-$ granulocyte population presented an eosinophil phenotype of $CD125^+$ $CD15^{+/-}$ $Fc\varepsilon R1^+$ (Fig. 4J and K). The HIV-1 spread mediated by neutrophils or eosinophils was much less extensive than that mediated by basophils (Fig. 4L), which might have been due to the much lower level of viral binding on neutrophils and eosinophils (Fig. 2B and C). Together, these data demonstrate that human blood-circulating basophils can mediate HIV-1 *trans*-infection of $CD4^+$ T cells through viral attachment factor-dependent viral binding on the cell surface.

Contact between basophils and T cells and formation of the infectious synapses facilitate viral *trans*-infection. To determine whether viral transfer requires direct contact between basophils and $CD4^+$ T cells, a transwell culture plate with a 0.4-μm-pore-size membrane was used to separate the virus-loaded basophils from the Hut/CCR5 target cells. The basophil-mediated transmission of both HIV-luc/HXB2 and HIV-luc/JRFL ceased in the transwell assay (Fig. 5A), suggesting that contact between basophils and $CD4^+$ T cells is required for viral transfer.

In the study of DC-mediated HIV-1 *trans*-infection of T cells, the infectious synapses formed between DCs and T cells appear to be crucial in viral *trans*-infection (39, 41, 56). The recruitment of HIV-1 to the basophil-$CD4^+$ T cell contact sites that form infectious synapses was also visualized using confocal microscopy. The Vpr-GFPs incorporating replication-competent HIV-1-AD8 viruses were found to be recruited to the basophil-$CD4^+$ T cell contact sites (Fig. 5B and C). The infectious synapses were also observed under TEM, and the basophils were characterized by the presence of typical cytoplasmic granules (Fig. 5D). Numerous HIV-1-AD8 viruses were observed at the basophil-$CD4^+$ T cell contact sites (Fig. 5D).

Analysis of infectious synapses between DCs and T cells have revealed the recruitment of HIV-1 receptors and coreceptors to conjugate sites (56); we also observed that CD4 molecules on T cells were recruited to the interface upon which the basophils concentrated viral particles (Fig. 5E). Moreover, the DC-SIGN receptor and even the FcεR1 receptor on the basophils were recruited to conjugate sites (Fig. 5C and F).

Together, these data suggest that direct contact between basophils and $CD4^+$ T cells and the formation of infectious synapses facilitate viral *trans*-infection where HIV-1 particles, viral receptors, and the virus-loaded HAFs have been concentrated.

The basophil frequency remained fairly stable during disease progression. To determine the effect of AIDS disease progression on granulocyte frequency, the complete blood counts of HIV-1 individuals with or without opportunistic infections were analyzed. As expected, patients with opportunistic pathogens displayed a significant reduction in levels of $CD4^+$ and $CD8^+$ T lymphocytes and a heightened level of viral load compared with HIV-1-infected individuals without opportunistic infections (Fig. 6A). HIV-1 infection was found to profoundly deplete the lymphocyte subpopulation, and this decline in lymphocyte count was exacerbated in patients with opportunistic infections (Fig. 6B).

transmission from seven donors and comparison with data on autologous MDDCs. (C) Enhancement assay. HIV-luc/JRFL-pulsed basophils were cocultured with Hut/CCR5 cells, or the same amounts of cell-free viruses were added directly to T cells, and viral infection was measured as described above after 2 days of culture. (D) PHA-P-activated primary $CD4^+$ T cells were used as the target cells to investigate basophil-mediated *trans*-infection by HIV-luc/HXB2. Results from two donors are shown. (E) Treating virus-loaded basophils with trypsin before coculture impairs HIV-1 transmission. (F, G, and H) Treatment with specific antibodies or mannan before viral inoculation and addition of EGTA during viral incubation diminish basophil-mediated HIV-1 transmission. (I) Procedure for the separation of neutrophils and eosinophils, using anti-CD16 antibody-coated magnetic beads. (J and K) Immunostaining with specific antibodies to identify the phenotypes of the neutrophils and the eosinophils by detection using flow cytometry. (L) Comparison of levels of HIV-1 transmission mediated by different types of granulocyte; results from one donor representative of seven are shown. cps, counts per second. *, $P < 0.05$; **, $P < 0.01$; **, $P < 0.001$ (representing statistically significant differences in a paired *t* test).

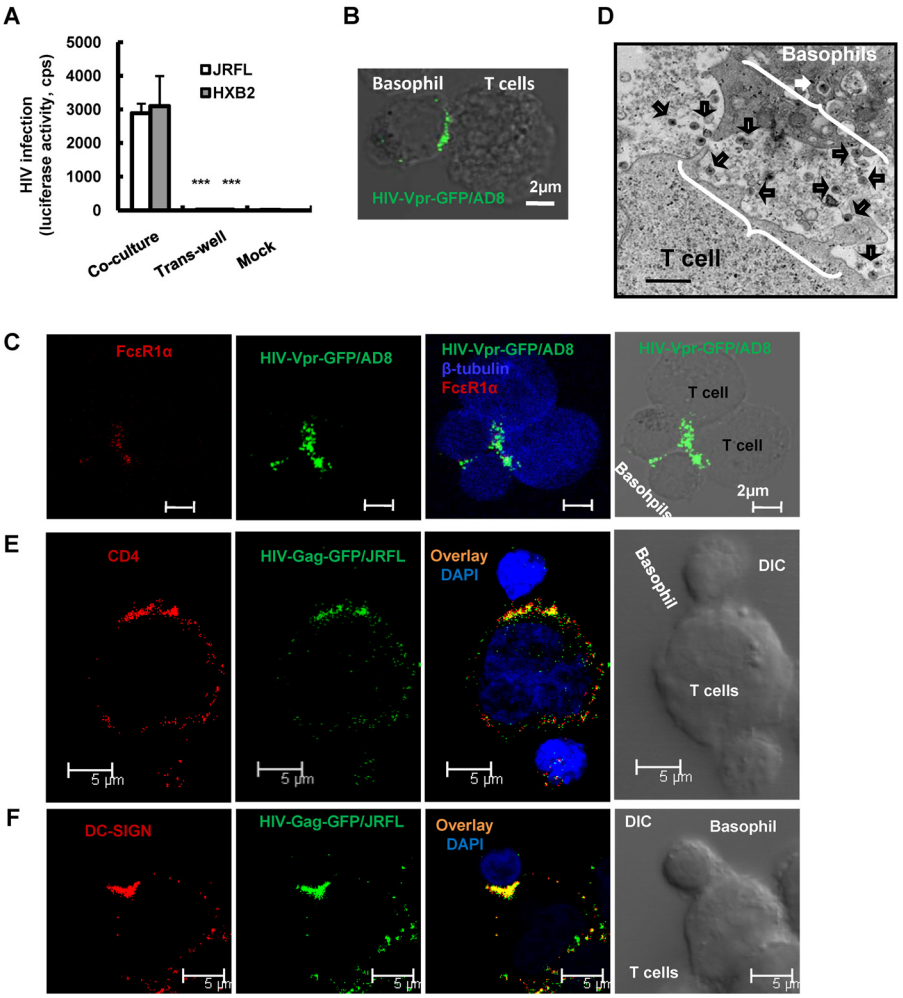

FIG 5 Infectious synapses formed between basophils and $CD4^+$ T cells appear necessary for HIV-1 *trans*-infection. (A) The separation of virus-loaded basophils from T cells terminates HIV-1 transmission. Transwell plates with 0.4-μm-pore-size membranes were used to separate the basophils from Hut/CCR5 cells, and viral infection was assessed. ***, $P < 0.001$ (representing statistically significant differences in a paired *t* test). Mock, the coculture of virus-unloaded basophils with Hut/CCR5. Results represent one experiment representative of four repeats. (B, C, E, and F) The recruitment of viruses, HAF, or viral receptor to conjugate sites. The basophils were incubated with HIV-1 Vpr-GFP/AD8 or HIV-Gag-GFP/JRFL (40 ng $p24^{gag}$) for 2 h at 37°C and cocultured with Hut/CCR5 cells for 30 min. Cells were fixed and immunostained first with specific antibodies and subsequently with the secondary antibodies. The nuclei were labeled with DAPI. Bar, 2 μm. (D) Formation of infectious synapses between basophils and $CD4^+$ T cells visualized under TEM. Replication-competent HIV-1-AD8 viruses in 5-ng $p24^{gag}$ amounts were cultured with basophils for 2 h at 37°C. The virus-loaded basophils were then washed and cocultured with Hut/CCR5 for 30 min. Cells were fixed, sectioned, and visualized by TEM. Empty arrows indicate HIV-1 recruited to the contact sites, and the white arrow indicates endocytosed viral particles. Bar, 0.5 μm.

HIV-1 infection and the acquisition of opportunistic pathogens had no obvious effects on basophil count (Fig. 6B), whereas a trend toward reduced numbers of eosinophils and neutrophils was seen in HIV-1-infected individuals compared with uninfected donors (Fig. 6B). Neutrophil death induced by HIV-1 infection has been reported previously (31, 32). However, counts of neutrophils showed an upward trend in patients upon their acquiring opportunistic infections (Fig. 6B). This accumulation of neutrophils may reflect the body's response to invading opportunistic pathogens.

The plasma $CD4^+$ T lymphocyte count was usually used to characterize disease progression. A significant increase in viral load and the progressive depletion of lymphocytes were observed in accompaniment with disease progression (Fig. 6C and D), whereas the frequencies of all three types of granulocytes remained fairly stable in patients with different levels of $CD4^+$ T cell counts (Fig. 6E). Taken together, these patient analyses revealed that the count of blood-circulating granulocytes, particularly of basophils, remains fairly stable during disease progression.

DISCUSSION

In this study, we investigated the role of peripheral blood granulocytes in HIV-1 dissemination. We demonstrated that circulating basophils can capture HIV-1 particles and mediate viral *trans*-infection of encountered $CD4^+$ T cells and that HAFs expressed on the cell surface mediate viral binding and transfer; that neutrophils can also capture HIV-1 particles and transfer them to T cells but do so less efficiently than basophils; and that eosinophils rarely if ever bind HIV-1 particles. Granulocyte cell counts, particularly those of basophils, were found to remain fairly stable during

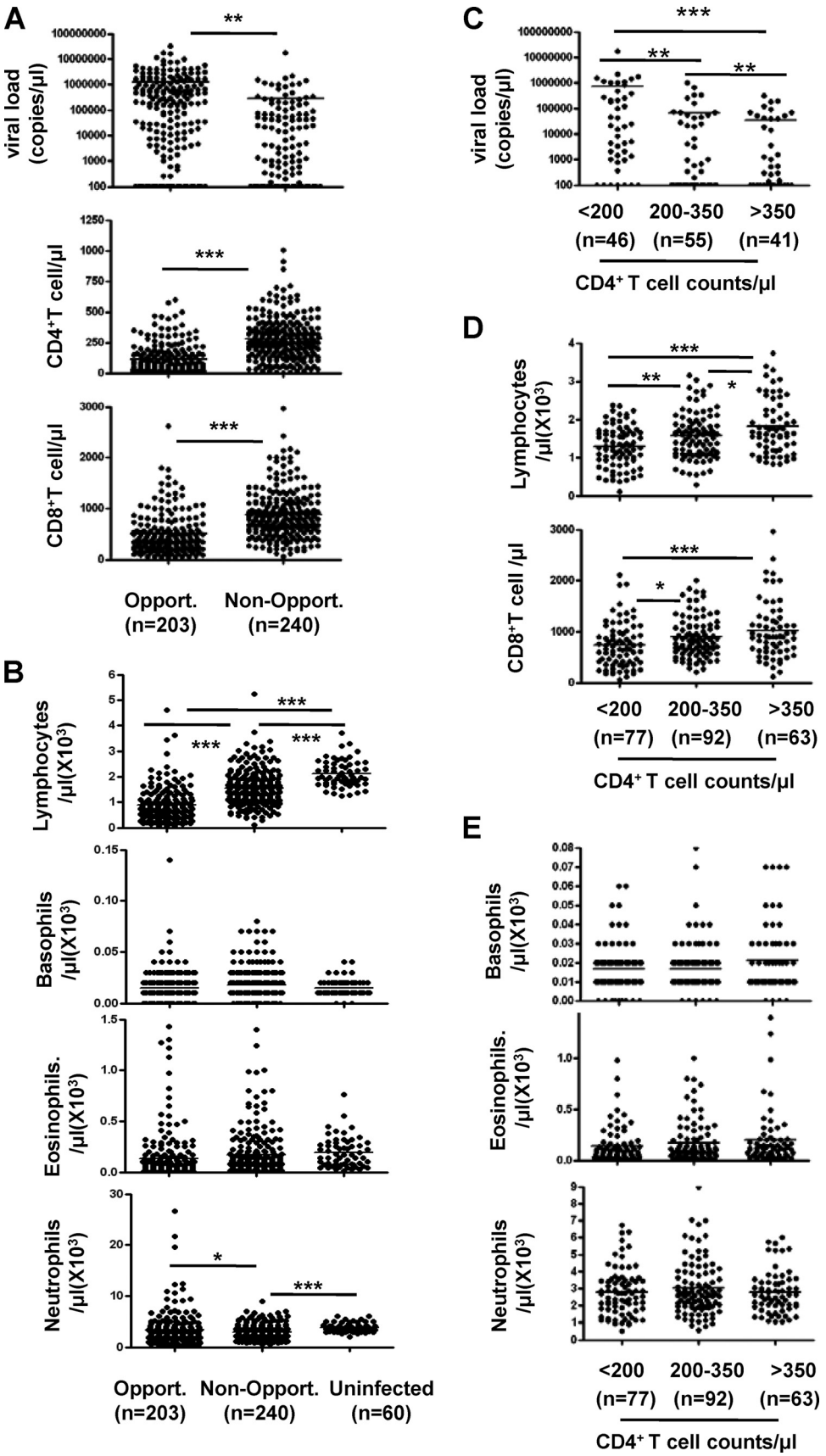

FIG 6 Analysis of granulocyte frequency in HIV-1-infected individuals. The blood counts of HIV-1-infected individuals and uninfected healthy donors were analyzed. (A and B) Lymphocyte and granulocyte counts of patients with or without the emergence of AIDS-associated pathogens compared with those of uninfected donors. Plasma viral load was also analyzed. Opport., opportunistic infections; Non-Opport., nonopportunistic infections. (C, D, and E) Plasma viral load (C), lymphocyte counts (D), and granulocyte counts (E) in patients with a range of $CD4^+$ T counts. *, $P < 0.05$; **, $P < 0.01$; **, $P < 0.001$ (representing statistically significant differences in a paired *t* test).

HIV-1 disease progression. This finding might suggest that granulocytes, particularly basophils, provide a stable cellular base for progressively capturing and thus spreading viruses.

The three types of granulocytes under study expressed one or several types of HAFs known to be responsible for HIV-1 capture and spread. Some viral attachment factors have greater binding affinity with gp120 than others (72), and some are better able to distinguish gp120 from other HIV-1 subtypes (54). DCIR displayed a lower binding affinity than DC-SIGN with gp120 (74), which may explain the much lower frequency of viral capture mediated by DCIR-expressing neutrophils than was mediated by DC-SIGN- and other HAF-expressing basophils. Activated α4β7 integrins have been shown to recognize different subtypes of HIV-1 gp120. The engagement of α4β7 by gp120 may rapidly activate leukocyte function-associated antigen 1 on $CD4^+$ T cells, facilitating the formation of cell-cell conjugation and viral spread (54). We also found that both basophils and eosinophils expressed α4β7 integrins. The naive stimulated eosinophils isolated in this study may have remained in the inactive form of α4β7 heterodimers, preventing them from performing HIV-1 binding. These viral attachment factors provide a feasible target of interventions to combat viral transmission. In a recent report, the administration of anti-α4β7 monoclonal antibodies in rhesus macaques prior to and during acute infection by SIVmac251 was found to block viral mucosal transmission and maintain $CD4^+$ T cell counts in both blood and gut-associated lymphoid tissue (75).

Surface-bound HIV-1 particles were found to account for the granulocyte-mediated transfer of HIV-1. The formation of infectious synapses to which numerous intact viral particles and viral receptors can be recruited is an efficient means of mediating viral transmission and thus occurs widely in host cells (56, 76, 77). Basophils can function as antigen-presenting cells, and allergen-stimulated basophils were found to upregulate major histocompatibility complex (MHC) class II expression and form immunological synapses with T cells to induce a Th2 response (6, 78–80).

Neutrophils, the most abundant WBCs, have been shown to interact in various ways with HIV-1 (27, 30, 81–83). In this report, we provide further evidence that neutrophils are capable of binding, transferring, and thus spreading HIV-1, although they do so less efficiently than basophils. Neutrophil-mediated HIV-1 binding and transmission can be enhanced by tumor necrosis factor alpha (TNF-α) stimulation (84).

Our analysis of HIV-1-infected individuals revealed that the basophil cell count remains fairly stable during disease progression. Therefore, basophils might provide a stable cellular base for progressive viral capture and spread. HIV-1 proteins gp120 and Tat have been reported to induce the degranulation of basophils and thus the release of chemokines and cytokines, contributing to the creation of an allergy-like Th2 bias in AIDS patients (34, 35). Further elucidation of the role of HIV-1 in modulating the release and *de novo* synthesis of various intracellular granules and cytokines from basophils and of the effect of HIV-1 on basophil functions such as phagocytosis would increase understanding of HIV-1 pathogenesis.

Taken together, our results offer novel insights into the roles of peripheral blood granulocytes, particularly basophils, in HIV-1 dissemination. Our findings suggest that blocking cell-associated HIV-1 transmission to combat viral spread is an important strategy of anti-HIV therapy.

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