CONTRIBUTION OF INNATE RESPONSES TO VIRAL CONTROL IN HIV-1 INFECTION

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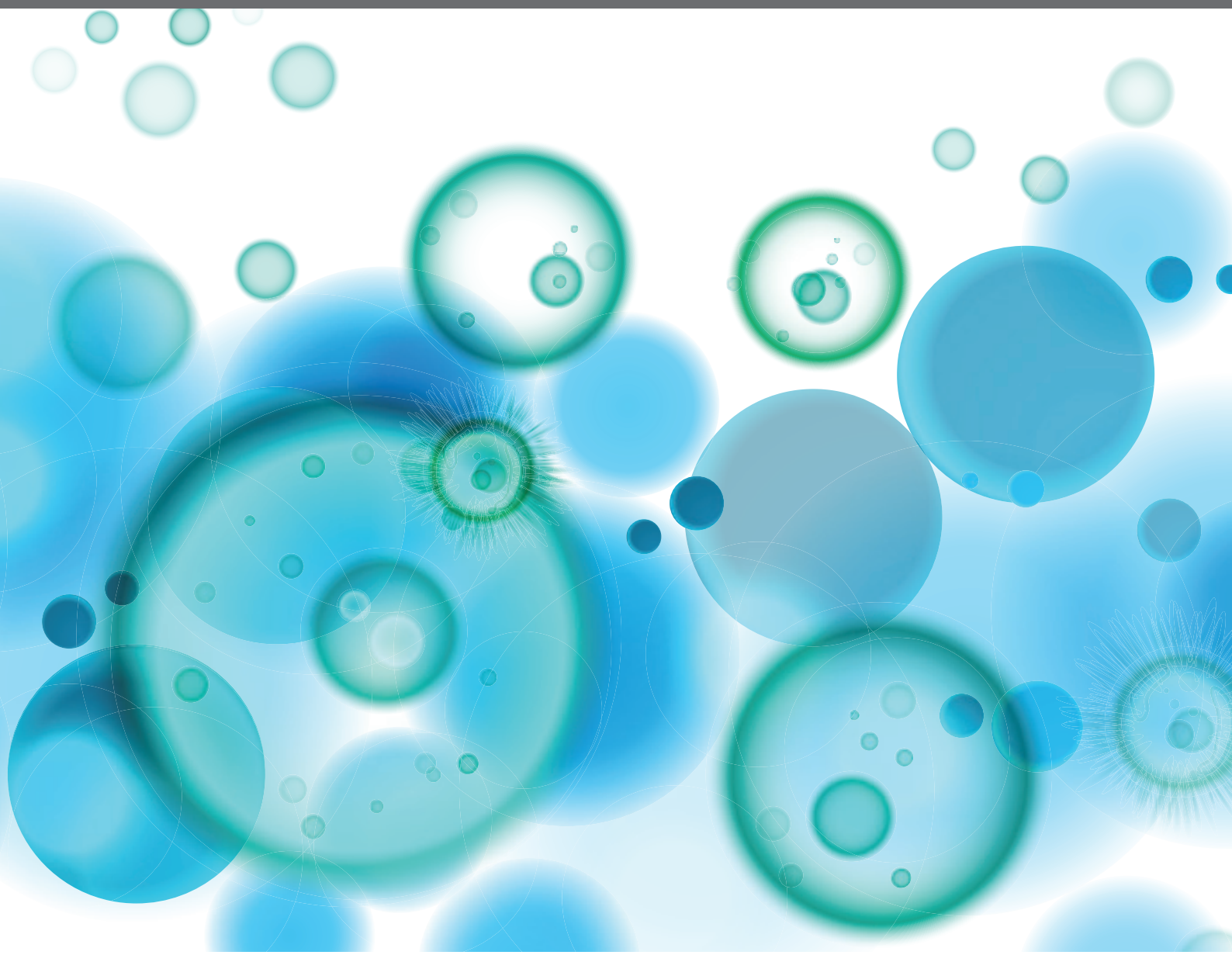

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CONTRIBUTION OF INNATE RESPONSES TO VIRAL CONTROL IN HIV-1 INFECTION

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ORIGINAL RESEARCH
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Mouse Liver Sinusoidal Endothelium Eliminates HIV-Like Particles from Blood at a Rate of 100 Million per Minute by a Second-Order Kinetic Process

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We crafted human immunodeficiency virus (HIV)-like particles of diameter about 140 nm, which expressed two major HIV-1 proteins, namely, *env* and *gag* gene products, and used this reagent to simulate the rate of decay of HIV from the blood stream of BALB/c male mice. We found that most (~90%) of the particles were eliminated (cleared) from the blood by the liver sinusoidal endothelial cells (LSECs), the remainder from Kupffer cells; suggesting that LSECs are the major liver scavengers for HIV clearance from blood. Decay was rapid with kinetics suggesting second order with respect to particles, which infers dimerization of a putative receptor on LSEC. The number of HIV-like particles required for saturating the clearance mechanism was approximated. The capacity for elimination of blood-borne HIV-like particles by the sinusoid was 112 million particles per minute. Assuming that the sinusoid endothelial cells were about the size of glass-adherent macrophages, then elimination capacity was more than 540 particles per hour per endothelial cell.

Keywords: liver sinusoidal endothelial cell, Kupffer cell, pinocytosis, endocytosis, clearance

NON-TECHNICAL SUMMARY

We have engineered a small particle that resembles a human immunodeficiency virus (HIV) in size and surface structure in order to learn how HIV travels in the blood circulation. We use the mouse as a model of the human. These particles, when infused into the blood stream, are removed from blood very rapidly, within minutes, mostly by a particular kind of cell that lines the blood vessels of the liver, a cell referred to as liver sinusoidal endothelial cell (LSEC) (ell-seck). The rate of removal from blood suggests complex details of the mechanism of removal. The capacity of LSEC to remove HIV-like particles is astonishingly high, namely, about 100 million HIV-like particles per minute. We can estimate that a single blood vessel-lining cell (LSEC) removes more than 500 particles per hour.

Abbreviations: LSECs, liver sinusoidal endothelial cells; KC, Kupffer cells; DIC, differential interference contrast; IF, immunofluorescence; RFI, relative fluorescence intensity; VLP, HIV-like particle.

Our findings have yet to be integrated into the understanding of the natural course of an HIV infection.

INTRODUCTION

A readily apparent but poorly understood aspect of the innate immune response is the rapid and copious removal (or clearance) and subsequent degradation of blood-borne virus by the endothelium of the liver sinusoids (LSEC). This capacity of LSEC to remove virus is far more robust than like clearance by Kupffer cells (KC), which instead appear responsible mostly for the removal of larger particles such as bacteria and autologous cellular material (1). LSEC clear small particles other than virus in a similar manner, particles such as virus-like particles expressing polyoma virus proteins (2), small immune complexes made of ovalbumin and IgG antibody (3), lipopolysaccharide (4), and very likely other nanoparticles. The LSEC thus constitutes an outpost of the innate immune system with which cytokines have been associated only rarely (5). The rapid elimination of such particles has prompted the liver to be referred to in common parlance as the "garbage dump" of the body.

The molecular mechanism by which these small particles are cleared by LSEC is only beginning to be known: Fc receptors for IgG on LSEC are required, we have found, for the clearance from blood of small immune complexes (1, 3). Scavenger receptor B-1 (SRB1) binds and likely facilitates the removal of hepatitis virus C (HVC) from circulation (6, 7). More mechanistic details are needed.

We now continue these studies by characterizing carefully the rate and extent of removal of a virus-surrogate, i.e., a non-infectious HIV-like particle that expresses the translation products of the *env* and *gag* genes of HIV and thus has structural and antigenic characteristics suitable for recognition by the immune system, both innate and adaptive. This reagent will give us the opportunity later to study the effects of anti-HIV-1 antibody on virus or VLP removal by the LSEC (8).

RESULTS

We engineered a small particle the size of a virus (HIV-like particle) that consisted of a viral membrane derived from cultured HEK-293 cells and expressed features of HIV, namely, the HIV CXCR4 envelope protein (gp160) and the *gag* protein p24, but lacked nucleic acid and accessory proteins required for replication (M&M). We refer to the particle as an HIV-like particle. The diameter of the particles, measured by high-resolution analysis of Brownian diffusion, was ~140 ± 5 nm, mode ± SE, $n = 43$ (M&M). The concentration of suspensions of HIV-like particles we measured using a p24 ELISA.

To ascertain the rate of removal (or clearance) of HIV-like particles from the mouse circulation, we infused HIV-like particles intravenously and assessed their concentration in peripheral blood periodically over the course of 30 min (**Figure 1**). The decay curve was plotted in four ways. First, plotting simply using linear measurements for both the vertical axis showing concentration of HIV-like particles in blood (mean ± SD) versus the horizontal axis showing time in minutes, the decay curve showed two phases, a sharp drop followed by a lengthy near-plateau, with nearly all HIV-like particles (~97%) being cleared within 10 min. The second phase showed a plateau suggesting nil or negligible clearance. It represented only ~3% of the infused dose and thus was not included in further analysis (**Figures 1D,E**). As the SD of the first data point at 1 min was large, we additionally show decay curves of three mice that represent the SD splay (**Figure 1B**). Plotting the data in the conventional log-linear manner, the decay was curvilinear, not at all characteristic of the anticipated pseudo-first order reaction, which would show a straight line relationship (**Figure 1C**). However, showing the data as log–log and inverse linear–linear plots, we see straight lines (**Figures 1D,E**) (see Discussion).

We found experimentally that the liver is the major organ clearing Cy3-HIV-like particles from blood (**Figure 2**): the organ distribution experiment showed that, in 10 min, the majority of total recovered Cy3-HIV-like particles was associated with liver (~80%), whereas less than 2% of the total recovered dose associated with spleen and kidney. The fraction of the dose of HIV-like particles that associates with blood shown in the bar graph is not statistical significantly different ($p > 0.05$) from the amount that we have estimated in the clearance curves using the unlabeled HIV-like particles (**Figure 1**). In addition, an experiment comparing the clearance of unlabeled HIV-like particles with that of the Cy3-HIV-like particles (data not shown) suggests that the clearance kinetics were not significantly different, assuring that the labeling of HIV-like particles with Cy3 did not change the clearance property of the particle. These data, indicating strongly that the liver is the major organ clearing circulating HIV-like particles, are in concert with several published studies showing liver to be the major site of removal of a variety of blood-borne viruses and virus-like particles, with minimal uptake occurring in the lung, spleen, and kidney (1, 2, 9–12).

Also, 3 min after the intravenous infusion of a dose, we determined the cellular localization within the liver of fluor-labeled HIV-like particles by examining 5 μm sections of mouse liver using immunofluorescence confocal microscopy, distinguishing LSEC with fluor-tagged anti-mannose receptor antibody, and KC with fluor-tagged anti-macrophage antibody. Visual microscopic inspection indicated that the HIV-like particles were situated on or in sinusoidal cells and were absent from the lumens of cross-sectioned sinusoids. The particles were much more abundantly associated with LSEC than with KC (**Figure 3A**). We have earlier illustrated the validity of these markers (1, 3). Substantiating this disparity quantitatively by estimating pixel fluorescence numbers and intensity, we found that 88% of HIV-like particles associated with LSEC while 12% associated with KC (**Figure 3B**). HIV-like particles were not found associating with hepatocytes. Additionally, the HIV-like particles associated with KC appeared aggregated while virus-like particles associated with LSEC appeared as fine puncta.

To ask whether liver uptake of HIV-like particles is saturable, we observed in **Figure 1** that nearly the entire dose (2×10^{10} HIV-like particles) was removed from blood in 10 min (~97%), which suggests that the clearance mechanism was not saturated. Further, increasing the dose to 4×10^{10} did not saturate the clearance mechanism as, again, nearly the entire dose was cleared

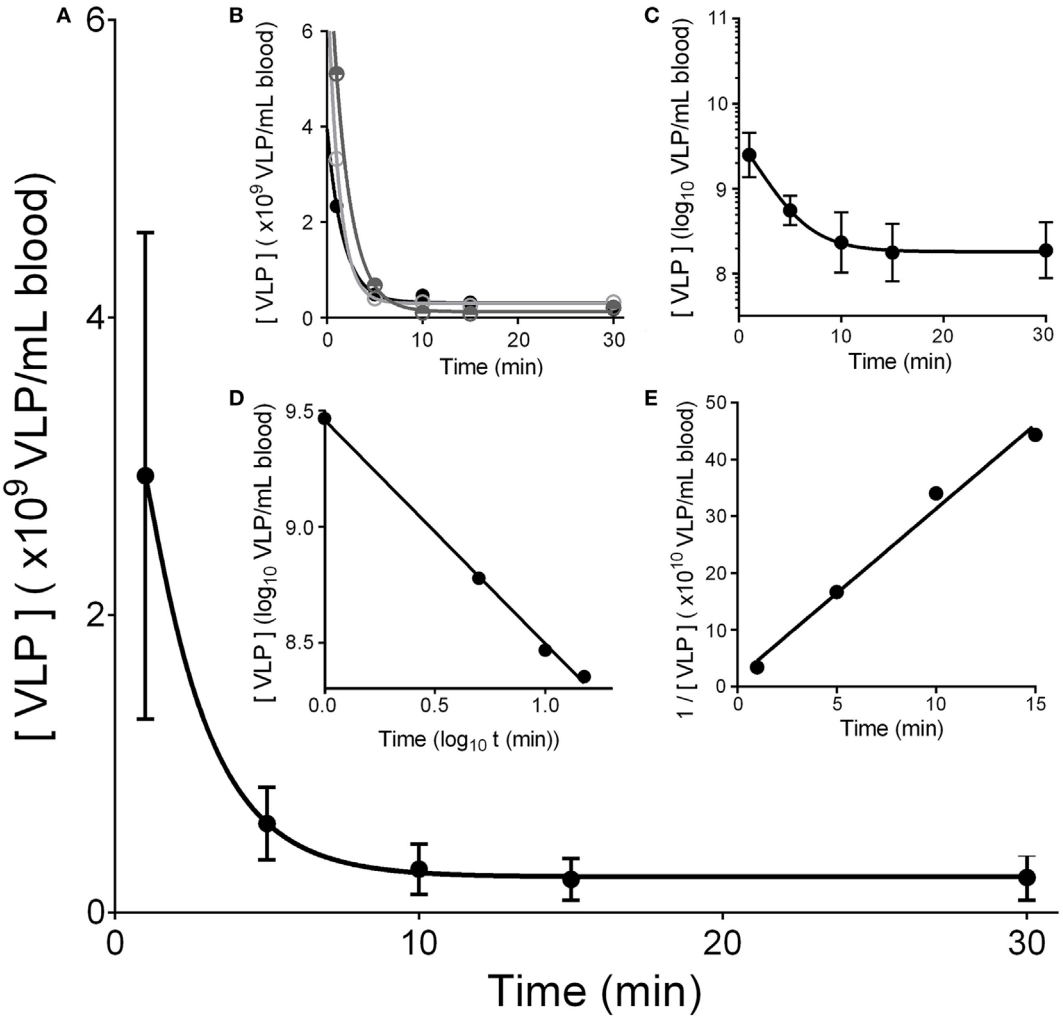

FIGURE 1 | HIV-like particles are cleared rapidly from murine circulation. Approximately 2×10^{10} HIV-like particles were intravenously infused by tail vein. The concentration of particles remaining in blood of the suborbital plexus over time was estimated using a p24 ELISA. **(A)** Shows a plot of mean ± SD of the decay curves. The curve was drawn using asymmetric sigmoidal five parameters simulation to smooth the connection of data points. **(B)** Shows decay curves of three different mice illustrating the splay of the SD in **(A)**; one high, one mid-level, and one low. **(C)** Shows a log–linear plot of the data illustrating no straight line. **(D)** Plots the data in log–log fashion to reveal a straight line of pseudo second order kinetics. **(E)** Shows a reciprocal plot of the same data, showing also a straight line. The 30-min data points that did not fall on the straight line are not shown; they represent less than 3% of the dose. Each data point represents mean ± SD of 26 BALB/c wild-type mice.

in 10 min. Re-evaluating a published study of ours showing decay curves of human recombinant adenovirus (rAd5) in mice, saturation was not achievable with an intravenous dose of 1.6×10^{11} rAd5 particles (1). Specifically, plotting vertically on a log scale three doses of rAd5 over a 2-log range versus, on the horizontal axis, the number of rAd5 removed in 10 min showed a straight line with a positive slope, indicating that the uptake mechanism was not saturated [curve not shown; data presented in **Figure 1A** of our paper (1)]. For technical reasons, we were unable to achieve higher doses of HIV-like particles; thus, with our present strategy failing to show saturation of the uptake mechanism, we can only assume that we are near saturation. For practical purposes, we assume, then, that a saturation dose is near 2×10^{10}.

Proceeding, we reasoned that the removal from circulation of a large dose of HIV-like particle (2×10^{10}) would leave the liver unable to remove a second dose as efficiently as the first; i.e., the liver may require time to recover its native removal ability. To calculate this refractory period that follows a first dose, we infused a second dose at varying times (1.5–12 h) after the first dose and plotted decay curves of the second dose, comparing the two decay curves performed simultaneously, the second with the first. As measures of differences both in the extent and rate of decay, we compared the numbers of HIV-like particle cleared at every point on the two curves, experimental, and control (**Figure 4**).

The decay curves performed 12 and 6 h after a first dose were fully superimposable on the initial control decay curves and were not statistically significantly different from the control decay

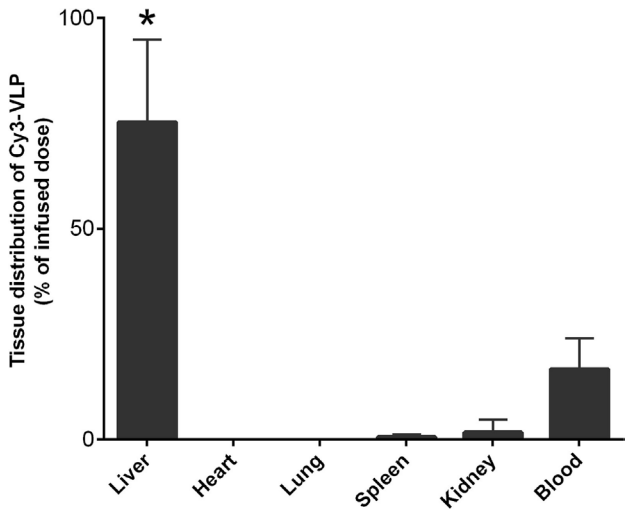

FIGURE 2 | The liver is the major organ clearing Cy3-HIV-like particles from blood. Mice were infused with 10^{10} Cy3-human immunodeficiency virus-like particles and, after 10 min of infusion, the Cy3 fluorescence was quantified in various organs as described in M&M. The bar graph expresses the percentage means and SDs of total Cy3 fluorescence that was recovered from the six organs studied. The asterisk represents data points where the *p*-Values were determined to be less than 0.05 using Student's *t*-test.

curves (**Figures 4A,B**). The decay curve at 3 h after the initial dose appeared to show disparity of later points, but the experimental and control points throughout the entire curves were not statistically significantly different (**Figure 4C**). However, at 1.5 h after the initial dose, the decay curve plateaued earlier than the control curve (**Figure 4D**); the two curves were statistically and significantly different at the 5 and 10 min points (asterisks).

DISCUSSION

The HIV-like particle used in this study was crafted to express multiple copies of two major HIV gene products, the surface-expressed gp160 *env* protein for eventual use as an antibody target, and the *gag* protein p24 for use with an immunoassay.

We have learned from these data that the rate and extent of removal of blood-borne HIV-like particle in the mouse shows a biphasic decay curve with a very rapid and extensive first phase and a negligible second phase. Thus, decay is similar to the removal of blood-borne virus as studied by us and others [see our paper (1) for brief review]. However, we perceive a distinct difference from the decay of Ad5. By analogy with standard chemical reactions, the straight lines of the graphs of **Figures 1D,E** are characteristic of pseudo-second order reactions with respect to HIV-like particle concentrations, assuming a constant concentration of the sinusoidal binding site for HIV-like particles. In standard chemical parlance, the reaction is represented as $2A + B \rightarrow AAB$. In contrast, adenovirus clearance using data from an earlier study plotted in log-linear fashion showed a straight line characteristic of a pseudo first order reaction (1).

We can only speculate what the apparent second order of VLP decay might mean. At face value, the data would suggest that two HIV-like particles are interacting as a unit with an LSEC target. We know of no reason why HIV-like particles should dimerize, and in fact, according to our Brownian motion detector, the HIV-like particles were monodisperse. Whether they dimerize in blood, we have no way of determining. It is also possible that the binding site on LSEC is a dimer, although we know of no evidence that CD4 or L-SIGN, both known to be expressed on LSEC (13, 14), dimerize. However, there is precedence for LSEC to express dimeric receptors; i.e., SRB-1, a receptor for HDL and HCV envelope protein E2 expressed on LSEC (6, 7, 15), is known to dimerize and oligomerize (16). What precisely second order means in the case of our **Figure 1** data awaits additional study.

We attempted to saturate the clearance mechanism of LSEC but were not at all successful. Doses of HIV-like particles as high as practicable, 4×10^{10}, were cleared nearly completely (97%) in 10 min, indicating that saturation had not yet been reached. Nor did we reach a saturating dose in our prior studies of human adenovirus clearance where doses equally high, 1.6×10^{11}, were infused (1). For technical reasons, we were unable to study higher doses; thus, we assume that saturation is 2×10^{10}.

Failing to find a saturating dose, we nevertheless were able to estimate a recovery time by examining closely the shape of the decay curves after a second dose of HIV-like particles. The decay curve of a second dose 1.5 h after the first was clearly different from the control, indicating, albeit tenuously, that the clearance mechanism was still recovering at 1.5 h. At 3 h, moreover, the decay curve looked abnormal in that it diverged from the control curve, but statistically it was no different than the control curve. By 6 h, however, the clearance mechanism had returned to superimposable on the normal curve and remained superimposable at 12 h. We have not calculated half-lives because half-life is a function of dose in second order reactions; we did not vary the dose substantially. For practical purposes, we arbitrarily let recovery time be 3 h.

Assuming that the greatest dose used in our experiments (2×10^{10}) is close to the saturation dose (S), and that the recovery time (R) is 3 h, we can then calculate the capacity (C) of the liver to remove HIV-like particles from circulation in an on-going, continuous fashion. We define capacity in units of particles per day as the saturation dose divided by the recovery time R ($C = S/R$). Specifically, every 3 h, 2×10^{10} HIV-like particles were removed from circulation. The capacity for clearance, therefore, is 6.7×10^9 ($2 \times 10^{10}/3$) HIV-like particles per hour per mouse.

Is this a biologically realistic number? Capacity expressed as functions of hourly or daily clearance are numbers too large for us to conceptualize. However, converting to clearance per minute gives an everyday number that most workers will appreciate; i.e., 112 million HIV-like particles per minute ($6.7 \times 10^9/60$ min). This number sounds realistic and substantial. In fact, the number sounds remarkably potent when compared to a lethal dose of an arbitrarily chosen widely known virus (influenza virus) which, given intratracheally, has been shown to be on the order of 10^4

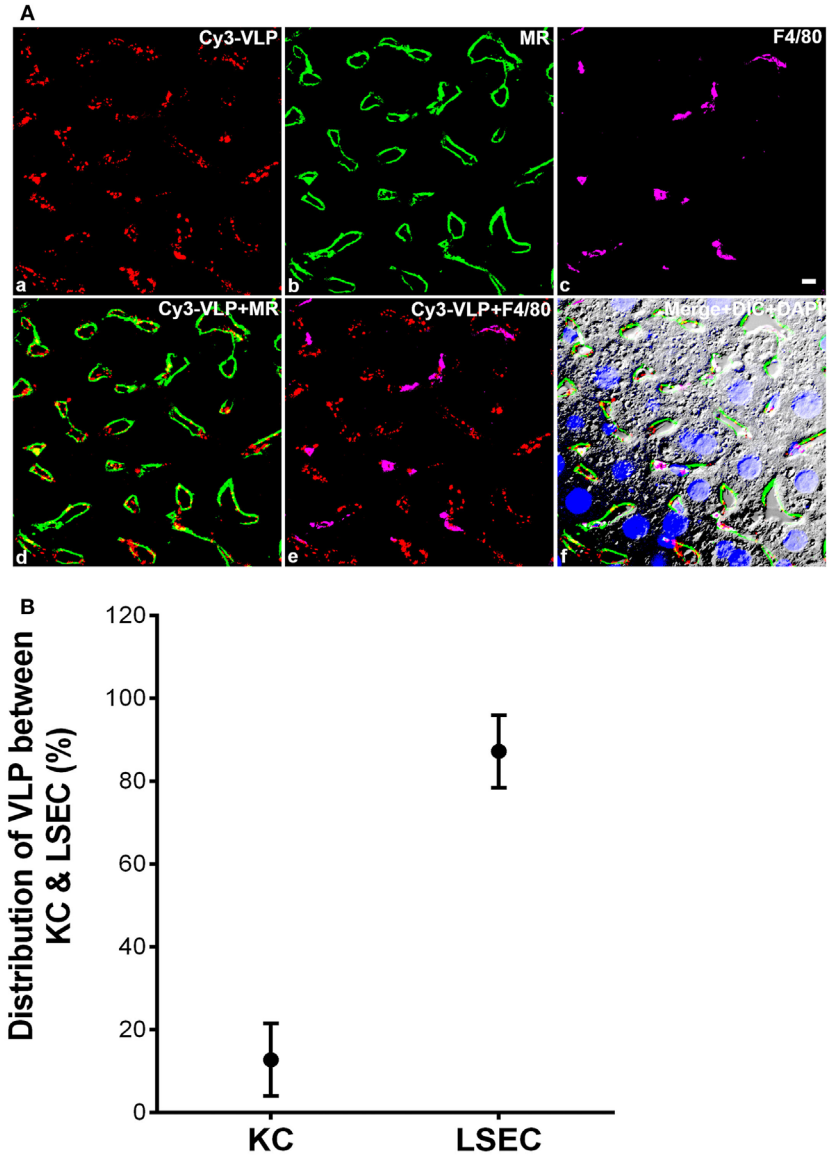

FIGURE 3 | The majority of human immunodeficiency virus (HIV)-like particles cleared by liver is localized to the liver sinusoidal endothelial cell (LSEC). **(A)** Four-color fluorescence microscopic images of 5 μm liver sections, 3 min after intravenous infusion of 2×10^{10} Cy3-HIV-like particles. (a) Red puncta show Cy3-HIV-like particles. (b) rabbit anti-mannose receptor (CD206) labeling of LSEC shown in green. (c) rat mab F4/80 labeling of KC shown in magenta. (d) Cy3-VLP (red) merged with LSEC marker. (e) Cy3-HIV-like particles (red) merged with KC marker. (f) Merged image showing Cy3-HIV-like particles (red), LSEC marker (green), and KC marker (magenta) plus DIC and DAPI staining of the nuclei (blue). Panels shown are representative of 160 images from three different mice. The scale bar in panel (c) signifies 5 μm. **(B)** Quantified association of HIV-like particles with cells of the liver. All HIV-like particles in the liver were associated with either LSEC or KC, indicating no association with hepatocytes. The total HIV-like particles was calculated as the pixel area × mean fluorescence intensity (red). HIV-like particles associated with the KC was subtracted from the total HIV-like particle to calculate LSEC association of HIV-like particle. The graph represents the mean ± SD of KC- and LSEC-association within liver. One hundred sixty images, roughly 50 from each of the three mice, were examined. The area totaled 30 mm^2 of sectioned liver tissue.

virus particles (personal communication, Ian Davis, The Ohio State University).

Can we put the clearance capacity into perspective by asking how large is the sinusoid area responsible for clearing 112 million particles/min? Calculating from the rat sinusoid area (17), assuming that mouse liver weight is 1 g, we find that the surface area of the mouse sinusoidal network is 5.8×10^{10} μm^2, or roughly half the area of the face of a tennis racquet.[1] Another way of rendering realistic the magnitude of the sinusoidal surface area is by thinking of it as covered by confluent adherent macrophages. Assuming that the area of an adherent macrophage

[1]http://officialtennisrules.com/official-dimensions-for-tennis-rackets/.

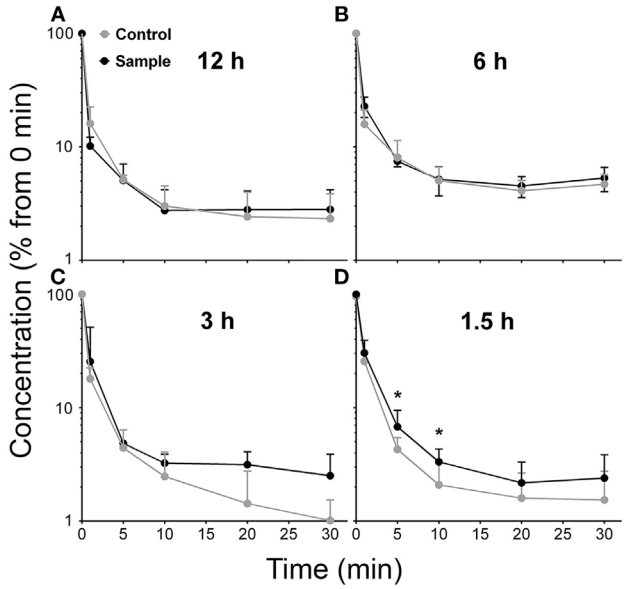

FIGURE 4 | Clearance capacity of human immunodeficiency virus (HIV)-like particles is fully recovered in about 3 h. Mice were infused intravenously with 2×10^{10} HIV-like particles, allowed to recover for the indicated time (1.5, 3, 6, and 12 h) and then were infused with an additional bolus of 2×10^{10} HIV-like particles. The blood concentration of HIV-like particles was determined as described in **Figure 1**; the (sample) curves are to be compared with decay curves performed simultaneously on mice that had not received the initial dose of HIV-like particles (control). Each data point represents mean ± SD of several BALB/c wild-type mice. The number of animals used at each time period 1.5, 3, 6, and 12 h was 6, 3, 3, and 4, respectively. The raw data were $\log_{10}$-transformed prior to running the random-effects linear regression model, described in M&M. The corresponding points on the two curves in panels (12, 6, and 3 h) were not statistically different, even the apparently divergent points at 3 h. However, the corresponding points on the curves in panel (1.5 h) were statistically significantly different at the 5 and 10 min points, but not at the others. Thus, the recovery time was estimated to be between 1.5 and 3 h.

is 4.5×10^3 μm² (Figure 5 of publication PMC3488130), then the mouse sinusoid would be covered by 13 million confluent glass-adherent macrophages. Assuming further that the area of the adherent macrophage is about the same as a sinusoidal endothelial cell, then we find that 540 HIV-like particles are cleared per LSEC per hour (calculations in Supplementary Material).

What is the fate of the HIV-like particles once cleared from blood and bound to the LSEC? We interpret our data to indicate that HIV-like particles, cleared from blood, associate mostly (88%) with LSEC, to a small extent with KC (12%), and not at all with hepatocytes. At 3 min after infusion (**Figure 3**), they appear on or within the endothelium but not luminal as evidenced by their absence in luminal cross-sections. At 10 min after infusion, we find virtually no HIV-like particles associating with LSEC or KC (data not shown). We presume, based on the fate of immune complexes (18, 19), that most bound particles are internalized and degraded, although we have not yet embarked on a formal study of HIV-like particles. The literature indicates that endocytosed particles of many sorts are degraded (2, 11); other studies suggest that a fraction of the endocytosed particles is degraded and a fraction is expressed back onto the surface of the endocytosing cell (18, 20). The general impression that endocytosed particles are disposed of quickly is consistent with the novelty of our suggestion; virus particles internalized by LSEC are not ordinarily described during the course of virus infections. In our recent study of LPS clearance from blood, we found no evidence that cleared LPS moved from LSEC to KC (4). A systematic study of the fate of cleared particles is needed.

A remarkable implication of this robust clearance capacity is that clearance may be fast enough to avoid detection in the blood by culture or nucleic acid assay while allowing hematogenous spread of infection. It would follow that blood cultures and nucleic acid assays would not become positive until the clearance capacity of the liver is saturated. These implications are testable.

Further, we would propose that two different viruses may compete for a single clearance mechanism, although to date no such evidence is available. Such studies will require a keener analysis of the clearance mechanism and its discrimination among various bound particles. In support of this speculation, it has been known for decades that small particles in blood such as thorotrast will block the LSEC uptake of virus (10).

Finally, this astonishingly rapid and robust removal of blood-borne virus would appear to have been overlooked by all but a few modern and early biologists studying virus turnover during infection (10–12, 21). To us, this mechanism appears to be a subdivision of the innate immune system that has received little attention but might very well be of immense value to the organism. Many additional consequences of our observation, we anticipate, will become clear with appropriate study.

As a postscript, we point out that the rapidity of particle clearance described herein is remarkably similar to the classical "distribution" phase of the decay curves of protein and drug clearance from blood, a phenomenon well described in the pharmacokinetics literature.[2] However, beyond rapidity, the similarity stops. Virus leaving the plasma compartment bound to the LSEC surface does not appear to be in equilibrium with the plasma compartment, which by definition it must be if the rapid portion of decay is to be considered "distribution" of kinetic decay. We imagine that the rapidity seen in our studies is simply quick clearance on a pathway toward ultimate degradation or processing by the sinusoidal cells.

MATERIALS AND METHODS

Animals

Wild-type male BALB/c mice of age 10–15 weeks were obtained from Taconic Biosciences. All studies were performed in accordance with appropriate guidelines and were approved by The Ohio State University Institutional Animal Care and Use Committee. All *in vivo* mouse procedures were performed under Isoflurane anesthesia.

[2]http://sepia.unil.ch/pharmacology/?id=93.

Plasmids

The pGag-EGFP plasmid (NIHARP cat #11468) used to prepare HIV-1 VLP, which directs Rev-independent expression of HIV-1 Gag-EGFP fusion protein (tier 1 clade B) to form VLP, was obtained from Dr. Marilyn Resh through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH. The pGag-EGFP plasmid was constructed by cloning Gag from pCMV55M1-10 (22) into the pEGFP-N1 plasmid (Clontech) (23). Plasmid DNA was amplified in *Escherichia coli* DH5α; DH5α-containing pGag plasmid was grown in LB medium supplemented with 25 μg/mL kanamycin. The pHXB2 envelope plasmid (NIHARP cat#1069), containing HXB2 gp160 under an SV40 promoter, was obtained from Dr. Kathleen Page and Dr. Dan Littman through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH. Plasmid DNA was amplified in *E. coli* DH5α; DH5α-containing pHXB2 env plasmid was grown in LB medium supplemented with 50 μg/mL ampicillin. Plasmid purification used the BenchPro 2100 Plasmid Purification System (Invitrogen).

Preparation of VLP-Containing HXB2 env

VLP were propagated using human embryonic kidney, 293T cells (ATCC). Cells were maintained in Dulbecco's Modified Eagle medium with 10% Fetal Bovine Serum. VLP-containing HXB2 envelope was produced by transient transfection of HEK 293T cells with pGag-EGFP and pHXB2 env using Lipofectamine 2000 transfection reagent (Life Technologies). Also, 10^7 cells in T175 flasks were transfected with 30 μg HXB2 envelope, 60 μg pGag-EGFP, and 360 μL Lipofectamine 2000 transfection reagent in serum/antibiotic-free medium. After 3–4 h of incubation at 37°C, the culture medium was replaced with DMEM + 10% FBS. VLP-containing supernatant was collected 72 h after transfection and clarified by centrifugation at 2,000 × *g* for 10 min. Clarified supernatant was further purified of cellular debris by 0.45 μm filtration. Purification of assembled VLP was completed by ultracentrifugation through a 20% sucrose pad at 122,000 × *g* for 2 h at 4°C. The VLP pellet was resuspended in filtered PBS.

Quantification of VLP

Two methods were used to quantify VLP concentrations, p24 ELISA and Nanoparticle Tracking Analysis (NTA). p24, a viral protein component Gag, was used to determine VLP concentration with the commercially available Zeptometrix p24 ELISA kit in accordance with the manufacturer's instructions. The lower limit of detectability in the assay was 10^7–2×10^7 particles/mL. Additionally, NTA was performed using a Nanosight NS300 (Malvern). VLP samples resuspended in filtered PBS were diluted to approximately 10^8–10^9 particles/mL; 1 mL of diluted VLP sample was injected into the Nanosight apparatus. Nanosight NTA 3.0 software was used to analyze nanoparticle tracking data. Five individual videos ranging from 30 to 60 s each were recorded and analyzed based on the VLP Brownian motion at room temperature. NTA analysis determined both particle size and concentration of VLP per milliliter. A ratio of p24 concentration (picograms per milliliter) to NTA (VLP per milliliter) was calculated for each VLP preparation.

Cy3 Labeling of VLP

VLP in PBS, pH 7.4, at a concentration of 10^{12} VLP/mL was adjusted to pH 9.4 by the addition of 0.5 M sodium carbonate bicarbonate. The Cy3 monoreactive dye pack (Amersham) was dissolved in 1 mL of VLP solution, pH 9.4, and incubated at room temperature for 30 min with constant stirring. The addition of 0.2% glycine stopped the labeling reaction, and the Cy3-labeled VLP was dialyzed against two changes of PBS, pH 7.4, at 4°C for 18 h. The efficiency of Cy3-conjugation was assessed by comparing the protein concentration (micrograms per microliter) of the dialyzed Cy3-conjugated VLP with the concentration of Cy3 (picomoles per microliter). The Cy3 concentration was converted to micrograms per microliter using the formula weight; the dye to protein ratio of Cy3-conjugated VLP was 0.02.

Immunofluorescence

BALB/c mice 11–14 weeks old were intravenously infused with 2×10^{10} Cy3-labeled VLP in PBS, pH 7.4. BALB/c livers were excised, cut into ~5 mm pieces, and fixed in 4% paraformaldehyde-PBS for 2 h at room temperature. Fixed tissue was washed with PBS and saturated with 20% sucrose-PBS overnight at 4°C. Upon sucrose saturation, tissue was embedded in tissue-freezing medium and stored at −80°C. Fixed and frozen tissue was sectioned at 5 μm thickness by Cyrostat sectioning and collected on Superfrost microscope slides. Tissue sections were rehydrated, blocked in 5% milk blocking solution for 1 h, and then incubated with primary antibodies in 5% blocking solution overnight at 4°C. Unconjugated primary antibodies were visualized using a 1:200 dilution of fluor-tagged secondary antibodies in 5% blocking buffer for 1 h at room temperature. DAPI staining was executed for 10 min and then tissue sections were mounted under coverslips with Prolong Gold Solution (Invitrogen). Isotype controls along with secondary antibodies were used to assess primary and secondary immunostaining.

Primary antibodies included rabbit polyclonal IgG anti-CD206 mannose receptor (Santa Cruz) and rat monoclonal IgG anti-F4/80 (Abserotec). Secondary antibodies from Invitrogen included Alexa 488-conjugated goat anti rabbit IgG and Alexa 680-conjugated goat-anti rat IgG.

Images were acquired using 405, 488, Cy3, and 680 laser settings on an Olympus Fluo View 1000 Laser Scanning Confocal microscope with a spectral detection system designed for finer separation of fluorochromes (FV 1000 Spectral).

Quantification of HIV-Like Particles in Various Organs

Approximately 10^{10} Cy3-labeled HIV-like particles were infused *via* the retro–orbital plexus of anesthetized mice and were sacrificed at 10 min. The mice were bled of ~20 μL *via* the retro–orbital plexus, and organs (liver, kidney, lung, spleen, and heart) were removed and weighed. The weighed portions of the organs were homogenized and lysed with organ lysis buffer (0.1% SDS, 10 mM Tris, pH 7.4, and 1 mM EDTA) (3). The Cy3 fluorescence in organ lysates and blood was measured using fluorimeter (2300 Enspire multimode reader). The amount of Cy3 fluorescence associated with each organ was estimated by factoring the total organ weight

and after subtracting both the blood volume of that particular organ (24) and the background organ fluorescence from uninfused age matched mice.

Quantification of the Association of Cy3-Labeled VLP with Liver Cells

Based on 4-color immunofluorescence analysis of liver sections, we determined that Cy3-labeled VLP associated with LSEC and KC, but not hepatocytes or large vessel endothelium. The association of VLP with LSEC and KC was quantified with Image J software as described previously in our work (1). Briefly, quantification was determined in a three-step process: (1) image threshold was adjusted to account for background intensity not associated with Cy3 VLP, and the total Cy3 fluorescence intensity was recorded for the image; (2) each Kupffer cell (KC) within the image, identified by anti-F4/80 staining, was partitioned, and the fluorescence intensity of Cy3 associated with individual KC was recorded; the VLP intensity associated with individual KC was summed to total KC-associated VLP; and (3) the total KC-associated VLP intensity was subtracted from the total VLP intensity of the entire image to give the total LSEC-associated VLP intensity. KC and LSEC association was averaged for a total of 160 technical replicates of the images over the three mice. Within each mouse, the technical replicates were averaged producing three KC and three LSEC associations. The mean and SD of the three KC- and the three LSEC-association observations are presented and no statistical testing was done since these observations are not independent of each other.

Clearance of VLP from the Bloodstream

Clearance is defined as elimination from blood. BALB/c male mice at age 14 weeks were infused intravenously *via* tail vein with 2×10^{10} VLP in PBS, pH 7.4. After infusion, 20 μL of blood was obtained *via* the retro–orbital plexus at 1, 5, 10, 20 (or 15), and 30 min using heparinized capillary tubes. The calculation of VLP concentration at the time of infusion (time 0) was based on the mouse weight and assumed total blood volume of ~2.58 mL/25 g mouse (25). Blood obtained at each time point was diluted and VLP were quantified using p24 ELISA (Zeptometrix) per the manufacturer's instructions. The concentrations of p24 in picograms per milliliter were converted to VLP per milliliter using the calculated conversion rate between pictograms per milliliter and NTA. Clearance kinetics were plotted as concentrations of VLP per milliliter of blood versus time. The curve was biphasic with a fast phase, an inflection at about 10 min, at which most of the VLP had been cleared (97%), and a second phase, a virtual plateau.

Statistics

The following method was used for the recovery time experiments. VLP concentration was $\log_{10}$-transformed, and all analyses were run on the transformed values. A random-effects linear regression model where $\log_{10}$-transformed VLP concentration was the dependent variable while group (study versus control mice), recovery period (1.5, 3, 6, 12, and 24 h), test time [0, 1, 5, 10, 20 (or 15), and 30 min], and all two-way interactions were included in the model as independent categorical variables. Random-effects regression was used due to the longitudinal nature of the observations where the outcomes ($\log_{10}$ VPL concentration) were nested within specific mice over time. Additionally, this method uses all of the results from all of the mice used in the study. After running the random-effects linear regression model, linear contrast statements were used to estimate differences between study and control mice for specific test times and recovery periods of interest. In addition to these differences, the 95% confidence interval of the differences and the *p*-Values are also produced by the linear contrast statements. All analyses were run using Stata 14.1, StataCorp LP, College Station, TX, USA.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: CA and LG. Performed the experiments: JM, ZY, AC, OS, MR, and GP Analyzed the data: JM, ZY, AC, OS, GP, JK, MR, JK, JR, LG, and CA. Contributed reagents/materials/analysis tools: JK and GP. Wrote the paper: CA.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2017.00035/full#supplementary-material.

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ORIGINAL RESEARCH
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Persistence of Activated and Adaptive-Like NK Cells in HIV$^+$ Individuals despite 2 Years of Suppressive Combination Antiretroviral Therapy

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Innate immune dysfunction persists in HIV$^+$ individuals despite effective combination antiretroviral therapy (cART). We recently demonstrated that an adaptive-like CD56^{dim} NK cell population lacking the signal transducing protein FcRγ is expanded in HIV$^+$ individuals. Here, we analyzed a cohort of HIV$^+$ men who have sex with men (MSM, $n = 20$) at baseline and following 6, 12, and 24 months of cART and compared them with uninfected MSM ($n = 15$) to investigate the impact of cART on NK cell dysfunction. Proportions of NK cells expressing markers of early (CD69$^+$) and late (HLA-DR$^+$/CD38$^+$) activation were elevated in cART-naïve HIV$^+$ MSM ($p = 0.004$ and 0.015, respectively), as were FcRγ$^-$ NK cells ($p = 0.003$). Using latent growth curve modeling, we show that cART did not reduce levels of FcRγ$^-$ NK cells ($p = 0.115$) or activated HLA-DR$^+$/CD38$^+$ NK cells ($p = 0.129$) but did reduce T cell and monocyte activation ($p < 0.001$ for all). Proportions of FcRγ$^-$ NK cells were not associated with NK cell, T cell, or monocyte activation, suggesting different factors drive CD56^{dim} FcRγ$^-$ NK cell expansion and immune activation in HIV$^+$ individuals. While proportions of activated CD69$^+$ NK cells declined significantly on cART ($p = 0.003$), the rate was significantly slower than the decline of T cell and monocyte activation, indicating a reduced potency of cART against NK cell activation. Our findings indicate that 2 years of suppressive cART have no impact on CD56^{dim} FcRγ$^-$ NK cell expansion and that NK cell activation persists after normalization of other immune parameters. This may have implications for the development of malignancies and co-morbidities in HIV$^+$ individuals on cART.

Keywords: NK cell, HIV, adaptive-like NK cell, immune activation, combination antiretroviral therapy

INTRODUCTION

Effective combination antiretroviral therapy (cART) suppresses HIV replication and prevents AIDS-related illness but does not eliminate HIV or fully restore immune function. Virologically suppressed HIV$^+$ individuals show phenotypic and functional evidence of persistent immune

dysfunction, particularly within the innate immune system (1). We and others have demonstrated that heightened HIV-related activation of NK cells (2, 3) and monocytes (4–8) persist in cART-treated individuals despite undetectable levels of HIV viremia (<20 HIV RNA copies/mL plasma). Markers of innate immune activation/inflammation are associated with co-morbidities such as cardiovascular disease, neurocognitive impairment, and malignancies in HIV$^+$ individuals [reviewed in Ref. (9)] and also predict mortality in this population (10). This suggests persistent innate immune activation may have a detrimental effect on the long-term health of HIV$^+$ individuals on cART. Elucidating the underlying mechanism of this effect is essential for preserving the health of the estimated 17 million HIV$^+$ individuals worldwide currently receiving cART.

Acute HIV infection triggers a short-lived expansion of the mature CD56^{dim}CD16$^+$ NK cell subset (which declines during progressive infection) and the emergence of a population of functionally anergic CD56$^-$ NK cells (11–13), but these defects are largely reversed by cART (14). In contrast, our cross-sectional study demonstrated increased NK cell activation and heightened spontaneous degranulation in both viremic and virologically suppressed HIV$^+$ individuals (2), indicating NK cell activation persists despite effective cART. However, the duration of this effect and its impact on co-morbid disease remain unknown.

Although considered innate immune cells, increasing evidence indicates NK cells also possess adaptive, memory-like properties similar to cytotoxic CD8$^+$ T cells (15). A rapid expansion of NK cells able to target murine cytomegalovirus (MCMV)-infected cells has been demonstrated following primary MCMV infection (16). Furthermore, NK cells exhibiting memory-like properties persisted in tissue for several months after MCMV infection and displayed rapid degranulation upon subsequent stimulation. A similar expansion and persistence of specific populations of NK cells also occurs in response to human CMV (HCMV) infection; HCMV seropositivity is associated with expansion of CD56^{dim} NK cells expressing the activating receptor NKG2C and a pattern of killer cell immunoglobulin-like receptors consistent with clonal expansion (17–19). Recent studies associate HCMV infection with expansion of multiple subsets of adaptive-like NK cells, including (but not limited to) those expressing NKG2C (20–22). These "imprinted" populations are stably maintained for at least 15 months following infection (23) and show enhanced antibody-dependent activation (21), consistent with an important role in protective immunity against viral infections. Clonal expansion of NK cells is also observed early after Chikungunya (24) and Hantavirus (25) infections; and we have previously reported expansion of adaptive-like FcRγ$^-$ NK cells in HIV infection (3); however, it remains unclear whether pre-existing CMV infection is a prerequisite for the observed expansion of specific NK cell populations in these settings.

NK cell receptor profiles are perturbed in viremic HIV$^+$ individuals, with increased expression of the activating receptor NKG2C and reduced expression of the inhibitory receptor NKG2A on NK cells (26, 27). The proportion of NKG2A$^+$ NK cells is restored in cART-treated HIV$^+$ individuals in some (27, 28) but not all studies (26); however, expansion of NKG2C$^+$ NK cells can persist in aviremic individuals (29) despite at least 2 years of viral suppression (27). HIV-associated expansion of NKG2C$^+$ NK cells appears to occur only in individuals seropositive for HCMV (27, 29), and HCMV seropositivity is also a prerequisite for NKG2C$^+$ NK cell expansion induced by other chronic viral infections such as hepatitis B and C infection (18). These findings suggest chronic viral diseases such as HIV and HCMV may act synergistically to heighten immune dysfunction. Accordingly, HCMV$^+$/HIV$^+$ individuals with >12 years of successful cART have higher levels of HCMV-reactive antibodies and T cells than HCMV$^+$/HIV$^-$ individuals (30, 31). These findings highlight the necessity to consider HCMV antibodies in studies of NK cell dysfunction and underscore the requirement for appropriate HIV$^-$ comparison groups when analyzing these defects in HIV$^+$ populations who carry a higher burden of HCMV than the general population.

In a recent cross-sectional study, we made the novel discovery that a population of CD56^{dim} NK cells lacking the intracellular signal transduction protein FcRγ is expanded in both viremic and virologically suppressed HIV$^+$ individuals (3). FcRγ is an immunoreceptor tyrosine-based activation motif-containing adaptor protein responsible for transducing signals through activating NK cell receptors such as CD16, and acting as a chaperone for these receptors. These CD56^{dim}FcRγ$^-$ NK cells have reduced expression of CD16 and the natural cytotoxicity receptors NKp30 and NKp46, but enhanced antibody-dependent cell-mediated cytotoxicity (ADCC) activity, and represent up to 90% of the NK cell population in some HIV$^+$ individuals (3). An analogous population of CD56^{dim}FcRγ$^-$ NK cells has previously been characterized in HIV$^-$/HCMV$^+$ individuals and shown to possess a memory-like phenotype with adaptive immune features including enhanced ADCC against target cells infected with HCMV or herpes simplex virus, implying a specialized role in antibody-dependent cross-protection (22, 32). We therefore investigated the HIV-related expansion of adaptive-like CD56^{dim}FcRγ$^-$ NK cells in a contemporary cohort of HIV$^+$ men who have sex with men (MSM). Given the abovementioned effect of HCMV infection on NK cell imprinting and the near ubiquitous HCMV seropositivity of HIV$^+$ MSM, we used a novel longitudinal MSM cohort to study the effect of HIV and cART on the prevalence of FcRγ$^-$ NK cells by comparison with appropriately matched HIV$^-$ MSM. Furthermore, we used latent growth curve modeling to quantify the rate at which NK cell activation is reversed following viral suppression as compared to activation of other immune cell compartments.

MATERIALS AND METHODS

Study Participants

Participants were identified from the Melbourne HIV Cohort, a prospective study of HIV-positive and HIV-seronegative men who self-report having sex with men. Participants in the Melbourne HIV Cohort were reviewed annually to assess co-morbidities. Peripheral blood mononuclear cells (PBMC) and plasma were prepared and archived from each visit. Baseline samples were analyzed from 20 cART-naïve HIV$^+$ MSM and 15 HIV$^-$ MSM matched for age with the HIV$^+$ MSM at the baseline time-point. HIV$^+$ individuals were recruited when they were cART-naïve and followed-up every 3 months for

12 months following cART-initiation, then annually thereafter. Of the 20 HIV$^+$ MSM, one initiated a cART regimen consisting of efavirenz, festinavir and lamivudine. The other 19 participants received a cART regimen of tenofovir and emtricitabine, plus either efavirenz ($n = 6$), rilpivirine ($n = 5$), raltegravir ($n = 3$), ritonavir + atazanavir ($n = 4$), or ritonavir + neviripine ($n = 1$); two individuals had their regimen altered (from raltegravir to dolutegravir and from efavirenz to raltegravir) during the follow up period. At the time of the study, 10 of the HIV$^+$ MSM had reached the 24-month post-cART initiation time-point and were included in the analysis. Exclusion criteria included co-morbid disease (e.g., cardiovascular disease, diabetes) and current use of statins, steroids, or other anti-inflammatory medications. For selected experiments, an additional 14 HIV$^-$ men of a similar age were recruited from the general community. Ethical approval for this study was obtained from the Alfred Hospital Research and Ethics Committee.

Sample Processing and Immunophenotyping

Cells and plasma were prepared from whole blood collected into acid citrate dextrose tubes. PBMC were collected following Ficoll density gradient centrifugation of blood and stored in liquid N_2. Cells were stained with LIVE/DEAD® fixable dead cell stain (ThermoFisher Scientific, Waltham, MA, USA) prior to immunophenotyping. Expression of surface receptors on NK cells, monocytes, and T cells were detected by staining with the following antibodies: CD56 APC (clone NKH-1) from Beckman Coulter (Brea, CA); CD14-V500 (clone M5E2), CD16 PE-Cy7 (clone 3G8), CD3 PerCP-Cy5.5 (clone UCHT1), CD38-PE (clone HB7), HLA-DR FITC or APC-H7 (clone G46-6), CD4 PE-Cy7 (clone RPA-TA), CD8 APC-H7 (clone SK1), all from BD Biosciences (San Jose, CA, USA); CD3 BV510 (clone OKT3), CD56 AF700 (clone HCD 56), CD57 Pacific Blue (clone HCD57), CD355 PerCP-Cy5.5 (NKp46, clone 9E2), CD337 AlexaFluor 647 (NKp30, clone CD337), all from Biolegend (San Diego, CA, USA). Expression of intracellular FcRγ was detected after labeling of surface antigens and following permeabilization with Perm/Wash buffer 1 (BD Biosciences), then staining with anti-FcRγ FITC (FcεR1, γ subunit, rabbit polyclonal, Millipore, Darmstadt, Germany). The specificity of the polyclonal anti-FcRγ FITC antibody has been previously demonstrated (3). Cells were acquired on a Fortessa LSR flow cytometer (BD Biosciences) and data analyzed using FlowJo software (version 10, FlowJo LLC, Ashland, OR, USA). Gating strategies for each cell type are depicted in Figure S1 in Supplementary Material.

Measurement of Plasma Inflammatory Markers and HCMV Antibody

Plasma concentrations of soluble CD163 (sCD163) and CXCL10 were measured using commercial ELISA kits as per manufacturer's instructions (Macro 163, IQ Products, Groningen, Netherlands and DIP-100, Quantikine ELISA, R&D Systems, Minneapolis, MN, USA, respectively). IgG reactive with HCMV was quantified using HCMV lysate, HCMV glycoprotein B (gB), and HCMV IE-1 antigens as previously described (3). CMV seropositivity was defined as >2 SD above the mean antibody levels for HCMV lysate derived for a set of 11 samples that had been deemed seronegative by the ARCHITECT CMV IgG assay (Abbott Diagnostics, IL). Data are presented in arbitrary units (AU) defined relative to a standard plasma pool run on each plate. Cross-reactivity of the CMV ELISA with other herpes viruses has not been formally assessed. Samples were measured over a range of dilutions to ensure accurate quantitation in the high range.

Statistical Analyses

Cross-sectional comparisons of marker levels between groups were made using Mann–Whitney *U*-test, while differences between baseline and post-cART time points in HIV$^+$ MSM were made using Wilcoxon matched pairs signed rank test (GraphPad Prism software, version 6.05). Multilevel modeling was used to estimate latent growth-curve models exploring the subject-specific nature of the association between each marker and time. Latent growth-curve models were also estimated on the natural log of each immunological marker and post-estimation non-linear equations using exponentiated model coefficients were estimated to provide proportional rates of immunological change at specific time-points. Latent growth-curve models comprised two-levels, HIV$^+$ individuals at level-2 (i.e., random intercept and coefficient for time) and their marker responses over-time at level-1 (see Supplementary Eq. 1 in Supplementary Material). Latent growth-curve modeling was extended to incorporate more complex bivariate outcome models (i.e., two outcomes), enabling simultaneous estimation of log-marker rates of change and post-estimation inference comparing rates of change between different markers (i.e., modeling the comparative mean percent change between two markers over-time, and accounting for the correlation between individuals' responses to these two markers across time). In these models, typically, random effects for heterogeneity in both participants' baseline marker levels (i.e., random intercept) and the nature of any change in marker level over time (i.e., random coefficient/slope) were estimated, with an unstructured covariance estimated between each random effect. A limitation of these analyses was that for markers measured using a binomial statistical understanding (i.e., outcomes where cell proportions were determined) the distributional assumptions of the linear mixed models used in longitudinal modeling were not entirely met. Nested model-based likelihood-ratio statistics were used to provide statistical inference for model fit when relaxing model constraints (random effects and the functional form of fixed effects for time). To assess the fit of the estimated latent growth-curve models, diagnostic plots comparing participants observed marker levels with Bayesian model-based (best linear unbiased predictions) predicted levels over time were produced and inspected. Contemporaneous (i.e., both outcome and factor variable responses from the same time-period were regressed) unadjusted longitudinal associations between selected factors and participant CD56^{dim} FcRγ$^-$ NK cell proportions were estimated using multilevel modeling. In these multilevel models, factors were estimated as time-varying fixed effects with a random intercept (level-2) to account for the dependency in the data given an individual's repeated marker

level measurement over-time. Statistical inference was assessed at the 5% level. Stata version 13.1 statistical package (StataCorp LP, College Station, TX, USA) was used for multilevel modeling and bivariate latent growth-curve modeling was undertaken using the user-written Stata program generalized linear latent mixed modeling (gllamm) [(33)].

RESULTS

MSM Show Expansion of FcRγ⁻ NK Cells and Elevated Plasma Levels of HCMV Antibodies as Compared to Non-MSM Individuals

In many developed countries, the HIV epidemic is concentrated in at-risk populations such as MSM, yet demographic and clinical differences that exist between these populations and the general community [i.e., prevalence of smoking, sexually transmitted infections, HCMV seropositivity (34)] are rarely considered in immunological studies. Given the association between HCMV infection and adaptive-like NK cell expansion in HIV seronegative individuals shown by ourselves and others (3, 22), we first asked whether proportions of FcRγ⁻ NK cells were influenced by MSM status. We compared samples from 14 non-MSM males (median age [IQR] 31.0 [30.0–39.0] years, 78.6% or 11/14 CMV-seropositive), and 14 MSM of a comparable age (35.0 [29.3–43.5] years, 85.7% or 12/14 CMV-seropositive) who were all HIV seronegative. MSM had higher levels of IgG antibodies reactive with the HCMV envelope protein gB (**Figure 1A**, $p = 0.047$) but also showed significantly increased proportions of FcRγ⁻ NK cells (median [IQR] 14.4% [4.8–17.8] for MSM vs 4.0% [1.8–6.8] for non-MSM; $p = 0.006$, **Figure 1B**). These differences persisted when only CMV seropositive individuals were compared ($p = 0.012$ and 0.037 for gB antibodies and FcRγ⁻ NK cells, respectively, not shown). These findings confirmed the importance of controlling for MSM sexual exposure and HCMV burden in our subsequent analyses of NK cell immunology in HIV infection.

Viremic HIV Infection in MSM Is Associated with a Further Expansion of Adaptive-Like FcRγ⁻ NK Cells with a Similar Phenotype to Those in HIV-Seronegative MSM

To assess the impact of untreated HIV infection on FcRγ⁻ NK cell expansion in an appropriately controlled study population, we analyzed baseline samples from cART-naïve HIV⁺ and HIV⁻ MSM of similar age ($n = 20$ and 15, respectively) from the Melbourne HIV cohort. Demographic characteristics and relevant clinical parameters are detailed in **Table 1**. Proportions of FcRγ⁻ NK cells were expanded in HIV⁺ MSM, with a median of 28.6% (IQR: 24.6–38.3%) compared to 14.4% (4.8–17.8%) in HIV⁻ MSM ($p < 0.0001$, **Figure 2A**). The phenotype of these cells in the two groups was similar; CD56^{dim} FcRγ⁻ NK cells had very low expression of the natural cytotoxicity receptors NKp30 and NKp46 and heightened expression of the maturation/differentiation marker CD57, which was statistically significant in HIV⁺ individuals; however, there was substantial inter-individual variation in the pattern of CD57 expression on FcRγ⁻ and FcRγ⁺ NK cells (Figure S2A–C in Supplementary Material). To further explore the phenotype of these cells and investigate whether they were the result of proliferative expansion, we analyzed expression of the chemokine receptor CXCR6 [indicative of tissue-homing NK cells (35)], and a proliferation marker (Ki-67) on FcRγ⁻ and FcRγ⁺ CD56^{dim} NK cells in a subset of HIV⁺ donors. Compared to FcRγ⁺ CD56^{dim} NK cells, FcRγ⁻ cells had significantly lower expression of CXCR6 (0.7 vs 5.8%; $p = 0.004$, data not shown) while both populations showed equal, low expression of Ki-67 (0.5 vs 1.0%, $p = 0.945$, data not shown). These data indicate that the proportion of adaptive-like FcRγ⁻ NK cells is expanded by HIV infection (in addition to the effects of MSM-related factors)

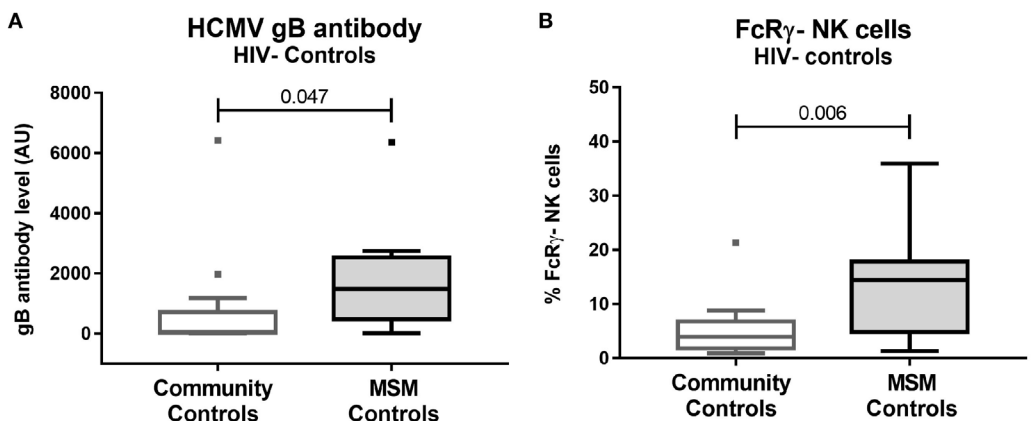

FIGURE 1 | CD56^{dim} FcRγ⁻ NK cell expansion and increased human CMV (HCMV) antibodies are associated with MSM-related factors. **(A)** Plasma antibodies to the HCMV glycoprotein B (gB) were quantified by ELISA in plasma from HIV⁻ male controls recruited from either the community ($n = 14$) or from the Melbourne HIV Cohort consisting of men who have sex with men (MSM, $n = 14$). **(B)** The proportion of CD56^{dim}CD16⁺ NK cells lacking the FcRγ⁻ signal transduction protein was measured in peripheral blood mononuclear cells from the same individuals as in **(A)** using intracellular staining and flow cytometry. Graphs show Tukey plots of median (bar), IQR (box), and 1.5x IQR (whiskers); outliers are indicated by squares. *p* values shown were determined by Mann–Whitney *U* test. AU, Arbitrary units.

TABLE 1 | Demographic and clinical characteristics of HIV$^-$ and HIV$^+$ MSM at baseline and longitudinally post-combination antiretroviral therapy (post-cART) initiation.[a]

	HIV$^-$ MSM	HIV$^+$ MSM			
			Post-cART follow-up time point		
Median (IQR)	Baseline	Baseline	6 months	12 months	24 months
n	15	20	20	20	10
Age (years)	34.0 (29.0–43.0)	32.0 (29.0–43.5)			
Current smoker, *n* (%)	1 (6.6%)	4 (20%)			
HCV$^+$ (antigen and PCR$^+$), *n* (%)	0	2 (10%)			
Human CMV seropositive	13 (86.7%)	20 (100%)[b]			
Nadir CD4 T cell count (cells/μL)	NA	385 (329–656)			
CD4 T cell count (cells/μL)	ND	452 (382–711)	580 (507–752)*	605 (480–942)*	741 (588–1037)*
ΔCD4 T cell count (cells/μL)	NA		135 (16–203)	129 (−22–209)	205 (53–462)
Viral load (RNA copies/mL)[c]	NA	41,050 (17,219–148,606)	20 (20–44)***	20 (20–20)***	20 (20–20)**
Undetectable viral load, *n* (%)	NA	0	13 (65%)	19 (95%)	10 (100%)

NA, not applicable; ND, not determined.

[a]*Longitudinal analysis was performed on HIV$^+$ MSM only.*

[b]*Not statistically different to HIV$^-$ MSM as determined by Chi-squared test.*

[c]*Values of <20 copies/mL were designated as 20 for the purpose of statistical analysis.*

**p < 0.05, **p < 0.01, ***p < 0.001 vs baseline from Wilcoxon matched pairs signed rank tests.*

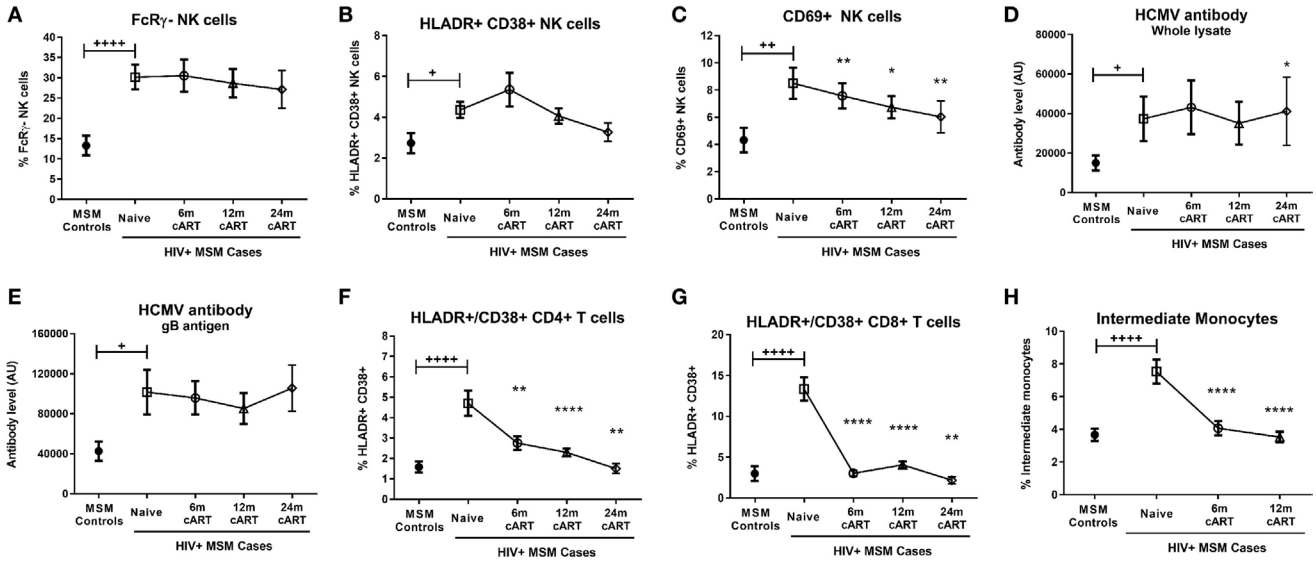

FIGURE 2 | Combination antiretroviral therapy (cART) reverses T cell and monocyte activation, but not NK cell dysfunction or human CMV (HCMV) antibody levels. The proportion of FcRγ$^-$ NK cells (**A**), activated HLA-DR$^+$CD38$^+$ **(B)**, and CD69$^+$ **(C)** NK cells, plasma levels of HCMV-specific antibodies to either whole HCMV lysate **(D)** or gB antigen **(E)**, the percentage of activated HLA-DR$^+$CD38$^+$ CD4$^+$ **(F)** and CD8$^+$ **(G)** T cells and the proportion of intermediate (CD14^{++}CD16$^+$) monocytes **(H)** was determined in HIV-uninfected MSM ($n = 15$) and HIV$^+$ MSM ($n = 20$) at baseline (cART-naïve) and after 6, 12, and 24 months of cART. Graphs show median and IQR. +, ++, and ++++ denote $p < 0.05$, 0.01, and 0.0001, respectively, as compared to HIV$^-$ MSM determined by Mann–Whitney *U* test. *, **, and **** and denote $p < 0.05$, <0.01, and <0.0001, respectively, as compared to the corresponding cART-naïve value determined by Wilcoxon matched pairs signed rank test. AU, arbitrary units.

and that this cell population does not appear to be the result of heightened proliferation or show increased expression of tissue-homing receptors but may represent a more mature NK cell subset.

Viremic HIV Infection Is Associated with Increased NK Cell Activation and Elevated Levels of HCMV Antibodies

Given our findings regarding the influence of MSM-related factors on FcRγ$^-$ NK cell expansion, it was important to confirm the effect of HIV on NK cell activation and other immune parameters in an adequately controlled cohort. Compared to HIV$^-$ MSM, viremic, cART-naive HIV$^+$ MSM had significantly increased levels of NK cell activation as indicated by proportions of either HLA-DR$^+$/CD38$^+$ (median [IQR] 4.5% [2.5–5.9%] in HIV$^+$ MSM vs 2.3% [1.4–3.0%] for HIV$^-$ MSM, $p = 0.015$) or CD69$^+$ NK cells (7.4% [4.5–10.8%] vs 3.2% [2.2–6.2%], $p = 0.004$) (**Figures 2B,C**, respectively). In addition to the increase in HCMV antibody levels associated with MSM status shown in **Figure 1**, viremic HIV infection was associated with a further increase in antibody

levels to both whole HCMV lysate ($p = 0.046$, **Figure 2D**) and the gB antigen ($p = 0.011$, **Figure 2E**), while antibody levels to HCMV IE-1 did not differ with HIV status (data not shown).

Analysis of T cell and monocyte activation markers confirmed the well-established effect of viremic HIV infection on heightened $CD4^+$ and $CD8^+$ T cell activation (as assessed by HLA-DR/CD38 coexpression; $p < 0.0001$ for both vs HIV^- MSM, **Figures 2F,G**), expansion of inflammatory intermediate $CD14^{++}CD16^+$ monocytes ($p < 0.0001$ vs HIV^- MSM, **Figure 2H**), and concomitant reduction in classical $CD14^{++}CD16^-$ monocyte proportion ($p = 0.008$, data not shown). Taken together, data from this unique MSM cohort confirm that untreated HIV infection is associated with increased NK cell activation and generalized adaptive and innate immune activation, and indicate that this occurs in addition to the effects related to MSM status shown in **Figure 1**. Although HCMV seropositivity was near-ubiquitous in this cohort and not significantly different between HIV^+ and HIV^- MSM (**Table 1**), a sub-analysis of only $HCMV^+$ MSM indicated the proportion of activated HLA-DR^+/$CD38^+$, $CD69^+$, and $FcR\gamma^-$ NK cells was significantly higher in HIV^+ vs HIV^- MSM ($p = 0.032$, 0.012, and 0.001, respectively, data not shown), indicating an effect of HIV independent of HCMV serostatus.

Viral Suppression Associated with cART Does Not Reverse NK Cell Activation or the Expansion of $FcR\gamma^-$ NK Cells

We undertook longitudinal analyses of pre- and post-cART samples from HIV^+ MSM to determine the extent to which cART was able to reverse HIV-associated defects to NK cells as compared to other cellular/immunological compartments. Sixty-five percent, 95%, and 100% of individuals achieved undetectable viral load (<20 copies/mL) after 6, 12, and 24 months of cART, respectively (**Table 1**), and significant increases in $CD4^+$ T cell counts observed at all post-cART time-points ($p < 0.05$ for all vs baseline, **Table 1**) confirmed the efficacy of cART in this cohort. The relatively high nadir $CD4^+$ T cell count of 385 [329–656] (median [IQR]) and baseline $CD4^+$ T cell count of 452 [382–711] cells/μL are typical of a contemporary HIV cohort where individuals initiate cART at higher $CD4^+$ T cell counts prior to experiencing significant immunological damage.

Descriptive statistical analysis revealed that cART had no impact on proportions of $FcR\gamma^-$ NK cells, which remained similar to pre-cART levels at 6, 12, and 24 months post-cART initiation (**Figure 2A**). Levels of activated HLA-DR^+/$CD38^+$ NK cells were also unaltered by cART and remained similar to levels in viremic, cART-naïve individuals at all follow-up time-points (**Figure 2B**). However, the proportion of NK cells expressing the early activation marker CD69 at 6, 12, and 24 months post-cART time-points were significantly lower than pre-cART levels ($p = 0.004$, 0.036, and 0.008, respectively; **Figure 2C**). Viral suppression associated with cART did not alter levels of HCMV antibodies to either whole lysate or gB antigens (**Figures 2D,E**, respectively). The lack of an effect of cART on NK cell dysfunction and HCMV antibody levels was in contrast to its effect on T cells and monocytes, where HIV-related $CD8^+$ T cell activation and intermediate monocyte expansion were reversed to levels observed in uninfected MSM within 6 months of cART initiation (**Figures 2G,H**, respectively), while $CD4^+$ T cell activation was resolved within 24 months of cART (**Figure 2F**). These descriptive analyses indicate cART is less effective at decreasing activation of NK cells compared to T cells and monocytes and demonstrate the persistence of NK cell dysfunction and elevated HCMV antibodies in HIV^+ individuals despite 24 months of viral suppression.

cART Reverses HIV-Related Activation of T Cells and Monocytes More Rapidly Than NK Cell Activation

To fully and quantitatively model the differential effect of cART on individual immune cell types indicated by the above descriptive analyses, we employed a mixed effects modeling framework to compare the rate of decline of HIV-related NK cell activation with other cellular compartments. This analytical approach has the additional benefit of accounting for the inherent variation in each individual's initial immunological status and the way they subsequently respond to therapy, permitting a more accurate modeling of the change to specific immunological parameters over time in response to cART than is achievable with simple descriptive statistical analyses.

Latent growth-curve modeling confirmed cART had no significant effect on proportions of activated HLA-DR^+/$CD38^+$ NK cells [Wald $\chi^2(1) = 2.3$, $p = 0.129$] or $FcR\gamma^-$ NK cells [Wald $\chi^2(1) = 2.5$, $p = 0.115$], while the HIV-related increase in activated $CD69^+$ NK cell proportion declined significantly over time on cART [Wald $\chi^2(1) = 9.1$, $p = 0.003$, **Table 2**]. Our modeling also confirmed that T cell activation [Wald $\chi^2(2) = 32.5$ and Wald $\chi^2(2) = 68.1$ for $CD4^+$ and $CD8^+$ T cells, respectively, $p < 0.001$ for both, **Table 2**] and inflammatory intermediate monocyte subset expansion [Wald $\chi^2(2) = 61.2$, $p < 0.001$] were significantly reduced over time on cART.

To compare the rate at which cART reversed $CD69^+$ NK cell, T cell and monocyte activation, the proportion of pre-cART immune activation, which remained after 6 and 12 months on cART, was calculated for each cell type (**Table 3**). After 12 months of cART, 60% (95% CI:53–67%) of activated $CD4^+$ and 30% (21–39%) of activated $CD8^+$ T cells remained, indicating a respective 40 and 70% reduction in T cell activation. Similarly, 12 months of cART was associated with a 53% (44–62%) and 85% (54–138%) reversal of the HIV-related alterations to intermediate and classical monocyte subset proportions, respectively. In contrast, reversal of activated $CD69^+$ NK cells was substantially slower, with only 20% (13–27%) of HIV-related activation reversed following 12 months of cART.

To determine whether differences in the rates at which cART reversed HIV-related activation of NK cells as compared to T cells and monocytes were significant, we estimated bivariate latent-growth curve models regressing each of the marker outcomes on respective functions of time simultaneously and allowing each individual's treatment responses in marker levels to correlate. This revealed significant differences in the

TABLE 2 | Latent growth-curve modeling[a] showing associations between immune parameter outcomes and time (linear and quadratic) post-combination antiretroviral therapy (post-cART) initiation in HIV+ individuals ($n = 20$).

Immune parameter outcome	*b* (SE)	95% CI	Wald χ^2	*p*-Value
Activated/adaptive-like NK cells				
NK cell (% CD69+)			$\chi^2(1) = 9.1$	0.003
Linear	−0.12 (0.04)	−0.21; −0.04		
Quadratic	–	–	–	–
NK cell (% HLA DR+/CD38+)	–	–	$\chi^2(1) = 2.3$[c]	0.129
Linear	−0.05 (0.03)	−0.11; 0.01	–	–
Quadratic	–	–	–	–
CD56dim FcRγ− NK cells			$\chi^2(1) = 2.5$	0.115
Linear	−0.22 (0.14)	−0.50; 0.05	–	–
Quadratic	–	–	–	–
T cell activation				
CD4+ T cell (% HLA DR+/CD38+)	–	–	$\chi^2(2) = 32.5$[b]	<0.001
Linear	−0.23 (0.05)	−0.34; −0.13	–	–
Quadratic	0.004 (0.002)	0.0004; 0.01	–	–
CD8+ T cell (% HLA DR+/CD38+)	–	–	$\chi^2(2) = 68.1$	<0.001
Linear	−1.08 (0.16)	−1.38; −0.77	–	–
Quadratic	0.03 (0.01)	0.01; 0.04	–	–
Monocyte subsets				
% Classical monocytes			$\chi^2(2) = 28.5$	<0.001
Linear	1.49 (0.39)	0.72; 2.26	–	–
Quadratic	−0.07 (0.03)	−0.13; −0.01		
% Intermediate monocytes			$\chi^2(2) = 61.2$	<0.001
Linear	−0.78 (0.11)	−1.00; −0.56	–	–
Quadratic	0.03 (0.01)	0.02; 0.05	–	–
% Non-classical monocytes			$\chi^2(1) = 10.9$	0.001
Linear	−0.30 (0.09)	−0.48; −0.12	–	–
Quadratic	–	–	–	–

Table shows regression coefficient (b), SE, 95% confidence intervals (95% CI), Wald tests (Wald χ^2) and probability value (p-value).
[a]Latent growth-curve modeling specifying a random intercept (baseline marker level) and coefficient (linear time) and unstructured covariance terms for random effects.
[b]Intercept and slope covariance term not able to be computed for this model.
[c]Random intercept model only—random coefficient model did not converge.
Note: Where a quadratic coefficient is not shown for an outcome, nested likelihood ratio tests did not reject the null hypothesis that the functional form of time on cART was linear.

rate of reversal of CD69+ NK cell activation as compared to inflammatory intermediate monocyte expansion [6 months: Wald $\chi^2(1) = 42.6$; 12 months: Wald $\chi^2(1) = 28.0$; $p < 0.001$ for both, **Figure 3A**]. Similarly, the proportion of activated CD69+ NK cells declined significantly more slowly after cART initiation than activated (HLA-DR+/CD38+) CD4+ T-cells [6 months: Wald $\chi^2(1) = 13.7$; 12 months: Wald $\chi^2(1) = 16.1$, $p < 0.001$ for both, **Figure 3B**] or CD8+ T-cells [6 months: Wald $\chi^2(1) = 58.3$; 12 months Wald $\chi^2(1) = 47.8$, $p < 0.001$ for both, **Figure 3C**]. These analyses indicate that cART has a differential effect on different arms of the immune system, and robustly confirm that NK cell activation resolves more slowly than T cell or monocyte dysfunction following cART initiation.

TABLE 3 | Exponentiated regression coefficient indicating percent change in log immunological parameters from baseline after 6 and 12 months of combination antiretroviral therapy from latent growth-curve modeling ($n = 20$).

	6 months	12 months
Immune parameter outcome	**Exp *b* (95% CI)[a]**	**Exp *b* (95% CI)[a]**
NK cell activation/function		
NK cell (% CD69+)	**0.89 (0.85,0.93)**	**0.80 (0.73,0.87)**
NK cell (% HLA DR+/CD38+)	0.94 (0.88,1.01)	0.89 (0.76,1.02)
CD56dim FcRγ− NK cells	0.94 (0.88,0.99)	0.88 (0.77,0.99)
T cell activation		
CD4+ (% HLA DR+/CD38+)	**0.77 (0.73,0.82)**	**0.60 (0.53,0.67)**
CD8+ (% HLA DR+/CD38+)	**0.50 (0.41,0.60)**	**0.30 (0.21,0.39)**
Monocyte subsets		
% Classical monocytes	**1.36 (1.23,1.51)**	**1.85 (1.54,2.38)**
% Intermediate monocytes	**0.56 (0.46,0.66)**	**0.47 (0.38,0.56)**
% Non-classical monocytes	**0.74 (0.64,0.84)**	**0.55 (0.40,0.70)**

*[a]Exponentiated regression coefficients (Exp b) and 95% confidence intervals (95% CI) from non-linear combined (i.e., linear and quadratic terms) effect estimation based on log-normal latent growth-curve models—coefficient (Exp b) represents the ratio of expected geometric mean difference in a marker for a specific length of time taking account of the functional form of the effect of time (i.e., % change in a marker per specified time-period). Ratios <1 indicate a percent decrease. Values in **bold** indicate marker levels exhibiting significant change over-time.*

Expansion of Adaptive FcRγ− NK Cells Is Not Associated with HCMV Antibody Levels or Other Immune Activation Markers in HIV+ Individuals

Given the persistence of both FcRγ− NK cells and elevated HCMV antibody levels in cART-experienced HIV+ MSM, and our previous observation of an association between these two parameters in HIV-uninfected individuals (3), we extended the modeling to investigate associations between FcRγ− NK cells and elevated HCMV antibody levels in HIV+ MSM. Contemporaneous time-varying associations between individuals' FcRγ− NK cell levels and other key markers were estimated from longitudinal data obtained up to 12 months post-cART initiation using linear mixed modeling, which permitted the analysis of repeated measures data from the same individuals. This analysis indicated that FcRγ− NK cell levels were not associated with levels of antibody to either HCMV lysate [Wald $\chi^2(1) = 0.23$, $p = 0.631$] or gB [Wald $\chi^2(1) = 0.89$, $p = 0.345$, **Table 4**]. Significantly, there was no association between the proportions of FcRγ− NK cells and NK cell activation (either HLA-DR+/CD38+ or CD69+ NK cells, $p = 0.705$ and 0.182, respectively, **Table 4**), nor with any other cellular or soluble immune activation marker measured. This lack of association was also observed when only baseline samples from cART naïve individuals were analyzed (data not shown). These findings are consistent with our previous cross-sectional study that indicated an association between HCMV antibody levels and adaptive-like FcRγ− NK cells in HIV− but not HIV+ individuals (3).

In contrast to levels of FcRγ− NK cells, NK cell activation, measured as either HLA-DR+/CD38+ or CD69+ NK cells, was significantly associated with CD4+ T cell activation [Wald $\chi^2(1) = 5.31$, $p = 0.021$ and Wald $\chi^2(1) = 9.55$, $p = 0.002$, respectively] and the

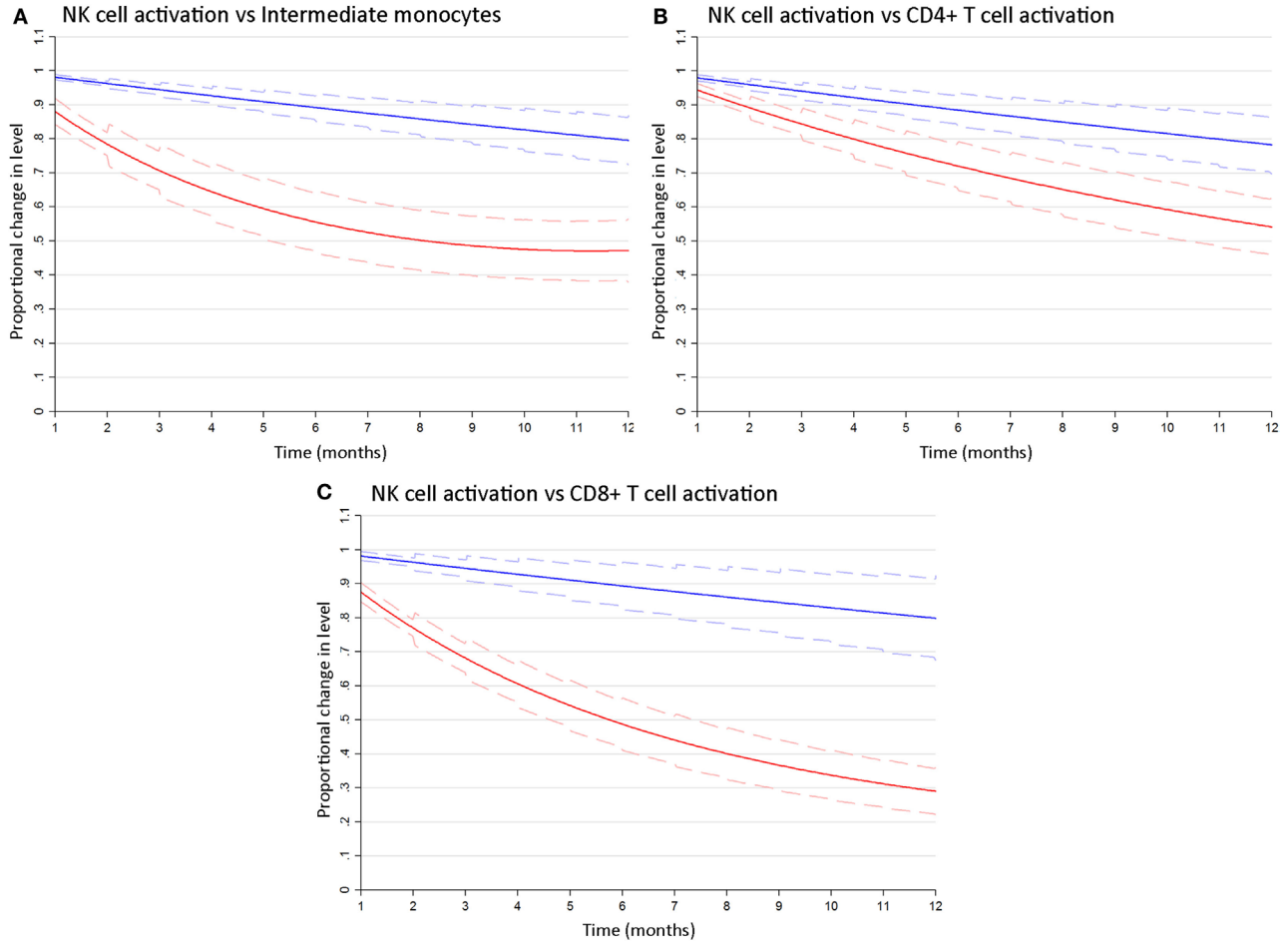

FIGURE 3 | NK cell activation decays more slowly following combination antiretroviral therapy (cART) initiation than T cell or monocyte activation. Bivariate latent growth curve models comparing the modeled rate of decline in the proportion of activated CD69$^+$ NK cells (blue lines) vs inflammatory intermediate CD14^{++}CD16$^+$ monocytes [red lines, **(A)**], activated HLA-DR$^+$/CD38$^+$ CD4$^+$ [red lines, **(B)**] or CD8$^+$ [red lines, **(C)**] T cells for 12 months after cART initiation. Mean exponentiated effects and 95% confidence intervals are shown (solid and dashed lines, respectively). Note: Plots show mean exponentiated linear/quadratic effects by time after cART initiation and 95% confidence intervals. Values <1 indicate a reduction in marker level over time.

proportion of intermediate [Wald $\chi^2(1) = 4.29$, $p = 0.038$ and Wald $\chi^2(1) = 6.02$, $p = 0.014$, respectively] and classical [Wald $\chi^2(1) = 5.71$, $p = 0.017$ and Wald $\chi^2(1) = 4.36$, $p = 0.037$, respectively] monocyte subsets (Table S1 in Supplementary Material). Activated CD69$^+$ NK cells were also significantly associated with CD8$^+$ T cell activation [Wald $\chi^2(1) = 9.24$, $p = 0.002$] and plasma levels of CXCL10 [Wald $\chi^2(1) = 6.66$, $p = 0.010$], whilst neither HLA-DR$^+$/CD38$^+$ or CD69$^+$ activated NK cells were associated with HCMV antibody levels ($p > 0.05$ for all). These findings suggest that whilst NK cell activation is associated with other markers of adaptive and innate immune activation in HIV$^+$ individuals, the expansion of adaptive-like FcRγ$^-$ NK cells is a discrete phenomenon.

DISCUSSION

HIV infection has a profound impact on NK cells including heightened cellular activation, imprinting of the NK cell receptor repertoire and expansion of NK cell subpopulations with an adaptive, memory-like phenotype (2, 3, 26–28). Studies investigating the impact of HIV infection and cART on immunological parameters are often limited by cross-sectional study designs and inappropriate HIV$^-$ comparator populations that do not adequately control for confounders such as heightened HCMV seropositivity. In this study, we used a unique and carefully controlled longitudinal cohort of HIV$^+$ MSM initiating cART with HIV-seronegative MSM recruited from the same primary care sites to investigate the impact of HIV infection and cART on NK cell dysfunction. We found the proportion of CD56^{dim} FcRγ$^-$ NK cells was significantly increased in cART-naïve HIV$^+$ MSM as compared to HIV$^-$ MSM, who in turn were immunologically distinct from community controls. Viremic HIV infection in MSM was also associated with significant activation of CD56^{dim} NK cells, as assessed using HLA-DR/CD38 co-expression as described previously (2) or CD69 expression as phenotypic markers of activation. CD69 ligation induces cytolytic activity in

TABLE 4 | Mixed modeling[a] showing unadjusted longitudinal associations between FcRγ$^-$ CD56^{dim} FcRγ$^-$ NK cell levels and immune parameters in HIV$^+$ individuals (n = 20).

Immune parameter	*b*(SE)	95% CI	Wald χ^2	*p*-Value
T cell activation				
CD4$^+$ (% HLA DR$^+$/CD38$^+$)	0.33 (0.66)	−0.96, 1.62	$\chi^2(1) = 0.26$	0.613
CD8$^+$ (% HLA DR$^+$/CD38$^+$)	0.01 (0.21)	−0.40, 0.41	$\chi^2(1) = 0.00$	0.977
NK cell activation/function				
NK (% CD69$^+$)	0.62 (0.46)	−0.29,1.52	$\chi^2(1) = 1.78$	0.182
NK (% HLA DR$^+$/CD38$^+$)	−0.24 (0.63)	−1.46, 0.99	$\chi^2(1) = 0.14$	0.705
Monocyte subsets				
% Classical monocytes	−0.13 (0.20)	−0.51, 0.26	$\chi^2(1) = 0.42$	0.519
% Intermediate monocytes	0.35 (0.46)	−0.55, 1.25	$\chi^2(1) = 0.59$	0.444
% Non-classical monocytes	0.14 (0.30)	−0.44, 0.72	$\chi^2(1) = 0.23$	0.631
Soluble markers				
HCMV lysate IgG (AU)	2.4×10^{-5} (4.9×10^{-5})	-7.3×10^{-5}, 1.2×10^{-4}	$\chi^2(1) = 0.23$	0.631
HCMV gB IgG (AU)	2.7×10^{-5} (2.9×10^{-5})	-2.9×10^{-5}, 8.4×10^{-5}	$\chi^2(1) = 0.89$	0.345
CXCL10 (pg/mL)	2.7×10^{-3} (0.01)	−0.02, 0.03	$\chi^2(1) = 0.05$	0.828
sCD163 (ng/mL)	3.5×10^{-3} (2.2×10^{-3})	-8.7×10^{-4}, 7.9×10^{-3}	$\chi^2(1) = 2.47$	0.116

Regression coefficient (b), SE, 95% confidence intervals (95% CI), Wald test (Wald χ^2), and probability value (p-value).

[a]*Linear mixed modeling specifying a random intercept for study participant to account for the dependency associated with repeated measurements (i.e., all participant observations were used in analyses).*

AU, arbitrary units.

NK cells (36), but it is also involved in retention of lymphocytes in lymphoid tissue (37) and is highly expressed on tissue-resident NK cells (38), suggesting HIV infection may be associated with greater trafficking of NK cells between blood and tissues. Expression of the maturation marker CD57 on CD56^{dim} NK cells was also unaltered by cART in this cohort (data not shown), which is consistent with our previous findings (31).

To robustly quantify the effect of virologic suppression on NK cell dysfunction, we used mixed effects modeling to account for inherent differences in each individuals' immunological response to HIV infection and subsequent therapy. Importantly, unlike T lymphocyte and monocyte activation, which declined significantly following cART initiation, the expansion of FcRγ$^-$ NK cells in HIV$^+$ MSM was not affected by cART and appeared to represent a stable population present in the setting of HIV infection. Similarly, Brunetta et al. found that proportions of NKG2C$^+$ NK cells (a population analogous to the FcRγ$^-$ NK cells described here (3)) remained consistently elevated despite 2 years of cART, while HIV-associated changes to the ratio of NKG2A$^+$/NKG2C$^+$ NK cells were normalized during this period (27). Together, these data indicate an enduring effect of HIV infection on NK cell dysfunction in virologically suppressed HIV$^+$ individuals, which persists for at least 2 years following cART initiation and long after normalization of other immune parameters.

The persistence of activated HLA-DR$^+$/CD38$^+$ NK cells in cART-treated HIV$^+$ MSM demonstrated by our latent growth curve modeling unequivocally confirms observations from our cross-sectional studies, which found heightened NK cell activation (measured using phenotypic markers or spontaneous degranulation) in virologically suppressed HIV$^+$ individuals (2, 3). This implies NK cell activation is driven by factors other than HIV viremia, although it is still possible that NK cells are more sensitive to residual viral replication (<20 RNA copies/mL detected using validated clinical assays) than other leukocyte types. The association between NK cell and monocyte activation observed here suggests monocyte activation, potentially resulting from endotoxemia, may contribute to NK cell activation in viremic HIV infection. However, the persistence of NK cell activation but not monocyte activation in cART-treated individuals implies either that other factors maintain NK cell activation in virologically suppressed individuals or that NK cells are more sensitive indicators of persistent immune dysfunction. Interestingly, we did not observe an association between NK cell activation and HCMV antibody levels, although HCMV antibody levels alone are an imperfect metric of an individual's HCMV burden and the frequency and magnitude of reactivation events, particularly in HIV infection, as antibody levels in individuals with advanced HIV disease can initially rise following cART-initiation, and then subsequently fall (39, 40). Quantitation of the burden of HCMV remains problematic as the virus replicates in tissue cells, so viral DNA may not be detectable in blood. Thus, it remains possible that HCMV replication may contribute to persistent NK cell activation in cART-treated HIV infection.

In contrast to HLA-DR$^+$/CD38$^+$ NK cells, there was a slow but significant decrease in CD69$^+$ NK cells following cART initiation, consistent with CD69 being an early marker of cellular activation known to decline during the convalescent stage of viral infections such as Hantavirus (41). However, the rate at which activated CD69$^+$ NK cells declined on cART was significantly slower than that observed for T lymphocyte and monocyte activation, indicating NK cells are a cell population very sensitive to inflammatory or immune stimulatory factors that remain after antiretroviral therapy. Monitoring NK activation may therefore be a robust indicator of residual immune dysfunction in cART-treated individuals and could also be a useful biomarker for monitoring the efficacy of future functional cure strategies aimed

at supressing HIV viremia and related immune activation in the absence of cART.

In HIV-uninfected individuals, FcRγ$^-$ NK cells are generated in response to HCMV infection (22, 42). We have reported that the frequency of these cells correlates strongly with HCMV antibody levels in HIV$^-$ individuals recruited from the community (3), suggesting that in the general population, levels of HCMV infection and/or reactivation (to the extent indicated by antibody responses) are driving the expansion of NK cells with adaptive immune properties. In contrast, we have shown both here and previously (3) that HCMV antibody levels do not correlate with FcRγ$^-$ NK cell frequencies in HIV$^+$ MSM. It remains possible that HCMV infection is a prerequisite for the HIV-associated expansion of FcRγ$^-$ NK cells observed here, similar to the effect of HIV on NKG2C$^+$ NK cell expansion, which is only observed in HCMV$^+$ individuals (27). Furthermore, HCMV reactivation may in fact be a primary driver of FcRγ$^-$ NK cell expansion in HIV infection, but the abovementioned limitations with HCMV antibody levels as indicators of HCMV burden may preclude detection of this relationship. We have previously shown the phenotype of adaptive-like NK cells expanded in response to HCMV infection in renal transplant patients differs from those expanded in HIV infection (31, 43), implying that HIV-related factors may drive expansion of adaptive-like NK cells in addition to the effects of HCMV. The lack of an association between FcRγ$^-$ NK cells and immune activation found in this study suggests adaptive-like NK cell expansion is driven by mechanisms distinct from immune cell activation.

Adaptive-like NK populations are characterized phenotypically by reduced expression of the inhibitory receptor NKG2A and signaling molecules such as FcRγ, Syk, and Siglec-7 and increased expression of the activating receptor NKG2C (23, 32). FcRγ$^-$ NK cells lack the cytotoxic receptors NKp30 and NKp46 (3) and have poor cytotoxic activity against tumor targets (32); thus, the increased proportion of these NK cells may contribute to the increased prevalence of non-AIDS malignancies seen in HIV$^+$ individuals, although this requires formal investigation. As NKp46 is required for killing of HIV-infected CD4$^+$ T cells by natural cytotoxicity (44), the accumulation of NKp46$^-$ FcRγ$^-$ NK cells in HIV$^+$ individuals may impair NK-mediated clearance of HIV-infected T cells, although this may be counteracted by their enhanced ADCC activity. The enhanced ability of FcRγ$^-$ NK cells to produce inflammatory factors such as TNF and IFNγ following antibody stimulation (22, 32) may also perpetuate inflammation and immune activation in cART-treated individuals. Given that adaptive-like NK cells are expanded and persist in response to chronic viral infection it is reasonable to speculate that they play a role in protective immunity, although their contribution to this process *in vivo* remains to be determined. Consistent with this, NKG2C^{bright} (45) and FcRγ$^-$ (22) NK cells expanded in HCMV$^+$ individuals show heightened antibody-mediated degranulation, cytokine production, and ADCC against not only HCMV but also HSV-1 targets, implying a role in antibody-dependent cross-protection. However, HIV$^+$/HCMV$^+$ individuals have higher levels of HCMV antibodies than individuals infected with HCMV alone (31), implying poor HCMV control. It is plausible that abundant antibody and FcRγ$^-$ NK cells together compensate for poor protective T-cell responses in HIV$^+$ individuals. We found FcRγ$^-$ NK cells isolated from HIV$^+$ individuals have increased *ex vivo* ADCC activity when stimulated by HIV peptides in the presence of heterologous HIV$^+$ serum (3), but whether this translates to enhanced killing of HIV-infected cells *ex vivo* or *in vivo*, and whether this affects HIV reservoirs, is an important question that warrants investigation.

This study presents unique longitudinal data examining HIV-related immune activation specifically in MSM by comparison to matched HIV$^-$ MSM controls. The concentration of the HIV epidemic in MSM populations in many developed countries including Australia (46) means that MSM are overrepresented in clinical HIV studies conducted in these settings, but MSM-related factors are rarely considered as potential confounders. Our finding of increased proportions of FcRγ$^-$ NK cells and elevated HCMV antibody levels in HIV-uninfected MSM as compared to community controls underscores the importance of using appropriately matched, MSM controls to study immunological changes in HIV$^+$ MSM.

This study has a number of limitations, including a relatively small sample size, although this cohort size was chosen since, with 20 participants, the study provides a minimum number of level-two units to reliably estimate fixed model parameters in longitudinal mixed modeling (47–49). Other limitations include the absence of female participants, the use of an exclusively MSM cohort, and a follow-up of only 2 years. Follow-up of the cohort is ongoing and future analysis of later post-cART time-points will be critical for determining whether periods of cART >2 years are able to mitigate FcRγ$^-$ NK cell expansion. This study has however highlighted a significant and enduring effect of chronic, virologically suppressed HIV infection on the activation and imprinting of NK cells. Identification of the mechanisms responsible for the creation and maintenance of the expanded adaptive-like NK cell population in HIV$^+$ individuals, and the clinical consequences of their expansion, will inform adjunct immunotherapies to adequately address persistent immune dysfunction in cART-treated HIV infection.

ETHICS STATEMENT

This study was approved by the Alfred Hospital Research and Ethics Committee and carried out in accordance with their recommendations. All subjects gave written informed consent in accordance with the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

AH, JZ, SB, MC, and TA generated experimental data; AH, PA, MG, PC, PP, JE, and AJ contributed to study design and interpretation of the data; and AH, PA, and AJ analyzed the data and prepared the manuscript (with approval from all authors).

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SUPPLEMENTARY MATERIAL

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Monocytes Phenotype and Cytokine Production in Human Immunodeficiency Virus-1 Infected Patients Receiving a Modified Vaccinia Ankara-Based HIV-1 Vaccine: Relationship to CD300 Molecules Expression

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A modified vaccinia Ankara-based HIV-1 vaccine clade B (MVA-B) has been tested for safety and immunogenicity in low-risk human immunodeficiency virus (HIV)-uninfected individuals and as a therapeutic vaccine in HIV-1-infected individuals on combined antiretroviral therapy (cART). As a therapeutic vaccine, MVA-B was safe and broadly immunogenic; however, patients still showed a viral rebound upon treatment interruption. Monocytes are an important part of the viral reservoir and several studies suggest that they are partly responsible for the chronic inflammation observed in cART-treated HIV-infected people. The CD300 family of receptors has an important role in several diseases, including viral infections. Monocytes express CD300a, c, e, and f molecules and lipopolysaccharide (LPS) and other stimuli regulate their expression. However, the expression and function of CD300 receptors on monocytes in HIV infection is still unknown. In this work, we investigated for the first time the expression of CD300 molecules and the cytokine production in response to LPS on monocytes from HIV-1-infected patients before and after vaccination with MVA-B. Our results showed that CD300 receptors expression on monocytes from HIV-1-infected patients correlates with markers of HIV infection progression and immune inflammation. Specifically, we observed a positive correlation between the expression of CD300e and CD300f receptors on monocytes with the number of CD4+ T cells of HIV-1-infected patients before vaccination. We also saw a positive correlation between the expression of the inhibitory receptor CD300f and the expression of CD163 on monocytes from HIV-1-infected individuals before and after vaccination. In addition, monocytes exhibited a higher cytokine production in response to LPS after vaccination, almost at the same levels of monocytes from healthy donors.

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Furthermore, we also described a correlation in the expression of CD300e and CD300f receptors with TNF-α production in response to LPS, only in monocytes of HIV-1-infected patients before vaccination. Altogether, our results describe the impact of HIV-1 and of the MVA-B vaccine in cytokine production and monocytes phenotype.

Keywords: human immunodeficiency virus, monocytes, CD300, CD300c, CD300f, therapeutic vaccine, lipopolysaccharide, HIV-1 vaccine

INTRODUCTION

The development of combined antiretroviral therapy (cART) has significantly improved the clinical outcome in human immunodeficiency virus (HIV)-infected patients. However, long-term cART poses considerable side effects and costs, and stopping the treatment generally causes rapid viral rebounds, mostly due to the latent viral reservoirs (1, 2). For this reason, several strategies are being studied in order to achieve a permanent control of HIV replication inducing an effective antiviral T cell response. Among the most immunogenic approaches for inducing HIV-specific CD8+ T cell responses have been poxvirus vector boost vaccines (3, 4). Recently, a modified vaccinia Ankara vector expressing HIV-1 antigens clade B (MVA-B) was tested as a therapeutic vaccine. MVA-B was first tested with healthy volunteers (RISVAC02), which demonstrated that this vaccine was safe, well tolerated (5) and induced polyfunctional and durable T cell responses in most individuals (6). Importantly, it has also been tested as a therapeutic vaccine in a phase-I clinical trial in HIV-1-infected individuals on cART (RISVAC03), and the vaccination with MVA-B vaccine was also safe and broadly immunogenic. Nevertheless, HIV-1-infected patients still showed a viral rebound upon treatment interruption, and vaccination did not affect the viral reservoir even in combination with disulfiram, a drug able to reactivate latent HIV-1 (7, 8). The viral rebound after removal of cART has been linked to the fact that vaccination with MVA-B tips the balance between activation and regulation toward regulation of the response of HIV-specific CD8+ T cells (9). Nevertheless, in order to design more effective therapeutic vaccines, more studies are required to completely understand the effects on the host of the MVA-B vaccination.

Although latently infected CD4+ T cells comprise the majority of the HIV reservoir, monocytes (mainly CD16+ monocytes) provide an important part of this reservoir and also perpetuate HIV replication through ongoing cell-to-cell transfer of virions and efficient infection of CD4+ T cells, even in the presence of cART (10). In addition, recent studies suggest that monocytes are also responsible for the chronic inflammation in cART-treated HIV-infected people (11). In fact, it has been described that monocytes of chronically HIV-infected subjects differ from monocytes of healthy people in subsets distribution (12), expression of different markers (e.g., CD163) (13), and cytokine production (e.g., IL-6) (11). All these findings emphasize the importance of studying the mechanisms that regulate the activation of monocytes in HIV-infected patients.

The human CD300 molecules (a, b, c, d, e, f, g, h) are type I transmembrane proteins that, with the exception of CD300g which is expressed on endothelial cells, are found in both lymphoid and myeloid cell lineages. CD300a and CD300f are inhibitory receptors while CD300b, CD300c, CD300d, CD300e, and CD300h are activating receptors (14–16). Inhibitory receptors contain a long cytoplasmic tail with immunoreceptor tyrosine-based inhibitory motifs (ITIMs) which are required for the inhibitory signaling. Activating receptors have a short cytoplasmic tail with a charged transmembrane amino acidic residue, that allows their association with adaptor proteins containing immunoreceptor tyrosine-based activating motifs and other activating motifs which induce activation signals (14, 16). CD300 molecules have an important role in several diseases, including viral infections (14, 16, 17). In the context of HIV infection, there are few publications describing the role of CD300 family. In HIV-infected patients, the expression of the CD300a inhibitory receptor is down-regulated on B lymphocytes, which may help to explain the hyperactivation and dysfunction of B cells observed in these individuals (18). Another important detail about CD300a involvement in the pathogenesis of HIV infection is given by the description of a positive correlation between mRNA levels of CD300a and the expression of BATF, a transcription factor that inhibit T cell function, in HIV-specific CD8+ T cells (19).

At least, monocytes express four members of this family: the CD300a and CD300f inhibitory receptors, and the CD300c and CD300e activating receptors. Among others, age and lipopolysaccharide (LPS) regulate the expression of these receptors (14, 16, 20). However, in HIV infection, the expression and function of CD300 receptors on monocytes is still unknown. In this work, we have analyzed the expression of CD300 molecules on monocytes from chronically HIV-1-infected patients and calculated the correlation with markers of HIV-1 infection progression (CD4+ T cell count) and immune inflammation (CD163 expression). Moreover, we investigated the effect of the vaccination with MVA-B in the cytokine production of monocytes stimulated with LPS in HIV-infected subjects and we studied the correlation with the CD300 family of molecules expression. Our results may contribute to a better knowledge of monocytes dysfunction in HIV-1 infection and the influence of the MVA-B therapeutic vaccine in these cells.

PATIENTS AND METHODS

Patients and Samples

Samples were obtained from HIV-1-infected patients enrolled in the RISVAC03 clinical trial (NCT01571466) (8). RISVAC03

is a double-blinded randomized phase-I trial in which cART-treated HIV-1-infected individuals received four intramuscular injections of MVA-B vaccine at weeks 0, 4, 16, and 36, combined with disulfiram for 3 months after the last dose of the vaccine. Specifically, in this study we have analyzed available frozen peripheral blood mononuclear cells (PBMCs) from eight HIV-1-infected patients before (week 0) and after last vaccination (week 48). Clinical data of HIV-1-infected patients are shown in **Table 1**. Frozen PBMCs from seven healthy donors (HD) available from the phase-I trial RISVAC02 (NCT00679497) (5) were also studied. Only cells from non-vaccinated healthy individuals were analyzed. The means of the percentages of viable cells after thawing were: 69.4 ± 4.55% (HD), 70.0 ± 3.33% (HIV-infected patients before vaccination), and 67.3 ± 3.59% (HIV-infected patients after vaccination). This study was approved by the Research Ethics Committee of Hospital Clìnic, Barcelona, Hospital Germans Trias i Pujol, Badalona and Hospital Gregorio Marañón, Madrid, Spain. All subjects that participated in RISVAC02 and RISVAC03 clinical trials provided written and signed informed consent (5, 8).

Flow Cytometry Analysis

The following anti-human fluorochrome conjugated antibodies were used for flow cytometric analysis: PE-Cy7 mouse anti-CD14 (clone MφP9), PerCP-Cy5.5 mouse anti-HLA-DR (clone G46-6), PE mouse anti-IL-1α (clone 364-3B3-14), and FITC rat anti-IL-6 (clone MQ2-13A5) from BD Biosciences; FITC mouse anti-CD16 (clone B73.1), BV421 mouse anti-CD163 (clone GHI/61), and APC mouse anti-TNFα (clone Mab11) from Biolegend; PE mouse anti-CD300a (clone E59.126) from Beckman Coulter; eFluor660 mouse anti-CD300c (clone TX45) from eBioscience; and APC mouse anti-CD300e (clone UP-H2) and PE mouse anti-CD300f (clone UP-D2) from Miltenyi Biotec. To test the viability of the cells, the 633–635 nm excitation LIVE/DEAD Fixable Near-IR Dead Cell Stain Kit (Life Technologies) was used. Frozen PBMCs from HD and HIV-1-patients were thawed, washed, and incubated at 37°C for 1–2 h in R10 (10% FBS and 1% Penicillin/Streptavidin in RPMI-1640 medium) medium with 10U of DNase (Sigma-Aldrich), in a concentration of 2×10^6 cells/ml. Afterward, cells were stained first with the LIVE/DEAD kit in order to detect dead cells, and then, they were incubated with different fluorochrome conjugated antibodies. Both steps were carried out for 30 min on ice protected from the light. PBMCs were fixed with 4% of paraformaldehyde (Sigma-Aldrich) for 15 min at 4°C and washed two times with PBS. A FACSCanto II flow cytometer (BD Biosciences) was used for sample acquisition and data was analyzed with FlowJo 10.0.7 software (TreeStar).

LPS Stimulation and Intracellular Cytokine Staining (ICS)

Peripheral blood mononuclear cells from HD and HIV-1-infected patients were cultured (10^6 cells/ml) in R10 medium with 1 ng/ml of LPS (Sigma) for 5 h at 37°C, in the presence of GolgiStop protein transport inhibitor containing monensin, following manufacturer's indications (BD Biosciences). After the stimulation, PBMCs were stained with LIVE/DEAD kit, followed by incubation with different fluorochrome conjugated antibodies for extracellular staining. In order to accomplish the ICS, cells were first permeabilized with Cytofix/Cytoperm Plus Kit following the manufacturer's protocol (BD Biosciences) and then they were incubated with different fluorochrome conjugated antibodies for the detection of cytokines. Sample acquisition and data analysis were carried out as described before.

Data Representation and Statistical Analysis

GraphPad Prism software (version 6.01) was used for graphical representation and statistical analysis. Data were represented in dot plot graphs and bar graphs showing the mean with SEM, and pie chart graphs. Values obtained from different subject groups were compared with non-parametric tests; the comparison between HD and HIV-1-infected patients' data was made with the unpaired Mann–Whitney test; and differences between HIV-1-infected patients before and after vaccination were evaluated with the Wilcoxon matched-pairs signed rank test. Correlation analyses were done using the same software. In the case of cytokine production data, percentages of poly-functional, mono-functional, and non-functional cells were obtained by a Boolean gate analysis with FlowJo software and the representation of these data were done using GraphPad Prism software.

TABLE 1 | Clinical data of HIV-1-infected patients.

Patient	Undetectable VL (years)	CD4+ T cells Nadir (cells/mm^3)	CD4+ T cells before ART (cells/mm^3)	CD4+ T cells baseline (cells/mm^3)	Age	Sex	Weight (kg)	Coinfection hepatitis C virus	Time of known HIV infection (years)
101	9	179	368	541	49	M	74	No	14
103	1	290	489	530	50	M	69	No	10
107	2	274	274	866	41	M	68	No	12
108	2	396	396	823	33	M	73	No	12
109	4	645	688	1,179	39	M	65	No	6
110	12	376	376	1,238	40	F	56	No	15
111	3	296	396	632	44	M	78	No	6
112	2	507	680	794	39	M	60	No	3

VL, viral load; ART, antiretroviral therapy; HIV, human immunodeficiency virus.

RESULTS

CD300 Receptors Expression on Monocytes from HIV-1-Infected Patients Correlates with Markers of HIV Infection Progression and Immune Inflammation

We first determined the expression of CD300a, CD300c, CD300e, and CD300f molecules on monocytes from HD and chronically HIV-1-infected subjects that are receiving cART at baseline, i.e., just before starting the RISVAC03 clinical trial. Monocytes were electronically gated based on their forward and side scatter properties, and the expression of CD14 and CD16; concretely, classical (CD14++ CD16−), intermediate (CD14++ CD16+), and non-classical (CD14+ CD16++) monocytes were analyzed (Figure S1A in Supplementary Material). As it has been described before (10, 12), the percentages of intermediate and non-classical monocytes were slightly increased in HIV-1-infected patients in comparison with HD (Figure S2 in Supplementary Material). The expression of four members of the CD300 receptor family was tested: the inhibitory receptors CD300a and CD300f, and the activating receptors CD300e and CD300c. We did not observed significant differences in the expression of CD300 receptors on monocytes of HIV-1-infected patients compared with HD (**Figure 1A**), not even when we separately analyzed each monocyte subpopulation (Figure S3 in Supplementary Material). In spite of that, we observed a tendency, although not statistically significant, of CD300c expression to decrease on monocytes of HIV-1-infected subjects [HD median fluorescence intensity (MFI) = 2,717 ± 630.4 vs HIV MFI = 1,596 ± 465.5] (**Figure 1A**), especially in non-classical monocytes (data not shown).

Next, we investigated the association between CD300 receptors expression and patients' clinical features. Clinical data, which consists mainly of CD4+ T cell numbers, are shown in **Table 1**. CD300a and CD300c receptor expression on monocytes did not correlate with the number of CD4+ T cells at baseline (data not shown); however, the expression of CD300e ($p < 0.05$, $r = 0.7820$) and CD300f ($p < 0.05$, $r = 0.7592$) receptors was positively correlated with the CD4+ T cell numbers (**Figure 1B**).

Afterward, the expression of the CD163 receptor was analyzed and calculated the correlation with CD300 molecules expression in monocyte subpopulations. CD163 is a scavenger receptor, expressed exclusively on monocytes and macrophages, that has been investigated as a potential inflammation marker in different infectious diseases (13). In fact, sCD163 plasma levels are elevated in chronically HIV-1-infected patients and this has been related to a higher risk of comorbid disorders (11). We saw that CD163 expression of classical (HD MFI = 1,025 ± 106.7 vs HIV MFI = 1,744 ± 243.8) and intermediate (HD MFI = 1,079 ± 175.3 vs HIV MFI = 1,200 ± 158.2) monocytes was higher in HIV-1-infected subjects than in HD; unlike non-classical monocytes, which exhibited a very low expression in both groups (**Figure 1C**). Correlation analysis showed that in monocytes of HD, CD163 and CD300 receptors expression were not associated (data not shown). In contrast, there was a positive correlation between CD163 and CD300c expression ($p < 0.05$, $r = 0.7234$) on monocytes of HIV-1-infected subjects, and also between CD163 and CD300f expression ($p < 0.01$, $r = 0.9559$) in intermediate monocytes of HIV-1-patients (**Figure 1D**).

Effects of MVA-B Vaccination on Monocytes from HIV-Infected Subjects

The safety and immunogenicity of the MVA-B vaccine in chronically HIV-1-infected patients and healthy people has been previously tested (6–8). This vaccine improves the magnitude of HIV-specific T cell responses (6, 7), although it does also tilt the balance between activation and regulation of T cell specific responses toward regulation (9), somehow explaining the viral rebound after removal of cART in patients that has received the vaccine. However, the effects of vaccination in other immune cells have not been studied. Considering that monocytes play an important role in chronic inflammation characteristic of HIV-1-infected subjects (11), we studied the phenotype and cytokine production of monocytes in HIV-1-infected patients after vaccination with MVA-B and we compared them with monocytes from the same patients before vaccination.

First, the expression of CD163 and CD300 surface receptors was determined in HIV-1-infected patients before and after the vaccination with MVA-B. The percentages of monocyte subpopulations in vaccinated HIV-1-infected individuals were very similar to the percentages found before the vaccination (Figure S2 in Supplementary Material). The expression of CD300 molecules was determined and we observed that the expression pattern in monocytes of HIV-1-infected patients before and after vaccination was almost identical (**Figure 2A**, left panel). CD163 expression on monocytes was not significantly different when compared before and after vaccination. However, on intermediate monocytes (HIV before vaccination MFI = 1,103 ± 153.4 vs HIV after vaccination MFI = 793.6 ± 173.8), CD163 tended, although not statistically significant, to be down-regulated in patients after vaccination, while in classical and non-classical monocytes CD163 expression was very similar before and after vaccination (**Figure 2A**, middle panel). Lastly, we analyzed the correlation between the expression of CD300 receptors and CD163 receptor, and no significant values were observed in any case, except for a positive correlation between the levels of CD300f and CD163 ($p < 0.05$, $r = 0.9275$) on intermediate monocytes, as it was found before vaccination (**Figure 2A**, right panel).

Afterward, PBMCs from HD and HIV-1-infected patients, before and after vaccination, were stimulated with 1 ng/ml of LPS for 5 h, followed by ICS in order to study IL-6, IL-1α, and TNFα production in monocytes. These were gated according to their forward and side scatter properties, and they were defined as CD14++ HLA−DR+. In our hands, monocyte subpopulations were not distinguished due to the down-regulation of CD16 receptor after LPS stimulation (data not shown). Positive cells for each cytokine were determined based on non-stimulated cells. First, we checked the level of cytokine production by the stimulated cells by MFI of cytokine staining, a value known to be correlated with the amount of cytokine produced by cells (21). We observed that monocytes from HIV-1-infected subjects produced less IL-6 and TNFα than monocytes from HD in response

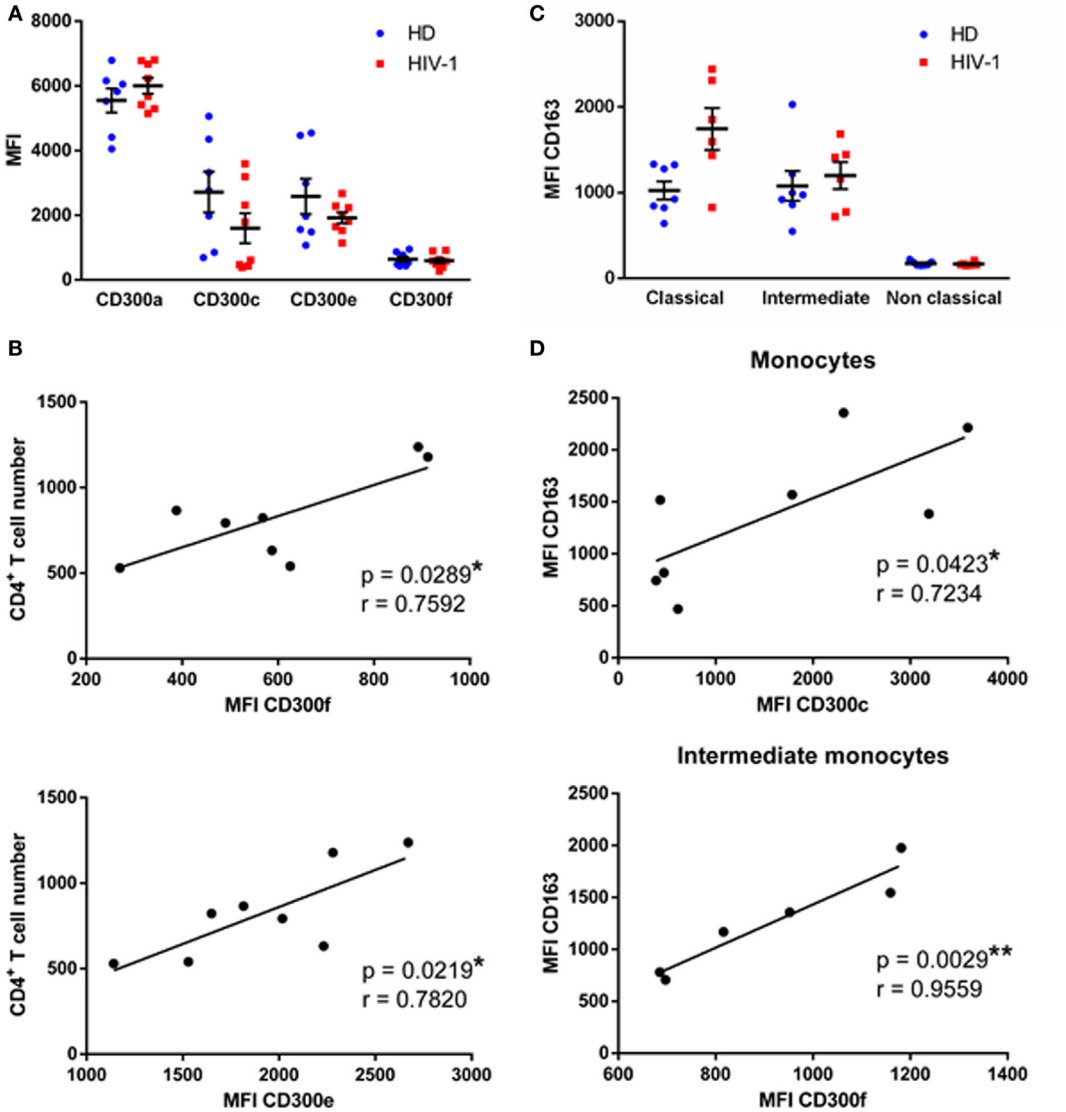

FIGURE 1 | CD300 receptors expression in human immunodeficiency virus (HIV)-1-infected patients. **(A)** Dot plot graph presenting the median fluorescence intensity (MFI) of CD300a, CD300c, CD300e, and CD300f receptors expression on monocytes from healthy donors (HD) and HIV-1-infected patients. Each dot corresponds to an individual and the mean with the standard error of the mean (SEM) is shown. **(B)** Correlation between CD4+ T cell number at baseline of the study and the MFI of CD300f and CD300e receptors expression on monocytes from HIV-1-infected patients is represented; the linear regression is shown. **(C)** Dot plot graph representing the MFI of CD163 receptor expression on classical, intermediate, and non-classical monocytes from HD and HIV-1-infected individuals. Each dot corresponds to an individual and the mean with SEM is shown. **(D)** Correlation between the MFI of CD163 and CD300c receptors expression on total monocytes and CD163 and CD300f receptors expression on intermediate monocytes from HIV-1-infected patients; the linear regression is shown. $^{*}p < 0.05$, $^{**}p < 0.01$.

to LPS. Interestingly, monocytes of vaccinated HIV-1-infected patients produced higher levels of IL-6, IL-1α, and TNFα in response to LPS after vaccination. Although IL-6 levels in vaccinated patients remained lower than in HD, TNFα production in vaccinated subjects reached the same levels as those from HD (**Figure 2B**). Moreover, analysis showed that the percentage of triple positive (IL-6+IL-1α+TNFα+) monocytes in response to LPS was higher in vaccinated HIV-1-infected subjects compared with the percentage of triple positive monocytes from the same patients before vaccination. On the other hand, the percentage of only double positive (IL-6-IL-1α+TNFα+) monocytes was higher in patients before the vaccination. These results indicate that monocytes of HIV-1-infected subjects were more polyfunctional in response to LPS stimulation after vaccination than before vaccination. As expected, although differences were not significant, probably due to the small sample, it was observed a higher percentage of non-cytokine (IL-6−IL-1α−TNFα−) producing monocytes from patients before vaccination than in monocytes after vaccination and from HD (HD = 7.63% vs HIV no vaccinated = 9.23% vs HIV vaccinated = 6.45%) (**Figure 2C**).

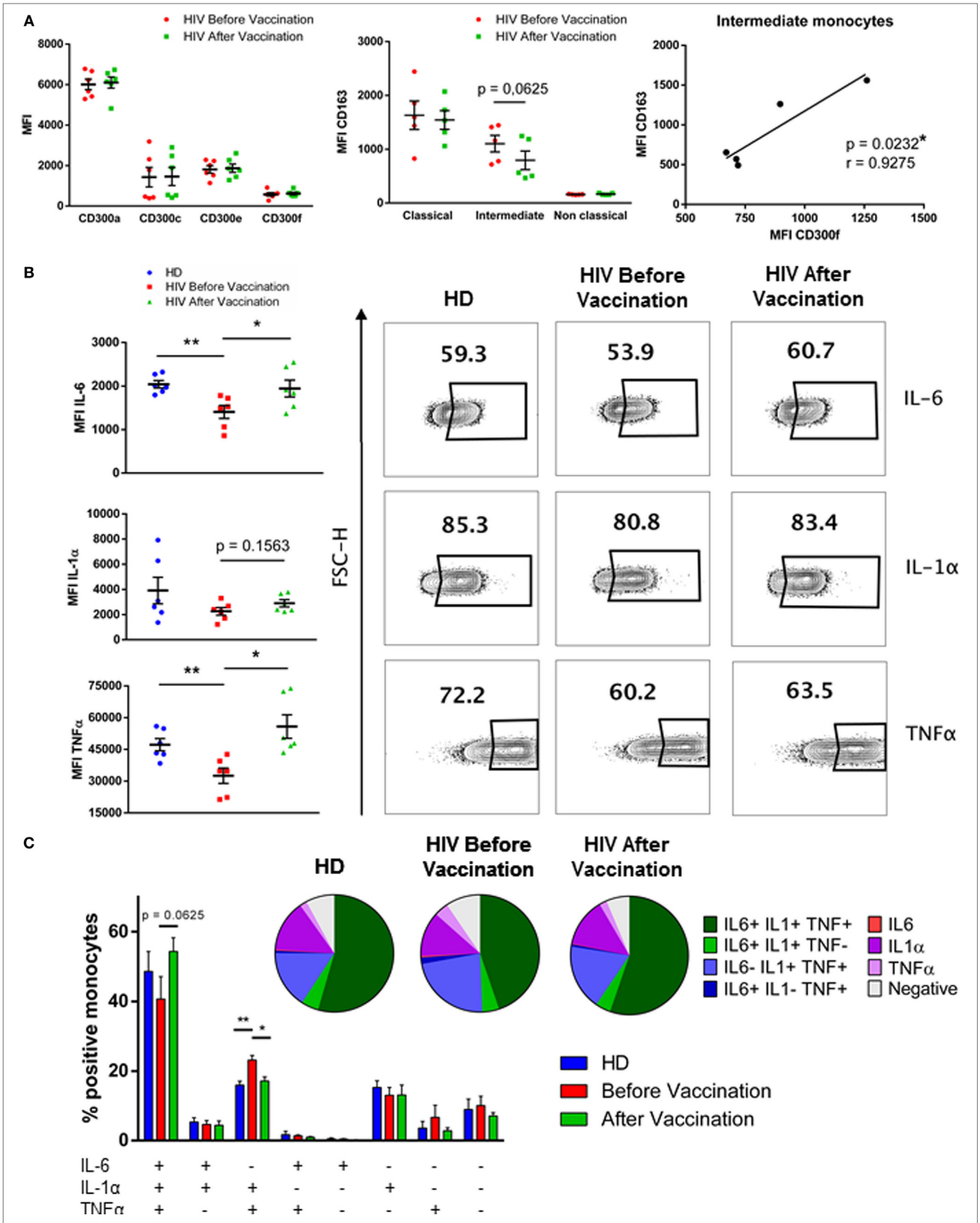

FIGURE 2 | Continued

FIGURE 2 | Continued
Phenotypical analysis and cytokine production of monocytes from HIV-1-infected patients after vaccination with MVA-B vaccine. **(A)** Dot plot graph (left panel) displaying the median fluorescence intensity (MFI) of CD300a, CD300c, CD300e, and CD300f receptors expression on monocytes from HIV-1-infected patients before (HIV before vaccination) and after (HIV after vaccination) vaccination. Each dot corresponds to an individual and the mean with SEM is shown. Dot plot graph (middle panel) representing the MFI of CD163 receptor expression on classical, intermediate, and non-classical monocytes from HIV-1-infected individuals before and after vaccination. Each dot corresponds to an individual and the mean with SEM is shown. The correlation between the MFI of CD163 receptor and the MFI of CD300f on intermediate monocytes of HIV-1-infected patients is represented (right panel); the linear regression is shown. **(B)** Dot plot graphs showing the MFI of positive monocytes for each cytokine; the mean with SEM is represented (left). Contour plots representing the percentage of positive monocytes for each cytokine after stimulation with lipopolysaccharide. Data from a representative healthy donor (HD) and an HIV-1-infected patient before and after vaccination are shown (right). **(C)** Boolean gate analysis representing the percentages of monocytes producing IL-6, IL-1α, and TNFα, in HD and HIV-1-infected patients before and after vaccination. Bar graphs showing the mean with SEM and pie charts are represented. $^{*}p < 0.05$, $^{**}p < 0.01$.

In conclusion, monocyte cytokine production in response to LPS in HIV-1-infected patients was higher after vaccination and resembled that observed in HD.

Relationship between CD300 Receptors Expression and Cytokine Production by Monocytes of HIV-1-Infected Patients Before and After Vaccination

The last step of the work was to investigate if the expression levels of CD300 molecules could have a correlation with the increased functionality found after the MVA-B vaccination in monocytes of HIV-1-infected individuals. We performed correlation analysis between CD300 receptors expression and cytokine production in response to LPS. The expression of CD300 molecules was not correlated with the percentage of IL-6+ monocytes in any case. In contrast, the expression of CD300e and CD300f correlated with IL-1α and TNFα production. The correlation with IL-1α production was only observed in monocytes from HD (data not shown); however, the expression of CD300e ($p < 0.05$, $r = 0.7505$) and CD300f ($p < 0.01$, $r = 0.8873$) was positively correlated with TNFα production in monocytes of HIV-1-infected patients before vaccination (**Figure 3B**). The percentages of TNFα+ monocytes of HD and vaccinated patients were not correlated with the MFI of CD300e and CD300f (**Figures 3A,C**). In fact, as it can be observed in the graphical representation (**Figure 3**), monocytes from HIV-1-infected patients are more similar to those from HD than to the monocytes from the same patients before vaccination. Taking altogether, we could propose that the monocyte phenotype and functional pattern in response to LPS stimulation of HIV-1-infected patients after vaccination with MVA-B are more similar to those found in monocytes from HD than from monocytes from HIV-1-infected subjects before vaccination.

DISCUSSION

Monocytes have been described as one of the cell types involved in the chronic inflammation characteristic of cART-treated HIV-1-infected people, which is currently the cause of death of the majority of HIV-1-patients (11). High numbers of circulating intermediate and non-classical monocytes have been associated with inflammation and immune activation during HIV infection (10). Furthermore, inflammatory mediators (e.g., IL-6) secreted by monocytes predict serious non-AIDS events in virologically suppressed HIV-infected subjects (11). Three main mechanisms have been proposed to explain the monocyte activation and consequently, the inflammation found in cART-treated HIV-infected patients: the microbial translocation, which augments LPS levels in plasma, the residual HIV viremia, and coinfection with human cytomegalovirus or some herpesviruses (11).

Since the CD300 family of receptors are able to modulate monocytes function (20, 22–24), our first objective was to investigate the CD300 receptors expression in monocytes from cART-treated chronically HIV-1-infected patients. Our results revealed that the expression pattern of CD300 molecules in monocytes from HD and in monocytes from HIV-1-infected people were not significantly different. However, we observed that the expression of CD300c tended, although not statistically significant, to be down-regulated in monocytes from HIV-1-infected patients, in comparison with monocytes from HD. This could be explained in part with the increase of the percentage of non-classical monocytes in HIV-1-infected patients, which express lower levels of CD300c than classical monocytes (Figure S3 in Supplementary Material) (20). It is important to keep in mind that many immunological abnormalities observed during the course of HIV infection can be reversed by cART, and therefore it is possible that the expression of CD300 molecules is altered in non-cART-treated patients with detectable viremia. More studies with blood samples from viremic patients are needed to obtain a more complete picture on the expression of the CD300 molecules during HIV infection. We did found a significant correlation between the expression of the activating receptor CD300e and the inhibitory receptor CD300f in monocytes with CD4+ T cell count in patients whose viremia is controlled by undergoing cART. These results may suggest that the levels of expression of CD300e and CD300f on monocytes could potentially be used as biomarkers of disease progression in combination with the well know predictive value of CD4+ T cell count (25, 26). Prospective studies with larger cohorts will confirm the predictive value of CD300e and CD300f expression on monocytes from HIV-infected patients.

We have not seen a significant increase in the expression of CD163 on monocytes from HIV-infected patients compared with monocytes from HD. Somehow, our results are different from those reported by others (13). We believe that this discrepancy is due to the low number of patients we have studied, since it is possible to observe a tendency, although not statistically significant, to increase CD163 cell surface expression on monocytes from HIV-infected individuals. Interestingly, there was a positive correlation between the expression of CD300f and CD163 in intermediate monocytes, a subset with a significant role in inflammation (27).

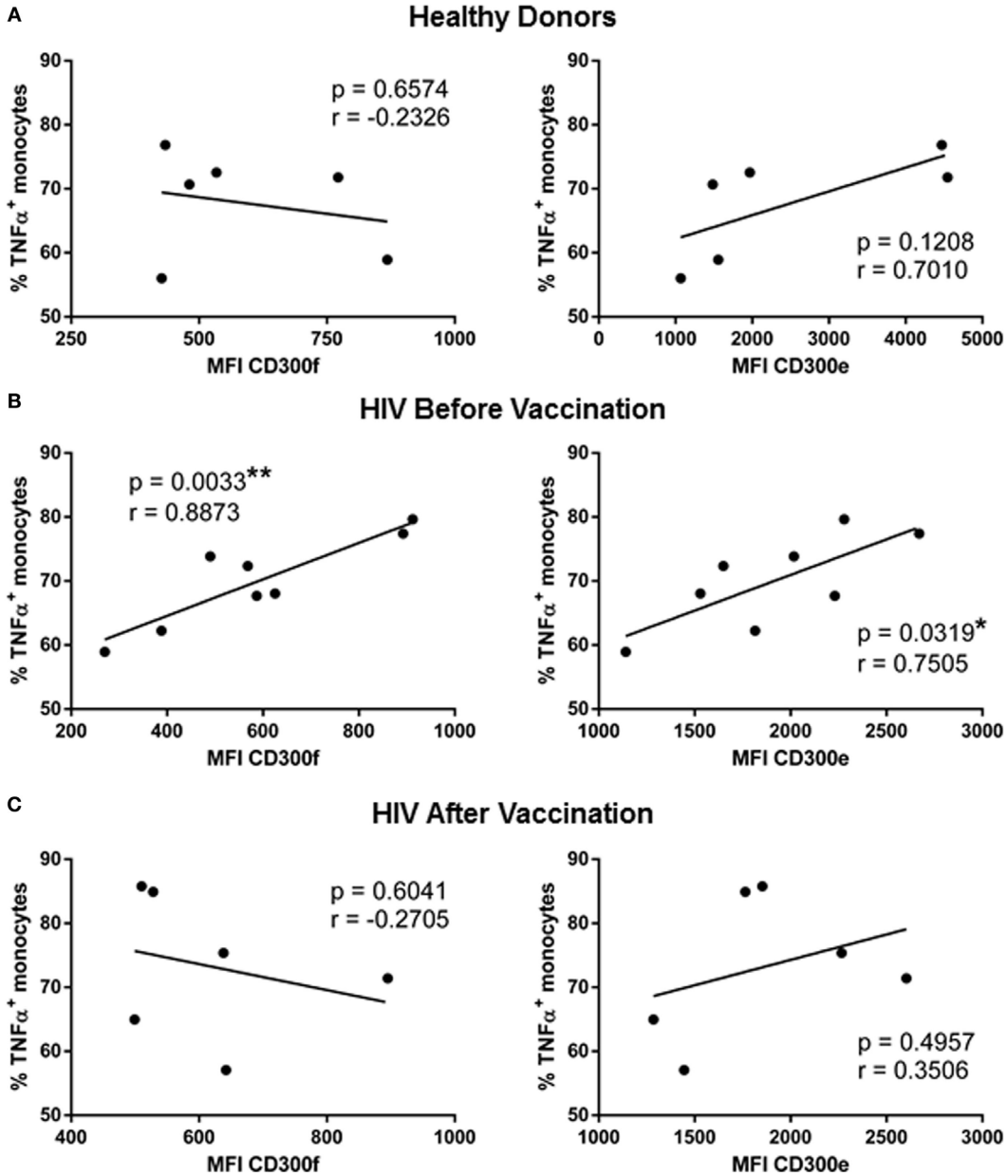

FIGURE 3 | Correlation analysis of TNFα production with the expression of CD300 receptors in human immunodeficiency virus (HIV)-1-infected patients before and after vaccination with MVA-B. Representation of the correlation between the percentage of TNFα positive monocytes and the median fluorescence intensity of CD300f and CD300e receptors expression, in healthy donors **(A)** and HIV-1-infected patients before **(B)** and after **(C)** vaccination with MVA-B; the linear regression is shown in each graph. *$p < 0.05$, **$p < 0.01$.

The positive correlation between the expression of CD300f and CD163 was maintained after vaccination. These results also suggest that the expression of CD300f, along with other markers, could be used as a biomarker of inflammation in HIV-infected patients. Human and mouse CD300f is commonly considered an inhibitory receptor because of the presence of ITIMs motifs in its intracellular tail (14). Several publications have shown its inhibitory role on monocyte cell lines (28–30). However, it has also been demonstrated that CD300f is able to deliver activating signals through motifs reported to bind the p85α regulatory subunit of PI3K (YxxM) (31–33). *In vivo* models in mice have shown that CD300f both inhibits and promotes the development of autoimmune diseases and allergic and inflammatory responses (34–39). This dual role of CD300f somehow may depend, not only on the cell type this intriguing receptor is expressed, but also on its described association with other receptors and adaptor proteins (33, 38, 40, 41). It would be of great interest to determine the signaling pathways of CD300f on monocytes during HIV infection, and determine if this receptor has different roles in monocytes from HD and HIV-1-infected patients.

Several therapeutic vaccines have been tested with the objective of controlling viral replication and to avoid viral rebound after treatment interruption in chronically HIV-1-infected patients (42, 43). MVA-B is an immunogenic vaccine which induces a T cell response in HIV-1-infected patients (7, 8). As expected, we did not observe any significant differences in the expression of CD300 molecules in monocytes of HIV-1-infected patients before and after vaccination. The most intriguing finding of this study was that the response of monocytes to LPS stimulation from patients after vaccination was different from the response before the vaccination, and at the same time similar to the response of monocytes from HD. Monocytes from non-vaccinated HIV-1-infected patients produced less cytokines in response to LPS than HD. This is in agreement with previous findings showing that HIV impairs TNFα production by human macrophages in response to Toll-like receptor 4 stimulation (44). Furthermore, this lower production of cytokines could also be due to the fact that monocytes when are chronically stimulated *in vivo* during chronic HIV infection become refractory to further stimulation with LPS *in vitro* (45), and it has been published that ART-treated infected patients exhibit higher levels of LPS in plasma than HD (46).

Vaccination with MVA-B induced higher levels of IL-6, IL-1α, and TNFα by monocytes in response to LPS. In fact, monocytes of vaccinated subjects exhibited a functional pattern more similar to the one of HD than to non-vaccinated HIV-1-infected patients. Furthermore, when we investigated if the expression of CD300 receptors might be correlated with the cytokine production levels, we also observed that the results were comparable between HD and HIV-1-infected patients after vaccination, and not between patients before and after vaccination. For example, the expression of CD300e and CD300f was positively correlated with TNFα levels in monocytes of HIV-1-infected subjects before vaccination, but not after vaccination or in monocytes of HD. We do not know the causes of this increase in the production of pro-inflammatory cytokines by monocytes in response to LPS after vaccination and if our results have some role in the lack of efficacy of the MVA-B vaccine as shown by a viral rebound after treatment interruption. It is possible that tipping the balance between activation and regulation toward regulation of the response of HIV-specific CD8+ T cells is not the only factor responsible for the lack of efficacy of the MVA-B vaccine. On the one hand, and considering our results showing lower CD163 expression on monocytes after vaccination, it seems that the administration of MVA-B vaccines may favor a less inflammatory environment. However, on the other hand, monocytes after vaccination have the potential to produce higher levels of pro-inflammatory cytokines and therefore could help to explain the lack of efficacy of the vaccine due to higher inflammation (10, 47–49). Also, it is important to remember that these patients have received disulfiram along with the MVA-B vaccine. Although the effect of disulfiram in monocytes of HIV-1-infected patients is unknown, several publications suggest that this drug have a role in decreasing the production of inflammatory mediators by monocytes. For example, it has been described that this compound diminishes the number of inflammatory cells and TNFα levels in the aqueous humor, in rats with endotoxin-induced uveitis (50). Furthermore, diethyldithiocarbamate, the active compound produced *in vivo* from disulfiram, impairs the release of oxygen metabolites and prostaglandins of human monocytes, two major pathways related to inflammatory processes (51). Undoubtedly, further research is required to delineate the role of monocytes in the efficacy of therapeutic vaccines.

In conclusion, our results have shown that vaccination with MVA-B, in addition to induce a specific T cell response, has also an effect on monocytes phenotype and their ability to produce cytokines after stimulation with LPS. We acknowledge that the number of patients included in this study is low and that it is very possible that a higher number of patients will provide more robust results. Clearly, more studies would be required to determine if the MVA-B mediated effect on monocytes favors the efficacy of the vaccine, or by the contrary is counterproductive. However, we believe that the results obtained with this work may form the basis of future studies to determine the functionality and phenotype of monocytes from patients enrolled in clinical trials testing therapeutic vaccines.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of Ethical Committee of Hospital Clìnic, Barcelona, Hospital Germans Trias i Pujol, Badalona, and Hospital Gregorio Marañón, Madrid, Spain with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Ethics Committee of Hospital Clínic, Barcelona, Hospital Germans Trias i Pujol, Badalona, and Hospital Gregorio Marañón, Madrid, Spain.

AUTHOR CONTRIBUTIONS

JV designed the study, designed and performed experiments, analyzed, and interpreted the data, designed the figures, and wrote the manuscript. OZ performed experiments and interpreted the data. IT designed the figures. MP participated in the design of the study and interpreted the data. AG participated in analysis and interpretation of the data. LL recruited and followed the patients and was responsible of vaccinations and clinical monitoring. JP interpreted the data. FG recruited and followed the patients and was responsible of vaccinations and clinical monitoring. FB conceived and designed the study, interpreted the data, and wrote the manuscript. All the authors critically reviewed, edited, and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

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REVIEW
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Natural Killer (NK) Cell Education Differentially Influences HIV Antibody-Dependent NK Cell Activation and Antibody-Dependent Cellular Cytotoxicity

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Immunotherapy using broadly neutralizing antibodies (bNAbs) endowed with Fc-mediated effector functions has been shown to be critical for protecting or controlling viral replication in animal models. In human, the RV144 Thai trial was the first trial to demonstrate a significant protection against HIV infection following vaccination. Analysis of the correlates of immune protection in this trial identified an association between the presence of antibody-dependent cellular cytotoxicity (ADCC) mediated by immunoglobulin G (IgG) antibodies (Abs) to HIV envelope (Env) V1/V2 loop structures and protection from infection, provided IgA Abs with competing specificity were not present. Systems serology analyses implicated a broader range of Ab-dependent functions in protection from HIV infection, including but not limited to ADCC and Ab-dependent NK cell activation (ADNKA) for secretion of IFN-γ and CCL4 and expression of the degranulation marker CD107a. The existence of such correlations in the absence of bNAbs in the RV144 trial suggest that NK cells could be instrumental in protecting against HIV infection by limiting viral spread through Fc-mediated functions such as ADCC and the production of antiviral cytokines/chemokines. Beside the engagement of FcγRIIIa or CD16 by the Fc portion of anti-Env IgG1 and IgG3 Abs, natural killer (NK) cells are also able to directly kill infected cells and produce cytokines/chemokines in an Ab-independent manner. Responsiveness of NK cells depends on the integration of activating and inhibitory signals through NK receptors, which is determined by a process during their development known as education. NK cell education requires the engagement of inhibitory NK receptors by their human leukocyte antigen ligands to establish tolerance to self while allowing NK cells to respond to self cells altered by virus infection, transformation, stress, and to allogeneic cells. Here, we review recent findings regarding the impact of inter-individual differences in NK cell education on Ab-dependent functions such as ADCC and ADNKA, including what is known about the HIV Env epitope specificity of ADCC competent Abs and the conformation of HIV Env on target cells used for ADCC assays.

Keywords: natural killer cells, antibody-dependent cellular cytotoxicity, antibody-dependent natural killer cell activation, natural killer cell education, CD16, inhibitory natural killer cell receptors, non-neutralizing antibodies, broadly neutralizing antibodies

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INTRODUCTION

There is great interest in developing an effective vaccine against HIV infection. It is generally acknowledged that inducing broadly neutralizing antibodies (bNAbs) would be a desirable goal for prophylactic HIV vaccines. The most potent bNAbs have been shown to protect against virus infection or to suppress viral replication in humanized mouse models and in rhesus macaques (1–6). Clinical trials conducted in HIV-infected humans, using the bNAbs VRC01 and 3BNC117, reduced HIV viral load by up to 2.5 logs (7, 8) and delayed viral rebound after antiretroviral therapy (ART) interruption (9, 10). However, there are still significant challenges to inducing such antibodies (Abs) through vaccination. BNAbs are rarely elicited in natural HIV infection and many exhibit high levels of affinity maturation (11–15). Despite this, progress has been made producing bNAbs in animal models using sequential cycles of boosting with defined immunogens (16). It is interesting to note that bNAbs able to protect humanized mice or rhesus macaques against challenge with HIV or simian/human immunodeficiency virus (SHIV) require an Fc region able to interact with Fc receptors (FcRs) on innate immune cells (17–19). One of these FcRs, FcγRIIIa, also known as CD16, is found on natural killer (NK) cells, macrophages, and monocyte subsets (20–22).

The HIV vaccine tested in the RV144 Thai trial is the only vaccine to date that conferred modest (approximately 31%) but significant protection against HIV infection (23). Protection was not associated with the presence of bNAbs or cytotoxic T cell responses (24). Rather, protection from HIV infection in trial participants was associated with the presence of anti-HIV envelope (Env) specific immunoglobulin G (IgG) non-neutralizing Abs (nNAbs) able to mediate Ab-dependent cellular cytotoxicity (ADCC) provided no potentially competitive IgA Abs were present (24–27). Follow-up analyses using systems serology approaches confirmed findings from correlation analyses and identified links between anti-Env V1/V2-specific IgG, IgG3, and IgG1, and Ab-dependent functions such as ADCC, Ab-dependent cellular phagocytosis, Ab-dependent complement deposition, and Ab-dependent NK cell activation (ADNKA) for secretion of IFN-γ and CCL4, and expression of CD107a in recipients of the RV144 vaccine (28). This raised the possibility that anti-HIV Env-specific nNAbs able to mediate ADCC and ADNKA activity may play a protective role against HIV infection.

Natural killer cells can be activated through Ab-dependent pathways that involve CD16 engagement by the Fc region of IgG1 and IgG3 Abs (29–35). They can also be activated by Ab-independent missing self recognition mechanisms based on how they were educated during development. Activating NK cells by either mechanism leads to secretion of chemokines and cytokines and to the release of cytotoxic granules that lyse target cells. ADNKA is the term used to describe the activation of NK cells for chemokine/cytokine secretion and degranulation by Ab-dependent stimuli. ADCC, on the other hand, denotes the lysis of target cells by NK cells in the presence of an Ab bridge. In the literature, these two activities have often been incorrectly referred to as ADCC. NK cells are important effector cells for these two Ab-dependent functions. Here, we will review recent findings on Ab-dependent functions mediated by NK cells and explore what is known regarding the influence of NK cell education on ADNKA and ADCC.

NK CELL EDUCATION

Tolerance to self and the state of activation of NK cells is determined by an ontogenic process termed education. NK cell education requires the interaction of inhibitory NK receptors (iNKRs) with their cognate human leukocyte antigen (HLA) ligands on neighboring cells (36, 37). The education of NK cells determines how these cells will respond to infected, transformed, stressed, and allogeneic cells in an Ab-independent fashion. Education is a complex process whereby functionality is tuned by the number of iNKRs engaged, the strength of interactions between iNKRs and their ligands, and whether activating NK cell receptors are also engaged (38–44). NK cells lacking iNKRs for self-HLA ligands remain uneducated and hyporesponsive (45). iNKRs involved in NK cell education include NKG2A and the killer immunoglobulin-like receptors (KIR)3DL1, KIR2DL1, KIR2DL2, and KIR2DL3 (see **Table 1**). NKG2A is a C-type lectin receptor that forms a heterodimer with CD94 (46, 47). It interacts with non-classical major histocompatibility complex class I (MHC-I) HLA-E antigens presenting 9-mer peptides cleaved from the leader sequence of several MHC-I proteins (48, 49). Both NKG2A and HLA-E have limited sequence variability and their effects on NK cell education were initially reported to be similar from one person to another (50). The inhibitory KIRs (iKIRs) recognize subsets of HLA antigens together with peptides (51). KIR3DL1 interacts with a subset of HLA-A and -B antigens belonging to the HLA-Bw4 (Bw4) group (52–54). Bw4 antigens differ from the remaining HLA-Bw6 (Bw6) HLA-B variants at amino acids 77–83 of the HLA heavy chain (55). Bw6 isoforms do not interact with KIR3DL1 receptors such that KIR3DL1$^+$ NK cells from individuals carrying no *Bw4* alleles are not educated through this receptor. KIR2DL3 and KIR2DL2 are encoded at the same locus and interact with HLA-C group 1 (C1) variants that have an asparagine at position 80 of the HLA heavy chain (56–58). The remaining HLA-C variants, belonging to the C2 group, have a lysine at this position and are ligands for KIR2DL1 (56). The KIR2DL3 receptor can also bind certain C2 variants, though with a lower affinity than either KIR2DL1 or KIR2DL2 (57, 59, 60). Therefore, KIR2DL3$^+$ NK cells from individuals expressing a C1 ligand are educated, but remain uneducated or modestly educated through this receptor in individuals who are negative for C1 ligands. By contrast, KIR2DL1$^+$ NK cells require the expression of a C2 ligand for education.

Genome-wide association studies (GWAS) confirm that genes influencing HIV viral load set point map to the *MHC-I* region on chromosome 6 (61, 62). MHC-I antigens encoded in this region form complexes with peptides, which are recognized by the T cell receptors on CD8$^+$ T cells (63). It is well established that CD8$^+$ T cells play an important role in HIV viral control (64–66). However, NKG2A and iKIR on NK cells also recognize MHC-I peptide complexes (48, 49, 52, 53, 56). Both epidemiological and functional studies have implicated iKIRs, particularly KIR3DL1, in combination with certain Bw4 variants in protection from HIV

TABLE 1 | Inhibitory natural killer (NK) cell receptors involved in NK cell education.

Receptor	Ligand	aa at position 80 of the human leukocyte antigen (HLA) heavy chain	Effect on education when ligand is present	Ligand levels in HIV-infected cells	Reference
NKG2A	HLA-E + leader peptide from HLA-A, -B, -C, and -G		Enhanced	Maintained	(48, 49)
Killer immunoglobulin-like receptors (KIR)3DL1	HLA-B*Bw4, HLA-A*23, *24, and *32	Isoleucine (*80I) or threonine (*80T)	Enhanced	Downmodulated	(52–54)
KIR2DL1	HLA-C2	Lysine	Enhanced	Maintained or downmodulated depending on HIV isolate	(56–60)
KIR2DL2	HLA-C1 (some HLA-C2)	Asparagine	Enhanced	Maintained or downmodulated depending on HIV isolate	(56–60)
KIR2DL3	HLA-C1 (some HLA-C2)	Asparagine	Enhanced	Maintained or downmodulated depending on HIV isolate	(56–60)

infection and slow disease progression in those already infected (67, 68). For example, individuals who are homozygous for *KIR3DL1 *h/*y* genotypes and co-carry *HLA-B*57* (**h/*y + B*57*) progress to AIDS more slowly and control HIV viral load better than *Bw6* hmz (67). *KIR3DL1 *h/*y* genotypes encode receptors expressed at high levels (69) while HLA-B*57 is a Bw4 variant that is also expressed on the cell surface at a high density and is a potent ligand for KIR3DL1 (44). The effect of this KIR/HLA combination on NK cell education is illustrated by the observation that KIR3DL1$^+$ NK cells from **h/*y + B*57* carriers, compared to those from *Bw6* hmz, have a superior functional potential upon stimulation with HLA null cells and inhibit HIV replication more potently in autologous-infected CD4$^+$ T cells through mechanisms that involve secretion of CC-chemokines (41, 70, 71). An upstream region of HLA-C that plays a role in determining HLA-C expression levels was also associated with HIV control in individuals of European American origin in GWAS studies (61, 62). While the mechanism underlying this association is related to HLA-C expression levels and the potency of CD8$^+$ T cell recognition of HLA-C-HIV peptide complexes, the potential involvement of NK cells has not been excluded (72).

A dimorphism at position −21 in the leader peptide of HLA-B antigens influences the delivery of peptides to either an NKG2A or iKIR focused NK cell response (73). The amino acid at this position corresponds to the HLA leader peptide's position 2, which is an anchor residue for HLA-E binding. A minority of HLA-B and all HLA-A and HLA-C antigens have a methionine at position −21 (−21M) of the leader sequence. −21M containing 9-mer peptides form stable complexes with HLA-E that are recognized by NKG2A. It is notable that the haplotypes carrying the −21M *HLA-B* alleles rarely encode Bw4 or C2 isoforms that are KIR3DL1 and KIR2DL1 ligands, respectively (73). By contrast, 9-mer peptides that have a threonine at the −21 (−21T) residue present in most HLA-B antigens, form poor complexes with HLA-E. Consequently, this −21M/T dimorphism defines two types of HLA haplotypes. One haplotype group, encoding −21M variants, is biased toward providing ligands for NKG2A and other group, encoding −21T variants, preferentially provides ligands for iKIR. This dimorphism appears to be clinically relevant in the context of HIV infection since the presence of −21M HLA-B antigens is associated with higher susceptibility to HIV infection in HIV-discordant couples and with poorer NK cell-mediated killing of HIV$^+$ cells than are −21T HLA-B antigens (74, 75). Together, these findings prompt a reconsideration of epidemiological and NK cell functional studies in the light of the contribution of NKG2A versus iKIR responses to the activation of NK cell populations expressing defined patterns of iNKR.

THE INFLUENCE OF NK CELL EDUCATION IN ADNKA

Antibody-dependent NK cell activation measures NK cell activation following incubation with Ab opsonized targets cells. Even though ADNKA depends on the presence of Ab, NK cell education can also influence NK cell activation through ADNKA. Many of the earlier reports describing a role for NK cell education in ADNKA used the CEM.NKr.CCR5 (CEM) cell line coated with recombinant HIV Env gp120 as target cells (76). CEM cells express the CCR5 co-receptor for HIV entry and are resistant to direct NK cell killing (77–79). CEM cells are negative for Bw4 and C2 antigens but express C1 antigens (80).

A higher frequency of KIR3DL1$^+$, than KIR3DL1$^-$ NK cells, from carriers of *KIR3DL1/Bw4* genetic combinations secrete IFN-γ and express CD107a in responses to anti-HIV Ab opsonized gp120-coated CEM. This differential activation of KIR3DL1$^+$ and KIR3DL1$^-$ NK cell populations also occurs when the stimulus is HIV-infected or gp120-coated allogeneic primary CD4$^+$ T cells (76). As well, a higher frequency of KIR2DL1$^+$ than KIR2DL1$^-$ NK cells from carriers of educating *KIR2DL1/HLA-C2* combinations secrete IFN-γ in response to HIV-infected autologous targets and gp120-coated CEM cells in the presence of anti-HIV Env-specific Abs in plasma from HIV$^+$ individuals (81). By contrast, if NK cells are from carriers of the non-educating *KIR/HLA* pair *KIR2DL1/C1* hmz, KIR2DL1$^+$ and KIR2DL1$^-$ NK cells respond to anti-HIV Ab-dependent stimulation equivalently (81). These observations implicate NK cell education in NK cell responses to anti-HIV Ab opsonized gp120-coated CEM cells, infected allogeneic CEM cells, and gp120-coated primary CD4$^+$ T cells. CD16 engagement is also important in ADNKA activity as NK cell activation is always higher in the presence versus absence anti-HIV-specific Abs.

Gooneratne et al. have speculated that ADCC activity directed at allogeneic HIV-infected cells may play a role in protecting against infection with allogeneic HIV-infected cells. Secretion of CCL4 from activated NK cells can bind the CCR5 HIV co-receptor and block HIV entry into new target cells (82). Activated NK cells also secrete cytotoxic granules that can lyse HIV-infected target cells (83). It is plausible that ADCC activity directed at allogeneic HIV-infected cells contributed to the modest protection conferred by the RV144 HIV vaccine trial, in which ADCC competent anti-Env-specific Abs were generated and to the protection conferred to infants who remain uninfected despite exposure to breast milk from HIV-infected mothers (24, 84).

There is a lack of consensus regarding whether educated NK cell populations respond more robustly than their uneducated counterparts to stimulation with anti-HIV opsonized autologous gp120-coated cells. KIR3DL1$^+$ and KIR2DL1$^+$ NK cells from carriers of *KIR/HLA* combinations able to support education through these receptors have been reported to respond better that their uneducated counterparts to HIV Ab-dependent activation (81, 85). These findings are consistent with results reported by Lang et al. (86). These observations have been interpreted as evidence that Ab-dependent activation of NK cells can overcome inhibitory signals mediated by the interaction of HLA ligand binding to self iKIR. However, this is not a general finding in that others have noted that ligands on autologous target cells to iNKR on educated NK cells suppress the activity of educated NK cells compared to that of their uneducated counterparts (87, 88). Further research is needed to understand what accounts for these discrepant results.

The experiments describing ADNKA in this section have used an inclusive gating strategy to compare how NK cell populations expressing, or not, one iNKR respond to anti-HIV opsonized target cells. When NK cells are stained inclusively for the presence of a single iNKR, the targeted population includes NK cells expressing other iNKRs not stained for. These other iNKRs could influence NK cell responses to HIV Ab opsonized target cells depending on which iNKR/HLA receptor ligand pairs contributed to the education of the NK cells studied. By using an Ab panel detecting KIR3DL1, KIR2DL1, KIR2DL3, and NKG2A on CD3$^-$CD56^{dim} NK cells, it will be possible to focus on NK cell populations expressing one of these iNKR to the exclusion of the others. Such Ab panels that also detect multiple NK cell functions using Abs conjugated with different fluorochrome have been designed (89, 90). In future studies, these Ab panels should be used to exclusively gate on NK cell populations expressing single iNKRs that detect functions induced by anti-HIV Ab opsonized target cells. Such an experimental approach will allow for a more precise definition of NK cell responses within population expressing single educating receptors to activation through missing self recognition of the ligands for these iKIR on allogeneic CEM cells in addition to signals received *via* ligation of CD16 (91).

The frequency of NK cells responding to stimulation in ADNKA assays displays inter-individual variation. One possible mechanism underlying the range of NK cell effector responses in ADNKA assays is likely related to inter-individual differences in iNKR/HLA ligand effects on NK cell education. KIR3DL1 allotypes differ in their cell surface expression levels, with high, low, and null expression allotype groups (69, 92–95). These KIR3DL1 allotypes also differ in their affinity for particular HLA-B allotypes (44, 96). KIR2D receptors differ in their affinity for C1 and C2 antigens (57, 60). HLA-A, -B, and -C antigens also differ in their cell surface expression levels (44, 72, 97). Thus, these factors, the number of iNKR/HLA pairs participating in NK cell education in each study subject, and the presence of ligands on CEM cells that provide, or that fail to provide, inhibitory signals to NK cells may all influence NK cell activation levels in ADNKA assays. Several authors have tested expression levels for HLA-B and C allotypes and have examined the avidity of interactions of high and low expression KIR3DL1 receptor groups for HLA-B antigens with either an isoleucine or a threonine at position 80 of the HLA heavy chain (44, 69, 72, 98, 99). The putative influence of inter-personal immunogenetics on ADNKA activity could be explored by correlating ADNKA activation levels with KIR3DL1/HLA-B, KIR2DL1/HLA-C2, and KIR2DL3/HLA-C1 affinity and expression levels as has been described by Boudreau et al. (44). For ADNKA, activation through education-dependent missing self-recognition and CD16 signaling influence NK cell activation while for ADCC the effect of education-dependent missing self-recognition is minimized due to the low frequency of single positive (SP) iKIR+ NK cells positive for CD16. The comparison of assay results where one or more of these receptor ligand interactions is blocked may provide further insights into the role of signaling through iNKR or CD16 in ADNKA and ADCC.

MEASURING ADCC ACTIVITY

As opposed to ADNKA, ADCC measures target cell phenomena arising from the bridging of effector and target cells by an Ab whose Fc portion binds CD16 on effector cells and whose Fab portion recognizes an antigen on target cells. In the context of ADCC function-directed HIV Env gp120-coated target cells, the target antigens recognized by ADCC competent Abs are HIV Env (30, 78, 100). ADCC activity directed to HIV infected may also recognize Tat (100).

Early versions of anti-HIV ADCC assays measured ^{51}Chromium release from target cells (101–103). These have been replaced by flow cytometry-based assays using either CEM cells coated with gp120 or gp140, HIV-infected CEM or HIV-infected primary CD4$^+$ T cells as target cells. Primary HIV-infected target cells have included reactivated CD4$^+$ T cells from HIV-infected subjects or CD4$^+$ T cells infected with transmitted/founder (T/F) HIV isolates (104–106). The GranToxiLux ADCC (GTL-ADCC) assay measures the delivery of granzyme B (GzB) to target cells, an early step in the pathway leading to target cell lysis (83, 107, 108). In the GTL-ADCC assay, target cells are labeled with fluorescent and viability dyes before incubation with effector cells, either peripheral blood mononuclear cells (PBMCs) or NK cells in the presence of HIV-specific ADCC competent Abs and a GzB substrate. If ADCC is induced following incubation with HIV-specific Abs, effector cells will release GzB that will enter target cells and hydrolyze the GzB substrate, activating its fluorescence, which can be detected by flow cytometry. Thus, the GTL-ADCC assay provides an estimate of ADCC activity

by measuring the number of viable targets that are positive for proteolytically active GzB.

Read outs for ADCC assays include the loss of target cells loaded with a fluorescent marker, infected with green fluorescent protein-tagged HIV, luciferase tagged HIV, or Gag p24$^+$ cells (105, 106, 108–113). The lactate dehydrogenase (LDH) release ADCC assay measures the loss LDH from dying target cells by ELISA (76, 114). The widely used rapid and fluorometric ADCC has been shown to not measure ADCC but rather the uptake of the membrane dye PKH-26 used to label target cells by monocyte-mediated trogocytosis (115, 116).

THE SPECIFICITY OF ANTI-HIV ADCC COMPETENT Abs

Both bNAbs and nNAbs can mediate ADCC activity provided they can stably bind to target cells (105, 106, 113, 117–121). HIV Env epitopes targeted by nNAbs include the immunodominant region of gp41 (122) and CD4-induced (CD4i) epitopes exposed by CD4 ligation of HIV Env on infected cells (111, 123, 124). Examples of prototypic anti-Env-specific Abs specific for a CD4i epitope is A32, which belongs to the anti-cluster A Ab group targeting the C1/C2 region and 17b, which recognizes the co-receptor binding site (CoRBS) (119, 125). Other nNAbs have been reported to recognize the CD4bs and the V3 loop of gp120, which are also targeted by bNAbs, though the nNAbs bind these epitopes in a manner that does not prevent HIV entry (126–130). At least some of the epitopes targeted by ADCC competent nNAbs are poorly exposed on CD4 unliganded cell surface Env trimers. This is mainly due to accessory proteins Nef and Vpu that downregulate cell surface CD4 making CD4i epitopes unavailable for Ab recognition (111, 120, 131, 132). Bruel et al. found that CEM cells infected with two laboratory-adapted HIV strains bound Abs from several classes of bNAb and nNAbs epitope specificity. If binding occurred, these Abs usually also mediated ADCC activity against these infected cells (106). However, when reactivated, HIV-infected cells from the reservoir of ART-treated HIV$^+$ individuals or CD4$^+$ T cells infected with T/F strains were used as target cells, several monoclonal nNAbs bound a lower frequency of infected cells with a lower affinity than did bNAbs. Furthermore, nNAbs, compared to bNAbs, exhibited poor ADCC activity against targets infected with such primary HIV strains (105, 106, 113, 117, 133). This phenomenon is likely related to the inability of nNAbs to access epitopes in the closed unliganded conformation of HIV Env (134).

Non-neutralizing Abs, particularly those specific for CD4i epitopes, preferentially bind HIV-uninfected bystander cells present in cultures with HIV$^+$ CD4$^+$ T cells (106, 135, 136). HIV-infected CD4$^+$ T cells can shed HIV Env gp120 leaving behind gp41 stumps (136). The shed gp120 binds CD4 on the surface of uninfected bystander CD4$^+$ T cells. This interaction has the potential to open the closed Env conformation exposing CD4i epitopes, making bystander enhanced targets for CD4i-specific ADCC competent Abs.

Strategies to improve the targeting of the open Env conformation by ADCC competent nNAbs has prompted exploring the use of CD4 mimetics to increase the susceptibility of HIV-infected cells to ADCC (106, 135, 137, 138). Richard et al. worked with CD4 mimetics that were unable to enhance the recognition of HIV-infected cells to A32 Abs by themselves (138). However, these small molecules initiated the opening of Env trimers enough to permit the binding of Abs such as 17b with specificity for a conserved epitope overlapping the CoRBS. Once 17b bound, the trimeric Env structure opened sufficiently to allow binding of A32 and susceptibility to ADCC activity (138).

It should be noted that most studies measuring anti-HIV ADCC activity have used gp120- or gp140-coated CEM cells as targets. While such targets are easy to prepare and convenient to use, the HIV Env on coated cells is monomeric and differs quantitatively and conformationally from trimeric Env found on the surface of HIV-infected cells. On coated cells, CD4 remains on the target cell surface while it is downregulated on infected cells unless Nef and/or Vpu HIV deletion mutants are used for infection. This needs to be kept in mind when interpreting the results of studies using coated cells as targets.

THE INFLUENCE OF NK CELL EDUCATION ON ADCC ACTIVITY

The GTL-ADCC assay using gp120-coated CEM cells as targets was used to show that education of effector populations through KIR3DL1 had no significant effect on the percent of GzB$^+$ (%GzB$^+$) target cells generated in a GTL-ADCC assay (139). There may be several explanations for this observation. One possibility is that NK cells are not the main effector cell in the GTL-ADCC assay. A drawback of using PBMCs as effector cells in ADCC assays is that it is difficult to draw conclusions regarding which effector population is responsible for GzB delivery to the target cells. Several Fcγ receptor-expressing cell types, including NK cells, monocytes/macrophages, and γδ T cells, are capable of mediating ADCC (107, 115, 140–144). To confirm that NK cells are the source of ADCC activity in the GTL-ADCC assay, Pollara et al. used effector PBMCs depleted of CD56$^+$CD16$^+$ NK cells and observed that ADCC responses declined by over 66% (145, 146). Purified NK cells and PBMCs from the same donors produced similar %GzB$^+$ target cells (107). Together, these findings indicate that the GTL-ADCC assay is measuring NK cell-mediated ADCC responses.

In the GTL-ADCC assay, PBMC effector cells are a heterogeneous population that includes NK cells educated through 1, 2, or more iKIR and/or NKG2A. An Ab panel detecting KIR3DL1, KIR2DL1, KIR2DL3, and NKG2A on CD3$^-$CD56^{dim} NK cells was used to gate exclusively on SPiNKR$^+$ NK cells. NK cells SP for iKIR had significantly lower frequencies of CD16$^+$ cells than did SPNKG2A$^+$ or NKG2A$^-$iKIR$^-$ NK cells (147). iKIR$^+$ NK cells are educated if they develop in a setting in which the iKIR's ligand is co-expressed. The implication of this observation is that educated SPiKIR$^+$ NK cells would be poor ADCC effector cells as a median of <5% of them are CD16$^+$ (**Figure 1**). This could account for the lack of an effect of KIR3DL1-mediated NK cell education on the %GzB$^+$ target cells generated in the GTL-ADCC assay (139, 147). Thus, it would be expected that NKG2A$^+$ NK cells are superior

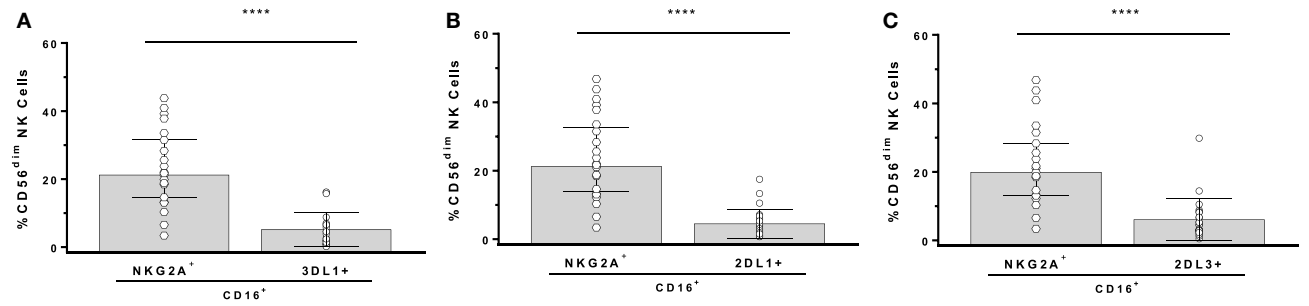

FIGURE 1 | Comparison of the frequency of CD16$^+$ cells among CD3$^-$CD56^{dim} natural killer (NK) cells stained for antibodies with NKG2A, KIR3DL1 (3DL1$^+$), KIR2DL1 (2DL1$^+$), and KIR2DL3 (2DL3$^+$). Comparison of single-positive (SP)NKG2A with SP3DL1 **(A)**, SPNKG2A with SP2DL1 **(B)**, and SPNKG2A with SP2DL3 **(C)**. Each point represents a single individual, bar height, and error bars represent median and interquartile range for the data set. Wilcoxon matched pairs tests were used to determine the significance of between group differences (****$p < 0.0001$).

to iKIR$^+$NKG2A$^-$ NK cells as effector cells in the GTL-ADCC assay. NKG2A/HLA-E interactions educate NKG2A$^+$ NK cells and these receptor ligand pairs are widely expressed with limited inter-individual variation. Their influence on NK cell education would have limited between-subject variation. If ADCC activity is an important correlate of protection against HIV, these findings suggest that inter-individual variation in NK effector cell education based on which iKIR/HLA receptor/ligand pairs are present would have a minimal impact on ADCC potency at the level of HIV-infected target cell lysis or suppression of replication. Together, these findings illustrate that the potency of NK cell education and functional activation of NK effector cells does not predict the %GzB$^+$ generated by ADCC.

In summary, factors important in determining ADNKA and ADCC activity differ from each other. The role of NK cell education in ADCC activity is limited by the low frequency of CD16$^+$ NK cells among SPiKIR$^+$ NK cells that have the potential to be educated through iKIR/HLA ligand interactions. Thus, a higher frequency of either uneducated NK cells or NK cells educated through NKG2A than those educated through iKIR are CD16$^+$ and able to mediate ADCC. On the other hand, both CD16 engagement and missing self-recognition contribute to ADNKA. The consequences of these findings for HIV vaccines is that NK cell education should contribute minimally to inter-individual differences in target cell lysis by ADCC. Furthermore, NK cell activation by Ab-dependent HIV-infected cell stimuli will vary depending on how NK cells are educated, the nature of the stimulatory cell and effect of HIV infection on cell surface MHC-I expression (90, 148).

CONCLUDING REMARKS

Arguing for a role for anti-HIV ADNKA and/or ADCC activity in protection from infection are the findings from the RV144 vaccine trial, which identified ADCC activity as a correlate of protection that was frequently linked to ADNKA activity (24, 27, 28). Moreover, antigenic drift from ADCC targeting Env epitopes has been documented, highlighting a role for ADCC being able to exert anti-HIV immune pressure (149). Of note, it is unlikely that bNAbs contributed to either of these findings as neither RV144 vaccinated individuals (24) nor most HIV$^+$ persons make HIV-specific bNAbs. Suppression of HIV viral load in HIV-infected persons receiving the bNAb 3BNC117 is likely not solely due to virus neutralization as this treatment also appears to clear infected cells (133). Also, the beneficial effect of treatment with several bNAbs depends on IgG Fc region effects (17–19). On the other hand, several attempts to show that nNAbs can protect against infection in rhesus macaques infected with SHIV have failed, though passive transfer of these Abs may have suppressed viremia or restricted the number of T/F viruses in some cases (122, 150–152). By contrast, the passive transfer of the most active bNAbs mediates sterilizing protection in primate models (1–6). The protective role of anti-HIV nNAbs and/or how to manipulate the ability of these Abs to protect from HIV infection or how to use them therapeutically is an active area of research with several questions left to answer.

AUTHOR CONTRIBUTIONS

Substantial contributions to the conception or design of the work (NB, ZK, AT-M, CL, SK, and FD). Drafting the work or revising it critically for important intellectual content (NB, ZK, AT-M, CL, SK, and FD). Final approval of the version to be published (NB, ZK, AT-M, CL, SK, and FD). Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved (NB, ZK, AT-M, CL, SK, and FD).

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REVIEW
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Are Evolution and the Intracellular Innate Immune System Key Determinants in HIV Transmission?

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HIV-1 is the single most important sexually transmitted disease in humans from a global health perspective. Among human lentiviruses, HIV-1 M group has uniquely achieved pandemic levels of human-to-human transmission. The requirement to transmit between hosts likely provides the strongest selective forces on a virus, as without transmission, there can be no new infections within a host population. Our perspective is that evolution of all of the virus–host interactions, which are inherited and perpetuated from host-to-host, must be consistent with transmission. For example, CXCR4 use, which often evolves late in infection, does not favor transmission and is therefore lost when a virus transmits to a new host. Thus, transmission inevitably influences all aspects of virus biology, including interactions with the innate immune system, and dictates the biological niche in which the virus exists in the host. A viable viral niche typically does not select features that disfavor transmission. The innate immune response represents a significant selective pressure during the transmission process. In fact, all viruses must antagonize and/or evade the mechanisms of the host innate and adaptive immune systems that they encounter. We believe that viewing host–virus interactions from a transmission perspective helps us understand the mechanistic details of antiviral immunity and viral escape. This is particularly true for the innate immune system, which typically acts from the very earliest stages of the host–virus interaction, and must be bypassed to achieve successful infection. With this in mind, here we review the innate sensing of HIV, the consequent downstream signaling cascades and the viral restriction that results. The centrality of these mechanisms to host defense is illustrated by the array of countermeasures that HIV deploys to escape them, despite the coding constraint of a 10 kb genome. We consider evasion strategies in detail, in particular the role of the HIV capsid and the viral accessory proteins highlighting important unanswered questions and discussing future perspectives.

Keywords: capsid, coevolution, HIV, innate immunity, restriction factors, sensing, transmission, Vpr

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INTRODUCTION

The primacy of transmission as a selective pressure favoring viral evasion of innate defenses is emphasized and reinforced by our understanding of the origins of HIV. The human lentiviruses HIV-1 and HIV-2 are zoonoses from simian ancestor viruses (1, 2). Antagonism of species-specific restriction factors likely determined the ability of the non-human primate viruses to cross into human hosts [reviewed in Ref. (3, 4)]. Indeed, innate effectors from both humans and non-human primates show

differential patterns of restriction for simian immunodeficiency viruses (SIVs) from divergent species, as well as for HIV-1 and HIV-2 [reviewed in Ref. (5, 6)]. SIV has been transmitted from apes to humans on at least four occasions, giving rise to the M, N, O, and P groups of viruses, but the distribution and incidence of these groups vary greatly and only HIV-1 M group is pandemic (7, 8).

In the case of HIV-1, crossing a mucosal surface during sexual transmission accounts for the vast majority of new infections. However, it is not clear whether the HIV-1 ancestral viruses, in chimpanzees and gorillas, or the HIV-2 parental viruses in Sooty Mangabeys (SIVsm), are sexually transmitted diseases (STDs), and it may be that HIV-1 M has uniquely adapted to be a highly effective STD. If, as we propose, the strongest evolutionary selective forces on a virus are applied during transmission then all conserved HIV-1–host interactions must favor sexual transmission across a mucosal surface. Importantly, we consider transmission to mean the events that lead to sustained infection in the new host and not, what we imagine are frequent, cases of viral replication after exposure, which do not lead to systemic viral dissemination and peak viremia. We expect this to be the window in which the innate immune response is particularly important in protecting the host. It is our view that there is a distinction between the forces driving viral evolution within a host, for example, usage of the co-receptor CXCR4 in 50% of all hosts, that do not favor transmission and are therefore do not become fixed from host-to-host, and those that do favor transmission, and are therefore inherited. We believe that viewing HIV pathogenesis and transmission from this evolutionary perspective is essential to fully understand the antagonistic interactions between HIV-1 and the intracellular innate immune system.

Evidence for a significant genetic bottleneck during sexual HIV-1 transmission comes from the low frequency of transmission per exposure (9). Furthermore, the identification of HIV-1 founder viruses reveals that sexual transmission is established by a surprisingly low number of transmitted viral sequences (10–12). In the case of heterosexual transmission, single founder clones are typically responsible for infection, whereas several clones are usually transmitted between men who have sex with men (MSM) (13). Larger numbers are observed in intravenous transmission by injecting drug users consistent with needle use bypassing protective barriers (14). A prominent feature of acute HIV-1 infection *in vivo* is a dramatic interferon (IFN) and pro-inflammatory cytokine response (15). The sensitivity of HIV-1 to the effects of IFNs is well-established *in vitro* (16, 17). Intriguingly, characterization of transmitted founder (T/F) clones has revealed that they are less sensitive to IFN as compared with viruses isolated during the chronic phase of infection (18–22). The molecular details of the IFN-induced restriction of HIV-1 are incompletely understood, and discussed later, but an important role for the interferon-induced transmembrane protein (IFITM) family during transmission has recently been proposed (20) and is reviewed in this issue. Together, these data show how IFN and the immune response can apply powerful selective pressures during mucosal transmission.

The primary cellular targets of HIV-1 infection during transmission remain unclear. Given their high frequency in mucosa and high permissivity to infection, macrophages are likely candidates, although recent work has revealed that T/F clones are particularly poorly tropic for macrophages (23). Transmission studies of SIVmac in rhesus monkeys have suggested that inflammatory responses lead to T-cell influx and early infection of activated CD4+ T cells [reviewed in Ref. (24)]. More recent work has implicated Th17 cells as the primary target of SIVmac during vaginal inoculation (25). However, we worry that studying mucosal transmission with an unnatural virus–host pair, such as SIVmac in rhesus monkeys, in which natural sexual transmission does not occur efficiently, might be misleading. Nonetheless, the tropism of T/F sequences for CD4+ T cells is good evidence for this cell type being among the earliest targets for infection (23). Dendritic cells (DCs) and Langerhans cells (LCs), both highly abundant in mucosal surfaces, have also been implicated as primary targets during transmission (26). However, these cells are unlikely to be productively infected by HIV-1 but can capture the virus *via* uptake dependent on C-type lectins, for example, DC-SIGN and Siglec-1 (27, 28). Subsequent migration of DC to lymph nodes is thought to promote infection of CD4+ T cells by transfer of the virus, in a process called trans-infection. Despite DC not being productively infected, it is thought that these cells, particularly plasmacytoid DC (pDC), generate the high levels of systemic type 1 IFNs and pro-inflammatory cytokines in the days immediately following HIV-1 infection (15, 29–33).

Despite the success of HIV-1 transmission, even the permissive host cell is a hostile environment for a virus. For example, the journey across the cytoplasm and into the nucleus is fraught with danger in the form of the cell-autonomous innate immune system. This intracellular immune arsenal entails a series of molecular tripwires that can mount an immediate response to invading pathogens if they are detected. Central to this defense system are pattern recognition receptors (PRRs): a diverse array of germline-encoded sensors that recognize pathogen-associated molecular patterns (PAMPs) and trigger a potent response to counteract infection, *via* activation of innate signaling pathways. This in turn induces the expression of a plethora of proteins with widespread antiviral functions that restrict infection at all stages of the viral lifecycle (**Figure 1**). For retroviruses such as HIV, the hazards of the cell-autonomous immune system are initially focused on the need to convert single-stranded RNA to double-stranded DNA between cell entry and integration: HIV must effectively smuggle a range of nucleic acid PAMPs past the host cell detection system. If HIV cannot negotiate these hazards it cannot replicate (**Figure 2**). The success story of HIV transmission therefore depends on its ability to antagonize or evade these host defenses. Every component of HIV can be defined by its individual role in the evolutionary arms race against human immunity: virus adaptation to host defenses is countered by evolution of the host cell proteins, and so on in cycles of counterevolution, described by the Red Queen Hypothesis (34), that are recorded in the genes of both organisms [reviewed in Ref. (35)].

In our view, all the host–virus interactions discussed in this review are driven by the selective forces at play during transmission. We invite the reader to consider all of the host–virus interactions we describe in the context of this perspective. Knowledge and understanding of the interactions between HIV-1 and the

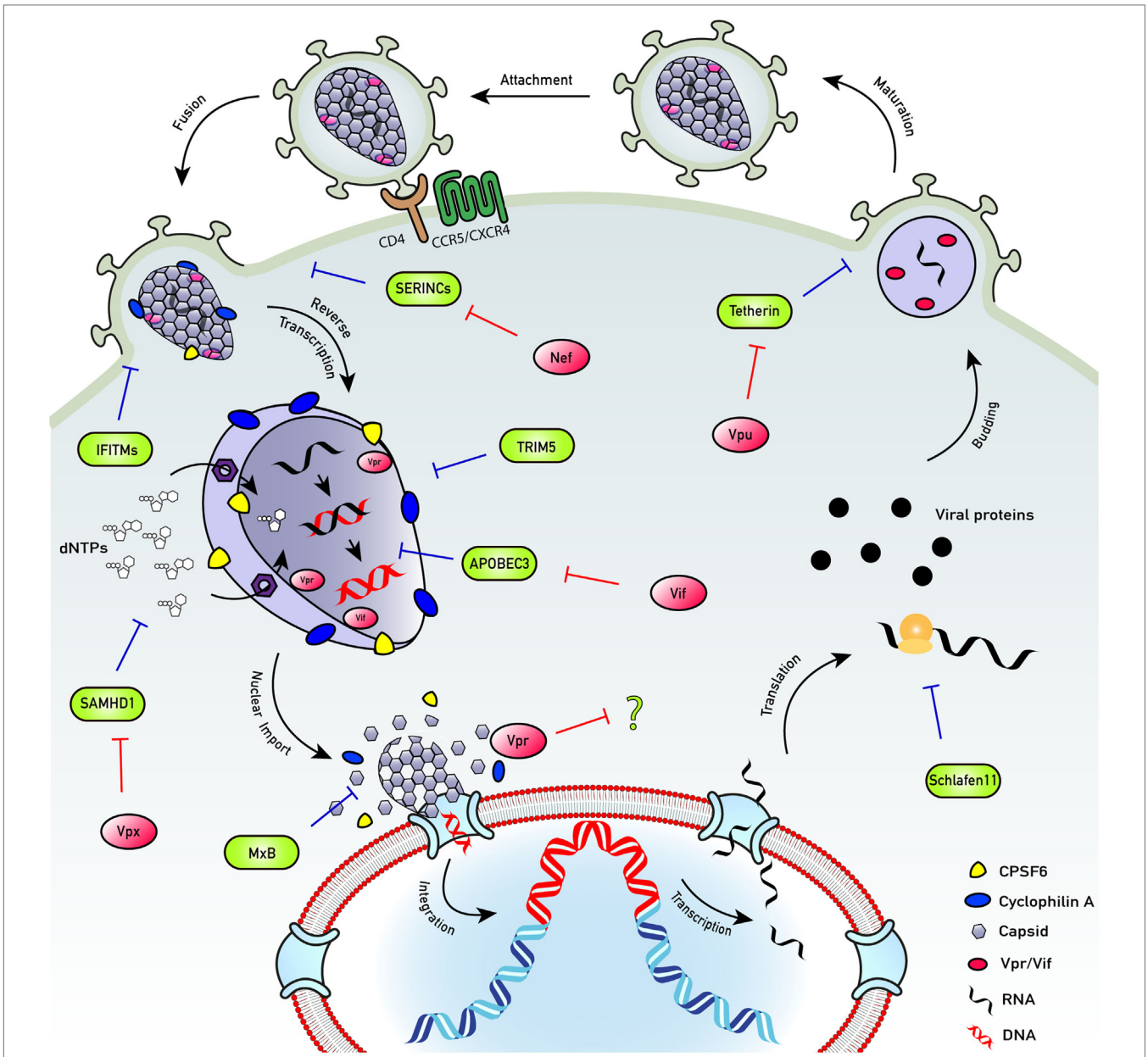

FIGURE 1 | HIV life cycle. The HIV life cycle comprises a complex series of immune evasion strategies that allow successful infection of host cells and transmission between them and between individuals. To enter cells, HIV engages its envelope glycoprotein gp160 trimers with cell surface protein CD4 and co-receptor (CXCR4 or CCR5). Co-receptor usage allows conformational masking of conserved binding domains of gp120 and avoids their exposure to neutralizing antibodies. Upon fusion, capsid is released into the hostile environment of the cell where it encounters numerous innate restriction factors. However, HIV employs several mechanisms to overcome the cellular assault. While the capsid traverses the hostile cytoplasm, nucleotides are transported into the capsid cone through an electrostatic nucleotide transporter to fuel reverse transcription. Encapsidated DNA synthesis shields the viral genome from DNA sensors as well as exonucleases, e.g., TREX1. Capsid recruits cellular proteins cyclophilin A (blue) and CPSF6 (yellow), which have a role in preventing detection of the viral reverse-transcribed DNA by DNA sensors, e.g., cyclic GMP–AMP synthase (cGAS). Uncoating of successfully infectious cores may happen late, at the nuclear pore complex, or in the nucleus, in an organized manner and the viral DNA is released. The viral DNA integrates close to the edge of the nucleus to perhaps prevent activation of DNA damage responses. Once integrated, the provirus is invisible to the host cell defenses and may become transcriptionally silent, or latent. Transcription and translation of the provirus result in viral protein expression. Viral assembly occurs at the cell surface. Immature virions bud off and are released. During maturation, the protease enzyme cleaves the structural polyprotein to form mature Gag proteins, resulting in the production of new infectious virions. SERINCs: prevent fusion of viral particles with target cells. Antagonized by Nef. IFITMs: impair virus entry into target cells. Antagonized by evolving IFITM3 insensitive Env proteins. TRIM5: forms a hexagonal lattice around the capsids. Targets them for proteasomal degradation and activates innate signaling. Antagonized by evolving TRIM5 insensitive viral capsid proteins. APOBEC3: suppresses viral DNA synthesis and induces mutations in the viral DNA. Antagonized by Vif-mediated degradation. SAMHD1: restricts infection by lowering nucleotide concentrations below those, which support viral DNA synthesis. Antagonized by Vpx-mediated degradation (SIVsm/HIV-2) or infection of inactive phospho-SAMHD1 positive cells (HIV-1). MxB: restricts HIV-1 nuclear entry and possibly integration. Schlafen 11: restricts HIV-1 protein translation. Tetherin: inhibits virus release from infected cells. Antagonized by Vpu-mediated degradation.

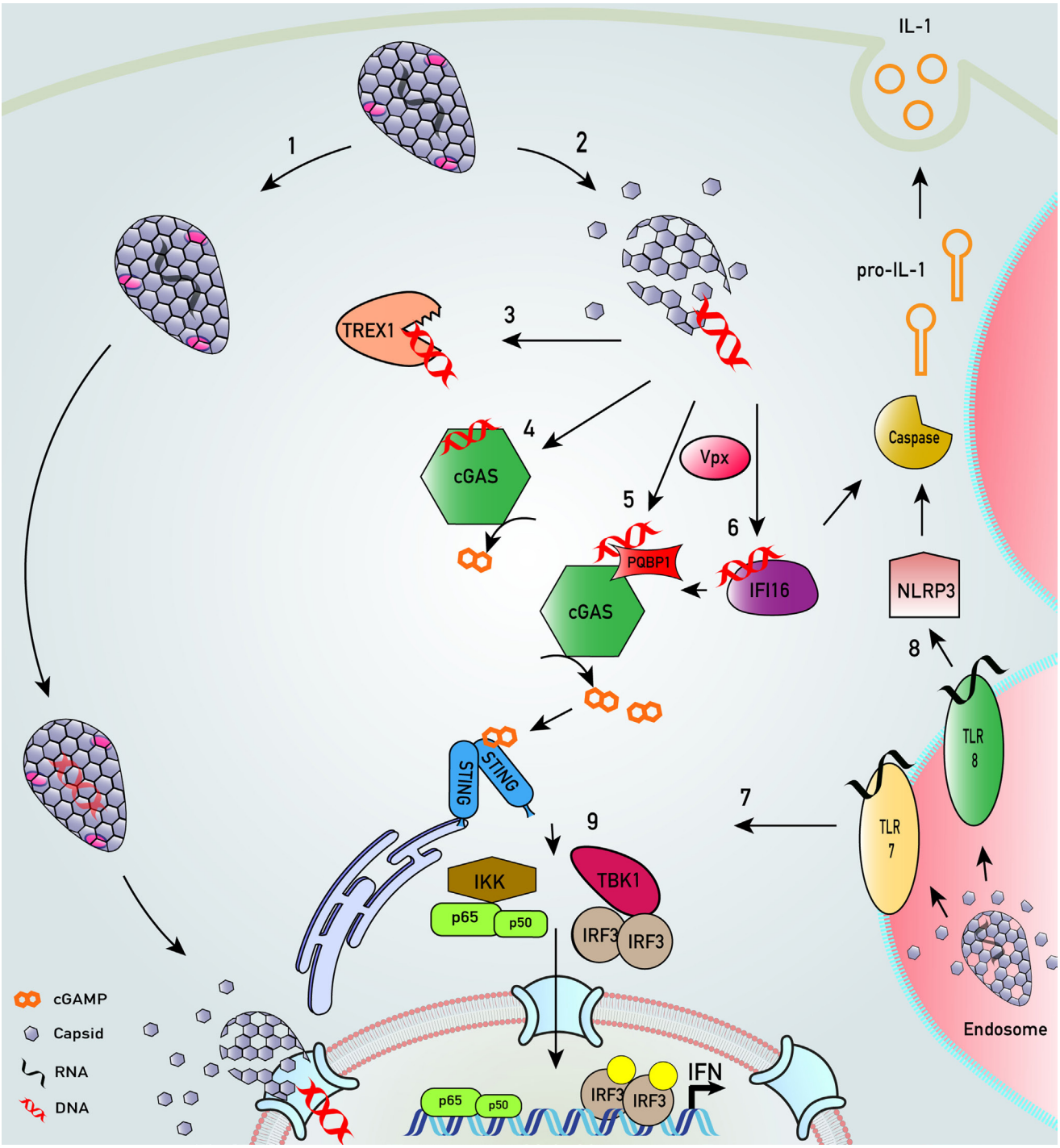

FIGURE 2 | Key innate sensing pathways activated by HIV-1 particles that do not establish productive infection. (1) HIV-1 disassembly may be stochastic. Some particles remain intact, perhaps through appropriate recruitment of cofactors. We envisage encapsidated DNA synthesis and uncoating in complex with the nuclear pore complex or even in the nucleus (33, 36–40). (2) Many particles disassemble, or are disassembled, by cellular defenses that are proteasome dependent (38, 41). (3) In macrophages and T cells, cytosolic exonuclease TREX1 digests escaped HIV-1 DNA that would otherwise trigger innate DNA sensing (42). (4) In TREX1-depleted cells, escaped HIV-1 DNA is sensed by DNA sensor cyclic GMP–AMP synthase (cGAS) (42, 43). (5) In monocyte-derived dendritic cell, after SAMHD1 degradation by viral protein x (Vpx), HIV-1 DNA products are sensed by polyglutamine-binding protein 1/cGAS (44). (6) Similarly, in the presence of co-transduced Vpx, interferon-γ inducible protein 16 (IFI16) may also sense HIV-1 DNA in monocyte-derived macrophages (45). (7) HIV-1 virions in endosomal compartments of myeloid cells may not lead to productive infection but may be sensed by toll-like receptor (TLR) 7 to trigger an innate immune response that may also drive interferon (IFN) production (30). (8) HIV-1 infection of monocytic cells may also lead to TLR8-dependent assembly of NLRP3 inflammasome to activate caspase-1, which cleaves pro-interleukin-1β (IL-1β) into bioactive IL-1β (46). (9) All sensing pathways described converge on activation of transcription factors IRF3 and NF-κB that drive IFN production.

cell-autonomous innate immune response have rapidly expanded in recent years, and as such have been the subject of numerous reviews (47–49). Here, we provide an overview to highlight recent developments with a focus on the intracellular arms race between HIV-1 and the cell-autonomous innate immune response, from the events that determine sensing, to the downstream signaling cascades, through to the mediators of intracellular restriction, and evasion and antagonism strategies of HIV-1. For an extensive review of the extracellular interactions between HIV-1 and the innate immune system, including the IFITMs, SERINCs, and tetherin, we refer the reader to another review in this edition by the Neil group.

INTRACELLULAR DETECTION OF HIV BY PRRs

Pattern recognition receptors fall into several families, defined either by their structure or the type of PAMP that they detect, and located in most cellular compartments including the plasma membrane, endosomes, the cytoplasm, and the nucleus (50). The PRRs implicated experimentally in the intracellular innate response against HIV are summarized in **Tables 1** and **2** and **Figure 2**. Engagement of PRRs by PAMPs initiates a complex cascade of protein interactions leading to activation of the inhibitor of κB kinases (IKK) and the IKK-related/TBK1 kinases (51). These activate transcription factors of the NF-κB and interferon regulatory factor (IRF) families, which together coordinate the expression of antiviral type I IFNs, pro-inflammatory cytokines, and other chemokines. IFN is secreted and signals back through the IFN receptor on the surface of the infected cell and bystander cells. This causes upregulation of so-called IFN-stimulated genes (ISGs) that encode numerous proteins with direct antiviral activity (52). Importantly, a subset of ISGs is activated directly by IRFs/NF-κB allowing a more rapid activation of their expression, which is then boosted by the wave of IFN receptor-dependent signaling (53). In addition to establishing the frontline antiviral state, triggering of innate immunity is crucial for the subsequent activation of a pathogen-specific adaptive immune response.

TABLE 1 | PRR detection of HIV in HIV target cells.

Cell type	PRR	How was the PRR implicated?	PAMP	Consequence	Reference
pDCs	TLR7	TLR7 antagonist	Purified genomic RNA	IFN, pro-inflammatory cytokines	(54)
Immature DCs	TLR8	Depletion by siRNA	ssRNA during infection	NF-κB activation, transcription of the integrated provirus	(55)
MDDC	cGAS	Depletion by shRNA, cGAMP production, and depletion by siRNA	RT products	CD86 expression, IFN and ISG induction	(32, 43, 44)
	PQBP1	Depletion by siRNA	RT products	ISG induction	(44)
	DDX3	Depletion by siRNA	Abortive RNA transcripts	IFN induction	(56)
MDM	cGAS	cGAMP production	RT products	NF-κB and IRF3 activation, IFN and ISG induction	(33, 43)
	IFI16	siRNA	RT products	Reduced replication and ISG induction	(45, 57)
	DDX3	Depletion by siRNA	Abortive RNA transcripts	IFN induction	(56)
Monocytes	NLRP3	Depletion by siRNA	Post-integration step	IL-1β and IL-18 production	(46, 58)
GECs	TLR2 and TLR4	Neutralizing Abs to TLRs	gp120	NF-κB activation and pro-inflammatory cytokine production	(59)
HLACs	IFI16	Depletion by shRNA	Abortive RT products	Pyroptosis	(60)
CD4+ T cells	DNA-PK	Chemical inhibitors	Viral integration	Cell death	(61)
	cGAS	Depletion by shRNA	Post-integration step	IFN and ISG induction	(62)
	cGAS	cGAMP production	Not determined	cGAMP production but no IFN response	(63)
	TLR7	Depletion by shRNA	Viral RNA	Anergy	(64)

IFN, interferon; DC, dendritic cell; pDC, plasmacytoid DC; PRR, pattern recognition receptor; PAMP, pathogen-associated molecular pattern; ISG, IFN-stimulated gene; TLR, toll-like receptor; MDDC, monocyte-derived dendritic cell; MDM, monocyte-derived macrophage; RT, reverse transcription; cGAS, cyclic GMP–AMP synthase; PQBP1, polyglutamine-binding protein 1; HLACs, human lymphoid-aggregated cultures; IFI16, interferon-γ inducible protein 16; GECs, Genital epithelial cells.

TABLE 2 | PRR detection of HIV in other cell types.

Cell type	PRR	How was the PRR implicated?	PAMP	Consequence	Reference
THP-1	cGAS	Depletion by shRNA	RT products	IRF3 activation, IFN and ISG induction	(43, 57)
	IFI16	Depletion by shRNA	RT products	IRF3 activation, IFN and ISG induction	(57)
	PQBP1	siRNA and hypomorphic mutation by CRISPR	RT products	ISG induction	(44)
	NLRP3	Depletion by shRNA	Post-integration step	IL-1β production	(46)
Huh7.5	RIG-I	Cell line is defective for RIG-I	Purified secondary-structured genomic RNA	ISG induction	(65)

IFN, interferon; PRR, pattern recognition receptor; PAMP, pathogen-associated molecular pattern; ISG, IFN-stimulated gene; RT, reverse transcription; cGAS, cyclic GMP–AMP synthase; PQBP1, polyglutamine-binding protein 1; IFI16, interferon-γ inducible protein 16.

The release of pro-inflammatory mediators recruits professional antigen presenting cells to the site of infection and aids their maturation. Upon migration to the local lymph nodes, these cells then prime adaptive T and B cell responses.

DETECTION OF HIV RNA

To date, endosomal members of the toll-like receptor (TLR) family including TLR3, TLR7, and TLR8 as well as the cytoplasmic RIG-I-like receptors (RLRs) have been described to sense RNA during infection with a range of viruses. TLR7 and TLR8 recognize ssRNA and are potent activators of NF-κB, acting *via* the signaling adaptor MyD88, whereas TLR3 recognizes dsRNA and engages the adaptor TRIF, allowing it to activate both NF-κB and IRF3. Members of the RLR family such as RIG-I and MDA-5 utilize MAVS to activate the IKK and TBK1 complexes, thus activating both the NF-κB and IRF3 arms of innate signaling [reviewed in Ref. (66)] (**Figure 2**).

Most studies implicating RNA sensing in the detection of HIV-1 have been based on transfection of either purified full-length HIV RNA or genome-derived oligos (**Table 1**). Evidence for whether these sensors are engaged during viral infection of target cells is lacking. In our view, a significant limitation of transfection-based sensing experiments is that they deliver naked RNA or genome-derived oligos directly into host cells, whereas during infection the virus uses complex evasion strategies, including wrapping the genomic RNA tightly into complexes with the nucleocapsid and other viral replicase proteins, and/or delivering it into the cell in intact protective viral capsids. Depending on the transfection method, the RNA may also be delivered to cellular compartments where it would not normally encounter sensors during infection. It remains unclear whether HIV-1 genomic RNA is accessible and can be sensed in the cytoplasm during infection. One study has indicated this is possible in monocyte-derived dendritic cells (MDDCs) and monocyte-derived macrophages (MDMs), as the RNA helicase DDX3 was able to detect abortive HIV-1 RNAs, which induced DC maturation and type I IFN responses dependent on the adaptor MAVS (56).

Using transfection methods, HIV RNA has been reported to be detected by members of both the TLR and RLR families. Guanosine- and uridine-rich ssRNA oligonucleotides derived from the HIV-1 LTR were found to stimulate both pDCs and macrophages to secrete IFNα and pro-inflammatory cytokines such as TNFα (67). Using murine cells deficient for various TLRs as well as TLR overexpression in 293T cells, the authors concluded that TLR7 and TLR8 were responsible for the sensing of HIV-1-derived ssRNA (67). TLR7 antagonists have been shown to inhibit cytokine release by pDC incubated with purified HIV-1 RNA (54).

The cytoplasmic RLRs have also been implicated in the detection of HIV-1 RNA through transfection-based studies. Secondary-structured genomic RNA induced ISG expression in peripheral blood mononuclear cells (PBMCs), independent of endosomal TLR signaling (65). MAVS and RIG-I were implicated in this study using murine bone-marrow derived macrophages deficient for MAVS, and Huh7.5 cells with defective RIG-I function. Purified monomeric and dimeric forms of HIV-1 genomic RNA were further shown to be potent PAMPs and inducer of ISGs in PMA-differentiated THP-1 cells (68). Using deficient MEFs, detection of this genomic RNA was shown to be RIG-I- but not MDA-5-dependent, although detection of HIV-1 RNA by these sensors was not demonstrated in human cells.

DETECTION OF HIV REVERSE TRANSCRIPTION (RT) PRODUCTS

A recently discovered and rapidly expanding arm of innate immunity research is the detection of viral DNA by cytoplasmic DNA sensors. Our knowledge of cytoplasmic DNA sensing has lagged behind that of RNA sensing, perhaps because, while a large proportion of tissue culture adapted cell lines are competent for sensing *via* RLRs and TLRs, the DNA-sensing pathways are generally defective in cell lines. For 293T and HeLa cells, some of the most transfectable cell lines, this has been attributed to expression of the viral oncoproteins E1A and E7, respectively, involved in transformation of the cell lines, which bind and inhibit STING, a central component of DNA signaling pathways, **Figure 2** (69). Indeed, transfectability may be dependent on defective DNA sensing. As a result, the use of primary cells, and the few cancer cell lines that are competent for DNA sensing (e.g., monocyte-like THP-1 cells), has been crucial to the expansion of our knowledge in this area. While cells such as THP-1s respond to a range of innate immune PAMPS and agonists, it remains unclear, even for these cells, whether they are as responsive to stimulation as primary macrophages.

Most DNA sensors that have been described to date utilize the ER resident signaling protein STING to activate NF-κB and IRF3 (70), **Figure 2**. STING is a direct sensor of cyclic dinucleotides (71), the best characterized of which, 2′–3′ cGAMP, is synthesized by the sensor cyclic GMP–AMP synthase (cGAS) upon binding to DNA in the cytoplasm (72–74). Upon engaging cGAMP, STING translocates *via* the Golgi to distinct perinuclear regions where it can activate the IKK and TBK1 complexes and thus drive a type I IFN response (75).

Some of the earliest evidence that HIV-1 produces a stimulatory DNA PAMP during infection was obtained in human CD4+ T cells and macrophages that had been depleted for the cytosolic exonuclease TREX1 (42). In this study TREX1 was suggested to digest unencapsidated HIV-1 DNA that would otherwise activate a type I IFN response in a STING-dependent manner. The sensor responsible for the detection of HIV DNA was later described by multiple groups to be cGAS (32, 33, 43, 57). Two groups measured cGAS- and STING-dependent ISG responses in monocyte-like THP-1 cells infected with VSV-G-pseudotyped HIV-1 vector, which were dependent on RT but independent of integration (43, 57). Gao et al. were able to measure cGAMP production in primary MDMs and MDDCs infected with HIV-1 in the presence of SIV virus-like particles (VLPs). SIV VLPs were used to deliver SIV accessory protein viral protein x (Vpx) to inhibit the restriction factor SAMHD1, thereby allowing HIV-1 infection. cGAS-dependent sensing of HIV-1 and HIV-2 has also been implicated in MDDCs pretreated with Vpx (32).

The function of cGAMP as a second messenger goes beyond the infected cell, as it can also pass through gap junctions and activate an antiviral response in neighboring cells in a STING dependent, but cGAS-independent manner (76). cGAMP can also be packaged in lentiviral virions themselves and is spread in this way to neighboring cells with infection (77, 78).

Interferon-γ inducible protein 16 (IFI16), a member of the PYHIN family, was originally described as a STING-dependent DNA sensor for transfected DNA and herpes simplex virus-1 (79). However, this sensor may be capable of detecting both single- and double-stranded HIV-1-derived DNA in THP-1 cells and primary MDMs (57). Depletion of IFI16 by siRNA in primary MDM led to enhanced replication of HIV-1, implicating this protein in the innate detection of HIV, although IFN or ISG induction in these cells was not measured in this study (57). Reduced ISG induction in IFI16-depleted primary MDM infected with HIV-1 BaL in the presence of SIV VLPs was, however, demonstrated in a follow-up study (45).

Polyglutamine-binding protein 1 (PQBP1) was recently identified in a targeted RNAi screen in MDDC and described as a DNA sensor that directly bound to reverse-transcribed HIV-1 DNA and interacted with cGAS to activate an ISG response (44). A role for this protein was also demonstrated in THP-1 cells, as silencing of PQBP1 led to reduced innate immune activation induced by HIV-1 VSV-G pseudotyped vector. In these experiments, co-infection with SIVmac VLPs antagonized SAMHD1. In both cell types, the authors measured a significant reduction in cGAMP production upon infection after PQBP1 depletion, leading them to conclude that it was required for an optimal cGAS/STING response to HIV-1 DNA in myeloid cells (44). A similar proximal role in augmenting the cGAS/STING pathway has now also been suggested for IFI16. In THP-1 cells, IFI16 enhanced cGAMP production upon DNA stimulation and aided the recruitment of TBK1 to STING to enhance IRF3 activation (45). Furthermore, IFI16 enhanced STING activation and signaling complex formation in keratinocytes, although in this study the authors did not find a role for IFI16 in cGAMP production, suggesting that cell type-specific roles for this protein may exist (80). These recent studies suggest that cGAS and STING constitute a central pathway that senses HIV-1 DNA in the cytoplasm, with proteins including PQBP1 and IFI16 somehow enhancing this signaling rather than acting independently as DNA sensors themselves (**Figure 2**).

An outstanding question in the field is which HIV-1 RT products are the major PAMP during infection. During RT, both single-stranded and double-stranded DNA are generated, as well as RNA:DNA hybrids. While both forms of DNA were recognized in an IFI16-dependent manner when transfected into THP-1 cells, Jakobsen and colleagues were not able to measure a significant innate response to RNA:DNA hybrids (57). By contrast, transfection of murine DCs and human PBMCs with RNA:DNA hybrids induced robust IFN and pro-inflammatory cytokine release, which was dependent on TLR9 (81). cGAS has also been implicated in the detection of RNA:DNA hybrids in PBMCs and PMA-differentiated THP-1 cells (82). Whether these transfection experiments reproduce the PAMP production and exposure seen during infection, and whether these sensors detect RT-derived hybrids during infection of relevant primary human target cells, remains to be determined.

DETECTION OF NON-NUCLEIC ACID COMPONENTS OF HIV

In addition to the detection of nucleic acids, some studies have suggested HIV proteins may act as PAMPs. The E3 ubiquitin ligase tripartite motif protein 5 (TRIM5α), is a capsid-binding restriction factor. A seminal study by Pertel et al. proposed that this protein also functions as a PRR to induce innate signaling upon recruitment of retroviral capsids (described in more detail below) (83). The restriction factor tetherin, which prevents newly synthesized virions from budding from the infected cell, has also been reported to act as a PRR that activates innate immune signaling cascades (84, 85), this is reviewed elsewhere in this edition.

ACTIVATION OF INFLAMMASOMES BY HIV

Inflammasomes are multiprotein complexes, found in myeloid cells and T cells, and activated by a wide variety of PAMPs and host-derived danger-associated molecular patterns. The innate sensors capable of activating inflammasomes include members of the NOD-like receptor family, the RLRs, and the DNA-sensing-associated proteins AIM2 and IFI16. Engagement of these receptors leads to the formation of a platform for caspase-1 activation and subsequent proteolytic maturation and secretion of the pro-inflammatory cytokines such as interleukin-1β (IL-1β) and IL-18, or induction of pyroptosis, an inflammatory form of programmed cell death [reviewed in Ref. (86)].

Two studies to date have described inflammasome activation in monocytic cells by HIV-1 (46, 58). IL-18 production by monocytes exposed to HIV-1 was dependent on endocytosis rather than infection, and both studies found that TLR-8 activation was required for induction of *pro-IL-1β* whereas cleavage into its active form and release was dependent upon NLRP3 and the inflammasome adaptor protein ASC (46, 58). Guo et al. further demonstrated that inflammasome activation occurred post-integration leading them to suggest HIV transcripts as potential PAMPs.

SENSING OF HIV DNA IN CD4+ T CELLS

In contrast to the classical ISG and pro-inflammatory cytokine response observed in HIV-infected cells of myeloid origin, innate immune sensing in CD4+ T cells has been described to lead to cell death. It is clear that HIV-1 replication in CD4+ T cells leads to massive cell death, but there are conflicting reports regarding the role of innate immune sensing and the mechanisms of this process (61, 87). One possible explanation for these discrepancies relates to the origin and activation status of the T cells used in each study.

Studies from the Greene lab using human lymphoid-aggregated cultures (HLACs) from tonsillar tissue showed that abortive HIV-1 infection of these cultures led to significant cell

death, which did not require viral integration (88). This study suggested that in many of the T cells in an infected culture, viral DNA synthesis occurred, but that the infection arrested before integration. The authors proposed that sensing of HIV-1 DNA in these abortively infected cells was responsible for the T cell death that drives T cell loss and eventually AIDS. A follow-up study demonstrated that death by apoptosis occurred only in the small percentage of cells in the culture that were productively infected and that the vast majority of cells died *via* caspase-1-mediated pyroptosis after abortive infection (87). They reported that IFI16 was the DNA sensor responsible for detecting the incomplete RT products in the abortively infected T cells (60). This group subsequently demonstrated that, in contrast to CD4+ T cells in HLACs, PBMC-derived CD4+ T cells are resistant to death by pyroptosis (89). They attributed this to the resting status of peripheral blood-derived CD4+ T cells, which could be overcome by coculture with lymphoid-derived cells, resulting in pyroptosis on HIV-1 infection (89). In contrast to these studies, the Nabel lab used PBMC-derived primary CD4+ T cells and found that, in these cells, HIV-1 induced cell death was associated with productive HIV-1 infection and dependent on integration (61). Cell death was accompanied by DNA-PK activity and phosphorylation of p53 and H2AX. The authors proposed that HIV-1 integration was detected by the DNA repair enzyme and DNA sensor, DNA-PK, as chemical inhibition of DNA-PK prevented cell death.

Interestingly, more recent publications are now beginning to address whether CD4+ T cells can in fact sense HIV-1 RT products in a manner more similar to myeloid cells. Again there are conflicting reports, with some studies measuring a type I IFN response after HIV-1 infection of T cells (60, 62, 88), while others have been unable to detect such a response (63, 90). Vermeire and colleagues observed an IFN and ISG response in PHA/IL-2-activated primary CD4+ T cells that was cGAS dependent and required provirus integration (62). In another study, cGAMP production was also detected in CD4+ T cells but in this case cGAMP did not lead to IFN production by the infected cells (63). Interestingly, the authors found that the cGAMP from the infected T cells could be transferred and activate a STING-dependent ISG response in macrophages through Env-induced membrane fusion sites, identifying an alternative mechanism by which T cell infection can contribute to local IFN production *via* macrophages.

INTRACELLULAR RESTRICTION OF HIV

Interplay between cellular restriction factors and HIV-1 occurs at every stage of its lifecycle and the virus uses a combination of evasion and antagonism strategies to achieve infection and replication (**Figure 1**). Our advancing understanding of the mechanisms of viral replication and innate immunity mean that any strict criterion for defining restriction factors rapidly becomes outmoded. It would be a shame for poorly thought out nomenclature to constrain creative thinking and understanding of innate immunity. We take the view that any protein with well-characterized antiviral activity can be considered a restriction or resistance factor. A current exciting research focus is the intersection between traditional direct-acting restriction factors and innate immune signaling. An emerging and important feature of restriction factors is to act a sensor for the presence of infection, as has been demonstrated for TRIM5, tetherin, and TRIM21 (83, 84, 91).

TRIM5α

TRIM5α targets incoming retroviral capsids soon after they enter the host cell to block infection before integration (**Figure 1**). It belongs to the large TRIpartite Motif (TRIM) family of proteins, encoded by over 100 genes in humans, which are involved in diverse cellular processes. Many TRIM proteins, including TRIM5α, are upregulated by type I and II IFNs and have direct antiviral and antimicrobial roles, in addition to less well defined regulatory roles in innate immunity in general [reviewed by Rajsbaum et al. (92)]. Among the TRIM family members, the TRIM5 locus exhibits the greatest rate of positive selection across primate genomes, probably due to selective pressure from direct interactions with retroviruses (93). TRIM5α has been extensively reviewed since it was discovered in 2004 (94, 95); however, recent reports have extended ideas on restriction specificity and have shed significant light on its antiviral mechanism and the role of ubiquitin in this process (96).

TRIM5α represents an important barrier to zoonotic retroviral transmission. It was first identified as an important contributor to the innate resistance of Old World Monkeys to HIV-1 infection, targeting incoming viral capsids to prevent RT (97). Further study of TRIM5 antiviral specificity revealed that each primate TRIM5 restricts a different subset of lentiviruses (97, 98). The importance of TRIM5α for species-specific restriction of HIV-1 is illustrated by the observation that the only monkeys permissive for SIV/HIV-1 chimeras bearing HIV-1 capsid are pigtailed macaques that are homozygous for a TRIMCyp protein that cannot restrict HIV-1 (99, 100).

The defining tripartite domain architecture of the TRIM family, comprises an N-terminal RING domain with E3 ubiquitin ligase activity, a B-box domain and a coiled-coil region that both mediate multimerization through protein–protein interactions (83, 101). TRIMs have various domain types at the C-terminus and, like many TRIMs, TRIM5α has a C-terminal PRYSPRY, also called a B30.2 domain, which is not present in splice variants that lack retroviral restriction activity. Restriction specificity is dependent on direct interaction between the viral capsid protein and the TRIM5α PRYSPRY (102–104). In an intriguing evolutionary arms race, TRIM5α has been modified independently in several simian species by swapping the PRYSPRY for a lentivirus-targeting cyclophilin A (CypA)-like domain. This is derived from retrotransposition of a CypA cDNA (99, 105–110). These observations indicate the importance of CypA to the virus and the plasticity of TRIM5α antiviral evolution.

Until recently, human TRIM5α has been thought to have poor restriction activity against HIV-1. This has been explained by a lack of interaction between the human TRIM5α PRYSPRY and the HIV-1 capsid. Indeed, human TRIM5α can be modified to restrict HIV-1 by a single point mutation in the PRYSPRY (104) or by replacing the whole domain with the rhesus

macaque TRIM5α PRYSRY (102). Certain primary isolates of HIV-1 have been found to be more sensitive to TRIM5α than lab strains. Indeed, it has been suggested that T cell escape mutations in the capsid target of TRIM5α may drive HIV-1 to be TRIM5α sensitive (111). Likewise, TRIM5α polymorphisms and expression levels have been associated with differential rates of HIV-1 acquisition and disease progression, supporting a role for human TRIM5α in HIV-1 transmission and pathogenesis *in vivo* (112, 113). Whether these observations are explained by direct TRIM5α restriction of HIV-1, or by its role in innate immune signaling (83) remain unclear.

A landmark study from the Geijtenbeek lab recently demonstrated that human TRIM5α contributes to restriction of HIV-1 in a cell type, and entry pathway, specific manner (114). LCs, resident in mucosal surfaces, are innately resistant to HIV-1 due to their unique C-type lectin receptor langerin, which mediates uptake of HIV-1 but directs virus into Birbeck granules for degradation (115). By investigating the specific mechanism of langerin-dependent restriction, Ribeiro et al. discovered a role for TRIM5α (114). Depletion of TRIM5α in primary LCs, or a Langerhans-like cell line (MUTZ-LCs), resulted in increased infection and enhanced transmission to cocultured CD4+ T cells. Critically, expression of langerin in a cell line (U87) allowed endogenous TRIM5α to restrict HIV-1, but only when langerin, and not the VSV-G receptor, was used as the virus entry receptor. Association of langerin and TRIM5α in cells was suggested by co-immunoprecipitation. This receptor mediated targeting to TRIM5α-dependent restriction was specific to langerin and was not observed when HIV-1 entered MDDC *via* the C-type lectin receptor DC-SIGN, or, of course, T cells *via* CD4 (114).

The study went on to show a role for autophagy in human TRIM5α-mediated restriction of HIV-1. TRIM5α was associated with components of the autophagy machinery in steady state conditions by co-immunoprecipitation and restriction led to an increase in autophagosome formation. Silencing autophagy proteins Atg16LI and Atg5 ablated the langerin-dependent TRIM5α-mediated restriction of HIV-1 (114). In our view, these data do not conflict with previous reports demonstrating receptor independent, PRYSPRY dependent, interaction between TRIM5α and capsid to define antiviral specificity. Rather, they provide evidence for a role for TRIM5α in restriction of HIV-1 by autophagy when langerin is utilized as an entry receptor. We expect that as the details of this restriction mechanism are uncovered the differences between this autophagy dependent, and previously described proteasome dependent, mechanisms will be clarified and a novel role for TRIM5α in autophagy defined.

Structural studies have shed significant light on the classical antiviral mechanism of TRIM5α. There is evidence that TRIM5α forms hexagonal assemblies on the surface of retroviral capsids, mimicking the organization of the hexameric capsomeres (116). Hexagonal lattice formation may position multiple C-terminal PRYSPRY domains, which interact with the capsid with low affinity and specificity, so as to promote binding through avidity effects. This observation was recently recapitulated using electron microscopy with recombinant full-length rhesus macaque TRIM5α proteins and purified native intact HIV-1 capsid cores. The B-box 2 domain appears responsible for mediating TRIM5α–TRIM5α interactions that drive higher order assembly of TRIM5α into multimers and are essential for restriction activity. The hexagonal TRIM5α nets are thought to have conformational flexibility enabling them to form on divergent retroviral capsid sequences, with different capsomere curvature and conformation. This model could explain the broad recognition of divergent viruses associated with TRIM5α antiviral activity (116, 117).

Formation of TRIM5α complexes on an incoming virion is reported to promote rapid capsid disassembly and premature uncoating (118). However, it is clear that the process of viral disassembly and disruption of viral DNA synthesis is dependent on ubiquitin-dependent recruitment of the proteasome (96, 119). Indeed, the fact that preventing proteasomal degradation of the TRIM5α–virus complex restored restricted viral DNA synthesis was the first hint that viral DNA synthesis occurs inside an intact capsid, a model that is gaining increasing traction (40). HIV-1 capsid uncoating is normally a highly regulated process and so premature uncoating by TRIM5α/proteasomes likely accounts for the observed block to RT.

A consequence of TRIM5α-capsid binding is activation of its RING domain E3 ubiquitin ligase activity (83). This results in complex TRIM5α autoubiquitination and enhanced proteasomal turnover, suggesting that TRIM5α targets capsids for proteasomal degradation (96, 120). Recent mapping of sequential TRIM5α autoubiquitination steps using a combination of biochemical and genetic approaches has implicated a series of E2 conjugation enzyme and ubiquitin linkages. Ube2W first attaches single Ub molecules to TRIM5α, which are then extended into polyUb chains through Lys63-linkages catalyzed by the heterodimeric E2 enzyme complex Ube2N/Ube2V2. Each of these steps was required for human TRIM5α restriction of murine leukemia virus (MLV) RT (96).

TRIM5α appears to serve as capsid PRR activating transcription factors NF-κB and AP-1 and resulting in pro-inflammatory cytokine synthesis that could contribute to the antiviral state and modulate adaptive responses (83). Inhibitors of TAK1 signaling, or depletion of pathway components, rescues some degree of TRIM5α restricted infections in myeloid cells suggesting this is a component of TRIM5α activity. A recent study further correlated the ability to induce signaling with retroviral restriction activity, although this was demonstrated using murine TRIM5α orthologs modified to be able to target HIV-1 capsids (121). It will be interesting to test whether activation of NF-κB and AP-1 pathways occurs after human TRIM5α inhibition of HIV-1 in LCs, and whether this contributes to restriction in this cell type.

Finally, it has recently been proposed that as well as amplifying the innate immune response, TRIM5α directly enhances the potency of CD8+ T cell responses to infected cells. Rhesus macaque TRIM5α restriction of HIV-1 boosted HIV-1 specific T cell activation and inhibition of infected cells *in vitro*. It is possible that TRIM5α mediated recruitment of virus to proteasomes may lead to increased peptide availability for MHC presentation (122).

SAMHD1

Sterile alpha motif and histidine–aspartate domain containing protein 1 (SAMHD1) was identified as the restriction

factor targeted by the SIV accessory protein Vpx in myeloid cells (123, 124). Shortly after this SAMHD1 was found to be a deoxynucleoside triphosphate triphosphohydrolase (dNTPase) that restricts infection by lowering nucleotide concentrations below those which support viral DNA synthesis (125). In the case of viruses such as SIVsm and HIV-2, Vpx directs proteasomal degradation of SAMHD1 by recruitment of the host cell cullin-4 ligase substrate receptor DDB1- and CUL4-associated factor 1, DCAF1, also known as Vpr-binding protein, for polyubiquitination (123). In this way, Vpx provided either packaged into VLPs for co-transduction or stably expressed in cell lines, is able to counteract SAMHD1 restriction of HIV-1 infection.

SAMHD1 comprises three main regions: the N-terminus, a catalytic core HD domain, and the C-terminus. Most reports attribute HIV-1 restriction to the dNTPase activity of the HD domain, which inhibits viral DNA synthesis by reducing the dNTP supply for RT (126). Mutations of key residues in the HD region cause SAMHD1 to lose its ability to restrict HIV-1 (124). Depletion of SAMHD1, using siRNA or by delivering SIV Vpx in *trans*, boosts both intracellular dNTP pools and HIV-1 replication. Indeed, SIV VLPs have regularly been used as a tool to deplete SAMHD1 thereby allowing the study of antiviral properties that would otherwise be masked by SAMHD1 activity. HIV-1 reverse transcriptase mutants with reduced dNTP affinity are consistently more sensitive to SAMHD1 restriction (127). Some studies have proposed additional antiviral activities for SAMHD1. For example, Ryoo et al. showed that overexpression of RNAse-active but dNTPase-inactive SAMHD1 mutants, identified through biochemical assays, are able to restrict HIV-1 (128). They also observed modest increases in HIV-1 RNA stability following transient SAMHD1 depletion. Other groups have suggested that RNase activity may be an artifact of contaminated samples (129, 130). Certainly, the SAMHD1 structural work is consistent with its role as a dNTPase (125).

SAMHD1 is widely expressed in diverse human tissues but *in vitro* appears to only restrict HIV-1 infection in non-dividing cells, perhaps because they typically have low nucleotide levels within the range of SAMHD1 control. Conversely, most rapidly dividing cell lines have high nucleotide levels that may exceed the inhibitory capacity of SAMHD1.

Unlike other restriction factors, where expression alone is typically sufficient to block infection, SAMHD1 antiviral activity is often not measurable in dividing cell lines. This may be because it is regulated by cell cycle-dependent phosphorylation as well as dNTP levels. SAMHD1 is inactivated by cyclin-dependent kinase (CDK)-mediated phosphorylation at C-terminal residue T592 (131). Structural studies have associated this inactivation with unstable tetramer structure and increased dissociation to catalytically inactive monomers and dimers (127, 129). The local dNTP environment also regulates SAMHD1 structure and function. Binding of dNTPs to the C-terminal allosteric regulation domains is required to activate tetramerisation and optimal catalytic activity (125, 132–134). SAMHD1 mutants that are unable to oligomerize are unable to restrict HIV-1 and this correlates with their inability to reduce dNTP pools (127, 133). One model to explain SAMHD1 activity in non-dividing cells is that the absence of CDK-mediated phosphorylation means that the small available dNTP pool is directed toward the C-terminal allosteric sites (127), leading to durable tetramer formation, dNTPase activity and HIV-1 restriction.

Recent work has revealed a crucial role for the CDK-mediated regulation of SAMHD1 in determining permissivity of myeloid cells to HIV-1 infection. Mlcochova et al. showed that T592 phosphorylation and thus SAMHD1 antiviral activity are dynamic in primary human MDM (135). They propose that macrophages, and likely other myeloid cells, exist in two states through which all of the cells periodically cycle. The first, a typical G0 state, characterized by active dephosphorylated SAMHD1, lack of the cell-cycle marker minichromosome maintenance complex component 2 (MCM2) and resistance to HIV-1; and the second, described as a G1-like state, permissive to HIV-1 and characterized by expression of MCM2 and inactive phosphorylated SAMHD1. Critically, though SAMHD1 phosphorylation in this model is CDK1 dependent and linked to MCM2 expression, both states exist without measurable DNA synthesis or cell division (135). These observations provide a plausible explanation for the ability of HIV-1 to infect myeloid cells despite the apparent presence of active SAMHD1 within the cell population. They may also explain the lack of an HIV-1 encoded SAMHD1 antagonist, though the question of why other viruses may have evolved one in Vpx remains open.

Inhibition of SAMHD1 restriction activity by phosphorylation is widely accepted, but some studies in non-permissive differentiated U937 cells or using biochemical assays have suggested that dephosphorylation does not affect dNTPase activity (136, 137). We note that the technical challenges of measuring intracellular dNTP levels, and, more particularly, direct enzyme activity across cell populations with unsynchronized cell-cycle status are consistently highlighted in the literature (127, 130).

There is consensus that SAMHD1 binds single-stranded nucleic acids (129, 138). However, whether there is specificity for this interaction remains unclear. In macrophages, HIV-1 RNA co-immunoprecipitates with SAMHD1 (128) and in biochemical assays ssRNA binds monomeric and dimeric SAMHD1 to inhibit oligomerization and dNTPase activity (130). This has not been recapitulated in cells but leaves open the possibility that binding of SAMHD1 to nucleic acids may represent a further restriction mechanism.

A number of SAMHD1 mutations are implicated in some cases of Aicardi–Goutieres syndrome, a condition characterized by elevated systemic IFN levels, dependent on innate sensing of endogenous retroviruses (139). This has been attributed to loss of SAMHD1-mediated restriction of LINE-1 (long interspersed element-1) retrotransposition that generates a DNA PAMP (140). Intriguingly, restriction of endogenous retroviruses was not sensitive to Vpx and was retained in the presence of a SAMHD1 catalytic site mutant, leading some to propose that SAMHD1 may sequester ssRNA to prevent sensing during homeostasis (141).

Unlike other restriction factors, SAMHD1 expression is not induced by type I IFN in human DCs, macrophages or CD4+ T cells (142, 143), the main target cells of HIV-1. SAMHD1 induction has been reported in HEK 293T and HeLa cells (144). It is possible that SAMHD1 is activated in response to type I IFNs, which have been shown to reduce phosphorylation at residue

T592 in MDMs and MDDCs, which would promote SAMHD1 tetramerization and catalytic activation (131). Together, these data implicate SAMHD1 as a component of a typical IFN-inducible antiviral response.

Lentiviral accessory proteins are often not conserved in their functions. For example, tetherin is antagonized by HIV-1 Vpu but the parental virus SIVcpz uses nef for this purpose. In a similar way, several viruses use Vpr, rather than Vpx, to antagonize SAMHD1. SAMHD1-degrading Vpr proteins are encoded by SIV syk (SIV that infects Sykes' monkey), SIV deb (De Brazza's monkey), and SIV agm (African green monkey) lineages (145). It is tempting to suggest that HIV-1 has gained advantage from avoiding a SAMHD1 degradation phenotype. Some have proposed that HIV-1 transmission *in vivo* is enhanced by avoiding sensing and activation of antiviral intracellular innate responses in dendritic and myeloid cells, perhaps evidenced by fewer cases of Vpx encoding HIV-2 than HIV-1 (146, 147). The model is that abrogation of SAMHD1 leads to HIV-2 DNA synthesis, which can then activate innate immune DNA sensing, particularly in DC. Consistent with this theory, HIV-1 infection of MDDC and MDM only results in cGAMP production when SAMHD1 is inhibited by pretreatment with VLPs containing Vpx (43, 148). Further, chronic HIV-2 infection is often characterized by stable CD4+ T cell counts, which may reflect an inability to efficiently establish high levels of infection in these cells *in vivo* (149). However, there are likely to be many differences between HIV-1 and HIV-2 that lead to the lower pathogenicity and transmission rates of HIV-2 as compared with HIV-1 and the role of Vpx in these differences remains poorly defined. Furthermore, there is as yet no good evidence that Vpx enhances replication in myeloid cells *in vivo*.

APOBEC3

Apolipoprotein B mRNA-editing, enzyme-catalytic, polypeptide-like 3 proteins (APOBEC3 or A3) belong to the family of single-stranded DNA deaminases. A3s are IFN-inducible and restrict HIV-1 primarily by suppressing viral DNA synthesis and inducing mutations in the viral DNA leading to replication incompetent proviruses (148, 150–152). Seven A3 enzymes have been identified: A3A A3B, A3D, A3F, A3G, and A3H are all active against HIV-1, and A3C may be inactive (4, 153). A3G is the most well defined anti-HIV APOBEC3 protein and was the first to be described to have a role in innate immunity through its ability to block HIV-1 replication (150). It is expressed in CD4+ T cells and MDM (154). The importance of APOBEC3 proteins in transmission and species-specific replication of HIV-1 is underlined by the observation that HIV-1 can be made to replicate in pigtailed macaques by changing only the APOBEC3-antagonizing HIV-1 accessory gene Vif (100).

To restrict HIV-1, A3 proteins must be packaged into viral particles and access the viral DNA in the infected cell (155). For example, A3A is not packaged but can be made to restrict HIV-1 by forcing incorporation into virions by fusing it to the packaged viral accessory protein Vpr (156). A3G is packaged into virus particles through its interaction with cellular or viral RNAs bound to the nucleocapsid domain of the Gag polyprotein (157). In the absence of the antagonistic viral accessory protein Vif (described below), A3G suppresses DNA synthesis and catalyzes the deamination of cytosines to form uracils in the minus strand of the reverse-transcribed single-stranded DNA, resulting in G to A mutations in the plus strand of the viral DNA (158, 159). The hypermutated proviral DNA that results is defective and unable to produce infectious progeny (160).

A3G disruption of HIV-1 DNA synthesis occurs at several steps. A3G prevents tRNA binding to the primer binding site in the viral RNA (161), minus and plus strand transfer (162), and primer tRNA processing and DNA elongation (152, 163). The studies reporting lack of HIV-1 restriction by the deaminase inactive A3G mutant (E259Q) should be considered in light of reports that show that the A3G E259Q mutant is also defective for RNA binding and therefore unable to inhibit HIV-1 DNA synthesis to the same extent as the wild-type A3G (164).

SUN2

SUN2 (also known as *UNC84B)* was originally identified as a potential innate immune effector with specific antiretroviral activity in an overexpression screen for ISGs against a range of different viruses (165). SUN2 is an integral membrane protein that spans the inner nuclear membrane and forms part of a multiprotein complex (LINC) that physically bridges the nucleoskeleton and cytoplasm (166). Several recent studies published in quick succession have suggested that manipulation of SUN2 can either inhibit or promote HIV-1 infection, depending on the level of expression (167–169).

Studies have found that SUN2 is constitutively expressed in human cells and is in fact not upregulated by IFNs (167–170). Several groups have confirmed that SUN2 overexpression leads to a block to HIV-1 infection and replication, as originally reported in Ref. (165). However, endogenous levels of SUN2 did not have antiviral activity. This suggests that SUN2 overexpression has antiviral activity through a dominant negative effect rather than through having specific innate antiviral properties.

SUN2 was included in the original ISG screen based on microarray data from primary chimpanzee PBMCs treated with IFNs (165, 167, 171). It is therefore possible that SUN2 could exert anti-HIV activity if induced in other species, although this remains to be tested. When overexpressed in human cells, SUN2 exerted strain-specific antiviral activity as T/F HIV-1 viruses were less susceptible (167). Infection was blocked after DNA synthesis, before or at the point of nuclear entry, and was associated with drastic changes in nuclear morphology resulting from SUN2 overexpression. It is not clear why evidently global effects on nuclear morphology, should specifically inhibit certain HIV-1 strains and not others. Serial passage of HIV-1 in the presence of overexpressed SUN2 resulted in resistant viruses, largely conferred by the single capsid mutation P207S (167). The host cell cofactor CypA, that is recruited to incoming virions, was also implicated in targeting the capsid to SUN2 restriction, as CypA inhibitors partially relieved the block to infection in the presence of overexpressed SUN2, consistent with the notion that capsid–CypA interactions guide virion nuclear import pathways (37, 172).

While silencing or depletion of SUN2 in cell lines has been shown to have either no impact or very modest impact on HIV-1 infection (167, 170), silencing in primary T cells inhibited infection and produced a large defect in replication assays, leading the authors to surmise that SUN2 acts as a cofactor for HIV-1 (168). This was again proposed to be dependent on CypA recruitment to the capsid, as CypA inhibitors had no additive effects with SUN2 silencing (168). However, the defect in primary T cells has since been convincingly attributed to defects in T cell proliferation, activation status and viability resulting from SUN2 silencing (169). Discrepancies in cell viability between the two studies could be explained by depletion efficiencies and the duration of silencing experiments. In summary, endogenous SUN2 appears to play a central role in T cell proliferation and activation, which indirectly makes it essential for HIV-1 infection of activated primary T cells in culture. Due to difficulties in infecting resting primary CD4+ T cells *in vitro*, it will be difficult to establish whether SUN2 has additional cofactor roles in infection.

MxB

MxA and MxB (Mx1 and Mx2 in mice) are ISGs that belong to the dynamin-like GTPase superfamily. Human MxA has broad antiviral activity against both RNA and DNA viruses, best characterized against influenza A viruses (173). By contrast, MxB has only been shown to have antiviral activity against certain retroviruses. Three different groups simultaneously reported that MxB is a potent inhibitor of HIV-1 and contributes to IFNα-induced anti-HIV-1 activity in a range of cell types (174–176). Nonetheless, type 1 IFNs typically suppress HIV-1 DNA synthesis, whereas MxB appears to act after HIV-1 has completed viral DNA synthesis. This suggests that MxB can act against HIV-1 but that in a typical type 1 IFN response, another, as yet unidentified factor(s) restricts HIV-1 before the MxB induced block. Consistent with this notion, some studies have shown that MxB knock out does not reduce the antiviral activity of type 1 IFN against HIV-1 (177).

Human MxB is active against various HIV-1 strains, including different subtypes and T/F viruses (178). In comparison, HIV-2 and some SIV strains are less susceptible, and unrelated retroviruses including MLV, feline immunodeficiency virus, and equine infectious anemia virus appear resistant to the human protein (174). Divergent primate MxB orthologs have been shown to have different patterns of restriction indicating some degree of species specificity (179), although this is not as clearly defined as, for example, for TRIM5α. Differences in viral susceptibility map to the capsid protein, suggesting it is the target of MxB antiviral activity. MxB resistant capsid mutants have been identified in naturally occurring primary isolates (180). The fact that MxB resistance mutations exist naturally, but are not universal, suggest uneven or incomplete selection pressure on HIV-1 from MxB, consistent with it having a minor role in the IFN response against HIV-1.

Most studies have reported that MxB expression inhibits nuclear entry, evidenced by a reduction in 2-long terminal repeat (2-LTR) circles, which are likely only formed in the nucleus by the uniquely nuclear non-homologous end joining pathway (181). A subsequent defect, implying a second block, can also be observed in the level of integrated proviral DNA (174, 175). Liu et al. reported a defect in integration, but not nuclear entry (176). These discrepancies prompted a thorough investigation by Busnadiego et al. who showed that MxB expression reduced 2-LTR circles, but that this defect did not fully account for the greater defect observed in infectivity (179). They suggested that MxB may therefore inhibit subsequent stages of infection in the nucleus. While they concluded that integrase activity was unaffected, MxB expression significantly altered the distribution of integrated proviral DNA away from gene-dense regions, although it is not clear if this also accounted for the remaining defect in infectivity. Similar effects on integration targeting have been observed for capsid cofactor binding mutants that are thought to have altered nuclear import pathways (37). Interestingly, the genomic position of integrated proviruses has recently been linked to differences in proviral expression and latency (182), although no study has yet demonstrated how retargeted integration by MxB may impact infectivity or replication in spreading infections. We speculate that the restriction activity of factors like MxB could have a greater impact on HIV-1 infection *in vivo* by retargeting integration, the full consequences of which may not be apparent in single round HIV-GFP infection assays in cultured cells.

The antiviral activity of MxB appears dependent on HIV-1 cofactors, including CypA, which are recruited to the incoming capsids. Like naturally occurring resistance, MxB resistance mutations, generated by repeat passage of HIV-1 in the presence of MxB, were found to map to the capsid, for example, to the CypA binding loop residue A88 (176). We note that HIV-1 CA A88 is very conserved in HIV-1 M isolates (183). RNAi mediated silencing of CypA and chemical inhibition of capsid–CypA interactions with cyclosporine rescue the MxB-mediated block to infection, consistent with a role for CypA (184). The N74D capsid mutant, which cannot bind the cytoplasmic cofactor CSPF6, or nuclear pore component Nup153, is also less susceptible to inhibition by MxB (175). Current thinking is that recruitment of cofactors to the incoming virion targets it into a pathway where it may encounter MxB in the context of an IFNα response, potentially at the cytoplasmic face of the nuclear pore where MxB is localized (185). Based solely on *in vitro* binding assays, the cofactors are not thought to be required for binding of MxB to the capsid as MxB-capsid interactions are not affected by cofactor binding mutations (184, 186). However, whether HIV-1 cofactors have a role in recruitment of MxB to the capsid during infection has yet to be determined.

The capsid residues that are targeted by MxB have not yet been mapped. Sites associated with resistance, found throughout the capsid, are thought to affect capsid stability suggesting they might not be directly targeted (179, 180). *In vitro*-binding assays suggest that MxB can only interact with capsid hexamers, rather than monomers, suggesting avidity effects and leading to suggestions that MxB may recognize hexameric capsid patterns (186).

The mechanistic details of MxB antiviral activity are therefore not yet fully understood. In trying to gain insight into the mechanism, numerous studies have probed the importance of each MxB domain through comparisons to MxA and structure-guided

mutagenesis. Like MxA, MxB has a GTPase domain, which is linked by a bundle signal element (BSE) to a carboxy terminal stalk domain (186). Surprisingly, and unlike MxA, neither the GTPase activity nor conformational communication through the BSE is required for MxB antiviral activity (175, 186, 187). MxB has an extended N terminal domain, not present in MxA, which is essential for *in vitro* binding to the capsid and antiviral activity (175, 179, 184, 187). Transfer of the human MxB N terminal domain (25 amino acids) onto canine MxB orthologs, and unrelated proteins, confers anti-HIV-1 activity, providing the chimeric protein is able to dimerize (188). This is consistent with structure-guided mutagenesis studies that have confirmed that MxB dimer or trimer formation, mediated by residues in the stalk domain, is required for anti-HIV-1 activity by increasing the avidity of MxB–capsid interactions (184, 189), much like TRIM5. A triple arginine motif in the N terminal domain has been suggested to directly bind to the capsid. This sequence is essential for restriction and introduction of the motif into non-restrictive MxB orthologs confers anti-HIV-1 activity (190).

The N terminal domain of MxB also contains a nuclear localization sequence (NLS), and MxB is able to shuttle between the nucleus and cytoplasm (191). Early studies with N-terminal truncation mutants that were unable to restrict HIV-1 led to conclusions that MxB nuclear localization is essential for activity (188). However, it is now thought that these studies were confounded by deletion of the MxB N-terminal capsid-binding motif. To deconvolute the two functions of the MxB N-terminus, a recent study made point mutations in the NLS, which did not compromise HIV-1 restriction, but prevented nuclear rim localization (188). This study also used leptinomycin B to prevent MxB nuclear export leading to an accumulation in the nucleus. This did not disrupt HIV-1 restriction. However, it is possible that residual cytoplasmic MxB was able to inhibit infection in these experiments and further studies are required to clarify these apparently contradictory reports and determine exactly where in the cell MxB restriction of HIV-1 takes place.

The N-terminal domain of MxB has been shown to be under diversifying positive selection in primates, consistent with a role in directly binding pathogens and with pathogen-driven evolution (192). However, the four amino acids found to be under positive selection did not include the triple arginine motif, or the NLS implicated in HIV-1 restriction. This suggests that MxB evolution may have been driven by other viral pathogens, implying broader antiviral activity (192). An alternative explanation is that we do not yet fully understand the interactions and mechanisms of inhibition of MxB against different lentiviruses. The N-terminal residues under selection were targeted by alanine scanning mutagenesis in a separate study with no apparent effect on HIV-1 inhibition (190). However, making evolutionary analysis-guided changes in MxB, rather than simply mutations to alanine, and testing antiviral specificity, may prove more informative.

An outstanding question regards the fate of MxB-restricted capsids in the infected cell. It has been proposed that MxB binding prevents uncoating, as accumulation of p24 capsid proteins has been observed with MxB expression (184). This was also based on indirect biochemical measurements using a "fate of capsid" assay, which compares the amount of "intact" viral cores that can be pelleted from infected cells in different conditions (193). Measuring uncoating in cells remains challenging and somewhat controversial, due to the rarity of infectious events and the possibility that the majority of events measured biochemically do not represent those leading to infection. Nonetheless, these experiments can be informative and understanding the effect of MxB on viral capsids as a whole is certainly worth pursuing.

SCHLAFEN (SLFN) 11

Schlafen genes are unique to mammalian cells; there are six human SLFN genes and all possess motifs shared with nucleic acid sensors RIG-I and MDA-5 (194). SLFN11 was originally suggested to restrict HIV-1 replication at the level of protein translation in human cell lines and activated primary CD4+ T cells (195). They suggest that SLFN11 counteracts HIV-1-induced changes in tRNA composition, which is presumed to relate to initiation of viral protein synthesis. These authors proposed that SLFN11 may exploit differential codon usage between viral and host proteins: lentiviral genomes have high frequencies of A nucleotides and favor rare codon usage, relative to host cells, with A/U in the third position. Thus, SLFN11 may exploit viral codon preferences to specifically attenuate viral protein synthesis. Electrophoretic mobility shift assays implied that SLFN11 might achieve this by binding and sequestering tRNA on which HIV-1 is dependent (195). More recent evidence suggests that overexpression of SLFN11 in HEK 293T cells reduces all protein production, including host protein translation in the absence of infection, with a bias toward genes that have not been codon optimized for the relevant host cell (196). SLFN11 gene expression is IFN induced and it may be more appropriate to consider SLFN11 as a broad-acting ISG that contributes to the antiviral state by mediating host cell translational shut-off, rather than a restriction factor specific to any particular virus or virus family (195, 197). The other human SLFN paralogs remain to be explored in this context.

IMMUNE EVASION STRATEGIES OF HIV

In comparison to large DNA viruses, such as herpes or pox viruses, which carry an armory of proteins capable of disabling all branches of the innate immune response, HIV-1 travels light, with just nine viral genes. The HIV accessory proteins, which are dedicated to antagonizing host defenses, are multifunctional and able to manipulate activity or expression of many target proteins (198–200). However, without the genetic capacity to initiate a global shutdown of host responses, evasion of detection is thought to be important for HIV-1 replication and particularly for transmission. As such HIV-1 has evolved a stealth strategy that operates throughout its lifecycle.

EVASION OF NUCLEIC ACID INNATE IMMUNE SENSING BY THE HIV-1 CAPSID

Studies from our lab and others have demonstrated that HIV-1 infection is silent in MDM and does not activate NF-κB or IRF3 signaling, or a type I IFN response, if the viral prep is purified

from inflammatory cytokines made by the viral producer cells (16, 33). This stealthy replication is in part dependent on the cytoplasmic exonuclease TREX1, which degrades HIV-1 reverse transcripts that would otherwise be sensed by cGAS leading to a type I IFN response (33, 42). In this way, HIV-1 exploits the negative regulatory role of TREX1 in modulating innate immune activation, which may have evolved to prevent sensing of mobile endogenous retrovirus genomes (201). Genetic polymorphisms that inactivate TREX1 cause some cases of Aicardi–Goutieres syndrome (mentioned earlier), a serious autoinflammatory condition characterized by high systemic levels of IFN (202).

The HIV-1 capsid plays a central role in evasion of cytoplasmic DNA sensing in MDM. The capsid recruits the cellular cofactors CypA and CPSF6, which somehow cloak HIV-1 replication and prevent detection of newly synthesized viral DNA during transit across the cytoplasm (33). CypA is a highly abundant cytoplasmic protein with prolyl-peptide isomerase activity, whose function is not well understood despite having been implicated in a range of cellular processes including innate immune signaling (203). CPSF6 is involved in mRNA processing in the nucleus, but can also be found in the cytoplasm (204). Both CypA and CPSF6 target the virus to particular nuclear import cofactors and influence integration site selection (37, 205). Both cofactors are essential for HIV-1 replication in MDM, as capsid mutants that are unable to recruit either CypA (P90A) or CPSF6 (N74D) trigger a type I IFN response that completely suppresses infection (33). RNAi-mediated depletion of CPSF6, or pharmacological inhibition of CypA, has the same effect. Blockade of IFN signaling rescues infection in each case, confirming the importance of innate immune evasion for successful infections. Sensing of the CypA binding mutant (P90A) was dependent on viral DNA synthesis and resulted in production of cGAMP. Non-immunosuppressive derivatives of cyclosporine A, which block CypA–capsid interactions, also triggered a type I IFN response that suppressed infection, demonstrating the potential for therapeutic intervention to promote innate immune responses.

We do not yet fully understand the mechanisms by which cofactor recruitment helps to cloak the incoming capsid and prevent sensing of viral DNA. An attractive hypothesis is that sequential cofactor binding acts as a "satnav," by regulating the coordinated processes of DNA synthesis and uncoating, ensuring they happen in the right intracellular location, and at the right time, to avoid detection. This hypothesis is supported by structural studies that revealed that pairs of cytoplasmic and nuclear cofactors, for example, CypA/Nup358 and CPFS6/Nup153, have overlapping binding sites on the surface of the capsid (39), suggesting that an exchange of cofactor binding may happen at the nuclear pore to control uncoating and protected DNA synthesis.

Structural analysis has also revealed that the CPSF6/Nup153 binding site spans multiple subunits within capsid hexamers, suggesting that interactions with Nup153 can only take place with intact capsid cores. Taken altogether, these studies have added to growing evidence that the capsid stays intact until it reaches the nuclear pore, contrary to dogma that proposes uncoating occurs soon after the capsid enters the cytoplasm. Encapsidated DNA synthesis would allow RT to occur within the safety of the core, shielded from cytoplasmic DNA sensors and from TREX1 degradation (40). Indeed, intact capsids have been observed docked at the nuclear pore by electron and light microscopy (36, 38).

For in-core RT to be possible, dNTPs must be able to enter intact cores to fuel DNA synthesis. Jacques et al. recently discovered that capsid hexamers form an electrostatic transporter that can transport dNTPs (40). They demonstrated that the channel, with its electrostatic core comprising a ring of positively charged arginines, allowed RT within intact cores *in vitro.* Mutation of the key arginine at position CA18 led to decreases in dNTP binding, RT, and infectivity. On the outside of the CA, the channel is opened and closed by a dynamic molecular iris formed by a beta-hairpin structure. The beta-hairpin exists in different conformations in X-ray structures, suggesting its acts as a lid to regulate the electrostatic channel. This could provide the virus with a means of controlling entry of dNTPs and DNA synthesis by CA binding cofactors. Of course, linked processes, such as uncoating, could also be controlled in this way. However, it remains to be defined as to whether and how the channel is regulated in the host cell cytoplasm. The contribution of the channel and beta-hairpin in encapsidated RT and the mechanisms of evasion of innate immune sensing also require further study.

ANTAGONISM OF INNATE IMMUNITY BY HIV-1 ACCESSORY PROTEINS

Viral Infectivity Factor (Vif)

HIV-1 Vif is essential for viral replication in CD4+ T cells and some T-cell lines (206, 207). Importantly, *in vivo* studies show that SIV lacking Vif is less infectious, with reduced pathogenicity (208). One of the reasons for this reduced infectivity is that Vif-deleted viruses are restricted by APOBEC3 proteins (150). The best characterized function of Vif is its ability to counteract the antiviral effects of APOBEC3 proteins by targeting them for degradation in infected cells. This prevents them from being packaged into nascent virions and circumvents their antiviral activity (209). To do so, Vif hijacks the Cullin5 (Cul5) E3 ubiquitin ligase complex by mimicking its cellular substrate recognition subunit, SOCS2 (210). As such, it links A3s to the Cullin5 E3 ubiquitin ligase complex containing elonginB, elonginC, and Rbx-2 for polyubiquitination and subsequent degradation by the proteasome. Structural studies have revealed that interactions of Vif with different A3 proteins are mediated by its N terminus, whereas the C-terminus recruits the Cul5 E3 ubiquitin ligase complex proteins (211). In crystal structures, Vif adopts an elongated cone-like shape, with two domains surrounding the zinc-binding region, when bound to the Cul5 E3 ligase complex (212). The zinc-binding region stabilizes Vif structure by coordinating zinc through an HCCH motif. Vif uses three distinct regions in its N terminus to bind A3 proteins, which affords it broad specificity. The ^{14}DRMR^{17} motif is used to interact with A3F, A3C, and A3D (213, 214). The ^{40}YRHHY^{44} motif is used to interact with A3G (215) and residues ^{39}F and ^{48}H are used to interact with A3H (216).

Although proteasomal degradation is the primary mechanism by which Vif antagonizes A3G, there is evidence that Vif

can also decrease translation of A3G mRNA (217), prevent A3G packaging into virions (218), and inhibit cytidine deamination activity of A3G (219). Various strategies used by Vif certainly hinder A3G packaging into virions; however, low levels of enzymatically active A3G can be detected in wild-type HIV-1 virions (220), resulting in sublethal deamination of the viral DNA (221). Several lines of research convincingly show that non-catastrophic increases in HIV-1 mutation rates, induced by low level A3G expression, may be beneficial for the virus and allow, for example, generation of antiretroviral resistance (222), escape from cytotoxic T lymphocytes (223) and co-receptor switching (224, 225).

Like other lentiviral accessory proteins, interaction of Vif with A3 proteins is species specific and is thought to present a cross-species transmission barrier. HIV-1 Vif degrades human but not simian A3G. Specificity can be determined by a single residue, for example, at position 128 of A3G, which dictates binding of Vif and therefore species-specific A3G antagonism (224). Species specificity of antagonism of A3G by Vif is indicative of the arms race between pathogens and their hosts, resulting in continuous selection pressure that drives evolution of this protein (226, 227).

Additional functions for Vif have recently been proposed by proteomic studies seeking additional targets for Vif degradation. Greenwood et al. identified host cell protein PP2A, which belongs to the B56 family of serine/threonine phosphatases involved in numerous cellular processes, as a novel Vif target (199, 228). By studying changes in the proteome of an HIV-1 infected T-cell line, they found that PP2A had the same pattern of temporal loss as APOBEC3 proteins suggesting PP2A as a Vif target. Subsequently, the authors confirmed that, indeed, Vif targets all five members of the B56 family for Cul5-dependent proteasomal degradation. In contrast to APOBEC3 antagonism by Vif, targeting of PP2A was found to be a conserved function of lentiviral Vif proteins as Vif proteins from different primate and non-primate lineages could target human PP2A. Currently, it is unclear why Vif targets the PP2A complex.

Vpr

Vpr, an accessory protein of around 96 amino acids, is packaged into viral particles *via* interactions with Gag derived p6 (229). Virion incorporation suggests it functions during viral entry or egress from infected cells. Although present in all primate lentiviruses, its sequence is highly variable between viruses and even within species. Numerous functions have been proposed for Vpr (230); however, its role in HIV-1 infection has remained poorly defined and its function remains enigmatic. This is partly because, while Vpr is typically dispensable for replication in cultured CD4+ T cells, there are reports of Vpr-dependent HIV-1 replication in MDMs (231), suggesting that its function might only be apparent under certain conditions. Here, we discuss only the proposed functions of Vpr relating to innate immunity.

Various studies have shown that Vpr modulates innate immune activation by regulating activation of transcription factors, IRF3 and NF-κB, during early stages of the HIV-1 life cycle. In TZM-bl cells reconstituted with STING, Vpr was found to inhibit sensing of HIV-1 by blocking translocation of IRF3 into the nucleus (232). On the other hand, in PBMCs, and the Jurkat T-cell line, Vpr was found to degrade IRF3 (233). In contrast to the effects of Vpr on IRF3, NF-κB has been described to be activated by Vpr, potentiating innate sensing of HIV-1 in CD4+ T cells and DCs (62, 234).

Like the Vpr related protein Vpx, Vpr usurps the Cul4-DCAF1 E3 ubiquitin ligase complex to target proteins for proteasomal degradation (235). The most extensively studied function of Vpr is to cause cell-cycle arrest at the G2 to mitosis (G2/M) transition. A 2014 study showed how Vpr can manipulate an endonuclease complex to arrest cell cycle and proposed that this prevents innate immune sensing of the viral DNA (236). The data suggested that Vpr interacts directly with SLX4, which is implicated in DNA damage repair pathways. SLX4 recruits structure-specific endonucleases (SSEs) MUS81-EME1, ERCC1–ERCC4, and SLX1 to form a complex (SLX4com) that repairs DNA damage. The activity of SSEs is kept under tight control during cell cycle. They are only activated at the G2/M transition, for example, by kinases such as polo-like kinase 1 (PLK1) leading to resolution of stalled replication forks and maintenance of genomic integrity (237). Laguette et al. proposed that Vpr recruits PLK1 to the SLX4com before the G2/M transition. PLK1 then prematurely activates SLX4com by phosphorylating EME1 resulting in abnormal processing of replication forks that eventually leads to replication stress and cell-cycle arrest at the G2/M transition. This function of Vpr is dependent on Cul4-DCAF1 ubiquitin E3 ligase complex as the DCAF1 binding mutant, VprQ65R, is unable to cause cell-cycle arrest. Furthermore, SLX4 was found to bind HIV-1 reverse transcripts only in the presence of Vpr suggesting that Vpr may recruit SLX4 to process HIV-1 reverse transcripts and prevent innate sensing.

These findings raise important questions of how Vpr manipulates the SLX4 complex to degrade viral DNA and evade innate sensing without suppressing productive infection. Importantly, the significance of SLX4 activation by Vpr during HIV-1 replication was not demonstrated in this study. The relevance of the Vpr interaction with SLX4 is undermined by the recent suggestion that Vpr from certain HIV-1 isolates are unable to interact with SLX4 (238). However, species-specific Vpr–SLX4 interactions support the importance of this interaction. SIV Vpr proteins from African green monkeys that do not arrest cell cycle in human cells can interact with the SLX4com in African green monkey cells and cause cell-cycle arrest (239). The role of SLX4 in HIV-1 replication and Vpr activity certainly warrants further investigation. It is also likely that Vpr targets, as yet unidentified, factors and pathways as evidenced by recent proteomic screens identifying putative Vpr targets (200, 240). A major challenge is to identify a reliable assay for Vpr function and a corresponding replication assay that links target degradation to viral replication as was the key to understanding the relationships between HIV-1 Vif and APOBECs, Vpu/Nef and Tetherin, and Nef and SERINC3/5 (241, 242).

Vpu

A detailed description of the roles of Vpu as an antagonist of the restriction factor tetherin, which prevents viral budding and

release from infected cells, and in the regulation of host transmembrane proteins, are described elsewhere in this edition (Neil lab review). Its anti-tetherin activity also implicates Vpu in innate intracellular signaling pathways, because tetherin also acts as a PRR that activates signaling cascades upon recruitment of HIV virions (84, 85). Intriguingly, another study examining tetherin signaling demonstrated that tetherin has a long and a short isoform, that are, as for MAVS, derived from alternate start codons (243, 244). Also like MAVS, the long form can activate an innate immune signal whereas the short form cannot. Intriguingly, Vpu preferentially targets the long signaling form of tetherin, despite the fact that the short form is competent for tethering newly formed virions (243). In the light of these data, we consider that the name tetherin is rather misleading, in fact, being tethered may not be disadvantageous to HIV-1, particularly given this feature aids cell-to-cell spread in T cells (245). Indeed, preferential targeting of the long form of tetherin by HIV-1 suggests that it is signaling and its consequences that exert the dominant pressure on the virus. This is consistent with a model in which the most important feature of restriction factors is their PRR function, which can protect many cells through initiating IFN responses, rather than their restriction function, which is focused on individual viral particles. Having said this, tetherin signaling may be a recent adaptation given that simian tetherin variants were found to be unable to activate signaling when expressed in 293T cells (84). Of course, these proteins may signal in their cognate species and the role of signaling in viral restriction by tetherin requires further study. Furthermore, cell-free virus is required for transmission and therefore antagonism of tetherin by Vpu is critical. Tethered viruses may also enhance antibody-dependent cell-mediated cytotoxicity (246).

In contrast to other accessory proteins, Vpu is exclusively encoded by HIV-1 lineage viruses and is absent from HIV-2 clades. It is not packaged inside the virion, and its Rev-dependent expression occurs late in the viral lifecycle (49). Vpu potently inhibits NF-κB activation and ensuing ISG expression; this likely requires viral integration in primary myeloid and CD4+ T cells (62, 247, 248). As mentioned earlier, activation of NF-κB occurs downstream of multiple innate sensing pathways to drive antiviral gene expression. Paradoxically, NF-κB activation is also implicated in driving HIV-1 and HIV-2 proviral transcription (249). Thus, primate lentiviruses may encode factors to closely regulate NF-κB activation at different stages in the lifecycle to strike a balance between shutting down antiviral responses and augmenting viral gene expression. In particular Nef and Tat, expressed at high levels early in the viral lifecycle, have been shown to increase virus replication by promoting NF-κB activation (250, 251). Intriguingly, Vpu's role as an antagonist of innate immune signaling is independent of tetherin antagonism and is apparently conserved between all lineages of SIV and HIV-1 containing Vpu (except HIV-1 group N) (62, 248). Several reports show that Vpu disrupts NF-κB activation downstream of a range of exogenous and overexpression stimuli that are not specifically related to innate signaling (62, 84, 85, 248). Besides NF-κB, there are also conflicting data regarding antagonism of IRF3 by Vpu (248, 252, 253). It seems likely that host cell type and activation status may significantly impact the role of Vpu in the context of intracellular innate immune responses to HIV, which has yet to be fully elucidated.

FUTURE PERSPECTIVES

The last decade has seen an extraordinary expansion in our understanding of HIV and its interaction with the cell-autonomous innate immune system, especially pertaining to the field of DNA sensing. We are beginning to understand the complexities of the cellular responses to HIV-1 and the subtleties of HIV evasion strategies in different cell types. Viruses are the masters of compromise, able to switch roles between viral accessory proteins or finely tune their behavior with as little as single amino acid changes. In our view, particularly pertinent studies are those that explain the differences in cofactor requirements or innate evasion strategies in cell lines versus primary cells. Of course, cell lines make tractable models for HIV infection, but we must remember that, in many cases, this is because of defects in cell-autonomous innate immunity related to their cancer origins, and so we must be cautious in interpreting experiments studying tropism in cells that cannot mount authentic responses. Also important are studies that take into account the fact that cells communicate through cytokine and cGAMP secretion (63, 72). Humanized mouse models are now allowing sophisticated and relatively cheap *in vivo* investigation of HIV therapeutics and innate responses (254–256). Similar studies considering innate immunity may eventually be more informative for HIV-1 than simian models given that simian lentiviruses, such as the Vpx encoding SIVmac, have different cellular tropisms and likely innate immune relationships with their hosts. Mouse models may be particularly effective for the study of HIV-1 tropism in human T cells *in vivo*, which must be activated for HIV-1 replication *in vitro*, thereby masking innate responses and cytokine secretion profiles in response to the virus itself.

It is clear that HIV adaptation to host defenses influences the HIV-1 lifecycle at every stage. As our understanding of HIV-1 innate immune evasion increases, comparative studies between the different SIV and HIV strains are becoming increasingly informative and may shed light on the determinants of pandemicity. Manipulation of these critical host–virus interactions offers tantalizing opportunities for development of novel therapies. However, excitement at the prospect of translation of our rapidly expanding knowledge base must be tempered by the contradictions and uncertainties in the field; there is still much to be understood. The literature tells us that there is great diversity in the innate immune capacities of each cell type relevant to HIV infection. One major and evolving challenge is understanding the dynamic relationship between intracellular immunity and the specific circumstances of each individual cell or cell population: cell location, cell-cycle status, relative cytokine exposure, for example, will variegate each cell's interaction with HIV. We view HIV as the ultimate tool for molecular cell biology, which, correctly deployed, will teach us a great deal of fundamental human biology and continue to transform our understanding of health and disease leading to innovative new tools and therapeutics.

AUTHOR CONTRIBUTIONS

All authors contributed to conception, drafting, and final approval and are accountable for the integrity of the final manuscript. RS and LT contributed equally to this article.

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Natural Killer Cells in Human Immunodeficiency Virus-1 Infection: Spotlight on the Impact of Human Cytomegalovirus

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Human cytomegalovirus (HCMV) has been closely associated with the human race across evolutionary time. HCMV co-infection is nearly universal in human immunodeficiency virus-1 (HIV-1)-infected individuals and remains an important cofactor in HIV-1 disease progression even in the era of effective antiretroviral treatment. HCMV infection has been shown to have a broad and potent influence on the human immune system and has been linked with the discovery and characterization of adaptive natural killer (NK) cells. Distinct NK-cell subsets, predominately expressing the activating receptor NKG2C and the marker of terminal differentiation CD57, expand in response to HCMV. These NK-cell populations engaged in the long-lasting interaction with HCMV, in addition to characteristic but variable expression of surface receptors, exhibit reduced expression of signaling proteins and transcription factors expressed by canonical NK cells. Broad epigenetic modifications drive the emergence and persistence of HCMV-adapted NK cells that have distinct functional characteristics. NKG2C$^+$ NK-cell expansions have been observed in HIV-1 infected patients and other acute and chronic viral infections being systematically associated with HCMV seropositivity. The latter is potentially an important confounding variable in studies focused on the cellular NK-cell receptor repertoire and functional capacity. Here, focusing on HIV-1 infection we review the evidence in favor of "adaptive" changes likely induced by HCMV co-infection in NK-cell subsets. We highlight a number of key questions and how insights into the adaptive behavior of NK cells will inform new strategies exploiting their unique properties in the fight against HIV-1.

Keywords: human immunodeficiency virus, human cytomegalovirus, natural killer cells, NKG2C, CD57, adaptive

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INTRODUCTION

Natural killer (NK) cells are a diverse group of innate lymphocytes residing at the crossroads of innate and adaptive immunity (1). Their remarkable effector agility is achieved via expression of a wide array of receptors and integration of signals that are finely attuned to ensure self-tolerance, while permitting effective responses against viral assaults and tumor transformation. In addition to important immunoregulatory functions (2, 3), a number of murine studies support that NK cells can acquire immunological memory similarly to B and T cells (4–7). While antigen-specific NK responses have been documented in mice and more recently in primates (8), clear evidence for NK-cell memory

in humans is lacking. The NK-cell compartment in humans displays phenotypic and functional heterogeneity encompassing populations at various stages of maturation with distinct receptor combinations (9–11). In recent years, it has become apparent that variegated expression of inhibitory and activating receptors at the single cell level leads to a more diverse NK-cell repertoire than previously envisaged. Cytometry by time-of-flight has enabled us to profile the healthy human NK-cell repertoire, uncovering between 6,000 and 30,000 unique NK-cell subsets per individual (12). This observed diversity is generated by a combination of factors including genetic contributions (13, 14), along with differentiation in reprogramming in response to local tissue milieu (15) and infections/environmental factors (12). The substantial influence of environmental factors is supported by twin studies demonstrating that non-heritable factors exert a more profound and cumulative influence compared to heritable traits (16, 17). One such factor is human cytomegalovirus (HCMV), a widespread β-herpesvirus with a prevalence ranging from 40 to 100% depending on age, socioeconomic factors, and geographical region (18). In immunocompetent hosts, HCMV infection is usually subclinical leading to latency, whereas in immunosuppressed patients, including human immunodeficiency virus-1 (HIV-1)-infected and transplant patients, it remains a significant cause of morbidity and potentially life threatening complications (18). HCMV has a broad impact on immunity (16) and has recently been associated with the expansion of adaptive or memory-like NK-cell subsets (19, 20).

In the context of HIV infection, HCMV is a highly prevalent (21) and well-recognized opportunistic pathogen responsible for significant morbidity and mortality prior to the introduction of antiretroviral treatment (ART) (22, 23). However, despite the roll-out of effective ART, HCMV remains a significant cofactor in HIV-1 disease progression (24–26), displaying a strong association with systemic inflammation (27, 28), cardiovascular disease (29, 30), reduced immune resilience (31), and immune senescence (27). A recent report has highlighted the role of HCMV replication in intestinal barrier dysfunction in asymptomatic HIV-1 infection and contribution to persistent immune activation (32). It is thus highly relevant to increase our understanding of the complex inter-relationship between HCMV and HIV-1 and of the effects that it bears on the effector immune response. The recent identification of distinct NK-cell subsets with adaptive properties induced by HCMV has raised a number of intriguing questions, including the ability of other viruses to induce them and their physiological relevance in different disease settings. Here, we summarize findings on the molecular signature of HCMV-adapted NK cells and discuss how NK-cell phenotypic and functional features described in HIV-1 infection could partly reflect the immunological fingerprint of HCMV.

FEATURES OF CMV-ADAPTED NK CELLS—EMPHASIS ON HCMV

Evidence from both murine and human studies has demonstrated an important role for NK cells in antiviral defense against herpesviruses, in particular HCMV (33), reinforced by elaborate viral evasion strategies (34).

Although NK cells have been originally described to represent short-lived innate lymphocytes, they can exhibit persistent memory in response to infections. This is best exemplified by mouse CMV (MCMV) infection, where naive NK cells that express Ly49H, recognizing the virally encoded glycoprotein m157, were reported to clonally expand and to subsequently contract forming a pool of long-lived memory cells (6). MCMV-primed memory NK cells mount a robust response upon secondary challenge with enhanced interferon-γ (IFN-γ) secretion and cytotoxicity (6), but display reduced "bystander" functionality to heterologous infection suggesting the specialized nature of these cells (35).

Congruent with animal models, HCMV infection has been shown to induce an adaptive reconfiguration of the NK-cell compartment. Seminal work by Lopez-Botet's group described a higher proportion of NK cells expressing the DAP-12 coupled NKG2C receptor in healthy individuals seropositive for HCMV (36, 37). These observations have been extended to hematopoietic stem cell transplantation (38, 39) and solid organ transplantation (40). Expansion of these subpopulations of NK cells and their subsequent longevity resembled clonal expansion of adaptive immune cells. Expanded NKG2C$^+$ NK cells display a differentiated phenotype characterized by expression of CD57, increased expression of the inhibitory CD85j (38, 40), and a preferential oligoclonal pattern of inhibitory killer immunoglobulin receptors (KIRs) for self HLA-C1 and/or C2 allotypes (41, 42). In addition, they lack NKG2A, the inhibitory counterpart of NKG2C sharing specificity for HLA-E, and express lower levels of natural cytotoxicity receptors (NCR: NKp30 and NKp46) (36), CD161, CD7, and Siglec-7 (43–45) and have higher expression of CD2 involved in their activation (46, 47). Expression of other receptors such as NKG2D is maintained (36). The phenotypic hallmarks of adaptive NK cells are summarized in **Figure 1**. Of note, the magnitude of the HCMV imprint on NK-cell subsets varies within seropositive individuals (i.e., the NKG2C^{bright} phenotype is found in 50% of HCMV$^+$ individuals) and the adaptive NKG2C$^+$ compartment can persist in high frequencies for years (41). Subclinical or tissue specific reactivations of HCMV during latency may contribute to the maintenance of NK$^+$NKG2C$^+$ pool in addition to NKG2C copy number and age-related changes in NK-cell differentiation (48, 49). The exact ligand involved in recognition and the cellular mechanisms driving the expansion of NKG2C$^+$ NK cells are yet to be elucidated. It remains unclear whether this is mediated through interaction with its cellular ligand HLA-E alone, HLA viral loaded peptide or an unknown ligand of host or viral origin (41, 50–52).

The large phenotypic heterogeneity of adaptive NK cells extending beyond the NKG2C$^+$ subset, is illustrated by the detection of NK-cell subsets sharing numerous attributes of adaptive NK cells in individuals independent of NKG2C or in the absence of NKG2C (KLRC2-deficient individuals) and in transplant recipients of NKG2C null grafts (41, 47, 53). Strikingly, these HCMV-driven expansions encompass activating KIRs (53), suggesting their potential role in the recognition and response to HCMV.

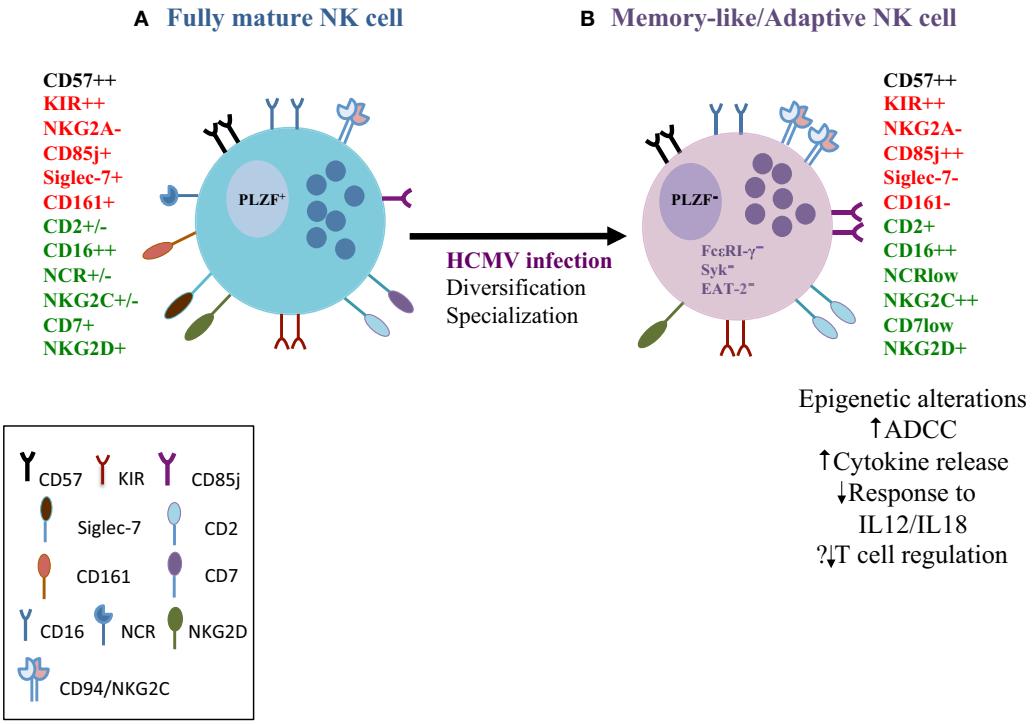

FIGURE 1 | The phenotypic, functional and molecular attributes of human cytomegalovirus (HCMV)-adapted natural killer (NK) cells. **(A)** As CD56dim NK cells go through the spectrum of differentiation they gradually lose expression of the inhibitory receptor NKG2A, natural cytotoxicity receptors and sequentially acquire more specific inhibitory receptors, such as inhibitory killer immunoglobulin receptors (KIRs) and CD85j. KIR acquisition is important in determining the functional fate of the NK cells. CD57 expression represents a terminal step in the differentiation process. Fully mature NK cells gain cytolytic ability and are efficient in mediating antibody-dependent cellular cytotoxicity (ADCC) **(B)** NK cells with adaptive features expanded in response to HCMV infection are distinct from conventional NK cells on the basis of expression of surface receptors, such as high expression of NKG2C, lower expression of the inhibitory Siglec-7, and down-regulation of the transcription factor promyelocytic leukemia zinc finger and key signaling molecules (FcεRI-γ, Syk, and EAT-2). Different combinations of expression patterns result in considerable heterogeneity among adaptive NK cells. Epigenetic diversification leads to altered target cell specificities and functional specialization that includes enhanced ADCC (increased IFN-γ and TNF-α against opsonized HCMV-infected targets) but reduced responsiveness to cytokine stimulation and reduced degranulation against autologous T cells. Red = inhibitory receptors; green = activating receptors.

Further reports described a subset of human NK cells deficient for the adaptor protein FcεRI-γ, which was strongly associated with HCMV seropositivity (54). FcεRI-γ$^-$ NK cells share a lot of the characteristics of adaptive NK cells, respond robustly to CD16 stimulation (55) and similar to NKG2C$^+$ cells display more vigorous effector responses to HCMV-infected targets, but only in the presence of HCMV-specific antibodies (54, 56). NK cells lacking FcεRI-γ expand in response to HCMV-infected targets accentuated by the presence of anti-HCMV antibody, highlighting the role of specific humoral immunity in also favoring their preferential expansion (57–59). Interestingly, these cells also responded to herpes-simplex virus-1 (HSV-1)-infected targets in the presence of HSV-1 plasma (54) demonstrating cross-protection to other viruses. The enhanced effector function of this subset was attributed to selective and more potent signaling through the CD3ζ chain, which has three immunoreceptor tyrosine-based activation motifs. Subsequently, CD2 has been identified as a key co-stimulatory receptor synergizing with CD16 to stimulate increased cytokine production in adaptive NK cells (47). Global epigenetic profiling has identified commonalities between adaptive NK cells and memory CD8 T cells (58, 60). These adaptive NK cells are marked by DNA methylation silencing of the transcription factor, promyelocytic leukemia zinc finger (PLZF), as well as stochastic down-regulation of several signaling molecules, such as Syk, EAT-2, and DAB-2 (58, 60). PLZF is known to interact with several target genes, including IL12RB2, IL18RAP, and KLRB1 (61), explaining the lack of responsiveness to IL12/IL18 stimulation (58). However, in comparison to conventional NK cells, adaptive NK cells display augmented IFN-γ and TNF-α production when triggered via antibody-dependent cellular cytotoxicity (ADCC); the hypomethylated IFN-γ and tumor necrosis factor (TNF) regulatory regions in adaptive NK cells provide a mechanism for increased cytokine production (58, 60). Interestingly, adaptive NK cells display reduced degranulation toward activated autologous T cells (58), which may impact on the regulation of immune responses.

Taken together, these results suggest the heterogeneity and functional specialization of adaptive NK cells in the immunosurveillance of infected cells and functional bias toward ADCC (**Figure 1**). Whereas the expansion of adaptive NK cells may serve as a strategy to control HCMV, during its life long interaction

with the host, it remains unclear whether other viral infections can induce adaptive properties in NK cells. Although potential cross-reactivity of adaptive NK cells could confer an advantage in the tumor setting such as reduced relapse risk in leukemia patients (62, 63), their role in the control of heterologous infections or post vaccination is less well defined (64, 65).

SKEWING AND ADAPTATION OF NK CELLS TO HIV-1 INFECTION: THE CONFOUNDING EFFECT OF HCMV

Accumulating data support an important role for NK cells in the control of HIV-1 infection and protection against disease acquisition (66–68). These stem from elegant genetic studies linking specific KIR/HLA combinations with HIV-1 outcome (66, 67), functional studies where protective KIR alleles are associated with enhanced NK-cell cytolytic function *in vitro* (69) and evidence of KIR-facilitated immune pressure on HIV-1 to escape NK-cell recognition (70). However, chronic HIV-1 infection is known to alter NK-cell composition and effector function. This has been documented by a number of studies with often conflicting results, which can be attributed to a number of factors including the influence of immunogenetics, disease state, and the cross-sectional nature of studies. The latter have not always adequately controlled for a number of confounding factors such as age, gender, ethnicity, and HCMV serostatus among HIV-1-infected and HIV-1-negative controls. Given the high prevalence of HCMV co-infection within HIV cohorts and the profound skewing and adaptation of NK cells to HCMV, this is an important variable to consider when interpreting findings.

HIV-1 viremia is associated with a significant and pathological redistribution of the NK compartment with the emergence of an aberrant $CD56^{-}CD16^{+}$ NK-cell subset (71, 72). This rare population displays phenotypic perturbations, including down-regulation of the activating NCRs, and features in common with mature $CD56^{dim}$ NK cells (72, 73). It has been proposed to represent an activated subset generated from chronic target engagement with impaired function. Recent studies have demonstrated that a decreased expression of the c-lectin-type inhibitory receptor, Siglec-7, on NK cells occurs early during HIV-1 infection and precedes the loss of CD56 (74). Expression of Siglec-7 is not affected in long-term non-progressors (LTNP), and ART leads to a progressive restoration of NK-cell subsets (74). Paralleling the observations in HIV-1 infection, HCMV reactivation in patients undergoing umbilical cord blood transplantation has been shown to induce the expansion of the $CD56^{-}/CD16^{+}/Siglec\text{-}7^{-}$ NK-cell subset (38). The expansion of hypofunctional $CD56^{-}$ NK cells following HCMV reactivation likely occurs when T-cell immunity is impaired and may also reflect the modulating effects of HCMV. It remains to be determined whether the $CD56^{-}/CD16^{+}$ subset represents a subgroup of NK cells with adaptive features that has become anergic following repeated stimulation.

A number of other studies have reported a variable degree of perturbations in the NK-cell repertoire consistent with a dichotomous effect of viremia, including down-regulation of activating NK-cell receptors and up-regulation of expression of inhibitory NK receptors (iNKRs) (75–77). Collectively, these changes have been described to contribute to defective NK-cell function described in HIV-1 infection (76, 77). Although the HCMV serostatus is not always considered in these studies, it is plausible that these changes are biased by HCMV co-infection and possible reactivation with increasing immunosuppression. Along these lines, the observed down-regulation of NCRs, stable expression of NKG2D, and higher levels of CD85j and skewing of inhibitory KIRs (although not consistently reported) bear phenotypic resemblance to NK-cell subsets with adaptive features described in HCMV infection. NK cells in HIV-1 infection exhibit a higher ratio of $CD57^{+}$ to $CD57^{-}$ due to the loss of $CD57^{-}$ cells in comparison to healthy controls; however, this comparison may be confounded by the HCMV status of these individuals, which was not reported (78). A shift toward a more mature terminally differentiated NK-cell phenotype is nonetheless supported by a study of HIV-1 infected individuals on effective ART, demonstrating that HCMV accelerates age-related increases in CD57 expression (79).

The most convincing evidence of the impact of HCMV co-infection on the NK-cell repertoire in HIV-1 infection comes from reports on NKG2C expression. Guma et al. originally proposed that HCMV co-infection is responsible for the expansions of $NKG2C^{+}$ NK cells encountered in HIV-1 infected individuals (80). These findings were further supported by additional studies when the HCMV serostatus was taken into consideration (81, 82). The dramatic expansion of $NKG2C^{+}$ NK cells in HIV-1 infected individuals was accompanied by a decrease in the expression of NKG2A leading to a low $NKG2A/C^{+}$ NK-cell ratio; these changes were attributed to concomitant infection and/or HCMV reactivation rather than being a consequence of HIV-1 infection alone (82). A number of reports describe $NKG2C^{+}$ NK-cell expansions in several acute and chronic viral infections, being systematically associated with HCMV co-infection (83–86). Although the relative increase in the proportions of $NKG2C^{+}$ NK cells between HIV-1-infected and HIV-1-uninfected HCMV seropositive individuals varies between studies and cohorts (80, 81), the data suggest that the impact of HCMV exposure is potentially greater in HIV-1 infection. It has been suggested by animal models that the differentiation of adaptive NK cells is driven by inflammation (87). Thus, it is plausible that adaptive NK-cell expansions may be inflated in HIV-1 infected individuals, as a result of lack of immune control, ongoing immune activation and higher infectious burdens, including HCMV. One could speculate that the size of the HCMV imprint represents a compensatory mechanism in antiviral defense especially when T-cell-mediated control is impaired (88). It remains uncertain whether HCMV reactivation occurs alongside acute infection or alternatively whether pre-existing HCMV primed NK-cell subsets expand in response to secondary viral infection alone. HIV-1 causes down-regulation of HLA-A, B while retaining HLA-E expression (89, 90), similar to HCMV maintaining/stabilizing HLA-E expression (91, 92). Thus, a direct effect of HIV-1 on $NKG2C^{+}$ NK-cell expansion is conceivable. The

recently reported down-regulation of HLA-C by most primary HIV-1 clones (93) raises questions about the ability of HCMV expanded NKG2C$^+$ NK cells, preferentially expressing self-HLA-C KIRs, to recognize "missing-self" on HIV-infected targets compared to mature educated NK cells.

Open questions remain regarding not only the mechanism but also the clinical implications of such HCMV-NK-cell interaction in terms of protection against acquisition and HIV-1 disease progression. NKG2C deletions have been linked to a higher risk of contracting HIV-1, in addition to accelerated disease progression and elevated pre-treatment viral load (94). Although these findings are interesting, this study did not report and correct for the influence of HCMV co-infection. One could speculate that the expansion of NKG2C$^+$ NK cells in HCMV seropositive individuals may confer protection against primary HIV-1; this notion is however not supported by some older observations that prior infection with HCMV is associated with low CD4 count, progression to AIDS and increased mortality (95). It has been suggested that maturation leads to divergence and increased NK-cell receptor diversity was found to be associated with an increased risk of HIV-1 acquisition in a small cohort of high-risk women (96). Given that viral challenge may increase receptor diversity, further work is required to determine whether this represents reduced plasticity to new challenging pathogens or whether it is linked to other immune characteristics such as exhaustion. Recently, a subpopulation of PD1$^+$ NK cells, mainly composed of fully mature NK cells, has been described in HCMV$^+$ individuals (97). It would be of interest to assess whether NK cells expanded in HCMV/HIV-1 co-infection succumb to continuous stimulation and examine the factors that may contribute to the induction of PD1 in this setting. PD1 signaling could therefore down-regulate not only T-cell-mediated responses but also innate responses, and this mechanism may be particularly prominent in HIV-1 infection (98).

Conversely, a link between a mature NK-cell compartment (CD57$^+$) and decreased levels of viral load and immune activation at the time of the primary HIV-1 infection has been reported. Those patients with a mature NK profile at inclusion showed a better early response to ART in comparison to patients with an immature NK profile (99). However, the HCMV serostatus of these individuals is not recorded and the status of NK cells at the point of infection is not known. Whether mature CD57$^+$ or NKG2C$^+$CD57$^+$ NK cells represent adaptive NK cells that contribute directly to better virus control during acute HIV-1 infection and how their role evolves during chronic infection remain unclear.

In agreement with the findings in HCMV seropositive individuals, an NK-cell population that lacks FcεRI-γ expression and has superior ADCC activity has been identified in HIV-1 viremic individuals and shown to persist following virological suppression with ART (100, 101). This subset shares some phenotypic characteristics with adaptive NK cells induced by HCMV (100). Although this subset is associated with HCMV antibody levels in the general population, in HIV-1-infected individuals correlates with inflammatory markers (100). The long-term effects of expansion of FcεRI-γ-deficient NK cells in HIV-1 infection needs to be further elucidated given a possible role in tumor surveillance. Nonetheless, the identification of a subset with enhanced ADCC activity in HIV-1 infection has potentially important implications for the design of vaccine strategies aimed at generating ADCC-promoting antibody responses.

These collective data demonstrate that a number of the phenotypic NK-cell features described in HIV-1 bear the trademarks of HCMV infection (**Figure 2**). With increased definition of the assortment of NK-cell subsets with adaptive features driven by HCMV infection and the increased appreciation of HCMV in driving ongoing immune activation even during effective ART, it would be important to reassess the NK-cell repertoire composition, their response potential in different phases of infection and stimulus-dependent functional properties. A comprehensive analysis of the transcriptional signatures and epigenetic modifications of NK cells in HIV-1 infection is lacking and worth exploring.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The potent effector function of NK cells and the rapidity of NK-cell response have identified them as key areas for research. Recent reports about the diversity of NK-cell repertoire and ability to assume adaptive features in response to HCMV infection and even display memory-like responses to cytokines (102) and antigen-specific responses in primates (8) have opened up prospects for the generation of new therapies. HCMV co-infection is highly prevalent in HIV-1 infected cohorts and remains an important cofactor in disease progression even in the era of ART. Both HIV-1 and HCMV as well as immune activation can further shape NK-cell responsiveness and differentiation. It is therefore important to capture the diversity of the NK-cell repertoire and identify potentially novel adaptive signatures of NK-cell subsets with preserved activation pathways. Whereas a number of questions remain regarding the epigenetic diversification,

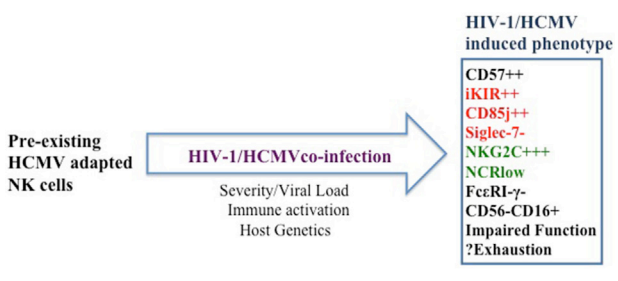

FIGURE 2 | Proposed model of the cumulative effect of human cytomegalovirus (HCMV) and ongoing immune activation on natural killer (NK) cells. Pre-existing HCMV-adapted NK cells expand during human immunodeficiency virus-1 infection to a variable degree depending on the tempo of HCMV reactivation, underlying level of immune activation, decreased T-cell-mediated control, and host genetics. HCMV co-infection accelerates NK-cell maturation and partly underlies the expansion of NK subsets with adaptive features in addition to the emergence of an aberrant CD56$^-$CD16$^+$ NK-cell subset. Whether these subsets become progressively dysregulated or exhausted remains to be determined.

development, and persistence of NK cells with adaptive properties, elucidating how clonal NK-cell populations can be directed or reshaped will critically inform our ability to harness NK cells toward a therapeutic goal.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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The Dynamic Interplay between HIV-1, SAMHD1, and the Innate Antiviral Response

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The innate immune response constitutes the first cellular line of defense against initial HIV-1 infection. Immune cells sense invading virus and trigger signaling cascades that induce antiviral defenses to control or eliminate infection. Professional antigen-presenting cells located in mucosal tissues, including dendritic cells and macrophages, are critical for recognizing HIV-1 at the site of initial exposure. These cells are less permissive to HIV-1 infection compared to activated CD4$^+$ T-cells, which is mainly due to host restriction factors that serve an immediate role in controlling the establishment or spread of viral infection. However, HIV-1 can exploit innate immune cells and their cellular factors to avoid detection and clearance by the host immune system. Sterile alpha motif and HD-domain containing protein 1 (SAMHD1) is the mammalian deoxynucleoside triphosphate triphosphohydrolase responsible for regulating intracellular dNTP pools and restricting the replication of HIV-1 in non-dividing myeloid cells and quiescent CD4$^+$ T-cells. Here, we review and analyze the latest literature on the antiviral function of SAMHD1, including the mechanism of HIV-1 restriction and the ability of SAMHD1 to regulate the innate immune response to viral infection. We also provide an overview of the dynamic interplay between HIV-1, SAMHD1, and the cell-intrinsic antiviral response to elucidate how SAMHD1 modulates HIV-1 infection in non-dividing immune cells. A more complete understanding of SAMHD1's role in the innate immune response to HIV-1 infection may help develop stratagems to enhance its antiviral effects and to more efficiently block HIV-1 replication and avoid the pathogenic result of viral infection.

Keywords: HIV-1, sterile alpha motif and HD-domain containing protein 1, infection, innate immunity, myeloid cells

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INTRODUCTION

Innate immunity is the cell-intrinsic defense mechanism that senses incoming pathogens and is characterized by type-I interferon (IFN-I) induction and the release of inflammatory cytokines that upregulate antiviral IFN-stimulated genes (ISGs) (1, 2). The activation of the innate response to pathogens is dependent on cellular pattern recognition receptors (PRRs) that detect pathogen-associated molecular patterns (PAMPs), including viral structures or nucleic acids. Interferon-inducible protein IFI16 and cyclic GMP-AMP synthase (cGAS) are cytosolic sensors of HIV-1 that detect viral DNA (3, 4). Recognition of PAMPs results in induction of IFN-I and ISGs to control initial infection and spread, while the concomitant induction of the inflammatory response and cytokines can initiate

adaptive immune responses (5, 6). Modulation of IFN-I activation is essential for viral clearance. However, overstimulation of IFN pathways can lead to inflammatory autoimmune disease (7).

HIV-1 is sensitive to ISGs and the IFN-induced antiviral response; so it is not surprising that HIV-1 is a poor inducer of IFN (8). HIV-1 benefits from evading innate immune activation and utilizes a variety of tactics to escape detection (9, 10). Professional antigen-presenting cells located in mucosal tissues, including dendritic cells (DCs) and macrophages, are critical for recognizing HIV-1 at the site of initial exposure. However, these cells are less permissive to HIV-1 infection compared to activated $CD4^+$ T-cells, mainly due to host restriction factors that control the establishment or spread of viral infection. Several host proteins can restrict HIV-1 at various points in the viral lifecycle, including APOBEC proteins, TRIM5α, and tetherin (11–13). However, HIV-1 can exploit innate immune cells and their cellular factors to avoid detection and clearance by the host immune system (13).

SAMHD1 is host protein capable of blocking replication of retroviruses and several DNA viruses in cells (14–18). SAMHD1 is constitutively expressed at various levels in all cell types and highly expressed in myeloid lineage and resting $CD4^+$ T-cells (14, 15, 19). IFN-I treatment increases SAMHD1 expression in certain cell types with low endogenous SAMHD1 levels (20, 21). SAMHD1 has been implicated as a negative regulator of the IFN-I inflammatory response (22–24), however, the underlying mechanism is not fully understood. While HIV-2 encodes the SAMHD1 antagonist Vpx, the more pathogenic HIV-1 does not. It was hypothesized that HIV-1 lacks a countermeasure against SAMHD1 because it is beneficial for infection. In this review, we will discuss the contributions of SAMHD1 to both the direct restriction of HIV-1 and to the modulation of the antiviral innate response and to analyze the hypothesis that HIV-1 restriction by SAMHD1 leads to a diminished induction of innate immunity.

INNATE IMMUNE SENSING OF HIV-1

Although HIV-1 can be sensed by the innate immune system, the prevailing theory is that HIV-1 avoids immune surveillance through poor replication in immune cells causing ineffective triggering of innate cytosolic sensors (25). Several studies have identified the molecular basis of cytosolic sensors important for targeting viral pathogens. Here, we focus on HIV-1 DNA as a trigger of the innate antiviral response. After sensing viral DNA, cGAS generates the second messenger, cyclic guanosine monophosphate-adenosine monophosphate (26–28), that activates the stimulator of IFN genes (STING) (6). STING activation leads to phosphorylation of TANK-binding kinase 1 (TBK1) and the subsequent phosphorylation and dimerization of IFN-regulatory transcription factors IRF3 and IRF7. Nuclear translocation of the IRF3/IRF7 homo-or-hetero dimers will activate IFN-I gene expression (**Figure 1**). This signaling cascade results in an upregulation of IFN-I and ISGs as a defense against viral infection (29, 30). Reverse transcribed HIV-1 DNA was identified as the trigger to the cGAS-STING pathway (3). Although cGAS is the primary sensor of cytosolic viral DNA, IFI16 can also act as a sensor of HIV-1 single-stranded DNA that induces an IFN-β response in macrophages by a cGAS-STING-dependent pathway (4).

INTRODUCTION TO SAMHD1

Human SAMHD1 is a 626-amino acid protein containing an N-terminal nuclear localization signal followed by a sterile-alpha motif and histidine/aspartic acid (HD) domain. SAMHD1 is a deoxynucleoside triphosphate triphosphohydrolase (dNTPase) (33, 34) that converts dNTPs into the constituent deoxynucleoside and inorganic triphosphate upon stimulation by dGTP or GTP (33–35). SAMHD1 and ribonuclease reductase, the enzyme responsible for *de novo* dNTP synthesis through the conversion of ribonucleotide diphosphates to deoxyribonucleotides (36), are allosterically regulated to achieve balanced intracellular dNTP levels in a cell-cycle-dependent manner (37). During G_1 to S-phase transition in actively proliferating cells, ribonuclease reductase expression increases, leading to expansion of the dNTP pool to facilitate DNA synthesis (38, 39). The activity of SAMHD1 is activated by high dNTP levels, and degradation of nucleic acids in the absence of DNA replication protects the cell from innate immune activation and cancer development (40, 41). Mutations in SAMHD1 that affect its enzyme activity are associated with Aicardi-Goutières syndrome (AGS), an encephalopathic autoimmune disease characterized by symptoms mimicking chronic viral infection (22). The accumulation of intracellular dNTPs caused by mutations in the genes encoding proteins involved in nucleic acid metabolism, including SAMHD1 and TREX1 (42), are sensed by PRRs, resulting in aberrant production of IFN-I (43). AGS patients present with increased production of IFN-α, the chemokine most characteristic of congenital virus infection. AGS patients with SAMHD1 mutations can present with signs of lupus erythematosus, with many symptoms mimicking those of HIV-1 infection (22, 44). Furthermore, cells isolated from AGS patients with homozygous *SAMHD1* mutation revealed that SAMHD1-deficient monocytes supported productive infection by HIV-1 (20), suggesting a link between SAMHD1 function in both autoimmunity and HIV-1 restriction.

Long interspersed element 1 (LINE-1) is the only autonomous and active human retroelement capable of producing new genomic insertions through its endogenous endonuclease and reverse transcriptase activities (45, 46). A study on AGS-related SAMHD1 mutations indicate that all disease-related mutations reduced LINE-1 inhibition in dividing cells (47). Recent work suggests that SAMHD1 potently blocks LINE-1 transposition in cycling cells by triggering the sequestration of LINE-1 ORF1p into stress granules (48). Impaired inhibition of LINE-1 retrotransposition may lead to triggering of the autoimmune response by stimulating toll-like receptors (TLRs) (49), although this has not been confirmed. Impaired dNTPase activity and LINE-1 suppression by mutant SAMHD1 could explain the chronic inflammatory response characteristics of AGS disease. These studies outlining the pathogenic effect of SAMHD1 deficiency on autoimmune disease implicate SAMHD1 as a negative regulator of the innate immune system.

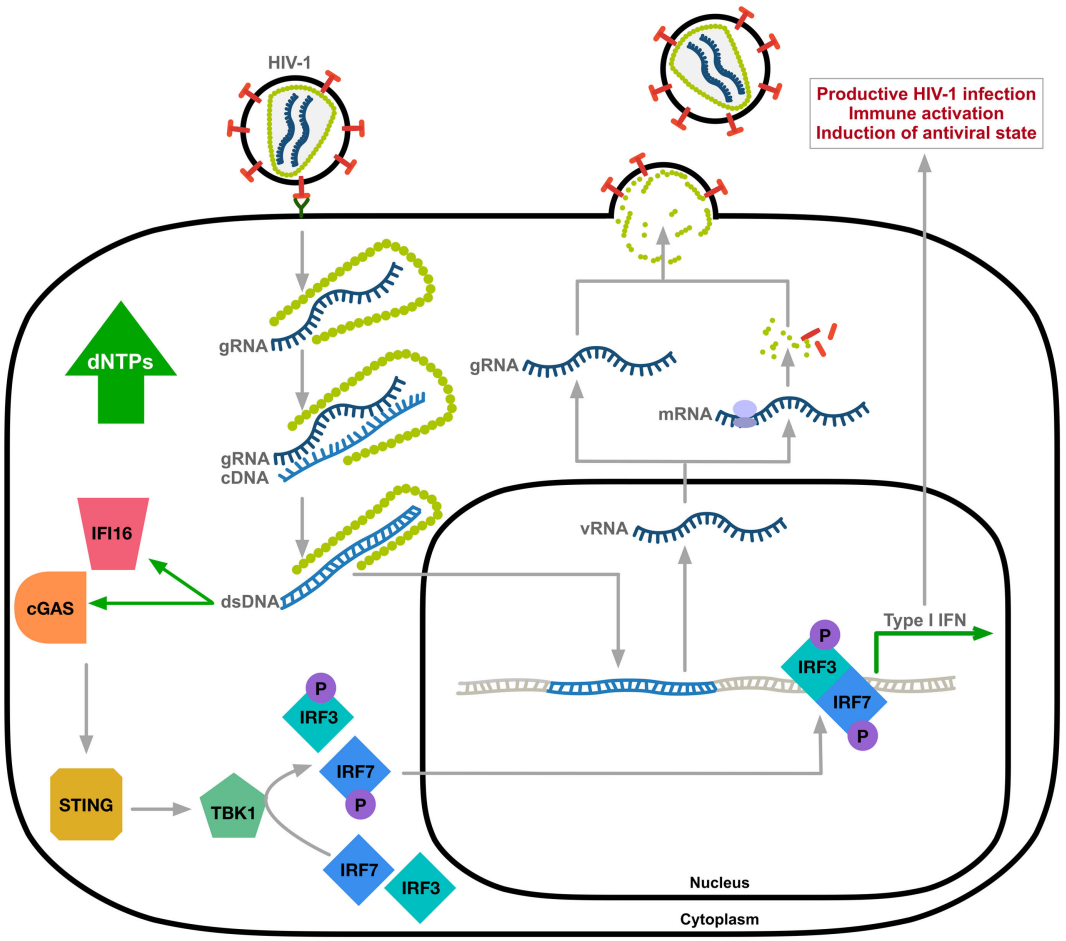

FIGURE 1 | Innate immune sensing of HIV-1 DNA. HIV-1 undergoes uncoating through the interaction between viral capsid and host factors (31, 32). Reverse transcribed HIV-1 DNA, mainly abortive transcripts, activates cytosolic DNA sensors IFI16 and cyclic GMP-AMP synthase (cGAS) resulting in TANK-binding kinase 1 (TBK1)-mediated phosphorylation and nuclear translocation of hetero-or-homo dimers of interferon regulatory factor-3 (IRF3) and IRF7 and induction of type-I IFN response. Expression of ISGs allows for immune activation and the induction of an antiviral state of the cell. gRNA, HIV-1 genomic RNA; cDNA, complementary DNA; vRNA, viral RNA; dsDNA, double-stranded DNA; STING, stimulator of IFN genes; the letter P indicates phosphorylation.

SAMHD1-MEDIATED HIV-1 RESTRICTION

HIV-1 replicates inefficiently in non-diving cells, such as quiescent CD4$^+$ T-cells, DCs, and monocytes. HIV-1 infection can be enhanced in these cells by Vpx, an accessory protein encoded by HIV-2 and certain lineages of simian immunodeficiency viruses (SIVs) (50, 51). This hinted at the existence of a cellular restriction factor counteracted by Vpx (50). SAMHD1 was identified as the mystery HIV-1 restriction factor by a mass spectrometry analysis of cellular proteins immunoprecipitated from cells expressing Vpx (14, 15). Vpx interacts with the C-terminal domain of SAMHD1, thereby initiating proteasomal degradation by an E3 ubiquitin ligase complex, and relieving SAMHD1-mediated lentiviral restriction (14, 15, 52, 53).

The mechanism and modulation of SAMHD1-mediated HIV-1 restriction is an area of intense scrutiny (**Figure 2**). Overexpression of SAMHD1 in PMA-treated monocytic U937 cells results in a depletion of dNTP levels (54). It was later confirmed that SAMHD1 restricts the replication of retroviruses and several DNA viruses by depleting the concentration of intracellular dNTPs to levels insufficient to support viral DNA synthesis (14–18, 54, 55). Structural studies strengthened a model of nucleotide-dependent tetramer assembly of SAMHD1 (56–58), where GTP binds to guanine-specific allosteric sites and dNTP binds to non-specific activator sites, initiating the formation of enzymatically active tetramers with the catalytic core of the HD domain (33, 34, 37, 59). Moreover, binding of single-stranded nucleic acids (ssNAs) to the dimer–dimer interface of SAMHD1 inhibits the formation of the catalytically active tetramer (60).

As SAMHD1 is also highly expressed in activated CD4$^+$ T-cells that support productive infection, several studies demonstrated posttranslational modification as a means of mechanistic regulation of SAMHD1 function in restricting HIV-1. SAMHD1 is phosphorylated at several residues; however, phosphorylation of threonine 592 was identified as essential for the negative

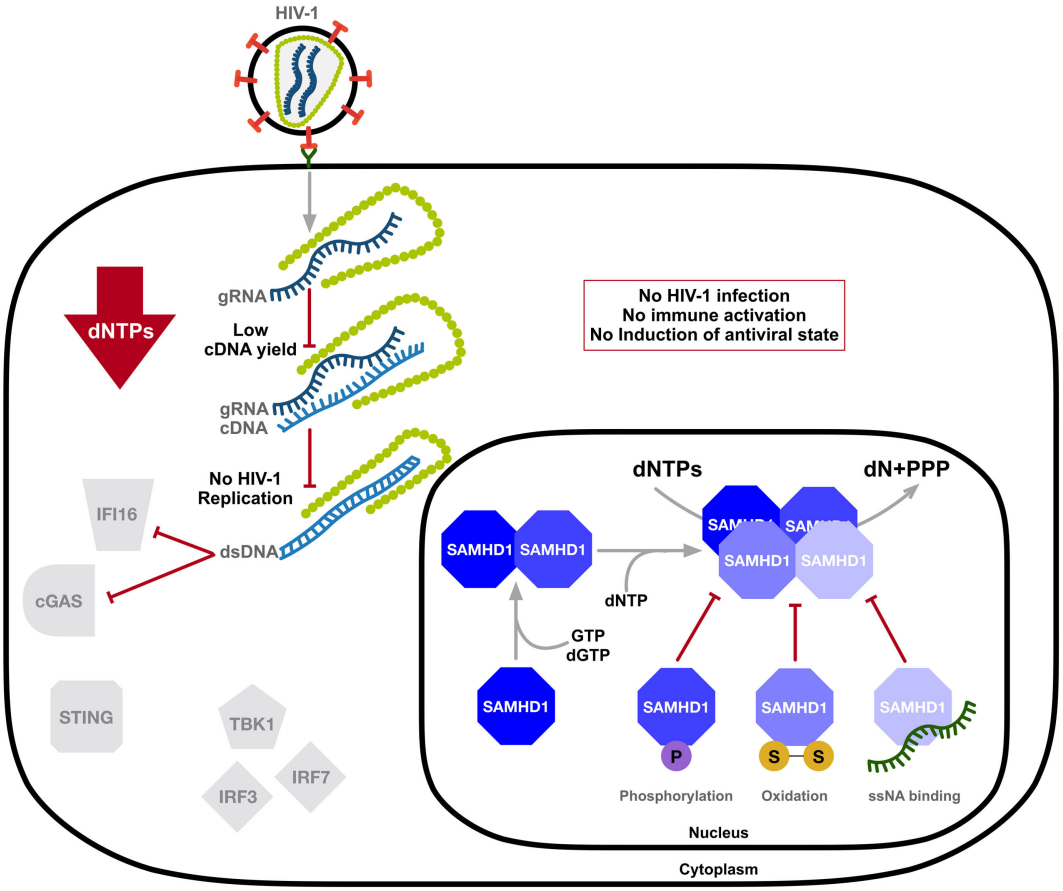

FIGURE 2 | SAMHD1 negatively regulates the innate immune sensing of HIV-1 DNA. SAMHD1 blocks HIV-1 infection through intracellular dNTP depletion, thus preventing the accumulation of viral DNA accessible to sensing by IFI16 and cyclic GMP-AMP synthase (cGAS) and the activation of the type-I interferon (IFN-I) response. The dNTPase activity of SAMHD1 is structurally regulated. Consecutive binding of dGTP/GTP and any dNTP to two allosteric sites provokes formation of the catalytically active tetramer, which can be destabilized by phosphorylation, oxidation, or the binding of single-stranded nucleic acids (ssNAs). dN, deoxynucleosides; PPPs, triphosphate; two linked letters S indicate the disulfide bond.

modulation of its HIV-1 restriction activity (61–65) and tetramer formation (66, 67). SAMHD1 is phosphorylated by cyclin-dependent kinase 1 (CDK1) and CDK2 in complex with cell cycle regulatory protein cyclin A. This regulation of SAMHD1 function is associated with the cell cycle, as CDK1 and cyclin A are highly expressed in dividing cells. Furthermore, S-phase requires elevated dNTP levels, indicating modulation of the dNTPase activity of SAMHD1 during the cell cycle (64). SAMHD1 protein levels may be altered during various stages of the cell cycle depending on different cell types (68, 69). Interestingly, proliferation-induced oxidation of SAMHD1 by hydrogen peroxide reversibly inhibits its dNTPase activity through the formation of tetramer-inhibiting disulfide bonds (70), suggesting a dynamic structure-based regulatory mechanism of SAMHD1's dNTPase activity that is influenced by the cell cycle (**Figure 2**).

Although the accepted consensus is that SAMHD1 restricts HIV-1 infection through the depletion of intracellular dNTPs, several studies suggested the existence of an additional yet-undiscovered mechanism of SAMHD1-mediated retroviral restriction. This undefined antiviral activity appears to be dependent on phosphorylation (61, 63, 65) and is not fully dependent on low dNTP levels (71). SAMHD1 acts as a ssNA binding protein that degrades single-stranded DNA and RNA *via* a metal-dependent 3′–5′ exonuclease activity *in vitro* (72–74). It has been suggested that SAMHD1 utilizes its nucleic acid binding potential to exert a ribonuclease activity against incoming HIV-1 genomic RNA in a phosphorylation-dependent manner (75). SAMHD1 was shown to restrict retroviruses though degradation of HIV-1 RNA in human monocyte-derived macrophages (MDMs), monocytes, and $CD4^+$ T-cells (75, 76). It was proposed that SAMHD1 degrades incoming HIV-1 gRNA, thereby restricting infection and preventing innate immune sensing of viral nucleic acids. However, recent studies have been unable to confirm the controversial findings (55, 77–79). As a nuclear-localized protein (80), incoming viral genomic RNA would be inaccessible by SAMHD1 for hydrolysis. Additional studies showed that dNTPase inactive SAMHD1 mutant retained exonuclease activities *in vitro*, indicating the exonuclease activity could not be attributed to the known dNTP-binding active site (77). Seamon et al. (77) suggested that the nuclease activity attributed to SAMHD1 was

due to contamination during purification. Cell-based assays also failed to recapitulate the findings, thereby confirming the lack of SAMHD1 RNase activity to restrict HIV-1 in infected cells (55, 78). Ryoo et al. suggested that the differences in experimental conditions are responsible for the conflicting results, including a shorter infection time and the use of RNaseH-defective reverse transcriptase (81). They further identified SAMHD1 as a phosphorolytic not hydrolytic ribonuclease (82).

THE INTERSECTION OF HIV-1, SAMHD1, AND THE INNATE ANTIVIRAL RESPONSE

SAMHD1 cDNA was originally identified as a ortholog of the mouse IFN-γ-induced gene *Mg11* in human DCs (83). A link to the innate immune response was strengthened by the discovery that cytokines, including toll-like agonists and IFNs, can induce SAMHD1 expression (84, 85). Cell lines treated with IFN-I (21, 86) and human primary monocytes treated with IFN-α and IFN-γ (20, 84, 87) show enhanced expression of SAMHD1. While SAMHD1 is highly expressed in MDMs, monocyte-derived dendritic cells (MDDCs), and primary $CD4^+$ T-cells, IFN treatment does not increase SAMHD1 protein levels further (21, 88–90). However, treatment of MDMs and MDDCs with IFN-I results in reduced phosphorylation of SAMHD1 at residue T592 (61), indicating a shift from catalytically inactive to active SAMHD1. Interestingly, the *SAMHD1* promoter is a direct target of IRF3. The overexpression and activation of IRF3 enhances SAMHD1 promoter activity in HeLa cells (86).

HIV-1 does not trigger a sterilizing immune response (91) and is a poor activator of inflammatory pathways (8), resulting in an impaired response to HIV-1 and the development of persistent infection. The DC response to HIV-1 infection contributes to this dysfunctional immune response (92). Myeloid cells constantly sample the cellular environment to identify pathogens and send out danger signals in the form of IFN-I. DCs are essential for activating the adaptive immune response to infection, as maturation leads to T-cell responses through antigen priming (91, 93). Interestingly, HIV-1 infects DCs without activating an effective antiviral response. As SAMHD1 limits HIV-1 cDNA synthesis in myeloid cells (14, 54), it was hypothesized that degradation of SAMHD1 by Vpx in DCs would result in productive HIV-1 infection and the synthesis of viral proteins that would directly enter antigen presentation, thereby strengthening the T-cell response to infection (94). This could be why the *vpx* gene was lost from the ancestor of HIV-1 during the coevolution of primate SAMHD1 and lentiviruses (95).

Vpx-mediated degradation of SAMHD1 in DCs leads to enhanced HIV-1 infection, and studies in primary MDMs and MDDCs indicate that Vpx-mediated SAMHD1 degradation results in cGAS stimulation and IRF3 activation (3). Early work suggested that enhanced infection by SAMHD1 depletion leads to DC maturation (94). A study utilizing coculture of autologous activated $CD4^+$ T lymphocytes with SAMHD1-deficient MDDCs infected with primary clinical HIV-1 isolates indicated enhancement of both infection and IFN response (96). Interestingly, cocultured primary T-lymphocytes, but not HIV-1, trigger a decrease in SAMHD1 expression in MDDCs independent of dNTP levels (96). This study suggests that crosstalk between lymphocytes and DCs induces downregulation of SAMHD1 expression, a requirement for stimulation of HIV-1 production in DCs, thereby inducing the innate sensing of HIV-1 and DC maturation (96).

Conversely, recent work indicates that DC maturation, measured by CD83 and CD86 expression, does not occur in SAMHD1-deficient cells due to additional manipulation of the innate immune system by HIV-1 (97). HIV-1 suppresses TLR-induced maturation of DCs independent of SAMHD1 expression, although Vpx-mediated depletion of SAMHD1 enhanced the effect of HIV-1 infection on lipopolysaccharide-induced DC maturation (97). Vesicular stomatitis virus G-protein-pseudotyped HIV-1 suppressed maturation similar to strains containing HIV-1 envelope protein, suggesting that viral replication, not envelope-receptor interactions, is required for suppression of maturation (97). Removing the SAMHD1-mediated block of reverse transcription resulted in a stronger suppression of maturation. Although infection and subsequent innate immune sensing in DCs is blocked by SAMHD1, HIV-1 maintains an additional SAMHD1-independent mechanism of suppressing DC maturation through downregulation of TLRs (97).

Two additional models suggest that, in MDDCs, HIV-1 attempts to hide its genomic RNA and newly synthesized cDNA from cytosolic sensors by obstructing the nucleic acids using viral capsid. The models differ with respect to the effect of recruitment of cellular cyclophillins and cleavage and polyadenylation-specific factor 6 (CPSF6) by capsid. One model suggests increased cyclophillin A (CypA) binding to the capsid increases sensitivity to innate sensing (94), while another proposes CypA binding coordinates uncoating, reverse transcription, and nuclear import of the preintegration complex (98), all to minimize the exposure of viral nucleic acids to cytosolic sensors. Future work is needed to clarify the contribution of CypA and SAMHD1 to the negative regulation of the innate immune response in myeloid cells to provide insight into HIV-1 mechanisms of evasion.

Non-cycling $CD4^+$ T-cells and macrophages are less permissive to HIV-1 because of SAMHD1. However, during HIV-1 infection *in vivo,* activated $CD4^+$ T-cells and macrophages are infected due to phosphorylation of SAMHD1. Although cytosolic HIV-1 DNA is abundant in these permissive cells, a cell-autonomous IFN response is not triggered (99). This is due at least in part to host protein TREX1. As a single-stranded DNA exonuclease, TREX-1 digests cytoplasmic DNA from retroviral DNA intermediates, thereby preventing the activation of mislocalized DNA by an innate immune sensor (99). Cytosolic HIV-1 DNA is accumulated in HIV-1 infected TREX1-deficient $CD4^+$ T-cells and macrophages, which leads to inhibition of TBK1-dependent IFN-I response (99). This suggests a competition between two DNA sensors: cGAS leading to antiviral effects, and TREX1 leading to enhanced viral replication (100).

REMAINING QUESTIONS

Although it is clear that HIV-1 utilizes a variety of mechanisms to evade myeloid cell activation, controversial questions still exist.

Conflicting reports could be due to the use of different cell types, and the differential use of clinical HIV-1 isolates, replication-competent lab strains, or pseudotyped virus. It is essential to confirm experimental findings with primary cells that accurately recapitulate *in vivo* mucosal infection sites. Further understanding of the strategies HIV-1 utilizes to evade the innate response will allow for better ideas on how to increase the innate immune response to HIV-1.

The existence of a yet-undiscovered mechanism of HIV-1 restriction that is dependent on phosphorylation cannot be overlooked (63). Pretreatment MDMs with Vpx enhances the rate of HIV-1 cDNA synthesis (101), suggesting that the decrease in reverse transcription kinetics conferred by SAMHD1-mediated modulation of dNTP levels negatively regulates the rate of proviral DNA synthesis in non-dividing cells. When transcription is silenced, integrated proviral DNA can lead to latency (102). Although SAMHD1 is highly expressed in cells purported to harbor latent provirus (19, 103) and the HIV-1 proviral promoter is activated by transcription factors (104), the effect of SAMHD1 expression on latency development or reversal has not been explored. It is possible that SAMHD1 utilizes its nucleic acid binding ability to restrict HIV-1 infection postintegration, although a recent study confirmed SAMHD1 exerts no effect on HIV-1 Gag synthesis, viral particle release, and virus infectivity in 293T cells transfected with a proviral DNA construct (55). SAMHD1 may exert a direct effect on proviral DNA through binding, as purified recombinant SAMHD1 was shown to bind *in vitro* transcribed fragments of *gag* and *tat* cDNA (72), or indirect effects may occur due to SAMHD1 modulation of inflammatory pathways. It is plausible that suppression of latency reactivation by SAMHD1 would further prevent activation of the innate antiviral response. Although viral nucleic acids can be sensed by IFI16 or cGAS in the absence of SAMHD1 (24, 105), whether other pro-inflammatory pathways are affected by SAMHD1 expression remains unknown.

Discovering the mechanisms used by HIV-1 to avoid innate immune sensors is critical for the design of new therapies to eradicate HIV-1 infection. Therapeutic strategies aiming to inhibit host factors that promote HIV-1 replication and to stimulate the immune response could diminish viral infection and transmission. Current work aims to determine whether a role exists for drugs targeting SAMHD1. Expression of SAMHD1 can increase the susceptibility of HIV-1 to nucleoside reverse transcriptase inhibitors by reducing the levels of competitive dNTPs (106–109), suggesting modulation of SAMHD1 function may be a means to enhance drug effectiveness. Conversely, as SAMHD1 expression enables immune evasion by HIV-1 (13), it is tempting to hypothesize that SAMHD1 could be used as a drug target to enhance the innate immune response to viral infection. However, research is just beginning to uncover mechanisms to modify the dNTPase activity of SAMHD1 (110, 111). Importantly, as an ISG and a negative regulator of the innate immune system, SAMHD1 may be involved in an unknown negative feedback loop aimed at modulating the complex and delicate system of inflammatory pathways.

The effect of SAMHD1 on IFN-I induction during viral infection should be further studied *in vivo*. Although initial robust IFN-I responses can lead to an upregulation of antiviral genes and a block in infection, chronic immune hyperactivity could lead to desensitization of IFN-I and an eventual suppression of antiviral gene expression. This phenotype was observed when Sandler et al. manipulated the IFN-α2a response to SIV infection in rhesus macaques (112). The dismantling of the antiviral state after long-term IFN-α2a treatment led to an increase in SIV reservoir size and an accelerated $CD4^+$ T-cell loss (112). Studies are necessary to determine whether stimulation of the IFN-I response through inhibition of SAMHD1 function leads to chronic inflammation and progression to AIDS *in vivo*.

CONCLUSION

The identification of SAMHD1 as a regulator of the innate immune response to viral infection has led to the development of an exciting field of research. The structural and functional studies of SAMHD1 connect the physiology of HIV-1 infection to the innate antiviral response and the dynamic regulatory mechanisms in cells. Further work will aid in the development of stratagems to enhance the antiviral effects of the intrinsic immune system.

AUTHOR CONTRIBUTIONS

JA wrote the manuscript with input and edits from CG and LW.

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REVIEW
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Natural Killer Cell Interactions with Classical and Non-Classical Human Leukocyte Antigen Class I in HIV-1 Infection

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Natural killer (NK) cells are effector lymphocytes of the innate immune system that are able to mount a multifaceted antiviral response within hours following infection. This is achieved through an array of cell surface receptors surveilling host cells for alterations in human leukocyte antigen class I (HLA-I) expression and other ligands as signs of viral infection, malignant transformation, and cellular stress. This interaction between HLA-I ligands and NK-cell receptor is not only important for recognition of diseased cells but also mediates tuning of NK-cell-effector functions. HIV-1 alters the expression of HLA-I ligands on infected cells, rendering them susceptible to NK cell-mediated killing. However, over the past years, various HIV-1 evasion strategies have been discovered to target NK-cell-receptor ligands and allow the virus to escape from NK cell-mediated immunity. While studies have been mainly focusing on the role of polymorphic HLA-A, -B, and -C molecules, less is known about how HIV-1 affects the more conserved, non-classical HLA-I molecules HLA-E, -G, and -F. In this review, we will focus on the recent progress in understanding the role of non-classical HLA-I ligands in NK cell-mediated recognition of HIV-1-infected cells.

Keywords: HIV-1, innate immunity, natural killer cells, killer cell immunoglobulin-like receptor, human leukocyte antigen class I, human leukocyte antigen-F, human leukocyte antigen-E

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INTRODUCTION

Untreated HIV-1 infection will lead to progressive, severe, and mostly fatal immune deficiency in the vast majority of individuals. Protective HIV-1 immunity is observed in a small subset of subjects whose immune system can naturally control HIV-1 infection and who are termed "elite controllers." Despite intense research in this area over the past decades, the correlates leading to protective immunity are still insufficiently understood. Host genetics alone can only explain approximately 20% of the variable outcomes between individuals observed in the natural course of infection (1). Nonetheless, a consistently documented key genetic determinant of HIV-1 control is the presence of particular human leukocyte antigen (HLA) class I alleles. This strong association between classical HLA-I alleles and HIV-1 disease outcome has been identified in genome-wide association studies (1, 2) as well as in large cohorts studying the immunogenetics of HIV-1 disease (3, 4). The protective effects of certain

HLA-I alleles have mostly been attributed to enhanced $CD8^+$ T lymphocyte-mediated immunity (5–7). HLA-I presentation of HIV-1 epitopes derived from conserved sequences of HIV-1 to $CD8^+$ T cells can pressure the virus to select for mutations in these epitopes, but viral escape can be associated with costs in viral fitness (8). Indeed, early $CD8^+$ T-lymphocyte responses contribute to the initial drop in HIV-1 peak viremia and with this, first HIV-1 escape mutations arise (9). Other protective factors in HIV-1 infection include enhanced proliferation potential of T lymphocytes (10, 11), polyfunctional immune responses (12, 13), variations in host restriction factors (14), and variants in HIV-1 coreceptors, in particular, of CCR5 (15, 16).

Over the past years, the role of antiviral innate immune responses mediated by natural killer (NK) cells in HIV-1 infection has been increasingly appreciated (17, 18). *In vitro,* NK cells can inhibit HIV-1 replication in autologous $CD4^+$ T cells as effectively as $CD8^+$ T cells (19). Additionally, the strong protective effect of host HLA-I alleles on disease progression has been linked to receptor families recognizing HLA-I. These include killer-cell immunoglobulin-like receptors (KIRs), predominantly expressed on NK cells (20), and leukocyte immunoglobulin-like receptors (LILRs), expressed on professional antigen presenting cells such as dendritic cells (DCs), monocytes, macrophages, and B cells, but also on T cells and NK cells (21). Indeed, accumulating data from population studies have identified certain *KIR*, *LILR*, and *HLA-I* allele combinations associated with slower HIV-1 disease progression (22–24), which has helped decipher a further piece of complex host genetics in HIV-1 disease variability.

Natural killer cells comprise 5–15% of the circulating lymphocytes (25) and their role in controlling viral infections has been long established (26). Two major subsets exist: $CD56^{bright}$ $CD16^{dim/neg}$ and $CD56^{dim}CD16^{pos}$ NK cells (25). These differ in their expression of key NK-cell receptors, response to soluble factors and cellular targets, capacity for cytotoxicity, and production of immunomodulatory cytokines (27). NK cells are a crucial first line of defense that detect infected cells before antigen sensitization has occurred (28, 29), and therefore, they precede adaptive immunity in the early phases of HIV-1 infection. Indeed, there is evidence that the early events following infection prior to the development of a specific immune response can determine the viral set point and influence the clinical course of infection (30). In acute HIV-1 infection, a rapid expansion occurs in predominantly cytotoxic $CD56^{dim}$ NK cells, prior to $CD8^+$ T cell expansion (31). On the other hand, in chronic HIV-1 infection, a redistribution of NK cells toward less functional subsets can be observed (32–35) and the presence of persistent viremia appears to deteriorate NK-cell function (19, 34, 36). Overall, the full extent of receptor-ligand interactions between NK cells and HIV-1–infected target cells in HIV-1 infection leading to either NK-cell expansion/killing or exhaustion is highly complex and not yet fully understood.

Natural killer cells, as members of the innate immune system, express a plethora of germline-encoded receptors, and their effector function is determined by integration of inhibitory and activating NK-cell receptor signaling, whereby inhibitory signals tend to be dominant (27). Major NK-cell receptor families are (i) natural cytotoxicity receptors (i.e., NKp46, NKp44, and NKp30), which deliver mainly activating signals, (ii) the KIR family, encompassing inhibitory and activating members and monitoring HLA-I, (iii) the C-type lectins with activating natural killer group 2D (NKG2D) and the heterodimers NKG2A-CD94 and NKG2C-CD94, and (iv) the FcγRIIIa receptor (CD16), which can bind to the Fc-region of IgG antibodies. Critical activating signals can also be delivered by other coreceptors including 2B4, DNAM-1, or CD2 (37, 38). Differential expression of activating and inhibitory receptors allows for a certain degree of specificity and shaping of NK-cell function in response to different stimuli. Ultimately, the stochastic expression of receptors on each NK cell leads to substantial NK-cell diversity and determines the differential response to target cells (39, 40).

HIV-1–infected cells can become vulnerable to NK cell-mediated killing by upregulation of stress signals recognized by activating NK-cell receptors and/or by downregulation of inhibitory NK-cell-receptor ligands. Of note, signaling *via* the FcγRIIIa receptor (CD16), which mediates antibody-dependent cellular cytotoxicity (ADCC), is sufficient to induce NK-cell activation on its own (37). However, the strength of CD16-mediated activation is dependent on tuning of NK-cell responsiveness through inhibitory interactions of KIR or NKG2A with HLA class I (41, 42). Stress ligands upregulated on HIV-1–infected cells are the major histocompatibility complex (MHC) class-I-chain-related proteins (MIC-) A and -B, the UL16-binding proteins (ULBPs) 1–3, which are the ligands for the activating NKG2D receptors (43, 44), and a yet unknown ligand for NKp44 (45, 46). In turn, HIV-1 encodes for multiple accessory proteins with pleiotropic functions to overcome host restriction factors and host immune responses (47–49). The upregulation of stress ligands such as ULBPs and MIC-A/B is counteracted *via* HIV-1 Nef (50) and the ligands for coactivating receptors such as NTB-A and DNAM-1 are downregulated *via* HIV-1 Vpu and partially Nef (51–53). The impact of HIV-1 Nef and Vpu on HLA class I expression will be discussed later. In this review, we will focus on the recent progress in understanding the interplay of HLA-I with HLA-I binding NK-cell receptors, and how this interaction either limits HIV-1 replication or is exploited by the virus to enhance pathogenesis.

KIR–HLA Interactions in HIV-1 Disease Progression and Acquisition

Classical and non-classical HLA-I genes (also known as HLA-Ia and HLA-Ib, respectively) are located within the MHC region p21.3 on chromosome 6, the most polymorphic region of the human genome. An extensive amount of allelic variation occurs within the region encoding for classical HLA-I genes (54). In contrast, non-classical HLA-I alleles display varying degrees of oligomorphism. To date, the classical *HLA-A*, *HLA-B*, and *HLA-C* loci comprise >10,000 alleles encoding for 8,662 distinct proteins, whereas the non-classical *HLA-E*, *HLA-F*, and *HLA-G* loci combined encode for 101 alleles and only 30 proteins (The Immuno Polymorphism Database, as of July 2017) (55, 56).

Classical HLA-I is ubiquitously expressed on nucleated cells. Given that the primary function of HLA-I is to present peptides derived from degradation of intracellular proteins (57), it is not surprising that variations mainly occur in regions surrounding

the peptide-binding groove (58) so as to maximize diversity of peptides presented across different gene and allele products. Under pathologic conditions such as malignant transformation or infection with intracellular pathogens, HLA-I presents antigenic peptides and thereby can elicit an immune response *via* HLA-I restricted cytotoxic CD8$^+$-T cells. Historically, it was thought that NK cells only respond to changes in surface levels of classical HLA class I [to missing-self (59)], but there is increasing evidence that KIR can bind differentially depending on the HLA-class I presented peptide (60–65).

In 2007, the first genome-wide association studies reported three protective single-nucleotide polymorphisms (SNPs) in HIV-1 disease (2). The presence of these SNPs was associated with lower viral set point in chronically HIV-1–infected subjects and together explained almost 15% of interindividual disease variability. Strikingly, all three SNPs were located in the MHC region of chromosome 6, emphasizing the crucial role of HLA class I in HIV-1 infection. The first SNP is in high linkage disequilibrium with HLA-B*57, a second SNP was located 35bp upstream of the HLA-C locus, and results in higher HLA-C expression levels. The last SNP was linked to an RNA polymerase subunit, ZNRD1 and affected the time to AIDS progression. Subsequent genome-wide association studies confirmed the first two SNPs and identified six additional SNPs associated with HIV-1 disease control in two different ethnic cohorts. Again, all SNPs were concentrated around the HLA-I region (1). Accordingly, the strongest HLA class I protective effects so far are reported for HLA-B*57 (66, 67) and HLA-B*27 (4, 68); two HLA class I alleles carrying the serologically defined Bw4 motif (determined by the amino acids 77–83). There is a strong association of HLA-Bw4 homozygosity with the ability to suppress viral replication of HIV-1 and with delayed time to AIDS progression (69).

The genes encoding for KIRs are located within the leukocyte receptor cluster on Chromosome 19q13.4, which additionally encodes Ig-like transcripts (ILTs) [also termed leukocyte Ig-like receptors (LIRs)], and leukocyte-associated inhibitory receptors (70). The KIR locus exhibits substantial polymorphism, in its degree only second to the MHC region in the human genome (71). KIRs can be subdivided into two different classes: KIRs with two extracellular Ig-like domains (KIR2Ds) and those with three domains (KIR3Ds). These Ig-like domains are classified as D0, D1, or D2. Type 1 KIR2Ds contain a D1 domain distal to a D2 domain, type 2 KIR2Ds (KIR2DL4 and KIR2DL5) have a D0–D2 domain organization, and KIR3Ds have all three domains as D0–D1–D2. In general, KIR2Ds bind to HLA-C and KIR3D bind to HLA-A and B-ligands (72, 73). Regarding signaling capacity, a long cytoplasmic tail (KIR-L) renders the KIR inhibitory as it contains immune tyrosine inhibitory motifs (ITIMs), whereas a short cytoplasmic tail (KIR-S) associates to adaptor molecules such as DAP12 and delivers activating signals (74). An exception to this is KIR2DL4, which holds an ITIM in its long cytoplasmic tail, but also associates with activating adaptor elements (73). KIRs are a major receptor family on NK cells, but are also expressed on CD4$^+$ and CD8$^+$ T cells (both αβ and γδ T cells) (75–80). Of note, expression of inhibitory KIR on T cells is increased following chronic immune activation, as was observed in the case of CMV reactivation in a posttransplantation setting (81, 82). Increased KIR expression on bulk CD8$^+$ T cells in HIV-1 infection has been reported, but barely detectable KIR expression was described, when investigating HIV-specific CD8$^+$ T cells (83, 84). Overall, little is known about a modulation of KIR-expression on T cells with or without CMV reactivation in HIV-1–infected subjects.

KIR3DS1/KIR3DL1 and HLA-Bw4^{I80}

The first study associating KIRs to HIV-1 control came from the laboratory of Mary Carrington in 2002. This study showed that possessing *KIR3DS1* and an *HLA-B* allele with a Bw4 motif and an isoleucine at position 80 (HLA-Bw4^{I80}) was associated with slower progression to AIDS, when compared to patients having only one or none of these alleles (22). A follow-up analysis by the same group reported a protective effect of combined KIR3DS1 and HLA-Bw4^{I80} against development of certain opportunistic infections in HIV-1–infected patients, also after controlling for presence of protective (e.g., *HLA-B*57* and *HLA-B*27*) and deleterious (*HLA-B*35*) alleles (85). The *KIR3DS1/KIR3DL1* locus is unique in that it encodes functionally divergent alleles (86). The inhibitory KIR3DL1 binds to HLA-I allotypes that possess a Bw4 motif (HLA-Bw4, which can derive from *HLA-A* or *HLA-B* alleles). Polymorphisms in position 80 of these HLA-Bw4 molecules have been shown to modulate the strength of binding to KIR3DL1 (87, 88). In addition, the interaction of KIR3DL1 with HLA-Bw4 is sensitive to the sequence of the HLA-Bw4–presented peptide (61). Contrary to KIR3DL1, a ligand for its activating counterpart, KIR3DS1, remained initially unknown.

In a cohort of recently infected individuals, Barbour et al. did not detect a synergistic protective effect of *KIR3DS1* and HLA-Bw4^{I80} assessing viral load and CD4$^+$ T cell loss. Nonetheless, encoding for at least one *KIR3DS1* allele was associated with higher CD4$^+$ T cell counts and encoding for HLA-Bw4^{I80} alleles correlated with lower viral load, suggesting a protective, but independent effect of *KIR3DS1* and HLA-Bw4^{I80} (89). A further epidemiologic study reported that HIV-1 viral load at set point correlated positively with the number of *KIR3DS1* gene copies in the presence of HLA-B Bw4^{I80} ligands. Higher copy numbers of the *KIR3DL1* gene also correlated with lower viral set point in the presence of HLA-Bw4^{I80} and at least one copy of *KIR3DS1* (90). In addition, a study by Jiang et al. (91) in a Chinese cohort showed that *KIR3DS1/KIR3DL1* heterozygotes were enriched in HLA-Bw4^{I80}–bearing long-term non-progressors with higher CD4$^+$ T cell counts and decreased viral loads as compared to KIR3DL1 homozygotes or individuals without HLA-Bw4^{I80} (91).

As KIRs are predominantly expressed on NK cells, Martin et al.'s first report associating a KIR to an outcome in HIV-1 infection (22) triggered multiple studies on NK-cell functionality attempting to elucidate the underlying protective mechanism of *KIR3DS1* in combination with HLA-Bw4^{I80} in HIV-1 disease. In line with the epidemiological data, functional studies reported that NK cells derived from donors possessing *KIR3DS1* combined with HLA-Bw4^{I80} inhibited viral replication in infected autologous CD4$^+$ T cells more potently than NK cells from donors having either or neither allele. Sorted KIR3DS1$^+$ NK cells degranulated significantly more in response to HIV-1–infected HLA-Bw4–expressing CD4$^+$ T cells compared to infected

HLA-Bw6$^+$ CD4$^+$ T cells (92). A second study showed that NK cells from individuals encoding for *KIR3DS1* displayed enhanced cytotoxic function compared to NK cells from individuals without *KIR3DS1*, but this was independent of the presence of HLA-Bw4^{I80} (93). Also, in acutely HIV-1–infected subjects a preferential expansion of KIR3DS1$^+$ NK cells—and to a lesser extent KIR3DL1$^+$ NK cells—was observed, which persisted only in subjects bearing HLA-Bw4^{I80} (94). Morvan et al. reported an expansion of KIR3DS1$^+$ NK cells in response to various non-specific stimuli, but KIR3DS1$^+$ NK-cell function was not influenced by the presence of HLA-Bw4 in this setting. Nonetheless, the frequency of KIR3DS1$^+$ NK cells and KIR3DS1 expression levels on NK cells were higher in healthy subjects with HLA-Bw4^{I80} than in those without HLA-Bw4^{I80} (95). Furthermore, HIV-1 viral inhibition assays demonstrated that in individuals encoding HLA-Bw4, having one copy of *KIR3DS1* and one or more copies of *KIR3DL1* resulted in increased antiviral capacity of bulk NK cells compared to individuals containing either *KIR3DS1* or *KIR3DL1* alone, which displayed the lowest amounts of viral inhibition (90). No differences were seen in HLA-Bw6 homozygous donors, whose NK cells had poor antiviral capacity. Having increasing copy numbers of *KIR3DL1* was correlated with elevated *KIR3DS1* transcript and frequency of KIR3DS1 expression on NK cells. Interestingly, this hinted at a *KIR3DL1*-related mechanism regulating the peripheral expansion and functionality of KIR3DS1$^+$ NK cells (90). A more recent study reported that NK cells from *KIR3DS1* and HLA-Bw4^{I80} cocarriers produced higher levels of chemokines after cell contact with infected CD4$^+$ T cells than NK cells derived from HLA-Bw6 homozygous donors, leading to superior inhibition of viral replication (96).

Understanding the mechanistic basis of the protective effect of *KIR3DS1* has proven difficult, as multiple attempts had failed to demonstrate a functional interaction of KIR3DS1 with its putative HLA-Bw4 ligand (74, 97) or for that matter, an interaction with any ligand. To add an additional layer of complexity, the combined genotype of high expressing *KIR3DL1*h* alleles and HLA-Bw4^{I80} (in particular *HLA-B*57*) conferred strong protection toward HIV-1 disease progression (23, 98). Indeed, increased target cell cytotoxicity was observed in NK cells derived from elite controllers with protective *KIR3DL1*h/*y* receptor genotypes along with its HLA-Bw4^{I80} ligand (99). As *KIR3DS1* homozygosity is rare, in the majority of studies investigating *KIR3DS1* and HLA-Bw4 epistasis, *KIR3DS1*-bearing subjects possessed also *KIR3DL1* as a potential confounding variable.

Protection by an inhibitory KIR in HIV-1 disease seems counterintuitive, but might be mediated through a process called NK-cell licensing or education. Expression of an inhibitory KIR during NK-cell development provides strong inhibitory signals in response to its specific HLA-I ligand, ensuring self-tolerance. This allows NK cells to acquire enhanced cytotoxic function, which becomes apparent once exposed to missing or altered self (100). KIR3DL1 allotypes indeed differ in their inhibition of NK-cell function, with an overall trend toward increasing inhibitory capacity in high-expressing KIR3DL1 allotypes (101). Thus, a potential explanation is that presence of high-expression *KIR3DL1*h* alleles together with HLA-Bw4^{I80} determines the increased cytotoxicity of KIR3DL1$^+$ NK cells toward HIV-1–infected targets (taking into account that HLA-B is downregulated *via* actions of the HIV-1 accessory protein Nef) (23). Indeed, a study in slow progressors to AIDS reported increased polyfunctionality of NK cells from donors carrying the *KIR3DL1*h/*y* allele together with its *HLA-B*57* ligand compared to HLA-Bw6 homozygous donors (102). Boudreau et al. recently demonstrated functionally that killing of HIV-1–infected targets *via* KIR3DL1$^+$ NK-cells was dependent on the strength of NK-cell education *via* distinct combinations of KIR3DL1 and HLA-Bw4, with highest cytotoxicity mediated by high-expressing KIR3DL1 and HLA-Bw4^{I80} interactions (103). Moreover, NK cell education not only leads to enhanced functionality (104), but signaling through inhibitory KIRs on NK cells can additionally promote NK-cell survival (105), potentially leading to accumulation of educated NK cells expressing inhibitory receptors in chronic viral infection.

Supplementary evidence comes from studies in highly exposed HIV-1 seronegative individuals. One study reported a significant overrepresentation of *KIR3DS1* homozygosity in high-risk uninfected individuals compared to seroconverted individuals, independent of HLA-Bw4^{I80} (106). This group also reported an association of the KIR3DL1*h/*y-HLA-B*57 combined genotype with protection from HIV-1 acquisition (107). Another study showed enrichment of the HLA-Bw4 carrier–*KIR3DS1* homozygous genotype in HIV-1-exposed seronegative subjects (108). In summary, whereas the results from epidemiological studies are not clear-cut, these studies point toward a potential dual effect of *KIR3DS1* (with or without HLA-Bw4^{I80}) on both the course of HIV-1 infection and HIV-1 acquisition.

HLA-C and KIR2Ds

Genome-wide association studies have clearly implicated the *HLA-C* locus in HIV-1 control, identifying a protective SNP associated with higher HLA-C expression levels (1, 2). Interestingly, HLA-C surface expression levels are only 10% of surface levels of HLA-A and -B (109), and *HLA-C* alleles demonstrate less polymorphism compared to *HLA-B* (56). Nonetheless, individuals with high HLA-C expression levels have been shown to have a higher likelihood of mounting an HLA-C-restricted CD8$^+$ T-cell response (110) and exhibit higher mutation rates in HLA-C–presented HIV-1 epitopes, indicating CD8$^+$ T-cell pressure *via* HLA-C (111). However, given that virtually all individuals encode for KIRs (i.e., KIR2Ds) able to recognize cognate HLA-C molecules, it was proposed that NK cells might play an additional role in mediating the protective effect of higher HLA-C expression. Inhibitory KIR2DL1 binds to HLA-C group 2 allotypes (HLA-C2, which contain Asn77 and Lys80), whereas inhibitory KIR2DL2 and KIR2DL3, which are allelic products of the same *KIR2DL2/3* locus, bind to HLA-C group 1 allotypes (HLA-C1, which contain Ser77 and Asn80). Notably, KIR2DL3 also recognizes HLA-B*46:01 due to an intergenic miniconversion between HLA-B*15:01 and HLA-C*01:02 (65). It was long believed that while HIV-1 Nef downregulated HLA-A and HLA-B surface expression to avoid recognition by cytotoxic CD8$^+$ T cells (112), it spared HLA-C surface expression to ensure inhibition of NK cells *via* engagement of KIR2DL. This paradigm—which

initially arose from studies performed with lab-adapted HIV-1 strains—was recently revised, when Apps et al. demonstrated that HLA-C is downregulated by HIV-1 Vpu variants derived from most primary HIV-1 isolates. HIV-1 Vpu-mediated downregulation of HLA-C was shown to subsequently impair the ability of HLA-C–restricted CD8$^+$ T cells to inhibit viral replication (113). Regarding NK-cell function, it was reported earlier that expression of HLA-C (and HLA-E) on activated, HIV-1–infected CD4$^+$ T cells impaired NK-cell killing, whereas blocking the HLA-C interaction with KIR2D enhanced NK-cell cytotoxicity toward HIV-1–infected CD4$^+$ T-cell blasts (114, 115). During primary HIV-1 infection, KIR2DL$^+$ NK-cell frequencies increased with the presence of their cognate HLA-C ligand (e.g., KIR2DL1$^+$ NK cells expanded in HLA-C2 homozygous individuals) and exhibited more polyfunctional responses, presumably due to a licensing effect (116). Downmodulation of HLA-C by various HIV-1 strains resulted in reduced binding of KIR2Ds to HIV-1–infected cells. Moreover, NK cells were able to sense alterations in HLA-C expression as measured by differing degrees of HIV-1-replication inhibition. Yet, remaining HLA-C surface levels were sufficient to inhibit antiviral function of licensed KIR2DL$^+$ NK cells (encountering their cognate HLA-C ligand) compared to unlicensed NK cells (117). Thus, although NK cells licensed through inhibitory KIR2D exhibit increased functionality against HLA-I–deficient target cells, first reports indicate that this subset does not have superior antiviral function against HIV-1–infected targets expressing self-HLA-C.

The Role of HIV-1 Peptides in KIR:HLA-I Interactions

HIV-1 exhibits an extraordinary ability to adapt to and evade host immune responses. The constant battle of the immune system attacking the virus and the virus evading leads to an extremely rapid accumulation of HIV-1 variants and quasispecies that, at least partially, escape from immune pressure (118, 119). Analyzing the major mechanisms of HIV-1 evasion and sites of sequence mutations provides direct insights into where the human immune system is able to apply critical pressure on the virus. A particular example is the rapid increase in HIV-1 mutations in HLA-I presented epitopes recognized by cytotoxic CD8$^+$ T cells (CTL), which allows the virus to overcome adaptive immune pressure. These mutations can abrogate CTL recognition, but sometimes also impair viral replication (120). By now, a substantial body of evidence from structural (62, 63) and functional studies (60, 61, 65, 121–123) shows that KIR binding is modulated by the sequence of HLA-I–presented peptides, and in particular, C-terminal residues of these peptides. Unlike T cells, NK cells have germ-line encoded receptors that do not undergo recombination nor are they "specific" at discriminating self from non-self peptides (27). Instead, they have a moderate degree of peptide "sensitivity," mediated in large part by KIR:HLA-I interactions, which allows NK cells to monitor for changes in the peptide repertoire expressed by target cells. In fact, common HIV-1 sequence variants can modulate binding of inhibitory KIR to HLA-I, and by this means modulate NK-cell function (61, 124, 125), which has also been demonstrated in the case of SIV (121). Alternatively, NK cells may respond to altered MHC-I peptide processing following induction of the immunoproteasome in response to viral infection. IFN-γ stimulation results in increased cleavage of peptides after hydrophobic and basic residues. Thereby, it alters the C-terminus of available peptides for HLA class I presentation [reviewed in Ref. (126)], which may ultimately affect KIR binding to HLA-I:peptide complexes presented on the cell surface of stressed cells.

Viral variants arising due to CTL-mediated pressure can in turn impact KIR recognition by (i) impairing binding to inhibitory KIRs (61, 127), (ii) reducing HLA-C surface levels (128), or (iii) enhancing binding to inhibitory KIRs directly, a mechanism termed as "double-escape" (129). Furthermore, several amino acid polymorphisms in the viral genome, which showed a significant enrichment in subjects having a specific *KIR* gene, have been identified (130). As one example, a polymorphism in the overlapping reading frame of *vpu* and *env* was associated with the presence of *KIR2DL2* in HIV-1–infected subjects. Antiviral activity of KIR2DL2$^+$ NK cells against this viral variant was reduced *in vitro* (130). However, a role for HLA-I in this process could not be determined due to small sample size. A subsequent study in a larger cohort of HIV-1 clade C–infected individuals identified two viral sequence variants, that were significantly enriched in individuals in the presence of the combined *KIR2DL3–HLA-C*03:04* genotype. One of the variants ($T_{gag303}V$) was contained within a CTL epitope and located at the C-terminal end of the nonamer (YVDRFFKVL), but did not mediate escape from recognition by HLA-C*03:04-restricted CTLs compared to the wild-type sequence (131). This viral variant, however, enhanced binding to KIR2DL3 and inhibited KIR2DL3$^+$ NK cells *in vitro* (132). Overall, these studies support the concept of KIR-mediated selection pressure on HIV-1 as an additional source driving viral evolution. Furthermore, a recent report showed that binding of KIR2DL2/3 to HLA-C1 allotypes is more selective to presented peptides than KIR2DL1 binding to HLA-C2 (60), further enhancing our mechanistic understanding of KIR:peptide:HLA-I interactions. Moreover, this study showed that certain peptides (including an HIV-1 Gag peptide) allow for binding of KIR2DLs to non-canonical HLA-C molecules (60). Taken together, whereas NK cells are not able to distinguish between self- and non-self peptides, KIR binding to HLA-I is certainly sensitive to changes in the peptide sequence presented on HLA-I molecules. This may in turn facilitate recognition of HIV-1–infected cells, potentially not only *via* presentation of viral peptides but also due to stressed-induced changes in the HLA-I-presented peptide repertoire.

Of note, the majority of studies evaluating the peptide sensitivity of KIR:HLA-I interactions to date have relied on external labeling with peptides, but overall, the abundance of viral peptides eluted from HLA-I compared to self-peptides is low (133, 134). Yet, antagonist peptides (i.e., peptides that are presented by HLA-I but abrogate KIR binding) can significantly interfere with KIR clustering at immune synapses and override NK-cell inhibition (135, 136). Therefore, HIV-1 infection may lead to NK-cell activation by causing a shift between antagonist and agonist peptides. Consequently, further investigations on how HIV-1 infection changes the HLA-I–presented peptide

repertoire and how this impacts NK-cell function are needed. Nonetheless, CTL pressure on viral sequence appears to be dominant, as the first escape mutations arise after peak viremia and following expansion of HIV-1–specific CTLs (9). KIR are also expressed on T cells and can modulate CTL activity (75, 83); therefore, a potential role of KIR$^+$ T cells in explaining *KIR/HLA* disease associations has to be considered. Overall, studies suggest a complex interplay between innate and adaptive immune pressures in driving HIV-1 sequence evolution, with HLA-I being central to the interaction with KIRs and TCRs.

KIR3DS1 and the Non-Classical HLA-F—A Non-Classical Stress Ligand?

Genetic evidence and functional data not only implicate KIR3DS1 in HIV-1 disease but indicate a widespread effect of KIR3DS1 in autoimmunity, transplantation, cancer, and other viral infections (137). Yet, for years, a definite ligand for this receptor that could account for these effects remained elusive. Only recently, we and others discovered that KIR3DS1 can bind open conformers (OCs) of HLA-F, a non-classical HLA-I molecule (138, 139). This was confirmed *via* surface plasmon resonance (SPR), pull-down experiments, HLA-F tetramer binding studies, as well as KIR3DS1$^+$ reporter cell assays (138, 139). Functionally, HLA-F OCs led to degranulation of KIR3DS1$^+$ NK cells, as well as cytokine production in response to HLA-F (138). HLA-F is a non-classical HLA-I molecule with a unique combination of features. It is (i) highly conserved with one dominant allele (140) [similar to *KIR3DS1* (71)], (ii) displays tight tissue specific regulation, with a mostly intracellular localization (141, 142), and (iii) is expressed on the cell surface of activated lymphocytes (143). HLA-F is known to bind to inhibitory KIR3DL2 (138, 144), as well as inhibitory LILRBs (145, 146), whereas the results on binding of HLA-F to KIRDS4 are conflicting (138, 139, 144, 146). In HLA-F, 5 of 10 residues, which are highly conserved in other HLA class I molecules, are substituted, resulting in an altered peptide groove (146). To date, there has been no structural data published describing the OC of HLA-F. Given that KIR3DS1, KIR3DL2 and KIR3DL1 (albeit weaker), bind to HLA-F, one could imagine a role of the D0 domain in contacting HLA-F OC as the D0 domain enhances KIR3DL binding to HLA class I and mediates binding to a non-HLA class I ligand (72, 147). Nevertheless, the contact residues of KIR3DS1 to OCs of HLA-F conferring specificity and high-affinity of the interaction are entirely unknown to date. SPR data suggest that KIR3DS1 additionally binds to OCs of classical HLA class I, but so far, the functionality of this binding remains to be demonstrated (139).

We previously demonstrated that HIV-1 infection causes upregulation of HLA-F at a transcriptional level in stimulated CD4$^+$ T cells. Therefore, KIR3DS1 binding to HLA-F expressed as a "stressed self" signal on HIV-1–infected cells might explain the superior ability of KIR3DS1$^+$ NK cells to inhibit viral replication in autologous CD4$^+$ T cells (92, 138). Thus, the interaction between KIR3DS1 and HLA-F upregulated on HIV-1–infected cells may have similarities to the well-reported upregulation of stress ligands such as ULPBs and MIC-A/MIC-B in HIV-1 infection, which are in turn recognized by the activating NK-cell receptor NKG2D (43).

Given the identification of HLA-F as a KIR3DS1 ligand, the following question remains unsolved: why is the strong genetic protective effect of *KIR3DS1* observed preferentially in combination with HLA-Bw4^{I80} in HIV-I infection? We can conceive four potential models that are not mutually exclusive and may explain this phenomenon (**Figure 1**):

(A) *KIR3DS1 binds to HLA-B*57:01 expressing particular HIV-1 peptides*: Only six residues differ in the extracellular domain of KIR3DS1 and KIR3DL1 (**013* versus **001* allele products, respectively) and one of these substitutions (L166R) abolishes binding to HLA-B*57:01. Nonetheless, one recent modeling study identified two HIV-1 derived peptides that can overcome the steric hindrance of R166 with HLA-B*57:01 R83 and allow for binding of KIR3DS1 to the HLA-B*57:01–peptide complex. Binding was of sufficient avidity to activate KIR3DS1$^+$ Jurkat reporter cells (148). Thus, a change in the peptide repertoire resulting from HIV-1 infection might therefore allow KIR3DS1 to engage certain HLA-Bw4^{I80} molecules and trigger KIR3DS1$^+$ NK-cell cytotoxicity. However, further studies are needed to confirm this and assess the functional relevance in natural HIV-1 infection.

(B) *HLA-Bw4^{I80} enhances HLA-F expression at the cell surface of HIV-1–infected cells*: HLA-I gene products differ in their ability to form homodimers on the cell surface. In particular, the protective HLA-B*27:05 allotype is commonly expressed as a β_2m-free disulfide-bonded homodimer (149, 150). Formation of HLA-I dimers in turn can affect recognition by immune receptors (151–153). HLA-F was reported to bind to OCs of other HLA-I to varying degrees and form heterodimers (154). Goodridge et al. discuss that the varying potential of different HLA-I gene products to interact as OCs with HLA-F may modulate HLA-F surface expression levels (144). Protective HLA-B allotypes (e.g., HLA-B*57:01) indeed demonstrate a higher degree of tapasin-dependent assembly and less stability as an OC compared to HLA-B allotypes associated with rapid progression (e.g., HLA-B*35: 03) (155). Thus, protective allotypes might differ from susceptible allotypes in their ability to interact as HLA-I OCs with HLA-F in a setting of HIV-1 infection, in turn enhancing or diminishing recognition by KIR3DS1$^+$ NK cells. This would indicate a KIR3DS1: HLA-Bw4^{I80}:HLA-F protective axis in HIV-1 infection that is independent of KIR3DL1.

(C) *KIR3DS1:HLA-F and KIR3DL1:HLA-Bw4^{I80} interactions are independently, but synergistically protective*: Martin et al. identified the protective effect of combined *KIR3DS1* and HLA-Bw4^{I80}, but the vast majority of individuals bearing *KIR3DS1* in this study were heterozygous and thus also encoded for *KIR3DL1* (22). Furthermore, Jiang et al. demonstrated that *KIR3DS1/KIR3DL1* heterozygosity in HLA-Bw4^{I80}–carrying individuals conferred superior HIV-1 disease control. Therefore, it might be the heterozygous state of *KIR3DS1/KIR3DL1* in the context of HLA-Bw4^{I80} that confers protection in HIV-1 infection, rather than *KIR3DS1* alone with HLA-Bw4^{I80} (91). Thus, protection could derive

from a synergistic but independent effect of KIR3DS1–HLA-F and KIR3DL1–HLA-Bw4^{I80} interactions. Long et al. showed that possessing *KIR3DS1* confers greater NK-cell functionality, also in absence of HLA-Bw4^{I80} (93). Under this model, the most effective NK cells against HIV-1–infected target cells would express both KIR3DS1 and KIR3DL1 and undergo activation *via* KIR3DS1-mediated engagement of HLA-F and KIR3DL1-dependent loss of inhibition due to HLA-B downregulation.

Yet, there is evidence that KIR3DS1 expression and function is not completely independent from KIR3DL1, as KIR3DS1 mRNA, and KIR3DS1$^+$ NK-cell frequency increases with more gene copies of *KIR3DL1* (90). Additionally, *KIR3DS1/KIR3DL1* individuals display superior viral inhibition activity than individuals with either KIR alone in the presence of HLA-Bw4 (90). Thus, there is a possibility of KIR3DL1-mediated epistatic regulation of KIR3DS1 expression and function. However, the existence of a KIR3DS1$^+$KIR3DL1$^+$ coexpressing NK-cell subset has not yet been definitively proven due to the limitations of current anti-KIR antibody cross-reactivity.

(D) *KIR3DL1:HLA-Bw4^{I80} interactions are necessary to limit KIR3DS1-HLA-F-mediated immune activation*: As chronic viral infections can drive inflammatory processes resulting from persistent immune activation (156), downmodulation of the immune response is important for host homeostasis and preventing immunopathology; especially in HIV-1 infection where immune activation can accelerate disease progression (157). Thus, it is conceivable that inhibition of NK cells *via* KIR3DL1:HLA-Bw4 interactions may be important to counteract an exuberant immune response mediated by KIR3DS1$^+$ NK cells recognizing HLA-F on "stressed"/infected cells. Moreover, education through inhibitory KIRs has been shown to promote increased survival of iKIR$^+$ NK cells (105). Increased survival of educated KIR3DL1$^+$ NK cells might counteract chronic immune activation that can result in disease progression. In line with this, the study of Martin et al. showed that *KIR3DS1* homozygosity without HLA-Bw4^{I80} was modestly associated with rapid progression to AIDS (22). Therefore, as supported by mouse models that implicate NK cells as "rheostats" in chronic viral infections (158), combined stimulatory and inhibitory signaling may result in a tunable antiviral response that confers optimal HIV-1 disease control without causing immunopathology.

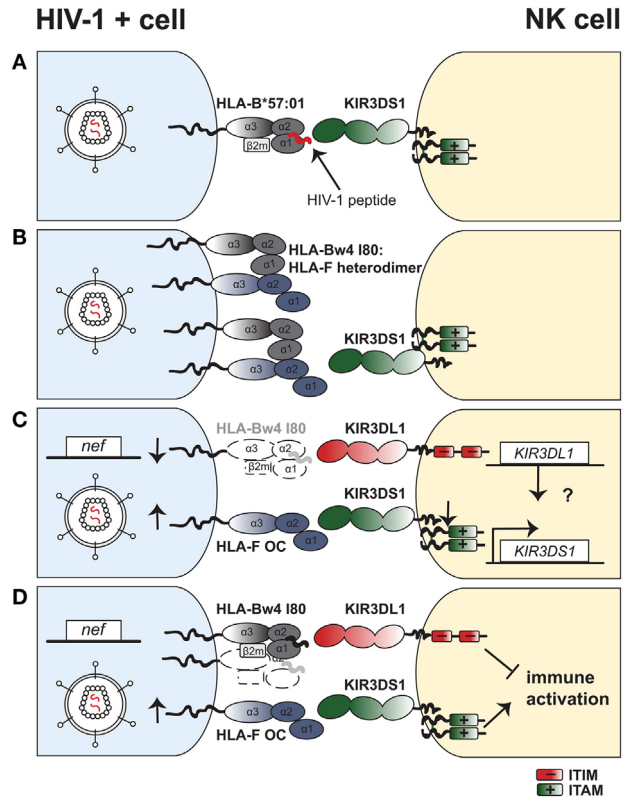

FIGURE 1 | Four models with potential mechanisms to explain the underlying protective effect of the combined *KIR3DS1*–HLA-Bw4 genotype in HIV-1 infection. **(A)** Viral peptides allow for KIR3DS1 binding to HLA-B*57:01 and trigger natural killer (NK)-cell activation. Presentation of viral peptides (in red) on HLA-B*57:01 upon HIV-1 infection of target cells (blue) enables binding of KIR3DS1 on NK cells (yellow). The short cytoplasmic tail of KIR3DS1 associates to the adaptor molecule DAP12, which bears two ITAMs. **(B)** HLA-Bw4 enhances human leukocyte antigen F (HLA-F) expression at the cell surface of HIV-1–infected cells. Open conformers of HLA-F exist as heterodimers with open conformers of HLA-Bw4 on the cell surface of HIV-1–infected cells. This enhances binding and triggering *via* KIR3DS1 on NK cells. **(C)** KIR3DS1:HLA-F and KIR3DL1:HLA-Bw4 interactions have independent but synergistic protective effects in HIV-1 infection. HIV-1 infection of target cells leads to downregulation of HLA-Bw4 from the cell surface *via* action of the accessory protein Nef. Loss of HLA-Bw4 on the infected cells leads to loss of inhibition *via* KIR3DL1. Simultaneously, cell stress induced by HIV-1 infection leads to upregulation of open conformers of HLA-F, which bind to KIR3DS1 and trigger NK-cell activation. A potential epistatic regulation of *KIR3DS1* gene expression *via* the *KIR3DL1* gene is depicted. **(D)** KIR3DL1:HLA-Bw4^{I80} interactions limit KIR3DS1:HLA-F-mediated immune activation. HIV-1 infection directly (and indirectly) causes cellular stress, which in turn upregulates surface expression of HLA-F open conformers on CD4$^+$ T cells and other cell types. OCs of HLA-F bind to KIR3DS1 and trigger NK-cell activation. On the other hand, KIR3DL1 binds to HLA-Bw4 molecules, which are present on HIV-1–infected cells, although at low levels due to HIV-1 Nef-mediated downregulation. Inhibitory signaling *via* KIR3DL1 limits NK-cell activation and inflammatory cytokine production, thus limiting activation *via* KIR3DS1.

In summary, our mechanistic understanding of how protection in HIV-1 disease is mediated in the context of combined KIR3DS1 and HLA-Bw4 is still limited and requires further study. Although we focus on NK cells, a potential role for HLA-Bw4^{I80}-restricted CD8$^+$ T cells expressing KIR3DS1 has also to be considered (84). So far, genetic studies of disease susceptibility have been extremely resourceful in guiding our understanding of the mechanisms involved in HIV-1 control. Therefore, HIV-1 disease association studies that are able to tease out the effect of *KIR3DS1* homozygosity in the context of HLA-Bw4^{I80} would be of great utility, but will require large sample sizes.

The Role of Peptide:HLA-F Complex

Major histocompatibility complex class I exists in two biologically relevant conformations on the cell surface: (i) as a membrane-bound heavy chain lacking peptide and β_2-microglobulin

(β_2m) termed open conformer (OC) or (ii) as a trimeric heavy chain:β_2m:peptide complex (159). Recently, thermal denaturation assays demonstrated that OCs of HLA-F are more stable (146) than OCs of other HLA-I gene products (160). Earlier findings assessing stability after cold treatment suggested an increased stability of HLA-F OCs compared to open conformers of classical HLA-I (141, 142). This—and the fact that no canonical peptides could be eluted from HLA-F—supported the notion that HLA-F is mainly expressed as an OC devoid of peptide (142, 154).

Recently, the crystal structure of HLA-F (in complex with β_2m and peptide) was solved, shedding first light onto the molecular structure of HLA-F (146). Surprisingly, this work showed that HLA-F has a unique peptide-binding grove that resembles the groove of classical HLA-I but does not anchor peptides at their N-terminus, allowing for binding of longer peptides. Indeed, peptides eluted from HLA-F and characterized by mass spectrometry had an extended length distribution compared to classical HLA-I molecules, peaking at 12 amino acids and with peptides up to 30 amino acids observed. This unconventional length rather resembles the length of HLA class II-presented peptides.

Moreover, new insights into the structure and docking mode of LILRB1 interacting with the HLA-F:β_2m:peptide complex were gained. The LILR family (also termed LIR, ILT, or CD85) are encoded on chromosome 19 within the leukocyte receptor complex along with the KIR locus. In total, 13 different LILRs have been identified. Similar to KIRs, LILRs can provide an either inhibitory (LILRB) or activating (LILRA) signal, depending on the presence of an ITIM or the association to ITAM-containing adaptor molecules, but also depending on the cellular context (161). LILRB2 is not expressed on NK cells and its implications in HIV-1 disease are reviewed elsewhere (24). LILRB1 recognizes most classical and non-classical HLA-I molecules, except for HLA-E (162–164), given that it binds to the conserved α3 domain of the HLA-I heavy chain as well as β_2m (165, 166). Intriguingly, the affinity of LILRB1 to peptide-bound HLA-F:β_2m is the highest observed so far compared to other HLA-I ligands (146, 167). LILRB1 is expressed on NK cells in varying percentages (0–50% with high interindividual variability), as well as on T cells and professional antigen presenting cells such as DCs, monocytes/macrophages, and B cells (146, 168). Engagement of LILRB1 *in vitro* leads to inhibition of cytotoxicity and cytokine production in a subset of NK cells (151, 169, 170), but interestingly, LILRB1$^+$ (but not LILRB1$^-$) NK cells are able to markedly suppress HIV-1 replication in infected monocyte-derived DCs in a manner independent of classical HLA-I (171), hinting at a possible role of HLA-F.

Looking at the binding footprint of LILRB1 on HLA-F, it is improbable that the interaction is sensitive to the nature of the presented peptide—in contrast to certain KIRs. In the case of KIR3DS1, it was shown that KIR3DS1$^+$ reporter cells responded to HLA-F OCs, but were not triggered by peptide-bound HLA-F complexes (146). This could be due to peptide-induced conformational changes in HLA-F structure or direct steric inhibition by the bound peptides. Furthermore, inhibitory KIR3DL2 recognizes OCs of HLA-F or HLA-I and posssibly heterodimers of HLA-F with HLA-I heavy chains, with the latter also being increasingly expressed on activated lymphocytes (144). This raises interesting possibilities for a cell-stress induced conformational change in HLA-F allowing binding to activating receptors, such as KIR3DS1, while abrogating binding to inhibitory receptors, such as LILRB1. Thus, although HLA-F is not expressed on the surface of lymphocytes in a resting state (143), it potentially can exist in various conformations on stressed cells (154) with differential impact on NK-cell function (**Figure 2**).

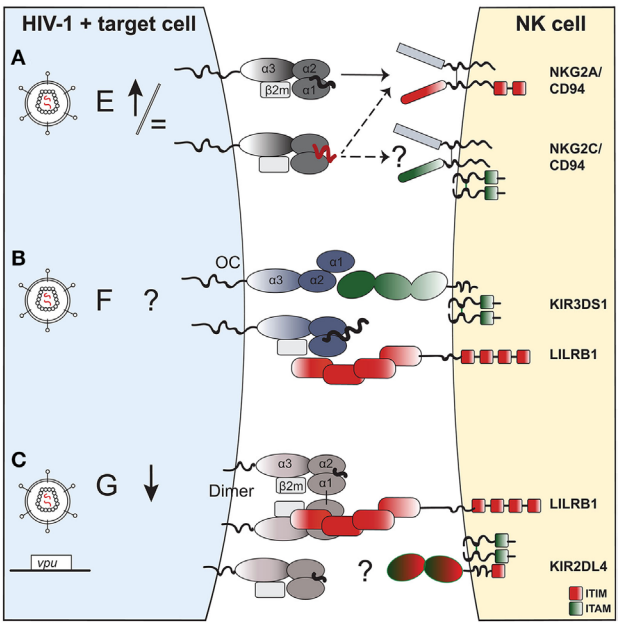

FIGURE 2 | The potential impact of HIV-1 infection on expression of non-classical human leukocyte antigen class I (HLA-I) molecules on a target cell and effect on natural killer (NK)-cell receptor binding. **(A)** *HLA-E.* HIV-1 infection of target cells leaves HLA-E surface levels either unchanged or slightly upregulated. HLA-E can present self-peptides (in black) that bind to the NKG2A:CD94 receptor complex, and inhibit NK-cell function. In the context of HIV-1 infection, HLA-E can present viral or "stress"-self-peptides (in red) that abrogate (or reinforce) binding to NKG2A:CD94 and modulate NK-cell activity. The potential role of viral or "stress" peptides presented on HLA-E that could trigger the activating NKG2C:CD94 receptor complex on NK cells is currently unknown. NKG2C associates to DAP12, an adaptor molecule containing two ITAMs. **(B)** *HLA-F.* The exact impact of HIV-1 infection on HLA-F surface expression in different cell types needs yet to be established. In general, HLA-F is expressed on activated or stressed cells, potentially in distinct functionally relevant conformations: (i) an open conformer that binds to the activating NK-cell receptor KIR3DS1 or (ii) a β_2m-bound complex presenting peptides of unusually long length for HLA-I, which allows binding of the inhibitory receptor LILRB1. **(C)** *HLA-G:* one study showed downregulation of HLA-G in monocyte-derived macrophages, potentially *via* HIV-1 Vpu, although this has not yet been confirmed in primary cells. Moreover, the functional relevance of HLA-G downregulation in antiviral immune responses has not been established to date, although HLA-G is thought to play a predominantly immunoregulatory role given its interaction with inhibitory receptors. HLA-G can form dimers on the cell surface *via* an interchain α1 disulfide bond, which enhances recognition by inhibitory LILRB1 on NK cells. KIR2DL4 binding to HLA-G remains controversial. KIR2DL4 has a dual activating and inhibitory effect on NK cells, given that its cytoplasmic tail contains an ITIM and it associates to ITAM-bearing FcRγ.

The Non-Classical HLA-G—An Immune Modulator?

Human leukocyte antigen-G is a non-classical HLA-I that displays a high degree of tissue restriction. It was first discovered in extravillous trophoblast cells in the fetal placenta (172), where HLA-G protein is abundant (173) and since then has been extensively studied in the context of reproduction. Further studies showed that under healthy conditions, HLA-G is expressed in other immune-privileged sites including the cornea (174), thymus (175), nail matrix, and on mesenchymal stem cells (176, 177). Under inflammatory conditions such as CMV infection or within a tumor microenvironment, HLA-G can be expressed on DCs and monocytes/macrophages (178, 179), and is reported to be upregulated in monocytes treated with IFN-γ (a potent inducer of HLA-I and -II expression) as well as IL-10 (180–182). Also, an increasing number of studies shows aberrant HLA-G mRNA expression by tumors (183, 184) [reviewed in Ref. (185)], but some of these findings remain controversial as in other studies no HLA-G protein was identified (179, 186) [reviewed in Ref. (187)]. Overall, there is evidence that HLA-G expression is induced on various immune cells under inflammatory conditions resulting from infections, allergies, or allogeneic stimulation following transplantation (188–191).

As a result of a premature stop codon in exon 6 (192), the cytoplasmic tail of HLA-G is truncated and the heavy chain has a molecular weight of only 39 kDa, compared to the 45 kDa weight of classical HLA class I heavy chain. In total, seven splicing variants of HLA-G have been described (193, 194). The predominant splice variant *in vivo* is HLA-G1, which encodes for the full-length, membrane-bound HLA-G protein (195, 196). Alternatively, soluble HLA-G (sHLA-G) can be generated from three splice variants or *via* proteolysis of the HLA-G1 isoform (197). Interestingly, sHLA-G can confer a protective effect to cells normally permissive to NK-cell killing (197). Apart from HLA-G1, three other alternatively spliced transcripts encode membrane-bound HLA-G, albeit in a truncated form: HLA-G2 lacks the α2 domain, HLA-G4 lacks the α3 domain, and HLA-G3 lacks both the α2 and α3 domains (198). These transcripts were reported to inhibit NK-cell function, although it remains unclear through which NK-cell receptors this occurs (198–200). Moreover, it was suggested that isoforms HLA-G2 and -G3 are expressed in individuals homozygous for the *HLA-G*0105N* null allele (201), possibly explaining the existence of healthy adults lacking full length HLA-G1 (201). Of note, all splicing variants encode the leader sequence enabling HLA-E expression (202) and thereby their expression in target cells can indirectly inhibit NK cells *via* NKG2A:CD94 (163).

To date, 18 distinct functional proteins of HLA-G have been identified, with the *HLA-G* gene encoding a total of 54 *HLA-G* alleles (including two *HLA-G* null alleles) (56). While most of the polymorphism of classical HLA-I genes lies in the α1 and α2 domains that bear the peptide-binding groove (203), HLA-G has a relatively conserved peptide-binding groove and has allelic variability occurring within the 3′UTR, which is important for posttranscriptional regulation of HLA-G (203, 204). Peptides eluted from HLA-G thus far appear to be derived from a restricted number of proteins (205) and a crystal structure demonstrates that these presented peptides are buried deep within the peptide-binding groove (206). The induction of an HLA-G-restricted $CD8^+$ T cell response against a human cytomegalovirus peptide in mice was described, but the cytolytic capacity of these T cells was limited (207). Overall, it seems that the immune modulatory functions of HLA-G mediated through binding of inhibitory receptors expressed on a variety of immune cells dominates over a potential role in presenting peptides.

NK-Cell Receptors Recognizing HLA-G

Human leukocyte antigen-G is recognized by LILRs with greater affinity than HLA-A, -B, or -C molecules (208). In addition, HLA-G is unique in possessing a cysteine at position 42 of its α1 domain, which allows for an unusual conformation of HLA-G as a homodimer of two β_2m-associated HLA-G complexes (152, 209, 210) (**Figure 2C**). This conformation dramatically enhances recognition and signaling of LILRB1 (151) and has been demonstrated to occur naturally on trophoblasts (173). Indeed, inhibition of $LILRB1^+$ NK-cell function is sensitive to the conformation of HLA-G, as the heavy chain of HLA-G alone does not inhibit $LILRB1^+$ NK cells (211). Studies measuring inhibition of $LILRB1^+$ NK-cell cytolytic function *via* HLA-G have to account for HLA-E expression as it is upregulated through the HLA-G leader peptide—an exception being the K562 cell line, which does not express HLA-E (212). Independent of HLA-E, HLA-G interferes with immunological synapse formation and inhibits NK-cell cytotoxicity (212, 213). Additionally, Riteau et al. demonstrated that HLA-G expression has a major inhibitory effect on NK cell lysis through LILRB1, also when coexpressed with other HLA-I ligands (214). Besides the inhibitory effect of HLA-G expression on NK-cell-effector function itself, HLA-G can impair NK-DC crosstalk. Pretreatment of DCs with sHLA-G leads to reduced activation and IFN-γ production by NK cells (215), while IFN-γ in turn triggers HLA-G surface expression (180, 181). This again supports the notion that HLA-G has tolerogenic properties.

In addition to LILRB1, HLA-G has been proposed to modulate NK-cell function *via* binding KIR2DL4. *KIR2DL4* is a framework gene within the KIR locus and thus is present in virtually all haplotypes, but there is a high frequency of alleles lacking the transmembrane domain or having truncated cytoplasmic tails (216). In peripheral blood, expression of KIR2DL4 is weak and restricted to the $CD56^{bright}$ subset, but can be induced on NK cells *in vitro* with stimulation (217, 218). KIR2DL4 has unique functional properties compared to other receptors of this family. A positively basic arginine residue in the transmembrane domain allows for association with the activating Fc receptor gamma protein (219), while the long cytoplasmic tail contains one immunoreceptor tyrosine-based inhibitory motif (ITIM). This results in mixed activating and inhibitory signaling, which has been shown to occur *in vitro* (219–222). In line with this, crosslinking of KIR2DL4 on peripheral blood NK cells induces IFN-γ production, and (albeit weaker) NK-cell cytotoxicity (217, 218, 223).

Newer reports provide conflicting evidence regarding the interaction of HLA-G with KIR2DL4 (224, 225). Although several groups reported binding using various techniques including cellular transfectants, SPR, and functional assays (169, 226–230), others have failed to reproduce KIR2DL4 binding *via* SPR, tetramers, or functional IFN-γ responses to sHLA-G (210, 231, 232). The crystal structure of the extracellular domains of KIR2DL4 solved by Moradi et al. (233) demonstrated oligomerization of KIR2DL4, uncharacteristic of other KIRs. In this study, no binding of KIR2DL4 to HLA-G was detected *via* SPR (233). An explanation might be that signaling *via* KIR2DL4 only occurs upon concentration of the ligand in endosomes [as discussed in Ref. (187)], since sHLA-G endocytosed into KIR2DL4-containing compartments was shown to induce cytokine secretion of NK cells (229, 234). Regardless of its binding to HLA-G, higher copy numbers of KIR2DL4 have been linked to better survival of $CD4^+$ T cells and increased IFN-γ responses from NK cells during acute SIV infection in rhesus macaques (235).

HLA-G Expression in HIV-1

Only a low percentage of immune cells in healthy subjects expresses HLA-G, whereas in HIV-1 infection a substantial upregulation of HLA-G has been observed in both peripheral blood monocytes and T-cell subsets (236). This was later partly attributed to antiretroviral treatment, as frequencies of $HLA\text{-}G^+$ monocytes decreased after treatment interruption (237). In fact, nucleoside reverse transcriptase inhibitors were found to increase HLA-G expression, whereas protease inhibitors did not (238). A role for $HLA\text{-}G^+$ HIV-1–restricted $CD8^+$ T cells has furthermore been described in HIV-1–infected subjects (239). Contrary to *in vivo* studies of high HLA-G expression levels on monocytes of patients undergoing HAART (236–238), one study showed downregulation of HLA-G1 surface expression in HIV-1–infected monocyte-derived macrophages *in vitro* (240). This downregulation was suggested to be mediated *via* HIV-1 Vpu (240), given that the truncated tail of HLA-G renders it resistant to HIV-1 Nef-mediated downregulation (241). Yet, this needs to be confirmed in primary cells. Overall, how HIV-1 directly impacts HLA-G expression in different cell types remains unclear.

In addition to inhibiting DC function *via* LILRB2 (242) and regulating $CD4^+$ T-cell proliferation (243), sHLA-G can inhibit NK-cell killing *in vitro* and may therefore suppress NK-cell function *in vivo* (197). sHLA-G plasma levels change during the course of HIV-1 infection and treatment, as two groups reported high sHLA-G levels in early infection (244) with a significant decrease after treatment initiation (245). In rapid progressors, however, levels of sHLA-G were persistently elevated even despite treatment initiation, while this was not the case for untreated normal progressors and long-term non-progressors (244). Furthermore, sHLA-G levels were higher in patients with opportunistic infections, indicating a potential role of sHLA-G as a surrogate marker of disease progression (246). In a cohort of female commercial sex workers from Benin, HIV-1–infected subjects were reported to have lower levels of sHLA-G in plasma (247) but higher levels of sHLA-G in the genital mucosa (248). Of note, levels of sHLA-G are also in part determined genetically by distinct *HLA-G* alleles (249). Thus, data on sHLA-G levels in HIV-1 infection need to be carefully controlled for confounding factors such as HAART (237, 238), *HLA-G* genetic background (249), sampling site (247, 248), or coinfections (246, 248). In summary, it is not known whether higher sHLA-G levels have direct functional consequences on HIV-1 disease progression *via* modulation of NK and other immune cells, or whether sHLA-G levels are rather a reflection of viremia and the antiviral immune response.

Genetic Evidence for a Role of HLA-G in HIV-1 Infection

Although HLA-G polymorphisms are limited, certain HLA-G alleles have been suggested to be involved in susceptibility to HIV-1 infection. In 2004, Matte et al. reported that the *HLA-G*0105N* allele, a null variant which does not encode functional HLA-G1, was protective in HIV-1 acquisition, whereas the *HLA-G*01:01:08* allele encoding for full-length HLA-G increased the risk of HIV-1 infection. They formulated the hypothesis that non-functional HLA-G proteins may allow for better NK-cell killing of HIV-1–infected cells (250). This observation was not consistent with findings of subsequent studies, which reported either enrichment of *HLA-G*0105N* in HIV-1–positive women (251) or did not identify *HLA-G*0105N* allele as a disease modifying factor (252). Other *HLA-G* alleles identified were *HLA-G*01:04:04*, which associated with susceptibility to HIV-1 infection, and *HLA-G*01:01:01*, which was enriched in HIV-1–resistant women (252). One study states that these conflicting findings may be explained by variation of *HLA-G* polymorphisms among different ethnic populations and reports no association of *HLA-G* polymorphisms to HIV-1 susceptibility except in African-American cohorts (253).

As HLA-G is an important player involved in maternal-fetal tolerance, *HLA-G* polymorphisms have been studied in the context of vertical HIV-1 transmission from mother-to-child. Mothers bearing the *HLA-G*01:03* allele were less likely to perinatally transmit HIV-1 (254). Upregulation of the functional isoform HLA-G1 mRNA in the placenta has been associated with increased risk of HIV-1 mother-to-child transmission (255). Further studies have assessed the risk of variants in the 5′ and 3′UTR of HLA-G, and in particular, the impact of the 14-bp insertion/deletion in the 3′UTR of HLA-G on mother-to-child transmission. In healthy subjects, the 14-bp insertion genotype (ins/ins) correlates with lower plasma levels of sHLA-G (256). *In vitro,* transfection of the 14-bp ins/ins HLA-G into K562 cells resulted in increased levels of membrane-bound HLA-G1 expression with higher mRNA stability and lower sHLA-G1 ratio (257). However, studies on the impact of the 14-bp insertion on HIV-1 vertical transmission risk report conflicting results (258–260). In horizontal transmission, the frequency of the 14-bp ins/ins genotype was enriched in HIV-1–infected patients in African (but not European) subjects (261). Overall, population studies attempting to shed light on the question whether functional versus non-functional *HLA-G* alleles are associated with HIV-1 susceptibility have painted an inconsistent picture. Moreover, posttranscriptional regulation of the *HLA-G* gene through variations in the 3′ and 5′ LTR and alternative splicing has to

be considered as an important genetic factor modulating HLA-G expression levels in these studies.

The Oligomorphic Interaction between HLA-E and NKG2:CD94—A Contrast to the Diversified HLA-KIR System

Inhibition of NK cells can be achieved either through highly diversified KIR:HLA-I interactions or through a second inhibitory system indirectly monitoring the level of overall HLA-I expression. This latter inhibitory mechanism is achieved *via* the well-conserved NK-cell receptor–ligand interaction of NKG2A/CD94 with HLA-E (262). Contrary to other non-classical HLA-I gene products, HLA-E is ubiquitously expressed (263), but at substantially lower levels as compared to classical HLA-A, -B, and -C (264). Its expression is dependent on the expression of other HLA-I, as it presents a nonamer peptide derived from the signal sequence of several HLA-A, -B, and -C gene products as well as HLA-G. HLA-F and HLA-E itself lack an HLA-E–presented leader peptide (265).

Human leukocyte antigen-E has restricted polymorphism with to date only 25 known alleles (56), of which two—HLA-E*01:01 and *01:03—are the most frequent in the human population and are believed to be in balancing selection (266, 267). HLA-E*01:01 encodes for an arginine at position 107 (HLA-E^R), whereas HLA-E*01:03 encodes for a glycine at this position (HLA-E^G). This substitution leads to higher surface expression levels of the latter, despite similar intracellular protein levels (160). HLA-E is highly relevant to innate immune responses due to its interaction with heterodimeric NKG2/CD94 type II transmembrane-anchored receptors, which are expressed on a large proportion of NK cells as well as on a subset of $CD4^+$ and $CD8^+$ T cells (268–270).

Natural killer group 2 receptors are a family of C-type lectin receptors encoded within the NK gene complex on chromosome 12p12-13 (271). Almost all NKG2 gene products heterodimerize with CD94, a non-signaling invariant glycoprotein also encoded within the NK gene complex. These include *NKG2A* [which produces NKG2A and NKG2B gene products *via* alternative splicing (272)], *NKG2C*, *NKG2E*, *NKG2F*, and *NKG2H*. The *NKG2D* gene is also located within the NK gene complex, but its gene product has low sequence homology to other NKG2 receptors and forms an NKG2D:NKG2D homodimer (without CD94) that binds to the stress ligands MIC-A, MIC-B, and ULBPs, but not to HLA-E (273). Unlike *KIR* genes, *NKG2* genes exhibit limited polymorphism (262, 274). Aside from being expressed widely on NK cells, they can also be expressed on subsets of T cells (275). Here, we focus on NKG2A:CD94 and NKG2C:CD94 receptor complexes, both of which bind HLA-E but have opposite effects on NK-cell function. While NKG2A signaling inhibits NK-cell cytotoxicity *via* two ITIMs in its cytoplasmic tail (276), NKG2C delivers activating signals through its associated adaptor molecule DAP12 (277).

Despite their similarity, the two major alleles of HLA-E differ in the subset of peptides they present (278). An example is the HLA-B*27–derived leader peptide, which stabilizes HLA-E^G, but does not bind detectably to HLA-E^R (160, 279). Similar to KIR:HLA interactions, binding of the NKG2:CD94 heterodimer to HLA-E is sensitive to the presented peptide (279, 280). The crystal structures of NKG2A:CD94 and NKG2C:CD94 receptor complexes bound to HLA-E presenting the HLA-G leader peptide (VMAPRTLFL; VL9) illustrate that both subunits (NKG2 and CD94) intimately interact with the peptide-binding domains (α1 and α2) of HLA-E. Interestingly, CD94 occupied the majority of the binding site, yet despite this, the NKG2A:CD94 complex had six times stronger binding affinity to HLA-E:VL9 than NKG2C:CD94 (280). Consequently, it is believed that CD94 is the main driver of HLA-E binding and peptide sensitivity, while the NKG2 subunit modulates affinity (and possibly sensitivity to some extent). Leader peptides of classical HLA-I presented on HLA-E do not trigger NK-cell activation through NKG2C, whereas $NKG2A^+$ NK cells are potently inhibited by a wide range of different HLA-I–derived leader peptides (281). Therefore, the NKG2A:CD94–HLA-E interaction allows NK cells to indirectly monitor for changes in overall HLA-I expression without causing aberrant immune activation through NKG2C:CD94. An exception to this is HLA-E in complex with the HLA-G leader peptide, which can engage NKG2C:CD94 and trigger activation (279, 281). As HLA-G displays high tissue-specific restriction, this nonetheless allows for tight regulation of NKG2C triggering. The amount of surface stabilization of HLA-E by various leader peptides does not strictly correlate with the level of inhibition through NKG2A:CD94, which emphasizes the role of specific peptides in the binding of NKG2A:CD94 to HLA-E (282).

Peptide Presentation by HLA-E in the Context of Viral Infections

Like classical HLA-I, HLA-E can also present virus- or "stress"-derived peptides. The leader sequences of heat shock protein 60 (HSP60), which is induced under stress conditions (283), stabilizes HLA-E, but disrupts binding to NKG2A:CD94 and thus disinhibits NK-cell function (284). HLA-E can also be the target of viral immune evasion. CMV, for example, encodes for a sequence identical to the HLA-C*03 leader peptide that can increase HLA-E expression and inhibit NK-cell cytotoxicity (285). Additionally, an HCV-derived epitope (HCV Core35-44) stabilizes HLA-E and inhibits NK-cell lysis (286). Cheent et al. showed that viral- or heat shock protein-derived peptides in isolation did not inhibit NK-cell lysis. However, these peptides enhanced inhibition in the presence of HLA-E–presented leader peptides and therefore were termed "synergistic peptides." Confocal microscopy has shown that these synergistic peptides act by recruiting non-signaling CD94 (without NKG2A) to the immunological synapse (262). Similar to peptide antagonism in KIR-HLA interactions (135, 136), this adds an additional layer of complexity to peptide-dependent modulation of NK-cell-effector function.

For HIV-1, the capsid-derived p24 aa14–22 epitope AISPRTLNA (AA9) has been described to stabilize HLA-E. One study by Natterman et al. demonstrated that AA9 inhibited NK cell-mediated cytolysis of peptide-pulsed HLA-E–transfected K562 cells (287), and that NK-cell killing could be restored *via* antibody blockade of either HLA-E or NKG2A. Contrary to this study, however, Davis et al. reported that HLA-E:AA9 tetramers

did not bind to NKG2A$^+$ CD56^{bright} NK cells (while HLA-E:VL9 tetramers did). Thus, the authors suggest a potential role for the AA9 peptide in abrogating HLA-E binding to NKG2A:CD94 on NK cells, explaining enhanced degranulation of NKG2A$^+$ NK cells against HIV-1–infected cells as compared to NKG2A$^-$ NK cells (288). In line with a role of HLA-E in HIV-1 infection, a genetic study in a cohort of Zimbabwean women demonstrated a four-fold reduced risk of HIV-1 acquisition in individuals homozygous for *HLA-E*01:03* (HLA-E^G) alleles compared to heterozygous or *HLA-E*01:01* homozygous individuals. Given that HLA-E^G is a high-expression allele, the authors speculated that increased presentation of HIV-1 peptides by HLA-E enhances NK cell cytotoxicity against HIV-1–infected target cells during the initial stages of infection (289).

Besides the role of HLA-E in innate immunity, increasing evidence demonstrates that HLA-E presentation of viral peptides derived from CMV, EBV, and HCV can elicit HLA-E–restricted CD8$^+$ T-cell responses (290–292). Furthermore, Hansen et al. (293) showed that inoculation of rhesus CMV-based SIV$_{gag}$ vectors leads to presentation of surprisingly diverse epitopes on MHC-E, inducing a broadly directed and protective CD8$^+$ T cell response in rhesus macaques (293). So far, HIV-1–specific HLA-E–restricted CD8$^+$ T cells have not been shown in humans (294), but the conserved nature of *HLA-E* alleles among different populations, its ability to present viral peptides, and its dual role in innate and adaptive immunity renders HLA-E an important target for future research.

NKG2A$^+$ NK Cells—A Subset with Enhanced (Not Reduced) Antiviral Capacity in HIV-1

Chronic HIV-1 viremia leads to a decrease in the proportion of NK cells expressing NKG2A (32, 295–297), and normal NKG2A levels are restored only after prolonged times of antiretroviral therapy (297). Subset analyses show, however, that NKG2A$^+$ cell frequency increases within the CD56^{dim}CD16^{bright} NK-cell subset over the course of HIV-1 disease progression, whereas NKG2A$^+$ cell frequency is decreased in the dysfunctional CD56$^-$ NK cell subset (298). Given that this highly dysfunctional CD56$^-$ NK cell subset with poor cytotoxic capacity expands in viremic subjects (33, 34), bulk NKG2A$^+$ NK-cell frequencies are reduced (298). Presence of viremia in patients with low CD4$^+$ T-cell counts correlated with significantly higher NKG2A$^+$ frequencies on CD56^{dim}CD16^{bright} NK cells compared to aviremic patients with low CD4$^+$ T cell counts (298), which may suggest a potential effect of long-term HIV-1 exposure itself on modulating NKG2A expression.

On the other side of the equation, HLA-E levels on CD4$^+$ T cells from HIV-1–infected patients increase with declining CD4$^+$ T cell counts *in vivo* (299). Upon HIV-1 infection or reactivation *in vitro*, HLA-E surface levels remain unchanged (36, 114, 288) or increase (287, 299). Functionally, blocking of the inhibitory NKG2A:CD94 interaction with HLA-E increases the ability of NK cells to kill HIV-1–infected CD4$^+$ T cells *in vitro* (114, 287). Similarly, blocking of NKG2A enhances ADCC of NK cells toward antibody-coated HIV-1–infected CD4$^+$ T cell blasts (115). Although these initial data implied the notion that HLA-E–NKG2A:CD94 interactions were inhibitory and detrimental to elimination of HIV-1–infected cells, recent experimental data demonstrated a superior ability of the NKG2A$^+$ NK-cell subset to degranulate in response to HIV-1–infected CD4$^+$ T-cell blast compared to NKG2A$^-$ subsets (288). Moreover, NKG2A$^+$ NK cells showed the highest polyfunctional responses with increased IFN-γ and MIP-1β, as well as higher CD107a expression against HIV-1–infected CD4$^+$ T cell blasts (300). This suggests that HLA-E-mediated inhibition of NK-cell function *via* engagement of NKG2A:CD94 is incomplete, potentially due to a skewed peptide repertoire in infected cells (288) (**Figure 2A**). Moreover, the increased functionality of NKG2A$^+$ NK cells highlights the concept that the inhibitory NKG2A:CD94 receptor is important in NK-cell education (301), as described later in more detail. Taken together, the factors driving an overall decline in NK-cell function in HIV-1–infected individuals are not entirely clear, although decreased frequency of NKG2A$^+$ NK cells may play a role.

NKG2C$^+$ NK Cells—A Role in HIV-1 Independent (or Dependent) of CMV?

It is conceivable that ligation of activating NKG2C:CD94 *via* HLA-E may enhance cytotoxicity toward HIV-1–infected cells, but this has not been demonstrated. In healthy subjects, NKG2C is expressed only at low-to-moderate frequencies depending on *NKG2C* zygosity and CMV status (288, 302). In HIV-1–infected subjects, an increased frequency of NKG2C$^+$ NK cells can be detected (295), independent of HIV-1 disease stage or presence of viremia (298), leading to a reversed NKG2A$^+$-to-NKG2C$^+$ NK-cell ratio in HIV-1–infected subjects compared to healthy controls (296). Additionally, NKG2C$^+$ NK cells form part of the dysfunctional CD56$^-$CD16$^+$ NK-cell population in HIV-1–positive viremic patients (303). Thus, NKG2C expression appears to be modulated by HIV-1 infection, but differences in NKG2C$^+$ NK-cell activity toward HIV-1–infected cells have not been demonstrated (**Figure 2A**).

It is important to note that CMV infection substantially skews the NK-cell repertoire toward NKG2C-expressing NK cells (304, 305). Furthermore, NK cells of CMV seropositive patients display enhanced cytotoxicity against target cells expressing HLA-E, which can be blocked by anti-NKG2C (306). Therefore, coinfection of CMV in HIV-1–infected patients is a highly relevant confounding factor when assessing NKG2C$^+$ frequencies and function on NK cells. In a cohort of HIV-1–positive aviremic individuals, the association between increased NKG2C expression and HIV-1 infection disappeared when accounting for CMV seropositivity (307). Furthermore, Brunetta et al. showed that NKG2C$^+$ NK-cell frequencies are higher in CMV seropositive individuals with HIV-1 infection compared to CMV seropositive HIV-1–negative subjects (297). Overall, the leading notion is that HIV-1 infection may render individuals more susceptible to CMV reactivation and impair immune control of CMV, potentially explaining the higher degree of CMV-driven expansion of NKG2C$^+$ NK-cell subsets in HIV-1–infected subjects (308, 309). Additional evidence for a potential role of

NKG2C comes from HIV-1 disease association studies, where homozygous deletion of *NKG2C* in a cohort of HIV-1–infected subjects was associated with increased risk of HIV-1 infection. Moreover, a genotype with two functional copies of *NKG2C* was significantly enriched in long-term non-progressors compared to normal progressors. This indicates a functional role for the NKG2C receptor in HIV-1 infection (310), which remains to be established experimentally.

CMV-driven expansion of NKG2C$^+$ NK cells has received great interest as it has been implied in conferring adaptive, memory-like functions to NK cells (311). Briefly, first evidence came from a study in hematopoietic stem cell transplantation (HSCT), where infusing NK cells from CMV-seropositive donors into CMV-seropositive HSCT recipients led to expansion of donor NKG2C$^+$ NK cells and production of increased amounts of IFN-γ in comparison to donor NKG2C$^+$ NK cells infused into CMV seronegative HSCT recipients (312). This hinted at a previous priming of donor NKG2C$^+$ NK cells leading to an enhanced antiviral response upon re-challenge with CMV in the CMV seropositive HSCT recipient (312). Additional evidence of adaptive NK-cell function in a rhesus macaque model demonstrated that splenic NK cells derived from previously SIV-infected macaques specifically lysed DCs pulsed with SIV Gag or Env *in vitro*. Remarkably, antigen-specific NK-cell cytotoxicity against Gag- or Env-pulsed DCs was reduced by blocking NKG2A and NKG2C, which suggests a potential role of these receptors in NK-cell memory (313). Taken together, in humans, the role of activating NKG2C:CD94 receptors in HIV-1 infection, either for increased recognition of HIV-1–infected target cells *via* HLA-E (independent of CMV) or for a potential HIV-1 specific NK-cell response remains to be further investigated.

HLA-E Is Affected by Dimorphism in the Leader Peptide of HLA-B

An additional factor impacting HLA-E surface expression is a dimorphism in the leader peptide of HLA-A, -B, and -C. *HLA-A* and *HLA-C* alleles encode for a methionine at position 2 of the leader sequence, whereas *HLA-B* alleles can either encode for methionine (-21M) or threonine (-21T) at this position. Leader sequences with threonine at P2 do not allow for stable induction of HLA-E surface levels and consequently fail to confer protection from NK cells through engagement of inhibitory NKG2A:CD94 (279). In HIV-1 infection, *HLA-B* alleles containing a Bw4 motif are associated with protection from AIDS (69) and all HLA-Bw4 alleles (with the exception of HLA-B*38:01) encode for the −21T polymorphism (42), whereas HLA-Bw6 alleles encode for either −21T or −21M. In a large cohort of serodiscordant Zambian couples, Merino et al. aimed to elucidate the impact of HLA-B leader peptide dimorphism independent of the Bw4 motif. Compound carriage of either Bw6/−21T or Bw4/−21T alleles displayed similar levels of protection in comparison to Bw6/−21M alleles, which were associated with increased risk of seroconversion. This indicates an independent protective effect of the −21T dimorphism on HIV-1 acquisition (314). Moreover, NK cells lysed HIV-1–infected CD4$^+$ T cells or HIV-1–infected monocyte-derived macrophages preferentially when target cells encoded for −21T/T over a range of various HIV-1 strains. Antibody-mediated blockade of HLA-E on −21M/M target cells increased NK-cell cytotoxicity, whereas no change was observed for −21T/T target cells. Surprisingly, in this study mean fluorescence intensity of HLA-E surface expression did not differ between the −21T/T, T/M, or M/M subsets (315). However, recent analyses employing mass cytometry revealed that donors with at least one copy of −21M displayed increased surface HLA-E levels compared to −21T homozygous donors. NK cells of −21M donors displayed reduced amounts and frequencies of NKG2A:CD94, but a higher phenotypic diversity (42). In this study, Horowitz et al. additionally showed that the increased availability of HLA-E peptides in −21M donors is important for NK-cell functionality (42).

Shaping of NK cell function *via* self-reactive inhibitory NK-cell receptors is a well-described process termed licensing or education (316). It is governed by two independent systems, the well-conserved interaction of NKG2A with HLA-E and the diversified interaction of HLA-I and inhibitory KIR (301). The presence of −21M leader peptides available for HLA-E stabilization indeed correlated with an increased polyfunctional NK-cell response in terms of ADCC, IFN-γ production and degranulation against the missing-self K562 target cell line compared to NK cells derived from donors with a −21T/T genotype (42). Based on the dimorphism in the HLA-B leader peptide, Horowitz et al. described the evolution of two distinct HLA-I haplotypes, which can be distinguished by the inhibitory receptor system operating in NK-cell education. The first, more ancient haplotype encoding the HLA-E permissive −21M and HLA-C1 alleles is skewed toward the supply of ligands for NKG2A:CD94, whereas the second haplotype encodes the non-HLA-E–permissive −21T dimorphism, *HLA-B* with a Bw4 motif and HLA-C2/C1 allotypes, hence being more skewed toward encoding strong KIR ligands (42). Studying the two potential routes of licensing, Bernard et al. reported that the NKG2A$^+$ NK cell subset mounted the highest polyfunctional response against infected CD4$^+$ T cells, without further modulation through coexpression of inhibitory KIR3DL1 (300). An earlier study reported a dual effect of NKG2A and inhibitory KIR coexpression in promoting NK cell education as well as survival (105). Coexpression of inhibitory KIR (with the presence of cognate ligand) with the activating NKG2C receptor following CMV reactivation after hematopoietic cell transplantation was required for robust cytokine production by NK cells (317). This raises the question of which combination of KIR and NKG2 receptors results in best possible NK-cell functionality and survival in combating HIV-1 infection. Overall, HLA-E—aside from presenting peptides—has clearly an additional function in NK-cell education through NKG2A. This in turn may explain the antiviral capacity of NKG2A$^+$ NK cells as observed *in vitro* following HIV-1 infection.

CONCLUDING REMARKS

Studies from the preantiretroviral treatment era suggest that early events in acute HIV-1 infection influence the rate of HIV-1 disease progression. NK cells, as first-responding innate effector cells, have been shown to expand in early HIV-1 infection and

kill HIV-1–infected cells, with genetic studies robustly linking variants in NK-cell receptors to HIV-1 acquisition and disease progression. Additionally, according to mouse models of chronic viral infections, NK cells have the potential to regulate adaptive immune responses, possibly even impairing an effective adaptive response (158, 318). Notably, the antiviral effector potential of NK cells is closely linked to HLA-I. HLA-I allows not only for effective NK-cell education, but also modulates NK-cell activity toward HIV-1-infected cells *via* changes in HLA-I surface expression and peptide presentation. While numerous studies have established a role for the KIR interaction with classical HLA-I in HIV-1, recent advances have increased our understanding of non-classical HLA-E, -F, and -G in HIV-1 infection. First, HLA-F was identified as a ligand for KIR3DS1, which is prominently associated with HIV-1 disease control. HLA-F may serve as a "stress" signal on HIV-1–infected cells, at best enhancing KIR3DS1$^+$ NK-cell killing of infected cells, and at worst mediating HIV-1–associated immunopathology (**Figure 2B**). Second, HLA-E expression levels are not downregulated in HIV-1, which is important as HLA-E is capable of presenting viral peptides. Moreover, HLA-E can tune NK-cell function through NKG2A in virtually all individuals, and is linked to a superior antiviral capacity of NKG2A$^+$ NK cells (**Figure 2A**). Third, HLA-G has predominantly immunomodulatory properties (rather than a peptide-presenting function), and although genetic studies are teasing apart the link between *HLA-G* polymorphisms and HIV-1 disease, the impact of HLA-G on NK-cell function in HIV-1 has yet to be determined (**Figure 2C**). Eventually, the unique properties of these non-classical HLA-I molecules and their conservation between individuals renders them an ideal target for new approaches aimed at harnessing innate immunity against HIV-1.

AUTHOR CONTRIBUTIONS

AH wrote the first draft of the manuscript, WG-B and MA have made substantial, direct, and intellectual contributions to the work and all authors approved it for publication.

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Innate Lymphoid Cells in HIV/SIV Infections

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Over the past several years, new populations of innate lymphocytes have been described in mice and primates that are critical for mucosal homeostasis, microbial regulation, and immune defense. Generally conserved from mice to humans, innate lymphoid cells (ILC) have been divided primarily into three subpopulations based on phenotypic and functional repertoires: ILC1 bear similarities to natural killer cells; ILC2 have overlapping functions with TH2 cells; and ILC3 that share many functions with TH17/TH22 cells. ILC are specifically enriched at mucosal surfaces and are possibly one of the earliest responders during viral infections besides being involved in the homeostasis of gut-associated lymphoid tissue and maintenance of gut epithelial barrier integrity. Burgeoning evidence also suggests that there is an early and sustained abrogation of ILC function and numbers during HIV and pathogenic SIV infections, most notably ILC3 in the gastrointestinal tract, which leads to disruption of the mucosal barrier and dysregulation of the local immune system. A better understanding of the direct or indirect mechanisms of loss and dysfunction will be critical to immunotherapeutics aimed at restoring these cells. Herein, we review the current literature on ILC with a particular emphasis on ILC3 and their role(s) in mucosal immunology and the significance of disrupting the ILC niche during HIV and SIV infections.

Keywords: innate lymphoid cells, innate immunity, HIV infections, SIV, mucosal immunity

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INTRODUCTION

Innate lymphoid cells (ILC) encompass a broad diversity of cell types including the nominal subtypes ILC1, ILC2, ILC3, and in some descriptions also include traditional natural killer (NK) cells and lymphoid tissue inducer cells, all of which arise from a common lymphoid progenitor. A common consensus in the field favors grouping of these cells based on the dependence on transcription factors, as well as by production of major cytokine classes (1–4). ILC1 and NK cells rely on the transcription factor T-bet and produce type I cytokines, such as IFN-γ and TNF-α, but notably ILC1 lack the complex cytotoxic functions inherent to NK cells. ILC2 are classified by their dependence on GATA3, and their production of IL-5 and IL-13 (5, 6), and finally ILC3 are generally identified by their dependence on RORγt and AHR, and secretion of IL-17 and IL-22 (7). Interestingly, through the expression of their respective cytokines and dependence on transcription factors for their development, the three ILC groups (1–3) show strong commonalities with TH1, TH2, and TH17/TH22 cells, respectively (8). It is important to note that the classification scheme remains somewhat fluid and grouping is not absolute, as NK cells and ILC1 do not always require T-bet (9), and ILC2 and ILC3 can both convert to ILC1 (10), underscoring the inherent plasticity of these cell types. To further complicate matters, innate subsets of lymphoid cells may also

include mucosal-associated invariant T cells (11), which express a semi-invariant T cell receptor and defined phenotypically as $CD3^+V\alpha7.2$ TCR^+CD161^{high} cells in humans (12, 13). In addition to their cellular and functional plasticity, ILC have a wide tissue distribution and thus are thought to be some of the earliest responders to infections and other inflammatory stimuli, but the full mechanisms involved are still poorly understood. Striking observations have revealed that lentiviral infection leads to the depletion of functional ILC3 in gut mucosae (14–16), and increased microbial translocation from the gut lumen and an overt disruption of epithelial tissue integrity in HIV^+ individuals is linked to a massive loss of IL-17-producing gut-resident lymphocytes (17). It is now becoming increasingly clear that reduced IL-17 and IL-22 production during infection cannot be attributed solely to the loss of TH17/TH22 cells and that early depletion of ILC may also contribute to this process.

ILC PHENOTYPES AND DISTRIBUTION

Although ILC are typified by their unique plasticity and their descriptive definitions are somewhat fluid, some generally accepted phenotypic nomenclatures have been established. ILC are usually identified as negative for common lymphocyte lineage markers (Lin^-) that are otherwise distinct from NK cells and can usually be distinguished as such by higher expression of the IL-7 receptor, CD127 (1–4). However, even these definitions can vary significantly as "Lin" markers differ depending on the animal species (**Table 1**). For instance, in mice the Lin group may include CD3, CD4, CD8, CD11b, CD11c, CD14, CD19, B220, FcεRI, TER119 antigen, and GR1, whereas in humans, the Lin group may include CD1a, CD3, CD11c, CD14, CD16, CD19, CD34, CD123, TCRαβ, TCRγδ, BDCA2, and FcεRI. Burkhard et al. (18) recommends using CD5 marker in order to exclude small levels of contaminating T cells, especially for analyzing ILC3 populations. Regardless, these exclusion criteria remove T, B, NK, and dendritic cells, as well as other myeloid/granulocyte-derived cells and stem cells. ILC in rhesus macaque models align most closely to patterns seen in humans, but partly due to variability in cross-reactive reagents, may be more simply defined by excluding CD3, FcεRI, CD14, CD20, and NK cell-related markers, such as NKG2A or NKp46 (15, 16, 19). It is also important to note that exclusion of Lin markers may vary significantly between laboratories. Several other factors are used to characterize ILC, including the presence of various cytokines mentioned above and utilization of key transcription factors and receptors (1–5, 20). The co-expression of NKp46 and NK1.1 classifies mouse ILC1 subsets including related NK cells from other ILC groups whereas the expression of transcription factors, namely T-bet and Eomes, can be used to distinguish ILC1 and NK cells from each other (21–23). Loosely, in mice, NK cells are $T\text{-}bet^+Eomes^+$ while ILC1 are $T\text{-}bet^+Eomes^-$ cells, although exceptions to this classification occur (9). Based on the nomenclature proposed by different reports (1, 2), ILC1 can be more comprehensively phenotyped as $Lin^-CD127^+ROR\gamma t^-T\text{-}bet^+IL\text{-}1R^+$ cells in mice, and $Lin^-CD127^+ICOS^+ROR\gamma t^-T\text{-}bet^+IL\text{-}1R^+$ cells in humans. ILC2 are described as $Lin^-CD25^+CD127^+ICOS^+THY1^+SCA1^+ST2^+$ cells in mice and $Lin^-CD25^{lo}CD127^+CD161^+ICOS^+CRTH2^+ST2^+$ in humans. Similarly, ILC3 may be identified as $Lin^-CD25^+CD127^+CD117^+THY1^+NKp46^{+/-}ROR\gamma t^+IL\text{-}1R^+$ in mice and $Lin^-CD127^+CD161^{+/-}CD117^+NKp46^{+/-}NKp44^{+/-}ROR\gamma t^+IL\text{-}1R^+IL\text{-}23R^+$ in humans. ILC may also be partially identified by receptors of cytokines to which they are responsive—$IL\text{-}12R\beta2^+$ (ILC1), IL-17RB$^+$ (ILC2), and IL-23R$^+$ (ILC3), but due to issues with antibody specificity may best be shown molecularly or in functional assays. Collectively, these phenotypic descriptions of ILC populations continue to evolve, and while there is generally a good consensus about the definition of ILC2 and ILC3, what truly defines ILC1 is still somewhat unclear. Currently there are no unique markers or complete phenotypes that uniquely identify ILC1, and the field is still limited to their identification *via* exclusion criteria—i.e., cells that are not NK cells, ILC2, or ILC3. Functionally ILC1 are identified as IFN-γ-producing cells that are distinct from NK cells through their low cytotoxic potential. Understandably, these factors make the study of ILC1 particularly cumbersome. Indeed, a recent profiling of ILC across tissues using mass spectrometry by Simoni et al. (24) indicated lack of ILC1 as described previously by other groups (25, 26). Instead, they described a unique intra-epithelial ILC1-like cells (ieILC1) that matched the description by Fuchs et al. (27).

Although ILC are generally found systemically, they are disparately distributed by subpopulation and are particularly enriched in mucosal sites and secondary lymphoid organs (**Table 1**). ILC have been identified in the lungs (ILC1, ILC2), colon (ILC3), small intestine (ILC1, ILC3), oral mucosae (ILC3), as well as in bone marrow, blood (ILC1, ILC2), lymph nodes (ILC3), liver (ILC1), and even in embryonic tissues (40), although the ILC-related NK cells tend to be much more broadly distributed (8). How ILC populations are maintained and replenished is unfortunately not well defined. Tissue-resident ILC predominantly replenish by

TABLE 1 | Phenotypic markers and tissue distribution for innate lymphoid cell (ILC) groups.

	ILC1	ILC2	ILC3	Reference
Mouse[a]	$Lin^-CD127^+ROR\gamma t^-T\text{-}bet^+IL\text{-}1R^+IL\text{-}12Rb2^+$	$Lin^-CD25^+CD127^+ICOS^+THY1^+SCA1^+ST2^+IL\text{-}17Rb^+$	$Lin^-CD25^+CD127^+CD117^+THY1^+NKp46^{+/-}ROR\gamma t^+IL\text{-}1R^+IL\text{-}23R^+$	(13, 20, 28–31)
Human[b]	$Lin^-CD127^+ICOS^+ROR\gamma t^-T\text{-}bet^+IL\text{-}1R^+IL\text{-}12Rb2^+$	$Lin^-CD25^{lo}CD127^+CD161^+ICOS^+CRTH2^+ST2^+IL\text{-}17Rb^+$	$Lin^-CD127^+CD161^{+/-}CD117^+NKp46^{+/-}NKp44^{+/-}ROR\gamma t^+IL\text{-}1R^+IL\text{-}23R^+$	(13, 20, 32–36)
Tissues distribution	Lungs, small intestines, blood, bone marrow, liver	Lungs, blood, bone marrow, skin, small intestines	Colon, small intestines, oral mucosae, lymph node, bone marrow, skin, spleen, thymus	(20, 25, 30, 32, 33, 35–39)

[a]*Lineage markers for mouse are CD3, CD4, CD8, CD11b, CD11c, CD14, CD19, B220, FcεRI, TER119 antigen, and GR1.*
[b]*Lineage markers for humans are CD1a, CD3, CD11c, CD14, CD16, CD19, CD34, CD123, TCRαβ, TCRγδ, BDCA2, and FcεRI.*

self-renewal (40), though evidence suggests that common precursor cells from the bone marrow, or elsewhere, may also contribute to ILC homeostasis *via* cell recruitment (22, 41).

ROLE OF ILC IN GASTROINTESTINAL (GI)-RELATED DISEASES AND REPAIR

Innate lymphoid cell populations are constitutively present in the GI tract and lymphoid tissues but differ in their compartmental distribution (42, 43). In healthy humans, ILC1 are the major population in the upper compartment of the GI tract while ILC3 are elevated in ileum and colon (44). The local distribution of ILC within the GI tract also differ—ILC1 predominate the intra-epithelial compartment of the intestine (27, 41, 42), while ILC2 are present in fat-associated lymphoid clusters in the intestinal mesentery and in significant numbers in lamina propria of small intestine where ILC3 is the dominant population (44–46). ILC3 are also enriched in the isolated lymphoid follicles, cryptopatches, and perifollicular area of Peyer's patches at steady state (20, 47).

In the healthy gut, ILC3 are thought to be one of the major cell populations contributing to overall homeostasis. This is, in part, because ILC3 produce large quantities of IL-22 and IL-17 (48), and directly interact with intestinal epithelial cells to maintain an intact barrier and modulate inflammation (49, 50). IL-22 protects intestinal epithelium from inflammation and promotes wound healing by inducing STAT-3 dependent increases in production of antimicrobials by epithelial cells and epithelial cell proliferation, thus maintaining barrier integrity (51–53). In a mouse model of dextran sulfate sodium-induced ulcerative colitis, microinjection-based gene delivery of IL-22 ameliorated local inflammation through activation of STAT-3 in colonic epithelial cells, stimulation of mucus production, and goblet cell restitution (54). In IL-22$^{-/-}$ mice, increased intestinal damage, bacterial burden, and mortality was observed on infection with *Citrobacter rodentium* (52), and in humans, IL-22 has been shown to protect intestinal epithelium in IBD (55). Specifically, IL-23 responsive, IL-17/22-producing ILC protected intestinal stem cells against intestinal inflammation leading to epithelial regeneration in graft versus host disease patients who underwent bone marrow transplantation (56).

Another mechanism by which ILC regulates intestinal homeostasis is through their interaction with the commensal and/or pathogenic microbiota (48, 50, 57). Several protective mechanisms exist in the gut for the containment of commensal bacteria within intestinal sites including tight epithelial junctions, production of mucus and antimicrobial peptides, and immunological mechanisms that include ILC- and IgA-mediated immune exclusion pathways (58–63). ILC3 prevent commensal bacterial dysbiosis by IL-22-mediated induction of antimicrobial proteins (RegIIIβ, RegIIIγ, and β-defensins), element-sequestering proteins (S100A8, S100A9, and lipocalin-2) and mucins in epithelial cells leading to a strengthened intestinal epithelial barrier (49, 64–66). For example, depletion of ILC in mice led to selective peripheral dissemination of a commensal bacteria originating from host lymphoid tissues, namely *Alcaligenes* spp. and alcaligene-specific immune responses were found to be associated with Crohn's disease and Hepatitis C virus-infected patients (63, 64). Further, ILC3 also are involved in the formation of gut-associated lymphoid tissues (GALT), including cryptopatches and isolated lymphoid tissues, which are important for protection against pathogens and act as niche areas of symbiosis for colonizing commensal microbiota (63). In turn, microbial products and signals were also found to be necessary for epigenetic modifications of ILC contributing to their diversity, plasticity, and maintenance of intestinal homeostasis (48, 57). This was evidenced by a study conducted by Manuzak et al. (67), describing the beneficial effects of probiotic therapy in healthy rhesus macaques by toll-like receptor (TLR) mediated downregulation of intestinal inflammatory markers and elevated ILC3 and T-follicular helper cells in colon.

Innate lymphoid cell can also act as a first line of defense at mucosal portals of entry due to their rapid production of cytokines following initial exposure to pathogens and recruitment of other innate and adaptive cells to sites of infection. ILC1 produce IFN-γ and TNF-α, both of which are important in the control of infections by intracellular pathogens such as *Toxoplasma gondii* (41) and *Listeria monocytogenes* (68). Furthermore, mice deficient in ILC3 were susceptible to intestinal pathogens including *Helicobacter* spp. and *Clostridium difficile* (69, 70). In helminthic infections, IL-25-mediated activation of ILC2 promotes a TH2 response which is important for an effective elimination of parasites (71). IL-17 is essential for the control of *Candida albicans* infection suggesting the importance of ILC3 in protection against oropharyngeal candidiasis in mice (72).

Given their critical roles in maintaining mucosal homeostasis, altered frequency or function of ILC during chronic disease could contribute to exacerbated intestinal inflammation. Indeed, intestinal ILC1 are elevated in IBD (73, 74), and production of IFN-γ by IL-15-activated ILC1 may play a major role in the pathogenesis of celiac and Crohn's disease (25, 75). ILC2 along with NKT cells can also promote IL-13-mediated inflammation in an oxazolone-induced model of colitis (76). Interestingly, IL-23 responsive ILC3 can play a pathogenic role in intestinal inflammation through the production of IL-17A and IFN-γ and are also increased in patients with IBD (25, 28, 73, 74, 77–79). Given the significant protective roles ILC mediate in the GI tract, it may be important to take into account various interactions with intestinal epithelium and microbiota in achieving a balance of positive and negative ILC-related functions.

LOSS OF ILC IN PATHOGENIC LENTIVIRUS INFECTIONS

One of the hallmarks of HIV and pathogenic SIV infection is early loss of gut integrity followed by massive and rapid translocation of microbial products from the lumen of the intestine into the lamina propria, blood, lymph nodes, and liver (80–83). Indeed circulating lipopolysaccharide (LPS), sCD14, and other microbial products are now well-established biomarkers for microbial translocation and immune stimulants associated with inflammation and chronic immune activation. Because ILC, particularly ILC3, play major roles in maintaining gut integrity, tissue modeling, and repair (53, 84–86), these cells are likely critical players in the pathophysiology of HIV/SIV disease.

Initial work in SIV-infected rhesus macaques by our group and others showed that ILC3 are generally restricted to mucosal tissue, express high levels of RORγt, and produce IL-17 and IL-22 much like their human counterparts, but they are depleted or otherwise dysfunctional in infection (15, 16, 87). Specifically, we showed that even 1 week following SIV infection there was up to a threefold reduction in ILC3 in colon and fourfold to ninefold reduction in jejunum and ileum (19) and that this loss was maintained during chronic infection. Surprisingly, SIV viral loads did not correlate with the loss of ILC3 (19), nor were ILC3 infected *in vivo* (15).

Functionally, ILC3 from SIV-infected animals took on a more cytotoxic phenotype and produced greater quantities of TNF-α, IFN-γ, and MIP-1β, but reduced levels of IL-17 (14). This cytokine profile suggests lentivirus infection may drive ILC3 plasticity toward ILC1, as has been previously described for mice (10). Similarly, a study by Xu et al. (16) clarified the kinetic changes in IL-17-producing ILC3 from intestinal epithelium by showing a reduction during acute pathogenic SIV infection (7–14 days postinfection) is followed by an increase in the total numbers of ILC (14–21 days postinfection) and eventually a gradual decline of ILC3 with disease progression after 28 days postinfection (16). Klatt et al., (87) also noted a significant depletion of all IL-17-producing lymphoid cells in rhesus macaques, but not in sooty mangabeys, where SIV replicates efficiently but does not cause significant mucosal barrier damage. This observation further underscores a potential role for ILC3 in maintaining gut homeostasis in HIV/SIV infections. Work in an HIV model of humanized mice by Zhang et al. (88) showed that persistent HIV-1 infection depleted ILC3 but effective antiretroviral therapy reversed this loss.

In human subjects, Kloverpris et al. (89) found that all three subgroups of ILC in blood were depleted during infection, but early administration of ART restored all ILC subsets. However, if ART was not administered within 5–14 days after infection, only ILC3 were partially restored while ILC1 and ILC2 remained depleted. Much like had been shown in SIV-infected macaque models (14), ILC3 loss did not occur in tonsil or other oral mucosal tissues (89). Surprisingly, they did not detect a reduction of ILC numbers in the gut, and a similar observation was made by Fernandes et al. (90). Although ILC levels in the gut during acute infection were not measured. The reason for this discrepancy between these studies and multiple macaque studies are not clear, but could be species specific. This could also be the reason for the contrasting observations made by Liyanage et al. (91), suggesting no restoration of NKp44$^+$ cells in the rectum after ART. More recently, a study by Kramer et al. (92) showed that intestinal ILC distribution is significantly perturbed in patients even on effective antiretroviral therapy and that levels of colonic ILC3 were inversely correlated to markers of microbial translocation.

One of the proposed mechanisms leading to mucosal inflammation in HIV infection is the interaction of viral envelope gp120 with polarized epithelial cells directly disrupting epithelial tight junctions (93–95). A closer look at the effect of viral infection on epithelial cells showed that HIV-1 directly reduces transepithelial resistance, a measure of epithelial cell monolayer integrity by 30–60% without affecting its viability (93). Furthermore, functions of tight junction proteins, such as claudin 1, 2, 4, occludin, and ZO-1, were also disrupted and significantly increased inflammatory cytokines, such as TNF-α, IL-6, MCP-1, and IL-1β (93). The resulting increase in cytokine production following T cell infection may also cause intestinal barrier breakdown [(96), reviewed in Ref. (97)].

The effect of HIV-2 on the other hand is less obvious. A previous study correlated both HIV-1 and HIV-2 with microbial translocation. However, a more recent study by Fernandes et al. (98) suggests no disruption of the epithelial tight junction by HIV-2 despite active replication. How ILC-mediated mucosal maintenance may differ in less pathogenic infections such as HIV-2 remains unstudied. Collectively, these data indicate that in both HIV-infected humans and pathogenic SIV-infected rhesus macaque models, ILC3 loss in the gut occurs early, is at least partially irreversible, and is linked to mucosal dysregulation and translocation of microbial products.

MECHANISMS OF ILC LOSS IN PATHOGENIC HIV/SIV INFECTIONS

While the loss of ILC during HIV/SIV infection is well established, multiple groups have pursued molecular and cellular mechanisms leading to this depletion. We had previously observed that the expression of IDO1, an enzyme upregulated during SIV infection [also observed in Ref. (99)] (**Figure 1**) correlates negatively with CD4$^+$TH17 cells as well as ILC3 (15). In HIV, IDO has been implicated in immunosuppressive activity (100) and dysbiosis during disease progression (101). Although the source(s) of IDO1 are not totally clear, the ability of HIV-1 TAT to induce production of IDO catabolites by dendritic cells has been described previously (102). Interestingly, increased levels of IDO1 in the gut showed a negative correlation of CD4$^+$ T cells and ILC3 but not with NK or CD8$^+$ T cells (15). This suggested IDO1 expression could be a negative regulator of ILC3 but not other effector cells. Furthermore, we were able to confirm that IDO catabolites caused numerical and functional depletion of ILC3 through a similar mechanism described for TH17 cells (99). Increased apoptosis leading to massive loss in total numbers of ILC3 was observed; however, the loss was not due to direct infection as no detectable SIV RNA was present in these cells. This is not surprising, as ILC do not express receptors for SIV/HIV.

Further studies indicated that the loss of ILC3 in the mucosae during acute infection was due to increased apoptosis and RORγt suppression induced by inflammatory cytokines, such as TGF-β, IL-2, IL-12, and IL-15 (19). In pathogenic SIV infection, we also showed previously that plasmacytoid DC (pDC) accumulates in the gut mucosa producing large quantities of IFN-α (103) (**Figure 1**). HIV-1 infection in a humanized mouse model and *in vitro* treatment of splenic ILC3 with IFN-α or HIV-1 significantly upregulated CD95 expression on ILC3 leading to apoptosis dependent on pDCs (88). RNA-seq analysis of ILC in human subjects with acute HIV-1 infection showed that there was a downregulation of genes associated with viability (89), and gene array analysis (87) showed that mucosal IL-17$^+$

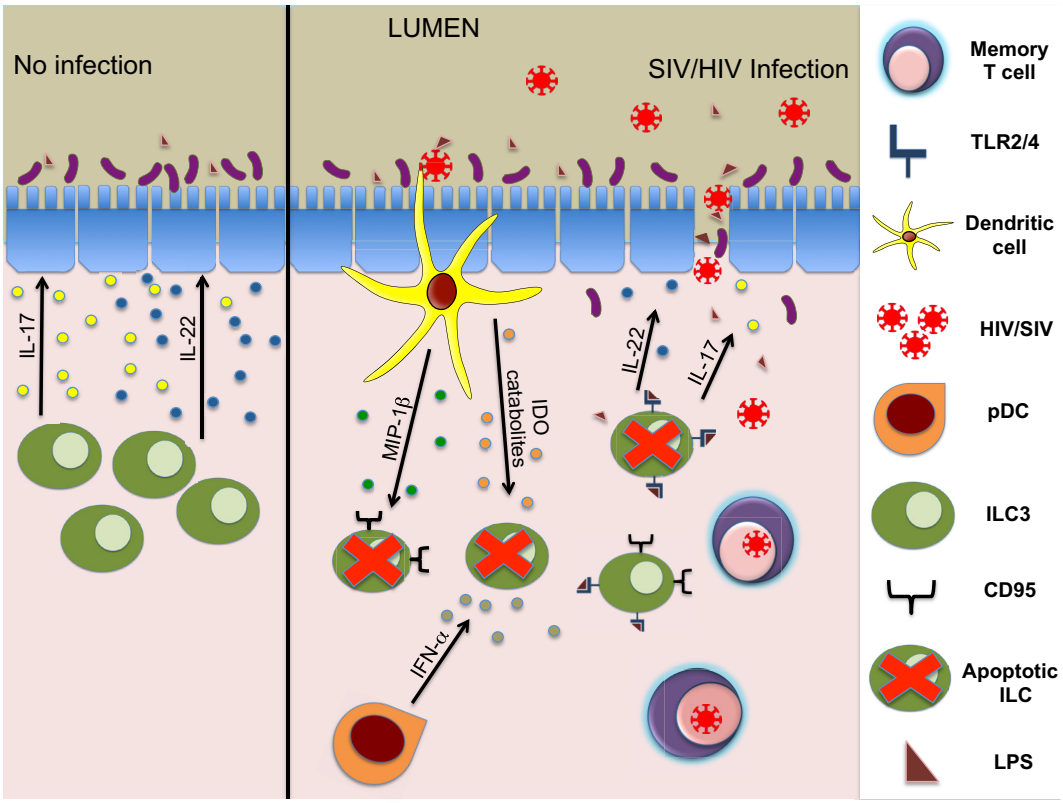

FIGURE 1 | Mechanisms of ILC3 depletion during HIV/SIV infections. ILC3 modulate structure and homeostasis of gut epithelial cells *via* secretion of IL-17 and IL-22 (left panel). During the acute phase of lentivirus infection, early innate responders (DCs and other cells) secrete cytokines leading to apoptosis of ILC3. Subsequently, reduced IL-17 and IL-22 production leads to damage of gut epithelial barrier and an influx of microbial products, causing further inflammation (right panel).

cells highly expressed TNF-receptor superfamily 4 (TNFRSF4, OX40), a co-stimulatory molecule involved in maintenance of mucosal lymphocytes, in comparison to IL-17$^-$ cells (104–106). Finally, ILC3 were shown to be depleted in lymphoid tissues mediated by TLRs in SIV-infected animals (107) (**Figure 1**). This study specifically showed that microbial translocation and resulting products like lipoteichoic acid or LPS *via* the TLR2/4 pathway can directly cause apoptosis in ILC3, further increasing HIV-induced disruption of GALT.

Altogether, these data indicated that a primary mechanism of ILC loss is likely apoptosis due to dysregulation of homeostatic elements on which ILC depend. One potential avenue that could be explored to restore ILC and gut integrity is IL-7-based therapies. Indeed in mice, IL-7 promoted IL-22 production during chronic LCMV infection (108); and in macaques, IL-7 therapy was shown to improve gut mucosal integrity in acute SIV-infected animals (109). Similarly, IL-7 immunotherapy in chronically infected HIV patients were associated with CD4$^+$ T cell protective functions (108, 110, 111) and led to an overall reduced systemic inflammation (110). While these studies suggest that IL-7 plays a key role in repairing gut immunity, the precise connection to ILC is clearly understudied and needs further evaluation. Interestingly, it was also recently shown that SIV-ALVAC in combination with multiple adjuvants could induce an expansion of ILC3 (112). Whether or not this modality could be used therapeutically to restore ILC or could contribute to protective vaccine efficacy remains to be elucidated.

CONCLUSION AND PERSPECTIVES

Innate lymphoid cell fill a unique and plastic niche of primarily tissue-resident cells that provide innate sources of typical T cell and NK cell produced cytokines, and although they clearly have a role in innate defense and homeostasis, many unknowns remain. Not the least of which being a recent report indicating that individuals lacking ILC may experience no obvious pathology as long as an intact T and B cell compartment remains (113). Specifically, regarding lentivirus infections, infection itself is not the source of depletion, but rather indirect or direct apoptosis, and while some potential mechanisms have been described herein this list is unlikely exhaustive or complete. It is also important to note that in several HIV studies no ILC depletion is observed in the gut. Regardless, whether loss is a virus-mediated subversion or an off-target effect of massive inflammation is unclear, and although ILC3 seemingly mediate gut homeostasis, their exact roles, both kinetically and functionally, in the perturbation and subsequent microbial translocation following HIV and pathogenic SIV infections are not obvious. And given the tight reciprocal relationship

between gut microflora and ILC3 in mice, it will be interesting to determine if ILC3 depletion also contributes to dysbiosis. Direct evidence for these phenomena will need to be confirmed by *in vivo* depletion strategies in macaques, should those reagents become available. Further, HIV/SIV clearly intersects with ILC3 but whether ILC2 and ILC1 also contribute against viral pathogenesis is less clear and will require further study. Nonetheless, despite a host of unknowns, the field as a whole can appreciate the novelty of these cell populations and conclude that manipulating ILC as early responders to infection could be an attractive target for multiple infectious as well as chronic conditions.

AUTHOR CONTRIBUTIONS

SS performed most of the writing and designed the figure. CM and DR contributed to writing of specific sections. RKR oversaw overall preparation of the manuscript, contributed to writing, and edited the final version.

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REVIEW
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Inhibiting the Ins and Outs of HIV Replication: Cell-Intrinsic Antiretroviral Restrictions at the Plasma Membrane

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Like all viruses, human immunodeficiency viruses (HIVs) and their primate lentivirus relatives must enter cells in order to replicate and, once produced, new virions need to exit to spread to new targets. These processes require the virus to cross the plasma membrane of the cell twice: once via fusion mediated by the envelope glycoprotein to deliver the viral core into the cytosol; and secondly by ESCRT-mediated scission of budding virions during release. This physical barrier thus presents a perfect location for host antiviral restrictions that target enveloped viruses in general. In this review we will examine the current understanding of innate host antiviral defences that inhibit these essential replicative steps of primate lentiviruses associated with the plasma membrane, the mechanism by which these viruses have adapted to evade such defences, and the role that this virus/host battleground plays in the transmission and pathogenesis of HIV/AIDS.

Keywords: human immunodeficiency virus, type I interferons, antiviral restriction, plasma membrane, tetherin/BST-2, serine incorporator, interferon-induced transmembrane

INTRODUCTION

A key feature of eukaryotic cells is the plasma membrane (PM), the single lipid bilayer that delimits the cytoplasm from the extracellular milieu (1). As well as acting as the physical boundary of the cell, the PM acts as a platform which plays a role in almost every cellular process, from regulating transport of small molecules and proteins in out of the cell, to cell mobility, and the response to its environment. As such, any infectious agent that seeks to gain access to the cell's cytosol must breach the PM or the limiting membranes of intracellular compartments. In the case of enveloped viruses, this entails an entry step in which viral envelope glycoproteins engage specific cellular receptors on the PM or undergo low pH-induced conformational changes upon endocytic uptake (2, 3). As a result of either of these processes, mechanisms intrinsic to the glycoproteins themselves mediate fusion between the viral and host cell membranes, allowing the viral genetic material to enter the cell and initiate the replication cycle. For lentiviruses, the replication cycle culminates in newly synthesized RNA genomes and viral structural proteins being targeted to the inner leaflet of the PM (4). With the aid of a multitude of cellular factors, new virus particles assemble and bud into the extracellular space, acquiring their lipid envelope from the host cell. Budding ends in a scission event that separates the new virion from the cell, allowing it to be released and infect new targets.

These unavoidable processes are common to all enveloped viruses. Moreover, the lipid envelope is the one component of the virus particle that is not encoded by the virus itself. It is perhaps unsurprising that the mammalian host has evolved multiple antiviral mechanisms whose role is to inhibit viral replicative processes that are associated with entry and exit (5–8), necessitating either the evolution of directly encoded countermeasures by the virus, or other mechanisms of resistance or avoidance. Furthermore, these mechanisms are often (but not always) regulated by type 1 interferons (IFN-I) and pattern recognition responses, linking these factors to the wider antiviral immune response.

OVERVIEW OF LENTIVIRAL ENTRY AND EXIT

The mediator of the entry of HIV-1 and its related viruses is the trimeric envelope spike [reviewed in Ref. (9)]. For HIV-1, this is comprised of three precursor Env proteins, gp160, that are proteolytically cleaved into a surface subunit, gp120, and a transmembrane subunit gp41. gp120 harbours the receptor binding components of the envelope spike whereas gp41 encodes the fusion machinery itself, buried within the trimer. gp120 consists of a series of conserved domains interspersed with variable loops and is heavily glycosylated on the outer faces of the trimer (10). There are surprisingly few spikes on the surface of the virion, with estimates of about 10–20 (11). Super-resolution microscopy imaging of HIV-1 particles has shown that these spikes cluster, which appears to be important for fusogenicity (12).

The Env trimer is a metastable structure, poised to mediate viral entry upon interaction with its receptor(s) (9). When gp120 binds to its cognate receptor, CD4 (13–15), on the target T cell or macrophage, structural rearrangements "open" the envelope to reveal a coreceptor binding site (16–18). This interacts with either CCR5 (19–21) or CXCR4 (22), and occasionally additional CC chemokine receptors. Upon coreceptor binding, further conformational changes expose the hydrophobic fusion peptide of gp41, which rapidly inserts in the target membrane. The extended conformation of the gp41 trimer collapses back to form a six-helix bundle common to diverse type 1 enveloped virus fusion proteins (9). This pulls the viral and cellular membranes together, and is sufficient to locally destabilise the membranes, allowing lipid mixing, fusion, and the release of the viral core into the cell (9).

The use of CCR5 appears to be essential for sexual transmission of HIV-1. Viruses that use CCR5 alone (R5), or more rarely CCR5 and CXCR4 [R5/X4 or dual tropic (23)], predominate in early infection (24, 25). Individuals homozygous for a 32 base pair deletion in CCR5 that disrupts its expression are largely HIV-1 resistant (26, 27). X4-using viruses tend to arise later in infection in some, but not all, individuals, and are associated with more rapid progression to AIDS. Whilst they can be transmitted by intravenous drug-use/transfusion, it is not clear why X4 viruses are almost never transmitted sexually given that target CD4+ T cells in the mucosa express CXCR4 (25). The selective pressures that produce the so-called coreceptor switch are not well understood, but it is associated with changes in the V3 loop of gp120 and perhaps arises through escape from certain classes of neutralizing antibody (28). Coreceptor usage in part determines the cellular tropism of the virus; R5 viruses infect predominantly subsets of antigen-experienced CD4+ T cells, whereas X4 usage expands this tropism to naïve cells (9). Macrophage-tropic viruses are almost exclusively R5 users, but importantly macrophage tropism is determined by changes in gp120 that allow it to use much lower cell surface concentrations of CD4 (29, 30). Thus most R5 isolates, including those transmitted between individuals (the so called transmitted-founder (TF) viruses) can only infect T cells (31). Quite why the majority of X4 viruses cannot infect macrophages which express abundant CXCR4 is not known (32).

Where entry occurs in the cell has been of some controversy. The pH-independence of HIV-1 entry would suggest that it occurs at the cell surface (2, 33). This was reinforced by early studies showing that endocytosis of CD4 was not necessary for productive viral entry (34). However, more recent studies have shown that HIV-1 entry is sensitive to certain endocytosis inhibitors, particularly those targeting the GTPase dynamin-2 (35). These effects may be cell-type dependent, as entry appears to be predominantly cell surface in T cell lines (36). Furthermore dynamin-2 may play a role in fusion, independent of its activity in endocytosis (37). Much further work, particularly with clinically relevant isolates, is required to fully rationalize many of these observations. However, the ability of certain membrane associated antiviral factors to differentially restrict HIV-1 entry dependent on their own subcellular localization may allow further insight into these issues.

The next encounter of HIV-1 with the limiting membrane of the cell is viral assembly [reviewed in Ref. (4, 38)]. For lentiviruses, this occurs exclusively at the plasma membrane. Small amounts of Gag and Gag-Pol polyproteins are targeted to the inner leaflet of the PM, bringing with them two copies of the viral genomic RNA. This allows more Gag/Gag-Pol to nucleate around them, and in doing so form a budding virion. Small peptide motifs in the p6 portion of Gag (termed late domains) interact with several members of the ESCRT pathway, a multi component protein machinery that resolves membrane-bound entities budding away from the cell's cytoplasm. The recruitment of the core ESCRT-I subunit TSG101 is the major event in initiating HIV-1 release, although other associated factors can also directly interact with Gag. This then leads to the recruitment of charged multivesicular protein (CHMP) subunits of ESCRT-III. The polymerization of these ESCRT-III subunits into filaments around the inside of the stem of the budding virions and their subsequent depolymerization by the AAA-ATPase VPS4, leads to the contraction of the neck of the bud and the final scission of the virus from the cell. During the budding process, mature Env trimers are recruited into the assembling virion, as well as a number of other host membrane proteins; some beneficial, others, as described below, less so. Co-incident with the latter stages of budding, dimerization of the protease component of the Gag-Pol polyprotein, driven by interactions between reverse transcriptase moieties, activates its catalytic activity. This then leads to the sequential processing of the Gag and Gag-Pol to generate the mature structural and enzymatic components of the infectious virion.

TYPE 1 INTERFERONS AND THE RESTRICTION OF HIV-1 REPLICATION

A burst of systemic inflammatory cytokines driven by type 1 interferons (IFN-I) is one of the earliest host responses detectable in HIV-1 infected individuals (39). Despite the virus being adept at avoiding host pattern recognition receptors in infected cells (see review by Sumner et al. in this issue), the consequence of the rapid increase in viral replication is that systemic IFN-I levels are detectable as early as 7 days after infection. Both alpha and beta interferons activate the same receptor, IFNAR1/2, expressed on the majority of somatic cells, and via the Jak/STAT pathway induce the transcription of hundreds of so-called interferon-stimulated genes (ISGs), many of which, like IFN-I themselves, are also activated directly by pattern recognition responses (40). In addition to the activation of systemic innate and adaptive immunity, a number of these ISGs have direct antiviral activity against the replicative stages of diverse mammalian viruses (7). These antiviral factors, sometimes called restriction factors, often target common pathways or structures that are essential for viral replication, and which cannot be simply mutated around. In the case of lentiviruses, several restriction factors have been identified that are targets of virally encoded accessory proteins (41), for example tetherin and Vpu described below. The evolutionary arms race between these countermeasures and species-specific orthologues of these restriction factors has shaped the adaptation of these viruses to new primate hosts, ultimately allowing chimpanzee and sooty mangabey simian immunodeficiency viruses to cross into humans to become HIV-1 and HIV-2 respectively (42). However, ectopic expression of a number of ISGs have a direct antiviral activity against HIV-1 with no obvious virally-encoded countermeasure (43). HIV-1 replication can be inhibited in primary CD4+ T cells and macrophages in culture by IFN-I treatment, indicating some of these ISGs may play a physiological role in early infection (43, 44). Furthermore, treating HIV-infected patients with pegylated-IFN leads to a transient reduction in viral loads (45). In macaques, although initial mucosal inflammasome activation may inhibit local ISG activation (46), early viremic control of SIVmac infection is dependent on systemic IFN-I responses (47). But perhaps the most powerful evidence of the importance of directly antiviral ISGs in HIV-1 pathogenesis comes from the observation that viruses that represent the most likely founder of an individual's infection, called transmitted/founder (TF) viruses, display a considerably higher resistance to the effects of IFN-I in their replication in primary CD4+ T cells than viruses isolated during the chronic phase (31, 48). While initially controversial in a replication study in subtype C infections using blood-derived viral sequences (49), these observations have been extended and now show that the TF virus sequence in a recipient partner is the most IFN-I resistant amongst the viral quasi-species that existed in the donor partners' genital secretions at the time of transmission in both clades B and C, thus indicating IFN-I resistance is a key attribute for transmission fitness (50). Curiously, as infection progresses, IFN-I resistance in circulating virus wanes (48). There are multiple molecular determinants of this difference in IFN sensitivity between TF and chronic viruses from the same the donor, suggesting a number of ISGs are involved (50). In the sections below, we will discuss host restriction factors and antiviral ISGs that target the entry and exit pathways of the virus.

THE INTERFERON-INDUCED TRANSMEMBRANE (IFITM) PROTEIN FAMILY

The interferon-induced transmembrane (IFITM) proteins are a family of antiviral factors that restrict the fusion of a number of pathogenic enveloped viruses with their target cells, including influenza A virus (IAV), Dengue virus (DENV), hepatitis C virus (HCV), Ebola virus (EBOV) and HIV (51–53). They are predominantly located at the PM and on endosomal membranes, the portals of entry for most viruses (54, 55). Recent studies have sought to identify the mechanisms of their antiviral restriction activities that may explain this broad spectrum activity, which primarily target the entry stages of the viral lifecycle.

Five members of the gene family have been identified in humans, *ifitm 1, 2, 3, 5 and 10*, all clustered on chromosome 11 (56, 57). Unlike *ifitms 1, 2* and *3*, *ifitm5* is not induced by type 1 or type 2 interferons but has been proposed to be involved in bone mineralization. A function for *ifitm10* has not been identified. In the mouse genome, the orthologues of the human *ifitm* genes are located on chromosome 7, with the pseudogene *ifitm4*, also not functional in humans, located in close proximity to *ifitm 1, 2 and 5*. Analogous genes have been identified in other mammals and in the avian species, where the IFITM proteins serve to inhibit influenza viruses.

IFITM Structure and Localisation

IFITMs are members of a larger superfamily of proteins found in both eukaryotes and prokaryotes, known collectively as dispanins (58). Structurally, the IFITMs each contain two hydrophobic domains that are separated by a short conserved intracellular loop (CIL) containing a CD225-like domain; speculation has however surrounded the topological conformation of the domains within the membrane. The current biochemical and cell biology evidence suggest that the IFITMs adopt a topology in which the N-terminus and CIL reside in the cytoplasm, with the first hydrophobic domain existing as an intra-membrane domain whilst the second hydrophobic domain spans the membrane such that the C-terminus resides in the extracellular space (**Figure 1A**) (54, 55, 59). The CIL domain also contains palmitoylation sites that likely stabilize this conformation (60–62). The intramembrane helices of the first hydrophobic domain are postulated to influence the curvature of the membrane in which the IFITM resides thus impacting the restriction activity (59). Evidence of self-association and intramolecular interactions between the IFITM proteins, via residues within the first transmembrane domain, has been reported, suggesting that higher order multimers may have functional implications (63).

Mammalian IFITMs are highly homologous at the amino acid level, and in particular IFITMs 2 and 3 in primates display highly complex positive selection signatures (64) suggesting that they are continually adapting to target pathogenic viruses (65). Such

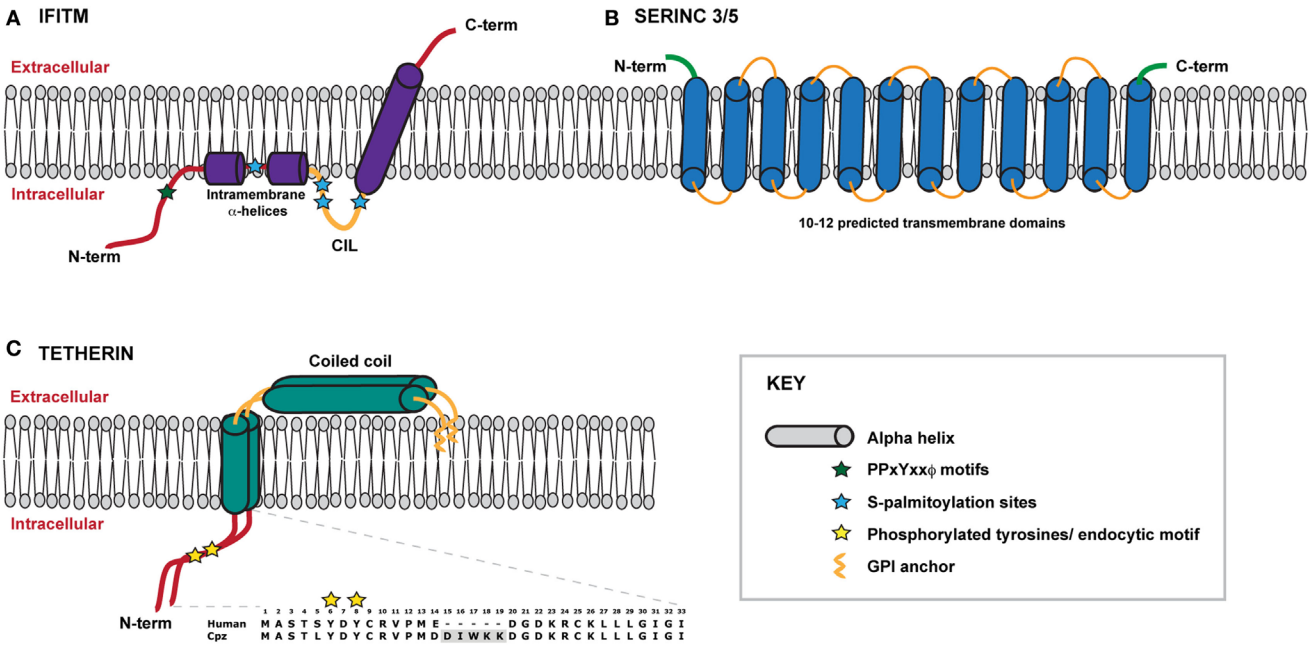

FIGURE 1 | Schematic representation of IFITMS, SERINC 3/5 and tetherin within a model membrane. **(A)** A model of the IFITM protein, which adopts a type II transmembrane protein topology in the membrane. The N-terminal domain lies within the cytoplasm and connects to two short intramembrane α-helices. IFITMs 2 and 3 possess a longer N-terminal domain that contains important trafficking motifs that determine protein localisation. The conserved intracellular loop (CIL) contains sites of palmitoylation that likely stabilise the conformation of the C-terminal transmembrane α-helix which spans the membrane, thus positioning the C-terminal domain within the extracellular space. **(B)** Very little information about the structures of SERINC proteins is currently known. SERINCs 3 and 5 are thought to possess between 10 and 12 transmembrane helices such that the N- and C-termini reside within the extracellular space. **(C)** Tetherin exists as a dimer anchored to the membrane via an N-terminal transmembrane domain and a C-terminal GPI anchor. The extracellular portion of tetherin is comprised of a coiled coil. The N terminal cytoplasmic tail contains a dual tyrosine motif that plays a role in both steady-state cycling of the protein and signal transduction following virus retention. Amino acid sequences of human and chimpanzee cytoplasmic tails are shown for comparison, highlighting the deletion of the DIWKK motif. The short isoform of human tetherin lacks the first 12 amino acids of the cytoplasmic tail.

selection raises the notion that they may be under pressure to provide a continuous barrier across the entry portals into the cell. Consistent with this, human IFITMs localize to distinct but overlapping cellular membranes (54). While IFITM1 appears to be mainly associated with the PM, the longer N-terminal cytoplasmic tail of IFITMs 2 and 3 contain a YxxΦ endocytic motif that permits their localization to early/recycling (IFITM3) and late (IFITM2) endosomal compartments (66, 67). This sorting signal overlaps with an endosomal degradation motif (PPxY) that regulates their turnover (**Figure 1A**) (68). Importantly, therefore, both endosomal IFITMs dynamically traffic via the cell surface to reach their major sites of localization. This localization is a key determinant of the antiviral spectrum which a given IFITM restricts because the mechanism of entry of different viruses (receptor requirements, pH thresholds of fusion etc.) define their sites of access to the cell. For example, mutation of the endocytic motif in IFITM3 such that it redistributes to the cell surface abolishes its antiviral activity against IAV (67). This has major implications for the discussion of their effects on HIV below.

Mechanism of IFITM Restriction

IFITMs appear to block the physical fusion of enveloped viruses with their target membranes, however the mechanism of action is not clear. It is widely postulated from the work of Brass, Liu and others particularly on IAV, that the mechanism of action is through modulation of the host cell membrane fluidity to block viral fusion (69–73). These "tough-membrane" models suggest a number of possible mechanisms: (1) Adjacent IFITM molecules may interact via their intramembrane domains thereby decreasing the fluidity of the host membrane and limiting the lateral movement of host entry receptors and formation of productive receptor complexes. (2) These intramolecular interactions may prevent the effective viral envelope clustering that is required particularly for IAV fusion and (3) the IFITM multiplexes could also form a "meshwork" within the outer leaflet of the membrane that not only decreases fluidity and imposes rigidity but induces an outward membrane curvature that opposes the forces exerted by the viral fusion machinery. These general mechanisms may account for the diversity of viruses inhibited, including non-enveloped viruses, such as reoviruses, that do not require fusion, but do need to disrupt the endosomal membrane to enter the cell (74). Such models are also consistent with observations of IAV and Semliki Forest virus (SFV) accumulating in endosomal compartments where the restricting IFITM resides, without affecting the pH-dependent exposure of the viral fusion machinery (70, 71, 75). Studies have demonstrated that IFITM-mediated restrictions of fusion can be overcome by antifungal drugs that target cholesterol metabolism, and oelic acid treatment that is predicted

to reverse the positive membrane curvature exerted by the IFITM (72, 73). Dye-dequenching transfer experiments using labelled IAV virions suggest that hemifusion, the mixing of lipids from the outer leaflets of viral and cellular membranes, still occurs in the presence of the IFITM (70, 72). However, whether this is generalizable to all enveloped virions is not known.

One related mechanism, suggested by Amini-Bavil-Olyaee et al., is that the direct interaction of IFITM3 with vesicle membrane protein associated protein A (VAPA) leads to a disruption of the VAPA-oxysterol binding protein (OSBP) function that acts to regulate intracellular cholesterol homeostasis (69). In the presence of IFITM3, endosomal membranes become cholesterol laden, less fluid and functionally impaired, thus blocking viral entry. However, other studies have failed to replicate the latter observation (70), and the lack of VAPA interaction with IFITM1 or 2 is difficult to reconcile with their antiviral properties. Lastly, a recent study has suggested that a ubiquitous zinc metalloprotease, ZMPSTE24, previously implicated in processing nuclear lamins, is an essential cofactor for IFITMs independent of its catalytic activity (76). As yet, the mechanism for its role is not known.

Restriction of HIV by IFITMs

All three IFITM proteins have been demonstrated to affect HIV-1 entry and replication, albeit to a lesser degree compared to their effects on other viruses. However, there has been some controversy over their potency and mode of action. The initial study from the Liang group, based on T cell lines ectopically expressing individual doxycycline-inducible IFITMs showed that IFITM2 and IFITM3 could block the entry of a model X4-using laboratory strain, but all three IFITMs could block spreading replication, suggesting multiple stages of the HIV-1 replication cycle were sensitive to IFITM restriction (53). While these differential effects on HIV-1 entry and replication were observed in the target cells, two further studies explored the role of IFITMs in HIV-1 producer cells (77, 78). Both groups observed that IFITMs were incorporated into viral particles, making the particles less infectious. They hypothesized that through cell-cell transmission, the virions were able to circumvent the effect of IFITMs in target cells but cell-free virus spread from infected producer cells is limited as the virions produced become increasingly less infectious through IFITM incorporation (**Figure 2A**). While IFITM3 was found to accumulate at sites of viral assembly on the PM, neither study reported a specific interaction with the envelope glycoprotein or an effect on envelope density due to IFITM incorporation. A third study reported that IFITM overexpression caused an infectivity defect to virions not because of their incorporation *per se*, but because they appeared to directly interact with nascent gp160 and block its processing to its mature subunits (79). The major caveat to all these studies is that the majority of the mechanistic data are based on un-physiological overexpression mediated either by transient transfection or drug-induction. Whilst all the studies performed RNAi-mediated depletion of IFITM expression levels (which is challenging because of high homology between the IFITMs) to show that a prototypical HIV-1 isolate replicates better in target cells, that this phenotype is because of the mechanisms proposed is unclear. In particular, the block to gp160 processing has not been reproduced by others under more physiological IFITM expression levels (65, 80). However, virion incorporation of IFITMs as a mechanism of reducing viral infectivity has been suggested for diverse enveloped viruses (81).

The subcellular site at which HIV-1 enters has been controversial. Recently, we wondered whether IFITM-mediated restriction might shed light on this controversy (80). Using a panel of model cell lines based on the neuroblastoma cell line U87-MG (long used in HIV-1 entry studies because they express no CD4 or endogenous major coreceptors), we expressed individual IFITMs at interferon-induced expression levels alongside CD4 and CXCR4 or CCR5. We found that IFITM restriction of HIV-1 was mediated by all three proteins but that there was a dependence on the viral co-receptor usage (**Figure 2A**). Virions that required the CCR5 co-receptor were more susceptible to inhibition by IFITM1 at the plasma membrane whilst CXCR4-using virions were more sensitive to IFITMs 2 and 3 that are predominantly localised within endosomal compartments. We therefore hypothesized that both properties of the viral envelope and that of the IFITM, in particular its subcellular localisation, dictated this "specificity" of inhibition. We showed that mutation of Y19/Y20 that mislocalises IFITMs 2 and 3 to the plasma membrane, or direct blockade of endocytosis, also modulates the restriction activity of these proteins against HIV-1 virus isolates that differ in their sensitivity to restriction by IFITM1 or IFITMs 2 and 3. HIV-1 envelope glycoproteins that were usually sensitive to restriction by IFITMs 2 and 3 were now insensitive in both one-round entry assays and spreading replication. The observation that this did not impair virion incorporation of the IFITM indicated that the primary mode of restriction was the blocking of viral entry by the IFITM expressed on the target cell membrane. These data implied that the pattern of IFITM-mediated restriction of a given envelope indicated different sites of entry—some viruses may fuse at the PM; others in, or en route to, endosomal compartments (**Figure 2A**). Three independent studies have also linked coreceptor use and IFITM sensitivity [(82, 83) #870] (81). In particular, Huang and colleagues (83) identified a putative splice variant (Δ20 IFITM2) of IFITM2 that lacks the N-terminal 20 amino acids of the full-length protein. They report higher endogenous expression of this isoform in monocytes and in CD4+ T-cells compared to the full-length protein, with localisation of the variant both at the plasma membrane and in endosomal compartments. They found that several R5-tropic viruses were resistant to inhibition by Δ20 IFITM2 with the cytoplasmic tail of CCR5, containing the major trafficking and signaling motifs, being a major determinant of this resistance. By contrast a diverse range of X4-tropic viruses were highly susceptible to inhibition. Whilst confirming that coreceptor usage also affected sensitivities to full length IFITM2 and IFITM3-mediated entry restriction, they found this was cell-type dependent, further highlighting the complexities of IFITM-mediated restriction of HIV entry. Interestingly, the authors showed that IFITM2 knockdown in primary dendritic cells led to a 2-fold increase in their permissivity to X4 viruses. Whilst it is unclear if this was a significant gain in replication capacity for myeloid cells, it raises the possibility that X4 viruses might lack macrophage tropism in part through active host restrictions that R5 viruses avoid, something previously suggested by Schmidtmeyerova et al. 20 years ago (84).

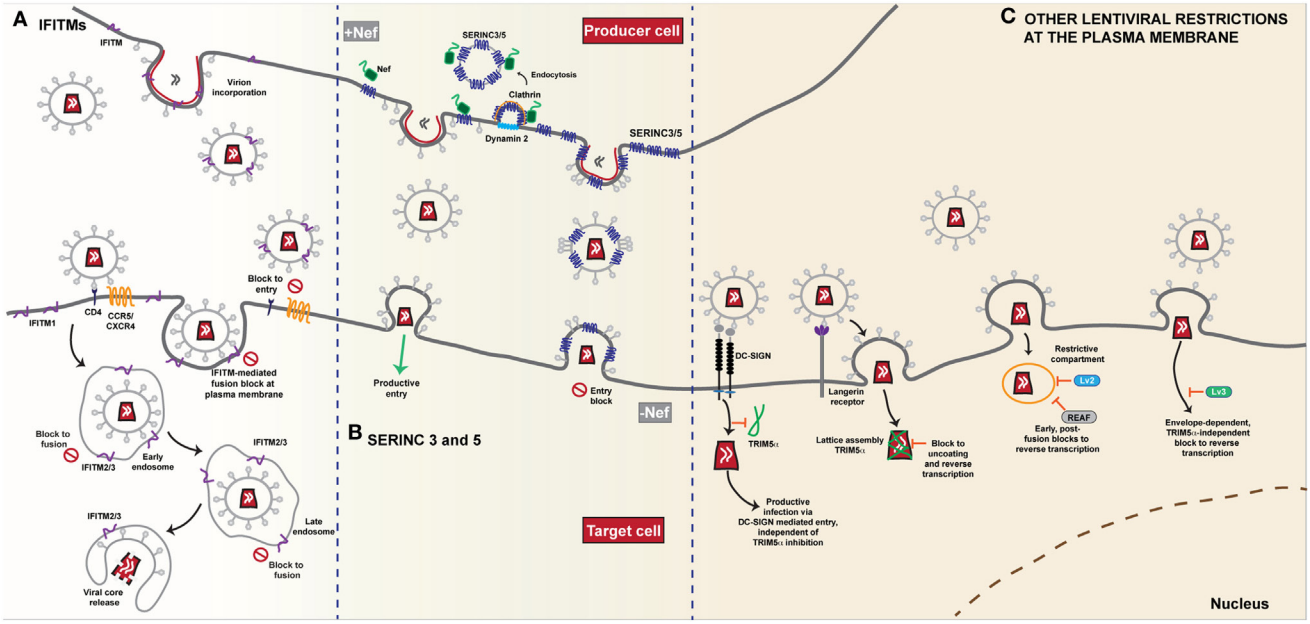

FIGURE 2 | Restriction of HIV-1 entry at the plasma membrane. **(A)** IFITMs. The antiviral restriction activity of the interferon induced transmembrane (IFITM) protein family appears to be linked to the site of viral fusion. The current general mechanism of action proposed, i.e. the physical fusion of the viral and host cell membranes is blocked, accounts for the diversity of viruses that IFITMs restrict. The influence of complex cellular trafficking pathways on this mechanism is yet to be determined. IFITMs 1, 2 and 3 are localised in different membrane compartments; IFITM1 primarily at the plasma membrane and IFITMs 2 and 3 in overlapping intracellular endocytic compartments-the sites of enveloped virus fusion. HIV-1 entry requires the CD4 receptor and co-receptors CCR5 or CXCR4 and it appears that HIV-1 sensitivity to IFITM restriction is influenced both by IFITM localisation and the site of fusion. Fusion that occurs at the plasma membrane is susceptible particularly to an IFITM1 mediated block. IFITMs 2 and 3 appear to restrict any fusion events that bypass the plasma membrane and occur within the intracellular compartments. IFITMs incorporated into viral particles during budding mediate their restriction on the target cell as the virus progeny become increasingly less infectious due to IFITM incorporation. **(B)** SERINC 3 and 5. The transmembrane proteins SERINC3 and SERINC5 are incorporated into budding HIV-1 particles from the membrane of the infected cell. In the absence of the Nef protein, HIV-1 infectivity in the target cell is restricted as delivery of the viral core is reduced due to a block to fusion. Conversely, in the presence of Nef, SERINC3/5 are relocalised from the plasma membrane through dynamin- and clathrin-dependent endocytosis, thus restoring viral infectivity and allowing for successful fusion of the progeny virions that lack SERINC3/5, with the target cell. **(C)** Other lentiviral restrictions at the plasma membrane. The post-entry restriction activity of lentivirus susceptibility factors 2 and 3 (Lv2/3) is dependent on the fusion events at the plasma membrane. Both envelope and capsid are determinants of Lv2 mediated restriction that blocks reverse transcription and nuclear entry. Likewise, RNA-associated early stage antiviral factor (REAF) which has been identified as a potent effector of Lv2, blocks reverse transcription in a similar manner dependent on the route of entry. The Lv3 block is a TRIM5α-independent process that is dependent on envelope interactions with viral entry receptors. The cell specific restriction factor TRIM5α, binds to capsid and forms a lattice leading to premature disassembly of the core. In Langerhans cells, HIV-1 uptake by the C-type lectin Langerin leads to recruitment of TRIM5α and a post-fusion block that occurs prior to integration. Conversely, in other DC subsets, interaction with DC-SIGN, induces a signalling cascade that facilitates reverse transcription and prevents TRIM5α restriction.

The importance of IFITM-Mediated Restriction in Transmission and Acute Infection

Amongst the viruses tested in our study, we showed that envelopes from R5-tropic TF viruses were uniformally resistant to IFITM restriction (80). Intriguingly, matched virus clones representing the majority species from the same individual at 6 months had gained substantial sensitivities to IFITM2 and 3 in particular. Again this was envelope determined, and was lost upon relocalization of the IFITM to the PM, suggesting that changes in Env during those 6 months had affected the route of viral entry, despite no change in coreceptor usage occurring. A major determinant of the IFITM-resistance of the TF virus was the cell surface level of CD4, suggesting that receptor engagement and density were key requirements. Consistent with this idea, selection of X4 HIV-1 resistance to IFITM1 by the Liang group yielded viruses with lesions in Vpu and changes in the CD4 binding site of Env (85). Such adaptations in culture will lead to a modulation of the envelope structure during assembly (see tetherin section below). The differences in Env between TF and 6 month viruses varied between individuals. It is well known that gp120 and gp41 are the targets for both T cell and antibody responses throughout infection *in vivo*. Hypothesizing that escape from such adaptive immune responses in Env might reveal IFITM sensitivity, we found that reversal of amino-acid changes in gp120 that arose through the escape of early neutralizing antibody responses (86) fully restored IFITM resistance to the 6-month virus. Furthermore, in primary human CD4+ T cells, knockdown of IFITM2 and 3 rescued much of the 6 month virus's replication after IFN-treatment. Thus it would appear that IFITM-resistance in Env is a major contributor to the overall IFN-I resistance of transmitted viruses, implying their evasion must be an important attribute for successful transmission. Moreover, once the virus

is systemically established, structural changes in Env that affect receptor/co-receptor interactions leading to IFITM sensitivity become tolerable if there is selective pressure applied by a competing adaptive immune response. This suggests that even host restrictions with a relatively small magnitude [by comparison to say APOBEC3G (87)] can have a major determining effect at transmission or in the early stages of systemic replication. Furthermore, because IFITM sensitivity appears to be dictated by Env/receptor interactions, these data further suggest a constraint on the envelope at transmission that endows it with IFN resistance, which itself may be an important consideration for vaccine design. Of note, recent studies on the adaptation of chimeric SIVs encoding HIV-1 envelopes (SHIVs) via sequential passage in macaques demonstrated a gain in IFN resistance mapping to Env, and particularly its level of virion incorporation (88, 89). Whether this is reflective of restriction by simian IFITMs, which do inhibit lentiviruses in culture (90), has yet to be determined.

The *ifitm* locus is complex and has not been well-annotated for genome wide association studies. However, SNPs in *ifitm3* have been implicated in the susceptibility to human disease. Of these, *rs12252* has generated much interest. Homozygosity for a very rare minor allele, *rs12252-C,* was strongly associated with the severity of H1N1 Swine Flu in the UK (91). This synonymous polymorphism changes a serine codon in the N-terminal cytoplasmic tail of IFITM3 from AG**T** to AG**C**. Initially, this was thought to lead to an alternatively spliced message that would express a N-terminally truncated IFITM3 protein lacking its endocytic YXXϕ motif. Such a truncated protein localizes to the PM and does not restrict IAV entry (91). However, no evidence of such a splice variant has since been found, raising questions about how this SNP exerts its effects. Reproduction of *rs12252-C* association with IAV pathogenesis has been mixed, but in Han Chinese populations, where the allele frequency is much higher (30–40%), a clear association with flu severity has been confirmed (92–97). At present it is not known whether other SNPs in the locus are in linkage disequilibrium with *rs12252-C* that might explain such discrepancies. In the same Chinese population *rs12252-C* is also strongly associated with rapid progression during acute HIV-1 infection, and in particular elevated viral loads and CD4+ T cell loss (96). Unlike IAV pathogenesis, this association was also observed in heterozygotes, suggesting the effect of *rs12252-C* is dominant. These intriguing results further highlight the importance the IFITMs in HIV-1 pathophysiology. The elucidation of the molecular bases for these observations will provide mechanistic insight to their role in HIV restriction.

SERINE INCORPORATORS 3 AND 5

The accessory protein Nef, common to all primate lentiviruses, has a multitude of functions in HIV-1 replication (42). Nef is myristoylated and associates with the inner leaflet of the PM and endosomal membranes. Here it promotes downregulation of various membrane proteins from the cell surface, predominantly to reduce the recognition of infected cells by adaptive immune responses. The most well-studied Nef targets are CD4, and class I and II MHC molecules, which protect infected cells from antibody-dependent cellular cytotoxicity (ADCC) (98) or recognition by antigen-specific T cells respectively, although several others have been identified (42), particularly amongst SIV Nef alleles. However, one conserved function of lentiviral Nef proteins that until recently remained unexplained, was its ability to promote the infectivity of the lentiviral virion (99).

Cells infected with HIV-1 mutants lacking Nef produce virions with reduced infectivity, even in the absence CD4 which itself interferes with envelope folding and trafficking (99). The magnitude of this CD4-independent effect on virion infectivity is variable amongst cell lines, but from lymphoid cells it can be reduced by as much as 50-fold (6). Pseudotyping virions with heterologous pH-dependent envelope proteins such as the glycoproteins from vesicular stomatitis virus or Ebola virus completely rescues the infectivity defect of HIV-1 Nef mutants (99). However, while this infectivity defect is manifest at an early entry or post-entry stage, it does not correlate with envelope incorporation into the virion. Furthermore, variations in gp120 variable domains, particularly the V1/V2 loops, affect the sensitivity of HIV-1 to Nef-dependent infectivity enhancement, implying that Nef regulates an intrinsic property of Env during the entry process (100). In keeping with this, Nef also affects the sensitivity of virions to certain neutralizing antibodies (101).

The first clue that this may be governed by a host restriction factor came from the observation that Nef interaction with dynamin 2 (dyn2), the major cellular GTPase that controls endocytosis, was essential to regulate particle infectivity (102). The requirement for dyn2 by Nef was during viral production, and its knockdown reduced virion infectivity to that of the Nef-defective mutant. Since Nef mediates the removal of other membrane proteins from the cell surface, one attractive hypothesis was that it was targeting an inhibitor of virion infectivity. This was further evidenced by the demonstration that in heterokaryons between human cells that had a high and low dependence on Nef for virion infectivity, the requirement for Nef was dominant (6). Intriguingly, the accessory protein of gamma retroviruses, a membrane-bound and glycosylated form of their major structural protein Gag (GlycoGag), can substitute for Nef activity and vice versa (103). GlycoGag is generated from a weak in-frame translational start site upstream of the regular Gag initiation codon, producing a Gag with an 88 amino acid N-terminal extension that results in its insertion in the ER membrane. As with Nef, GlycoGag promotes MLV infectivity in a dyn2 and endocytosis dependent manner (104), thus indicating that they target a common factor or pathway.

In 2015 two groups cloned the factor(s) responsible for this phenotype by complementary approaches. In the first, Massimo Pizzato and colleagues performed a large scale gene expression analysis of cells where the virus dependence on Nef varied, looking for mRNAs whose abundance correlated with the magnitude of the infectivity enhancement (6). In the second, Heinrich Gottlinger's group performed proteomic analyses of HIV-1 virions purified from human T cells in the presence or absence of Nef and/or GlycoGag expression, hypothesizing that a Nef-regulated inhibitor of infectivity may be incorporated into virions of Nef-defective viruses (8). Both groups identified members of the serine incorporator (SERINC) family of multi-pass membrane transporters, SERINC5 and SERINC3 respectively. Shortly afterward, a further proteomic study documenting global

changes to the cell surface proteome of HIV-1 infected T cell lines identified both SERINCs as differentially regulated by wild-type and Nef-defective viruses (105). Both proteins were shown to inhibit Nef-defective virus infectivity upon ectopic expression in "low Nef-responsive" cells, with SERINC5 being the most potent (6, 8, 106). Conversely, CRISPR/Cas9 knockout of both SERINC5 and SERINC3 fully restored Nef-defective virus infectivity from CD4+ T cells. In the presence of Nef, SERINC5 is relocalized from the PM to endosomal compartments dependent on Nef interaction with the clathrin adaptor AP-2 (**Figure 2B**). Moreover, SERINC5 was also counteracted by various SIV Nef alleles as well as MLV GlycoGag and VSV-G (6, 8), thus recapitulating the known features of the proposed restriction factor. Additionally, the S2 accessory protein of the distantly related lentivirus, equine infectious anemia virus (EIAV), also counteracts SERINC5 (107). Interestingly, unlike the IFITMs or tetherin (see below), SERINCs are neither significantly regulated by IFN-I, nor do they display evidence of positive selection in mammals (6, 108).

At the time of writing almost nothing is known about the mechanism by which SERINC5 exerts its antiviral activity. SERINCs are PM proteins with 12 predicted TM domains (**Figure 1B**). They are conserved from yeast to man, but only SERINCs 3 and 5 restrict retroviral infectivity (6, 8). Whilst there are several predicted isoforms of SERINC5 derived from putative splice variants, the majority mRNA species encodes the longest form (109). SERINCs were originally named for their proposed ability to incorporate serine into membranes as phosphatidylserine or sphingolipids (110), although how they do this or even whether this activity is relevant for viral restriction is not known. Direct incorporation of SERINC5 into the virion seems to be essential, and as a result of Nef-mediated internalization, SERINC5 is excluded from the assembling virion (6, 8, 111). However, this is not sufficient to explain the antiviral activity as VSV-G pseudotyping of the virus confers complete SERINC5 resistance without blocking incorporation (6, 8). What has been shown is that the block mediated by SERINC5 occurs at the fusion stage (6, 8). Both particle-associated beta-lactamase (BLAM) or CRE recombinase transfer to target cells is reduced in the presence of SERINC5, however the magnitude of this block to fusion does not fully match that of the infectivity defect or levels of reverse transcription. Whilst this has been interpreted as a potential block to fusion pore expansion rather than the initiation of fusion, it could also simply be a reflection of the difference in the dynamic range of assays that measure entry and post-entry events. Interestingly, SERINC5 sensitivity of primary R5 tropic viruses is variable in the absence of Nef (8). Exchange of the gp120 V1/V2 or V3 loops between these and prototypic X4 viruses swaps these phenotypes. This in part maps to variable N-linked-glycosylation sites in gp120 that are thought to stabilize the envelope glycoprotein (100). A very recent study indicates that while there is no evidence yet of direct Env/SERINC5 interaction, sensitive envelopes appear to be inactivated, exposing epitopes that would normally require receptor interactions (112, 113). Thus SERINC5 may be affecting the intrinsic stability of the Env trimer, thus blocking fusion. It is interesting to note the potential parallels here with those of the restriction of HIV-1 by IFITMs, with the relative resistance of R5 envelopes again highlighting that constraints on the envelope glycoprotein may be driven by selection for their resistance to intrinsic antiviral restriction mechanisms.

As noted above, SERINC3/5 expression appear not to be regulated by inflammatory stimuli and there is no evidence of the positive selection in mammalian SERINCs that is a common feature of other viral restriction factors (6). There is no apparent species specificity in antagonism, with a given HIV-1, HIV-2 or SIV Nef counteracting both human and primate SERINC5 orthologues (114). This conservation of function in Nef would in itself imply its importance. However, further observations have hinted that the efficiency of Nef-mediated SERINC antagonism by HIV and SIV Nef alleles may correlate with prevalence of a given virus in its host primate species (114). If so, then the selective pressure on Nef that gives rise to this variation in activity will be more complex than simply Nef/SERINC5 interaction, and may reflect, for example, impacts of envelope variation in SIVs or other properties of SERINCs in lentiviral replication yet to be discovered.

OTHER LENTIVIRAL "ROUTE OF ENTRY" RESTRICTIONS

Aside from IFITMs and SERINCs, other restrictions have been reported that affect post-entry events in lentiviral replication dependent on the route of viral entry. These restrictions, termed Lv2 and Lv3 [Lv1 being the name of the post-entry restriction activity later shown to be conferred by species-specific variants of TRIM5α (115, 116)], operate in human and primate cells respectively. Lv2 manifests as a block to reverse transcription and nuclear entry, and was originally demonstrated for HIV-2 in certain human cell lines and primary macrophages (**Figure 2C**) (117). The viral determinants of this restriction mapped both to the viral capsid and envelope proteins, but the entire restriction can be bypassed by VSV-G, suggesting that the post-entry block depends on where in the cell the virus fuses (118). Consistent with this, Lv2 restriction can be relieved by blocking endocytosis or mis-localizing CD4 at the PM (119). Moreover, these restriction patterns can also be seen for a variety of X4-using HIV-1 strains and be in part conferred to a heterologous core by envelope pseudotyping (119), a phenotype that bears some similarity to those for IFITM-mediated restriction (80). However, more recently regulation of nuclear pre-mRNA domain-containing protein 2 (RPRD2), termed by the authors REAF (RNA-associated early stage antiviral factor), has been proposed to be the effector of Lv2 restriction (120, 121). REAF appears to interact with the incoming genome to block reverse transcription, but is dependent on the pseudotyping envelope (**Figure 2C**). Whether REAF is differentially localized along the endocytic network, or whether restrictions during fusion (or avoidance thereof) predispose the incoming virus to REAF-mediated restriction remains to be determined. Similarly, Lv3 is a post-entry block to HIV replication in macaque cells that is distinct from TRIM5α and again depends on the Env CD4/coreceptor interactions (122). Again, this bears superficial similarities to IFITM restrictions, but the block appears to be manifest at reverse transcription and can be saturated (**Figure 2C**).

A third very recent example of "route of entry restrictions" has been described in a human dendritic cell subset, Langerhans cells (LCs), that are resistant to HIV-1 infection due to the interaction between the virus and the C-type lectin langerin (123). This mediates virion targeting to Birkbeck granules and prevents viral replication at an early post-entry stage. This turns out to be dependent on human TRIM5α, the capsid-binding restriction factor to which HIV-1 was thought to be resistant. In the presence of langerin, the authors propose that TRIM5α is recruited to the site of entry and targets the incoming virus to an autophagic degradation pathway. In other DC subsets, virion engagement with a different lectin, DC-SIGN, prevents the recruitment of TRIM5α upon virion internalization (**Figure 2C**).

Type II IFNs (IFNγ) have an under-appreciated direct antiviral activity on HIV-1 (124). In part, this again maps to the envelope protein, and in particular the V1/V2 loop (124). The initial results suggest that TF viruses may be more resistant to the effects of IFNγ, but the factors involved are not yet known.

TETHERIN

At the other end of the viral lifecycle, the most prominent antiviral inhibitor of lentiviral replication associated with the plasma membrane is tetherin (also known as bone marrow stromal cell antigen 2—BST2 or CD317). Tetherin's antiviral activity was discovered as the target of the HIV-1 accessory protein Vpu (125, 126), long known to play a role in the efficient release of new retroviral particles from infected cells. Tetherin is an IFN- and pattern recognition-regulated gene and has a general antiviral function against diverse enveloped viruses [reviewed in Ref. (5)]. Amongst the primate lentiviruses, tetherin antagonism is a highly conserved attribute (127). Furthermore, the adaptation of HIV-1 Vpu to target the human tetherin orthologue was a key event in the development of the HIV/AIDS pandemic. In this section we will focus only on the role of tetherin in lentiviral pathogenesis.

Tetherin-Mediated Restriction of Viral Release

Tetherin is a type 2 membrane protein whose distinctive topology is indicative of its primary mode of action: the retention of fully-formed virions on the PM of infected cells and their subsequent removal to endosomes (128, 129). Tetherin exists in the PM as disulfide-linked dimers that constitutively recycle via the Golgi apparatus (130, 131). The extracellular domain of tetherin forms a rod-like coiled-coil, with a hinge towards its N-terminal transmembrane domain to allow a degree of rotational flexibility (**Figure 1C**) (132–134). The C-terminus is covalently attached to the lipid of the PM by a glycophosphatidyl-inositol (GPI) linkage, giving the mature protein two membrane anchors (**Figure 1C**) (128, 130). As the nascent virus buds through the PM, tetherin dimers are recruited to the virion membrane (128, 135, 136). The C-terminal GPI anchor appears to be preferentially incorporated into the virion whilst the N-terminal TM domain is retained outside the bud (**Figure 3**) (129). When the ESCRT pathway mediates the scission of viral and cellular membranes, tetherin dimers retain the new viral particle via a stable protease-sensitive crosslink (125, 129, 137, 138). Leaky scanning of the tetherin mRNA leads to two isoforms being expressed at apparently equal levels, differing in the length of their cytoplasmic tails (139). Depending on the species orthologue, the shorter isoform lacks the first 12–17 amino acids that encompass the major subcellular trafficking signal—a dual tyrosine-based motif that engages clathrin adaptors AP1 and AP2 (131). Both isoforms can form homo- and heterodimers and both can potently restrict viral release (139, 140). However, the longer human isoform has a pro-inflammatory signalling activity associated with it (see below), and is also more sensitive to Vpu (139, 140).

Tetherin expression is induced by both type I and II IFNs, as well as pattern recognition signals, in many cell types (141, 142). It is expressed on activated T cells and is constitutively expressed by plasmacytoid dendritic cells. Tetherin expression is upregulated on peripheral blood mononuclear cells during the acute phase of HIV infection (143), and by treatment of HIV-infected individuals with IFNα (45). Its expression is enriched on tissues with barrier function, further suggesting an important role in host defence (144).

Tetherin Counteraction by Primate Lentiviruses and Its Role in Limiting Cross-Species Transmission

Tetherin targets a part of the virus that it cannot mutate to evade restriction, therefore the virus must evolve a countermeasure. Although the virally-encoded protagonist and mechanism differ, the ability to counteract tetherin is conserved among primate lentiviruses (5).

SIVs are naturally prevalent in a wide range of African non-human primates [reviewed in Ref. (145)]. For the most part each species is infected with a monophyletic strain of SIV (indicated by a suffix denoting the host species e.g. SIVsmm in sooty mangabeys), signifying predominantly within-species spread, with some notable examples of cross-species transmissions. Over 40 primate lentiviruses have been identified, and of these three have crossed the species barrier into humans: SIVcpz, SIVgor and SIVsmm, from chimpanzees, gorillas and sooty mangabeys respectively (145).

The precursors to HIV-1 were transmitted from chimpanzees to humans on at least 2 separate occasions, giving rise to HIV-1 groups M and N (146, 147), and twice from gorillas to humans resulting in HIV-1 groups O and P (148, 149). The precursors to HIV-2 crossed from sooty mangabeys into humans at least 8 different times (or at least their sequence diversity suggests independent cross-transmissions), resulting in HIV-2 groups A-H (150–152). These 12 groups of viruses have had vastly different impacts on the human population, ranging from single-case HIV-2 infections to the millions of people infected with Group M since its first predicted zoonotic infection in the early 1900s (145). While environmental and social factors inevitably played a role in the outcome of these zoonoses, extensive work dissecting host-pathogen relationships reveals a role for tetherin in influencing the course of cross-species infections.

Most SIVs counteract their host's tetherin using the accessory protein Nef (127, 153, 154). Notable exceptions to this are SIVs

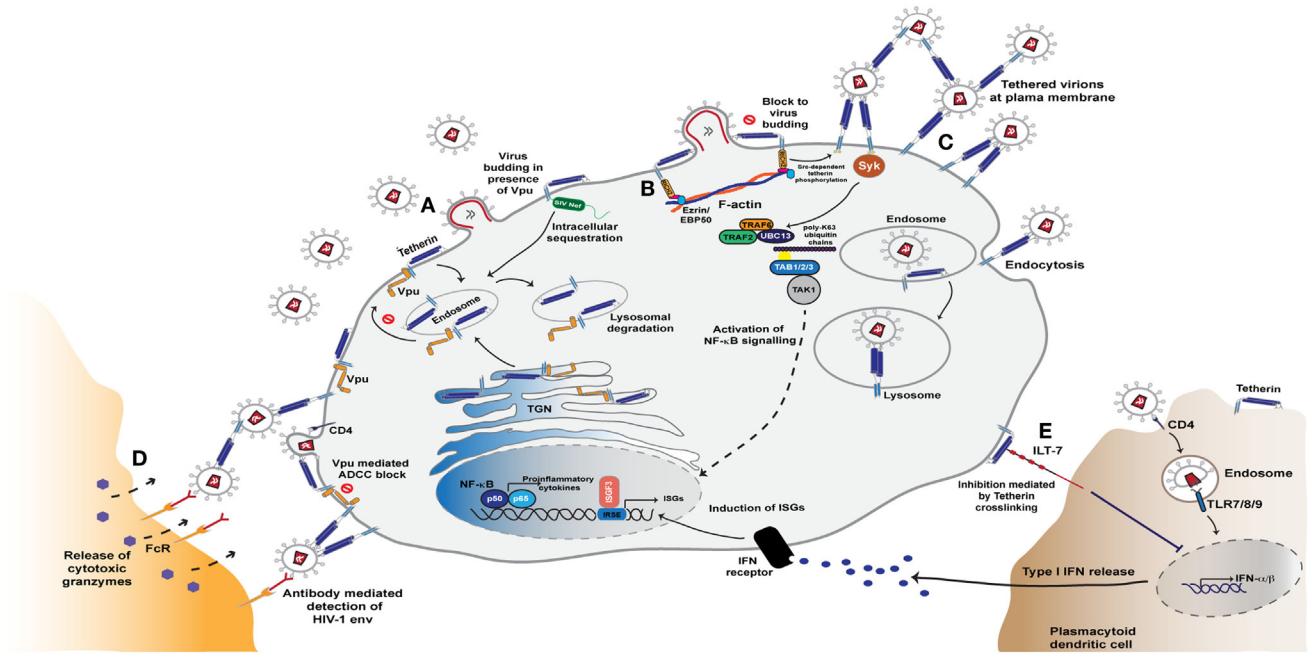

FIGURE 3 | Tetherin mediated restriction of HIV-1 assembly and release. **(A)** Tetherin resides in the plasma membrane and is constitutively recycled from the plasma membrane via endosomes and the trans-Golgi network (TGN). The antiviral activity of tetherin is counteracted by the Vpu and Nef proteins. During HIV-1 infection, Vpu binds to the transmembrane domain of tetherin in the endoplasmic reticulum and Golgi network and sequesters it in endosomal regions in a clathrin-dependent manner. This rerouting of tetherin from the plasma membrane leads to its degradation in lysosomes via the ESCRT pathway. SIV Nef, too, is able to antagonise tetherin activity by sequestering tetherin from the sites of virus assembly, leading to lysosomal degradation of the entrapped molecules. **(B)** In the absence of Vpu and Nef activity, tetherin accumulates at virus assembly sites and blocks virus release from the plasma membrane. This clustering of retained virions triggers a signalling cascade mediated by tetherin's cytoplasmic tail that leads to NF-κB activation and release of proinflammatory cytokines. This sensing of retention is reliant on tetherin association with the actin cytoskeleton via the adaptor protein RICH2. **(C)** Signalling that ultimately leads to NF-κB activation triggered by the phosphorylation of tetherin monomers and recruitment of the Syk kinase. This in turn leads to recruitment of the E3 ligases TRAF2 and TRAF6, that with the E2 enzyme UBC13, and K63-ubiquitin-mediated activation of the kinase TAK1 to activate NF-κB **(D)** Virions tethered at the cell surface are also exposed to anti-Env antibodies that thereby sensitise the infected cell to anti-HIV antibody-dependent cell-mediated cytotoxicity (ADCC) responses from Fc-receptor expressing myeloid and NK cells. **(E)** Tetherin is able to further modulate the cell's innate immunity through activation of the plasmacytoid dendritic cell specific leukocyte inhibitory receptor ILT7. Interaction with this ligand results in dampened TLR signals that thereby decrease type I interferon (IFN-I) production and enhancement of immune responses.

from greater spot-nosed, mustached and mona monkeys (SIVgsn, mus and mon respectively) which are unique among SIVs in possessing the accessory protein Vpu, capable of antagonising tetherin in a species-specific manner (127). Although SIVcpzPtt and SIVgor are also among the subset of SIVs that possess a *vpu* gene, their Vpus lack the ability to counteract chimpanzee, gorilla and human tetherin, although they still maintain function in the form of robust CD4 downregulation (127). These viruses use Nef as an antagonist, which stimulates the AP2-dependent clathrin-mediated endocytosis of tetherin, removing it from the site of virus assembly. The use of Nef rather than Vpu as a tetherin antagonist may be explained by the origins of SIVcpz—a chimaeric virus originating from recombination between an ancestral strain of the SIVgsn/mus/mon lineage and red-capped mangabey SIV (SIVrcm) (155). Inheriting two tetherin antagonists appears to have resulted in SIVcpz losing counteractivity in one.

The deletion of a five amino acid stretch (G/DIWKK) in the cytoplasmic tail of tetherin (**Figure 3**) between 1 and 6 million years ago—after divergence from chimpanzees but before the divergence of Denisovans and Neanderthals—has rendered the human protein resistant to SIV Nef antagonism (127, 153, 154, 156). Consequently, establishing a successful infection in humans requires an alternative mechanism of tetherin counteraction, either by adapting a different antagonist or adjusting the action of Nef. As detailed below, the mechanism and/or the extent of the adaptation differs in each known case of cross-species transmission.

HIV-1 group M Vpu efficiently deals with both tetherin's physical virus restriction and subsequent antiviral signalling by escorting nascent tetherin into a defunct cellular pathway and triggering its degradation (5). Vpu and tetherin interact via their transmembrane domains, with the interactive face of Vpu consisting of highly conserved alanines and a tryptophan (**Figure 3**) (157–160). Moreover, it is this interacting face that was likely to have been the key adaptation that led to human tetherin counteraction by the prototypic group M HIV-1 as revealed by the Vpu sequences of its closest extant SIVcpzPTT relatives (161). Tetherin/Vpu complexes are then targeted to late endosomes for degradation (162). This complex process requires the phosphorylation of the Vpu cytoplasmic tail that facilitates

the formation of a ternary complex between tetherin, Vpu and the clathrin adaptor AP-1, and perhaps AP-2, promoting their targeting to late endosomes (163–165). This mechanism allows Vpu to engage both newly synthesized and recycling tetherin pools. Concomitant with this process, the dual-serine phosphorylation site of Vpu, a conserved DSGxxS motif, interacts with an SCF E3 ubiquitin ligase, predominantly through the adaptor protein βTRCP2 (162, 166–169). This leads to multiple ubiquitination events in the Vpu cytoplasmic tail (170–172) that target it for ESCRT-mediated degradation (164, 173–175). This final rerouting and degradation of tetherin requires the major endocytic motif in its cytoplasmic tail (163, 175). Thus the short isoform of tetherin cannot be degraded or downregulated from the surface by Vpu (140). However, physical interaction with Vpu does reduce its incorporation into virions, counteracting tetherin at lower expression levels.

Primary HIV-1 group M Vpus are highly active antagonists of tetherin and efficient inhibitors of tetherin-mediated NF-κB signalling, and these functions are conserved in transmitted viruses and throughout the course of infection, and across the clades (127, 176–178). Suboptimal Vpus are rapidly selected against *in vivo*, and robust anti-tetherin function is maintained even years after infection (177). Studies of viruses with mutations in Vpu rendering them specifically unable to counteract tetherin but otherwise unaffected, demonstrate that these viruses are compromised compared to wildtype viruses in the presence of high concentrations of IFN-I (179). Likewise, selective pressure provided by upregulated tetherin expression during IFNα treatment of HIV-infected individuals may select for changes in Vpu (45). Thus, Vpu-mediated tetherin counteraction contributes to the overall viral interferon resistance.

Interestingly, it appears that Group M Nefs are able to acquire moderate ability to counteract human tetherin in certain circumstances (180). Although this does not represent a common activity amongst Group M Nefs, the association of a proportion of the active Nefs with viruses harbouring defective Vpus further underlines the importance of tetherin antagonism *in vivo* (180).

Fewer than 20 cases of Group N infections have been documented to date, and their adaptation to human tetherin represents a mixed and developing picture. For the most part they display some ability to counteract tetherin and enhance infectious virus release from cells, but activity is poor compared to the typical levels of Group M Vpus (127). However, a highly pathogenic Group N virus isolated from a French individual—the first case of Group N infection found outside Cameroon—demonstrated Vpu activity on a par with that of Group M. This French/Togo Vpu contains functional domains known to contribute to activity in Group M Vpus, whilst these are lacking in other known weak Group N Vpus (181). The mixed success of Group N Vpus to combat human tetherin is counterbalanced by its total inability to perform another major function of Vpu, the downregulation of CD4.

HIV-1 Group O infections represent a substantial epidemic, with an estimated 100,000 people infected. The majority of Group O Vpus tested demonstrate poor tetherin antagonism (127, 182, 183); instead, Group O Nef has adapted to target a different region of human tetherin, circumventing the 5 amino acid deletion that confers resistance to inhibition by SIV Nefs (184). The activity of the Group O Nefs is species-specific, being more efficient at downmodulating human compared to gorilla tetherin. Interestingly, a single example of a Group O Vpu able to counteract tetherin has recently been reported (185).

HIV-1 group P viruses have been isolated from only two individuals to date, both from Cameroon (186, 187). These viruses appear to be poorly adapted to humans, with no tetherin counteractivity detected in either their Vpu, Nef or Env proteins (183, 188).

Like most SIVs, the SIVsmm precursor to HIV-2 uses Nef to antagonise tetherin in its sooty mangabey host (127). Similar to SERINC5 antagonism, SIV Nefs bind to their cognate primate tetherin dependent on the G/DWIKK motif and promote its AP-2-mediated endocytosis from the cell surface (189, 190). While HIV-1 Group M evolved efficient tetherin antagonism by Vpu, and Group O Nefs evolved to target a different region of tetherin (184), HIV-2 employs a different strategy of antagonism, using the Env protein (191). The extracellular domains of both proteins interact, and again this stimulates endocytic removal of tetherin from the cell surface through Env's interaction with AP-2 (191–194). Tetherin antagonism appears to be a conserved attribute of HIV-2 isolates tested to date (195), although the potency of HIV-2 Env in enhancing virus release is weaker than that of HIV-1 group M Vpus, insofar as *in vitro* assays are a true reflection of activity. Whether there is a fitness and efficacy cost associated with using a major structural protein, also under pressure to evade antibody responses, to carry out a role more commonly performed by accessory proteins remains to be seen.

In Vivo Relevance—Evidence from Experimental Infections

The importance of tetherin *in vivo* is demonstrated by the remarkably diverse strategies enlisted by viruses to overcome this barrier (5). Simple demonstrations of this arms race in action come from experimental infections of primates, of which there are several examples demonstrating pathogenesis associated with acquisition of tetherin counteractivity. Studies of chimpanzees infected with HIV-1 for the purposes of vaccine studies in the 1980s were revisited in order to investigate readaptation to a previous host species. Examination of the readapted viruses revealed that, although the Vpu maintained function, tetherin antagonism was also acquired in Nef, with the virus using both proteins to overcome chimpanzee tetherin (196). The minimal changes required to restore anti-chimpanzee tetherin activity to the HIV-1 Nef were just 2 amino acids, and the region of chimpanzee tetherin targeted by the adapted Nef was mapped to the DIWKK region deleted in human tetherin (196). It therefore appears that lost accessory gene functions can be reacquired relatively easily. Similarly, serial passage of modified simian tropic HIV-1 in pigtail macaques resulted in a virus that could replicate efficiently and cause AIDS in these otherwise unsusceptible hosts (197). The modified virus used in the original inoculum was endowed with resistance to macaque APOBEC3 restriction factors, but unable to counteract monkey tetherins. Four passages resulted in a pathogenic virus that was able to efficiently counteract macaque tetherin while maintaining anti-human tetherin activity. The amino acid changes responsible

for this adaptation were mapped to the transmembrane region of the Vpu—the region that interacts with tetherin—and involved only two amino acid changes (197).

Infection of rhesus macaques with Nef-deleted SIV (SIVmacΔnef) usually results in attenuated infection, with persistent but low-level viral replication. After serial passage these viruses can revert to pathogenicity, leading to high viral loads and progression to disease (198). Analyses of the pathogenic revertant viruses confirmed that these viruses had adapted to counteract rhesus tetherin, with determinants mapping to the cytoplasmic tail of the envelope protein gp41. The minimal changes required to endow Env with this Nef-like activity involved just five amino acids (199). Acquisition of tetherin counteraction in SIV envelopes has been documented in the SIVtan envelope, most likely through passage in human cells (200). More recently, such an adaptation has also been observed in an *in vivo* for a highly neurotropic SIVsm (201).

Studies in tetherin knock-out mice provide direct evidence of tetherin's antiviral role *in vivo*, with increased replication and pathogenicity of a murine retrovirus observed in the absence of tetherin (202). Otherwise normal development of −/− mice, including no detectable adverse effects on the immune system, further support the primary function of tetherin as an antiviral effector protein. Indeed, most mammalian tetherin orthologues possess antiviral activity, and the role of tetherin as an ancient immune effector molecule is supported by the demonstration of identifiable tetherin orthologues with antiviral activity in reptiles and as far back as the coelacanths (203, 204).

Tetherin's Role in Linking Innate and Adaptive Immunity

Tethering viruses to the producer cell membranes and preventing their release is an obvious obstruction to virus propagation. However, the major mode of HIV transmission in cultured T cells is via synaptic conjugations between infected and uninfected cells. These virological synapses are driven by Env/CD4 interactions and result in polarized secretion of new virions across the synaptic cleft (205). While very potent at blocking cell free virus release, the inhibitory effects of tetherin on cell-to-cell spread via the virological synapse structures is weak. In primary human CD4+ T cells, Vpu-defective viruses even spread faster due to tetherin-mediated cell-associated virus accumulation, despite lower cell-free virion release (206). Given the high selection pressure to maintain tetherin counteraction in lentiviruses, it has therefore been of particular interest to determine whether the consequences of restriction have wider ramifications than simply the physical prevention of dissemination. Viruses tethered to the cell surface are exposed to anti-Env antibodies, particularly those targeting CD4-induced epitopes, and this sensitizes the infected cell to ADCC-mediated elimination by Fc-receptor bearing myeloid and NK cells (**Figure 3**) (98, 207–209). This effect is enhanced by treatment of cells with IFNα due to increased tetherin expression. In turn it is effectively suppressed by HIV-1 Vpu and Nef, which play dual roles by counteracting tetherin and by degrading CD4, therefore protecting the nascent Env trimers from exposing CD4-dependent epitopes and reducing the numbers of cell-associated virions (98, 207–209). Importantly, tetherin therefore acts as a link between innate and adaptive immunity, enhancing the potency of antiviral antibodies and increasing the pressure on the virus to maintain efficient tetherin antagonism.

The clustering of cell surface tetherin molecules due to virus retention triggers signalling events mediated by its cytoplasmic tail, leading to NF-κB activation and the release of pro-inflammatory cytokines (139, 210, 211). These cytokines could potentially serve to further amplify tetherin's role in ADCC by recruiting effector cells to the site of infection. Tetherin's signaling activity is restricted to homodimers of the long isoform (139). In this context the major endocytic site, a dual tyrosine motif YDYCRV, acts as a hemi-immuno-tyrosine activation motif (212). Upon virion retention, tyrosines on both L-tetherin monomers become phosphorylated by Src-family kinases and present an SH2-domain for the recruitment of the kinase Syk (212). This in turn recruits a signaling complex including TRAF2, TRAF6 and TAK1, ultimately activating NF-κB (**Figure 3**) (211, 212). Thus in addition to retaining virions at the cell surface, tetherin acts akin to a pattern recognition receptor in sensing virus restriction. This sensing is dependent on tetherin's link to the cortical actin cytoskeleton via an adaptor protein RICH2 (AHRGAP44) (212, 213). There appears to be some primate species specificity in tetherin's signaling activity. The deletion that occurred in chimpanzee tetherin that rendered the human orthologue resistant to Nef antagonism, and serves as a highly effective barrier to cross-species transmissions, also appears to have contributed to the efficiency with which human tetherin initiates proinflammatory signalling (210). In human cells this correlates with primate tetherin phosphorylation efficiency and Syk recruitment (212). Whether this is truly an neofunctionalization of tetherin during primate evolution, or reflects species incompatibilities in experimental cellular systems is not clear. However, in mice knocked-in for constitutive somatic human tetherin expression, runting and early lethality is observed consistent with chronic inflammatory signaling (214).

A further intriguing link between tetherin and innate sensing of viruses is its identification as a ligand for the leukocyte inhibitory receptor, ILT7, expressed on plasmacytoid dendritic cells (pDCs). Interaction between tetherin and ILT7 induces an inhibitory signal that dampens responses by TLR ligands (**Figure 3**) (215). Recent data from the Cohen group suggests that the ILT7/tetherin interaction acts akin to a 'missing self' signal when a pDC encounters a cell infected with a tetherin-sensitive virus (216). The recruitment of tetherin into budding virions occludes its ability to interact with ILT7 on the pDC, thereby enhancing the responsiveness of the pDC if simultaneously encountering extracellular RNA. The authors postulate that differential surface removal of long and short tetherin isoforms by HIV-1 group M Vpu (and some extent Group O Nefs) ensures a sufficient pool tetherin at the PM to deliver this inhibitory signal at the same time as counteracting its antiviral effects (216, 217). Whether this is a universal function of tetherin is unclear; mice lack an ILT7, and a functional orthologue has yet to be identified. However, the upregulation of tetherin on some cancers may suggest that ILT7 interaction is important for tumor-cell immune evasion (215).

Together these observations indicate that tetherin's antiviral activity *in vivo* is not limited to the physical reduction in cell free virus produced from the infected cell. Rather, virion-tethering to the cell has important knock-on effects on how it is perceived and dealt with by both the innate and adaptive immune response. This linkage between direct antiviral activity and the augmentation of downstream immune responses would thus further explain the high level of selective pressure on viruses such as HIV-1 not only to counteract tetherin for efficient transmission, but to maintain this activity after the establishment of systemic infection where the physical impairment of viral release has only minor effects on spread to new target cells.

OTHER INHIBITORS OF HIV-1 RELEASE AND ASSEMBLY AT THE PLASMA MEMBRANE

Whilst the most prominent, tetherin is unlikely to be the only antiviral factor that targets HIV during the assembly and release stage. In principle many adhesion molecules or lectins could exert an antiviral effect on virus release provided they, or their ligand, are incorporated into viral particles. Indeed, in the absence of both Vpu and Nef, CD4/Env interactions can limit HIV release (218) as well as exposing epitopes for ADCC.

The T-cell immunoglobulin and mucin domain (TIM) family of phosphatidylserine (PS) receptors have been implicated as important attachment for a variety of enveloped viruses (219). The exposure of PS on the surface of the PM of apoptotic cells (220) is important for their clearance by phagocytes, and it is thought that diverse enveloped viruses hijack PS exposure to facilitate attachment and entry into target cells (219). TIM family members are variably expressed on myeloid and activated T cell subsets. In the case of HIV-1, expression of TIM-1 in target cells enhances virion entry. This may be by upregulating CD4/coreceptor levels, but very recent evidence has shown that PS exposure on the target cell is important for HIV-1 fusion. Conversely, overexpression of TIM family members restricts virion release by mediating a phenotype remarkably similar to tetherin (220). Of note, TIM-3 silencing in primary macrophages enhances virion release 2–4 fold, suggesting these observations maybe of relevance *in vivo*. Interestingly, the mucin domain of TIM-1 is highly polymorphic and homozygosity for a 6 amino acid in-frame deletion variant (delMTTTVP) has been associated with reduced HIV-1 disease progression (221) and replication in *ex vivo* cultured CD4+ T cells (222). Whether this is because of an inhibitory effect or a reduced entry-enhancing activity is yet to be determined.

The inhibition of processing and incorporation of Env into nascent virions was suggested as an antiviral mechanism of IFN-I against HIV-1 many years ago (223). Recent studies have implicated this process as a target for two ISGs (224, 225). LGALS3BP/90K, a cysteine rich secreted scavenger receptor that has a role in regulating cell adhesion, is strongly upregulated by IFN-I and IFN-II and is present at high concentrations in most bodily fluids. Expression of cell-associated 90K blocked envelope incorporation and gp160 processing dependent on its BR-C, ttk, BOZ/Poxvirus Zinc finger (BTB/POZ) domain (225). 90K does not generally inhibit furin-like proteases that cleave a number of viral glycoproteins, nor does it have antiviral activity against murine retroviruses. Neither was 90K found to directly associate with gp160 in the secretory pathway. However, 90K depletion in both T cells and macrophages enhanced HIV-1 replication. A similar activity has been associated with guanylate binding protein 5 (GBP5), a member of a family of IFN-induced GTPases (224). As with 90K, expression of GBP5 blocked the processing and incorporation of gp160 as well as other retroviral envelope proteins. This required the ability of GBP-5 to localize to the Golgi network, but appears independent of its GTPase activity. Furthermore, GBP5 expression levels in primary macrophages inversely correlated with viral replication. Interestingly, Env expression levels were a key to HIV-1 GBP-5 sensitivity. Mutations in the start codon of *vpu*, which is expressed from the same mRNA, enhances Env expression levels and confers partial GBP5 resistance. Since Vpu is essential to counteract tetherin (see below), the authors speculate that balancing the expression of Vpu and Env allows for optimal viral replication in the face of these two IFN-induced restrictions. As yet, little further mechanistic understanding of 90K or GBP-5-mediated effects on Env are known, or indeed whether they are related given their phenotypic similarities.

The assembly and budding of the nascent virion at the PM has been suggested as a target for IFN-I-mediated restriction. 2′,3′-cyclic-nucleotide 3′-phosphodiesterase (CNP) was identified in an overexpression screen of ISGs that restrict viral release (226). CNP, a membrane-associated enzyme, bound to Gag in membrane fractions and inhibited particle formation independent of its enzymatic activity. While most mammalian CNP orthologues tested had antiviral activity against HIV-1, a single amino acid difference in murine CNP accounted for its lack of retroviral restriction. Selection of CNP-resistant viruses resulted in a single point mutation (E40K) in the matrix (MA) domain of the Gag polyprotein, which alongside the murine CNP species-specific difference, governed CNP/Gag interactions. Interestingly, the equivalent position in MA is a K in some HIV-2 and SIV isolates and this correlates with their resistance to CNP. However, whether CNP ever gets the opportunity to restrict HIV-1 *in vivo* is unclear. It is expressed mainly in oligodendrocytes and epithelial cells, with some expression in DCs, but is not detectable in primary CD4+ T cells.

Finally, the ESCRT-mediated release of the virus has been suggested as a target of IFN-mediated restriction. The interferon-induced ubiquitin-like modifier, ISG15, has a broad role in antiviral defence (227). The ESCRT-III complex constricts the neck of the budding virion to the point of scission. This requires the polymerization of its charged multivesicular protein (CHMP) components into helical polymers on the internal surface of the neck, followed by their regulated disassembly by the AAA-ATPase VPS4 and its cofactor, LIP5 (4). Direct conjugation of ISG15 (ISGylation) to various CHMPs blocks their interaction with VPS4/LIP5, thereby stalling retrovirus budding (228, 229). ISGylation of CHMP5 appears to be essential for this process as in its absence, no other CHMP becomes modified (228). CHMP5 is dispensable for ESCRT-III function itself, raising the possibility that it is a regulator that can rapidly inhibit ESCRT

function after IFN treatment. Whether CHMP5 ISGylation is a major mechanism of antiretroviral defence under physiological conditions is not yet clear. Another ESCRT-III regulating factor, CC2D1A, binds to the ESCRT-III CHMP4B and blocks polymer formation, thereby dominantly interfering with HIV-1 assembly (230, 231). CC2D1A itself is an ISG (7), although whether it acts in a directly antiviral capacity is not known given that it has also been identified as a regulator of TBK1, a major kinase in the pattern recognition signaling cascade (232).

CONCLUDING REMARKS

Negotiating the limiting membranes of the cell represent the first and last stages of HIV-1 replication. As analogous processes are common to all enveloped viruses, the evolution of antiviral factors that inhibit them present general first line defences against HIV-1 and related viruses. Their importance is reflected in the resistance mechanisms that primate lentiviruses have evolved to avoid them, and the evidence that their antiviral activities present significant barriers to viral transmission, systemic spread and augmentation of other immune responses. This suggests that targeting the virus's resistance to PM-based host restrictions may have therapeutic or vaccine-relevant potential. Their study also reveals fundamental new understanding of the basic processes of viral entry and exit from the cell.

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Human Intestinal Epithelial Cells Release Antiviral Factors That Inhibit HIV Infection of Macrophages

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As a rich source of CD4+ T cells and macrophages, the gastrointestinal (GI) tract is a major target site for HIV infection. The interplay between GI-resident macrophages and intestinal epithelial cells (IECs) constitutes an important element of GI innate immunity against pathogens. In this study, we investigated whether human IECs have the ability to produce antiviral factors that can inhibit HIV infection of macrophages. We demonstrated that IECs possess functional toll-like receptor 3 (TLR3), the activation of which resulted in induction of key interferon (IFN) regulatory factors (IRF3 and IRF7), IFN-β, IFN-λ, and CC chemokines (MIP-1α, MIP-1β, RANTES), the ligands of HIV entry co-receptor CCR5. In addition, TLR3-activated IECs release exosomes that contained the anti-HIV factors, including IFN-stimulated genes (ISGs: ISG15, ISG56, MxB, OAS-1, GBP5, and Viperin) and HIV restriction miRNAs (miRNA-17, miRNA-20, miRNA-28, miRNA-29 family members, and miRNA-125b). Importantly, treatment of macrophages with supernatant (SN) from the activated IEC cultures inhibited HIV replication. Further studies showed that IEC SN could also induce the expression of antiviral ISGs and cellular HIV restriction factors (Tetherin and APOBEC3G/3F) in HIV-infected macrophages. These findings indicated that IECs might act as an important element in GI innate immunity against HIV infection/replication.

Keywords: human intestinal epithelial cells, HIV, macrophages, toll-like receptor 3, interferons, IFN-stimulated genes, exosomes

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INTRODUCTION

The gastrointestinal (GI) tract has the largest mucosal surface in the body and serves as an important barrier between pathogens in the external environment and the body's sterile internal environment (1). Tight epithelial junctions together with the GI immune system protect the host from pathogenic invasion. The GI tract is rich in HIV target cells, mainly activated CD4+ T cells and macrophages. Therefore, the GI tract is a major site for HIV infection. As first layer cells in the GI tract, intestinal epithelial cells (IECs) constantly exposed to HIV or HIV-infected cells, which could have a profound impact on the immune and barrier functions of the GI tract (2). In addition, IECs express galactosylceramide and HIV co-receptor CCR5 (3), which facilitate translocation of CCR5-tropic HIV from the apical to the basolateral surface *via* vesicular transcytosis (4, 5).

Central to the capacity of IECs to maintain barrier and immunoregulatory functions is their ability to act as frontline sensors to their microbial encounters and to integrate commensal bacteria-derived signals into antimicrobial and immunoregulatory responses (6). Studies have shown that the IECs express pattern-recognition receptors (PRRs) that enable them to act as dynamic sensors of the microbial environment and as active participants in directing mucosal immune cell responses (7). Among PRRs, toll-like receptor 3 (TLR3) in conjunction with TLR7 and TLR9 constitutes an effective system to monitor viral infection and replication. TLR3 is known to recognize viral double-stranded RNA (dsRNA), while TLR7 and TLR9 detect single-stranded RNA (ssRNA) and cytosine phosphate guanine DNA, respectively (8). Therefore, expressing functional TLR3, 7 and 9 in IECs play a crucial role in virus-mediated GI innate immune responses (9).

Macrophages present in the GI system constitute a major cellular reservoir for HIV due to the abundance of these cells at mucosal sites. GI-resident macrophages represent the largest population of mononuclear phagocytes in the body (10). In the rectum, there are more than three times as many $CD68^+$ macrophages expressing CCR5 as those in the colon (4). The high expression of CCR5 on rectal macrophages suggests that the most distal sections of the gut may be especially vulnerable to HIV infection. Macrophages constitute up to 10% of infected cells in HIV-infected individuals (11, 12). HIV-Infected macrophages can transfer virus with high-multiplicity to $CD4^+$ T cells and reduce the viral sensitivity to antiretroviral therapy and neutralizing antibodies (13, 14). In mucosa infiltrating, macrophages also play a role in systemic HIV spread (5). Macrophage activation contributes to HIV-mediated inflammation, as they can produce and release inflammatory cytokines that induce systemic immune activation, a hall marker of HIV disease progression. Conversely, macrophages play an important role in the host defense against HIV infection. Macrophages are a major producer of type I interferons (IFNs). Our early investigations (15, 16) showed that TLR3 activation of macrophages produced multiple intracellular HIV restriction factors and potently suppressed HIV infection/replication. However, the ability of macrophages to produce type I IFNs are significantly compromised by HIV infection. HIV blocks IFN induction in macrophages by inhibiting the function of a key kinase (TBK1) in the IFN signaling pathway through viral accessory proteins (Vpr and Vif) (17). In addition, HIV infection downregulates the antiviral IFN-stimulated genes (ISGs) (ISG15, OAS-1, and IFI44) in primary macrophages (18).

Exosomes play a key role in intercellular communication and innate immune regulation. A recent study showed that exosomes are formed in an endocytic compartment of multivesicular bodies (19). Exosomes are involved in many biological processes such as tissue injury and immune responses by transfer of antigens, antigen presentation (20), and the shuttling of proteins, mRNAs, and miRNA between cells (21). As such, it has been postulated that exosomes mediate intercellular communication by delivering functional factors to recipient cells (22). IEC lines also can secrete exosomes bearing accessory molecules that constitute a link between luminal antigens and local immune system (23). Studies have documented that the bystander cells can produce and release the exosomes, which contain multiple antiviral factors that can inhibit viral replication in target cells, including hepatitis B virus (24), HCV (25), and HIV (26, 27).

Evidently, the interplay between GI-resident macrophages and IECs has a key role in the GI innate immunity against viral infections. Unlike macrophages, IECs are not a host for HIV infection/replication, and it is unlikely that HIV has a direct and negative impact on functions of IECs. However, because IECs in the GI tract have to encounter a number of stimuli and immune cells, including HIV-infected macrophages (28), the activation of these non-immune cells in the GI tract is inevitable. Recent studies (19, 29) have shown that IECs can be induced to express and secrete specific arrays of cytokines, chemokines, and antimicrobial defense molecules, which is crucial for activating intestinal mucosal innate and adaptive immune responses. However, there is little information about whether the IECs are involved in the GI innate immunity against HIV infection. Specifically, it is unknown whether the IECs possess functional TLRs that can be immunologically activated to produce anti-HIV factors. Therefore, this study aimed to determine whether IECs have the ability to mount TLR3-IFN-mediated antiviral activities against HIV infection of macrophages.

MATERIALS AND METHODS

Reagents

All culture plastic ware were obtained from Corning (Corning, NY, USA). Lyovec transfection reagent and Polyinosinic-polycytidylic acid (Poly I:C) (TLR3 ligand), Imiquimod (TLR7 ligand), ssRNA40 (TLR8 ligand), ODN2006 (TLR9 ligand) were purchased from InvivoGen (San Diego, CA, USA). All culture reagents were purchased from Gibco (Grand Island, NY, USA). Exosome-depleted fetal bovine serum (FBS) was purchased from System Biosciences, Inc. (Mountain View, CA, USA).

Cell Culture

The human intestinal epithelial cell line (NCM460), originally derived from the normal colonic mucosa of a 68-year-old Hispanic male, were expanded in RPMI-1640 medium (30). Cells were cultured at 37°C with 5% CO_2 and 100% humidity, and culture medium was changed every 3 days. To polarize IECs, we used a transwell system (31, 32), in which IECs (1×10^5 cells/well) were grown on a 0.4 μm pore sized, 6.5 mm diameter transwell insert. The transepithelial electrical resistance was measured by Ohm meter. The cell cultures were considered to constitute a polarized epithelial monolayer when resistances were $\geq 600\ \Omega \times cm^2$ and stable (33). Purified human peripheral blood monocytes were purchased from Human Immunology Core at the University of Pennsylvania (Philadelphia, PA, USA). The Core has the Institutional Review Board approval for blood collection from healthy donors. Freshly isolated monocytes were cultured in the 48-well plate (2.5×10^5 cells/well) in DMEM containing 10% FBS. Macrophages refer to 7-day cultured monocytes.

TLRs Activation

Lyovec was used for the transfection of the TLR ligands. IECs seeded on 48-well plates (5×10^4/well) were transfected with Poly I:C (10 μg/ml), Imiquimod (10 μg/ml), ssRNA40 (10 μg/ml), ODN2006 (5 μM). Lyovec-treated cells were used as a vehicle control.

Exosome Isolation

Intestinal epithelial cells were transfected with poly I:C (0.1, 1, 10 μg/ml) for 4 h and fresh-culturing medium containing 10% exosome-free FBS was added. At 48 h post-transfection, IECs supernatant (SN) was collected and exosomes were isolated through multiple rounds of centrifugation and filtration as previously reported (24). Briefly, 10 ml of SN were centrifuged at $300 \times g$ for 10 min to remove floating cells, then at $2{,}000 \times g$ for 10 min, and $10{,}000 \times g$ for 30 min to remove cell debris, shedding vesicles, and apoptotic bodies. Finally, exosomes pellet were collected by ultracentrifugation at $100{,}000 \times g$ for 70 min. For further purification, the pellets were washed with phosphate buffered saline (1× PBS) (Gibco, NY, USA) and centrifuged at $100{,}000 \times g$ for 70 min. The pellet was resuspended in 100 μl 1× PBS, then immediately stored at −80°C until use.

Immunofluorescence of Exosome

Macrophages were cultured at a density of 2.0×10^5 cells/well in 48-well plates. Isolated exosomes from IECs SN were labeled with PKH67 Fluorescent according to the manufacturer's protocol (Sigma-Aldrich). Purified PKH67 exosomes were incubated with macrophages and cultured at 37°C for 18 h in a CO_2 incubator. Macrophages were then stained with a PKH26 Fluorescent for membrane and Hoechst 33342 for nuclei and washed three times with 1× PBS. The cells were photographed under a confocal microscope (Nikon A1R, Nikon, Japan).

qRT-PCR Quantification of mRNA and miRNA

Total RNA from cultured cells was extracted with Tri-Reagent (Molecular Research Center, OH, USA) as previously described (34). Total RNA (1 μg) was subjected to reverse transcription (RT) using reagents from Promega (Promega, WI, USA). The RT system with random primers for 1 h at 42°C. The reaction was terminated by incubating the reaction mixture at 99°C for 5 min, and the mixture was then kept at 4°C. The resulting cDNA was then used as a template for qPCR quantification. The qPCR was performed with iQ SYBR Green Supermix (Bio-Rad Laboratories, CA, USA) as previously described (35). Thermal cycling conditions were designed as follows: initial denaturation at 95°C for 3 min, followed by 40 cycles of 95°C for 10 s, and 60°C for 1 min. miRNA was extracted from IECs-derived exosomes using the miRNeasy Mini Kit (Qiagen, CA, USA) in accordance with the manufacturer's instruction and reverse-transcribed with a miScript Reverse Transcription Kit (Qiagen, CA, USA). qRT-PCR was carried out using miScript Primer Assays and miScript SYBR Green PCR Kit from Qiagen as previously described (36). Synthetic caenorhabditis elegans miRNA-39 (cel-miR-39) was used as a spiked-in miRNA for normalization.

Western Blot

Total cell lysates of IECs transfected with Poly I:C was prepared by using the cell extraction buffer (Thermo Fisher Scientific, MA, USA) according to the manufacturer's instructions. Equal amounts of protein lysates (30 μg) were separated on 4–12% sodium dodecyl sulfate polyacrylamide gel electrophoresis precast gels and transfected to an Immunobiolon-P membrane (Millipore, Eschborn, Germany). The blots were incubated with primary antibodies in 5% nonfat milk in PBS with 0.05% Tween 20 (PBST) overnight at 4°C (IRF3, 1:1,000; Phospho-IRF3, 1:1,000; IRF7, 1:1,000; Phospho-IRF7, 1:1,000; GAPDH, 1:5,000; β-actin, 1:5,000; EEA1, 1:1,000; CD63, 1:1,000; LAMP2, 1:2,000; Alix, 1:1,000; ISG15, 1:1,000; ISG56, 1:1,000; GBP5, 1:1,000; Viperin, 1:1,000; MxA, 1:1,000; MxB, 1:1,000; OAS-1, 1:1,000). All antibodies were obtained from Cell Signaling Technology (Cell Signaling Technology, MA, USA) Horseradish peroxidase-conjugated appropriate second antibodies were diluted at 1:2,000 to 1:8,000 in 2% nonfat milk PBST. Blots were developed with SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, MA, USA).

ELISA

Interferon-β and IFN-λ protein levels in IECs culture SN were measured with ELISA (IFN-β: Invitrogen; IFN-λ1/3, IFN-λ2: R&D system Inc., MH, USA). Assays were carried out according to the manufacturer's instructions.

Cytometric Bead Array (CBA) Assay

The CBA assay was performed to simultaneously measure CC chemokines (MIP1-α, MIP1-β, and RANTES) levels in cell culture supernatant, according to the instructions of the manufacturer (BD Biosciences, CA, USA).

Macrophage Treatment and HIV Infection

Macrophages were pretreated for 24 h with SN (10%, v/v) or exosomes (2 μg/ml, equal to the amount of 10% SN) from IECs cultures collected at 48 h post-stimulation with Poly I:C. HIV Bal strain was obtained from the AIDS Research and Reference Reagent Program at the National Institution of Health (NIH). Macrophages were incubated with cell-free HIV Bal (p24, 20 ng/ml) overnight, and cells were then washed three times with fresh DMEM. During the postinfection period, SN or exosomes were added to the macrophages where appropriate. At day 8 postinfection, cell and SN samples were collected for HIV GAG gene expression. To determine whether the polarized stimulation of IECs could mediate HIV inhibition in macrophages. Poly I:C (1 μg/ml) was added to the upper or lower chamber of IECs cultures. Culture SN was collected 48 h after Poly I:C transfection. Cell-associated HIV GAG gene expression in macrophages treated with 10% [volume to volume ratio (v/v)] of indicated SN was measured by qRT-PCR at 96 h post-infection. To deplete exosomes, the SN from Poly I:C-stimulated IECs were incubated with anti-CD63 antibody-conjugated Dynabeads overnight at 4°C and then separated in a magnetic field. For detection of early products of Strong-Stop DNA in macrophages, SN from TLR3-activated IECs cultures was added to macrophages cultures 24 h

prior to infection with DNase I-treated HIV Bal for 3 h. Cellular DNA, including genomic and viral DNA products, was then isolated with the Tri-Reagent. Strong-stop DNA, the first product of HIV RT, was analyzed by the qPCR with primers specific for strong-stop DNA. The DNA concentrations of the each sample were normalized by equal DNA loading confirmed with primers for GAPDH.

Data Analysis

Data were presented as the mean ± SD from at least three independent experiments, and statistical significance was measured by Student's *t*-test or one-way analysis of variance followed by the Newman–Keul's test where appropriate. Statistical significance was defined as $P < 0.05$ or $P < 0.01$.

RESULTS

TLR3 Signaling of IECs Induces IFNs

Activation of TLRs 3, 7, and 9 could trigger intracellular IFN-mediated innate immunity against virus infections (37). Therefore, we first examined the expression of TLRs in IECs. As shown in Figure S1A in Supplementary Material, IECs expressed mRNAs for all known human TLRs except TLR5. To investigate whether the antiviral TLRs (TLR3, 7, 9) are biologically functional in IECs, we transfected the cells with the ligands to TLR3 (Poly I:C), TLR7 (Imiquimod), TLR8 (ssRNA40), and TLR9 (ODN2006). As shown in Figure S1B in Supplementary Material, the IECs expressed functional TLR3 and TLR8, as the ligands to these TLRs could induce the expression IFN-β and IFN-λ. In contrast, the ligands of TLR7 and TLR9 had little effect on IFN induction. TCI, a TLR3 complex inhibitor, could significantly block the effect of Poly I:C (Figure S2 in Supplementary Material). We thus focused on the impact of TLR3 signaling on IFN induction in IECs in the subsequent experiments.

As shown in **Figure 1**, TLR3 activation of IECs induced IFN-β and IFN-λ at both mRNA (**Figure 1B**) and protein (**Figure 1C**) levels. These effects of Poly I:C stimulation on IFN-β and IFN-λ expression in IECs were dose- and time-dependent (**Figures 1A,B**). We next examined whether IRF3 and IRF7, key regulators of the IFN signaling pathway, are involved in the TLR3 action on IFN induction by IECs. As shown in **Figure 2**, TLR3 signaling of IECs induced the phosphorylation of both IRF3 and IRF7, which were positively associated with the dose of Poly I:C transfected into IECs.

IECs-Derived Exosomes Can Be Taken up by Macrophages

Exosomes released from donor cells could deliver their cargo to recipient cells and subsequently modulate host cell function (21). We thus isolated and characterized the exosomes from activated IECs cultures by detecting the common exosome-carried proteins (Alix, CD63, and LAMP2) (**Figure 3A**). To determine whether macrophages (recipient cells) can take up the exosomes released from IECs, we incubated macrophages with exosomes labeled with green fluorescent dye PKH67. As shown in **Figure 3B**, PKH67-labeled exosomes were observed within macrophages treated with SN from activated IECs cultures.

IECs-Isolated Exosomes Carry the Antiviral ISGs and miRNAs

Next, we investigated whether the exosomes from activated IECs contained the antiviral ISGs and miRNA. As shown in **Figures 4A,C**, TLR3 signaling of IECs induced the expression of ISG15, ISG56, OAS-1, MxA, MxB, GBP5, and Viperin at both mRNA and protein levels. In addition, there were elevated levels of these ISGs in the exosomes isolated from Poly I:C-stimulated IECs (**Figure 4D**). We also found that the anti-HIV miRNAs: miRNA-17, miRNA-20, miRNA-28, miRNA-29 family members (miR-29a, 29b, and 29c) and miRNA-125b were increased in the exosomes (**Figure 4B**) from Poly I:C-stimulated IECs.

TLR3 Signaling of IECs Inhibits HIV Infection of Macrophages

As shown in **Figure 5A**, macrophages treated with SN from Poly I:C-stimulated IECs cultures had less HIV infection-induced

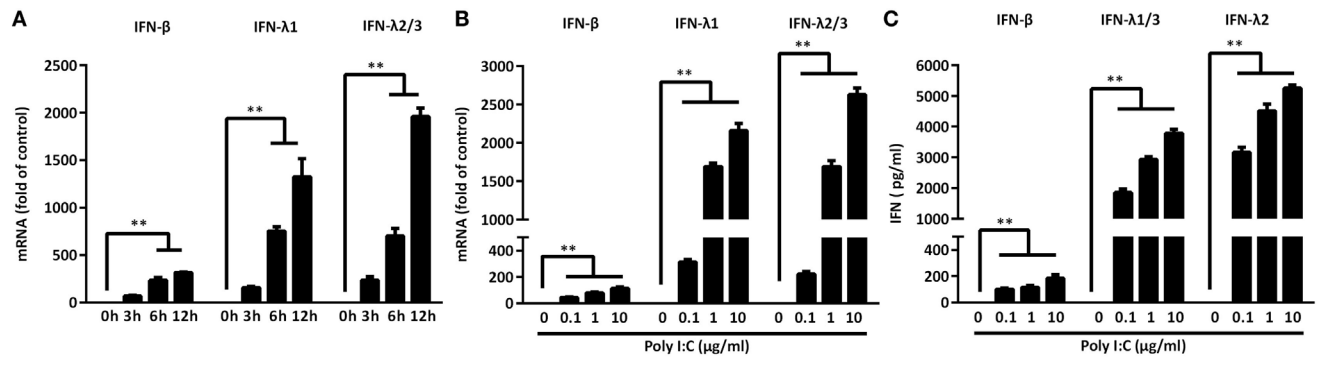

FIGURE 1 | Toll-like receptor 3 signaling induces interferon (IFN)-β and IFN-λ expression. **(A)** Intestinal epithelial cells (IECs) were transfected with Poly I:C (1 μg/ml) for the indicated times. Dose-dependent effect of Poly I:C on IFN induction of IECs at **(B)** mRNA and **(C)** protein levels. Data shown were the mean ± SD of three independent experiments. Asterisks indicate that the differences between the indicated groups are statistically significant (**$P < 0.01$).

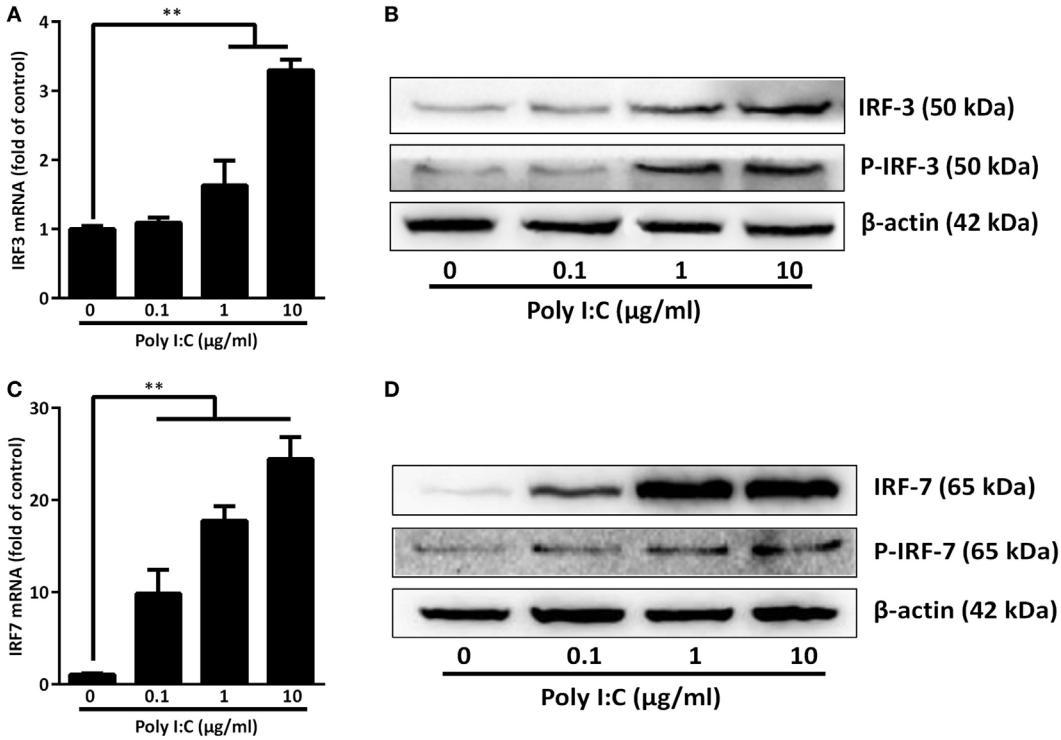

FIGURE 2 | Effect of toll-like receptor 3 activation on IRFs. intestinal epithelial cells were transfected with or without Poly I:C at indicated concentrations for 3 or 6 h. **(A,C)** For mRNA quantification, total cellular RNA was collected at 3 h post-transfection and subjected to the qRT-PCR. **(B,D)** For protein quantification, cellular proteins were collected at 6 h post-transfection and subjected to immunoblot. β-actin serves as the loading control. P-IRF3:Phospho-IRF3; P-IRF7:Phospho-IRF7. Data shown were the mean ± SD of three independent experiments. Asterisks indicate that the differences are statistically significant (**$P < 0.01$).

syncytia than untreated cells. In addition, HIV GAG gene expression was suppressed in macrophages pretreated with SN from Poly I:C-stimulated IECs cultures (**Figures 5B–E**). This IECs SN-mediated HIV inhibition was positively associated with the concentrations of Poly I:C used to activate IECs (**Figures 5B,D**) and the percentage of IECs SN added to macrophage cultures (**Figures 5C,E**). To decipher the roles of each subtype of IFNs in IECs-mediated anti-HIV activity, we used the neutralization antibody against IFN-β to pretreat the IECs SN or antibody against IFN-λ receptor to pretreat macrophages, respectively. As shown in **Figure 5F**, antibody to IFN-β significantly reduced the anti-HIV activity of SN from activated IECs cultures. In addition, anti-IL10 receptor β (IL-10Rβ a subunit of IFN-λ receptor) antibody pretreatment of macrophages could also block the anti-HIV activity of the IECs SN. We then investigated whether ISGs could be induced in macrophages treated by IECs SN. As shown in **Figure 5F**, TLR3 signaling of IECs induced the expression of ISG (ISG15, ISG56, OAS-1, OAS-2, MxA, MxB, GBP5, and Viperin) and several known HIV restriction factors, including Tetherin and APOBEC3G/3F in macrophages.

To ensure the IECs cultures are polarized (38), we used the transwell system to determine whether the polarized stimulation IECs could mediate HIV inhibition in macrophages. As shown in **Figure 6**, HIV GAG gene expression was suppressed in macrophages treated with SN from either upper (apical side) or lower (basolateral side) chambers of the transwell cultures. No significant difference in HIV inhibition was observed between SN from the upper level chambers and those from the lower level chambers.

IECs-Derived Exosomes Contribute to HIV Inhibition in Macrophages

To evaluate the role of the exosomes in IECs-mediated anti-HIV activity in macrophages, we added the activated IECs-derived exosomes to macrophage cultures. As shown in **Figures 7A–D**, macrophages treated with the exosomes showed less expression of cell-associated as well as extracellular HIV GAG gene as compared with untreated macrophages. We then examined the anti-HIV potency of IECs SN with or without exosome depletion. As indicated in **Figure 7E**, SN from Poly I:C-stimulated IECs significantly suppressed HIV, while the depletion of exosomes from IECs SN diminished IECs-mediated anti-HIV activity in macrophages.

TLR3 Signaling of IECs Induces CC Chemokines

CC chemokines (MIP-1α, MIP-1β, RANTES) are the ligands of the HIV entry co-receptor, CCR5. We examined whether IECs upon the TLR3 activation can produce these CC chemokines. As shown in **Figure 8**, Poly I:C treatment of IECs dose-dependently induced the CC chemokines at both mRNA

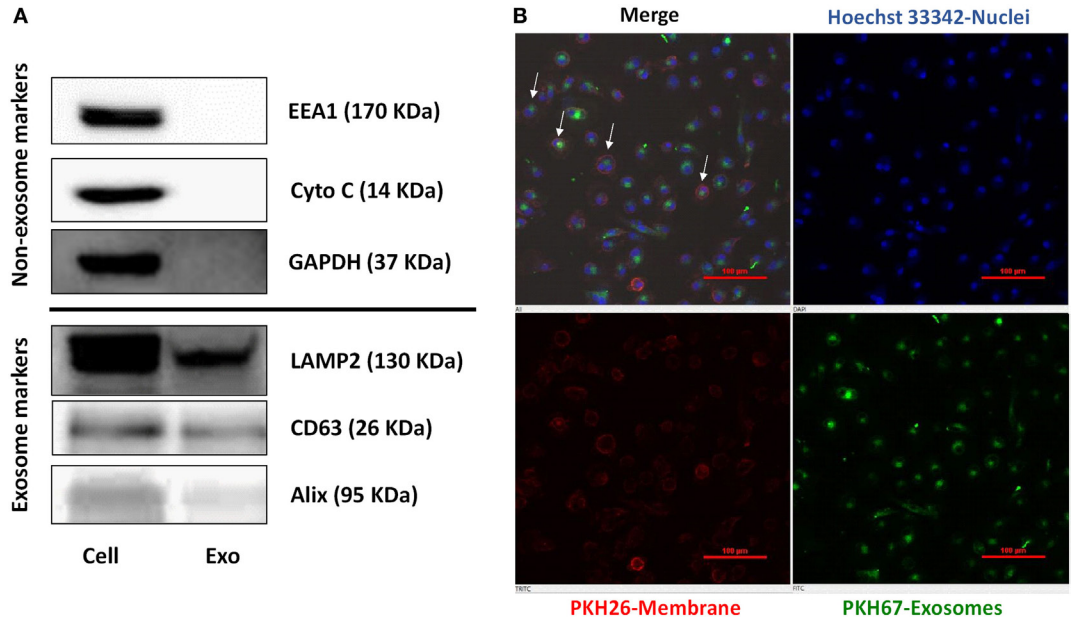

FIGURE 3 | Characterization of exosomes and delivery of intestinal epithelial cells (IECs) exosomes (Exo) to macrophages. **(A)** The expression of exosomal markers (Lamp2, Alix, CD63), and nonexosomal markers (EEA1, Cytochrome C, GAPDH) was determined by immunoblot. **(B)** The uptake of IECs exosomes labeled with PKH67 by macrophages. Macrophages were incubated with PHK67-labeled IECs exosomes (green) for 24 h and then stained with PKH26 for general cell membrane labeling (red) and Hoechst 33342 (blue) for nuclei. Data are representative of three independent experiments. Scale bar, 100 μm.

(**Figure 8A**) and protein (**Figure 8B**) levels. We then examined the ability of IECs SN to block HIV entry into macrophages. As shown in **Figure 8C**, the pretreatment of macrophages with the IECs SN resulted in a marked decrease in strong-stop DNA of HIV.

DISCUSSION

HIV infection provides ample pathogen-associated molecular patterns that can be detected by a variety of PRRs of the innate immune system (39). Among the PRRs, TLR3 is implicated in sensing dsRNA structures during viral infections, including HIV (40). While it has been reported that intestinal epithelial cell lines Caco-2 and HT-29 express functional TLR3 (41), there is little information about TLR3 activation of IECs and its role in antiviral activity against HIV infections of macrophages. We demonstrated that human IECs expressed functional TLR3, the activation of which resulted in the production of multiple antiviral factors, including the type I and III IFNs (**Figure 1**), ISGs, HIV restriction miRNAs (**Figure 4**), and CC chemokines (**Figure 8**). Importantly, we found that when added to primary human macrophage cultures, SN from the activated IECs cultures could potently suppress HIV infection and replication. In our early work of studying factors that influence the activation efficiency of TLR3 by Poly I:C (15), we found that the direct addition of Poly I:C to the cultures of primary macrophages or a neuroplastoma cell line could effectively activate TLR3. However, the transfection was necessary and needed in order to have efficient TLR3 activation by Poly I:C in the human hepatic cell line (Huh7) and brain microvascular endothelial cell line (hCMEC/D3). In addition, we demonstrated that the efficiency of TLR3 activation by high molecular mass Poly I:C was significantly higher than that by low molecular mass Poly I:C. These findings indicated that cell types and the size of Poly I:C are the crucial factors in Poly I:C-mediated TLR3 activation. As demonstrated in Figure S3 in Supplementary Material, we examined difference in the TLR3 activation efficiency between the direct addition and transfection of Poly I:C in IECs, showing that the levels of induced IFNs were significantly higher in IECs transfected with Poly I:C as compared to direct Poly I:C treatment. Therefore, we used the transfection technique for Poly I:C stimulation of IECs in this study to conceptually prove that as non-immune cells in GI tract, IECs can produce antiviral factors that can be transported through exosomes to macrophages, inhibiting HIV replication. The HIV inhibition in macrophages was also seen in macrophages treated with SN from either apical side or basolateral side of the polarized/activated IEC cultures (**Figure 6**). It was reported that there were little differences in TLR3 expression at different sites or between non-inflamed and inflamed mucosae in tissues from ulcerative colitis patients (42). Also, the polarized IECs responded to the TLR ligands, including TLR3, secreting IL-8 into the basolateral chamber, either exclusively on basolateral stimulation, or on apical stimulation. In non-polarized IECs, as expected, there was no difference in the response to all of these ligands (33).

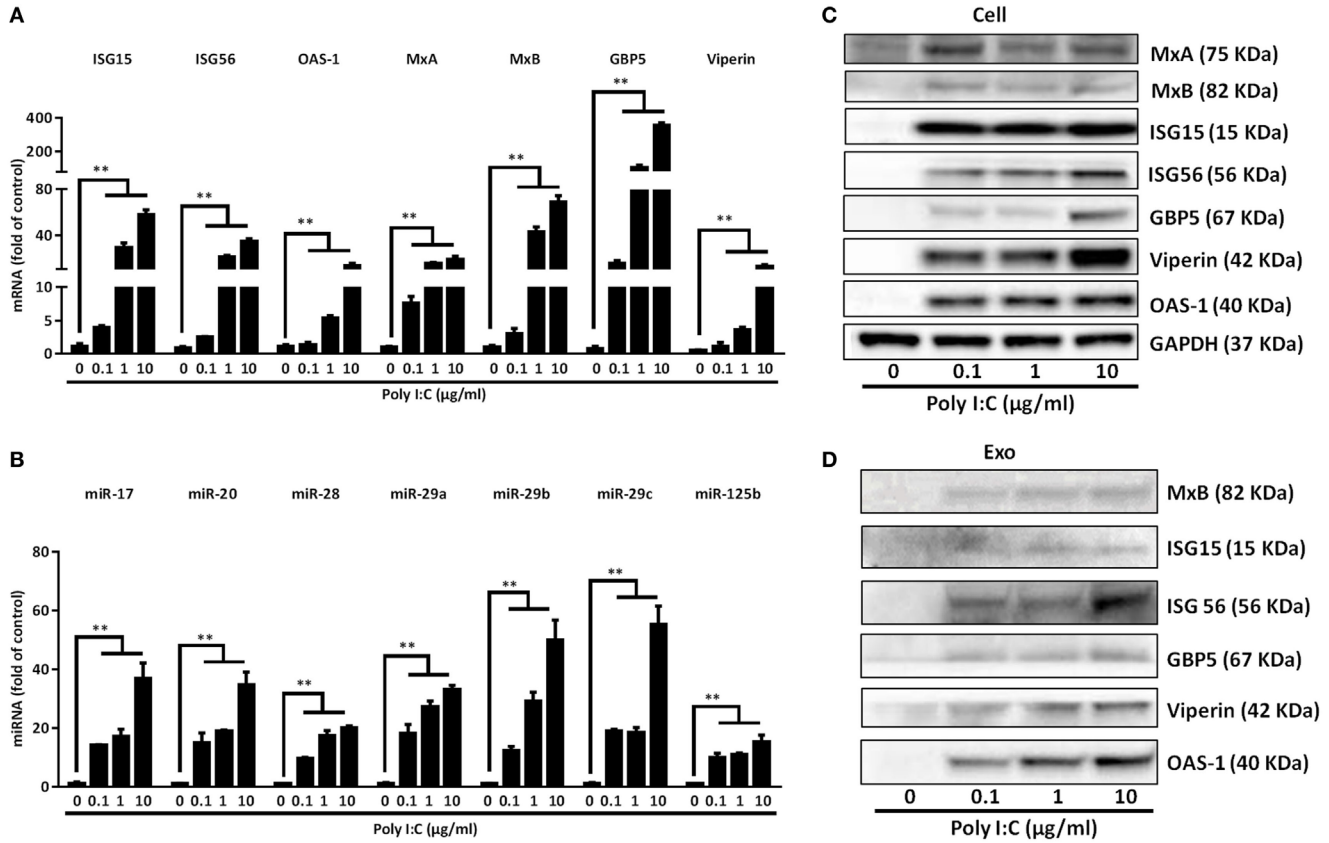

FIGURE 4 | Characterization of the antiviral factors in the cells and exosomes of toll-like receptor 3 signaling of intestinal epithelial cells (IECs). IECs were transfected with or without Poly I:C at indicated concentrations. **(A)** For IFN-stimulated genes (ISGs), mRNA quantification, total cellular RNA was collected at 12 h post-transfection and subjected to the qRT-PCR. **(B)** IECs cultured in exosome-free media were transfected with or without poly I:C (1 μg/ml) for 48 h. miRNA in secreted exosomes from IECs supernatant were quantified by qRT-PCR. Synthetic caenorhabditis elegans miRNA-39 (cel-miR-39) was used as a spiked-in miRNA for normalization. Levels of miRNAs were plotted as fold of control. **(C)** For protein quantification, cellular proteins were collected at 24 h post-transfection and subjected to immunoblot. GAPDH serves as the loading control. **(D)** Exosomal protein was collected at 48 h and subjected to immunoblot with indicated ISGs antibodies. 20 μg of total exosome loaded. Data shown represent the mean ± SD of three independent experiments. Asterisks indicate statistically significant differences. (**$P < 0.01$).

Although IECs are non-immune cells, they are able to produce IFN-driven antiviral factors, including ISGs. Studies have shown that the ISGs, including ISG15, ISG56, MxA, MxB, OAS-1, OAS-2, and GBP5 have anti-HIV activities (43–45). ISG15 plays a crucial role in the IFN-mediated inhibition of late stages of HIV assembly and release (46); MxB inhibits HIV infection by inhibiting the capsid-dependent nuclear import of subviral complexes (47); GBP5 reduces HIV infectivity by interfering with Env processing and incorporation (48). In addition to the ISGs, Poly I:C-stimulated IECs expressed HIV restriction miRNAs (**Figure 4**), including miRNA-17, miRNA-20, miRNA-28, miRNA-29 family members (miR-29a, 29b, and 29c), and miRNA-125b. It is known that miRNA-28 and miRNA-125b can target the 3'UTR of HIV transcripts (49). miRNA-29 family members interfere with virus replication, as they can target a highly conserved site in various HIV subtypes (50). Studies have shown that miRNA-17 and miRNA-20 target p300/CBP associated factor (PCAF), a cellular cofactor of the HIV Tat protein (51). Furthermore, we found that CC chemokines (MIP-1α, MIP-1β, RANTES), ligands of HIV entry co-receptor CCR5, were induced in activated IECs (**Figure 8**). The observation evidenced the role of CC chemokines in IECs-mediated HIV inhibition that SN from TLR3-activated IEC cultures could block HIV entry into macrophages. IFN-β and IFN-λ in IECs SN appeared to be responsible for the induction of these anti-HIV factors, as the antibodies to IFN-β and IFN-λ receptors could block the inhibitory effect of IECs SN on HIV (**Figure 5**).

The investigation on the mechanisms for the induction of IFNs showed that there was upregulation of IRF3 and IRF7 in activated IECs (**Figure 2**). IRF3 and IRF7 are the key regulators of type I and III IFNs during viral infections (52). IRF3 and IRF7 phosphorylation is a crucial step in activating type I and III IFNs-mediated antiviral response (53). Both IRF3 and IRF7 require phosphorylation-induced activation in order to translocate to the nucleus to activate IFNs (54). Specifically, during viral infections, IRF3 is important in the early phase of inducing the transcription of IFN-α and IFN-β, which then can

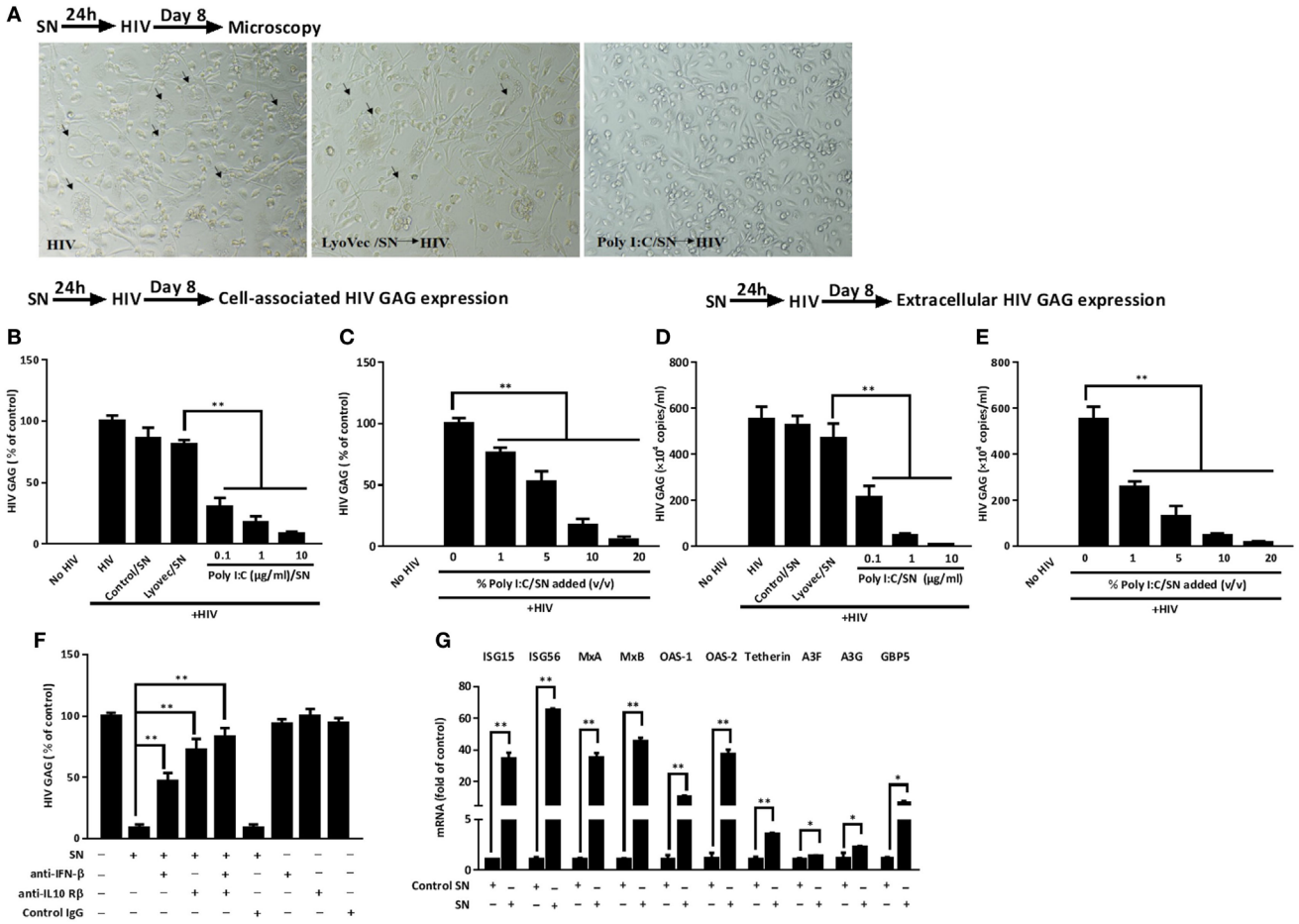

FIGURE 5 | Effect of supernatant (SN) from intestinal epithelial cells (IECs) cultures inhibits HIV replication in macrophages. **(A)** Morphologic observations of HIV-infected macrophages with mock treatment or pretreated with either LyoVec/SN or Poly I:C/SN (arrows indicate syncytium, magnification × 100). **(B,C)** Cell-associated and **(D,E)** extracellular HIV GAG gene expression level in macrophages with 10% [volume to volume ratio (v/v)] of indicated SN pretreatments or with indicated volumes of 1 μg/ml Poly I:C-stimulated IECs SN pretreatments was measured by qRT-PCR at 8 days postinfection. **(F,G)** Roles of interferon (IFN)-β and IFN-λ in IECs SN-mediated anti-HIV activity and the effect of IECs SN on the expression of IFN-stimulated genes (ISGs) in macrophages. **(F)** Effect of neutralization antibodies (Abs) to IFN-β or IFN-λ receptor on IECs culture SN-mediated anti-HIV activity. IECs SN was preincubated with anti-IFN-β (10 μg/ml) for 1 h and then used to treat macrophages 24 h prior to HIV Bal infection (p24, 20 ng/ml). For IFN-λ receptor pretreatment, the anti-IL10Rβ neutralization antibody (10 μg/ml) was added to macrophage cultures for 1 h prior to the addition of SN. HIV GAG expression was then measured by qRT-PCR for 8 days postinfection. **(G)** Effect of Poly I:C-stimulated IECs culture SN on ISG expression of macrophages. IECs were stimulated with Poly I:C for 48 h and culture SN was collected for treatment of macrophages (10% v/v) for 12 h. RNA was extracted, and the expression of ISGs was measured by qRT-PCR. Representative data were the mean ± SD of three independent experiments using macrophages of three donors. Asterisks indicate that the differences between the indicated groups are statistically significant (*$P < 0.05$, **$P < 0.01$).

activate IRF7. Similar to IFN-β, IFN-λ1 gene is regulated by virus-activated IRF3 and IRF7, whereas IFN-λ2/3 gene expression is mainly controlled by IRF7 (55). IRF7 not only induces IFNs, but also actives many ISGs, among which PKR, OAS, and the Mx protein have been well characterized for their antiviral activities (56).

As one of the primary targets for HIV infection and persistence, macrophages have been implicated as an important HIV reservoir. Our early investigations (26, 57) showed that TLR3 activation of macrophages potently suppressed HIV infection and replication through multiple antiviral mechanisms at both the cellular and molecular levels. Despite being a major producer of type I IFNs, the biological functions of macrophages are significantly compromised in IFN induction upon HIV infection (17, 18). In contrast to macrophages, IECs are not the target of HIV. Therefore, it is unlikely that HIV has a direct and negative impact on IECs. As the first line of cells in the GI system, the IECs have to encounter a number of stimuli and immune cells, including HIV-infected macrophages (58). Thus, the activation of these nonimmune cells in the GI tract is inevitable. We found that activated IECs SN could induce the expression of several key HIV restriction factors in macrophages, including Tetherin and APOBEC3G/3F (**Figure 5**). Tetherin is a transmembrane protein that specifically inhibits HIV release from infected cells (59), APOBEC3G/3F are single-stranded DNA deaminases that inhibit HIV replication

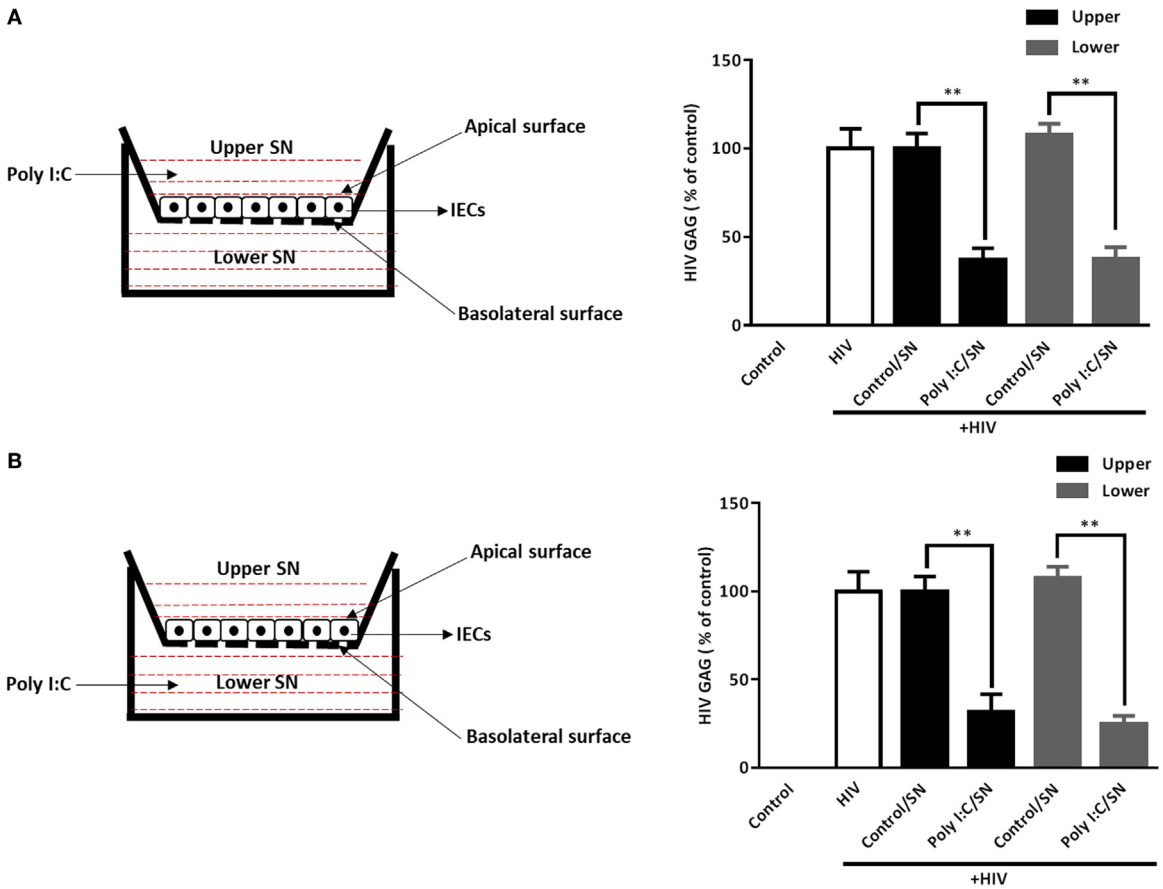

FIGURE 6 | Supernatant (SN) of polarized intestinal epithelial cells (IECs) cultures inhibits HIV replication in macrophages. IECs were seeded onto a transwell insert at a density of 1×10^5 cells/insert and cultured for 72 h prior to use. The integrity of the IEC monolayer in each well was assessed for the development of transepithelial electrical resistance (TEER). Poly I:C (1 μg/ml) was then added to the upper [**(A)**, apical level] or lower [**(B)**, basolateral level] chamber of the IECs cultures. The SN was collected 48 h after Poly I:C treatment. Cell-associated HIV GAG gene expression in macrophages treated with 10% [volume to volume ratio (v/v)] of indicated SN was measured by qRT-PCR at 96 h postinfection. Data shown were the mean ± SD of three independent experiments. Asterisks indicate statistically significant differences (**$P < 0.01$).

through deaminating cytidine to uracil on the minus strand of the HIV proviral DNA (60). Thus, the activation of IFN-mediated antiviral responses by IECs should be beneficial for GI protection. As a non-HIV target cell in the GI tract, it is unlikely that the ability of IECs to mount an IFN-mediated anti-HIV response would be compromised by HIV infection. We as well as others have shown that IFNs were produced not only by the immune cells but also by the nonimmune cells in the CNS, such as neurons and astrocytes (34, 61). In contrast to Poly I:C induction of both IFN-α and IFN-β in the immune cells, TLR3 signaling of IECs induced only IFN-β expression. This finding is consistent with the report by Starace et al. showing that Poly I:C induced IFN-β but not IFN-α in mouse Sertoli cells (62). These observations along with the findings of this study support the notion that IECs and other nonimmune cells in the GI tract could be important bystanders in mounting effective antiviral responses, which may have a key role in restricting HIV infection/replication in the GI system.

To understand how IECs could transport the antiviral factors to macrophages, we examined whether IECs can produce and release exosomes which are known to have the ability to shuttle biologically active molecules. Exosomes have a vital role in a variety of biologic processes, such as cell proliferation, apoptosis, and immune responses (63, 64). A major recent study in the intestinal mucosa field unveiled the capacity of exosomes to mediate the functional transfer of genetic materials (mRNAs and miRNAs) between immune cells (65). We found that IECs-derived exosomes could be taken up by infected macrophages, inhibiting HIV replication (**Figure 7**). We also observed that exosomes from Poly I:C-stimulated IECs were enriched with antiviral cellular ISGs and miRNAs (**Figure 4**), including miRNA-17, miRNA-20, miRNA-28, miRNA-29 family members (miR-29a, 29b, and 29c) and miRNA-125b. miRNA-28 and miRNA-125b are known to target 3'UTR of HIV transcripts (66). miRNA-29 family members interfere with virus replication, as they can target a highly conserved site in various HIV subtypes (50). Studies by several groups

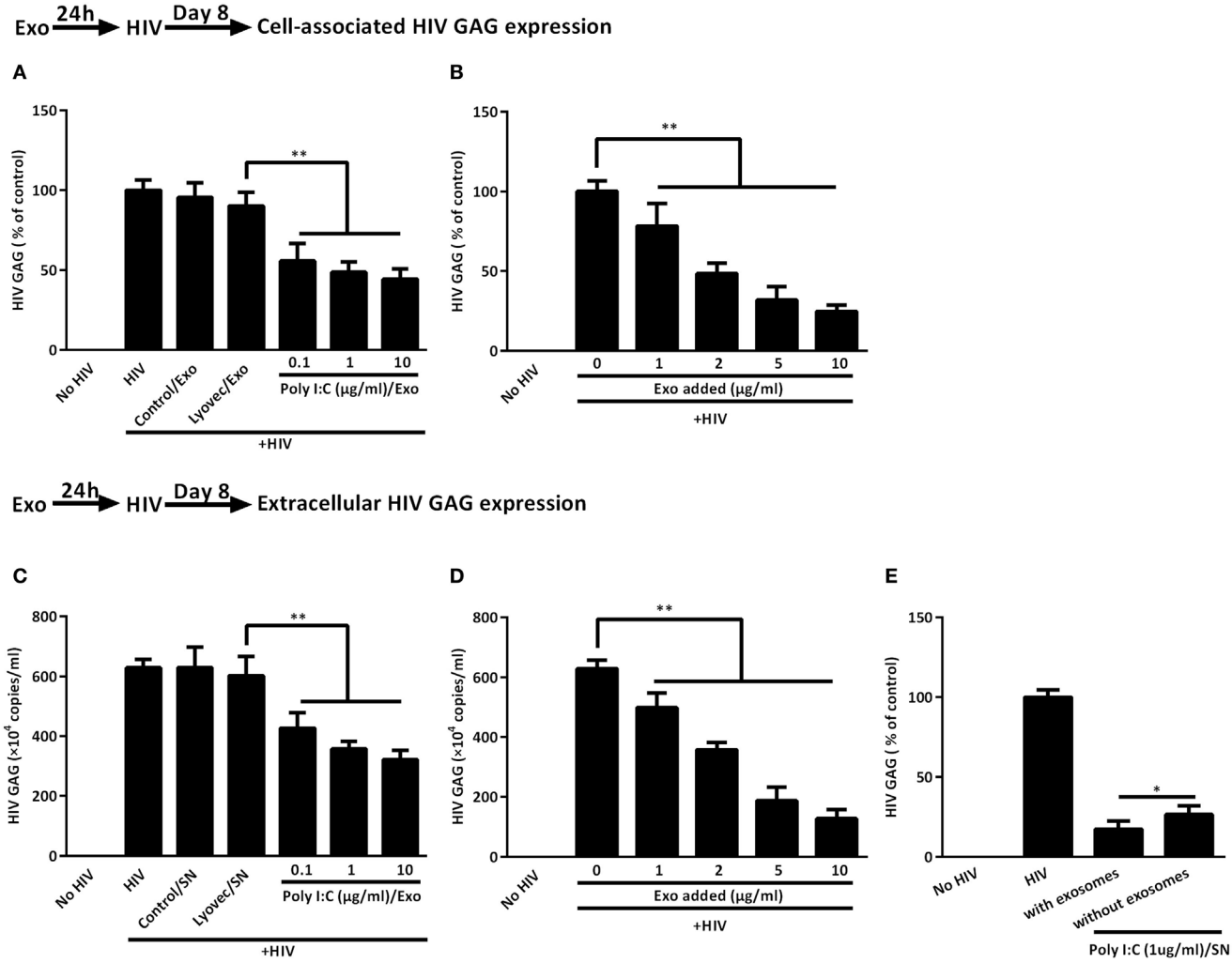

FIGURE 7 | Intestinal epithelial cells (IECs)-derived exosomes contribute to IECs supernatant (SN)-mediated HIV inhibition in macrophages. **(A,B)** Cell-associated and **(C,D)** extracellular HIV GAG gene expression in macrophages with 2 μg/ml of indicated exosomes pretreatments or with the indicated concentration of 1 μg/ml Poly I:C-stimulated IECs exosomes pretreatments were measured by qRT-PCR for 8 days postinfection, respectively. **(E)** The inhibition of HIV replication by IECs culture SN with or without exosome depletion. To deplete exosomes, the SN from Poly I:C-stimulated IECs were incubated with anti-CD63 antibody-conjugated Dynabeads overnight at 4°C and then separated in a magnetic field. Representative data were the mean ± SD of three independent experiments using macrophages of three donors. Asterisks indicate that the differences between the indicated groups are statistically significant (*$P < 0.05$, **$P < 0.01$).

showed that miRNA-17 and miRNA-20 target p300/CBP associated factor (PCAF), a cellular cofactor of the HIV Tat protein (67).

Collectively, we have provided the experimental evidence that TLR3 activation-induced antiviral factors in IECs could be transported to macrophages through exosomes released by IECs and internalized by macrophages (**Figure 9**). Because HIV has evolved several mechanisms to evade TLR3 mediated intracellular innate immunity in target cells, such as macrophages (68, 69), anti-HIV support from non-immune bystander cells is helpful in restoring the HIV-suppressed system in infected cells. Given that macrophage is an important cellular reservoir for HIV infection/persistence, to control and eradicate HIV in macrophages is clinically significant. Although the precise cellular and molecular mechanisms by which activated IECs could inhibit HIV replication in macrophages remain to be determined, the induction of IFNs, antiviral ISGs, HIV restriction miRNAs, and CC chemokines should account for much of IECs-mediated anti-HIV activity. However, further *in vitro* and *in vivo* investigations are necessary in order to determine whether the TLR3 signaling of IECs is indeed beneficial in protecting GI macrophages from HIV infection. Currently, the therapeutic TLR agonists are being developed for the treatment of cancer, allergies and viral infections. A number of TLR agonists are now in clinical or preclinical trails such as the anti-HIV TLR3 agonist (Poly I:C 12U) (70–72). These studies support the notion for further developing a TLR3 agonist-based therapy for HIV disease in which host cell innate

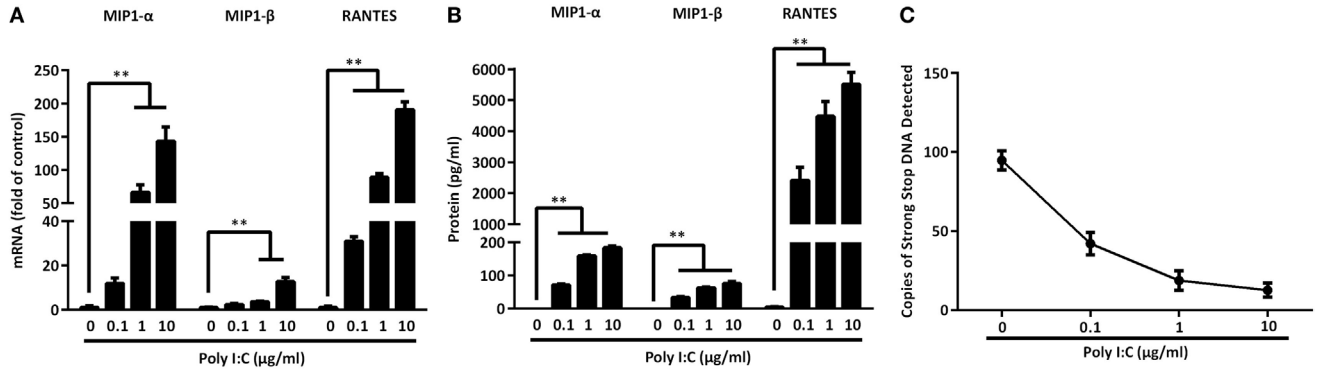

FIGURE 8 | Toll-like receptor 3 signaling of intestinal epithelial cells (IECs) induces CC chemokines. IECs were transfected with or without Poly I:C at indicated concentrations for 12 h (mRNA) or 48 h (protein). **(A)** Cellular RNA was collected and subjected to the qRT-PCR. **(B)** MIP-1α, MIP-1β, and RANTES proteins were analyzed by Cytometric Bead Array with the specific kits according to the manufacturer's instructions. **(C)** HIV strong-stop DNA was detected in macrophages with 10% (v/v) of supernatant from indicated doses of Poly I:C-treated IECs cultures. Representative data from at least three donor macrophages was shown. Asterisks indicate that the differences between the indicated groups are statistically significant (**$P < 0.01$).

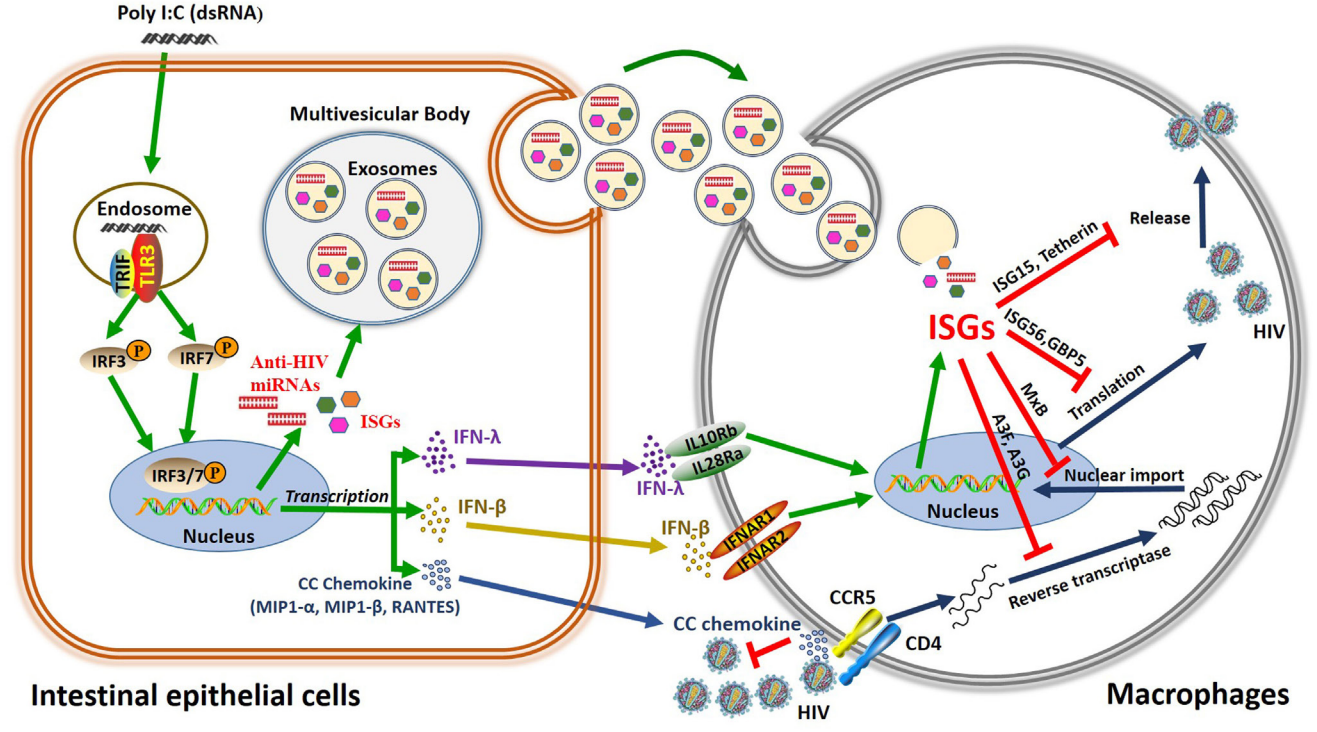

FIGURE 9 | Schema of the anti-HIV mechanism of toll-like receptor 3 (TLR3) signaling of intestinal epithelial cells (IECs). Stimulation of IECs with double-stranded RNA (Poly I:C) activates TLR3 pathway, which facilitates phosphorylation and translocation of IRF3 and IRF7, initiating the transcription of interferon (IFN)-β, IFN-λ, and CC chemokine and releasing exosomes in the IECs. CC chemokines bind to HIV entry co-receptor CCR5 and block HIV entry. In addition, IFN-β and IFN-λ released from IECs can bind to their receptors in macrophages, inducing anti-HIV IFN-stimulated genes (ISGs) (ISG15, ISG56, MxA, MxB, OAS-1, OAS-2, GPB5, Tetherin, and APOBEC3G/3F), and exosome delivery of ISGs and miRNA to HIV-infected macrophages, which inhibit HIV at different steps of viral replication.

immune responses are significantly compromised by the virus. These future studies are critical for the design and development of TLR3 activation-based immune treatment for people with HIV infection.

ETHICS STATEMENT

In this *in vitro* study, we obtained primary human monocytes from the Immunology Core at the University of Pennsylvania

School of Medicine. The Core has the Institutional Review Board approval for blood collection from healthy donors. Anyone who obtains human cells from the Core is considered as secondary use of de-identified human specimens, which does not subject to human subject review by both NIH and IRB.

AUTHOR CONTRIBUTIONS

LG, LZ, XW, J-LL, and W-ZH designed the study. LG, X-QX, R-HZ, J-BL, BZ, and HL performed the experiments. W-ZH supplied reagents needed for this study. LG analyzed and interpreted the data and wrote the manuscript. LG and W-ZH reviewed and revised the manuscript. All the authors have read, reviewed, and edited the manuscript and agreed for submission to this journal.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/articles/10.3389/fimmu.2018.00247/full#supplementary-material.

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