differentiate into cDC in the presence of Flt3L, and monocytes differentiate into either macrophages or DC, presumably depending on the inflammatory stimuli present (Cheong et al., 2010).

With regards to lineage, there is considerable difference between mouse and human DC subsets, as defined by surface markers. Five major DC subsets are known in mice: $CD8^+$DC, $CD4^+$DC, and pDC in lymphoid tissues, and $CD103^+$ and $CD11b^+$DC in non-lymphoid tissues (Steinman and Idoyaga, 2010). Alternately, the source of DC in humans is more limited, originating from either skin or blood. In human skin, three DC subsets have been identified: epidermal $CD207^+$ Langerhans cells (LCs), $CD14^+$ dermal DC, and $CD14^-CD207^-CD1a^+$ DC. Amongst them, LC are the most potent inducer of Th2 cytokines and able to cross prime $CD8^+$ effector cells (Klechevsky et al., 2008). With respect to the origin of LC localized in the skin epidermis, Sere et al. (2012) have recently reported that LC are replenished in two waves, one by monocyte-derived, short lived LC upon inflammation, and the other by non-blood monocyte origin with long life expectancy (Sere et al., 2012). In other tissues and blood, two subsets of human cDC are known, which express either CD1c (BDCA-1) or CD141 (BDCA-3). The other group, pDC, expresses human-specific pDC markers, BDCA-2 and BDCA-4, in addition to CD123 [interleukin-3 receptor (IL-3R)], and produces a large amount of type I interferon (IFN; Steinman and Idoyaga, 2010).

A unique and important function of DC is their cross-presentation ability by phagocytosing dead cells, tumor cells, or infected cells, which results in the activation of MHC class I-restricted effector $CD8^+$ T cells (Bevan, 2006). A particular subset of DC is known to efficiently cross-present antigens. In mice, splenic CD8α DC are the most potent DC subset for cross-presentation, and the human counterpart of mouse CD8α has been recently identified (Bachem et al., 2010; Jongbloed et al., 2010; Poulin et al., 2010).

The minor DC population in human blood, $CD141^+$ DC, shares several phenotypic and functional properties with mouse CD8α DC, such as: (1) the expression of DC natural killer (NK) lectin group receptor-1 (DNGR-1) or CLEC9A, a sensor of necrotic cells (Sancho et al., 2009), and (2) the selective expression of the chemokine receptor XCR1 (Bachem et al., 2010), transcriptional factor ATF (activating transcription factor)-like-3, and IFN regulatory factor 8 (IRF-8). $CD141^+$ DC also express a high level of Toll-like receptor 3 (TLR3) and upon TLR3 stimulation, produce IL-12 and IFN-β. More recently, by using comparative genomics to align human and mouse cell types, Haniffa et al. (2012) demonstrated that cutaneous $CD141^{hi}$ $CLEC9A^{hi}XCR1^+$ DC, which are closely related to blood $CD141^{hi}$ $CLEC9A^{hi}XCR1^+$ DC, are much more potent at cross-presentation than LC. Taken together, in humans, $CD141^+$DC, $CD1c^+$DC, and pDC localize within lymphoid tissues, while the former two types of DC and $CD14^+$ DC are present in non-lymphoid tissues. In addition, human tissue $CD141^+$ DC are functional homologues of mouse $CD103^+$(α chain of the $\alpha_E\beta_7$ integrin) non-lymphoid DC, with high cross-presentation activity (Haniffa et al., 2012).

However, the relationship between DC and monocytes/macrophages can sometimes be confusing when based only on cell surface markers and functional properties. Two types of human monocytes are present in the blood: "patrolling" $CD14^{dim}CD16^+$ and inflammatory $CD14^+$ $CD16^-$ or $CD14^+CD16^+$monocytes, which resemble mouse Gr^- ($Ly6C^{low}$) and Gr^+ ($Ly6C^{high}$) cells, respectively (Auffray et al., 2009; Cros et al., 2010). When tissue inflammation occurs, $CD14^+$inflammatory monocytes migrate to the site of inflammation, and may differentiate into $CD11b^+CD14^+$ monocyte-derived DCs (MDDCs) as demonstrated in mice (Cheong et al., 2010).

Currently, four major DC subsets comprise and make up the category of DC: cDC, pDC, LCs, and MDDCs (Satpathy et al., 2012). A recent large scale collaborative study focusing on the transcriptional network of DC and monocyte/macrophage subsets, highlighted on some lineage-specific key transcriptional factors and the mutual relationship between DC and monocytes/macrophages (Miller et al., 2012), though the definition of DC by molecular signatures has raised further discussion (Hume et al., 2013).

Incorporating recent findings, the development and lineage relationship of human myeloid precursors is illustrated in **Figure 1**.

OVERVIEW OF ANTIVIRAL IMMUNE RESPONSES IN ACUTE AND CHRONIC HIV INFECTION

The natural infection of HIV-1 mostly occurs through vaginal or rectal routes. Because these submucosal spaces are rich in DC, they have been argued to be the primary targets of HIV-1 infection (see reviews by Wu and KewalRamani, 2006; Piguet and Steinman, 2007). However, detection of HIV-1-producing DC in these tissues is rare, which is in contrast to the rapid and massive HIV/simian immunodeficiency virus (SIV) infection detected in $CD4^+CCR5^+$ memory T cells (Brenchley et al., 2004; Mehandru et al., 2004; Li et al., 2005; Mattapallil et al., 2005). Nonetheless, the scenario that mucosally residing, non-producing HIV-infected DC or phagocytosed HIV-1-infected DC migrate to regional lymph nodes, and subsequently spread HIV-1 infection there to contacting T cells, in association with nominal antigen presentation, well reflects what maybe occurring in lymphoid organs (Wu and KewalRamani, 2006; Piguet and Steinman, 2007).

In a chronic stage of HIV-1 infection, a major reservoir of latent HIV-1 infection is considered to be the circulating resting memory T cells, carrying integrated HIV-1 DNA (review by Pierson et al., 2000). When resting memory cells enter a lymphoid organ, they interact with resident DC, which may induce T cell activation and concomitant transmission of reactivated HIV-1 to DC. There is a possibility that newly HIV-infected DC can transmit virus to intact naïve or memory T cells. Although these HIV-1-infected T cells and DC can be eliminated by a cytotoxic T cell (CTL) response, a continuous cycle of such aforementioned events may cause intermittent surges of plasma viral load under chronic infection. Henceforth, lymphoid organs are considered to be major sites of HIV-1 production.

During the induction of primary immune responses in lymphoid organs, an inflammatory response can occur, as an innate immune mechanism at the invasion site, attracting a variety of immune cells, including T cells and monocytes. As previously described, so-called inflammatory monocytes are recruited into peripheral tissues subsequently, differentiating into inflammatory

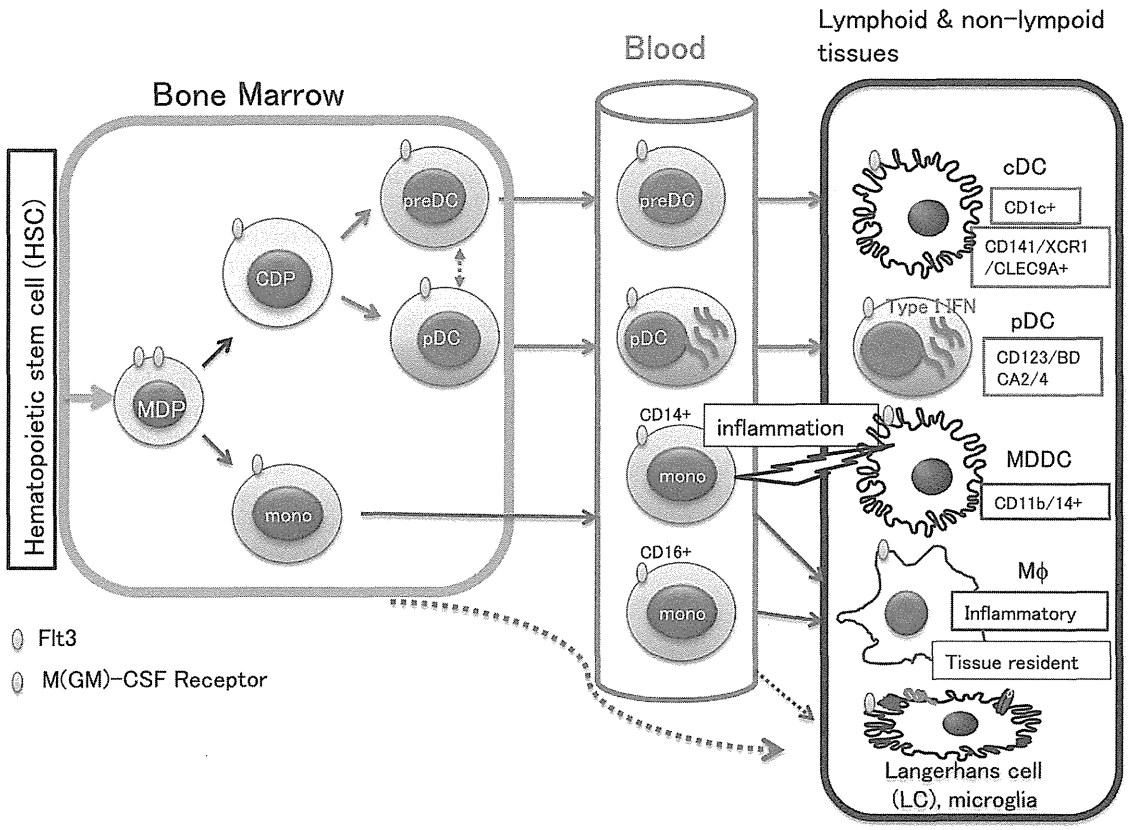

FIGURE 1 | Development of myeloid cells and lineage relationship between DC subsets. Hematopoietic stem cells in the bone marrow differentiate into monocyte/macrophage and DC precursor (MDP), which commits to a distinct lineage cell, either becoming a monocyte or common DC precursor (CDP). CDP differentiates to preDC or pDC, and migrates to the peripheral tissues through blood. In the lymphoid or non-lymphoid tissues, preDC can become either of two types of cDC: $CD1c^+$ DC and $CD141^+/XCR1^+/CLEC9A^+$ DC, the latter of which has high cross-presenting activity. In the blood, $CD14^+$ or $CD16^+$ monocytes are also circulating. Blood monocytes are source of macrophages (Mϕ), LC, and microglias. The other LC precursor in the bone marrow, was also shown to migrate to the skin. Upon tissue inflammation, $CD14^+$ monocytes migrate to the inflamed tissue and differentiate into either migratory MDDC or inflammatory Mϕ. It is known that the cells committed to become DC express Flt3, whereas those that commit to monocytes/macrophages express M-CSF receptor. Therefore, Flt3L and M-CSF are key molecules for differentiation and proliferation of DC and monocytes/macrophages, respectively.

MDDC which can regulate immune responses locally (Cheong et al., 2010).

It is well-known that effector $CD8^+$ T cells play an essential role regarding protection against HIV/SIV infection (Appay et al., 2008). Once infection primes in lymphoid organs, memory $CD8^+$ T cells can act as effector cells by circulating in the blood, in order to migrate to infected tissues upon re-exposure, and quickly eliminate infected cells. The importance of long-term resident memory $CD8^+$T (T_{RM}) cells has been highlighted with respect to efficacy of the local memory response (Gebhardt and Mackay, 2012). By utilizing an elegant mouse model of herpes simplex virus (HSV) infection, memory $CD8^+$ T cells were shown to be initiated in extra-lymphoid tissues, independent of migratory memory $CD8^+$ T cells (Wakim et al., 2008). In this model, HSV-infected dorsal root ganglia (DRG) was surgically transplanted to naïve mice, because DRG is known to be latently infected with HSV, after acute virus resolution, and to be reactivated by surgical extraction. Stimulation of these $CD8^+T_{RM}$ cells was dependent on recruited DC in the DRG, and $CD4^+$T cell help was required. These $CD8^+$ T_{RM} cells represent a self-renewing and highly protective population of memory T cells distinct from circulating memory $CD8^+$ T cells, and express CD103 which is known to be widely expressed in non-lymphoid tissues (Gebhardt et al., 2009). Interestingly, a study using intravital two-photon microscopy revealed that while memory $CD4^+$ T cells are trafficked rapidly, $CD8^+$ T_{RM} cells are removing slowly in the original skin site during HSV infection (Gebhardt et al., 2011).

The functional properties of $CD8^+$ T_{RM} cells have just begun to be elucidated in influenza virus infection. The innate antiviral function of IFN-induced transmembrane protein 3 (IFTM3) was first discovered by using RNA interference screening for factors modifying influenza virus infection (Brass et al., 2009). The IFITM family is: (1) made up of IFN-stimulated genes (ISGs) with diverse biological functions, (2) comprises of multiply closed members of four genes in both humans and mice, and (3) restricts various virus infections at a site of viral fusion (Diamond and Farzan, 2012).

Recently, IFITM3 was reported to be expressed in lung $CD8^+$ T_{RM} cells after influenza virus infection, endowing greater resistance to the secondary influenza virus infection (Wakim et al., 2013). Sustained expression of IFITM3 is intrinsically regulated in $CD8^+$ T_{RM}, but not $CD4^+$ memory T cells, and enhances the survival of $CD8^+$ T_{RM} cells only at sites of viral infection. Although the involvement of recruited DCs on the activation of $CD8^+$ T_{RM} cells is demonstrated (Wakim et al., 2008), the mechanisms in which memory and effector $CD8^+$ T cells develop and localize to target peripheral tissue such as intestinal mucosa and the involvement of intestinal DCs need to be addressed in future for the development of effective anti-HIV vaccines.

The overview of this section is illustrated as **Figure 2**.

DISTINCT SUSCEPTIBILITY OF DCs TO HIV: *CIS*- AND *TRANS*-INFECTION MODES

When MDDC are activated by various stimuli, such as lipopolysaccharide (LPS), TLR ligands, cytokines like type I IFN, DC express higher levels of MHC class II and other costimulatory molecules, thereby engaging in antigen presentation rather than antigen uptake. This maturation status and difference in lineage can profoundly affect the susceptibility of DC to HIV-1 infection (see review by Wu and KewalRamani, 2006).

Dendritic cell express various C-type lectin receptors (CLRs) which bind to HIV-1, such as DC-SIGN (dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin), Langerin, and dectin (Turville et al., 2002), and recently discovered CLEC9A (Caminschi et al., 2008; Huysamen et al., 2008; Sancho et al., 2009). Each lectin receptor appears to have a unique and distinct function. HIV-1 transfer in *trans*, through the capture of virus by a CLR, such as DC-SIGN, occurs early in *in vitro* experiments (Cavrois et al., 2007; Dong et al., 2007; Izquierdo-Useros et al., 2007), and is designated *trans*-infection. However, DC lectin receptors are important molecules involved in the presentation of foreign antigens. In fact, the most of the HIV-1 virions captured by DCs were known to be rapidly degraded (Turville et al., 2004; Nobile et al., 2005). Moreover, the interaction of leukocyte-specific protein 1 (LSP-1), a protein directing internalized virus to the proteasome, with the cytoplasmic region of DC-SIGN may further facilitate the degradation of HIV-1 (Smith et al., 2007).

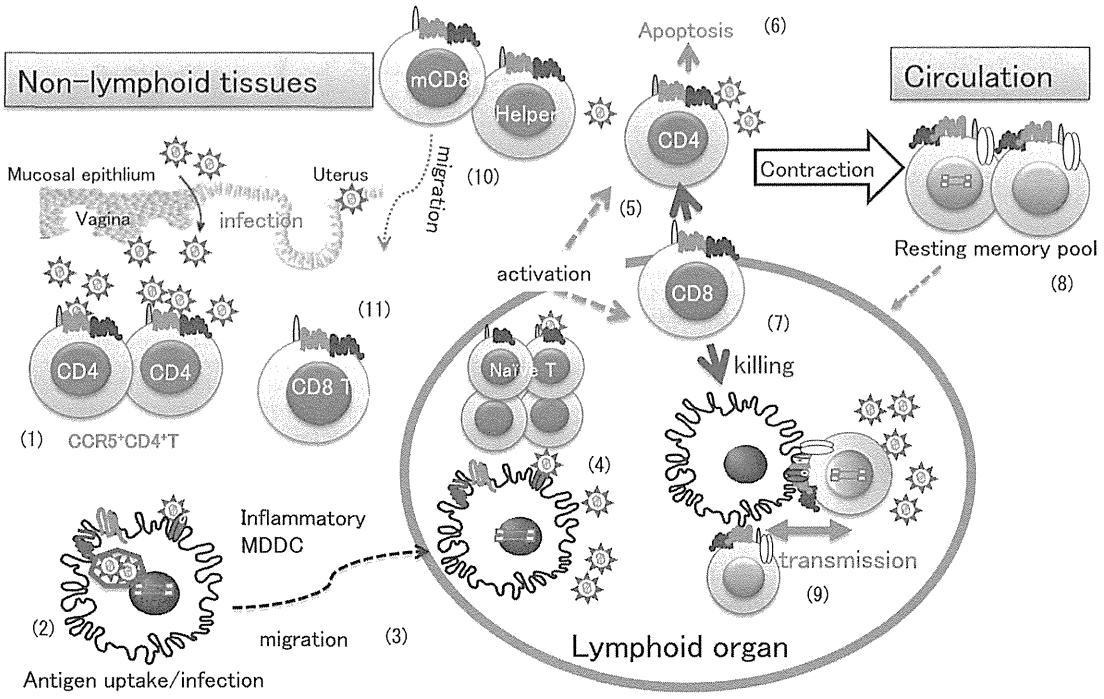

FIGURE 2 | HIV infection and T cell differentiation during primary and memory antiviral immune responses. A general view of antiviral immune responses focusing on HIV-1 infection is illustrated. Once HIV invades the vaginal mucosa, infection mostly occurs in activated $CD4^+CCR5^+$ T cells (1). Resident DCs in the submucosa engulf virions or HIV-infected cells (2), and then migrate to the draining lymph nodes (3), where an antiviral immune response is initiated (4). In a primary immune response, antigen-specific T cells are differentiate into effector cells. During the effector phase, memory T cells are also produced, the mechanism of which is not fully understood. Once activated, $CD4^+$ T cells are infected with HIV (5), however, most effector cells will die of apoptosis (6), or killed by effector $CD8^+$ T cells (7). When virus-infected cells are eliminated in the infection site, the immune response enters into the contraction phase. After some time, memory T cells revert to quiescent or resting state (8) and circulate back to peripheral tissues for checking for next infection. Latent infection of HIV is known to occur in such quiescent memory $CD4^+$ T cells. When these latently HIV-infected memory $CD4^+$ T cells interact with uninfected DCs and T cells, for secondary antigen exposure, HIV-1 may spread to $CD4^+$ T cells in the lymphoid organ through DC–T cell interaction (9). In the case of circulating memory $CD8^+$ T cells (mCD8), they may quickly enter into tissues to eliminate the secondary infection (10). However, recent evidence indicate that tissue resident $CD8^+$ T cells (T_{RM}) (11) primed *in situ* play a more important role than circulating mCD8 in protective immunity, which may be a distinct feature from memory $CD4^+$ T cells.

On the other hand, HIV-1 replication in DCs, but not DC-SIGN, is required for long-term transfer of HIV from DCs to $CD4^+$ T cells (Lore et al., 2005; Nobile et al., 2005; Burleigh et al., 2006; Wang et al., 2007). Although DCs can support only minimal replication of HIV-1 (DC restriction, see Restrictions in DCs), it is considered that the antigen-dependent close DC–T cell contact, forming IS, would support the efficient virus transmission followed by massive virus replication in $CD4^+$ T cells (Tsunetsugu-Yokota et al., 1995; Lore et al., 2005). We assume that such *cis*-infection is more likely to occur *in vivo*, as opposed to *trans*-infection.

Thus, the *trans*-infection *in vitro* needs to be discriminated from HIV-1 transmission in *cis* or *cis*-transmission through genuine infection via DC as shown in **Figure 3**.

THE ROLE OF TETHERIN IN HIV TRANSMISSION: IMMUNOLOGICAL SYNAPSE VERSUS VIROLOGICAL SYNAPSE

Various intrinsic antiviral mechanisms or factors have evolved in eukaryotes to fight against virus invasion (see review by Yan and Chen, 2012). To countermeasure these intrinsic cellular factors (restriction factors), HIV-1 evolved to encode several accessory proteins, such as Vpu, Vif, Vpr, and Vpx. BST-2/CD317/tetherin was recently identified as one such cellular factor, antagonized by HIV-1 Vpu (Neil et al., 2008; Van Damme et al., 2008). Normally, BST-2/tetherin inhibits virion release by anchoring virus at the cell membrane, but Vpu can directly bind to BST-2/tetherin, internalizing and degrading it, probably in the endolysosomal compartment (Arias et al., 2012).

It has been known for some time that vpu mutant virus can be efficiently spread by T cell to T cell transfer (Gummuluru et al., 2000) in a rapid turnover HIV-1 replication culture. As such, this observation raises the important question of whether Vpu affects cell-to-cell spread of HIV-1 by inhibiting the membrane tethering function of BST-2/tetherin? This issue was addressed under the setting of a VS, using HIV-infected cells directly contacting with an uninfected target cell system. Jolly et al. (2010) found that Vpu-defective HIV-1 disseminates more efficiently through cell-to-cell contact, despite the premise that BST-2/tetherin inhibits cell-free virion release, which corroborates with a previous report by Gummuluru et al. (2000), in which the authors speculated that BST-2/tetherin either mediates the accumulation of virions or regulation of VS integrity. On the other hand, Casartelli et al. (2010) studied virus transfer to T cells (targets) from HeLa or 293T cells, expressing a high level of BST-2/tetherin either naturally or by transfection (donors). They observed that BST-2/tetherin does not prevent VS formation, but assists the accumulation of Gag at the contact zone in the absence of Vpu (Casartelli et al., 2010). Important to note, however, is since the authors used mostly vesicular stomatitis virus (VSV)-pseudotyped HIV-1 for the infection of donors, their system is a transient infection event without new infectious virus production and reflect a cell-to-cell *trans*-infection as discussed in a previous section. Regardless, in both studies (Casartelli et al., 2010; Jolly et al., 2010), the accumulation of HIV-1 virions, in the presence of a high level of tetherin expression was observed.

Alternately, Kuhl et al. (2011) reported that a Vpu-mediated effect in viral spread among $CD4^+$ T cells is independent of

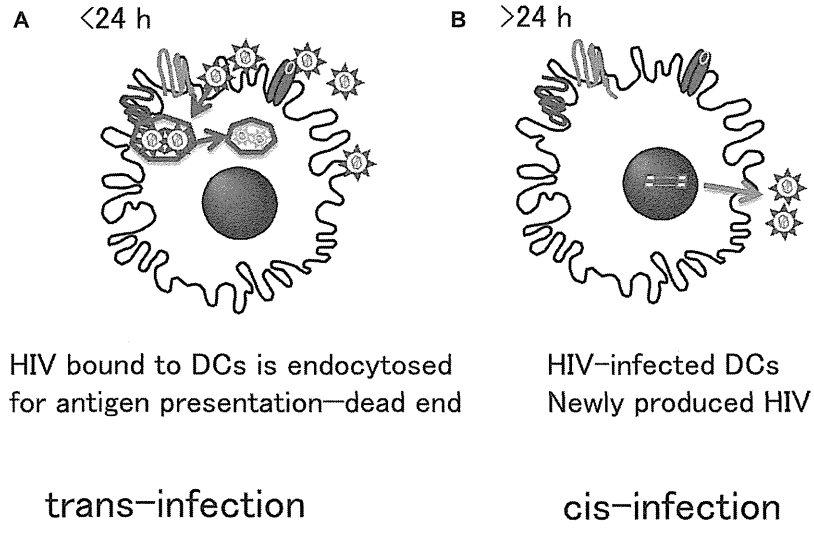

FIGURE 3 | Distinct HIV transmission mode by DCs: *Cis*- and *Trans*-infection. There are two modes of transmission of HIV from DC to T cells. **(A)** *Trans*-infection: DCs express various receptors to bind foreign antigens, which is important to efficiently endocytose and process for antigen presentation. Such machinery is highlighted for HIV transfer *in trans* in *in vitro* experiments. However, because endocytosed antigens are quickly degraded within 24 h (Turville et al., 2004), infectious HIV particles may not retain their infectivity for a long time. Thus, the physiological relevancy of *trans*- infection *in vivo* remains unclear. **(B)** *Cis*-infection: On the other hand, because DC express CD4 and chemokine receptors essential for HIV infection, DC are naturally susceptible to HIV infection. Although HIV replication is limited in DC, their strong antigen-presenting activity allows HIV to replicate more in antigen-specific $CD4^+$ T cells tightly interacting with DC. This DC–T cell interaction is a genuine immunological synapse (IS). The precise molecular mechanism of HIV transmission from DC to T cells has yet to be clarified, but the *cis*-infection mode, is what most likely occurs *in vivo*.

the extent of Vpu-mediated BST-2/tetherin cell surface down-modulation. They postulated the presence of an additional Vpu-independent mechanism for BST-2/tetherin cell surface down-modulation following HIV-1 infection in T cell lines, which is consistent with a report by Miyagi et al. (2009).

Vpu interacts with BST-2/tetherin to promote virion release, whereas BST-2/tetherin normally causes accumulation of virus particle on the membrane surface. However, the efficiency of cell-to-cell HIV-1 transfer or transmission appears largely dependent on experimental conditions. As previously described, DC–T cell contact is not equal to the VS form of contact. Therefore, we should perhaps consider the physiological function of BST-2/tetherin, under the setting of an antigen-mediated IS instead.

In this context, Coleman et al. (2011) reported that immature MDDC does not express BST-2/tetherin, however, after HIV-1 infection, BST-2/tetherin expression was upregulated by HIV-1 Nef. Therefore, HIV-1 transmission from DC to T cells does not appear to be restricted by BST-2/tetherin (Coleman et al., 2011). However, because their target is the HUT/CCR5 T cell line and culture conditions favor *trans* infection (immediate co-culture after HIV-1 infection), the question of whether cell surface expression of BST-2/tetherin assists or inhibits virus transmission to $CD4^+$T cells via IS needs to be clarified.

RESTRICTIONS IN DCs

The poor replication of HIV in DC is partly explained by a block during virus fusion with MDDC (Cavrois et al., 2006). The restriction of HIV-1 infection in DC can also occur at a post-entry level. Several cell factors known to interfere with HIV-1 infection and/or replication step in DC are illustrated in **Figure 4**. APOBEC3 was originally discovered as a potent intrinsic antiviral factor interacting with HIV-1 Vif (Sheehy et al., 2002). APOBEC3G (A3G) is a member of the cytidine deaminase family, which edits C to U in a single stranded HIV DNA, causing G-to-A hyper mutation of the HIV-1 genome. HIV-1 Vif counteracts this deaminase function by inhibiting A3G incorporation into virions and promoting A3G degradation by ubiquitination (Sheehy et al., 2003).

Myeloid cells differentiate from monocytes to either macrophages or immature MDDC, and susceptibility to HIV-1 infection amongst these cell types has been inversely correlated to

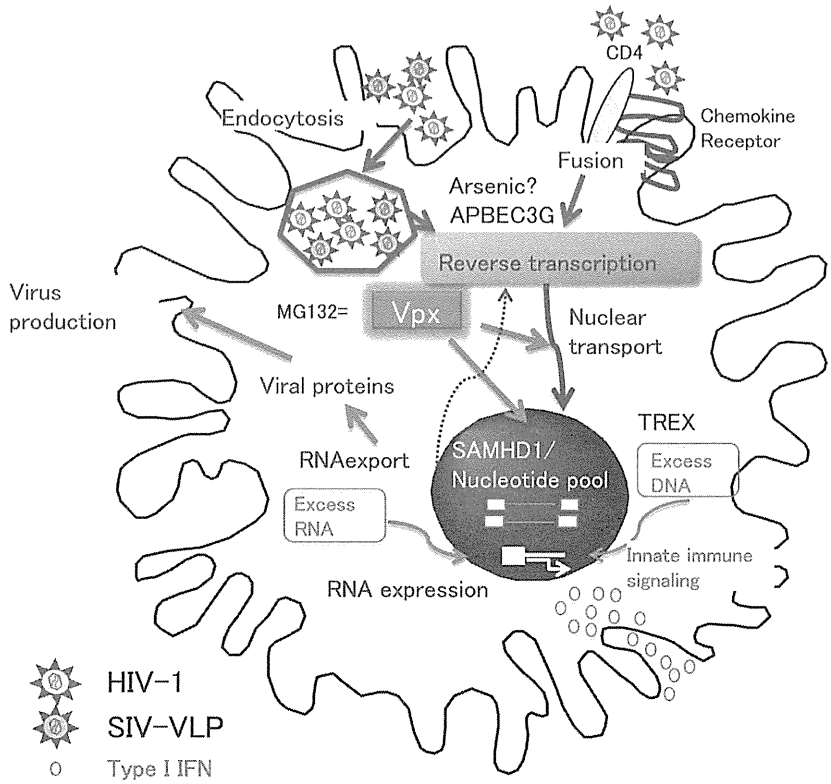

FIGURE 4 | DC restriction and type I IFN response. HIV-1 utilizes CD4 and chemokine receptors for entry. The restriction of cDC to HIV-1 infection occurs at the entry (fusion) and post-entry level. Of note, in order to investigate the step at the post-entry level, VSV-pseudotyped virus is frequently utilized, which enters cells by endocytosis. After entry, APOBEC3G and arsenic sensitive factor inhibit reverse transcription. However, Vpx can counteract this restriction in DC by either degrading (1) SAMHD1-nucleotide pool-reverse transcription pathway or (2) nuclear transport. In addition, because the proteasome inhibitor MG132 works in a similar fashion to Vpx, it is possible that Vpx interferes with the proteasomal degradation step of endocytosed virions by degrading DC factors other than SAMHD1. Whatever the case, Vpx causes the accumulation of numerous virus products in the cytosol (red arrows). Importantly, the accumulation of viral DNA and RNA may trigger the innate immune signaling, resulting in type I IFN production, followed by the maturation of DC.

A3G expression, with highest A3G expression and lowest HIV-1 susceptibility in monocytes (Peng et al., 2007). In MDDC, A3G and APOBEC3F were shown to restrict HIV-1 infection at a post-entry step by Pion et al. (2006) and can be mainly ascribed to the deaminase-independent A3G function that inhibits reverse transcription (Bishop et al., 2008). However, Pion et al. (2006) also observed that arsenic trioxide enhanced the reverse transcription of HIV-1 in MDDC and $CD1c^+$ blood cDC in an A3G-independent manner (Pion et al., 2007). Although the mechanism of arsenic in DC is currently unknown, these results suggest that cellular factors, other than APOBEC3, are definitely playing important roles in the restriction of HIV-1 replication, most likely at a reverse transcription step, in DC.

Coincidentally, a novel DC restriction factor was discovered from a study on Vpx. SIV virus like particles (VLPs) containing Vpx are known to enhance the expression of VSV-G-pseudotyped lentivirus in DC (Santini et al., 2000), and this finding lead to the identification of SAMHD1, a myeloid cell restriction factor (Caminschi et al., 2008; Hrecka et al., 2011). SAMHD1 is a potent triphosphohydrolase that converts deoxynucleoside triphosphates (dNTPs) to deoxynucleoside and triphosphate (Goldstone et al., 2011). Therefore, the current working hypothesis for the relationship between Vpx and SAMHD1 is that virus containing Vpx can replicate well in macrophages by degrading SAMHD1, which would otherwise reduce the dNTP pool and inhibit reverse transcription (Lahouassa et al., 2012). In SIV_{SM} or HIV-2 infection, Vpx was shown to be essential for viral replication and critical for reverse transcription of the viral RNA genome in macrophages (Fujita et al., 2008). However, a lack of Vpx in HIV-1 has negligible consequence with HIV-1, which can substantially replicate in macrophages. Therefore, Fujita et al. (2012) hypothesized that because of the lower activity of reverse transcriptase in HIV-2 versus HIV-1, HIV-2 had evolved to carry Vpx for compensation (Fujita et al., 2012).

However, it should be mentioned that immature MDDC do not support HIV-1 replication, in a similar manner, as macrophages do in R5-type HIV-1 infection (Tsunetsugu-Yokota et al., 1995) and that the susceptibility of HIV-2 containing native Vpx is even lower in MDDC and blood cDC (Smith et al., 2007). Furthermore, although remarkable Vpx-induced enhancement is observed in the case of VSV-pseudotyped lentivirus infection, it is not so obvious when native HIV was co-infected with SIV-VLP (Manel et al., 2010). Because it is known that the way virus enters via HIV envelope and/or VSV glycoprotein can lead to distinct outcomes in $CD4^+$ T cells (Yu et al., 2009), the HIV envelope and its signaling in DC may contribute to different effects of Vpx.

There is no convincing explanation as to why the DC restriction, common to natural HIV-1 and HIV-2 infections, would necessarily be mediated by Vpx causing SAMHD1 degradation *per se*. Considering that the proteasome inhibitor, MG132, exerts similar effects with Vpx (Groot et al., 2006), we speculate that Vpx delivered into endosomes may inhibit endosomal degradation of endocytosed VSV-based lentivirus vector, which results in the release of numerous viral particles into the cytosol followed by production of a high copy number of RT products, thereby enhancing all subsequent steps of HIV-1 infection (integration, RNA synthesis, nuclear export, etc.). This scenario still needs to be proven and validated. Determination of whether Vpx induces the degradation of DC factors, other than SAMHD1, will prove to be an interesting future endeavor.

Vpx AND TYPE I IFN

Another interesting feature of Vpx is that SIV-VLP containing Vpx induces a high level of Type I IFN in DC (Manel et al., 2010). Genetic diseases lacking SAMHD1 and TREX, a recently identified 3′-exonuclease which can suppress excess DNA accumulation in the cell (Yan et al., 2010), are known to develop similar autoimmune diseases due to a high level of type I IFN production (Lim and Emerman, 2011), indicating that these two distinct cellular proteins are important to regulate type I IFN responses. Thus, it is safe to assume that SAMHD1 degradation by Vpx can contribute to the induction of a high level of type I IFN production in DC. However, because Vpx enhances intracellular virus replication events as described in the previous section, excess accumulation of proviral DNA, as well as viral RNA, will occur, which might act as triggers for innate immune signaling in DC as depicted in **Figure 4**.

In contrast to other RNA viruses such as measles and influenza infection (Zilliox et al., 2006), HIV-1 infection, and other retroviruses also, do not induce type I IFN responses in DC (Luban, 2012). As regards to the mechanism, IRF-3, a pathogen-sensing pathway component, was shown to be depleted in HIV-infected T cells (Doehle et al., 2009) but not in DC, whereas Vpr-dependent inhibition of IRF-3 nuclear translocation was reported to occur in DC (Harman et al., 2011). The stimulation of DNA- and/or RNA-sensing pathway by Vpx may overcome this Vpr effect in DCs.

Finally, it is well-known that type I IFN is one of a multitude of cytokines which can induce the maturation of DC (Santini et al., 2000), and the maturation of MDDC has been shown to block HIV-1 infection at a post-entry level (Dong et al., 2007). However, in pDC, type I IFN production is induced by HIV-1 infection, as with other virus infections, resulting in inhibition on HIV-1 replication (Groot et al., 2006). Therefore, the lack of innate immune responses in cDC will be compensated *in vivo* in the early phase of HIV infection. Surprisingly, Schlafen 11 (SLFN11), a molecule induced by type I IFN, was shown to inhibit the translation of HIV-1 based on its virus-specific codon usage (Li et al., 2012). This finding indicates that there are still many undiscovered factors related to type I IFN signaling in DC which may be exploited to fight against retrovirus infections. Alternately, from the HIV side of things, by counteracting these restriction factors, HIV is able to establish a low level infection in DC. However, once type I IFN is induced in DC, it will help to generate more potent antigen-presenting DC, as demonstrated by Manel et al. (2010). Vaccine strategies utilizing Vpx for the enhancement of antigen presenting cell (APC) function in DC have already began and look promising (Durand et al., 2013).

CONCLUSION

We now recognize that the majority of recently identified cellular factors interacting with HIV accessory proteins are related to the type I IFN or innate immune response. The remarkable antiviral activity of type I IFN has been well-known for a long time, but we

are just beginning to understand its precise mechanism, which is not necessarily common to all cell types, especially in DC. It is has been postulated that HIV has evolved to escape from potent antigen presenting activity of DC, and took advantage of subverting within DC with a minimum level of replication, for easy transmission of virus to T cells, during the T cell activation process. As described in this review, our knowledge concerning the biology of various subsets of DC has advanced enormously. However, we need to further apply this basic knowledge and understanding to manipulate DC by placing them in crucial sites (tissues), within proper time frames, for the development ofa protective acquired immunodeficiency syndrome (AIDS) vaccine.

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Sensitive detection of measles virus infection in the blood and tissues of humanized mouse by one-step quantitative RT-PCR

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Live attenuated measles virus (MV) has long been recognized as a safe and effective vaccine, and it has served as the basis for development of various MV-based vaccines. However, because MV is a human-tropic virus, the evaluation of MV-based vaccines has been hampered by the lack of a small-animal model. The humanized mouse, a recently developed system in which an immunodeficient mouse is transplanted with human fetal tissues or hematopoietic stem cells, may represent a suitable model. Here, we developed a sensitive one-step quantitative reverse transcription (qRT)-PCR that simultaneously measures nucleocapsid (N) and human RNase P mRNA levels. The results can be used to monitor MV infection in a humanized mouse model. Using this method, we elucidated the replication kinetics of MV expressing enhanced green fluorescent protein both *in vitro* and in humanized mice in parallel with flow-cytometric analysis. Because our qRT-PCR system was sensitive enough to detect MV expression using RNA extracted from a small number of cells, it can be used to monitor MV infection in humanized mice by sequential blood sampling.

Keywords: measles virus infection, humanized mouse, quantitative RT-PCR, EGFP expression, flow cytometry

INTRODUCTION

Measles, a highly contagious childhood disease caused by the measles virus (MV), affects more than 20 million people each year. MV infection is characterized by a high fever with typical Koplik's spots followed by the appearance of a generalized maculopapular rash, and is often associated with respiratory and neuronal complications (Griffin, 2007). Since the implementation of vaccination programs using an effective live attenuated MV vaccine, global measles deaths have decreased dramatically. Nevertheless, measles is still one of the leading causes of death among young children under the age of 5 years, especially in countries with weak health infrastructures, and approximately 158,000 measles death occurred in 2011 (http://www.who.int/mediacentre/factsheets/fs286/en/). The ongoing global vaccination strategy aims to protect small children at high risk.

The MV vaccine is safe, effective, and inexpensive. Based on its long and successful vaccination history, several groups have taken advantage of reverse-genetics technology to utilize the live attenuated MV vaccine strain as a viral vector to elicit immune responses against foreign antigens from various pathogens, such as Env or Gag of human immunodeficiency virus (HIV; Lorin et al., 2004; Stebbings et al., 2012), hepatitis B surface (S) antigen (Singh et al., 1999; Reyes-del Valle et al., 2009), fusion protein of respiratory syncytial virus (Sawada et al., 2011), and envelope glycoprotein of West Nile virus (Despres et al., 2005; Brandler et al., 2012). MV is a human-tropic virus that uses CD46, signaling of lymphocyte activation molecule (SLAM, CD150), and the recently identified epithelial-cell receptor nectin-4 (PVRL4, see review in Kato et al., 2012) as receptors. To test the immune response against MV-based recombinant vaccines, both MV receptor-transgenic mice (Singh et al., 1999; Lorin et al., 2004; Despres et al., 2005) and non-human primates have been used as animal models (Reyes-del Valle et al., 2009; Brandler et al., 2012; Stebbings et al., 2012).

Although non-human primates are susceptible to MV, and they develop pathologies similar to those that occur in humans, the expense of using monkeys in research limits the number of animals that can be used for studies. To overcome such practical problems, various types of human MV receptor-transgenic mice expressing CD46 or CD150 have been developed (review in

Sellin and Horvat, 2009). Unfortunately, MV infection of all of these human MV receptor-expressing mouse models is severely restricted by the presence of murine type I IFN; to establish MV infection, it is necessary to introduce the IFNα receptor knockout into the MV receptor-transgenic mice, even in strains expressing CD150 driven by a native human promoter (Ohno et al., 2007). The IFNα receptor knockout/CD150 knock-in mouse is highly susceptible to MV infection and reproduces some aspects of MV infection in humans, including immunosuppression (Koga et al., 2010). This makes it a useful mouse model for study purposes. However, one problem is the lack of an initial innate immune response, which may modify the outcome of MV infection. Thus, the model may not truly reflect the outcome in humans.

In the early 2000s, a series of immunodeficient mice were developed that allow efficient transplantation of human cells or tissues; these systems are collectively termed "humanized mice." A large number of studies have described the development of human hematopoietic cells and their immunological functions in humanized mice, and technical modifications have been made for the study of various human diseases (Ito et al., 2012). Currently, humanized mouse systems are widely used as alternatives to non-human primate models, especially for the study of human-tropic infectious diseases such as HIV, human T cell leukemia virus (HTLV), dengue virus, HCV, and EB virus (Akkina, 2013). Of the different humanized mice models, the BM/Liver/Thymus transplanted (BLT) mouse, which is transplanted with human fetal liver and thymus tissue in addition to hematopoietic stem cells (HSCs), is recognized as the model that most closely mimics the human immune response (Wege et al., 2008). However, the use of this model is limited, mainly because of the ethical issues surrounding human fetal organs/tissues.

We have recently established an HIV infection model in NOD/SCID/Jak3null (NOJ) mouse transplanted with human cord blood HSCs (Terahara et al., 2013). To study MV infection in humanized NOJ (hNOJ), we infected an MV vaccine strain (AIK-C) expressing enhanced green fluorescent protein (EGFP) into hNOJ and analyzed the MV-infected cells by flow cytometry. The hNOJ mouse is highly susceptible to MV infection; in that study, we observed that GFP^+ cells were present in systemic lymphoid tissues and bone marrow (BM). Because it is important to assess MV infection kinetics in an animal without sacrificing the infected mouse, we developed a highly sensitive one-step quantitative reverse transcription-PCR (qRT-PCR) system to monitor MV infection in human peripheral blood mononuclear cells (PBMCs) circulating in the blood of humanized mice. In this study, we describe how this monitoring system works and demonstrate that the results obtained reflect the actual frequency of MV-infected cells, as determined by flow cytometry.

MATERIALS AND METHODS

CELL FRACTIONATION OF PBMCs

Peripheral blood mononuclear cells were obtained from human blood samples of healthy volunteers. Samples were collected after obtaining the approval of the institutional ethical committee of the National Institute of Infectious Diseases (NIID; No. 350) and written informed consent from each subject. PBMCs were separated by Ficoll–Hypaque density-gradient centrifugation (Lymphosepal; IBL, Gunma, Japan).

To obtain monocyte-derived dendritic cells (MDDCs), monocytes were enriched from PBMCs using CD14 microbeads (Miltenyi Biotec) and cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, and antibiotics in the presence of interleukin-4 (IL-4) and granulocyte–macrophage colony-stimulating factor (GM-CSF; both 10 ng/ml, from PeproTech Inc., London, UK) for 1 week. T cells were isolated from CD14-negative PBMCs using the Total T Cell Enrichment Kit (STEMCELL technologies, Vancouver, BC, Canada).

PREPARATION OF RNA

Total RNA was extracted from mouse blood, BM, and spleen of humanized mice, human PBMCs, and Jurkat cells expressing human SLAM (Jurkat/hSLAM) using the RNeasy Mini Kit (QIAGEN, Valencia, CA, USA) or the Total RNA Isolation Mini Kit (Agilent Technologies, Santa Clara, CA, USA).

To prepare a standard of MV RNA, the cDNA encoding measles virus nucleocapsid (N) (MV-N: AB052821) was subcloned into the pBluescript II vector, and then MV-N RNA was produced by *in vitro* RNA transcription using the T7 RiboMAX^{TM} Express Large Scale RNA Production System (Promega, Madison, WI, USA). The RNA product was purified by DNase treatment, followed by phenol–chloroform extraction and ethanol precipitation, according to the protocol supplied by the manufacturer. The final concentration of RNA was measured using an ND-1000 spectrophotometer (Thermo, Waltham, MA, USA).

PREPARATION OF STANDARD TEMPLATE DNA

To prepare a standard template DNA, cDNAs of human CD45 (hCD45: NG_007730) and RNase P (NM_006413) were synthesized from total RNA of CEM cells by reverse transcription (RT)-PCR using SuperScript III RT/Platinum Taq Mix (Invitrogen, Carlsbad, CA, USA). The products were further amplified by PCR using TaKaRa Ex Taq Hot Start Version (TAKARA, Otsu, Shiga, Japan) for hCD45, or AmpliTaq Gold 360 (Applied Biosystems, Carlsbad, CA, USA) for RNase P. These PCR products of hCD45 and RNase P were subcloned into plasmids using the pGeneBLAzer TOPO TA Expression kit (Invitrogen) and pGEM-T (Easy) Vector Systems (Promega), respectively.

REAL-TIME RT-PCR ASSAY

To perform real-time qRT-PCR, SuperScript III Platinum One-Step Quantitative RT-PCR system (Invitrogen) was used according to the manufacturer's instructions. Briefly, each reaction contained 1× reaction mix, ROX reference dye, SuperScript III RT/Platinum TaqMix, 0.2 μM specific primers, and 0.1 μM TaqMan probe. Reactions were performed on an Mx3000P qPCR system (Agilent Technologies). Thermocycling parameters included a RT step at 50°C for 20 min, followed by a DNA polymerase activation step at 95°C for 2 min and 50 PCR cycles (95°C for 20 s, 60°C for 30 s). Threshold cycle (C_t) values were calculated for each reaction; C_t represents the cycle at which a statistically significant increase in the emission intensity of the reporter relative to the passive reference dye is first detected.

For detection of hCD45 mRNA, the following sequences were used: forward primer, 5′-GGA AGT GCT GCA ATG TGT CAT T-3′; reverse primer; 5′-CTT GAC ATG CAT ACT ATT ATC TGA TGT CA-3′; TaqMan probe; 5′-FAM-ACA ACT AAA AGT GCT CCT CCA AGC CAG GTC T-BHQ1-3′ (Hamaia et al., 2001). For detection of RNase P mRNA: forward primer, 5′-AGA TTT GGA CCT GCG AGC G-3′; reverse primer, 5′-GAG CGG CTG TCT CCA CAA GT-3′; TaqMan probe, 5′-FAM-TTC TGA CCT GAA GGC TCT GCG CG-BHQ1-3′ (Kimberly et al., 2005). For detection of MV-N RNA: forward primer, 5′-CGA TGA CCC TGA CGT TAG CA-3′; reverse primer, 5′-GCG AAG GTA AGG CCA GAT TG-3′; TaqMan probe, 5′-FAM-AGG CTG TTA GAG GTT GTC CAG AGT GAC CAG-BHQ1-3′ (Hummel et al., 2006).

GENERATION OF HUMANIZED MICE

Humanized NOD/SCID/JAK3null mice were established as described previously (Terahara et al., 2013). In brief, NOJ mice were transplanted with human HSCs ($0.5–1 \times 10^5$ cells) enriched from human umbilical cord blood cells into the livers of irradiated (1 Gy) newborn mice within 2 days after birth. All mice were maintained under specific pathogen-free conditions in the animal facility at NIID and were treated in accordance with the guidelines issued by the Institutional Animal Care and Committee of NIID.

Human umbilical cord blood was donated by the Tokyo Cord Blood Bank (Tokyo, Japan) after obtaining informed consent. The use of human umbilical cord blood cells was approved by the Institutional Ethical Committees of NIID and the Tokyo Cord Blood Bank. Human HSCs were isolated using the CD133 MicroBeads Kit (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity was approximately 90% as assessed by flow cytometry.

PREPARATION AND INFECTION OF MV

Recombinant wild-type MV (IC323: AB016162) expressing EGFP (IC323-EGFP; Hashimoto et al., 2002) and a recombinant vaccine strain of MV (AIK-C: S58435) expressing EGFP (AIK-C-EGFP; Fujino et al., 2007) were grown in Vero/hSLAM cells. Virus titers were determined by plaque assay using Vero/hSLAM cells.

Jurkat/hSLAM cells were infected with various doses of MV [multiplicity of infection (MOI) = 0.25, 0.05, and 0.01] by incubation at 37°C for 1 h, washed twice with phosphate buffered saline (PBS), and seeded on 24-well plates. Cells were harvested immediately after washing (time 0) or 6, 12, 18, or 24 h later. The harvested cells were either lysed for RNA extraction or analyzed by flow cytometry.

Humanized NOD/SCID/JAK3null mice were challenged intravenously (i.v.) with different doses [200, 2,000, 10,000, or 20,000 plaque-forming units (pfu)] of AIK-C-EGFP. Peripheral blood was obtained from MV-infected hNOJ mice at 3, 5, 7, 10, 14, and 21 days post-infection (p.i.). In some experiments, MV-infected hNOJ mice were sacrificed at day 7 p.i. At the time of sacrifice, peripheral blood, BM, spleen, and mesenteric lymph nodes (MLNs) were harvested, and red blood cells were lysed in ACK buffer (0.15 M NH_4Cl, 1 mM $KHCO_3$, and 0.1 mM EDTA-2Na; pH 7.2–7.4).

FLOW-CYTOMETRIC ANALYSIS OF MV-INFECTED CELLS

PE-conjugated anti-human CD150 (A12) and Pacific Blue-conjugated anti-hCD45 (HI30) monoclonal antibodies (mAbs) were purchased from BioLegend Inc. (San Diego, CA, USA). Cells were stained with these mAbs, fixed with 2% formalin/PBS for 15 min at room temperature, washed, and kept at 4°C prior to flow-cytometric analysis. Dead cells were stained with a LIVE/DEAD Fixable Dead Cell Stain Kit (L34957; Invitrogen). Data were collected using a FACScanto (BD Biosciences, San Jose, CA, USA) and analyzed using the FACSDiva (BD Biosciences) or FlowJo (Tree Star, San Carlos, CA, USA) software.

STATISTICAL ANALYSIS

Non-parametric one-way ANOVA was performed to compare cell type-specific differences in hCD45 and RNase P mRNA expression. Spearman's rank correlation coefficient test was also performed to compare the level of MV-N expression and frequency of MV-infected cells. Prism ver.5 software (GraphPad Software, San Diego, CA, USA) was used for all analyses. $P < 0.05$ was considered statistically significant.

RESULTS

HUMAN-SPECIFIC qRT-PCR SYSTEM FOR THE DETECTION OF MV INFECTION

For the detection of MV infection in clinical specimens, Hummel et al. (2006) established a sensitive qRT-PCR system that used primer and probe sets targeting the MV-N gene. In our humanized mouse model, it is necessary to analyze endogenous mRNA expression in human PBMCs to determine the level of human cell-associated MV infection in mouse blood. We initially assumed that hCD45 expression would be suitable to discriminate human hematopoietic cells from co-existing mouse hematopoietic cells *in vivo*. On that basis, we designed human-specific primer and TaqMan probe sets for hCD45 and compared their usefulness with a primer/probe set for a widely used housekeeping gene, RNase P. RNA was extracted from humanized (hu-mouse) or non-humanized (non-hu-mouse) murine splenocytes, and the level of mRNA was measured by one-step qRT-PCR. Both hCD45 and RNase P primer/probe sets detected mRNA expression of target genes from human PBMCs present in hu-mouse spleen, at similar sensitivities, but neither set detected expression in non-hu-mouse (**Figure 1A**). Thus, both primer/probe sets are human-specific. Next, we enriched $CD14^+$ monocytes and T cells from PBMCs by positive and negative magnetic-bead selection, respectively, and then determined the copy numbers of hCD45 and RNase P in these cell fractions from each of five donors. In **Figure 1B**, the expression levels of hCD45 (left panel) and RNase P (right panel) in monocytes and T cells are depicted relative to the level in each donor's PBMCs. Because RNase P expression was less affected by cell type than CD45 expression ($^*P < 0.05$), in subsequent experiments we exclusively used RNase P primer/probe sets as an endogenous control for mRNA expression.

PARALLEL INCREASE IN THE TIME COURSE OF MV-INFECTED CELL FREQUENCY AND MV-N RNA LEVEL *IN VITRO*

Because wild-type MV mainly utilizes SLAM as the receptor for entry into lymphoid cells (Tatsuo et al., 2000), the kinetics of MV

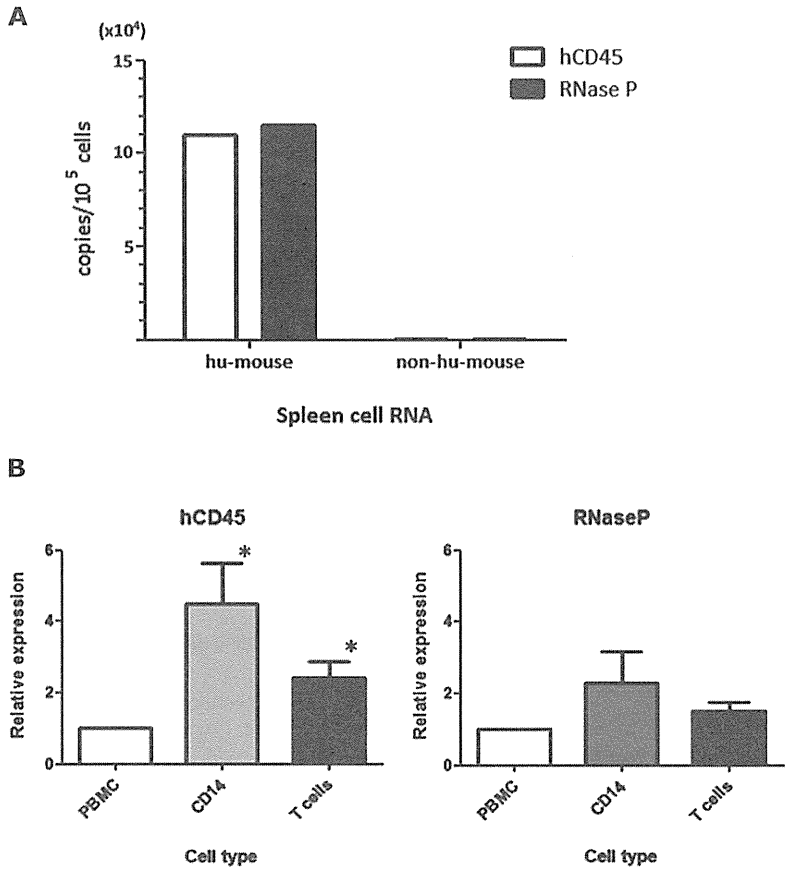

FIGURE 1 | Selection of an endogenous control for the analysis of MV-infected human PBMCs. (A) RNA was extracted from spleen cells of hNOJ and non-humanized NOJ, and one-step qRT-PCR was performed using primer and probe sets designed against the human-specific hCD45 and RNaseP mRNAs. To calculate copy numbers of these genes, the PCR products of human CD45 and RNase P were subcloned into plasmids and used as standard DNAs. **(B)** Human PBMCs from five donors were fractionated into $CD14^+$ monocytes and T cells. RNA from these cell populations was extracted, and the expression levels of hCD45 and RNase P were analyzed by qRT-PCR. The graph depicts the expression levels in these fractionated cells relative to the levels in PBMCs (defined as 1). Statistical differences in hCD45 and RNase P expression among these cell populations were evaluated by non-parametric one-way ANOVA test ($*P<0.05$).

infection in Jurkat/hSLAM cells can be clearly visualized by flow cytometry. We infected Jurkat/hSLAM cells with a wild-type MV encoding EGFP (IC323-EGFP) at MOI of 0.01, 0.05, and 0.25. Cells were washed and harvested at 6, 12, 18, or 24 h after MV infection. A subset of the cells in each sample was analyzed by flow cytometry, and the remainder of the sample was used for RNA extraction. The mRNA levels of MV-N and RNase P were determined by qRT-PCR, and the level of MV-N mRNA relative to RNase P RNA was calculated. Representative results of three experiments are shown in **Figure 2A** (flow cytometry) and **Figure 2B** (qRT-PCR). Because of the rapid and strong cytopathic effect by MV at the highest MOI (0.25), we omitted the flow cytometry data corresponding to that condition. At MOI 0.01, a similar frequency of GFP^+ cells was detectable at 12 and 18 h p.i., whereas at MOI 0.05, the GFP^+ cell frequency was already high at 12 h p.i. Note that the level of hSLAM was not down-modulated by MV infection. Over the time course, relative MV-N expression level at all three MOIs increased in parallel over two orders of magnitude, indicating that these two methods yield comparable results (as shown in **Figure 2C**) and are useful for monitoring the replication kinetics of MV infection *in vitro*.

PARALLEL INCREASE OF MV-INFECTED CELL FREQUENCY AND MV-N RNA LEVELS *IN VIVO*

We then applied these detection systems *in vivo* in MV-infected hNOJ mice. hNOJ mice were infected with an MV vaccine strain expressing EGFP (AIK-C-EGFP) at 2000 pfu, and the animals were sacrificed 7 days later. Blood PBMCs and BM cells were washed with PBS, and a subset of the cells in each sample were stained with anti-hCD45 mAb. Representative results of flow-cytometric analysis of BM cells from three mice are shown in **Figure 3A**. The percentages of GFP^+ cells in mice 127-1, 127-4, and 127-5 mice were low (0.002%), high (0.35%), and intermediate (0.028%), respectively. The number of human PBMCs obtained from mouse blood was not sufficient to determine GFP^+

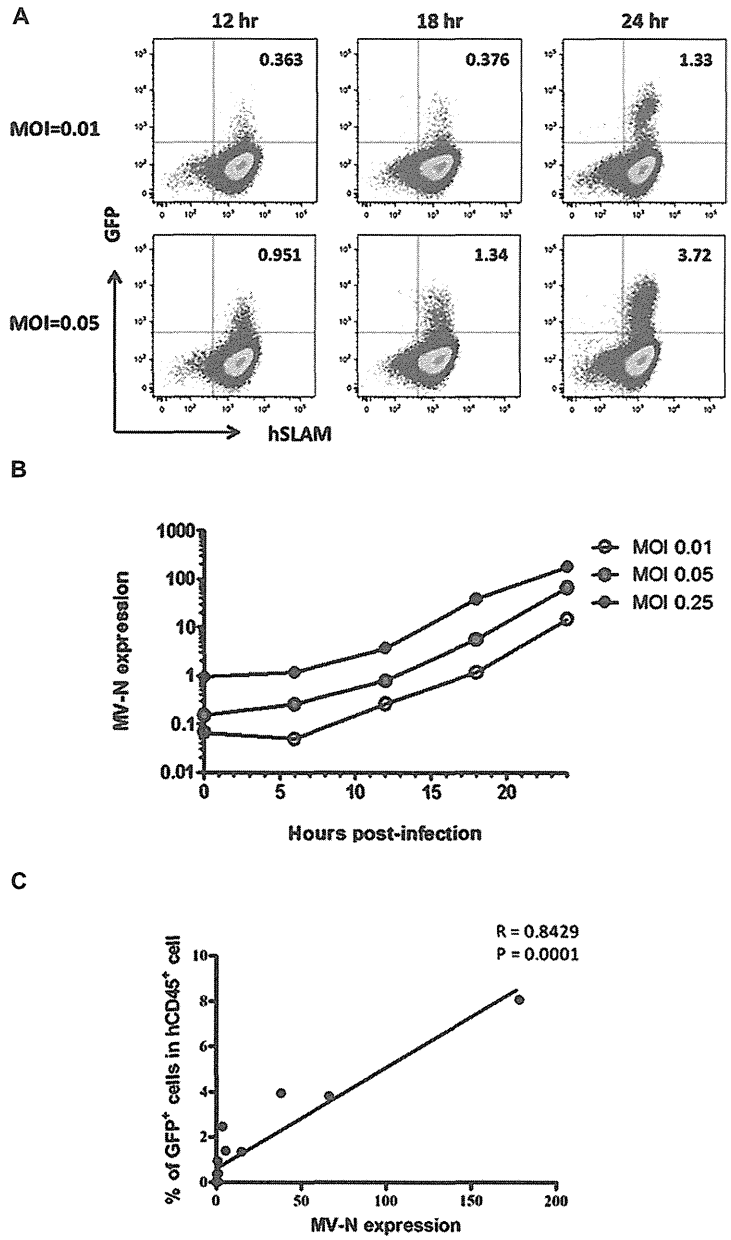

FIGURE 2 | Time course of MV infection *in vitro*. Jurkat/hSLAM cells were infected with wild-type MV IC323-EGFP at MOI of 0.01, 0.05, and 0.25, washed, and harvested at the indicated time points. **(A)** Cells were stained with PE-conjugated anti-hSLAM mAb, fixed with 2% formalin/PBS, and GFP expression was analyzed. **(B)** RNA was extracted from cells, and expression levels of MV-N and RNase P were analyzed by one-step qRT-PCR. The copy numbers of MV-N and RNase P were determined, and the ratio of MV-N copies to RNase P copies is depicted on the vertical axis. **(C)** Correlation between the percentage of GFP^+ Jurkat/SLAM cells and the time course of MV-N expression. Spearman's rank correlation coefficient was used for statistical analysis.

cell frequencies by flow cytometry. Next, we extracted RNA from PBMCs and BM cells and analyzed MV-N expression by qRT-PCR, as described in the previous section. MV-N expression paralleled the GFP^+ frequencies in BM (**Figure 3B**). Notably, a high level of MV-N expression was also detected in PBMCs of mouse 127-4, suggesting that the level of MV-N expression per single hematopoietic cell is similar between blood and BM. We plotted the GFP^+ frequency and MV-N expression level in BM cells of eight mice. As shown in **Figure 3C**, these values were well correlated ($R = 0.9286$). Taken together, these data indicate that MV infection *in vivo* is detectable in BM by both flow cytometry and MV-N RNA qRT-PCR analysis, but only MV-N RNA qRT-PCR is

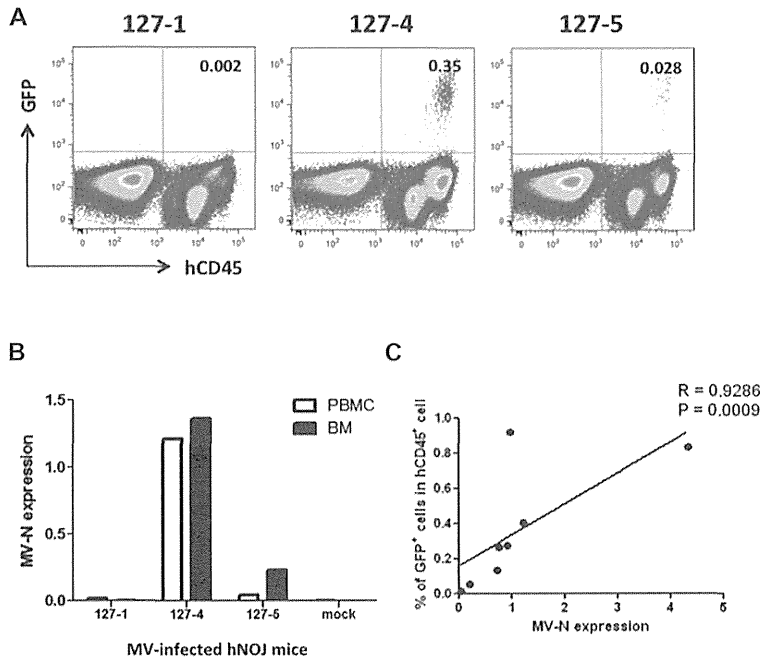

FIGURE 3 | Analysis of MV infection *in vivo*. Three hNOJ mice (127-1, -4, and -5) were infected intravenously with 2,000 pfu of the MV vaccine strain, AIK-C-EGFP. Mice were sacrificed at day 7 post-infection, and blood and bone marrow cells (BM) were obtained. **(A)** BM cells were stained with PB-anti-human CD45 mAb, fixed with 2% formalin/PBS, and GFP expression was analyzed. **(B)** PBMCs from blood and BM cells were lysed, and RNA was prepared. The expression MV-N and RNase P was analyzed as described in the legend for **Figure 2B**. **(C)** Correlation between the percentage of GFP^+ cells among $hCD45^+$ cells in BM and the level of MV-N expression in MV-infected hNOJ mice, at day 7 ($n = 4$) or day 10 ($n = 4$) p.i. Spearman's rank correlation coefficient was used for statistical analysis.

sensitive enough to detect PBMC-associated MV infection in the blood.

KINETICS OF MV GROWTH CAN BE MONITORED IN THE BLOOD OF hNOJ MOUSE

Finally, we measured MV growth kinetics *in vivo* by qRT-PCR analysis using sequential blood samples obtained from MV-infected hNOJ mice; it was not feasible to perform these measurements by flow cytometry because of the paucity of human PBMCs in the blood. Two or three hNOJ mice in each group were infected intravenously with 200, 2000, or 20,000 pfu AIK-C-EGFP and followed up to 21 days p.i. The level of PBMC-associated MV RNA in individual mice is shown in **Figure 4A**. We noticed two peaks of MV replication, the first at around day 3 p.i., and the second at day 10 p.i., irrespective of the initial inoculum. Two mice infected with 20,000 pfu MV exhibited a high level of MV replication that peaked at day 10 p.i. One mouse infected with 2,000 pfu exhibited a high level of MV replication at day 3 p.i., followed by a small peak at day 10 p.i. For some mice, we counted the number of human cells per 50 μl of blood used for RNA extraction. The data are shown in **Figure 4B**. We were able to detect high levels of MV in samples containing less than 2,000 cells, indicating that the qRT-PCR system is sensitive enough to detect low numbers of MV-infected human cells.

Although MV replication was not obvious in three mice infected with the smallest dose (200 pfu), one of these animals exhibited an increase in MV RNA expression at day 21 p.i. (gray circle). We sacrificed this particular mouse and used flow cytometry to analyze GFP expression in its blood, spleen, MLN, and BM. As shown in **Figure 4C**, GFP^+ cells were present in spleen (0.308%) and all the other tissues, albeit at a lower frequency, indicating that MV infection can occur even at a low dose (200 pfu) and spread slowly in the systemic lymphoid tissues of hNOJ.

It may be necessary to acquire at least 30,000 events to be sure of having > 10,000 cells for flow cytometry analysis. This is because of the substantial amount of sample loss that occurs in this system. The flow cytometry data presented in **Figure 4C** were obtained by analyzing ~0.4 ml blood from a sacrificed mouse. However, even under these conditions, the proportion of MV-infected cells detected was only 0.056%; indeed, the cells are barely visible on the plot. Therefore, it appears that flow cytometry is not a suitable method for the sequential monitoring of infected (GFP^+) cells. Thus, the qRT-PCR system we have developed here allowed us to monitor systemic MV replication using a small volume of blood from humanized mice.

DISCUSSION

Based on a highly sensitive MV-N RNA detection method previously developed by Hummel et al. (2006), which could detect one copy of synthetic MV RNA/reaction, we developed a novel one-step real-time qRT-PCR system for the purpose of monitoring MV replication in the blood of MV-infected humanized mice.

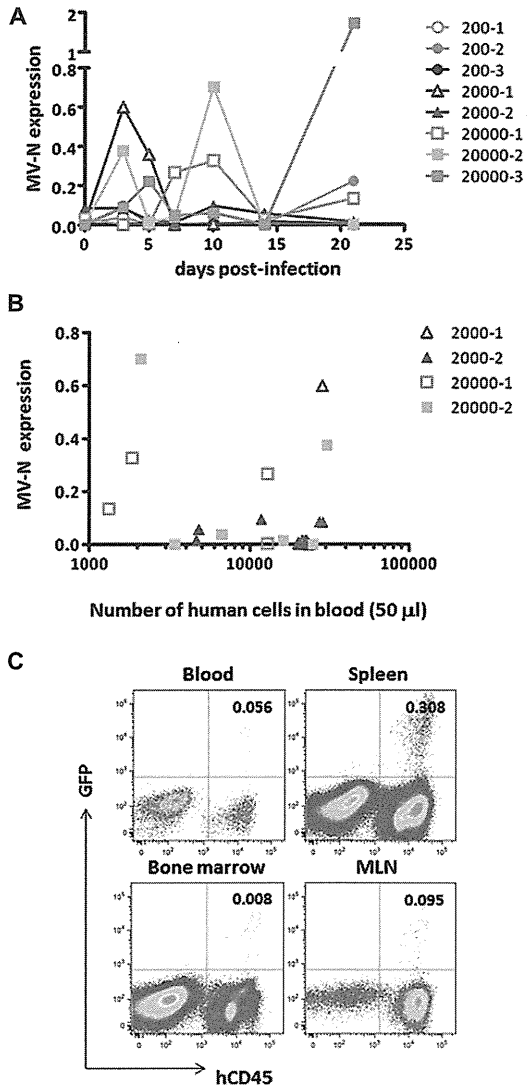

FIGURE 4 | Monitoring of MV replication *in vivo*. Two to three hNOJ mice per group were challenged with MV AIK-C-EGFP at 20,000 (squares), 2,000 (triangles), or 200 pfu (circles). The PBMCs of these mice were collected at day 3, 5, 7, 10, 14, and 21 p.i. **(A)** The level of MV-N expression in the blood of infected hNOJ. Vertical axis shows the level of MV-N relative to that of RNaseP, as described in the legend for **Figure 2B.(B)** For some mice (depicted using the same symbol as **(A)**), the number of human cells per 50 µl of blood used for RNA extraction and analyzed for MV-N expression was plotted on the X-axis. **(C)** An hNOJ mouse infected with MV at a low dose (gray closed circle, 200-2) exhibited an increased level of MV-N. At day 21 p.i., the mouse was sacrificed; cells from blood, spleen, BM, and mesenteric lymph node (MLN) were prepared. Cells were stained and analyzed as described in the legend for **Figure 2A**.

Because MV replication usually occurs in association with cells (Griffin, 2007), it is necessary to evaluate the endogenous RNA expression level of human PBMCs that co-exist with mouse blood cells. To this end, we designed human-specific primer/probe sets for the CD45 and RNase P mRNAs. When we analyzed the detection efficiencies of these two primer/probe sets using distinct cell types present in human PBMCs, we found that RNase P expression was less dependent than CD45 expression on cell type. Using this qRT-PCR system with RNase P as an internal control, we can reliably detect MV replication with high sensitivity in humanized mice *in vivo*. When MV expressing GFP was used for infections *in vitro* or *in vivo*, the level of MV-N RNA was closely correlated with the frequencies of GFP^+ MV-infected cells determined by flow cytometry.

Our qRT-PCR system allowed us to follow MV replication *in vivo* using a small amount of blood, with no need to sacrifice mice at each time point. Although flow-cytometric analysis provides valuable information, such as the proportions of various cell types and the surface phenotypes of MV-infected cells, the small number of human cells circulating in the mouse blood may not be sufficient for precise estimation of MV-infected cells by flow cytometry. By contrast, our qRT-PCR system was able to detect MV-N RNA in fewer than 2,000 human PBMCs (**Figure 4B**). This is an important technological advantage considering that individual humanized mice exhibit variable levels of human cell engraftment, i.e., chimerism (Terahara et al., 2013); moreover, there may exist donor-to-donor variation in susceptibility to MV infection. Thus, it should be possible to select humanized mice with a degree of MV infection appropriate for the purpose of a given experiment.

In this study, MV was inoculated through the tail vein, and infected cells were distributed to systemic lymphoid tissues as well as BMs, where human hematopoietic cells localize in humanized mice (Traggiai et al., 2004). MV may also be distributed to other organs, such as lung and intestinal tissue, as demonstrated in the case of HIV infection using the BLT mouse (Sun et al., 2007). To our surprise, by monitoring MV replication in PBMCs of humanized mice, we noticed two peaks of MV replication, at around 3 and 10 days p.i., in some mice. This pattern of MV replication did not depend on the initial dose of MV inoculum. We do not know why MV replication showed two peaks in many animals. However, it was recently reported in a monkey model that MV RNA persists in PBMCs for more than 1 month after primary infection, and declined in three phases (Lin et al., 2012). The authors of that study hypothesized that both T cells, including regulatory T cells (Treg), and antibody responses contributed to the dynamics of MV replication *in vivo*. Although hNOJ mice are reported to show poor immune responses, the role of regulatory T cells should be considered. This is because these cells regulate HIV-1 infection in humanized mice (Jiang et al., 2008). Alternatively, it may be that the intravenous injection of MV rapidly kills the target cells (probably those showing an activated phenotype) within 3 days. The low number of MV-infected cells then gradually transmits the virus to the human cells that are replenished from the BM stem cell pool. Further investigations are required to clarify this issue.

The humanized mouse model is expected to be a useful tool for studying virus infection (Akkina, 2013). Although the human immune system is not fully reconstructed by the transplantation of human HSCs alone, we believe that further improvements are possible, which will allow us to utilize this mouse model to not only evaluate vaccine and drug efficacy but also to increase our understanding of the pathogenesis of MV infection. The described novel method of monitoring MV-infected human cells in the blood will

be useful for studying MV-based vaccines in humanized mouse models without the need to sacrifice the mice.

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RETROVIROLOGY

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DNA damage enhances integration of HIV-1 into macrophages by overcoming integrase inhibition

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Abstract

Background: The prevention of persistent human immunodeficiency virus type 1 (HIV-1) infection requires the clarification of the mode of viral transduction into resting macrophages. Recently, DNA double-strand breaks (DSBs) were shown to enhance infection by D64A virus, which has a defective integrase catalytic activity (IN-CA). However, the mechanism by which DSBs upregulate viral transduction was unclear. Here we analyzed the roles of DSBs during IN-CA–independent viral transduction into macrophages.

Results: We used cellular systems with rare-cutting endonucleases and found that D64A virus integrated efficiently into the sites of artificially induced DSBs. This IN-CA-independent viral transduction was blocked by an inhibitor of ataxia telangiectasia mutated protein (ATM) but was resistant to raltegravir (RAL), an inhibitor of integrase activity during strand transfer. Moreover, Vpr, an accessory gene product of HIV-1, induced DSBs in resting macrophages and significantly enhanced the rate of IN-CA-independent viral transduction into macrophages with concomitant production of secondary viruses.

Conclusion: DSBs contribute to the IN-CA–independent viral infection of macrophages, which is resistant to RAL. Thus, the ATM-dependent cellular pathway and Vpr-induced DNA damage are novel targets for preventing persistent HIV-1 infection.

Keywords: DNA damage, HIV-1, Integrase inhibitor, Integration, Resting macrophages, Vpr

Background

The prognosis of individuals infected with human immunodeficiency virus type 1 (HIV-1) has improved due to the development of combination antiretroviral therapy (cART) [1]. However, several lines of evidence revealed that the current regimen does not block viral replication completely [2], which promotes the emergence of drug-resistant mutant viruses. Recently, new anti-retroviral drugs that target viral entry or the integration of viral DNA into the host genome have been applied clinically [3,4], which allows the possibility of overcoming viruses that are resistant to conventional cART. Moreover, an advanced study directed at the development of novel anti-HIV-1 compounds attempted to identify the cellular proteins that associate with HIV-1 proteins [5]. Macrophages are less sensitive to the toxic effects of HIV-1 and they function as persistent producers of the virus [2]; therefore, it is important to develop novel anti-HIV-1 compounds that target viral transduction into resting macrophages.

Integrase, a 288-amino-acid and 32-kDa HIV-1 protein, promotes strand-transfer reaction [6], where the reverse-transcribed double-stranded viral DNA is integrated into the host genome. The integrase catalytic activity (IN-CA) excises two nucleotides from the 3′-end of the viral DNA and the CA-3′-OH is ligated to the 5′-*O*-phosphate end of the genomic DNA [6]. All these strand transfer steps depend on the presence of a D,D(35)E motif in the central domain and any mutations in this motif abrogate the activity required for the strand-transfer process [7]. Notably, single-strand gaps are produced in both regions flanking the viral DNA and it was postulated that cellular factors repair these gaps because viral proteins have a low DNA damage repair activity [8].

Initially, Daniel *et al.* proposed that DNA-dependent protein kinase was a cellular factor involved in gap-repair [9], and then ataxia telangiectasia mutated (ATM), ataxia telangiectasia and Rad3-related (ATR), Nijmegen

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breakage syndrome 1 (NBS1), and poly(ADP-ribose) polymerase 1 (PARP1) have also been nominated as cellular proteins involved in efficient viral transduction [10-13]. Using KU55933, a specific ATM inhibitor, Lau *et al.* proposed that ATM is also involved in HIV-1 transduction [14], whereas Sakurai *et al.* demonstrated that DNA damage repair enzymes are involved in multiple steps of retroviral infection [15]. These observations support the importance of DNA double-strand breaks (DSBs) in viral transduction, although their roles are controversial [16-19]. A possible explanation for discrepancies in reported observations is that the single-strand gaps are repaired in a redundant fashion by DNA damage repair enzymes, the expression of which varies among cells [20]. It is also possible that DSBs have modest effects on viral transduction, which may be overwhelmed by the infectivity of the wild-type (WT) virus. This suggests that it is important to evaluate the effects of DSBs using more sophisticated experimental approaches.

Here we focused on the role of DNA damage (DSBs), particularly in integration of viral DNA. Interestingly, HIV-1 DNA integrated into artificially induced DSBs in an IN-CA–independent manner and DNA damaging agents upregulated the infectivity of IN-CA–defective virus. The positive effects of DSBs on viral integration were resistant to raltegravir (RAL), an IN-CA inhibitor. Moreover, Vpr, an accessory gene product of HIV-1, mimicked DNA damaging agents and increased IN-CA–independent viral transduction into monocyte-derived macrophages (MDMs). Even when the catalytic activity of IN was impaired, infectious secondary virus was generated without any mutations that yielded phenotypes resistant to RAL.

Based on these observations, we propose that the ATM-dependent mode of DSB-specific integration of viral DNA and the Vpr-induced DSBs are novel targets for anti-HIV compounds that inhibit viral transduction into MDMs, a persistent reservoir of HIV-1 infection.

Results

HIV-1 integrates into the sites of artificially induced DSBs

To understand the roles of DSBs in integration of viral DNA into macrophages, we established a system using THP-1 cells, a human monocytic leukemia cell line that differentiates into macrophage-like cells after treatment with phorbol myristate acetate (PMA) (Figure 1A) [21]. We transfected THP-1 cells with plasmid DNA that contained the recognition sequence for I-*Sce*I, a rare-cutting endonuclease [22] and obtained clones with the I-*Sce*I site after drug selection. Using the experimental procedures outlined in Figure 1A, the frequency of viral DNA integration into I-*Sce*I sites was evaluated. After PMA-treated cells were infected with VSVG-pseudotyped WT virus (NL-Luc-E(−)R(−)) together with adenovirus-expressing I-*Sce*I, provirus DNA was detected in the I-*Sce*I provirus (Ad-I-*Sce*I) site or its vicinity (Figure 1B, Additional file 1: Figure S1A). PCR amplification targeting the junction of the I-*Sce*I site and the 5′-end of the integrated proviral DNA (Figure 1A) selectively generated PCR amplicons from the Ad-I-*Sce*I-infected samples (compare the upper and lower panels of Figure 1B). Sequence analysis of several independent clones detected the presence of provirus DNA in the I-*Sce*I site (Figure 1C, each arrowhead indicates the integration site of individual clones analyzed). Notably, KU55933 blocked I-*Sce*I site-targeted integration (Figure 1D, lower panel) [14].

Similar results were obtained using a different system with another rare-cutting endonuclease, I-*Ppo*I (Figures 1-E–H and Additional file 1: Figure S1B). The recognition sites of I-*Ppo*I are present in the human genome, although the mammalian genome has no gene that encodes the enzyme [23]. In this experiment, we used a lentiviral vector to ensure the generality of our observations (Figure 1E). As shown in Figure 1F, the viral DNA reproducibly integrated into the I-*Ppo*I site, which was confirmed by PCR amplification and sequence analysis (Figure 1G and H). The data clearly indicated that the viral DNA was inserted in the DSB sites.

Integration into DSB sites was independent of the catalytic activity of integrase

Interestingly, analysis of the nucleotide sequence of the viral DNA inserted in the I-*Sce*I site revealed that both the 5′- and 3′-long terminal repeat (LTR) ends of the provirus DNA had adenine and cytidine (pAC) dinucleotides (Figure 1I) [6], suggesting that the viral DNA integrated into DSBs in an IN-CA–independent manner (Additional file 1: Figure S2). To confirm this, similar experiments were performed using D64A mutant virus, which is defective in integrase, co-infected with Ad-I-*Sce*I (Figure 2A). PCR amplification followed by sequence analysis consistently detected the presence of pAC in the 5′ ends of the integrated viral LTR (Figure 2B).

We then estimated the frequency of viral integration into the DSB sites in the total number of provirus DNA. Intriguingly, we observed that more than half of the integrated D64V lentiviruses were present in the I-*Ppo*I site (approximately 53%) when viral infection was conducted using HT1080 cells that had been cultured in 0.1% FBS (Figure 2C, Additional file 1: Figure S3A). In contrast, the DSB-specific integration of the viral DNA was reduced to approximately 18% in a similar experiment performed in the presence of 10% FBS. FACS analysis of HT1080 cells that had been pulse-labeled with BrdU revealed that the population of cycling cells decreased from 43% to 18% when cells were cultured in

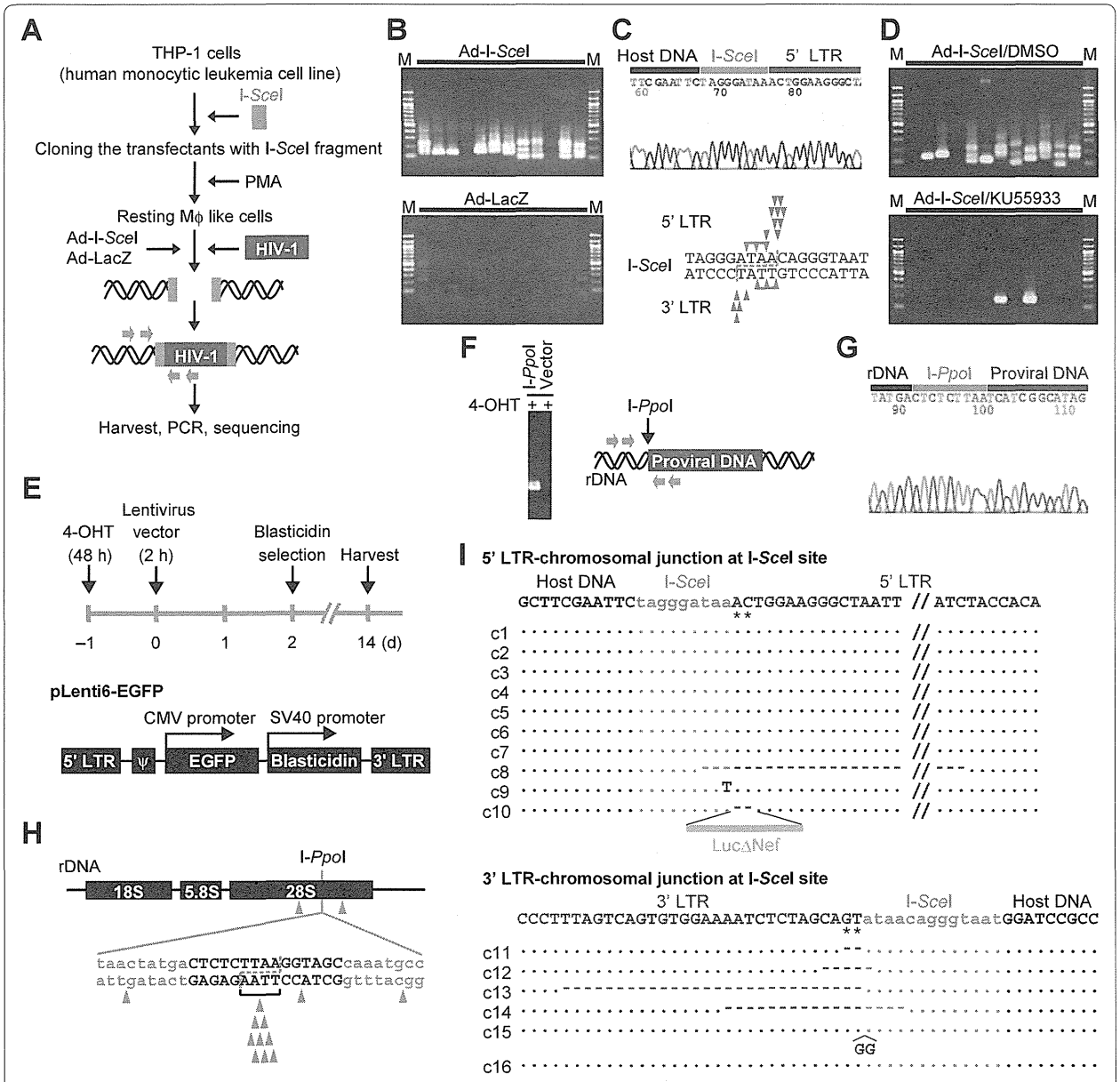

Figure 1 HIV-1 DNA integrates into a DSB site. (**A**) An experimental procedures for (**B**)–(**D**) and (**I**). Red arrows indicate the primers used in (**B**) and (**D**). (**B**) PCR amplification of WT provirus DNA integrated in the I-*Sce*I site. Each lane depicts each result of twelve samples independently infected with WT virus and Ad-I-*Sce*I (upper panel) or Ad-LacZ (lower panel). M, molecular marker. (**C**) Upper panel, representative sequencing chromatogram of the PCR amplicon in samples, which were shown in upper panel of (**B**). Lower panel, summary of viral DNA integration sites. The 18-bp recognition sequence of the I-*Sce*I site is shown. When digested with I-*Sce*I, a 3′-protruding end of 4 nucleotides is formed (dotted red line). Each arrowhead indicates an actual integration site of viral DNA in samples shown in (**B**). Integration sites were identified on most of clones except for two clones, which are indicated by arrowheads with a horizontal bar. (**D**) Effect of KU55933 on viral DNA integration into the I-*Sce*I site. (**E**) Schematic outline of the I-*Ppo*I-PCR experimental design in (**F**)–(**H**) (Top panel). The lentiviral vector was used in this study (bottom panel). (**F**) PCR amplification of lentiviral vector inserted in the I-*Ppo*I site. Primers are shown by red arrows (**G**) A representative result of sequence analysis of proviral DNA integrated in the I-*Ppo*I site. (**H**) Summary of integration sites of the lentiviral vector. Each arrowhead depicts each result of independent clones. The dotted line indicates I-*Ppo*I site with a 3′-protruding end of 4 nucleotides. (**I**) Summary of the I-*Sce*I-PCR sequence data. A representative nucleotide sequence was shown at the top of each panel. Asterisks indicate the pAC that would be normally removed during IN-mediated integration (see Additional file 1: Figure S2). Dots indicate identical sequence to that of the representative sequence. Dashes indicate deleted nucleotides.