Activation of latent HIV-1 provirus by different dendritic cell subsets isolated from blood.

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ABSTRACT
HIV-1 latency remains a major obstacle towards virus eradication from infected individuals. The major contributors to the long-lived reservoir are latently infected resting T lymphocytes. However, we have recently demonstrated that PHA-activated T lymphocytes with an effector phenotype can also harbor latent HIV-1 provirus. The provirus could be purged from latency by co-culturing of the HIV-1 infected T lymphocytes with monocyte-derived dendritic cells (MDDCs). In this study we investigated whether other cells were capable of purging HIV-1 from latency. We show that provirus could not be purged from latency by co-culturing of the T lymphocytes with B lymphocytes, monocytes or plasmacytoid dendritic cells. Monocyte-derived macrophages type I and II moderately activated latent provirus. However, HIV-1 provirus was very efficiently purged out of latency by co-culturing of the T lymphocytes with CD1c⁺ or CD141⁺ myeloid dendritic cells. Interestingly, maturation of primary myeloid DCs with several TLR ligands showed that these matured myeloid DCs activated latent provirus as efficiently as immature myeloid DCs, except for poly(I:C) matured cells which almost lost purging properties. Knowledge on ‘natural’ purging mechanisms may contribute to eradication strategies targeting the long-lived viral reservoir.
INTRODUCTION
HIV-1 latency remains a major barrier towards eradicating virus from the infected individual and therapeutic attempts to purge these reservoirs have thus far been unsuccessful. The pool of latent proviruses is established early during infection and forms a steady source of integrated proviral DNA that last for the lifetime of infected individuals. Even after many years of successful antiretroviral therapy, reducing the viral load to undetectable levels, the virus can rebound from latently infected cellular reservoirs and re-establish systemic infection upon therapy interruption. The majority of the reservoir consists of latently infected resting T lymphocytes. However, recently we and others have demonstrated that actively proliferating effector T lymphocytes can frequently harbor latent HIV-1 provirus as well. A latently infected proliferating T lymphocyte might escape recognition by the immune system, as no viral peptides are expressed and presented, and could revert into resting T lymphocytes, thus contributing to the long-lived latent viral reservoir. These latently infected proliferating T lymphocytes did not start virus production upon treatment with known anti-latency drugs, such as TNFα, prostratin, TSA and NaBut. However, activation of latent provirus was mediated by co-culturing of T lymphocytes with monocyte-derived dendritic cells (MDDCs), demonstrating that a natural mechanism based on cell-cell interactions can purge HIV-1 from latency. In this study, we investigated whether other cells from the myeloid lineage as well as different peripheral blood cells could induce expression from the latent HIV-1 provirus in proliferating T lymphocytes. Understanding the natural mechanisms that activate latent HIV-1 provirus may lead to novel intervention therapies to overcome latency.

RESULTS
MDDCs are more potent in activating HIV-1 provirus from latency than MDMØs. We previously demonstrated that HIV-1 quite frequently establishes a latent provirus in actively proliferating T lymphocytes with an effector phenotype. Co-culture of these latently infected T lymphocytes with monocyte-derived dendritic cells (MDDCs) efficiently purged the provirus from latency, resulting in active virus production. The results implied that encounter of DCs with HIV-1 infected T lymphocytes at contact sites in mucosal tissue or lymph nodes might reverse the HIV-1 latent phenotype in T lymphocytes. To determine whether other monocyte-derived cell types could purge the HIV-1 provirus from latency, monocytes were isolated from peripheral blood and differentiated into MDDCs and monocyte-derived macrophages (MDMØs) with a type I or type II phenotype. Cell imaging with confocal microscopy showed the cell morphology of MMDC and MDMØ (Fig. 1A). The cells were stained with antibodies to determine the expression levels of different immune phenotype markers by flow cytometry. The MDDC were negative for CD14, expressed intermediate levels of the MHC class II molecules and high levels of CD1c, CD11c and DC-SIGN, indicating an immature MDDC phenotype (Fig. 1B). Type I MDMØs
expressed low levels of CD14 and CD163 and high levels of CD206, while MDMØs type II expressed high levels of CD14, CD163 and CD206, confirming that they were indeed MDMØs with a type I or II phenotype (Fig. 1C).

To study whether the two MDMØ cell types could activate HIV-1 provirus from latency in the same way that MDDCs would, we performed a previously described latency assay\textsuperscript{15,16}. To study HIV-1 proviral latency, the transcriptionally silent provirus must be distinguished from a defective provirus. This can be achieved by purging silent provirus out of latency and measuring production of viral proteins such as the major structural protein Gag or its CA-p24 domain. The latency assay is based on single round infection because subsequent infections are blocked by the use of fusion inhibitor T1249. PHA-activated T lymphocytes were infected with HIV-1 and after 4 h unbound virus was washed away. One day after infection the cell culture was split into 4 individual cultures which were further incubated with 4 different cell types: mock (no cells), type I MDMØs, type II MDMØs, or MDDCs. By comparing the percentages CA-p24 positive cells in the co-culture with the percentages of CA-p24 positive cells in the mock culture a fold activation from latency can be calculated. After 24 h of co-culturing, the cells were fixed, stained for CD3 (to detect T lymphocytes) and intracellular CA-p24, and analyzed by flow cytometry. In the mock treated culture, 2.5% of the T lymphocytes were CA-p24 positive (Fig. 2A). Upon co-culturing the T lymphocytes with type I MDMØs, the percentage CA-p24 positive T cells mildly increased to 3.6% (Fig. 2B), yielding a 1.4-fold activation of latent provirus (Fig. 2C). A similar induction level was obtained by co-culturing HIV-1 infected T lymphocytes with type II MDMØs, yielding a 1.3-fold activation. Co-culturing of the T lymphocytes with MDDCs induced the provirus from latency more efficiently than the MDMØs: to 5.8% (2.3-fold activation), consistent with what we reported previously\textsuperscript{13}. Thus, macrophages have some capacity to purge the HIV-1 provirus from latency in T lymphocytes, but that capacity is lower than that of MDDCs.

**Monocytes have no effect on latent HIV-1 provirus.** To investigate if the activation of latent provirus by MDMØs could also be obtained with undifferentiated monocytes, HIV-1 infected T lymphocytes were either mock treated or co-cultured with monocytes that were freshly isolated from blood. Isolation of monocytes with αCD14 labeled magnetic beads yielded a 98% pure monocyte population (Fig. 3A). Co-culturing of the T lymphocytes with monocytes did not change the percentages of CA-p24 positive cells compared to mock treated cultures (Fig. 3B). As positive control, the T lymphocytes were also co-cultured with MDDCs that again induced a significant 2.4-fold activation (Fig. 3D). These results show that cell-cell interactions between monocytes and T lymphocytes do not induce activation of HIV-1 provirus from latency.
Myeloid DCs isolated from blood can activate HIV-1 provirus from latency. To investigate whether peripheral blood DCs can activate the latent provirus, two different subsets of myeloid dendritic cells (mDCs) were directly isolated from blood, using a ‘Dendritic Cell Isolation Kit’. The conventional mDC population (CD1c+), characterized by high expression levels of CD1c and CD11c, but negative for the general blood cell lineage markers CD3, CD14, CD16, CD19, CD20 and CD56, yielded a
purity of 68% (Fig. 4A). Isolation of cross-presenting mDCs, expressing high levels of CD141 and CD11c while negative for the different general blood cell lineage markers, yielded 76% purity (Fig. 4B). Co-culturing of the HIV-1 infected T lymphocytes with CD1c+ and CD141+ mDCs (mDC:T cell ratio 1:3) increased the percentage of CA-p24 positive cells from 2.6% in the mock treated culture to 5.6% and 4.7%, yielding an average activation of 1.9- and 1.8-fold, respectively (Fig. 4C and D). Thus, similar to MDDCs, myeloid DCs directly isolated from blood can activate provirus from latency.
B lymphocytes do not induce activation of latent provirus. Similar to MDDCs and MDMØs, B lymphocytes perform antigen presenting functions and frequently contact T lymphocytes in blood and draining lymph nodes. To assess whether B lymphocytes have the capacity to activate the HIV-1 provirus from latency, B lymphocytes were isolated from PBMCs with CD19 specific beads, reaching a purity of ~98% as determined by FACS flow cytometry (Fig. 5A). Co-culturing of HIV-1 infected T lymphocytes with B lymphocytes did not increase the percentage of
Fig. 4. Myeloid DCs isolated from blood can activate HIV-1 provirus from latency. A: Representative dot-plot of blood isolated myeloid DCs positive for CD1c, CD11c and negative for the markers CD3, CD14, CD16, CD19, CD20 and CD56 ("lineage"). B: Representative dot-plot of blood isolated myeloid DCs positive for CD141, CD11c and negative for the markers CD3, CD14, CD16, CD19, CD20 and CD56 ("lineage"). C: Percentage of intracellular CA-p24 positive T lymphocytes with or without CD1c⁺ mDC or CD141⁺ mDC co-culture. Shown is a representative experiment with two different donors performed in triplicate (n=6). D: Analyses of the percentage of CA-p24 positive T lymphocytes shown as the mean fold activation. Results are the mean values (± sem) of three independent experiments with six donors and each experiment was performed in triplicate or duplicate (CD1c⁺ n=16: CD141⁺ n=18).
CA-p24 positive cells compared to mock treated T lymphocytes (Fig. 5B). As positive control the T lymphocytes were co-cultured with MDDCs and this induced the expected 2.4–fold activation (Fig. 5C).

We also tested whether unstimulated T lymphocytes such as CD4/CD8 T cells and NK cells were able to induce virus production from latently infected T lymphocytes. For this we used the remainder of the PBMC fraction from which the CD14 positive

Fig. 5. B lymphocytes cannot activate the HIV-1 provirus from latency. A: Representative dot-plot of isolated B lymphocytes positive for CD19. B: Representative dot-plot of HIV-1 infected T lymphocytes positive for CA-p24 and CD3 after a 24 hour mock treatment or co-culture with B lymphocytes or MDDCs. C: Analyses of the percentage CA-p24 positive T lymphocytes shown as mean fold activation. Results are the mean values (± sem) of two independent experiments with four different donors and each experiment was performed in triplicate (n=12).
monocytes, CD19-positive B cells and myeloid DCs were depleted. This bulk ‘left-over’ T cell fraction could not alleviate proviral latency in infected T lymphocytes (Fig. S1).

**Plasmacytoid DCs cannot activate HIV-1 provirus from latency.** To investigate whether activation of latent provirus is specifically induced by DCs of myeloid origin, we set out to determine if plasmacytoid DCs (pDCs) could activate the provirus from latency. After pDC isolation from blood, the cells were stained with antibodies to detect expression of different immune phenotype markers. Flow cytometry analyses demonstrated that 99.9% of the isolated cells expressed the IL-3 receptor CD123 and 92.3% expressed the pDC marker CD304 (BDCA-4), while being negative for different general blood cell lineage markers (Fig. 6A). Co-culturing of HIV-1 infected T lymphocytes with pDCs did not affect the percentage CA-p24 positive cells as compared to the mock treated culture (Fig. 6B and C). These results show that pDCs can not activate the HIV-1 provirus from latency in T lymphocytes.

In conclusion, our results show that the latent HIV-1 provirus in proliferating effector T lymphocytes cannot be activated by monocytes, plasmacytoid DCs, B lymphocytes or T cells, but HIV-1 provirus can be purged from latency by CD1c+ and CD141+ myeloid DCs, MDDC, and MDMØ.

**Fig. 6. Plasmacytoid DCs isolated from blood cannot activate the HIV-1 provirus from latency.** A: Representative dot-plot of blood isolated plasmacytoid DCs positive for CD123, CD304 and negative for markers CD3, CD14, CD16, CD19, CD20 and CD56 ('lineage'). B: Percentage of intracellular CA-p24 positive T lymphocytes with or without pDC co-culture. Shown is a representative experiment with two different donors performed in triplicate (n=6). D: Analyses of the percentage of CA-p24 positive T lymphocytes shown as the mean fold activation. Results are mean values (± sem) of two independent experiments with four donors, each experiment being performed in triplicate (n=12).
Fig. 7. Maturation of MDDCs or myeloid DCs affects the activation of latent provirus differently. A: Representative mean fluorescent intensity (MFI) histogram of unstimulated iMDDCs (filled grey) expressing low levels CD83, CD86 and intermediate levels of MHC class II (HLA-DR), LPS stimulated (bold black line) or poly(I:C) stimulated (grey line) mMDDCs expressing high levels of CD83, CD86 and MHC class II. B: Fold activation of the percentage CA-p24 positive T lymphocytes after 24 hour mock treatment or co-culture with iMDDC, mMDDC<sub>LPS</sub> or mMDDC<sub>poly(I:C)</sub>. Results are mean values (± sem) of two independent experiments with two donors and each experiment was performed in triplicate (n=6). C: Fold activation after 24 hour mock treatment or co-culture with unstimulated CD14<sup>+</sup> mDC, CD14<sup>+</sup> mDC<sub>LPS</sub> or CD14<sup>+</sup> mDC<sub>poly(I:C)</sub>. Results are the mean values (± sem) of two independent experiments with three donors and each experiment was performed in triplicate (n=9). D: Fold activation after 24 hour mock treatment or co-culture with unstimulated CD11<sup>+</sup> mDC, CD11<sup>+</sup> mDC<sub>LPS</sub> or CD11<sup>+</sup> mDC<sub>poly(I:C)</sub>. Results are the mean values (± sem) of a single experiment with two donors, performed in duplicate (n=4).
Mature MDDC-mediated activation of latent provirus is less potent than immature MDDC-mediated activation. Blood circulating mDCs can undergo a maturation process after recognition of pathogen-associated molecular patterns (PAMPs) derived from pathogens such as viruses or bacteria by pathogen recognition receptors (PPRs) and Toll like receptors (TLR). Maturation induces mDC migration to the lymph nodes to initiate an adequate immune reaction. To investigate if mature and immature DCs have different effects on transcriptional activation of latent HIV-1 provirus in T lymphocytes, latency activation properties of mature and immature MDDCs were tested in the assay. MDDCs were stimulated with lipopolysaccharide (LPS, mMDDC_{LPS}), for maturation via the TLR4 receptors, or with poly(I:C) (mMDDC_{poly(I:C)}), for maturation via TLR3, or left untreated to remain immature (iMDDC). Maturation of the MDDCs was analyzed by measuring the expression of MHC class II (HLA-DR), CD83 and CD86 on the plasma membrane by flow cytometry. Expression of these markers was strongly increased after MDDC stimulation with LPS or poly(I:C), confirming their mature status (Fig. 7A). As described above, co-culturing of HIV-1 infected T lymphocytes with iMDDC induced a significant 2.8-fold increase in the percentage of CA-p24 positive cells (Fig. 7B). T lymphocytes co-cultured with mMDDC_{LPS} or with mMDDC_{poly(I:C)} showed significantly reduced activation, yielding 1.8- and 1.6-fold activation, respectively. Thus, MDDC maturation reduces their ability to activate HIV-1 provirus from latency in T lymphocytes.

To investigate if this reduced activation due to cellular maturation of the MDDC is also observed for primary myeloid DCs, CD1c^+ mDCs were stimulated with LPS or poly(I:C) prior to the co-culture with HIV-1 infected T lymphocytes. Co-culturing of the HIV-1 infected T lymphocytes with immature CD1c^+ mDCs activated latent provirus 1.9-fold (Fig. 7C). Stimulation of CD1c^+ mDCs with poly(I:C) almost abolished activation of latent provirus (1.2-fold activation). In contrast, LPS stimulated CD1c^+ mDCs increased the number of CA-p24 T lymphocytes, yielding a 2.3-fold activation. Similar results were obtained when T lymphocytes were co-cultured with matured CD141^+ mDCs. More T lymphocytes became positive for CA-p24 when co-cultured with immature (2.3-fold) or LPS stimulated CD141^+ mDCs (3.0-fold), whereas poly(I:C) stimulated CD141^+ mDCs showed significant reduction of activation (1.2–fold) compared to the immature CD141^+ mDCs (Fig. 7D). Collectively, these results indicate that the DC maturation status can affect the DC-mediated HIV-1 activation from latency. Furthermore, opposite effects are observed when comparing blood myeloid DCs and MDDCs upon stimulation with LPS (Fig. 7B-D), the latter losing their capacity to revert latency.

Latency activation after maturing MDDCs and myeloid DCs with different ligands for TLR or the NOD2 receptor. As discussed above, stimulating DCs with LPS derived from bacteria or poly(I:C), a mimic of double stranded viral RNA, altered the DCs capacity to activate latent HIV-1 provirus in T lymphocytes. This suggests that HIV-1 latency in T lymphocytes may also be influenced by other pathogens or PAMPs.
triggering DC activation. To test this, MDDCs were stimulated with PAM₃CSK₄ (a ligand for TLR1/2), peptidoglycan (PGN, ligand for TLR2), poly(I:C) (TLR3), LPS (TLR4), flagellin (TLR5), CLO97 (TLR7/8), R848 (TLR8), or muramyldipeptide (MDP, NOD2 receptor), or not treated to preserve the immature DC phenotype (see also Fig 8A). The MDDC maturation was verified by measuring increased expression levels of CD83, CD86 and HLA-DR (Fig. S2). Co-culturing of the HIV-1 infected T lymphocytes with immature MDDCs increased the percentage CA-p24 positive cells 2.2-fold.

![Diagram](image)

**Fig. 8. Activation upon maturation of MDDCs and myeloid DCs with different ligands for TLR or the NOD2 receptor.**

A: HIV-1 infected T lymphocytes were mock treated, co-cultured with immature MDDCs or co-cultured with MDDCs that were stimulated with different TLR ligands to induce DC maturation. The percentage of CA-p24 positive T lymphocytes was analyzed by flow cytometry and fold activation was calculated. Results are the mean values (± sem) of two experiments with three donors and each experiment was performed in duplicate or triplicate (n=7). B: Fold activation after 24 hour mock treatment or co-culture with unstimulated CD1c⁺ mDC or CD1c⁺ mDC stimulated with different TLR ligands. Results are the mean values (± sem) of a single experiment with two donors performed in duplicate (n=4).
(Fig. 8B). Co-cultures of T lymphocytes with LPS or poly(I:C) maturated MDDCs reduced this to 1.5- or 1.0-fold, respectively. Stimulation of the MDDCs with the other compounds increased the percentage CA-p24 positive cells, ranging from 1.7-fold for flagellin stimulated MDDCs to 3.0-fold for MDP stimulated MDDCs. T lymphocytes treated with the different stimuli alone did not change the number of CA-p24 positive cells (Fig. S3). These results illustrate that maturation stimuli strongly influence the capability of MDDCs to purge latent HIV-1 provirus and imply that this capability does not depend on maturity of the DC per se.

To investigate whether conventional CD1c+ DCs maturated with the different TLR antagonists have similar effects on the activation of latent provirus, HIV-1 infected T lymphocytes were co-cultured with the differently maturated CD1c+ DCs and fold activation from latency was determined. Co-culturing of the T lymphocytes with immature CD1c+ DCs induced a 2.3-fold activation of latent provirus (Fig. 8C). All mature CD1c+ DCs induced comparable induction of latent provirus, ranging from 2.2-fold for CLO97 and R848 stimulated DCs to 2.8-fold for PGN stimulated DCs. Except for poly(I:C) stimulated CD1c+ DCs, as these DCs activated the provirus from latency only by 1.2-fold.

Collectively, our results show that DCs can purge HIV-1 provirus from latency in proliferating T lymphocytes. The extent of purging can differ between DCs, such as MDDCs or primary myeloid CD1c+/CD141+ DC that circulate in peripheral blood. Furthermore, the DC maturation status influences the purging potency, but this is dependent on the specific maturation stimulus.

**DISCUSSION**

Dendritic cells are important antigen presenting cells (APCs), inducing immune system activation in response to pathogens. In blood, lymph nodes, spleen and inflamed mucosal tissues, DCs constantly interact with T lymphocytes. In HIV-1 pathology, DCs are known to be hijacked by HIV-1 for virus transmission to T lymphocytes\(^\text{17}\). In this study we describe an additional role for DCs in HIV-1 pathogenesis and show that different DC subsets isolated from blood can activate HIV-1 from latency.

The HIV-1 reservoir predominantly consists of resting T lymphocytes that harbor latent provirus\(^7,12\). However, we and others recently reported that effector T lymphocytes can also harbor latent HIV-1 provirus and that this occurs quite frequently\(^13,14\). Co-culturing of HIV-1 infected T lymphocytes with monocyte-derived dendritic cells (MDDCs) purged HIV-1 provirus from latency. In this study we show that the provirus can also be purged from latency by co-culturing infected T lymphocytes with blood isolated CD1c+ or CD141+ myeloid DCs, but not by co-culturing with monocytes, B lymphocytes or plasmacytoid DCs. Monocyte-derived
macrophages (MDMØ) moderately purged the provirus from latency but not as strongly as MDDCs. These results suggest that purging of the latent provirus in T lymphocytes is mediated by differentiated cells of the myeloid lineage.

Stimulating MDDCs with LPS (triggering the TLR4 receptor) or poly(I:C) (TLR3) reduced the DC purging properties. In contrast, maturation of the MDDCs via triggering of other LTRs or the NOD2 receptor activated the provirus from latency with equal or slightly increased efficiency compared to immature MDDCs. Immature CD1c⁺ and CD141⁺ myeloid DCs isolated from blood purged the provirus from latency

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**Fig. 9. Schematic representation showing purging properties of immature and mature myeloid DCs.**

**A:** Co-culturing of HIV-1 infected T lymphocytes with immature myeloid DC purges HIV-1 provirus from latency in primary activated T lymphocytes.

**B:** LPS-induced (via TLR4) matured myeloid DCs purge the HIV-1 provirus from latency. Similar results were obtained with DCs matured by stimulating the TLR1, 2, 5, 7, 8 or the NOD2 receptor.

**C:** Poly(I:C)-induced (via TLR3) matured myeloid DCs do not purge the HIV-1 provirus from latency.
Maturated myeloid DCs showed equal or slightly increased activating properties, including TLR4-induced maturated myeloid DCs. An exception was observed for poly(I:C) stimulated myeloid DCs that completely lost their purging properties.

TLR1, 2, 5, 7 and 8 signal via the universal adaptor protein MyD88 to activate the NF-κB and MAPK pathways. TLR3, in contrast, signals via the TIR-domain-containing adapter-inducing interferon-β (TRIF) that apart from NF-κB and MAPK signaling routes also activates the IRF3 pathway, leading to production of interferon-β. TLR4 signals via both MyD88 and TRIF adaptor proteins, dependent on its location. Cell surface localized TLR4 signals via MyD88, endosome localized TLR4 signals via TRIF. Our observation that poly(I:C) stimulated DCs (TLR3) have lost their anti-latency properties suggests that this reduced purging property is linked to the TRIF pathway. The different anti-latency profiles observed for LPS maturated MDDCs and myeloid DCs, may suggest that external or internal TLR4 signaling differs between these two cell types.

Poly(I:C) can also activate protein kinase RNAactivated (PKR). PKR is an anti-viral kinase, phosphorylating translation initiation factor EIF2A, thus preventing mRNA translation and subsequent virus production. Additionally, PKR can activate transcription factors that induce expression of cytokines so that an anti-viral response is signaled to other cells. We are currently investigating whether the reduced purging associated with poly(I:C) stimulation of DCs occurs via TLR3 or PKR.

In our in vitro experiments the T lymphocytes are cultured outside their natural habitat. Our observations that viral latency in T lymphocytes is influenced differently by various DC subsets suggest that viral latency in vivo might depend on the specific location of the T lymphocytes. For instance, production of virus particles from the latent reservoir may be beneficial for virus replication in the lymph node or other lymphoid organs, where cART levels may be suboptimal. Combined, this may allow for low level virus replication to occur. Reduced latency levels (i.e. higher productive infection) may also help explain the high HIV-1 replication rate in the intestine of infected patients. The intestinal lamina propria is an important reservoir for HIV in which viral DNA can be detected even when viral load is not detectable in plasma. Damage of gut mucosa is associated with microbial translocation in the intestine and circulating LPS is significantly increased in chronically HIV-infected individuals and in simian immunodeficiency virus (SIV)-infected rhesus macaques. Immature DCs residing in the lamina propria may mature via LPS-induced triggering of the TLR4 receptor. Subsequent contact with latently infected T lymphocytes in the intestinal lamina propria, germinal centers in Peyer’s patches or local lymph nodes would then allow efficient purging of latent HIV-1 (as illustrated in Fig. 10). Virus production from latently infected cells may be beneficial for the virus, allowing it to maintain or expand the reservoir (i.e. establishing more latent proviruses in other cells).
increase the chance of viral escape from drug or immune pressure (by constant low-level virus replication).

When DCs engulf viral particles, the DC will maturate by dsRNA triggering the TLR3 receptor and migrate to secondary lymphoid organs to encounter T lymphocytes. In our study, poly(I:C) stimulated myeloid DCs hardly activate latent provirus. Others have reported that myeloid DCs induce HIV-1 latency in resting T lymphocytes\textsuperscript{29}. Our assay is not optimal for measuring the induction of latency. We use intracellular HIV-1 CA-p24 as marker for virus production. CA-p24 protein is relatively stable and decreases in steady state are probably not observed over the time frame of our assay. For these reasons we did not study the induction of latency by DCs, but the observations that dsRNA maturated myeloid DCs have reduced purging capability or might even induce viral latency become most intriguing considering virus transmission by DCs. The DC that ferries the virus to infect the T lymphocyte may at the same time prevent virus production by inducing HIV-1 latency. Particularly during primary infection this would be a very useful Trojan horse strategy for the virus,
allowing establishment of latent reservoirs not recognized by the immune system as no viral peptides are expressed and presented.

In our study we use PHA-activated effector T lymphocytes to investigate viral latency. We primarily focused on determining which myeloid lineage derived cells and which peripheral blood cells could induce gene expression from latent HIV-1 provirus in proliferating T lymphocytes. In HIV-1 infected patients the long-lived HIV-1 reservoir consists predominantly of resting T lymphocytes. A study by Marini et al. describes a role for maturated MDDC in activating HIV-1 provirus from latency in resting T lymphocytes. Future research should answer the question whether immature MDDCs or the CD1c+ and CD141+ myeloid DCs can also purge provirus from latency in resting T lymphocytes. Knowledge regarding ‘natural’ purging mechanisms may contribute to eradication strategies targeting the long-lived viral reservoir.

**MATERIALS AND METHODS**

**Cells.** HEK 293T cells were grown as a monolayer in Dulbecco’s minimal essential medium (Gibco, BRL, Gaithersburg, MD) supplemented with 10% (v/v) fetal calf serum (FCS), 40 U/ml penicillin, 40 μg/ml streptomycin and nonessential amino acids (Gibco, BRL, Gaithersburg, MD) at 37°C and 5% CO₂.

Human peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats (Central Laboratory Blood Bank, Amsterdam, The Netherlands) by use of a Ficoll gradient and frozen in multiple vials. When required, PBMCs were thawed, activated with phytohemagglutinin (PHA, Remel, 2 μg/ml) and cultured in RPMI medium supplemented with 10% FCS and recombinant IL-2 (rIL-2, Novartis, 100 U/ml). On day 3 of culture, CD4+ T lymphocytes were enriched by depleting CD8+ T lymphocytes using CD8 immunomagnetic beads (Dynal, Invitrogen). The CD4+ T lymphocytes were cultured for 3 days in RPMI medium (Gibco, BRL, Gaithersburg, MD) with rIL-2 and 10% FCS.

Monocytes were isolated from PBMCs with a CD14 selection step using a magnetic bead cell sorting system (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) according to the manufacturer’s protocol. Purified monocytes were cultured in RPMI 1640 medium containing 10% FCS and differentiated into immature monocyte-derived dendritic cells (iMDDCs) by stimulation with 45 ng/ml interleukin-4 (rIL-4; Biosource, Nivelles, Belgium) and 500 U/ml granulocyte macrophage colony-stimulating factor (GM-CSF; Schering-Plough, Brussels, Belgium) on day 0 and 2, and used on day 6. Mature monocyte-derived DCs (mMDDCs) were obtained on day 6 after stimulating iMDDCs on day 5 with poly(I:C), LPS or other compounds (as indicated). To prepare monocyte-derived macrophages (MDMØ), purified monocytes were cultured in RPMI 1640 medium containing 10% FCS and differentiated by stimulation with 5 ng/ml GM-CSF for a type I phenotype or with 5 ng/ml macrophage
colony-stimulating factor (M-CSF; Immunotools) for a type II phenotype on day 0 and 3, and used on day 7.

B lymphocytes were selected from PBMCs using CD19+ magnetic beads according to manufacturer’s protocol (Miltenyi Biotec) and were maintained in RPMI medium supplemented with 10% FCS after isolation.

Myeloid DCs (CD1c or CD141) were isolated from PBMCs using the CD1c (BDCA-1) Dendritic Cell Isolation Kit, or CD141 (BDCA-3) MicroBead Kit from Miltenyi Biotec according to manufacturer’s protocol. Plasmacytoid DCs were isolated with the Diamond Plasmacytoid Dendritic Cell Isolation Kit II (Miltenyi Biotec). Briefly, CD14+ monocytes and CD19+ B lymphocytes were depleted from freshly isolated PBMCs and CD1c+ and CD141+ myeloid DCs were subsequently isolated. The remaining PBMC fraction was treated with a non-PDC microbead cocktail II (Miltenyi Biotec) to deplete T lymphocytes and NK cells, followed by a positive selection for pDC with BDCA-4 labeled magnetic beads. mDCs and pDCs were cultured in RPMI medium supplemented with 10% FCS and respectively stimulated with 500 U/ml GM-CSF (Schering-Plough, Brussels, Belgium) or 10 ng/ml IL-3 (Invivogen).

Phenotypes of cells were analyzed by determining specific marker protein expression with FACS flow cytometry. Monocytes and B lymphocytes expressed high levels of CD14 or CD19, respectively. Immature MDDC were negative for CD14, expressed low levels of MHC class II (HLA-DR), CD83 and CD86 with high levels of DC-SIGN, whereas mature MDDC expressed high levels of MHC class II (HLA-DR), CD83 and CD86 but low levels of DC-SIGN. MDMØ type I expressed low levels of CD14 and CD163 with high levels of CD206, whereas type II MDMØ expressed high levels of CD14, CD163 and CD206. CD1c+ mDCs expressed high levels of CD1c/BDCA-1 and CD11c, but were negative for the general blood cell lineage markers CD3, CD14, CD16, CD19, CD20 and CD56. CD141+ mDCs expressed high levels of CD141/BDCA-3 and CD11c and were negative for the general blood cell lineage markers. pDCs expressed high levels of CD304/BDCA-4 and CD123 and were negative for the general blood cell lineage markers.

**Virus.** Plasmid DNA encoding the CXCR4-using HIV-1 LAI primary isolate was transiently transfected in HEK 293 T cells with the calcium phosphate method as described previously. Virus supernatant was harvested 2 days after transfection, sterilized by passage through a 0.2 μm filter and stored in aliquots at -80°C. The concentration of the virus stocks was determined by CA-p24 ELISA.

**Extracellular CA-p24 ELISA.** Culture supernatant was heat inactivated at 56°C for 30 min in the presence of 0.05% Empigen-BB (Calbiochem, La Jolla, USA). The CA-p24 concentration was determined by twin-site ELISA with D7320 (Biochrom, Berlin, Germany) as capture, and alkaline phosphatase-conjugated anti-CA-p24 monoclonal
antibody (EH12-AP), as detection antibody. Quantification was performed with the lumiphos plus system luminescence reader (Lumigen, Michigan, USA) in a LUMIstar Galaxy (BMG labtechnologies, Offenburg, Germany). Recombinant CA-p24 produced in a baculovirus system was used as standard.

Reagents. The fusion inhibitor T1249 was obtained from Pepscan (Therapeutics BV, Lelystad, The Netherlands) and used at a final concentration of 0.1 μg/ml. 0.1 μg/ml LPS (Invivogen), 20 μg/ml Poly(I:C) (Sigma-Aldrich, St. Louis, MO), 10 μg/ml PAM3/CSK4 (Invivogen), 10 μg/ml PGN (Invivogen), 1 μg/ml flagellin (Invivogen), 5 μg/ml CLO97 (Invivogen), 5 μg/ml R848 (Invivogen), 10 μg/ml MDP (Invivogen) was used to stimulate the different TLRs or the NOD2 receptor.

HIV-1 latency assay. HIV-1 infected cells were used in the latency assay as described previously. In short, PHA-activated CD4+ T lymphocytes (1.5 × 10^6 or 2.0 × 10^6 cells) were infected with HIV-1 (20 ng CA-p24). Excess virus was washed away after 4 hours and the cells were cultured in the presence of the fusion inhibitor T1249 to block new infections. At 24 hr after infection the CD4+ T lymphocytes (1.5 × 10^5/well) were either mock treated or co-cultured with MDDCs, MDMØs, B lymphocytes, monocytes, CD1c+ myeloid DCs, CD141+ myeloid DCs or plasmacytoid DCs (all at a concentration of 0.5 × 10^5/well). After another 24 hr, cells were harvested, stained for CD3 and intracellular CA-p24 and analyzed by FACS flow cytometry. The percentage of CA-p24 positive cells in the treated culture was divided by the percentage of CA-p24 cells in the mock treated culture and used to measure proviral latency (fold activation). One Way ANOVA and student T test (2-tailed) were used to evaluate if observed differences between groups were significant (Graphpad Prism, version 5). P values * = p<0.05, ** = p<0.01, *** = p<0.001.

FACS flow cytometry. For CA-p24 analyses, cells were fixed in 4% formaldehyde for 10 min at room temperature and subsequently washed with 2% FACS buffer (PBS supplemented with 2% FCS). The cells were permeabilized with BD Perm/Wash™ buffer (BD Pharmingen) and antibody staining was performed in BD Perm/Wash™ or FACS buffer for 1 hr at 4°C. Unbound antibody was removed and the cells were analyzed on a BD FACSCanto II flow cytometer with BD FACSDiva Software v6.1.2 (BD biosciences, San Jose, CA) in FACS buffer. The T lymphocyte population was defined based on forward/sideward scatter and expression of CD3 (T cell receptor). Virus production on the gated T lymphocyte population was determined by measuring the intracellular viral CA-p24 protein. Gate settings were fixed between samples for each experiment.

Confocal Microscopy. MDDCs, MDMØs type I and II were cultured on 10 mm Ø glass coverslips (VWR, Germany). Cells were fixed with 3.7% paraformaldehyde (PFA) for 20 minutes. PFA was quenched with 50 mM NH₄Cl and cells were permeabilized with 0.1% saponin (Riedel de Haen, Germany), 10 mM NH₄Cl, and 1% BSA in PBS for 30
min. Subsequently, cells were stained with Hoechst 33258 (Sigma-Aldrich). Excess of Hoechst was removed by washing twice with permeabilization buffer, once with PBS, and twice with millipore filtered water. Cells were embedded in Vectashield and fluorescence was analyzed by confocal microscopy. Fluorescent images were made with a Leica DM SP2 AOBS confocal microscope with a X63 HCX PL APO 1.32 oil objective. The whole cell was scanned (1024 x 1024) with a pixel size of 232 nm and a step size of 340 nm, acquired with the Leica confocal imaging processing software with a line average of four scans per image.

**Antibodies.** For intracellular CA-p24 measurement RD1-PE was used (clone KC57, Coulter). For CD3 staining CD3-APC (BD Bioscience) was used. For MDDC staining purified α-CD83-APC (BD Bioscience), α-CD86-PE (BD Pharmingen), α-HLA-DR-PerCPCy5 (BD Bioscience), α-CD14-FITC (BD Pharmingen) and α-DC-SIGN-PE (R&D Systems) antibodies were used. For MDMØ staining purified α-CD14-FITC (Becton), α-CD163-APC (BioLegend) and α-CD206-PE (BioLegend) were used. For monocyte staining α-CD14-FITC (BD Pharmingen) was used. For B lymphocyte staining CD19-APC (BD Pharmingen) was used. For mDC and pDC staining purified PE conjugated α-BDCA-1, -3 or -4 (Miltenyi Biotec), α-CD11c-APC (BD Bioscience), α-CD123-PE (BD Pharmingen) and α-Lineage-FITC (BD Bioscience) were used.

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REFERENCES

1. Imamichi H, Degray G, Asmuth DM, Fischl MA, Landay AL, Lederman MM and Sereti I. 2011. HIV-1 viruses detected during episodic blips following interleukin-7 administration are similar to the viruses present before and after interleukin-7 therapy. *AIDS* 25:159-64.


SUPPLEMENTARY DATA

**Fig. S1.** Unstimulated CD4/CD8 positive T lymphocytes and NK cells do not activate HIV-1 provirus from latency. **A:** Representative dot-plot of the peripheral blood ‘left-over’ bulk after CD14, CD19, CD1c and CD141 depletion. The majority is positive for the lineage markers CD3, CD14, CD16, CD19, CD20 and CD56 but contains predominantly CD3+ T lymphocytes. **B:** Percentage of intracellular CA-p24 positive T lymphocytes with or without ‘left-over’ bulk co-culture. Shown is a representative experiment with a single donor performed in triplicate (n=3). **C:** Analyses of the percentage of CA-p24 positive T lymphocytes shown as mean fold activation. Results are mean values (± sem) of a single experiment with two different donors and the experiment was performed in triplicate (n=6).
Fig. S2. Maturation of MDDCs with different TLR ligands. Representative mean fluorescent intensity (MFI) histogram of unstimulated MDDCs (iMDDC, filled grey) expressing low levels CD83, CD86 and intermediate levels of MHC class II (HLA-DR) or stimulated MDDCs with different TLR ligands for a maturated MDDC phenotype (mMDDC, bold black line) expressing high levels of CD83, CD86 and MHC class II.
**Fig. S3. TLR stimulating compounds do not activate the HIV-1 provirus from latency.** HIV-1 infected T lymphocytes were either mock treated, or cultured with LPS, poly(I:C), PAM3/CSK4, PGN, flagellin, CLO97, R848 or MDP in the latency assay. The results presented are mean values (± sem) obtained with two independent experiments. In each experiment a different T lymphocyte donor was used and each experiment was performed in triplicate (n=6).