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TGFβ Induces a SAMHD1-independent Post-Entry Restriction to HIV-1 Infection of Human Epithelial Langerhans Cells.

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Short title: **TGFβ Induced HIV-1 Restriction in Langerhans Cells**

Abbreviations: TGFβ Transforming growth factor-beta, MDDC Monocyte-Derived Dendritic Cells, MDLC Monocyte-Derived Langerhans Cells, SAMHD1 SAM and HD domain-containing protein 1

Abstract

SAMHD1 was previously identified as a critical post-entry restriction factor to HIV-1 infection in myeloid dendritic cells (DC). Here we show that SAMHD1 is also expressed in epidermis-isolated Langerhans Cells (LC) but degradation of SAMHD1 does not rescue HIV-1 or VSV-G-pseudotyped lentivectors infection in LC. Strikingly, using Langerhans cells model systems (MuLC, MDLC and freshly isolated epidermal LC), we characterize previously unreported post-entry restriction activity to HIV-1 in these cells, which acts at HIV-1 reverse transcription, but remains independent of restriction factors SAMHD1 and MX2. We demonstrate that TGF β signalling confers this potent HIV-1 restriction in MDLC during their differentiation and blocking of SMAD2 signalling in MDLC restores cells infectivity. Interestingly, maturation of MDLC with a TLR2 agonist or TNF α significantly increases cells susceptibility to HIV-1 infection, which may explain why HIV-1 acquisition is increased during co-infection with sexually transmitted infections (STIs). In conclusion, we report a SAMHD1-independent post-entry restriction in MDLC and LC isolated from epidermis, which inhibits HIV-1 replication. A better understanding of HIV-1 restriction and propagation from LC to CD4⁺ T cells may help in the development of new microbicides or vaccines to curb HIV-1 infection at its earliest stages during mucosal transmission.

Introduction

Transforming growth factor-beta (TGF β) drives the development of immature Langerhans cells (LC) from hematopoietic progenitor cells (Borkowski et al, 1996, Geissmann et al, 1998). This pleiotropic cytokine shapes the function and phenotype of LC (Geissmann et al, 1999, Letterio and Roberts, 1998). TGF β exerts a strong immunosuppressive effect on immune cells (Kobie et al, 2003, Letterio and Roberts, 1998, Li et al, 2006), which facilitates the progression of

HIV-1 infection in patients (Card et al, 2012). Due to their mucosal distribution, LC are likely to be early cellular targets for HIV-1 during sexual transmission (Hladik et al, 2007, Kawamura et al, 2005, Piguet and Steinman, 2007). In common with other myeloid dendritic cell (DC) subsets, LC do not readily support robust virus replication, unlike activated CD4⁺ T cells. The relatively low HIV-1 infection of LC was previously attributed to the presence of Langerin (a LC-specific C-type lectin receptor), which forms a protective barrier against the virus (de Witte et al, 2007, Kawamura et al, 2000). Other DC subsets restrict HIV-1 infection due to the presence of cellular restriction factors, such as SAM and HD domain-containing protein 1 (SAMHD1) (Berger et al, 2011, Hrecka et al, 2011, Laguette et al, 2011). SAMHD1 was shown to be highly expressed in cells of myeloid origin, in which it mediates depletion of the cellular deoxynucleoside triphosphate pool, leading to a drastic impediment of HIV-1 reverse transcription (Goldstone et al, 2011, Lahouassa et al, 2012). Additionally, in mature DC, SAMHD1 imposes an antiviral activity independent of its effect on deoxynucleotide levels (Reinhard et al, 2014). Little is known about post-entry restrictions to HIV-1 in LC. Previous work indicates that high viral titres of HIV-1 can lead to productive LC infection, despite the presence of Langerin, and consequently, increased HIV-1 transmission to T cells (de Witte et al, 2007). Importantly, *de novo* HIV-1 production in LC is also reported in cells that are matured in response to sexually transmitted infections (STIs) (de Jong et al, 2008, Ogawa et al, 2009, Ogawa et al, 2013). In this study, we investigated post-entry restriction mechanisms to HIV-1 infection in LC. Since abrogating SAMHD1 expression and function in immature LC did not result in a significant increase in HIV-1 infection, we show that the predominant HIV-1 restriction in LC operates independently of SAMHD1 and is induced by TGF β . Importantly, this post-entry HIV-1 restriction in LC can be partially relieved following cell maturation with TLR2 agonists or TNF α and fully eliminated by inhibitors of TGF β signalling. This previously unreported finding sheds light on our understanding of the post entry restriction to HIV-1 in

different cell types and opens the possibility to utilize this knowledge in order to prevent new HIV-1 transmissions.

Results

Vpx-mediated degradation of SAMHD1 uncovers a HIV-1 restriction in immature Langerhans cells

To explore the role of SAMHD1 in LC, we used two cellular models of LC in addition to primary LC migrating from epidermal sheets. The cellular models were monocyte-derived LC (MDLC) and Mutz-3-derived LC (MuLC) (de Jong et al, 2010, Masterson et al, 2002, Santegoets et al, 2006). Both models were previously reported to be relevant to analyse interactions between LC and HIV-1, and display phenotypic and functional similarities to skin-resident LC (Geissmann et al, 1998, Ginhoux et al, 2006). MuLC, autologous MDLC and MDDC express the SAMHD1 protein which is down-regulated by the SIV3 lentivector encoding the Vpx gene (Vpx) in each cell type as previously described (Hrecka et al, 2011, Laguette et al, 2011, Miyagi et al, 2009). Densitometry quantification on western-blotting experiments showed that SAMHD1 was down-regulated on average by 95.2% in MDLC (SEM=1.2%), 94.8% in MDDC (SEM=2.8%) and 96.7% in MuLC (SEM=1.6%) (Figure 1 and Supplementary Figure S1). However, we noticed a significantly lower HIV-1 infection of MDLC (mean=5.9%, SEM=1.84) compared to MDDC (mean=55.5%, SEM=6.84) (Figure 1a). The same resistance to HIV-1 infection was observed when using the MuLC cell line (Supplementary Figure S1). Langerin can bind HIV-1 envelope and has been shown to protect LC from HIV-1 infection by capturing and targeting HIV-1 for degradation in Birbeck granules (de Witte et al, 2007). Therefore, in order to evaluate if HIV-1 restriction occurred at entry or post-entry level, we transduced MDDC and MDLC with Vesicular Stomatitis Virus G protein (VSV-G)-pseudotyped HIV-1 lentiviral vectors encoding green fluorescent protein (GFP)

(VSV-G HIV-GFP) (Dull et al, 1998). After down-regulation of SAMHD1, we observed that 61.6% (SEM=5.8%) of MDDC were expressing GFP compared to only 10.7% (SEM=3.6%) for MDLC (Figure 1b) and 17.1% in MuLC (SEM=1.76) (Supplementary Figure S1). Interestingly, in the absence of SAMHD1, viral restriction in MDLC could not be overcome with increasing doses of VSV-G HIV-GFP (Figure 1d). These findings were confirmed in a more physiological system by comparing VSV-G HIV-GFP infection levels in primary epidermal LC and dermal DC freshly isolated from skin. Using antibodies against characteristic lineage markers, we were able to select dermal DC and epidermal LC populations and evaluate VSV-G HIV-GFP infection in these subsets (Figure 2a). Infection experiments demonstrated that dermal DC were significantly more susceptible to VSV-G HIV-GFP infection (14.04% GFP⁺ cells) compared to epidermal LC (3.99% GFP⁺ cells) after Vpx-mediated removal of SAMHD1 (Figure 2b, c and d). Our results demonstrate that the SAMHD1-independent HIV-1 restriction activity found in *in vitro* derived immature LC is present in primary LC isolated from skin, and operates at a post-entry level.

A SAMHD1-independent reverse-transcription restriction operates in MDLC

To investigate the step of viral replication affected in MDLC depleted of SAMHD-1, we performed qPCR analysis of the extracted DNA in order to measure early and late reverse transcription products as well as 2-LTR circles accumulation in infected cells. Using established primers (Anderson and Hope, 2008, Butler et al, 2001, Münk et al, 2002), we observed a higher accumulation of viral DNA in MDDC infected with VSV-G HIV-GFP+Vpx as compared to autologous MDLC (Figure 3a-c). Vpx-mediated removal of SAMHD1 resulted in a substantial increase of early and late RT products in MDDC (early RT 6hpi mean=8404.6, SEM=1677.35; late RT 12hpi mean=98850.916, SEM=21245.97) which remained significantly lower in MDLC (early RT 6hpi mean=3693.75, SEM=307.29; late RT 12hpi

mean=40711.91, SEM=15911.23). Analysis of later stages of virus reverse transcription revealed a 8.4 fold difference in 2-LTR circles accumulation between VSV-G HIV-GFP +Vpx infected MDDC (mean=531.2, SEM=82.94) and MDLC (mean=63.3, SEM=4.47) which correlated with a lower amount of integrated provirus in MDLC (MDLC mean=229.3 as compared to MDDC mean=2097) (Figure 3d).

IFN α does not account for HIV-1 infection restriction in LC

Interferon alpha (IFN α) is a potent antiviral cytokine which increases expression of several HIV-1 cellular restriction factors and interferon stimulated genes (ISG) (Blanchet et al, 2013, Goujon et al, 2013, Kane et al, 2013, Van Damme et al, 2008) and restricts HIV-1 infection in myeloid cells. To investigate whether VSV-G HIV-GFP restriction in MDLC is dependent on type I interferon, we infected MDLC and MDDC with VSV-G HIV-GFP after addition of Vpx. We observed, that at early points of infection (6-12 hrs), VSV-G HIV-GFP +Vpx does not significantly stimulate IFN β mRNA synthesis in MDLC (mean 6hrs 1.47 and mean 12hrs 4.09) or MDDC (mean 6hrs 1.38 and mean 12hrs 3.94) compared to controls (Figure 4a). However, significant increase of IFN β mRNA levels was demonstrated in MDDC at 48 and 72 hours post VSV-G HIV-GFP transduction (mean 48 hrs 9.9-fold, 72 hrs 8.1-fold) and at 24 hours in MDLC (24 hrs mean 17.72). This effect correlated with partial maturation of MDDC and MDLC at 24 hours post infection (Figure 4b). Recently, MX2 was described to act as an IFN α inducible HIV-1 restriction factor in myeloid cells (Goujon et al, 2013, Kane et al, 2013). We show that MX2 was not up-regulated at mRNA level in MDLC until 24 hours post infection with VSV-G HIV-GFP (+Vpx) (Figure 4c) in line with IFN β stimulation results. Late expression of MX2 in MDLC transduced with VSV-G HIV-GFP (+Vpx) confirms that an early block to lentivirus replication in MDLC is independent of MX2 and type I IFN function. In contrast, addition of IFN α prior to viral challenge, further decreased VSV-G HIV-GFP (+Vpx)

infection in MDLC and in MDDC (Figure 4d-f) as expected. IFN α stimulates expression of RIG I and HIV-1 restriction factors including tetherin, APOBEC3G and MX2 in MDDC and MDLC (Figure 4d). These results show that type-I IFN is not involved in early inhibition of HIV-1 in infected MDLC, however, an involvement of viral sensing in MDLC might contribute to limiting virus replication at later stages of infection (e.g. 48 hrs post infection).

HIV-1 post-entry restriction in LC is partially abolished by TLR2 agonists and TNF α

Previous reports suggested that stimulation of LC with TNF α or TLR2 agonists promoted HIV-1 infection of these cells (de Jong et al, 2008, Ogawa et al, 2009). This is relevant in the context of co-infections between HIV-1 and other STIs, including gram-positive bacteria and HSV. We tested if the increased infection of LC observed during bacterial co-infection or TNF α exposure was explained by the modulation of a post-entry viral restriction in LC. We observed a significant enhancement of VSV-G HIV-GFP infection of LC pre-treated with Pam3CSK4 (TLR2 agonist) (mean=6.01%, SEM=1.32 p=0.024) and TNF α (mean=9.25, SEM=1.01, p=0.0001). In contrast, the TLR3 agonist, poly I:C (Figure 4b) rendered MDLC significantly more restrictive to VSV-G HIV-GFP (Mean=1.01%, SEM=0.27, p=0.31) (Figure 5a and b). We detected no significant changes in expression of HIV-1 restriction factors or APOBEC3G, except for SAMHD1 levels which were slightly decreased after TNF α treatment (Figure 5c). TLR agonists and pro-inflammatory mediators signal through the NF κ B pathway. Therefore, we investigated NF κ B activation in MDLC and MDDC following LPS or TNF α . We noticed increased levels of phosphorylated NF κ B (p-NF κ B p65) in MDDC and MDLC after both LPS and TNF α treatment (Figure 5d). These results correlated with a decrease of expression of I κ B α , a negative NF κ B regulator. Although, NF κ B signalling was efficiently induced in MDLC after TNF α stimulation, rescue of VSV-G HIV-GFP infection was incomplete after SAMHD1 depletion (Figure 5b). We therefore suggest that a post-entry restriction to VSV-G HIV-GFP,

independent of SAMHD1, APOBEC3G or Langerin expression, is present in immature LC and that this restriction can be partially down-regulated by TLR2 agonists and TNF α .

TGF β induces an early reverse transcription block to HIV-1 infection in LC.

The main difference between MDDC and MDLC is the addition of TGF β to monocytes which induces differentiation into MDLC. In order to investigate the role of TGF β in the post-entry resistance of MDLC to HIV-1 infection, we infected monocytes with VSV-G HIV-GFP (+Vpx) at different time points during differentiation (Figure 6a). MDLC progenitors supplemented with TGF β became more resistant to VSV-G HIV-GFP infection after 1 day of differentiation (Figure 6b). and after 5 days of differentiation as only 7.4% of differentiating MDLC became GFP positive, in contrast to more than 30% for monocytes derived without TGF β . In agreement with the critical role of TGF β in MDLC post-entry restriction to HIV-1, pharmacological inhibition of its downstream signalling molecule SMAD2 by LY2109761 restored infectivity of LC (Figure 6c and e). Strikingly, in the presence of LY2109761, an average of 80.7% of MDLC become GFP positive when transduced with VSV-G HIV-GFP +Vpx, compared to 15.1% of untreated MDLC (Figure 6d). This highlights a critical role for TGF β signalling in the restriction to HIV-1 in LC.

Discussion

In this study, primary skin-resident epidermal LC, MDLC and the LC-like cell line (MuLC) have been used to investigate HIV-1 restrictions in LC. Our results show that the cellular restriction factor SAMHD1 is expressed in LC and that Vpx mediates its degradation. However, in contrast to other myeloid DC subsets, SAMHD1 degradation in LC was not associated with an increased susceptibility to HIV-1 R5 infection. VSV-G pseudotyped GFP expressing HIV-lentivectors, which bypass Langerin-mediated binding and HIV-1 surface

receptors/co-receptors were used. Exploiting an *ex vivo* model of skin-resident primary epidermal LC, and LC model systems, we show restriction of VSV-G HIV-GFP infection in LC, even after Vpx-mediated SAMHD1 degradation. We could not detect a significant induction of IFN β mRNA at early time points after transducing MDLC with VSV-G HIV-GFP (+Vpx), indicating that HIV-1 sensing is not a prerequisite for virus restriction in LC prior integration. We did observe, however, a partial maturation of both MDDC and MDLC at later times of VSV-G HIV-GFP +Vpx (after 48 hrs).

Importantly, we showed that TGF β induces HIV-1 restriction in MDLC and blocking of SMAD2 signalling makes LC highly susceptible to infection. In agreement, it has been shown that TGF β restricts HIV-1 replication in monocytes and macrophages (Chen et al, 2005, Poli et al, 1991). Quantitative PCR analysis on DNA isolated from MDDC or MDLC transduced with VSV-G HIV-GFP (+Vpx) revealed that VSV-G HIV-GFP post-entry restriction in MDLC occurs during early reverse transcription and prior to nuclear translocation as reflected by the lower amount of 2-LTR circles and impaired integration (Figure 2c). We investigated expression levels of known HIV-1 restriction factors, APOBEC3G (A3G) and MX2, acting prior viral integration in MDDC and MDLC. A3G is a deaminase that when incorporated into budding HIV-1 particle in producer cells inhibits viral infection of the target cell by prevention of virus DNA elongation (Bishop et al, 2008). However, the infectious viruses used in our experiments express HIV-1 Vif when produced, therefore preclude such A3G effect *in trans* (Sheehy et al, 2003, Stopak et al, 2003). Additionally, we did not observe any difference in A3G expression in MDDC and MDLC. We also observed that MX2 was induced at the mRNA level only after 24 hours post-transduction, thus suggesting a MX2-independent restriction in LC at early time points.

HIV-1 capsid was shown to play an important role in shielding of viral nucleic acids from cellular sensing (Gao et al, 2013). In addition, interaction of capsid with cellular proteins such

as Cleavage and polyadenylation specific factor 6 (CPSF6), Cyclophilin A (CypA) and TNPO3 regulate viral replication by seemingly acting at the nuclear entry level (Rasaiyaah et al, 2013, Sokolskaja et al, 2006, Towers et al, 2003) and disruptions of this interactions leads to an impairment of viral replication (Schaller et al, 2011, Zhang et al, 2010). It is possible that CPSF6 and TNPO3 might contribute to HIV-1 infection in MDLC prior to viral integration, however, this would not explain the replication block at RT in LC. One hypothesis is that TGF β signalling in MDLC influences the interactions of viral capsid with some host proteins either by directly changing the expression of host proteins or via modulation of cell activation. We showed that infection could be partially reversed after LC maturation with TLR2 or TNF α possibly due to downregulation of the restriction in LC or alternatively by up-regulation of viral dependency factor(s) in immature MDLC. The MuLC cell line expresses Langerin but is impaired in IFN type-I responses (data not shown and (Rasaiyaah et al, 2009). MDLC, which functionally resemble LC, are closer representatives of short-lived LC (Romani et al, 2012, Seré et al, 2012) and thus their susceptibility to viral infection may mimic inflammatory rather than resident LC. We also extended our infection experiments to MDLC and MuLC as well as skin-isolated epidermal LC. Despite their semi-matured phenotype (data not shown) (Chu et al, 2012) induced by the walkout procedure, epidermal LC represent resident population and confirm a LC-specific HIV-1 restriction. Extension of these studies to other LC models would be valuable as there might be as many subtle differences to LC subtypes in specific tissues and mucosa exposed to different microenvironment (vaginal LC, penile LC, rectal LC...) (Bouschbacher et al, 2008, Ganor et al, 2010, Sivard et al, 2004). For instance, reconstructed vaginal models (Bouschbacher et al, 2008, Sivard et al, 2004) that comprise CD34+-derived LC in between human primary epithelium and the lamina propria could be potentially good models. Alternatively, penile/vaginal explants or even humanized mice would be interesting systems to use to extend our observations. Reports suggested a functional difference between

LC from the skin and oral mucosa LC when comparing their capacity at stimulating allogeneic T cells in vitro (Hasséus et al, 2004) therefore infection studies in LC subsets would prove valuable. Nevertheless, the reproducibility of the results obtained from the three LC model systems used here strongly argue in favour of a HIV-1 restriction in TGF- β -derived LC.

We demonstrated, in this study, that TGF-beta signalling induces a potent HIV-1 restriction occurring at reverse transcription in MDLC. We conclude its dependency on SMAD2 signalling but not type-I IFN or known HIV-1 restriction factors such as SAMHD-1 and MX2. Finally, we showed that pharmacological modulation of TGF β -mediated signalling pathway drastically influences LC susceptibility to HIV-1. Our data highlight a previously unreported post-entry restriction to HIV-1 in LC. These findings are relevant for the development of vaccines or microbicides preventing HIV-1 transmission due to the critical localisation of LC in mucosal tissues.

Materials & Methods

Cells

Buffy coats from healthy donors were obtained from the Welsh Blood Bank service. CD14⁺ monocytes isolation and differentiation to MDLC were performed as previously described (Blanchet et al, 2010, Blanchet et al, 2013). MDLC were obtained from CD14⁺ monocytes upon 6 days incubation with 500 U/ml GM-CSF, 500 U/ml IL4 and 10 ng/ml TGF β in RPMI-1640 medium supplemented with 10% FBS 100 U/ml penicillin, 100 μ g/ml streptomycin and 2 mM L-glutamine. MuLC were derived from Mutz-3 as previously described (de Jong et al, 2010). When stated, LY2109761 (Selleckchem) was added to differentiating MDLC 15 min prior to addition of GM-CSF, IL-4 and TGF- β .

HIV-1 infection and VSV-G HIV-GFP transfection

Cells seeded in U-bottom 96-well plates at 1×10^5 cells/well in 100 μ l of medium were treated, when indicated, with Vpx-expressing lentivectors (Vpx) at least 4 hours prior to infection with indicated dose of HIV-1 R5 virus or VSV-G HIV-GFP. For IFN α treatment, cells were incubated with 1000 U/ml of IFN α 2a (Sigma-Aldrich) for 20 hours before addition of Vpx and consequent infection.

Conflict of Interest

The authors state no conflict of interest.

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Figure Legends

Figure 1. SAMHD1-independent restriction activity in HIV-1 R5 infected LC. Autologous MDLC and MDDC were infected with full length HIV-1 (strain R8Bal at 25ng p24/10⁵ cells) or VSV-G HIV-GFP (30ng p24/10⁵ cells) (\pm Vpx) or left uninfected (NI) for 3 days. Quantification of (a) HIV-1 R5 ($n=3$) and (b) VSV-G HIV-GFP ($n=10$) infection in MDLC and MDDC and representative FACS plots are shown. The horizontal line represents the mean values. (c) A successful Vpx-mediated degradation of SAMHD1 in MDLC and MDDC after minimum 4 hrs incubation is shown. Densitometry quantification of SAMHD1 signal intensity was normalized to actin levels in cell lysates and is given ($n\geq 5$). (d) Titration of VSV-G HIV-GFP dose infection of MDDC and MDLC pre-treated with Vpx ($n=5$). * $p<0.05$, ** $p<0.01$, and **** $p<0.0001$.

Figure 2. Skin-isolated epidermal LC remain refractory to VSV-G HIV-GFP in presence of Vpx. (a) Primary *ex vivo* DC/LC isolated from dermal and epidermal skin sheets were phenotyped. (b) Cells were infected with VSV-G HIV-GFP (30ng p24/10⁵ cells) with or without Vpx pre-treatment and infection levels were measured 3 days post infection. A representative experiment and a quantification of GFP⁺ cells from all donors ($n=4$) are shown. (c) A successful Vpx-mediated degradation of SAMHD1 in dermal and epidermal skin walkout cells after 24 hrs incubation is shown. (d) Data were normalised to VSV-G HIV-GFP values in order to eliminate inter-patient variability and to show fold-change in infection occurring in the presence of Vpx. NI indicates non-infected cells. NT indicates non-treated cells. * $p<0.05$.

Figure 3. A potent post-entry restriction to VSV-G HIV-GFP in Langerhans cells operates at two stages of viral reverse transcription. Autologous MDLC and MDDC were

infected with 30ng VSV-G HIV-GFP (\pm Vpx) for 6, 12 or 18 hours. **(a,b,c)** DNA isolated from samples was used for qPCR analysis of early and later reverse transcription products and 2-LTR circles levels at 6 and 12 hours post infection ($n=5$). **(d)** Integration efficiency in samples was measured at 18 hours post infection ($n=3$). NI indicates non-infected cells. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ and **** $p <0.0001$.

Figure 4. Type I IFN further restricts VSV-G HIV-GFP infection in MDLC, but it is not required for virus inhibition in immature MDLC. **(a)** QPCR analysis of the IFN β mRNA expression levels in MDLC and MDDC after 0, 6, 12, 24, 48 and 72 hrs of VSV-G HIV-GFP (+Vpx) infection are shown ($n=3$). **(b)** A representative experiment showing expression of maturation marker CD86 on MDLC and MDDC after 48 hrs. **(c)** MX2 mRNA expression in VSV-G HIV-GFP +Vpx infected MDLC and MDDC is shown. Poly I:C (25 μ g/ml) or poly dA:dT/LyoVec (1 μ g/ml) (positive controls). **(d)** Analysis of cellular restriction factors expression in MDLC and MDDC after 24 hrs of IFN α treatment and **(e)** infection analysis of MDLC is shown ($n=4$) and **(f)** a representative analysis.

Figure 5. TLR agonists and TNF α regulate VSV-G HIV-GFP infection of LC. MDLC were pre-treated with TLR agonists: Pam3CSK4 (5 μ g/ml), poly I:C (25 μ g/ml) or TNF α (0.1 μ g/ml) for 8-16 hrs, then transduced with Vpx and infected with VSV-G HIV-GFP for 3 days. Cells were fixed, washed and GFP expression was analyzed by flow cytometry. **(a)** A representative infection profile for MDLC and **(b)** a graph representing the infection profile for each of 6 donors are depicted. The horizontal line represents the mean percentage of GFP positive cells. NI indicates non-infected cells, NT indicates non-treated cells. **(c)** Western blot analysis for expression of APOBEC3G and SAMHD1 after TNF α or PAM3CSK4 treatment is shown. **(d)**

Western blot showing activation of NF κ B pathway in stimulated MDLC and MDDC after 1 hour treatment with LPS or TNF α . * $p < 0.05$ and **** $p < 0.0001$.

Figure 6. A potent post-entry restriction to VSV-G HIV-GFP in MDLC is fully eliminated by the TGF β signalling inhibitor LY2109761. Monocytes obtained from the same donor were seeded for differentiation into MDDC (GM-CSF+IL4+ β -Mercaptoethanol (β 2M)) and MDLC (GM-CSF+IL4+TGF β). At day 0, 1, 3 and 5 of differentiation, cells were infected with VSV-G HIV-GFP in absence of SAMHD1 (+Vpx). (a) A schematic design of the experiment is presented. (b) Pooled data for MDLC and MDDC after 4 days is represented ($n=3$). (c) LY2109761-mediated inhibition of SMAD2 phosphorylation in MDLC at 5 or 10 μ M in two donors is shown. Actin served as loading control. (d) Pooled data ($n=3$) and (e) a representative infection of flow cytometry analysis of VSV-G HIV-GFP infection (\pm Vpx) of MDLC derived in the presence of LY2109761 (5 μ M) are shown. NI indicates non-infected cells. NT indicates non-treated cells. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Supplementary Methods

Human Skin Samples

Human skin samples were obtained from female patients undergoing mastectomy or breast reduction surgery with informed written patient consent and local ethical committee approval (South East Wales Research Ethics Committees Panel C, Reference: 08/WSE03/55). Human skin samples were transported following surgery as previously described (Pearson et al, 2010). Subcutaneous fat and excess lower dermis were removed by blunt dissection. The upper layers of the skin were subsequently removed using a dermatome set to a depth of 300 μm to collect the epidermis and upper papillary dermis. Skin sheets were cut into 1 cm^2 pieces and incubated with agitation in shaking water bath (at 175 strokes/minute) in RPMI containing collagenase A (10 mg/ml), DNase I (20 U/ml) and Dispase II (10 mg/ml) for 30 minutes at 37°C, after which the epidermis was mechanically separated from the dermis using forceps. Epidermal and dermal sheets were cultured separately in RPMI with 10% human AB serum and 1% Penicillin/Streptomycin/Fungizone solution (DC-RPMI) for 48 hours, after which migratory cells were collected from the media.

Dermal DC were positive for HLA-DR, the leukocyte marker CD45 and expressed CD11c, a dermal DC lineage marker. The expression of a typical mDC2 marker, Thrombomodulin/CD141/BDCA-3 (Chu et al, 2012), varied in different donor tissues, ranging from 4% to 19.6% of the cells isolated. All analysed epidermal walkout LC were HLA-DR⁺/CD1a⁺/Langerin⁺ (Figure 2a) and constituted relatively low percentage of total epidermal cells walkout population.

Virus stocks

All lentivectors and viruses were produced by calcium phosphate transfection of HEK293T cells (HEK293T cells) with corresponding plasmids as described previously (Blanchet et al, 2013). The VSV-G pseudotyped GFP expressing lentivector VSV-G HIV-GFP was obtained by co-transfection of pMD.G (vesicular stomatitis virus envelope protein, VSV-G) expression vector (Naldini et al, 1996), pR8.91 (gag-pol) expression vector (Naldini et al, 1996) and plox-EWdeltaSalGFP (a retroviral expression vector encoding green fluorescent protein) (Salmon et al, 2000). VSV-G-pseudotyped SIV3 lentivector encoding the Vpx gene (Nègre et al, 2000) was produced by co-transfection of pMD.G and SIV3⁺ packaging construct. Proviral plasmid pR8Bal, encoding HIV-1 R5 fully infectious virus was used for wild type HIV-1 virus production. A Lenti-X p24 titre ELISA kit (Clontech) was used to quantify HIV-1 p24gag accordingly to the manufacturer's instructions.

QPCR and Integration Assay

For MX2 and IFN β mRNA levels analysis, 1x10⁶ MDDC and MDLC per condition were used for RNA extraction using RNeasy Mini Kit (Qiagen) including DNase I treatment accordingly to manufacturer instructions. Isolated RNA was immediately reverse transcribed to cDNA by qPCRBio cDNA Synthesis Kit (PCR Biosystems) accordingly to manufacturer protocol. QPCR was performed on ViiA7 Real-Time PCR system ($\Delta\Delta$ Ct methods) using qPCRBio SyGreen Mix Lo-Rox (PCR Biosystems), IFN β forward primer 5'-AGCACAGGATGAACTTTGAC-3', and IFN β reverse primer 5'-TGATAGACATTAGCCAGGAG-3', MX2 forward primer 5'-AAGCAGTATCGAGGCAAGGA-3', MX2 reverse primer 5'-TCGTGCTCTGAACAGTTTGG-3' (Eurofins MWG Operon, Germany). Results were analysed using ExpressionSuite Software v1.0.3, and normalized to GAPDH expression (Hs_GAPDH_1_SG QuantiTect Prime primer, QIAGEN). The levels of early and late reverse transcription products and 2-LTR circles in cells were quantified by SYBR-green based qPCR

analysis using primer sets and protocol described previously (Anderson and Hope, 2008, Butler et al, 2001, Münk et al, 2002).

Lenti-X Provirus Quantitation Kit (Clontech) was used accordingly to the manufacturer instructions, to determine the copy number of integrated lentivectors in MDLC and MDDC.

Flow cytometry staining

Flow cytometry analysis was performed on cells fixed with 2% formaldehyde and intracellularly stained in Perm buffer (BD Biosciences) at 4°C for 30-45 minutes.

Analyses were performed on FACS Canto II (BD Biosciences) as previously reported (Blanchet et al, 2010).

Antibodies and TLR agonists

Antibodies used for flow cytometry were obtained from Beckman Coulter: anti-Langerin-PE, anti-p24gag-FITC; BD Bioscience: anti-DC-SIGN-APC; anti-CD1a-FITC, anti-CD141-PacificBlue, anti-CD45-PE and anti-HLA-DR-APC; Cambridge Biosciences: anti-CD11c-APC-Cy7; Miltenyi Biotechnology: anti-CD14-PerCP. Antibodies against SAMHD1 (Abcam), APOBEC3G (Abcam), actin (Millipore), MX2 (Santa Cruz Biotec), and BST2/tetherin (kindly provided by K. Strebel (Miyagi et al, 2009) were used for western blotting. For signalling studies all antibodies come from Cell Signalling. Goat anti-rabbit (Bio-Rad) and goat anti-mouse (Dako) conjugated to HRP were used for immunoblotting. TLR agonists were purchased from InvivoGen.

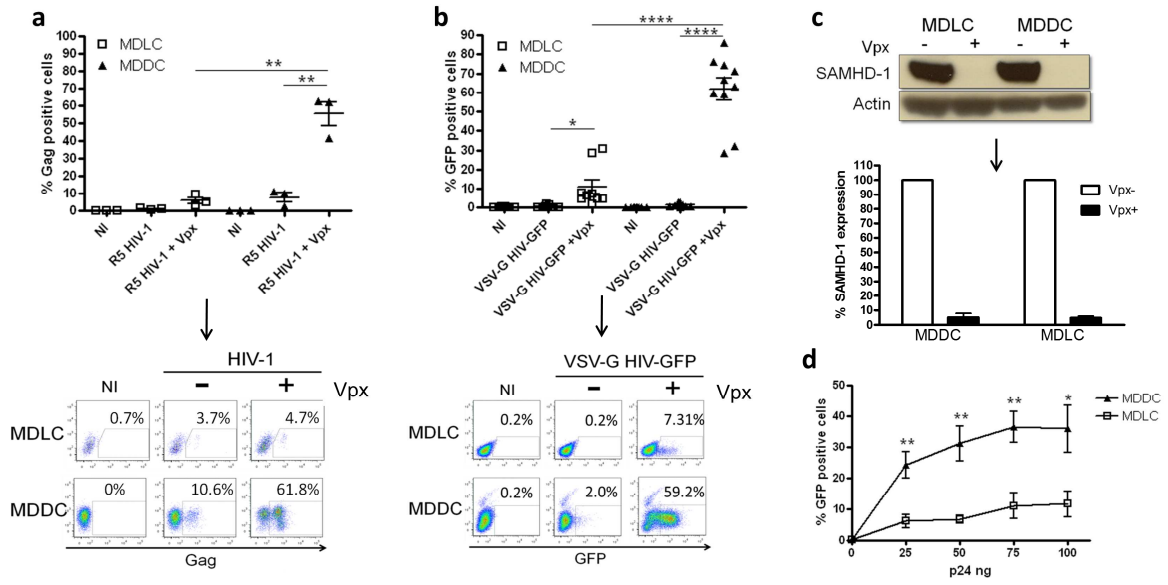
Immunoblotting

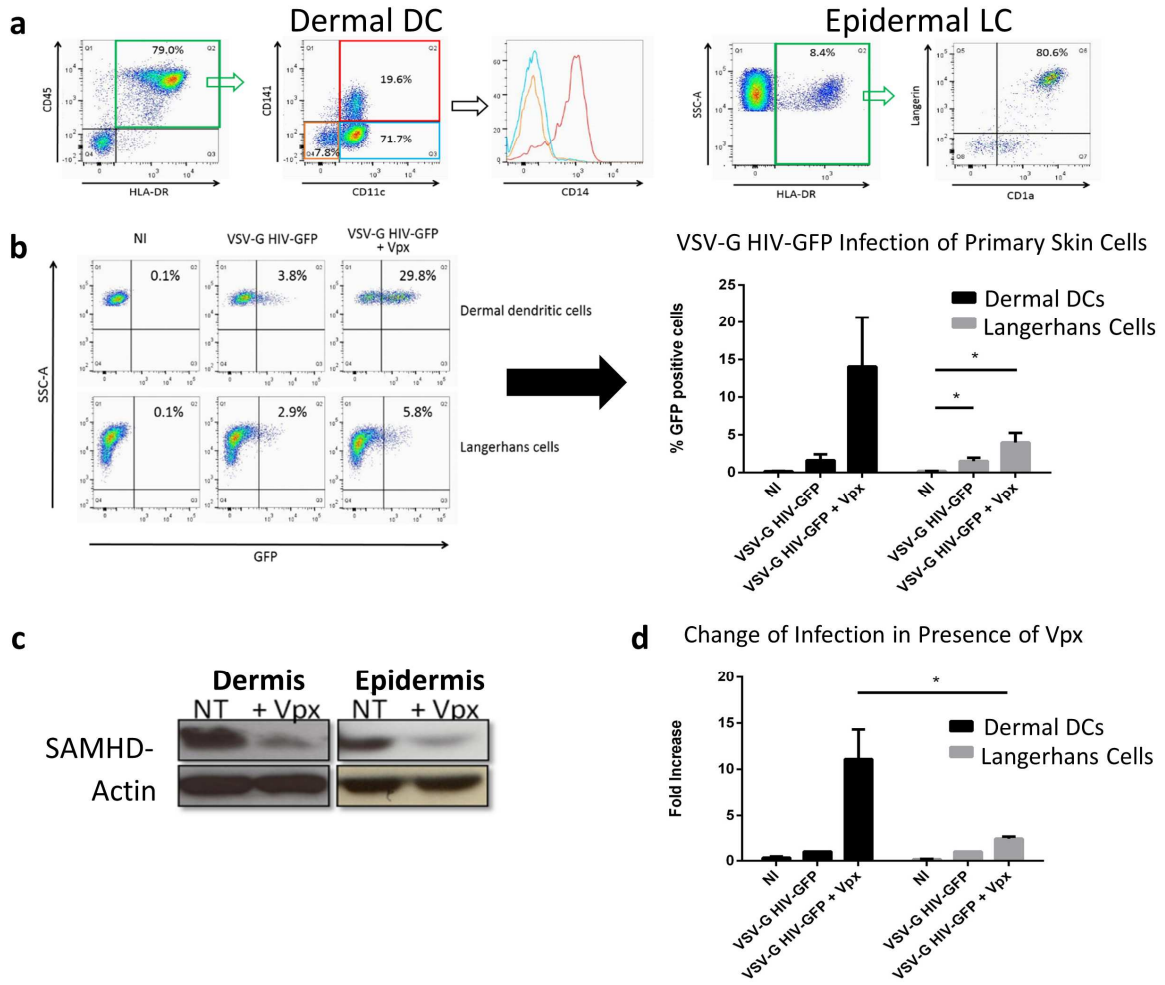
Cell lysates were prepared and Western blot analysis performed as described previously (Blanchet et al, 2010). Data were analysed and quantified using Image J.

Statistical analysis

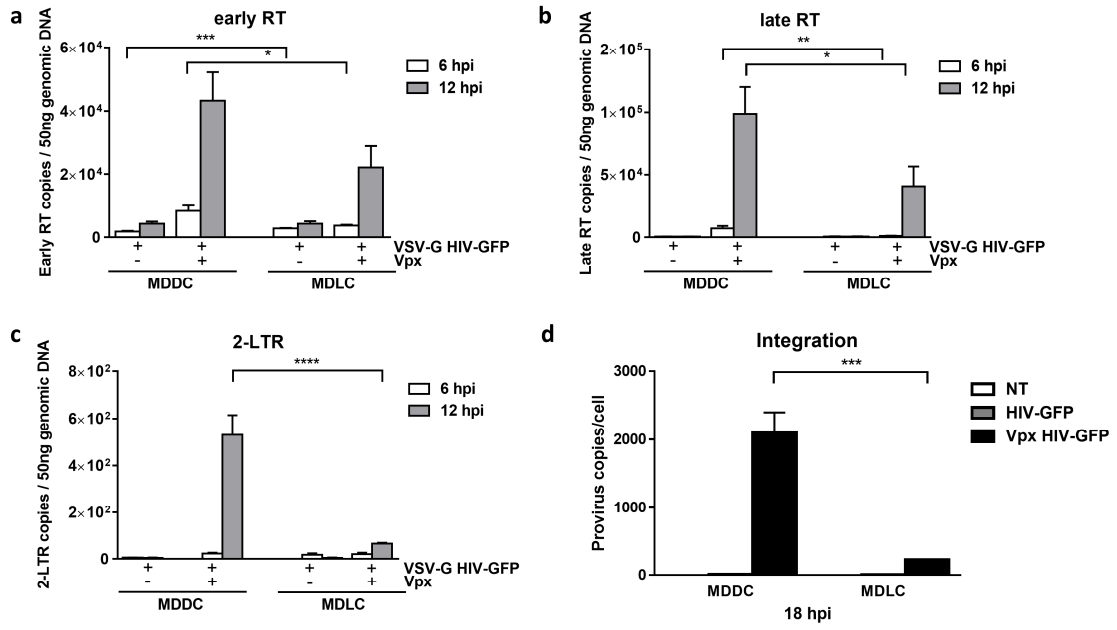
All results are displayed as means \pm standard errors of the means (SEM). Student's t-tests were used to evaluate the significance of differences between experimental groups.

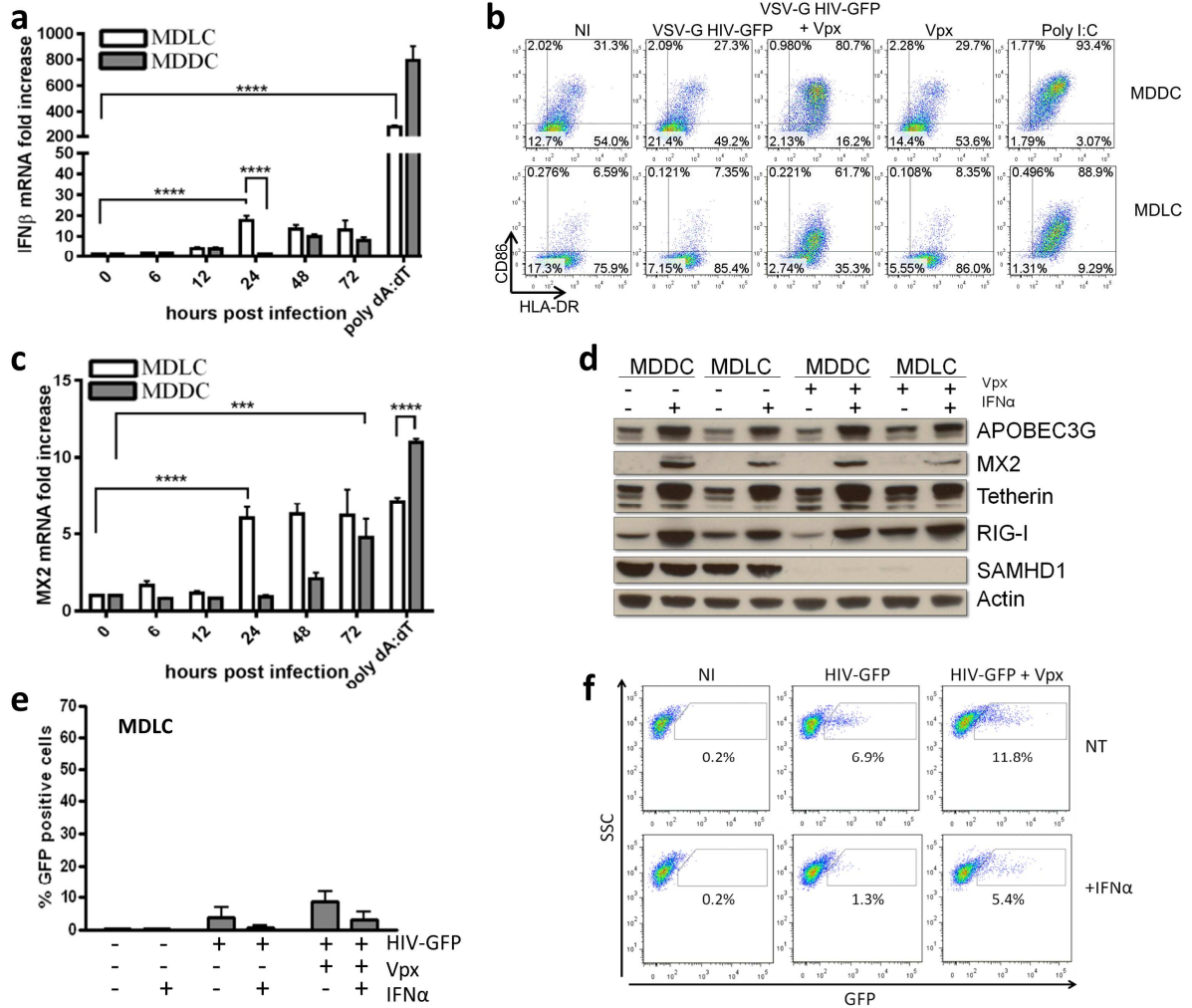
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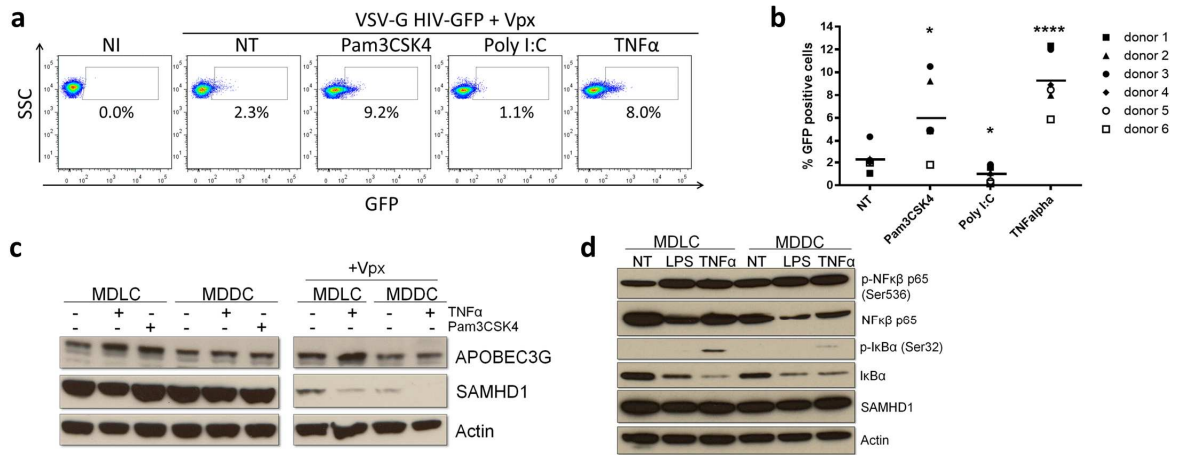




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