

HIV-1 exploits innate signaling by TLR8 and DC-SIGN for productive infection of dendritic cells

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Pattern-recognition receptors (PRRs) elicit antiviral immune responses to human immunodeficiency virus type 1 (HIV-1). Here we show that HIV-1 required signaling by the PRRs Toll-like receptor 8 (TLR8) and DC-SIGN for replication in dendritic cells (DCs). HIV-1 activated the transcription factor NF- κ B through TLR8 to initiate the transcription of integrated provirus by RNA polymerase II (RNAPII). However, DC-SIGN signaling was required for the generation of full-length viral transcripts. Binding of the HIV-1 envelope glycoprotein gp120 to DC-SIGN induced kinase Raf-1-dependent phosphorylation of the NF- κ B subunit p65 at Ser276, which recruited the transcription-elongation factor pTEF-b to nascent transcripts. Transcription elongation and generation of full-length viral transcripts was dependent on pTEF-b-mediated phosphorylation of RNAPII at Ser2. Inhibition of either pathway abrogated replication and prevented HIV-1 transmission. Thus, HIV-1 subverts crucial components of the immune system for replication that might be targeted to prevent infection and dissemination.

Dendritic cells (DCs) are crucial in the induction of antiviral immunity to human immunodeficiency virus type 1 (HIV-1)¹. DCs express pattern-recognition receptors (PRRs) that sense invading pathogens and trigger signaling pathways, which lead to type I interferon responses as well as adaptive immunity^{2,3}. PRRs such as Toll-like receptors (TLRs), C-type lectins and nucleotide-binding oligomerization domain proteins recognize highly conserved structures expressed on microorganisms called pathogen-associated molecular patterns^{4–6}. Both TLRs and C-type lectins are involved in HIV-1 recognition through different pathogen-associated molecular patterns. The endosomal receptors TLR7 and TLR8 (A002300) recognize HIV-1 single-stranded RNA (ssRNA) and induce activation of the transcription factor NF- κ B that is dependent on the adaptor MyD88 (refs. 7–9). Whereas TLR7 expressed by plasmacytoid DCs senses HIV-1 ssRNA and induces type I interferon responses that have a central role in HIV-1-induced activation of the immune system^{10,11}, little is known about the role of TLR8 in HIV-1 infection. C-type lectins such as DC-SIGN and langerin are also involved in HIV-1 recognition by binding the HIV-1 envelope glycoprotein gp120 (refs. 12,13). DC-SIGN–HIV-1 interactions modulate TLR-induced adaptive immune responses to HIV-1 (refs. 13–15). Data have shown that HIV-1 activates the kinase Raf-1 (A002008) through the DC-SIGN signalosome, which modulates cytokine responses to HIV-1 (ref. 15). Activation of the Rho guanine nucleotide-exchange factor LARG by DC-SIGN also facilitates the formation of DC–T cell infectious synapses, which enhances HIV-1 transmission¹⁶. It is unclear whether innate signaling induced by interactions of HIV-1 with PRRs on DCs is involved in HIV-1 infection.

DC infection by HIV-1 has an important role in the transmission of HIV-1. Sexual transmission of HIV-1 is the main route of infection worldwide^{17,18}. DCs are the first cells to encounter HIV-1 in the mucosal genital tissues¹⁹. Infected DCs migrate from those tissues to the lymph nodes, where HIV-1 is efficiently transmitted to CD4⁺ T cells^{19,20}. DC infection is essential to the long-term survival of HIV-1 (ref. 21). Moreover, increased HIV-1 replication in DC–T cell cocultures has been linked to the enhanced acquisition of HIV-1 observed in the STEP vaccine trial²², which further emphasizes the importance of HIV-1 replication in DCs. Thus, better understanding of HIV-1 replication in DCs is important for the prevention of HIV-1 infection and transmission.

HIV-1 replication is a tightly controlled process that requires host as well as viral factors^{23,24}. The binding of HIV-1 to CD4 and chemokine receptors such as CCR5 and CXCR4 (ref. 20) results in fusion with the cell membrane, which leads to viral uncoating, reverse transcription of HIV-1 ssRNA and subsequent integration of HIV-1 into the human genome²⁴. HIV-1 transcription is initiated from the 5' HIV-1 long terminal repeat (LTR) by cellular transcription factors such as Sp1 and NF- κ B^{25,26}. Assembly of the transcription complex at the LTR induces transcription initiation by RNA polymerase II (RNAPII). However, the generation of full-length HIV-1 transcripts requires that RNAPII proceed beyond transcription initiation to the elongation stage²⁷. HIV-1 encodes a transcription activator, Tat, that drives transcription elongation by recruiting the transcription-elongation factor pTEF-b²⁸. It has been shown that pTEF-b phosphorylates RNAPII at Ser2 in the C-terminal domain repeats, a modification that promotes

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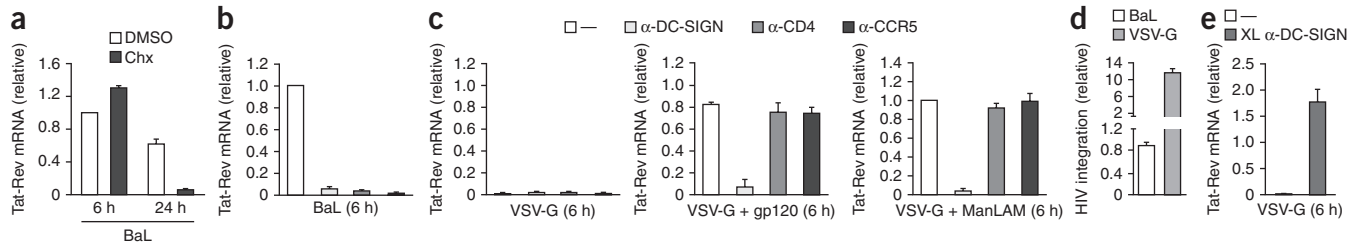


Figure 1 Binding of DC-SIGN by gp120 is essential for early HIV-1 transcription. **(a)** Quantitative real-time PCR analysis of Tat-Rev mRNA expression in DCs infected for 6 or 24 h with HIV-1 BaL in the presence (Chx) or absence (DMSO (dimethyl sulfoxide)) of the translation inhibitor cycloheximide. **(b)** Quantitative real-time PCR analysis of Tat-Rev mRNA expression in DCs infected for 6 h with CCR5-tropic HIV-1 (BaL) in the presence or absence (–) of blocking antibody to (α-) DC-SIGN, CD4 or CCR5. **(c)** Quantitative real-time PCR analysis of Tat-Rev mRNA expression in DCs infected for 6 h with VSV-G-pseudotyped HIV-1 (VSV-G) in the presence or absence of blocking antibodies as in **b** and simultaneously stimulated with the DC-SIGN ligands gp120 or ManLAM. **(d)** HIV-1 integration into DCs infected for 6 h with HIV-1 BaL or VSV-G-pseudotyped HIV-1, determined by Alu-PCR and presented relative to HIV-1 integration in HIV-1 BaL-infected cells, set as 1. **(e)** Quantitative real-time PCR analysis of Tat-Rev mRNA expression in DCs infected for 6 h with VSV-G-pseudotyped HIV-1 in the presence (XL α-DC-SIGN) or absence (–) of crosslinked anti-DC-SIGN (H-200). Tat-Rev mRNA expression (**a–c,e**) is presented relative to the expression of GAPDH (glyceraldehyde phosphate dehydrogenase). Data are representative of at least four (**a–c**) or two (**d,e**) independent experiments (mean and s.d.).

transcription elongation²⁹. In the absence of Ser2 phosphorylation, RNAPII is released from its HIV-1 DNA template shortly after initiation. This premature termination of transcription results in short RNAs, which are often found with latently integrated proviruses^{30,31}. Thus, Tat is crucial for HIV-1 transcription. Paradoxically, it is unclear how transcription elongation is initially induced and full-length transcripts are generated from the integrated provirus before Tat proteins are synthesized.

Here we demonstrate that HIV-1 subverts the innate signaling pathways by TLR8 and DC-SIGN for its replication in DCs and subsequent transmission to T cells. TLR8 triggering by HIV-1 ssRNA activated NF-κB, which was required for transcription initiation of the integrated HIV-1 genome by RNAPII. However, this stimulation led to abrogative transcription in the absence of a second signal required for transcription elongation. After binding of DC-SIGN by HIV-1 gp120, transcription elongation by RNAPII resulted in full-length HIV-1 transcripts and productive DC infection. DC-SIGN–HIV-1 interactions induced Raf-1-dependent phosphorylation of the NF-κB subunit p65 (A001645) at Ser276. Notably, p65 phosphorylated at Ser276 recruited pTEF-b to the LTR, which resulted in phosphorylation of RNAPII at Ser2. Inhibition of either TLR8- or DC-SIGN-induced signaling prevented productive HIV-1 infection of DCs and subsequent transmission of HIV-1 to T cells. Thus, HIV-1-induced innate signaling via TLR8 and DC-SIGN controls transcription initiation and elongation from the integrated provirus and enables HIV-1 replication in DCs.

RESULTS

HIV-1 replication requires DC-SIGN signaling by gp120

We investigated the role of innate signaling events in the generation of the first Tat transcripts early after HIV-1 infection. We infected immature DCs with small amounts of CCR5-tropic HIV-1 (HIV-1 BaL; multiplicity of infection, 0.1) and measured mRNA production of its early gene products, Tat and Rev. We observed that protein synthesis, and therefore *de novo* Tat protein, was not required for the generation of Tat transcripts early after infection, as the translation inhibitor cycloheximide did not inhibit the production of Tat-Rev mRNA during the first 6 h infection, whereas transcription was affected after 24 h (**Fig. 1a**). On DCs, HIV-1 interacts with CD4 and CCR5, which is essential for virus entry, and DC-SIGN, which functions as an attachment receptor to enhance the binding of HIV-1 to CD4 (refs. 32,33). The production of Tat-Rev mRNA after HIV-1 infection was inhibited by antibodies to DC-SIGN (anti-DC-SIGN), anti-CD4

and anti-CCR5 (**Fig. 1b**). As these antibodies most probably prevented the production of Tat-Rev mRNA by blocking viral entry, we used VSV-G-pseudotyped NL4.3-Δenv HIV-1, which does not contain HIV-1 envelope glycoproteins, to investigate the requirement for innate signaling via these receptors, as VSV-G envelope proteins do not interact with CD4, CCR5 or DC-SIGN³³. Notably, infection with VSV-G-pseudotyped virus did not result in any Tat-Rev transcripts early after infection (**Fig. 1c**), whereas we easily detected integration of VSV-G-pseudotyped HIV-1 into the host genome that was several-fold greater than of HIV-1 BaL, as determined by the Alu-PCR integration assay³⁴ (**Fig. 1d**). These data suggest that HIV-1 gp120 is required for HIV-1 transcription, as the entry and integration of VSV-G-pseudotyped HIV-1 occurred efficiently. Notably, infection of DCs with VSV-G-pseudotyped HIV-1 in the presence of recombinant HIV-1 gp120 induced substantial production of Tat-Rev mRNA that was inhibited by blocking antibodies to DC-SIGN but not by anti-CD4 or anti-CCR5 (**Fig. 1c**). Likewise, infection with VSV-G-pseudotyped virus in the presence of ManLAM, a mycobacterial mannose-specific ligand of DC-SIGN (**Fig. 1c**), or crosslinking of DC-SIGN by the activating DC-SIGN antibody H-200 (ref. 16; **Fig. 1e**) induced production of Tat-Rev mRNA. These data suggest that DC-SIGN-induced signaling by HIV-1 is required for the initial production of Tat mRNA and therefore early HIV-1 replication in DCs.

HIV-1 replication requires Raf-1 signaling

DC-SIGN triggering by HIV-1 and *Mycobacterium tuberculosis* induces a Raf-1-dependent signaling pathway to tailor adaptive immunity^{14,15}. Therefore, we investigated whether Raf-1 is involved in HIV-1 replication. Inhibition of Raf-1 by the small-molecule inhibitor GW5074 completely blocked Tat-Rev mRNA production in DCs infected with CCR5-tropic HIV-1 (BaL) or CXCR4-tropic HIV-1 (LAI), even after 24 h (**Fig. 2a**). Raf-1 inhibition did not affect HIV-1 integration (**Fig. 2b**). To confirm specificity of the Raf inhibitor, we silenced Raf-1 in DCs by RNA-mediated interference (RNAi; **Supplementary Fig. 1**). Similar to Raf-1 inhibition by GW5074, production of Tat-Rev mRNA was abrogated in DCs in which Raf-1 was silenced (**Fig. 2c**). These data demonstrate that Raf-1 signaling is required for early transcription of integrated HIV-1.

Recombinant gp120 and ManLAM, both DC-SIGN ligands, induce phosphorylation of Raf-1 at Ser338 and Tyr340–Tyr341 (refs. 14,15), which is required for Raf-1 activation³⁵. Similarly, infection of DCs with CCR5- or CXCR4-tropic HIV-1 induced Raf-1 phosphorylation,

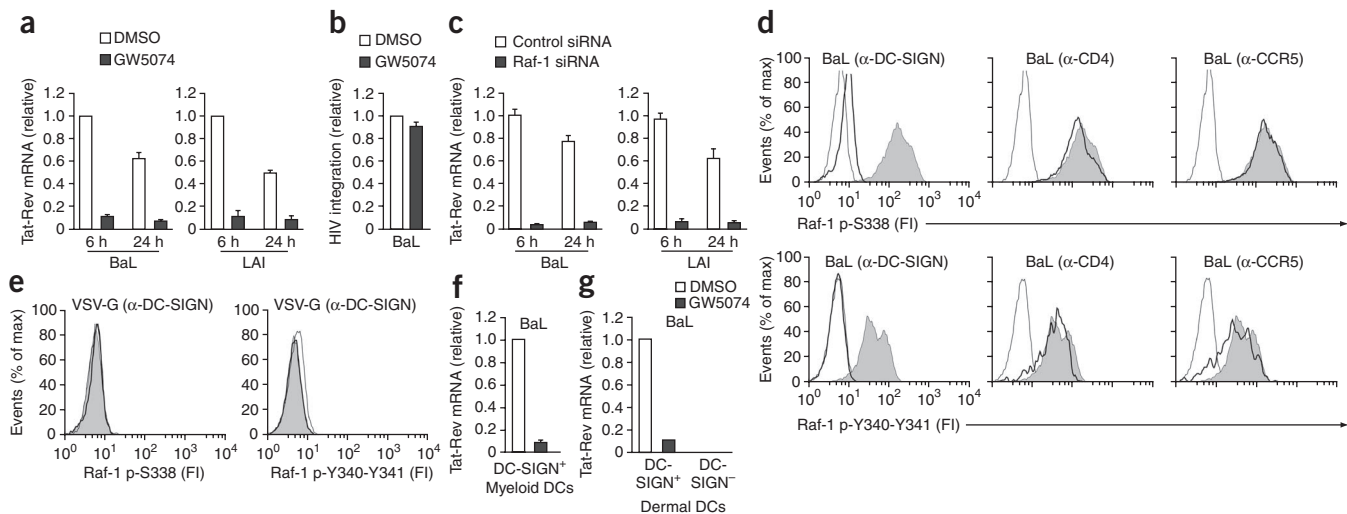


Figure 2 Raf-1 signaling by HIV-1-DC-SIGN interactions is essential for HIV-1 replication. **(a)** Tat-Rev mRNA expression in DCs infected for 6 or 24 h with CCR5-tropic HIV-1 (BaL) or CXCR4-tropic HIV-1 (LAI) in the presence or absence of the Raf-1 inhibitor GW5074, assessed as described in **Figure 1a**. **(b)** HIV-1 integration into DCs infected with HIV-1 BaL in the presence or absence of GW5074, determined by Alu-PCR as described in **Figure 1d**. **(c)** Tat-Rev mRNA expression in DCs treated with Raf-1-specific or control small interfering RNA (siRNA) and infected for 6 or 24 h with HIV-1 BaL or HIV-1 LAI, assessed as described in **Figure 1a**. **(d, e)** Flow cytometry analysis of Raf-1 phosphorylation at Ser338 (p-S338; **d**) or Tyr340-Tyr341 (p-Y340-Y341; **e**) in unstimulated DCs (thin lines) or DCs stimulated for 10 min with HIV-1 BaL (**d**) or HIV-1 LAI (**e**) in the presence (thin lines) or absence (filled histograms) of blocking antibodies (in parentheses above); FI, fluorescence intensity; max, maximum. **(f, g)** Tat-Rev mRNA expression in DC-SIGN⁺ myeloid DCs (**f**) and DC-SIGN⁺ and DC-SIGN⁻ dermal DCs (**g**) infected for 6 h with HIV-1 BaL in the presence or absence of GW5074, assessed as described in **Figure 1a**. Data are representative of at least six (**a, c**) or two (**b, d-f**) independent experiments (mean and s.d., **a-c**) or one independent experiment (**g**).

and blocking antibodies to DC-SIGN inhibited Raf-1 phosphorylation, but blocking antibodies to CD4 or CCR5 did not (**Fig. 2d** and **Supplementary Fig. 2**). As expected, VSV-G-pseudotyped virus did not induce Raf-1 phosphorylation (**Fig. 2e**), as it does not bind to DC-SIGN. Thus, HIV-1 infection leads to Raf-1 activation through the interaction of HIV-1 gp120 with DC-SIGN.

We next isolated DC-SIGN⁺ myeloid and dermal DCs from blood and skin, respectively, to investigate whether HIV-1 replication in primary DC populations is similarly dependent on Raf-1-induced

signaling. Notably, Raf-1 inhibition in both DC-SIGN⁺ myeloid DCs and DC-SIGN⁺ dermal DCs abrogated the production of Tat-Rev mRNA (**Fig. 2f, g**), whereas a DC-SIGN⁻ dermal DC population was not productively infected by HIV-1 (**Fig. 2g**). These data support the idea of an important role for DC-SIGN signaling in HIV-1 replication.

HIV-1 induces NF- κ B activation through TLR8

We next investigated NF- κ B activation, as Raf-1 signaling has been shown to modulate NF- κ B activity^{14,15}, whereas NF- κ B has

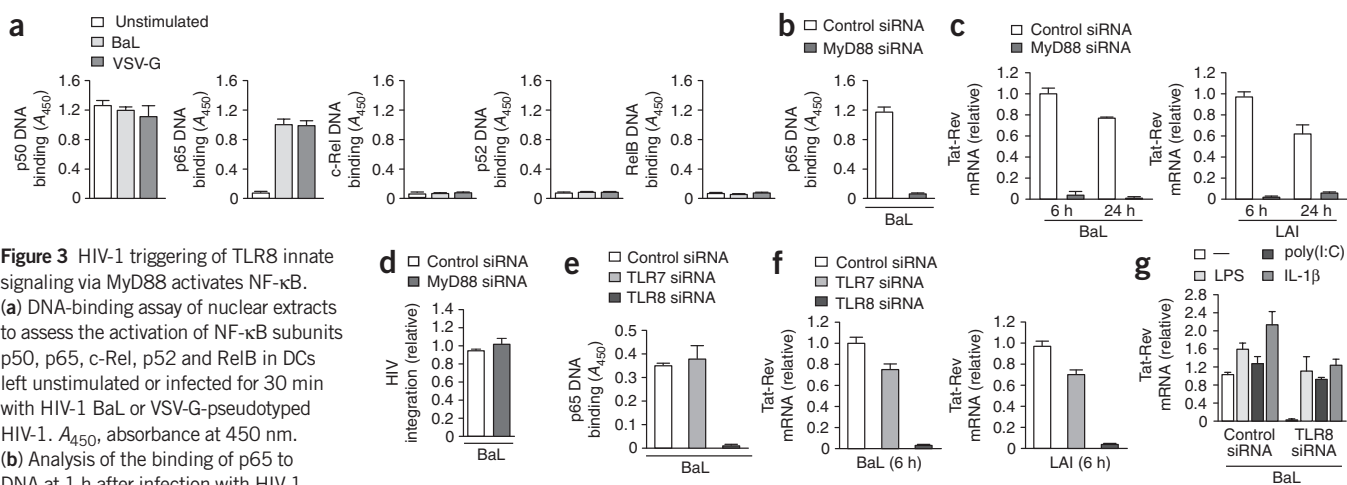
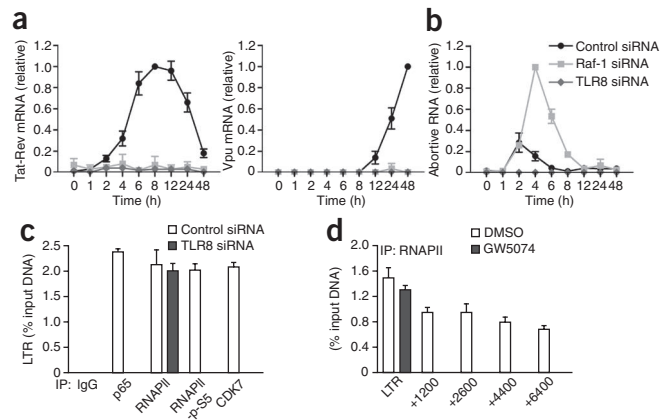


Figure 3 HIV-1 triggering of TLR8 innate signaling via MyD88 activates NF- κ B.

(a) DNA-binding assay of nuclear extracts to assess the activation of NF- κ B subunits p50, p65, c-Rel, p52 and RelB in DCs left unstimulated or infected for 30 min with HIV-1 BaL or VSV-G-pseudotyped HIV-1. A_{450} , absorbance at 450 nm. **(b)** Analysis of the binding of p65 to DNA at 1 h after infection with HIV-1 BaL in nuclear extracts of DCs treated with control or MyD88-specific siRNA. **(c)** Tat-Rev mRNA expression in DCs infected with HIV-1 BaL or HIV-1 LAI and treated with control or MyD88-specific siRNA, assessed as described in **Figure 1a**. **(d)** HIV-1 integration in DCs treated with control or MyD88-specific siRNA, detected as described in **Figure 1d**. **(e)** Analysis of the binding of p65 to DNA at 1 h after infection with HIV-1 BaL in nuclear extracts of DCs treated with control or TLR7- or TLR8-specific siRNA. **(f)** Tat-Rev mRNA expression in DCs infected with HIV-1 BaL or HIV-1 LAI and treated with control or TLR7- or TLR8-specific siRNA, assessed as described in **Figure 1a**. **(g)** Tat-Rev mRNA expression in DCs treated with control or TLR8-specific siRNA and infected for 6 h with HIV-1 BaL and costimulated with the TLR4 agonist lipopolysaccharide (LPS), the TLR3 agonist poly(I:C) or the interleukin 1 receptor ligand IL-1 β , assessed as described in **Figure 1a**. Data are representative of at least two (**a, b, d, e**), three (**g**) or four (**c, f**) independent experiments (mean and s.d.).

Figure 4 HIV-1 exploits TLR8 signaling for transcription initiation, whereas DC-SIGN signaling is essential for transcription elongation by RNAPII. (**a,b**) Quantitative real-time PCR analysis of early Tat-Rev and late Vpu mRNA (**a**) and short (abortive) RNA (**b**) in cells infected with HIV-1 BaL and treated with control or Raf-1- or TLR8-specific siRNA, presented relative to GAPDH expression (**a**) or 18S rRNA expression (**b**); expression at the time of maximum expression of each RNA is set as 1. (**c**) ChIP assay of the recruitment of NF- κ B, RNAPII, RNAPII phosphorylated at Ser5 (RNAPII-p-S5), and CDK7 to the HIV-1 LTR (% DNA input) in DCs infected for 4 h with HIV-1 BaL and treated with control or TLR8-specific siRNA. IgG, immunoglobulin G (negative control). (**d**) ChIP assay of the presence of RNAPII (% DNA input) at various positions across the integrated HIV-1 genome (LTR; distance in base pairs from the transcription-initiation site along horizontal axis) in DCs infected for 4 h with HIV-1 BaL in the presence of absence of GW5074. Data are representative of two independent experiments (mean and s.d.).



been linked to HIV-1 replication by binding to the LTR²⁶. We first determined whether HIV-1 infection induced NF- κ B activation. Infection with HIV-1 or VSV-G-pseudotyped HIV-1 activated NF- κ B dimers consisting of subunits p50 (A002937) and p65 (**Fig. 3a**). HIV-1 ssRNA can activate NF- κ B through the endosomal MyD88-dependent receptors TLR7 and TLR8 (refs. 7–9). Silencing MyD88 by RNAi (**Supplementary Fig. 3**) abrogated NF- κ B activation (**Fig. 3b**), which supports the idea of a role for TLR7 and/or TLR8. Notably, silencing of MyD88 abrogated HIV-1 replication (**Fig. 3c**) but did not interfere with HIV-1 entry and subsequent integration (**Fig. 3d**). We next silenced both TLR7 and TLR8 by RNAi (**Supplementary Fig. 3**). Silencing TLR8 completely abrogated both activation of p65 (**Fig. 3e**) and production of Tat-Rev mRNA (**Fig. 3f**). In contrast, even though TLR7 is expressed by DCs, silencing TLR7 did not affect p65 activation (**Fig. 3e**) and resulted in only slightly less production of Tat-Rev mRNA after infection (**Fig. 3f**). Cytokine production in response to the TLR7-specific ligand R-837 (imiquimod) was completely abrogated in DCs in which TLR7 was silenced but not those in which TLR8 was silenced (**Supplementary Fig. 3**). These data demonstrate that TLR8, but not TLR7, specifically recognizes HIV-1 ssRNA during HIV-1 infection; thus, HIV-1 exploits TLR8 signaling to activate NF- κ B for the production of Tat-Rev mRNA.

We next investigated whether other NF- κ B-inducing stimuli could substitute for HIV-1-induced TLR8-mediated activation of NF- κ B. The TLR3 agonist poly(I:C) and TLR4 agonist lipopolysaccharide, as well as the interleukin 1 receptor ligand IL-1 β , restored the production of Tat-Rev mRNA in DCs in which TLR8 was silenced after HIV-1 infection (**Fig. 3g**), further emphasizing the idea that TLR8 activation by HIV-1 is required for NF- κ B activation. Notably, both HIV-1 and VSV-G-pseudotyped HIV-1 induced NF- κ B activation (**Fig. 3a**), but VSV-G-pseudotyped HIV-1 did not induce early production of Tat-Rev mRNA in the absence of DC-SIGN signaling (**Fig. 1c**). These data show that although TLR8-MyD88-dependent activation of NF- κ B was required for Tat mRNA production, Raf-1 signaling by DC-SIGN was equally essential for HIV-1 transcription (**Fig. 2a,c**).

HIV-1 exploits TLR8 and DC-SIGN for transcription

To elucidate the contributions of the TLR8 and DC-SIGN pathways to HIV-1 transcription, we silenced TLR8 and Raf-1 in DCs by RNAi and measured both Tat-Rev mRNA and short RNAs as markers of productive and abortive transcription, respectively. In DCs infected with HIV-1, we detected Tat-Rev transcripts (a marker of productive transcription) as early as 4 h after infection (**Fig. 4a**). Tat-Rev mRNA abundance peaked between 8 and 12 h after infection, after which expression decreased gradually because of nuclear export of unspliced or singly

spliced transcripts by Rev³⁶ (**Fig. 4a**). This result was supported by the appearance of singly spliced transcripts of the late gene *Vpu*²⁷ at 12 h after infection, which increased with time and is indicative of productive infection (**Fig. 4a**). We also detected transient accumulation of short RNAs (a marker of abortive transcription); this peaked at 2 h after infection and gradually decreased thereafter (**Fig. 4b**). In DCs in which TLR8 was silenced, we did not observe any HIV-1 transcription, including abortive transcription (**Fig. 4a,b**), which demonstrated that NF- κ B activation by TLR8 triggering is an absolute prerequisite for HIV-1 transcription initiation by RNAPII. We observed the same effect in DCs in which MyD88 was silenced (data not shown). Notably, in DCs in which Raf-1 was silenced, the production of both Tat-Rev and Vpu mRNA was completely abrogated (**Fig. 4a**), but we observed production of short RNAs that was more than fourfold greater (**Fig. 4b**). The increase in short RNAs without the formation of Tat-Rev mRNA reflects transcription initiation in the absence of transcription elongation. The transient induction of short RNAs after Raf-1 silencing probably reflected transient TLR8-mediated activation of NF- κ B. These data suggest that whereas activation of NF- κ B by TLR 8 is required for transcription initiation, DC-SIGN-mediated activation of Raf-1 is crucial for transcription elongation.

To elucidate how TLR8 signaling induces initiation of transcription of the HIV-1 provirus, we did a chromatin-immunoprecipitation (ChIP) assay to assess the recruitment of p65 and RNAPII to the LTR, as well as phosphorylation of the RNAPII C-terminal domain at Ser5, a modification that is an absolute requirement for transcription initiation²⁹. We detected binding of p65 to the LTR after TLR8 triggering by HIV-1, whereas RNAPII recruitment did not require TLR8 signaling (**Fig. 4c**). However, phosphorylation of RNAPII at Ser5 was completely absent in cells in which TLR8 was silenced (**Fig. 4c**). The general transcription factor TFIID has a vital role in transcription initiation through the phosphorylation of RNAPII at Ser5 by its cyclin-dependent kinase CDK7–cyclin H subcomplex²⁹. Recruitment of CDK7 to the LTR coincided with p65 recruitment and phosphorylation of RNAPII at Ser5 and was similarly abrogated by silencing of TLR8 (**Fig. 4c**). These data suggest that TLR8-induced binding of p65 to the HIV-1 LTR is required for the recruitment of TFIID to phosphorylate RNAPII at Ser5 and initiate transcription.

To further investigate the role of Raf-1 activation in transcription elongation, we determined the progress of RNAPII across the integrated provirus by ChIP assay. We detected RNAPII over the whole 10-kilobase HIV-1 genome at 4 h after infection (**Fig. 4d**). Notably, Raf-1 inhibition prevented RNAPII progress and transcription elongation, as we did not detect RNAPII beyond the LTR (**Fig. 4d**); this supported the finding of greater abortive transcription and absence of productive transcription

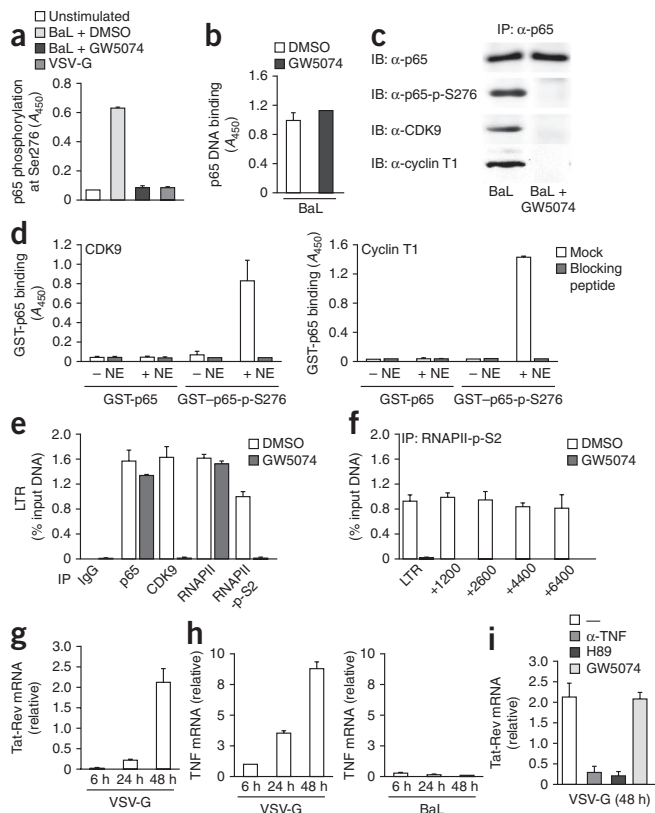


Figure 5 HIV-1-induced phosphorylation of p65 at Ser276 is essential for pTEF-b recruitment and transcription elongation. (a) ELISA of p65 phosphorylation at Ser276 in DCs infected for 30 min with HIV-1 BaL or VSV-G-pseudotyped HIV-1 in the presence or absence of GW5074. (b) Analysis of p65 DNA binding in nuclear extracts of DCs treated with GW5074, assessed 30 min after infection with HIV-1 BaL. (c) Immunoblot analysis (IB) of the association of CDK9 and cyclin T1 (pTEF-b) with p65 after coimmunoprecipitation (IP) from nuclear extracts (NE) of HIV-1 BaL-infected DCs with anti-p65 in the presence or absence of GW5074. (d) Precipitation of CDK9 and cyclin T1 (pTEF-b) from DC nuclear extracts with a fusion of GST and nonphosphorylated p65 (GST-p65) or p65 phosphorylated at Ser276 (GST-p65-p-S276) in the presence or absence (Mock) of a blocking peptide containing the phosphorylated Ser276 sequence of p65. (e) ChIP assay of the presence of RNAPII phosphorylated at Ser2 across the integrated HIV-1 genome, as described in **Figure 4d**. (g,h) Expression of Tat-Rev mRNA (g) or TNF mRNA (h) in DCs infected for 6, 24 or 48 h with VSV-G-pseudotyped HIV-1 (g,h) or HIV-1 BaL (h), assessed as described in **Figure 1a**. (i) Tat-Rev mRNA expression in DCs infected for 48 h with HIV-1 VSV-G in the presence or absence of the MSK1 inhibitor H89, neutralizing antibodies to TNF, or GW5074, assessed as described in **Figure 1a**. Data are representative of three (a,c-e) or two (b,f-i) independent experiments (mean and s.d.).

of HIV-1 infection, the LTR of integrated HIV-1 was occupied by p65, CDK9 and RNAPII (**Fig. 5e**); 62% \pm 7% of the RNAPII recruited to the LTR was phosphorylated at Ser2, a marker of elongating RNAPII (**Fig. 5e**). Inhibition of Raf-1 prevented the recruitment of CDK9 and consequently abrogated the phosphorylation of RNAPII at Ser2, although recruitment of both p65 and RNAPII to the LTR was unaffected (**Fig. 5e**). Next we tracked the progression of RNAPII phosphorylated at Ser2 over the HIV-1 genome by ChIP assay. RNAPII phosphorylated at Ser2 was present throughout the whole HIV-1 genome, whereas inhibition of Raf-1 abrogated the progression of RNAPII beyond the LTR (**Fig. 5f**). Thus, our data demonstrate that HIV-1-induced signaling by DC-SIGN is essential for Raf-1-mediated phosphorylation of p65 at Ser276, which recruits pTEF-b to the HIV-1 LTR to phosphorylate RNAPII at Ser2. Phosphorylation of RNAPII at Ser2 drives the transcription elongation and production of HIV-1 Tat transcripts.

Although our data demonstrated that VSV-G-pseudotyped HIV-1 did not induce the production of Tat-Rev mRNA after 6 h because of the lack of DC-SIGN signaling (**Fig. 1c**), VSV-G-pseudotyped HIV-1 has been shown to infect DCs³⁹. Therefore, we monitored the production of Tat-Rev mRNA after infection with VSV-G-pseudotyped HIV-1 over time. We detected small amounts of Tat-Rev transcripts at 24 h after infection, which increased considerably by 48 h after infection (**Fig. 5g**). These data suggest that the induction of Tat-Rev transcripts is a secondary effect. As infection with VSV is known to induce substantial production of tumor necrosis factor (TNF)⁴⁰ and as TNF-induced signaling also induces phosphorylation of p65 at Ser276 (ref. 41), we hypothesized that infection with VSV-G-pseudotyped HIV-1 might indirectly activate its own replication through the production of TNF. Indeed, we observed considerable TNF production after infection of DCs with VSV-G-pseudotyped HIV-1 but not after infection with wild-type HIV-1 (**Fig. 5h**). Notably, neutralizing anti-TNF, but not Raf-1 inhibition, blocked late production of Tat-Rev mRNA (**Fig. 5i**), which demonstrated that TNF is required for productive transcription after infection with VSV-G-pseudotyped HIV-1. TNF-induced phosphorylation of p65 at Ser276 involves a pathway dependent on the kinase MSK1 but independent of Raf-1 (ref. 41). The MSK1 inhibitor H89 abrogated HIV-1 transcription after infection with VSV-G-pseudotyped HIV-1 (**Fig. 5h**). These data further support the idea of a crucial role for phosphorylation of p65 at Ser276 in HIV-1 replication.

after Raf-1 silencing (**Fig. 4a,b**). Thus, DC-SIGN-mediated activation of Raf-1 is crucial for transcription elongation by RNAPII.

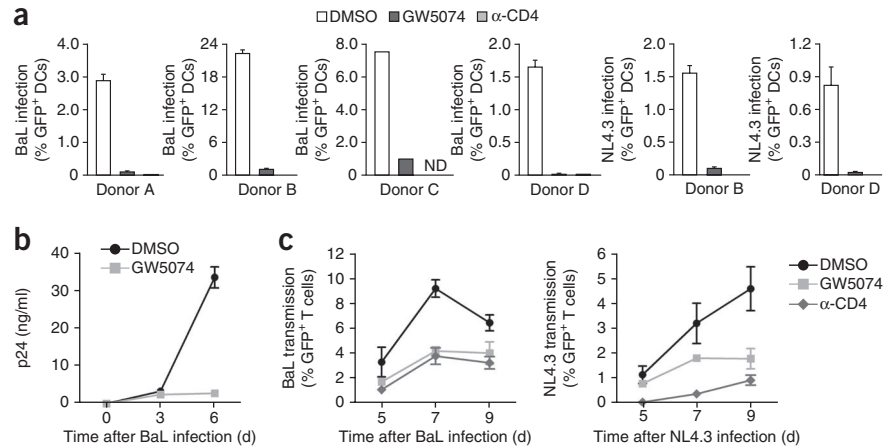
HIV-1 recruits pTEF-b via p65 phosphorylation

Recruitment of pTEF-b to the HIV-1 LTR is essential for transcription elongation²⁸. It is known that pTEF-b consists of cyclin T1 and the cyclin-dependent kinase CDK9, which phosphorylates the RNAPII C-terminal domain at Ser2; this promotes transcription elongation²⁹. We hypothesized that DC-SIGN signaling induces transcription elongation through the Raf-1 pathway by inducing recruitment of pTEF-b to the HIV-1 LTR. Raf-1 activation leads to phosphorylation of p65 at Ser276 (refs. 14,37), which has been shown to recruit pTEF-b to specific cytokine promoters³⁸. We found that HIV-1 infection induced phosphorylation of p65 at Ser276, which was abrogated by Raf-1 inhibition (**Fig. 5a**). Raf-1 inhibition did not interfere with p65 activation (**Fig. 5b**), which suggests that HIV-1 induces phosphorylation of p65 at Ser276 through Raf-1. DC infection with VSV-G-pseudotyped HIV-1 did not result in phosphorylation of p65 at Ser276 (**Fig. 5a**), as VSV glycoprotein did not bind to DC-SIGN to induce Raf-1 activation (**Fig. 2e**).

We next investigated whether pTEF-b is recruited to p65 phosphorylated at Ser276. Both CDK9 and cyclin T1 immunoprecipitated together with p65 phosphorylated at Ser276 in experiments using anti-p65 from nuclear extracts of HIV-1-infected DCs, whereas this association was abrogated by Raf-1 inhibition (**Fig. 5c**). Furthermore, a fusion of glutathione S-transferase (GST) and p65 phosphorylated at Ser276, in contrast to a fusion of GST and nonphosphorylated p65, precipitated both cyclin T1 and CDK9 from DC nuclear extracts, and this was inhibited by a p65-derived peptide containing the phosphorylated Ser276 sequence (**Fig. 5d**). These data demonstrate that phosphorylation of p65 at Ser276 is required for the binding of pTEF-b to p65 after HIV-1 infection.

We next used ChIP assays to demonstrate that p65 phosphorylated at Ser276 recruits pTEF-b to the HIV-1 LTR early after infection. After 4 h

Figure 6 Raf-1 activation by HIV-1 is required for productive DC infection and transmission to CD4⁺ T cells. (a) Flow cytometry of GFP⁺ cells among DCs obtained from donors A–D, assessed 5 d after infection of cells with eGFP-expressing HIV-1 BaL or HIV-1 NL4.3 in the presence or absence of GW5074 or CD4-blocking antibodies (to assess HIV-1 infection of DCs). ND, not determined. (b) ELISA of p24 in DCs infected with HIV-1 BaL in the presence or absence of GW5074 (to assess virus production). (c) Flow cytometry of GFP⁺ T cells among DCs infected for 48 h with eGFP-expressing HIV-1 BaL or HIV-1 NL4.3 in the presence or absence of GW5074 or CD4-blocking antibodies, then cultured with CD4⁺ cell-enriched T cells (to assess HIV-1 transmission to T cells). Data are representative of four independent experiments (mean and s.d. of triplicate measurements).



DC-SIGN signaling is required for HIV-1 infection

We next investigated whether Raf-1 signaling is essential to productive DC infection. We inoculated DCs with CCR5-tropic (BaL) or CXCR4-tropic (NL4.3) HIV-1 strains containing a gene encoding enhanced green fluorescent protein (eGFP). Productive infection with these strains leads to eGFP expression⁴²; we assessed infection by flow cytometry after 5 d. DCs were productively infected with both CCR5- and CXCR4-tropic HIV-1, as measured by eGFP expression, although the efficiency was donor dependent (Fig. 6a). As observed for Tat-Rev mRNA production (Fig. 2a,c), DC infection was completely blocked by Raf-1 inhibition, similar to blocking by anti-CD4 (Fig. 6a). Similarly, viral p24 production by infected DCs was completely abrogated by Raf-1 inhibition (Fig. 6b). Thus, DC-SIGN-dependent Raf-1 signaling is required for productive infection of DCs.

DC infection is involved in HIV-1 dissemination, as infected DCs efficiently transmit HIV-1 to T cells^{19,20}. Therefore, we investigated whether Raf-1 signaling is involved in HIV-1 transmission by DCs. We incubated DCs for 48 h with CCR5- or CXCR4-tropic HIV-1 expressing eGFP in the presence or absence of Raf-1 inhibitor or anti-CD4. We stringently washed away unbound virus, antibodies and inhibitor before adding target CD4⁺ T cells and measured HIV-1 transmission to T cells over time by flow cytometry. Both CCR5- and CXCR4-tropic HIV-1 were transmitted by DCs to T cells (Fig. 6c). Notably, Raf-1 inhibition and blocking antibodies to CD4 blocked the transmission of HIV-1 to T cells to a similar extent (Fig. 6c). Together these data demonstrate that DC-SIGN-mediated Raf-1 signaling is crucial for the productive infection of DCs and effective transmission of HIV-1 to T cells.

Coinfection enhances HIV-1 transcription via Raf-1

M. tuberculosis and *Candida albicans* trigger Raf-1 signaling through C-type lectins, which leads to phosphorylation of p65 at Ser276; this

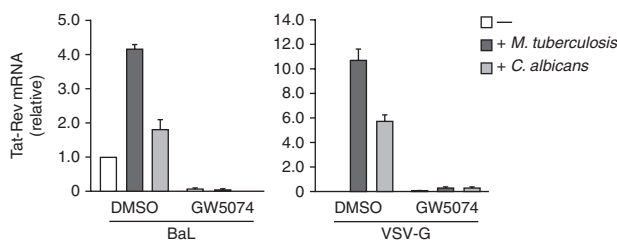


Figure 7 Coinfection enhances HIV-1 replication via Raf-1 signaling pathways. Tat-Rev mRNA expression in DCs coinfecting for 6 h with HIV-1 BaL or HIV-1 VSV-G in combination with *M. tuberculosis* or *C. albicans* in the presence or absence of GW5074, assessed as described in Figure 1a. Data are representative of at least two independent experiments (mean and s.d.).

shapes adaptive immune responses^{15,37}. Coinfection with *M. tuberculosis* or *C. albicans* increases HIV-1 replication in cells from patients with AIDS both *in vitro* and *in vivo*^{43–45}, whereas infection with *C. albicans* has also been shown to increase HIV-1 transmission⁴⁶. Therefore, we investigated whether these pathogens increase HIV-1 replication in DCs through Raf-1-dependent signaling. Infection with *M. tuberculosis* or *C. albicans* resulted in more Tat-Rev mRNA production after infection of DCs with HIV-1 (Fig. 7). Notably, HIV-1 transcription was completely abrogated by Raf-1 inhibition (Fig. 7), which demonstrates that *M. tuberculosis* and *C. albicans* induce Raf-1-dependent signaling to enhance HIV-1 replication. Infection of DCs with VSV-G-pseudotyped HIV-1 did not result in early production of Tat-Rev mRNA at 6 h after infection, as observed before (Fig. 1c), but coinfection with either *M. tuberculosis* or *C. albicans* strongly induced HIV-1 transcription, which was abrogated by Raf-1 inhibition (Fig. 7). We obtained similar results with DC-SIGN⁺ myeloid DCs infected with HIV-1 (Supplementary Fig. 4). These data suggest that innate signaling is not only required for HIV-1 infection but is also involved in enhancing HIV-1 transcription after microbial coinfection.

DISCUSSION

Innate signaling by PRRs is crucial to the induction of antiviral responses to HIV-1 (refs. 10,15). Here we have demonstrated that HIV-1 subverted the innate signaling pathways induced via TLR8 and DC-SIGN for its replication in DCs and subsequent transmission to T cells. HIV-1 infection led to the TLR8 triggering by HIV-1 ssRNA that is required for transcription initiation from the integrated provirus. The HIV-1 envelope glycoprotein gp120 induced a second signal through its interaction with DC-SIGN, mediated by Raf-1 signaling, that was crucial for transcription elongation and productive HIV-1 transcription. Notably, coinfection with *M. tuberculosis* or *C. albicans* induced similar Raf-1 signaling that enhanced HIV-1 replication. Our data have shown that DC-SIGN-dependent signaling was important not only for infection of monocyte-derived DCs with HIV-1 but also for infection of primary DC-SIGN⁺ DCs in blood and peripheral tissues with HIV-1. The identification of two host signaling pathways exploited by HIV-1 for its replication might provide a molecular basis for strategies to prevent HIV-1 transmission.

Effective immunity to pathogens requires the induction of the differentiation of helper T cells, which is determined by specific cytokine profiles^{1,2,6}. PRR signaling is crucial in the induction and tightly control of cytokine expression⁶. Here we found that HIV-1 exploited TLR8 signaling for transcription initiation of its integrated provirus. HIV-1 infection of DCs led to activation of p50-p65 NF- κ B dimers

through TLR8. Although both TLR7 and TLR8 recognize viral ssRNA and have been linked to immune responses to HIV-1 (refs. 7,8), HIV-1 selectively triggers TLR8 for its replication. Endosomal routing of HIV-1 is thought to be involved in HIV-1 degradation and antigen processing as well as the transmission of HIV-1 to T cells²⁰. Our data suggest that HIV-1 hijacks the internalization routing to initiate HIV-1 transcription via endosomal TLR8 signaling.

Transcriptional regulation of gene expression is a strictly controlled process with a key role for RNAPII. Transcription factors and cofactors control RNAPII activity through dynamic phosphorylation of its C-terminal repeat domain; phosphorylation of Ser5 and Ser2 is required for transcription initiation and elongation, respectively²⁹. Notably, we have demonstrated that TLR8-dependent activation of NF- κ B by HIV-1 resulted in transcription initiation but not elongation. NF- κ B p50-p65 bound to the LTR of integrated HIV-1 and thereby recruited the general transcription factor TFIID. The CDK7 subunit of TFIID phosphorylated RNAPII at Ser5 to induce transcription initiation. However, RNAPII was not phosphorylated at Ser2 and progression of RNAPII was abrogated, which resulted in short HIV-1 RNAs but not full-length HIV-1 transcripts. Thus, triggering of TLR8 signaling by HIV-1 leads to transcription initiation; however, a second signaling pathway is needed to induce the elongation of nascent HIV-1 transcripts.

Our data have shown that the binding of HIV-1 to DC-SIGN provided the signal required for transcription elongation. The DC-SIGN-induced Raf-1-dependent signaling pathway resulted in phosphorylation of RNAPII at Ser2, which drove the progression of RNAPII over the entire HIV-1 genome, as demonstrated by ChIP assay. Consequently, full-length HIV-1 transcripts were produced, which were then initially spliced into Tat- and Rev-encoding mRNAs and later were processed into singly spliced and unspliced transcripts required for the production of new virus particles. Raf-1 inhibition did not affect the binding of p65 to the HIV-1 LTR or recruitment of RNAPII but did attenuate phosphorylation of RNAPII at Ser2 and therefore transcription elongation of nascent HIV-1 transcripts. Thus, DC-SIGN-induced signaling to Raf-1 is essential for productive HIV-1 transcription. Our data have identified a crucial function for gp120; HIV-1 gp120 is already known to be essential for initial interactions between HIV-1 and DCs for membrane fusion²⁴, but here we found that gp120 is also essential for HIV-1 replication and productive infection of DCs by triggering DC-SIGN signaling.

Raf-1 activation by DC-SIGN induces the phosphorylation of NF- κ B p65 at Ser276 that allows acetylation of p65, which shapes adaptive immunity^{14,15}. Here we found that phosphorylation of p65 at Ser276 was crucial for HIV-1 transcription through the recruitment of pTEF-b to the HIV-1 LTR. The transcription-elongation factor pTEF-b consists of cyclin T1 and CDK9; CDK9 is responsible for phosphorylation of RNAPII at Ser2 to promote transcription elongation by RNAPII (ref. 29). A similar requirement for NF- κ B-mediated recruitment of pTEF-b has been reported for the expression of several cytokine genes³⁸. The finding that attenuation of phosphorylation of RNAPII at Ser2 after Raf-1 inhibition coincided with a block in phosphorylation of p65 at Ser276 as well as recruitment of cyclin T1 and CDK9 to the LTR provides further support for the idea of an essential role for DC-SIGN-induced, Raf-1-mediated signaling in HIV-1 transcription. Thus, productive HIV-1 transcription requires TLR8 signaling to induce transcription initiation through NF- κ B binding and TFIID recruitment, whereas DC-SIGN signaling drives transcription elongation through phosphorylation of p65 at Ser276 and subsequent pTEF-b recruitment.

Viral Tat protein is crucial for HIV-1 transcription, as Tat binds to the transactivation response element in nascent HIV-1 transcripts

and recruits pTEF-b to stimulate transcription elongation by RNAPII (refs. 28,29). However, Tat protein is not a part of the HIV-1 virion, and the conundrum of how productive transcription is initiated before Tat synthesis has remained unsolved. Several studies investigating latently infected T cells have suggested that NF- κ B has a role in HIV-1 transcription, without answering the question how transcription elongation is triggered. Our data have now provided evidence that two innate signaling pathways are subverted by HIV-1 to induce phosphorylation of p65 at Ser276, which recruits pTEF-b and induces transcription elongation. Once Tat protein is produced, it will sustain late HIV-1 transcription independently of NF- κ B by recruiting pTEF-b.

Coinfection with *M. tuberculosis* or *C. albicans* increases HIV-1 replication both *in vitro* and in HIV-1-infected patients⁴³⁻⁴⁵. Such coinfection results in a higher rate of progression of HIV-1 disease⁴⁷. Here we have demonstrated that coinfection enhanced HIV-1 replication in a Raf-1-dependent manner in both monocyte-derived DCs and DC-SIGN⁺ myeloid DCs. *M. tuberculosis* and *C. albicans* trigger Raf-1-dependent phosphorylation of p65 at Ser276, which shapes the adaptive immune responses to these pathogens^{14,15,37}. Those data suggest that coinfection enhances HIV-1 transcription by stimulating transcription elongation via Raf-1, which further supports our conclusion that phosphorylation of p65 at Ser276 is a crucial requirement for efficient HIV-1 replication. They also suggest that preventive measures should be taken to avoid triggering of DC-SIGN in patients infected with HIV-1. Moreover, vaccination strategies with gp120 will also result in activation of DC-SIGN and possibly reactivation of HIV-1 replication.

Latently integrated proviruses in T cells are efficiently reactivated by TNF⁴⁸, which is attributed to activation of NF- κ B by TNF signaling. However, TNF signaling also induces p65 phosphorylation at Ser276 through MSK1 (ref. 41). Our data suggest that the molecular mechanism behind this reactivation involves stimulation of transcription elongation via recruitment of pTEF-b mediated by p65 phosphorylation at Ser276. Notably, our data have demonstrated that infection of DCs with VSV-G-pseudotyped HIV-1 showed delayed kinetics relative to infection with HIV-1, as it does not trigger DC-SIGN signaling. The delayed production of Tat-Rev mRNA after 48 h was dependent on TNF signaling in an MSK1-dependent manner. These observations support the idea of crucial role for the phosphorylation of p65 at Ser276 in HIV-1 replication.

Our data have shown that DC-SIGN signaling is crucial for HIV-1 infection of primary DC populations present in blood and peripheral tissues, as infection of primary isolated DC-SIGN⁺ myeloid DCs as well as dermal DCs was abrogated after Raf-1 inhibition. These data suggest that these pathways might be important for infection via blood or sexual transmission. Infection of plasmacytoid DCs, macrophages and T cells with HIV-1 also leads to productive infection of these cells. Although macrophages express DC-SIGN²⁰ and might use DC-SIGN signaling to initiate productive HIV-1 transcription, it remains unclear how HIV-1 transcription in plasmacytoid DCs and T cells is initiated early after infection in the absence of Tat protein. Our data have suggested that recruitment of pTEF-b to the LTR is a crucial determinant for early productive transcription; this could also apply to T cells. The transcription factor IRF1 is a candidate for the recruitment of pTEF-b to the LTR; IRF1 has been shown to be induced after HIV-1 infection and to sustain pre-Tat productive HIV-1 transcription in T cells⁴⁹. It will be of interest to determine whether IRF1 interacts with pTEF-b.

Here we have demonstrated that HIV-1 has evolved a strategy to turn PRR signaling in DCs to its own advantage. Our study has identified crucial roles for both HIV-1 ssRNA and HIV-1 gp120 in inducing two complementary signaling pathways by TLR8 and DC-SIGN, respectively,

for HIV-1 replication and productive DC infection. As DC infection is involved in HIV-1 dissemination during sexual transmission, inhibitors of the innate signaling pathways identified might represent new antiretroviral drugs for the prevention of HIV-1 infection and transmission.

METHODS

Methods and any associated references are available in the online version of the paper at <http://www.nature.com/natureimmunology/>.

Accession codes. UCSD-Nature Signaling Gateway (<http://www.signaling-gateway.org>): A002300, A002008, A001645 and A002937.

Note: Supplementary information is available on the Nature Immunology website.

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AUTHOR CONTRIBUTIONS

S.I.G. designed, executed and interpreted most experiments and prepared the manuscript; M.v.d.V. executed HIV-1 stimulation, infection and transmission experiments, isolated myeloid DCs and helped with the nuclear extract preparations; L.M.v.d.B. isolated dermal DCs; J.d.D. helped with flow cytometry; M.L. helped with GST-p65 purification and setting up the GST enzyme-linked immunosorbent assay (ELISA); and T.B.H.G. supervised all aspects of this study, including study design, execution and interpretation, and manuscript preparation.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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- Steinman, R.M. & Banchereau, J. Taking dendritic cells into medicine. *Nature* **449**, 419–426 (2007).
- Medzhitov, R. Recognition of microorganisms and activation of the immune response. *Nature* **449**, 819–826 (2007).
- Akira, S., Uematsu, S. & Takeuchi, O. Pathogen recognition and innate immunity. *Cell* **124**, 783–801 (2006).
- Kanneganti, T.D., Lamkanfi, M. & Núñez, G. Intracellular NOD-like receptors in host defense and disease. *Immunity* **27**, 549–559 (2007).
- O'Neill, L.A.J. When signaling pathways collide: positive and negative regulation of Toll-like receptor signal transduction. *Immunity* **29**, 12–20 (2008).
- Geijtenbeek, T.B.H. & Gringhuis, S.I. Signalling through C-type lectin receptors: shaping immune responses. *Nat. Rev. Immunol.* **9**, 465–479 (2009).
- Meier, A. *et al.* MyD88-dependent immune activation mediated by human immunodeficiency virus type 1-encoded Toll-like receptor ligands. *J. Virol.* **81**, 8180–8191 (2007).
- Beignon, A.S. *et al.* Endocytosis of HIV-1 activates plasmacytoid dendritic cells via Toll-like receptor-viral RNA interactions. *J. Clin. Invest.* **115**, 3265–3275 (2005).
- Heil, F. *et al.* Species-specific recognition of single-stranded RNA via Toll-like receptor 7 and 8. *Science* **303**, 1526–1529 (2004).
- Meier, A. *et al.* Sex differences in the Toll-like receptor-mediated response of plasmacytoid dendritic cells to HIV-1. *Nat. Med.* **15**, 955–959 (2009).
- Hardy, A.W., Graham, D.R., Shearer, G.M. & Herbeval, J.P. HIV turns plasmacytoid dendritic cells (pDC) into TRAIL-expressing killer pDC and down-regulates HIV coreceptors by Toll-like receptor 7-induced IFN- α . *Proc. Natl. Acad. Sci. USA* **104**, 17453–17458 (2007).
- de Witte, L. *et al.* Langerin is a natural barrier to HIV-1 transmission by Langerhans cells. *Nat. Med.* **13**, 367–371 (2007).
- Geijtenbeek, T.B.H. *et al.* DC-SIGN, a dendritic cell-specific HIV-1-binding protein that enhances trans-infection of T cells. *Cell* **100**, 587–597 (2000).
- Gringhuis, S.I. *et al.* C-type lectin DC-SIGN modulates toll-like receptor signaling via Raf-1 kinase-dependent acetylation of transcription factor NF- κ B. *Immunity* **26**, 605–616 (2007).
- Gringhuis, S.I., den Dunnen, J., Litjens, M., van der Vliet, M. & Geijtenbeek, T.B.H. Carbohydrate-specific signaling through the DC-SIGN signalosome tailors immunity to *Mycobacterium tuberculosis*, HIV-1 and *Helicobacter pylori*. *Nat. Immunol.* **10**, 1081–1088 (2009).
- Hodges, A. *et al.* Activation of the lectin DC-SIGN induces an immature dendritic cell phenotype triggering Rho-GTPase activity required for HIV-1 replication. *Nat. Immunol.* **8**, 569–577 (2007).
- Royce, R.A., Sena, A., Cates, W. & Cohen, M.S. Sexual transmission of HIV. *N. Engl. J. Med.* **336**, 1072–1078 (1997).
- UNAIDS. Global AIDS epidemic just beginning. *AIDS Policy Law* **20**, 1 (2005).
- de Witte, L., Nabatov, A. & Geijtenbeek, T.B. Distinct roles for DC-SIGN⁺-dendritic cells and Langerhans cells in HIV-1 transmission. *Trends Mol. Med.* **14**, 12–19 (2008).
- Wu, L. & KewalRamani, V.N. Dendritic-cell interactions with HIV: infection and viral dissemination. *Nat. Rev. Immunol.* **6**, 859–868 (2006).
- Turville, S.G. *et al.* Immunodeficiency virus uptake, turnover, and 2-phase transfer in human dendritic cells. *Blood* **103**, 2170–2179 (2004).
- Perreau, M., Pantaleo, G. & Kremer, E.J. Activation of a dendritic cell-T cell axis by Ad5 immune complexes creates an improved environment for replication of HIV in T cells. *J. Exp. Med.* **205**, 2717–2725 (2008).
- Goff, S.P. Host factors exploited by retroviruses. *Nat. Rev. Microbiol.* **5**, 253–263 (2007).
- Greene, W.C. & Peterlin, B.M. Charting HIV's remarkable voyage through the cell: Basic science as a passport to future therapy. *Nat. Med.* **8**, 673–680 (2002).
- Perkins, N.D. *et al.* A cooperative interaction between NF- κ B and Sp1 is required for HIV-1 enhancer activation. *EMBO J.* **12**, 3551–3558 (1993).
- Nabel, G. & Baltimore, D. An inducible transcription factor activates expression of human immunodeficiency virus in T cells. *Nature* **326**, 711–713 (1987).
- Peterlin, B.M. & Trono, D. Hide, shield and strike back: how HIV-infected cells avoid immune eradication. *Nat. Rev. Immunol.* **3**, 97–107 (2003).
- Barboric, M. & Peterlin, B.M. A new paradigm in eukaryotic biology: HIV Tat and the control of transcriptional elongation. *PLoS Biol.* **3**, e76 (2005).
- Egloff, S. & Murphy, S. Cracking the RNA polymerase II CTD code. *Trends Genet.* **24**, 280–288 (2008).
- Adams, M. *et al.* Cellular latency in human immunodeficiency virus-infected individuals with high CD4 levels can be detected by the presence of promoter-proximal transcripts. *Proc. Natl. Acad. Sci. USA* **91**, 3862–3866 (1994).
- Kao, S.Y., Calman, A.F., Luciw, P.A. & Peterlin, B.M. Anti-termination of transcription within the long terminal repeat of HIV-1 by tat gene product. *Nature* **330**, 489–493 (1987).
- Burleigh, L. *et al.* Infection of dendritic cells (DCs), not DC-SIGN-mediated internalization of human immunodeficiency virus, is required for long-term transfer of virus to T cells. *J. Virol.* **80**, 2949–2957 (2006).
- Kwon, D.S., Gregorio, G., Bitton, N., Hendrickson, W.A. & Littman, D.R. DC-SIGN-mediated internalization of HIV is required for trans-enhancement of T cell infection. *Immunity* **16**, 135–144 (2002).
- Brussel, A. & Sonigo, P. Evidence for gene expression by unintegrated human immunodeficiency virus type 1 DNA species. *J. Virol.* **78**, 11263–11271 (2004).
- Wellbrock, C., Karasarides, M. & Marais, R. The RAF proteins take centre stage. *Nat. Rev. Mol. Cell Biol.* **5**, 875–885 (2004).
- Yedavalli, V.S.R.K., Neuveut, C., Chi, Y.h., Kleiman, L. & Jeang, K.T. Requirement of DDX3 DEAD box RNA helicase for HIV-1 Rev-RRE export function. *Cell* **119**, 381–392 (2004).
- Gringhuis, S.I. *et al.* Dectin-1 directs T helper cell differentiation by controlling noncanonical NF- κ B activation through Raf-1 and Syk. *Nat. Immunol.* **10**, 203–213 (2009).
- Nowak, D.E. *et al.* RelA Ser276 phosphorylation is required for activation of a subset of NF- κ B-dependent genes by recruiting cyclin-dependent kinase 9/cyclin T1 complexes. *Mol. Cell Biol.* **28**, 3623–3638 (2008).
- Pion, M. *et al.* APOBEC3G/3F mediates intrinsic resistance of monocyte-derived dendritic cells to HIV-1 infection. *J. Exp. Med.* **203**, 2887–2893 (2006).
- Publicover, J., Ramsburg, E., Robek, M. & Rose, J.K. Rapid pathogenesis induced by a vesicular stomatitis virus matrix protein mutant: viral pathogenesis is linked to induction of tumor necrosis factor α . *J. Virol.* **80**, 7028–7036 (2006).
- Vermeulen, L., De Wilde, G., Van Damme, P., Vanden Berghe, W. & Haegeman, G. Transcriptional activation of the NF- κ B p65 subunit by mitogen- and stress-activated protein kinase-1 (MSK1). *EMBO J.* **22**, 1313–1324 (2003).
- de Jong, M.A. *et al.* TNF- α and TLR agonists increase susceptibility to HIV-1 transmission by human Langerhans cells ex vivo. *J. Clin. Invest.* **118**, 3440–3452 (2008).
- Toossi, Z. Virological and immunological impact of tuberculosis on human immunodeficiency virus type 1 disease. *J. Infect. Dis.* **188**, 1146–1155 (2003).
- Ranjbar, S. *et al.* HIV-1 replication is differentially regulated by distinct clinical strains of *Mycobacterium tuberculosis*. *PLoS ONE* **4**, e6116 (2009).
- Lawn, S.D. AIDS in Africa: the impact of coinfections on the pathogenesis of HIV-1 infection. *J. Infect.* **48**, 1–12 (2004).
- Hester, R.A. & Kennedy, S.B. Candida infection as a risk factor for HIV transmission. *J. Womens Health* **12**, 487–494 (2003).
- Collins, K.R., Quinones-Mateu, M.E., Toossi, Z. & Arts, E.J. Impact of tuberculosis on HIV-1 replication, diversity, and disease progression. *AIDS Rev.* **4**, 165–176 (2002).
- Kim, Y.K. *et al.* Recruitment of TFIID to the HIV LTR is a rate-limiting step in the emergence of HIV from latency. *EMBO J.* **25**, 3596–3604 (2006).
- Sgarbanti, M. *et al.* Modulation of human immunodeficiency virus 1 replication by interferon regulatory factors. *J. Exp. Med.* **195**, 1359–1370 (2002).

ONLINE METHODS

Cells, stimulation, inhibition and RNAi. Immature DCs and peripheral blood lymphocytes were isolated and cultured as described^{12,37}. DC-SIGN⁺ myeloid DCs⁵⁰ were isolated by positive selection from peripheral blood mononuclear cells after Ficoll separation with the CD209 MicroBead kit according to the manufacturer's instructions (Miltenyi Biotec). DC-SIGN⁺ dermal DCs were isolated from skin obtained from healthy donors after plastic surgery. Split skin grafts 0.3 mm in thickness were digested for 45 min at 37 °C with dispase (2 µg/ml; Invitrogen) for separation of the dermis from the epidermis. The dermis was floated on medium for 3 d and then was digested for 30 min at 37 °C with collagenase III (0.2 mg/ml; Sigma) to obtain single-cell suspensions. DC-SIGN⁺ dermal DCs were isolated as described above. This study was in accordance with the ethical guidelines of the Academic Medical Center.

DCs were stimulated with recombinant HIV-1 BaL gp120 (10 µg/ml; obtained through the AIDS Research and Reference Reagent Program of the National Institute of Allergy and Infectious Diseases), ManLAM (10 µg/ml; isolated from *M. tuberculosis*; provided by J. Belisle), *M. tuberculosis* (4 µg/ml; provided by J. Belisle), heat-killed *C. albicans* (multiplicity of infection, 10), plate-coated anti-DC-SIGN (20 µg/ml; H-200; Santa Cruz Biotechnology), lipopolysaccharide from *Salmonella typhosa* (10 ng/ml; Sigma), poly(I:C) (10 µg/ml; Invivogen), R-837 (10 µg/ml; Invivogen) and/or IL-1β (50 ng/ml; Miltenyi). Cells were preincubated for 2 h with blocking antibodies or inhibitors. Final concentrations of blocking antibodies and inhibitors were as follows: 20 µg/ml of anti-DC-SIGN (AZN-D1; generated in-house⁵¹), 20 µg/ml of anti-CD4 (RPA-T4; BioLegend), 20 µg/ml of anti-CCR5 (2D7; BD Pharmingen), 20 µg/ml of anti-TNF (3052; Biovision), 1 µM GW5074 (Raf inhibitor⁵²; Calbiochem), 1 µM H89 (MSK1 inhibitor⁴¹; Calbiochem) and 2 µg/ml of cycloheximide (translation inhibitor⁵³; Sigma). Raf-1 inhibition did not affect cell viability or induce apoptosis, as determined by CellTiter Glo and Caspase-Glo 3/7 assays (Promega; **Supplementary Fig. 1**).

DCs were transfected with 50 nM siRNA with the transfection reagent DF4 (Dharmacon) and were used for experiments 72 h after transfection. The siRNA (SMARTpool; Dharmacon) was specific for the following: Raf-1 (M-003601-02), MyD88 (M-004769-01), TLR7 (M-004714-01) and TLR8 (M-004715-01). Nontargeting siRNA (D-001206-13; Dharmacon) served a control. This protocol resulted in a transfection efficiency of nearly 100%, as determined by flow cytometry of cells transfected with siGLO RISC-Free Control siRNA (D-001600-01; Dharmacon), and did not induce interferon responses, as determined by quantitative real-time PCR analysis³⁷. Silencing of expression was verified by real-time PCR and flow cytometry. Silencing of Raf-1 did not influence cell viability or induce apoptosis, as determined by CellTiter Glo and Caspase-Glo 3/7 assays (Promega; **Supplementary Fig. 1**).

Viruses, infection and transmission. NL4.3-BaL, LAI, NL4.3-eGFP-BaL, NL4.3-eGFP and VSV-G-pseudotyped NL4.3-Δenv HIV-1 have been described^{42,54,55}. NL4.3-BaL, NL4.3-eGFP-BaL, NL4.3-eGFP and VSV-G-pseudotyped NL4.3-Δenv HIV-1 were produced as described⁴². HIV-1 LAI was propagated on SupT1 human lymphoma cells and was concentrated with Amicon Ultra-15 Ultracel 100K filter tubes (Millipore). Virus concentrations were determined by titration with indicator TZM-bl cells (human cervical cancer cells). DCs were infected at a multiplicity of infection of 0.1. DC infection was assessed by flow cytometry at 5 d after infection by measurement of the frequency of eGFP⁺ cells or by measurement of p24 in culture supernatants by ELISA (Perkin Elmer Life Sciences). For analysis of the transmission of HIV-1 to T cells, DCs were stringently washed 2 d after infection and peripheral blood lymphocyte samples enriched for CD4⁺ cells were added.

RNA isolation and quantitative real-time PCR. First, mRNA was isolated with an mRNA Capture kit (Roche). Short abortive HIV-1 RNAs were isolated from cell lysates from which the mRNA fraction had been removed with the mRNA Capture kit, followed by an additional 1 h of incubation in streptavidin-coated plates (Sigma) to ensure complete removal of complexes of mRNA and biotin-labeled oligo(dT)₂₀. RNA was then isolated with the miRNeasy 96 kit (Qiagen), and cDNA was synthesized with a reverse-transcriptase kit (Promega). Samples were amplified by PCR with SYBR Green as described³⁷. Specific primers were designed with Primer Express 2.0 (Applied Biosystems;

Supplementary Table 1). Tat-Rev primers recognize HIV-1 early gene transcripts encoding either Tat or Rev. Vpu primers are specific for the HIV-1 late gene transcript encoding Vpu but do not recognize other single-spliced transcripts. All HIV-1 primers were designed to recognize viral sequences of both NL4.3 and LAI origin. Strict control experiments were done with the non-mRNA fraction to ensure that none of the signal obtained for the short abortive HIV-1 primers could be accounted for by contaminating mRNA or DNA in the isolation: fractions were tested with primers spanning the *GAPDH* promoter region and primers to detect *GAPDH* mRNA or Tat-Rev mRNA as well as full-length HIV-1 transcripts. The cycling threshold (C_T) value is defined as the number of PCR cycles in which the fluorescence signal exceeds the detection threshold value. The normalized amount of target mRNA (N_t) was calculated from the C_T values obtained for both target and *GAPDH* mRNA with the equation $N_t = 2^{CT(\text{control}) - CT(\text{target})}$. The expression of mRNA or short HIV-1 RNA is presented relative to the abundance of control *GAPDH* mRNA or 18S rRNA, respectively. For relative RNA expression, N_t in HIV-1-infected cells was set as 1 in one experiment and for each donor, except for the time-course experiment in **Figure 4**, in which expression at the time point at which Tat-Rev mRNA, Vpu mRNA or short RNA expression was at its maximum was set as 1.

HIV-1-integration Alu-PCR assay. Total cell DNA was isolated at 6 h after infection with a QIAamp blood isolation kit (Qiagen). Integrated HIV-1 DNA was measured by two-step Alu-PCR as described with minor modifications³⁴. In the first round of preamplification PCR, Alu-LTR sequences were amplified with an HIV-1-specific primer (LTR R region) in combination with a primer that anneals to the abundant genomic Alu repeats. The HIV-1-specific primer was extended with a marker region at the 5' end, which was used for specificity in the second-round PCR. The second round was nested quantitative real-time PCR of the first-round PCR products with primers annealing to the aforementioned marker region in combination with a HIV-1-specific primer (LTR U5 region). Primer sequences were as follows: first round, HIV-1 LTR R forward, 5'-ATGCCACGTAAGCGAACTGCTGGCTAACTAGGGAACCCACTG-3' (marker sequence underlined); Alu reverse, 5'-TCCCAGCTACTGGGGAGGCTGAGG-3'; second-round marker forward, 5'-ATGCCACGTAAGCGAACTG-3'; HIV-1 LTR U5 reverse, 5'-CACACTGACTAAAGGGTCTGAGG-3'. Samples were assayed at two concentrations to ensure that PCR inhibitors were absent. Dilutions were prepared with genomic DNA from uninfected cells to ensure that the number of Alu sites per reaction mixture remained constant. For monitoring of the signal contributed by unintegrated HIV-1 DNA, the first-round PCR was also done with the HIV-1-specific primer alone as a control. HIV-1 integration was normalized relative to *GAPDH* DNA. For relative HIV-1 integration, N_t for NL4.3- or BaL-infected cells was set as 1 in one experiment and for each donor.

Raf-1 phosphorylation. Phosphorylation of Raf-1 at Ser338 or Tyr340-Tyr341 was assessed by flow cytometry as described¹⁵.

NF-κB DNA binding, p65 phosphorylation and p65-pTEF-b association. Nuclear extracts of DCs were prepared with the NucBuster Protein Extraction kit (Novagen) and NF-κB DNA binding was determined with a TransAM NF-κB Family kit (Active Motif). Phosphorylated p65 was detected by ELISA as described before¹⁴ or by immunoblot analysis after immunoprecipitation with anti-p65 (3034; Cell Signaling).

The association between cellular p65 and pTEF-b was assessed by immunoblot analysis with anti-cyclin T1 (sc-10750; Santa Cruz) or anti-CDK9 (sc-8338; Santa Cruz) after immunoprecipitation of p65 from nuclear extracts with anti-p65. For analysis of the role of Ser276 phosphorylation in this association, GST-p65 precipitation assays were used. GST-p65 (amino acids 1–305) expressed in *Escherichia coli* DH5α was purified with the B-PER GST Fusion Protein Purification kit (Pierce). *In vitro* kinase reactions (30 min at 30 °C) were done with 100 ng purified GST-p65 and 1 µg recombinant protein kinase A (Active Motif). Phosphorylation of GST-p65 at Ser276 was verified as described for cellular p65. Phosphorylated or nonphosphorylated GST-p65 was mixed with nuclear extracts from DCs in the presence or absence of a p65-derived peptide containing the phosphorylated Ser276 sequence (Phospho-NF-κB p65 (Ser276) Blocking Peptide; Cell Signaling) and was incubated for 2 h before GST-p65 and

bound proteins were captured with anti-GST-coated wells (Pierce). Associated CDK9 and cyclin T1 were detected with anti-cyclin T1 and anti-CDK9.

ChIP assay. The ChIP-IT Enzymatic kit (Active Motif) was used for ChIP assays to determine occupancy of the integrated HIV-1 genome by regulatory proteins. Cells were infected with HIV-1 NL4.3 or HIV-1 BaL and after 4 h were fixed with 1% (vol/vol) paraformaldehyde. Nuclei were isolated and chromatin DNA was fragmented by enzymatic shearing for 10 min at 37 °C. After lysates were precleared with salmon sperm-saturated protein G agarose beads, protein-DNA complexes were immunoprecipitated overnight at 4 °C with anti-p65, anti-CDK9, anti-CDK7 (sc-856; Santa Cruz), antibody to RNAPII phosphorylated at Ser2 (H5; Covance), antibody to RNAPII phosphorylated at Ser 5 (H14; Covance), antibody to total RNAPII (included in the ChIP-IT kit; Active Motif) or immunoglobulin G (negative control; included in the ChIP-IT kit; Active Motif). HIV-1 DNA was quantified by real-time PCR. Input and immunoprecipitated DNA was purified after reversal of crosslinks. Real-time PCR was then done with primer sets spanning the NF- κ B-binding sites and transcription-initiation site in the HIV-1 LTR⁵⁶ or downstream of the transcription-initiation site for monitoring of RNAPII progress (primer sequences, **Supplementary Table 1**). In addition, as a negative control, amplification was done with primers spanning genomic DNA at cytogenetic location 12 p13.3 (included in the ChIP-IT kit; Active Motif). For normalization to input DNA, a sample of each

condition that had not undergone immunoprecipitation with specific antibody (input DNA) was analyzed; results are presented as percentage of input DNA.

Statistical analysis. Student's *t*-test for paired observations was used for statistical analyses. Statistical significance was set at a *P* value of less than 0.05.

50. Geijtenbeek, T.B.H. *et al.* DC-SIGN-ICAM-2 interaction mediates dendritic cell trafficking. *Nat. Immunol.* **1**, 353–357 (2000).
51. Geijtenbeek, T.B. *et al.* Identification of DC-SIGN, a novel dendritic cell-specific ICAM-3 receptor that supports primary immune responses. *Cell* **100**, 575–585 (2000).
52. Lackey, K. *et al.* The discovery of potent cRaf1 kinase inhibitors. *Bioorg. Med. Chem. Lett.* **10**, 223–226 (2000).
53. Obrig, T.G., Culp, W.J., McKeehan, W.L. & Hardesty, B. The mechanism by which cycloheximide and related glutarimide antibiotics inhibit peptide synthesis on reticulocyte ribosomes. *J. Biol. Chem.* **246**, 174–181 (1971).
54. Gallay, P., Hope, T., Chin, D. & Trono, D. HIV-1 infection of nondividing cells through the recognition of integrase by the importin/karyopherin pathway. *Proc. Natl. Acad. Sci. USA* **94**, 9825–9830 (1997).
55. Chatterji, U. *et al.* Naturally occurring capsid substitutions render HIV-1 cyclophilin A independent in human cells and TRIM-cyclophilin-resistant in Owl monkey cells. *J. Biol. Chem.* **280**, 40293–40300 (2005).
56. Duh, E.J., Maury, W.J., Folks, T.M., Fauci, A.S. & Rabson, A.B. Tumor necrosis factor α activates human immunodeficiency virus type 1 through induction of nuclear factor binding to the NF- κ B sites in the long terminal repeat. *Proc. Natl. Acad. Sci. USA* **86**, 5974–5978 (1989).