HIV-1 Vpr-induced DNA damage activates NF-κB independent of cell cycle arrest and CRL4A$^{DCAF1}$ engagement

Carina Sandoval$^1$ and Oliver I. Fregoso$^{1,2}$

$^1$ Molecular Biology Institute, University of California, Los Angeles, California, USA
$^2$ Department of Microbiology, Immunology, and Molecular Genetics, University of California, Los Angeles, California, USA

Correspondence: Oliver I. Fregoso
Tel: 310.794.1424
Email: ofregoso@mednet.ucla.edu
ABSTRACT

Lentiviral accessory genes enhance replication through diverse mechanisms. HIV-1 accessory protein Vpr modulates the host DNA damage response (DDR) at multiple steps through the degradation of host proteins, cell cycle arrest, DNA damage, and both activation and repression of DDR signaling. Vpr also alters host and viral transcription; however, the connection between Vpr-mediated DDR modulation and transcriptional activation remains unclear. Here, we determined the cellular consequences of Vpr-induced DNA damage using Vpr mutants that allow us to separate the ability of Vpr to induce DNA damage from CRL4A\textsuperscript{DCAF1} complex dependent phenotypes including cell cycle arrest, host protein degradation, and repression of DDR. In both tissue-cultured U2OS cells and primary human monocyte-derived macrophages (MDMs), we found that Vpr induces DNA breaks and activates DDR signaling in the absence of cell cycle arrest and CRL4A\textsuperscript{DCAF1} complex engagement. Moreover, through RNA-sequencing, we found that Vpr-induced DNA damage alters cellular transcription via activation of NF-κB/RelA signaling. NF-κB/RelA transcriptional activation was dependent on ATM-NEMO, as inhibition of NEMO resulted in loss of NF-κB transcriptional upregulation by Vpr. Furthermore, HIV-1 infection of primary MDMs validated NF-κB transcriptional activation during infection. Both virion delivered and \textit{de novo} expressed Vpr induced DNA damage and activated NF-κB transcription, suggesting that engagement of the DDR can occur during early and late stages of viral replication. Together, our data support a model where Vpr-induced DNA damage activates NF-κB through the ATM-NEMO pathway, independent of cell cycle arrest and CRL4A\textsuperscript{DCAF1} engagement. We propose this is essential to overcoming restrictive environments, such as macrophages, to enhance viral transcription and replication.
INTRODUCTION

The DDR is a signaling cascade activated in response to exogenous and endogenous genotoxic stress, such as DNA breaks. The DDR consists of sensor proteins that sense the damaged DNA, mediator and transducer proteins that transmit the signal of damaged DNA, and effector proteins that elicit a cellular response. Typically, the DDR is divided into three main pathways based on the mediator kinases activated: Ataxia telangiectasia and Rad3 related (ATR), ataxia telangiectasia mutated (ATM), and DNA-dependent protein kinase (DNA-PK). Single-strand DNA breaks (SSBs) are primarily sensed by RPA to activate ATR signaling, while double-strand DNA breaks (DSBs) can be sensed by the MRN (MRE11, RAD51, and NBS1) or Ku70/80 complexes to activate either ATM or DNA-PK signaling, respectively (1,2). However, a significant amount of crosstalk exists between these pathways. DDR signaling leads to various cellular responses, such as DNA repair, cell cycle arrest (3), transcriptional changes (4,5), apoptosis, or senescence (6).

Many diverse RNA and DNA viruses have evolved to modulate the DDR to enhance viral replication (7,8). Primate lentiviruses, such as HIV-1, primarily engage the DDR through the accessory protein Vpr (9,10). Vpr is evolutionarily conserved amongst extant primate lentiviruses and is required for replication in vivo (11,12) and in macrophage (13,14) and dendritic cells (15) in vitro. Vpr is largely unique among the lentiviral accessory genes in that it is delivered by the incoming virion, allowing it to act early in viral replication, and is also expressed de novo from the integrated provirus, allowing it to act later in viral replication (16). Although the primary conserved function of Vpr remains elusive, viruses lacking Vpr have decreased proviral transcription in monocyte derived macrophages (MDMs) and dendritic cells (15), suggesting an important role for Vpr in viral transcription.

Emerging literature suggests that Vpr engages the DDR at multiple, potentially unique, steps. ATR activation by Vpr leads to cell cycle arrest and requires recruitment of the CRL4A\textsuperscript{DCAF1} complex, which has a primary role in DNA repair (17). CRL4A\textsuperscript{DCAF1} complex recruitment by Vpr also leads to the degradation of many host proteins involved in the DDR, including CCDC137 (18), HLTF (19), UNG2 (20,21), SAMHD1 (22), Mus81/EME1 (23), EXO1 (24), TET2 (25), and MCM10 (26), as well as more global proteome remodeling (27). Moreover, CRL4A\textsuperscript{DCAF1} complex recruitment is required for Vpr-mediated repression of DSB repair (28). In contrast, we have previously shown that the ability of Vpr to induce DNA damage does not correlate with cell cycle...
arrest or repression of DSB repair (28), suggesting that Vpr-induced DNA damage may have unique roles in enhancing lentiviral replication.

Both DNA damage and HIV-1 Vpr promote transcriptional changes involving NF-κB (29,30). In response to DSBs, ATM signaling promotes NF-κB transcriptional upregulation through the ATM-NEMO pathway (31). ATM and NEMO interact in the nucleus before translocating to the cytoplasm (32,33) to subsequently activate RelA nuclear translocation, promoter binding, and NF-κB transcriptional activation. Previous literature suggests that HIV-1 Vpr modulates NF-κB pathways through phosphorylation and ubiquitylation of TAK1 to enhance NF-κB signaling (34) and altering the availability of the NF-κB p50-RelA heterodimer to inhibit NF-κB signaling (35). This proposes a testable model where Vpr-induced DNA damage alters the cellular environment to enhance viral replication by altering transcription through ATM-NEMO and NF-κB. Here, we aimed to identify the cellular consequences of Vpr-induced DNA damage and the connection between DNA damage and ATM activation, CRL4A<sup>DCAF1</sup> engagement, and transcriptional changes.

To determine the consequences of Vpr-induced DNA damage on cellular transcription, we used Vpr mutants that allow us to uncouple DNA damage from cell cycle arrest and CRL4A<sup>DCAF1</sup> complex mediated host protein degradation. We found that Vpr does not require CRL4A<sup>DCAF1</sup> engagement to induce DNA breaks and activate DDR signaling in U2OS tissue-culture cells and primary human MDMs. RNA-sequencing (RNA-seq) identified that wild-type HIV-1 Vpr and Vpr mutants that do not induce cell cycle arrest or engage the CRL4A<sup>DCAF1</sup> complex alter NF-κB associated cellular transcription. In support of this, we showed that only Vpr proteins that induce DNA damage activate RelA nuclear translocation and upregulate NF-κB target genes, such as BIRC3 and CXCL8. We further assessed the requirement for ATM and NEMO signaling in Vpr-mediated NF-κB activation. We found that inhibition of NEMO resulted in loss of NF-κB transcriptional upregulation in primary MDMs. HIV-1 infection and virus-like particle (VLP) delivery of Vpr in MDMs validated the upregulation of NF-κB target genes early during infection. Together, our data support a model where Vpr-induced DNA damage activates NF-κB through the ATM-NEMO pathway, independent of cell cycle arrest and CRL4A<sup>DCAF1</sup> complex mediated host protein degradation. This study further informs how lentiviral accessory proteins engage the DDR at multiple and unique steps, which remolds host environment and immune pathways to promote viral replication.
RESULTS

HIV-1 Vpr induces DNA damage and alters cellular transcription independent of CRL4ADCAF1 engagement and cell cycle arrest

Given the connections between the DDR and cellular transcription, we set out to understand whether the ability of Vpr to induce DNA damage gives rise to transcriptional changes, and whether these transcriptional changes are distinct from those caused by Vpr-mediated cell cycle arrest and/or degradation of host proteins. We have previously shown that a Vpr mutant that is unable to cause cell cycle arrest (HIV-1 Q23-17 S79A) causes DNA damage (28). However this mutant still engages the DCAF1 component of the CRL4ADCAF1 E3 ubiquitin ligase complex (36). To test the connection between transcriptional changes, cell cycle arrest, and host protein degradation, we used the HIV-1 Q23-17 Vpr mutant H71R, which does not bind the DCAF1 component of the CRL4ADCAF1 E3 ubiquitin ligase complex and thus loses the ability to cause cell cycle arrest and degrade host proteins (28) (Fig. S1A). U2OS cells were infected with rAAV expressing either HIV-1 Q23-17 Vpr wild type (WT), Vpr H71R, Vpr Q65R, a mutant of Vpr that is largely functionally dead (37), or etoposide (positive control). DDR activation was assessed by immunofluorescence while DNA damage was assessed by the comet assay 24 hours post infection. Consistent with our previous results, HIV-1 Vpr WT induces DNA breaks (Fig. 1A) and activates the DDR marker γH2A.x (Fig. 1B), while the nonfunctional Q65R mutant does not. In addition, we found that H71R induces DNA breaks (Fig. 1A) and activates γH2A.x (Fig. 1B) at levels similar to Vpr WT. To validate this, we knocked-down DCAF1 in U2OS cells with shRNAs and found that Vpr can still induce DNA breaks and activate DDR signaling when DCAF1 is knocked down (Fig. S1B-D). Together, our data show that Vpr does not require DCAF1 or associated cellular responses to induce DNA damage and activate the DDR, and that the H71R Vpr mutant allows us to investigate the ability of Vpr to induce DNA damage in the absence of host protein degradation and cell cycle arrest.

Next, we asked if Vpr mutants that induce DNA damage in the absence of cell cycle arrest (H71R and S79A) and CRL4ADCAF1 recruitment (H71R) alter cellular transcription. U2OS cells were infected with rAAV expressing HIV-1 Vpr WT, H71R, S79A, and Q65R, and RNA was collected at 12, 24, and 36 hours post infection. RNA-seq identified that Vpr WT, H71R and S79A, but not Q65R or empty vector, significantly alter cellular transcription 36 hours post infection when compared to untreated cells (Fig. 1C-E and Fig. S2A-B). Sixty-eight differentially expressed genes with pvalue <0.01 and FDR < 4.50E-05 were shared among Vpr WT, H71R, and S79A, indicative
of genes potentially altered by Vpr-induced DNA damage (Fig. 1D and 1E). Gene ontology indicated the shared upregulated genes were enriched for positive regulation of NF-κB signaling and NF-κB signaling pathways, and transcriptional regulatory relationships unravelled by sentence-based text-mining (TRRUST) analysis (38) further identified RelA/NF-κB as a top transcription factor activated by Vpr (Fig. 1F-G, Fig. S2 A-B and Supplemental Files S1 & S2). Finally, we identified differentially expressed genes exclusively altered in Vpr WT expressing cells, Vpr WT and S79A expressing cells, and unique to both Vpr H71R and S79A mutant expressing cells (Supplemental Files S1 & S2). Collectively, our data show that Vpr alters cellular transcription in the absence of cell cycle arrest and host protein degradation, and further suggest that Vpr-induced DNA damage specifically alters RelA/NF-κB-mediated transcription.

HIV-1 Vpr-induced DNA damage activates RelA and promotes NF-κB transcription

We next directly assayed whether Vpr mutants that can only induce DNA damage activate RelA/NF-κB. NF-κB activation was first validated by qRT-PCR for two NF-κB target genes identified in our RNA-seq, BIRC3 and CXCL8 (Fig. 1E), which are important for innate immunity and cell survival (39,40). U2OS cells were infected with rAAV expressing Vpr WT, H71R and Q65R for 24 or 36 hours. Consistent with the RNA-seq, Vpr WT and H71R upregulate BIRC3 and CXCL8 compared to untreated cells or Q65R mutant (Fig. 2A). qRT-PCR for BIRC3 and CXCL8 in DCAF1 knock-down cells further confirmed that DCAF1 recruitment is not required for HIV-1 Vpr to activate these two NF-κB target genes (Fig. S1E). To directly assess NF-κB activation, we assayed for RelA localization by immunofluorescence. As RelA is cytoplasmic when inactive and nuclear when active, we expect that Vpr WT and H71R will lead to nuclear translocation of RelA. Indeed, we found that Vpr WT and H71R activate nuclear translocation of RelA similar to the positive control, etoposide, while Vpr Q65R and empty vector do not (Fig 2B). These data demonstrate that Vpr activates RelA and NF-κB transcription in the absence of cell cycle arrest, DCAF1 recruitment and host protein degradation, suggesting that Vpr-induced DNA damage is sufficient to drive NF-κB activation.

HIV-1 Vpr-induced DNA damage activates ATM-NEMO signaling

NF-κB is activated in response to many pathways and signals involved in DNA repair and innate immunity (41). One such pathway is ATM-NEMO, where ATM stimulates NEMO to activate RelA and thus NF-κB signaling (33). Previous work has shown that Vpr activates markers of both ATR and ATM signaling (28,42). While ATR is required for Vpr-induced cell cycle arrest, the extent of ATM activation and the cellular consequences of ATM activation without cell cycle arrest are

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unclear. We hypothesized that Vpr-induced DNA damage activates the ATM-NEMO pathway, which consequently promotes RelA/NF-κB transcription. To determine if Vpr-induced DNA damage activates ATM signaling in the absence of cell cycle arrest and DCAF1 recruitment, we measured activation of multiple DDR proteins including two DNA damage sensors, NBS1 and MRE11, and two downstream signaling transducers, 53BP1 and γH2A.x. U2OS cells stably expressing NBS1-GFP or 53BP1-GFP were infected with rAAV expressing Vpr WT, H71R, and Q65R to assess DDR activation through live-cell imaging throughout infection. We found that Vpr WT and H71R promote the formation of NBS1 and 53PB1 foci at 26, 33, and 48 hours post infection (hpi), similar to the positive control etoposide, while the non-functional Q65R Vpr mutant was indistinguishable from untreated control cells (Fig. 3A and Fig. S3A-C). As many viruses activate or dysregulate DDR signaling through relocalization or sequestration of markers of the DDR, we next assayed for colocalization of the DNA damage sensors MRE11 and NBS1, and transducers 53BP1 and γH2A.x. Both damage sensors MRE11 and NBS1, and transducers 53PB1 and γH2A.x, colocalize in Vpr WT and H71R, similar to etoposide treated cells (Fig. 3B&C), suggesting that Vpr-induced DNA damage activates, but does not dysregulate, classical ATM signaling.

To determine if NEMO is required for Vpr to upregulate NF-κB transcription, we inhibited NEMO using a cell-permeable NEMO binding domain (NBD) inhibitor peptide (43). U2OS cells were pretreated with NBD peptide and infected with rAAV expressing Vpr WT and mutants. We assessed upregulation of the NF-κB target genes BIRC3 and CXCL8 using qRT-PCR. As before, Vpr WT and H71R upregulated both BIRC3 and CXCL8, while Q65R and empty vector did not (Fig. 4). With the addition of the NBD, Vpr WT and H71R mutant lost the ability to upregulate BIRC3 and CXCL8 (Fig. 4), suggesting that Vpr requires NEMO activation to upregulate transcription of NF-κB target genes.

Virion delivery of Vpr, through VLPs or HIV-1 ΔENV infection, in primary MDMs activates ATM-NEMO signaling and promotes RelA/NF-κB transcription

Together our data proposes a model where Vpr-induced DNA damage activates ATM and NEMO signaling resulting in RelA nuclear translocation and NF-κB transcriptional upregulation. We next tested this model in primary human MDMs, where Vpr enhances viral transcription and replication (14). Primary MDMs (Fig. S4A) were infected with virus-like particles (VLPs) carrying physiological levels of Vpr WT, H71R, or Q65R (Fig. S4B), and assayed for DNA damage, NF-κB activation, and NEMO dependence (Fig. 5A). Similar to overexpression of Vpr in U2OS tissue
culture cell lines, Vpr WT and H71R mutant induced DNA breaks and activated γH2A.x compared to Vpr Q65R and empty VLPs (Fig. 5B-C). Consistent with our data in U2OS cells, Vpr WT and H71R, but not Q65R or empty VLPs, upregulated BIRC3 and CXCL8 similar to the TNFα positive control when compared to empty cells (Fig. 5D and Fig. S4C). Finally, NBD delivery prior to VLP infection blocked Vpr WT and H71R-mediated BIRC3 and CXCL8 transcriptional activation, but not Vpr-induced DNA damage (Fig. 5C-D and Fig. S4C). These data indicate that physiological levels of virion-delivered Vpr induces DNA damage that activates NF-κB transcription in a NEMO dependent manner in primary MDMs.

To determine if HIV-1 infection activates NF-κB target genes, we infected primary human MDMs from six different donors with HIV-1 ΔEnv and assayed for transcriptional changes at early (8 hours), intermediate (16 hours), and late (24 and 48 hours) timepoints post infection. HIV-1 infection was monitored by flow cytometry for HIV-1 core proteins and qRT-PCR for unspliced transcripts (Fig. S4D-E), while NF-κB activation was measured by qRT-PCR for CXCL8 and BIRC3. TNFα was used as positive control (Fig. S4F). HIV-1 ΔEnv infection resulted in upregulation of CXCL8 and BIRC3 in MDMs as early as 8hpi in all donors, despite some donor-to-donor variability in the magnitude of upregulation (Fig. 5E and Fig. S4G-H), suggesting that HIV-1 infection upregulates Vpr-induced NF-κB target genes early during infection, consistent with VLP delivery of Vpr. Jointly, our data support a model where both incoming HIV-1 Vpr and de novo produced Vpr induces DNA damage that activates ATM-NEMO signaling to upregulate NF-κB transcription (Fig. 6).

DISCUSSION

In this study, we tested the hypothesis that HIV-1 Vpr alters cellular transcription through induction of DNA damage and activation of DDR signaling. By leveraging well-characterized Vpr mutants that separate Vpr-induced DNA damage from cell cycle arrest and CRL4A\(^{DCAF1}\) complex mediated host protein degradation, we identified a current model where Vpr-induced DNA damage activates ATM-NEMO signaling, which stimulates RelA and upregulates NF-κB mediated transcription. Using U2OS and primary human MDMs, which allowed us for the first time to investigate how HIV-1 Vpr engages the DDR in this important cell type, we showed that Vpr induces DNA breaks and activates markers of ATM signaling independent of cell cycle arrest, CRL4A\(^{DCAF1}\) complex recruitment, and host protein degradation. Moreover, we showed that Vpr-induced DNA damage correlates with RelA/NF-κB activation, as assayed by bulk RNA-seq, and RelA
immunofluorescence. We validated the upregulation of two NF-κB target genes, BIRC3 and CXCL8, and showed that inhibition of NEMO ablates the ability of Vpr to upregulate NF-κB transcription. Finally, VLP delivery of Vpr and HIV-1 infection show that incoming virion-associated Vpr is sufficient to induce DNA damage and activate RelA/NF-κB transcription. In complement, RAAV expression of Vpr show that de novo expressed Vpr can also induce DNA damage and activate RelA/NF-κB transcription. These data suggest that Vpr-induced DNA damage can play roles in both early and late stages of viral replication. Overall, our data support a model where Vpr-induced DNA damage activates ATM-NEMO signaling and upregulates RelA/NF-κB transcription in cell lines and primary human MDMs.

Although Vpr is a multifunctional and enigmatic protein, the DNA damage response is central to many of the phenotypes associated with Vpr. Previous reports from our lab and others have worked to untangle how and why Vpr engages the DDR. A consensus is emerging that Vpr engages the DDR at multiple, potentially unique, steps. Through recruitment of the CRL4A<sup>DCAF1</sup> ubiquitin ligase complex, Vpr degrades various DDR proteins, activates ATR signaling, represses double-strand DNA break repair, and causes cell cycle arrest (28,44). However, as shown here, Vpr induces DNA damage and alters cellular transcription independent of its ability to engage the CRL4A<sup>DCAF1</sup> ubiquitin ligase complex and associated phenotypes. Functions independent of host protein degradation are unique among most lentiviral accessory genes, as their typical primary functions depend on subverting antiviral mechanisms through host protein degradation (45). These data further define the multifunctional nature of Vpr in enhancing lentiviral infection and support the model where Vpr engages the DDR at multiple independent steps.

Here, we have also shown that Vpr-induced DNA damage directly correlates with NF-κB transcriptional activation that is dependent on ATM signaling. We have demonstrated that Vpr activates markers of ATM signaling, such as MRE11, NBS1, γH2A.x, and 53PB1, in a manner that resembles host activation rather than dysregulation. Antagonism or dysregulation of DNA damage sensors through relocalization or sequestration is a conserved mechanism among many viruses to evade innate immune detection (46). While we have shown that Vpr-induced DNA damage activates markers of ATM signaling in a manner that seems to facilitate classical ATM activation, with the end goal of activating transcription, it remains to be seen whether Vpr-mediated activation of ATR, which correlates with CRL4A<sup>DCAF1</sup> recruitment and cell cycle arrest, is classically activated or dysregulated. Moreover, how Vpr induces DNA breaks leading to both ATM and ATR activation is not understood.
Complementary studies have shown that Vpr modulates cellular (47) and viral transcription (48,49). In the context of cellular transcription, Vpr activates genes associated with innate immunity and proliferation in CD4+ T cells (50) and promotes the expression of proinflammatory cytokines in MDMs and monocyte-derived dendritic cells (MDDCs) (15,25). Together this suggests that Vpr has a vital role in transcriptional reprogramming to potentially create a proinflammatory environment that is conducive for viral replication. Consistent with these studies, we identified by bulk RNA-seq that Vpr regulates innate immune pathways that are either independent of or dependent on cell cycle arrest and CRL4A\textsuperscript{DCAF1} engagement and host protein degradation. Our data show that Vpr downregulates TASOR target genes (Fig. S2 D-F), which is consistent with the literature suggesting that SIV and HIV-2 Vpr counteract the human silencing hub (HUSH) repressor complex (51,52). Furthermore, we found that Vpr downregulates CXCL14, which aligns with the data reporting that human papillomaviruses downregulate CXCL14 to enhance replication (53).

Our data also indicate that Vpr activates the RelA/NF-κB immune pathway without induction of cell cycle arrest and CRL4A\textsuperscript{DCAF1} recruitment and host protein degradation. This is consistent with previous reports showing that Vpr activates NF-κB transcription via phosphorylation of TAK1 (34), an upstream regulator of NF-κB. We further found that Vpr-induced DNA damage activates ATM-NEMO signaling to upregulate RelA/NF-κB transcription, adding to the mechanistic understanding of NF-κB activation by Vpr. Through engagement with the CRL4A\textsuperscript{DCAF1} complex, Vpr has also been found to repress NF-κB activation by altering the availability of the NF-κB p50-p65 heterodimer, thus limiting proinflammatory cytokine expression (35). While it remains unclear what differentiates Vpr-mediated NF-κB activation from repression, whether Vpr engages the CRL4A\textsuperscript{DCAF1} complex seems to be a distinguishing factor. Furthermore, it is clear that in all cases Vpr carefully modulates NF-κB activation without globally activating interferon, which would inhibit viral replication. Thus, further studies understanding how Vpr manages to activate only aspects of NF-κB signaling (54,55) will help to define how Vpr may contribute to the ability of HIV to subvert the innate immune response.

Although various studies have shown that Vpr also alters viral transcription the mechanisms are unclear and disparate. For example, Vpr-mediated LTR activation is associated with cell cycle arrest where the LTR is most transcriptionally active (56,57), degradation of host proteins such as CCDC137 (18), and CRL4A\textsuperscript{DCAF1} independent mechanisms (58). However, studies have
shown that Vpr also promotes LTR transcription in noncycling cells (59,60) where induction of cell cycle arrest is absent and the necessity for degradation of specific host proteins in LTR activation has not been extensively examined. One benefit of Vpr activating NF-κB signaling via ATM is the potential direct enhancement of LTR transcription, as the HIV-1 LTR contains multiple NF-κB binding sites essential for viral gene expression. In addition to NF-κB, we further identified that Vpr activates CEBPB and JUN transcription factors involved in LTR activation. This is similar to Vpr-mediated de-repression of the LTR by removal of the transcriptional repressor ZBTB2 following ATR activation (61). Overall, Vpr WT and mutants that induce only DNA damage globally alter cellular transcription, upregulate NF-κB signaling and transcription factors that promote LTR-driven transcription, suggesting that Vpr-induced DNA damage is important for promoting LTR transcription to enhance viral replication.

Overall, our data suggest that during HIV infection, incoming Vpr and de novo expressed Vpr prime the cellular environment by activating RelA/NF-κB signaling to promote LTR transcription and enhance viral replication in macrophages. Our data align with the growing body of literature that supports the role of accessory proteins modulating the host environment and the DDR to promote viral replication through mechanisms independent of host protein degradation.
**FIGURE LEGENDS**

**Figure 1:** HIV-1 Vpr induces DNA damage and alters cellular transcription independent of cell cycle arrest and CRL4A<sup>DCAF1</sup> engagement. (A) Comet assay of U2OS cells infected with rAAV expressing 3xFLAG-tagged Vpr WT, H71R, Q65R, untreated (negative control), or 50μM etoposide (positive control). Percent tail DNA was quantified at 24 hours post infection (hpi) using the OpenComet plug-in for the ImageJ software. Each circle represents one cell. N=3, one representative experiment shown. (B) Representative immunofluorescence images of U2OS cells infected under the same conditions as Fig. 1A; γH2A.x (magenta) and nuclei stained with DAPI (blue). Images were taken at 63x magnification. N=3, one representative experiment shown. EV, empty vector. (C) RNA-seq of U2OS cells at 36 hpi. Heat map displays Log₂ fold changes of upregulated genes in red and downregulated genes in blue. Each column represents a biological replicate, n=3 (D) Venn diagram of the top 100 upregulated and top 100 downregulated genes among Vpr WT, S79A, H71R, and Q65R. E) Dot plot of the 68 conserved differentially expressed genes among Vpr WT, S79A, and H71R. BIRC3 and CXCL8 are highlighted as the two most upregulated genes (Log₂ fold change) under all conditions. (F) Gene ontology and (G) TRRUST analysis of the 30 upregulated genes performed with Metascape software. Asterisk indicate statistical significance compared to untreated control, as determined by one-way ANOVA test (NS, nonsignificant; *** P< 0.0004, **** P< 0.0001). Related to Figures S1 and S2.

**Figure 2:** HIV-1 Vpr activates RelA and promotes NF-κB transcription independent of cell cycle arrest and CRL4A<sup>DCAF1</sup> engagement. (A) qRT-PCR validation of upregulated NF-κB target genes BIRC3 and CXCL8. Normalized expression (ΔΔCt) was calculated by normalizing to GAPDH followed by calculating fold change to untreated or empty vector treated cells. Cells treated under the same conditions as Fig. 1C. n=3, one representative experiment shown. (B) Representative immunofluorescence images of U2OS cells infected under the same conditions as Fig. 1A; RelA (red) and nuclei stained with DAPI (blue). Images were taken at 63x magnification. n=3, one representative experiment shown. Asterisk indicate statistical significance compared to untreated control, as determined by one-way ANOVA test (NS, nonsignificant; *** P< 0.0003, **** P< 0.0001).

**Figure 3:** Vpr-induced DNA damage activates ATM signaling independent of cell cycle arrest and CRL4A<sup>DCAF1</sup> engagement. (A) Live-cell imaging of U2OS cells stably expressing NBS1-GFP or 53PB1-GFP. Cells were infected under the same conditions as Fig. 1A and
representative images were taken at 33 hpi at 63x magnification. n=3, one representative experiment shown. (B) Representative images of co-localization of DNA damage sensors MRE11 (magenta) and NBS1 (yellow) in U2OS NBS1-GFP or (C) DNA damage transducers γH2A.x (magenta) and 53PB1 (yellow) in U2OS 53PB1-GFP cells infected under the same conditions as Fig. 1A. Representative images taken at 24 hpi at 63x magnification. n=3, one representative experiment shown. Related to Figure S3.

**Figure 4:** Vpr upregulates NF-κB transcription dependent on NEMO, yet independent of cell cycle arrest and CRL4A<sup>DCAF1</sup> engagement. qRT-PCR of BIRC3 and CXCL8 in the presence or absence of Nemo-Binding Inhibitor peptide (NBD) at 36 hpi. U2OS cells were pretreated with 100uM of NBD for 2 hours prior to infection with rAAV expressing 3xFLAG-tagged Vpr WT, H71R, Q65R, empty vector (negative control). Normalized expression to GAPDH. Asterisk indicate statistical significance compared to untreated control, as determined by one-way ANOVA test (NS, nonsignificant; **** P< 0.0001).

**Figure 5:** Vpr-induced DNA damage activates ATM-NEMO signaling and NF-κB transcription in primary MDMs early during infection. (A) Experimental schematic depicting the isolation and differentiation of primary human monocyte-derived macrophages (MDMs) from peripheral blood mononuclear cells (PBMCs) followed by delivery of Vpr via Virus-like Particles (VLPs) or HIV-1 infection. Infected MDMs were assayed for induction of DNA damage, activation of DDR signaling, and the upregulation of NF-κB target genes in the context of NEMO inhibitor. (B) Comet assay of MDMs treated with VLPs packaging 3xFLAG-tagged Vpr WT, H71R, Q65R, empty VLPs (negative control), or 25uM Mitomycin C (positive control) for 8 hours. Comet assay analysis was performed as in Fig. 1A. n=2, one representative experiment shown. (C) Representative immunofluorescence images of MDMs infected with VLPs as in Fig. 5B and treated or not with Nemo-Binding Inhibitor peptide (NBD); γH2A.x (magenta) and nuclei stained with DAPI (blue). Images were taken at 100x magnification. n=3, one representative experiment shown. (D) qRT-PCR for CXCL8 of MDMs treated with NBD as in Fig. 5C with TNFα as positive control. n=3, one representative experiment shown. (E) qRT-PCR for CXCL8 of MDMs infected with HIV-1 ∆Env for 8, 16, 24, and 48 hpi from four separate donors. Normalized expression to GAPDH. Asterisk indicate statistical significance compared to untreated control, as determined by one-way ANOVA test (NS, nonsignificant; * P<0.03, ** P < 0.005, **** P< 0.0001). Related to Figure S4.
Figure 6: Model showing Vpr-induced DNA damage activates ATM and NEMO signaling resulting in RelA nuclear translocation and NF-κB transcriptional upregulation. 1. Vpr induces double-strand DNA breaks independent of cell cycle arrest and CRL4A<sup>DCAF1</sup> complex engagement. 2. Vpr-induced DNA damage activates markers of ATM signaling including MRE11, NBS1, γH2A.x, and 53PB1. 3. ATM-DDR activation results in NEMO relieving RelA inhibition from inhibitor of NF-κB (IκBα and IκBβ). 4. RelA translocates into the nucleus. 5. RelA binds to NF-κB promoters and activates transcription (txn) of NF-κB target genes such as BIRC3 and CXCL8. (Schematic made on Biorender).

SUPPLEMENTAL FIGURE LEGENDS

Figure S1: Knockdown of DCAF1 does not abrogate the ability of Vpr to induce DNA damage, activate DDR signaling, or upregulate NF-κB target genes. (A) Schematic representation of Vpr mutants used in these studies to decouple some Vpr functions. Vpr S79A and Vpr H71R do not induce cell cycle arrest, and H71R also does not bind DCAF1 of the CRL4A<sup>DCAF1</sup> complex to degrade host proteins or remodel the proteome. Vpr Q65R (not shown) is a largely non-functional negative control. (B) Western blot of endogenous DCAF1 U2OS cells to test stable knockdown with shRNA. Control (ctl) shRNA and two DCAF1-specific shRNAs (shRNA1 and shRNA2) were used. Actin is used as a loading control. (C) Comet assay of DCAF1 knock-down cells infected with rAAV and analyzed as in Fig. 1A. n=1. (D) Representative immunofluorescence images of DCAF1 knock-down U2OS cells infected under the same conditions as Fig. 1A.; γH2A.x (magenta) and nuclei with DAPI (blue). Images were taken at 63x magnification. n=1, one representative image shown. (E) qRT-PCR for CXCL8 and BIRC3 in DCAF1 knock-down U2OS cells infected under the same conditions as Fig. 1A. n=1. Asterisk indicate statistical significance compared to untreated control, as determined by t-test (NS, nonsignificant; * P< 0.02, ** P<0.004, *** P<0.0003, **** P<0.0001). EV, empty vector negative control; Etop., etoposide positive control. Related to Figures 1 and 2.

Figure S2: Vpr alters cellular transcription through diverse mechanisms that are either dependent or independent of cell cycle arrest and CRL4A<sup>DCAF1</sup> engagement. (A) Gene ontology using Metascape displaying TRRUST analysis for upregulated genes. (B) Principal component analysis (PCA) of the RNA-seq data showing the relatedness of the three biological replicates compared to empty vector or untreated cells. Related to Figure 1.
**Figure S3: Vpr-induced DNA damage activates markers of ATM signaling.** (A) Live-cell imaging taken on the IncuCyte of U2OS NBS1-GFP or 53PB1-GFP cells infected as in Fig. 1A for 48 hrs. n=2, one representative experiment shown. (B & C) Quantification of DNA damage induced in the live-cell imaging experiment in Fig. 3A at 8, 26, 33, and 48hpi. Foci per cell and foci size were quantified using ImageJ software. Images were taken at 63x magnification using the LSM 900. n=3, one representative experiment shown. EV, empty vector; AU, arbitrary units. Related to Figure 3.

**Figure S4: Infection of primary MDMs with VLPs packaging Vpr protein or HIV-1 infection upregulate NF-κB transcription dependent on NEMO.** (A) Flow cytometry plots displaying differentiation of PBMC-derived monocytes to macrophages. Primary MDMs are CD14+, CD45+ and CD16+. n=6, one representative experiment shown. (B) Western blot of 3X FLAG-Vpr WT and mutants packaged in Virus-like particles (VLPs). (C) qRT-PCR for BIRC3 of MDMs treated with NBD as in Fig. 5C with TNFα as positive control. n=3, one representative experiment shown. (D) Histogram quantifying percent of infected MDMs with HIV-1 ΔEnv at 48hpi. n=6, one representative experiment shown. (E) qRT-PCR for unspliced HIV-1 RNA at 8, 16, 24 and 48hpi. n=6, one representative experiment shown. (F) qRT-PCR for TNFα of MDMs infected with HIV-1 ΔEnv at 8, 16, 24 and 48hpi. n=6, one representative experiment shown. (G & H) qRT-PCR for BIRC3 or CXCL8 of MDMs infected with HIV-1 ΔEnv at 8, 16, 24 and 48hpi in separate donors. n=6, (G) is all 6 donors, (H) is donors 5 and 6 in addition to those shown in Fig. 5E. Asterisk indicate statistical significance compared to untreated control, as determined by one-way ANOVA test (NS, nonsignificant; **** P<0.0001). Related to Figure 5.

**Files S1 & S2: HIV-1 Vpr WT and mutants alter cellular transcription.** Analysis of all upregulated (File S1) and downregulated (File S2) genes in the RNA seq analysis that passes statistical significance of Log2 (1.25), p < 0.01, and FDR < 4.50E-05. Counts per million reads mapped (CPM) Log2 fold changes were calculated relative to untreated control. Related to Figure 1 and Figure S2.
MATERIALS AND METHODS

Plasmids
pcDNA-3xFLAG-Vpr and pscAAV-mCherry-T2A-Vpr WT and mutant plasmids were generated as previously described (28). For rAAV production, pHelper and pAAV-2.5 capsid plasmids were used (Addgene and (28). For VLP production, psPAX2 and pmD2.G were used (Addgene). For HIV-1 ΔENV production, Bru-GFP ΔENV was generated as previously described (62) and pmD2.G (Addgene). For lentivirus stable knock-down of DCAF1, pLKO.1 (Addgene), psPAX2(Addgene) and pmD2.G (Addgene) were used.

Generation of Viruses
rAAV vectors packaging the pscAAV-mCherry-T2A-Vpr WT or mutant plasmids were generated by transient transfection of HEK 293 cells using polyethyleneimine (PEI) as previously described (63). Virus-like particles (VLPs) packaging Vpr WT or mutant proteins were generated by transient transfection of HEK 293T using TransIT-LT1 (Mirus). VLPs were harvested 48hrs post transfection, concentrated through a 25% sucrose cushion at 24,000 rpm for 3 hrs at 4°C, and resuspended in PBS. Protein packaging was validated through western blot. HIV-1 ΔENV pseudotyped with VSV-G were generated by transient transfection of HEK 293T using TransIT-LT1 (Mirus). Virus was collected 48hrs post transfection. Lentivirus expressing DCAF1 shRNAs were generated by transfection of HEK 293T using TransIT-LT1 (Mirus). shRNA1:

AATTCAAAAAGCTGAGAATACTCTTCAAGAACTCGAGTTCTTGAAGAGTATTCTCAGC
shRNA2: AATTCAAAAAGCACTTCAGTTATCATCAACTCGAGTTGTAGATAATCTGAGGTCT.

Media was changed 18 hours post transfection. Lentiviruses were harvested 48 hours post media change and filtered through a 0.45 μm PES filter.

Cell Lines and Cell Culture
Human bone osteosarcoma epithelial (U2OS), Human embryonic kidney (HEK) 293, and HEK 293T cells were cultured as adherent cells directly on tissue culture plastic (Greiner) in Dulbecco’s modified Eagle’s medium (DMEM) growth medium (high glucose, L-glutamine, no sodium pyruvate; Gibco) with 10% fetal bovine serum (FBS) (Gibco) and 1% penicillin-streptomycin (Gibco) at 37°C and 5% CO2. All cells were harvested using 0.05% trypsin-EDTA (Gibco). The panel of U2OS cells stably expressing 53PB1-GFP (64) and NBS1-GFP (65) were kindly provided
by Claudia Lukas (University of Copenhagen, Denmark). Stable DCAF1 knock-down cells were generated by delivering lentiviruses expressing DCAF1 shRNAs or empty vector to U2OS cells. U2OS with integrated lentiviral construct were selected for with 1ug/ml of Puromycin. Knock-down of DCAF1 was assessed through western blots.

Monocyte-Derived Macrophages (MDMs)

Human peripheral blood mononuclear cells (PBMCs) were obtained from human donors at the UCLA/CFAR Virology Core Laboratory. Primary monocytes were isolated from PBMCs by negative selection using the EasySep™ Human T Cell Isolation Kit (STEM CELL) and were differentiated into monocyte-derived macrophages (MDMs) by stimulation with 20ng/mL macrophage colony-stimulating factor (M-CSF) (R&D Systems). MDMs were cultured in Roswell Park Memorial Institute (RPMI) 1640 growth medium (L-Glutamine) with 10% fetal bovine serum (Gibco) at 37°C and 5% CO2 for 7 days while replenishing media every 3 days.

Alkaline Comet Assay

The alkaline comet assay and data analysis was performed as previously described (28), with minor changes. MDMs were infected with VLPs delivering Vpr WT and mutants at equal protein levels or 25uM Mitomycin C (Cayman). Cells were harvested with Accutase (STEM CELL) at 10 hrs post infection and resuspended in 0.5% low-melting-point agarose at 37°C. Images were acquired on the Zeiss Axio Imager Z1. Images were analyzed using the OpenComet plug-in for ImageJ.

RNA-sequencing

Total RNA from U2OS cells was isolated using TRIzol® reagent (Invitrogen). RNA integrity was assessed using the Bioanalyzer TapeStation 4200 RNA High Sensitivity (Agilent). Library Preparation was completed using the KAPA mRNA HyperPrep Kit (Illumina) enriching for Poly(A) RNA with magnetic oligo-dT beads and attaching unique dual indexed adapters. Quality control of the library was done with the Bioanalyzer TapeStation 4200 D1000 High Sensitivity (Agilent). RNA was sequenced using the Hiseq3000 1x50 at the UCLA Technology Center for Genomics & Bioinformatics core. Reads were aligned to the Human h38 STAR genome and gene counts were determined using edgeR. log2 reads per kilobase of transcript per million reads mapped (rpkms) were calculated for samples compared to untreated cells. Heat map was generated using heatmap hierarchical clustering on z-score log2 rpkms. Gene ontology and TRRUST analysis were done on Metascape.
**Quantitative Reverse Transcription PCR (qRT-PCR)**

Total RNA was isolated using the PureLink™ RNA Mini Kit (Invitrogen). RNA was reverse transcribed using SuperScript IV First-Stand Synthesis System (Invitrogen) with Oligo(dT) primers. qRT-PCR was performed with PowerTrack SYBR Green Master Mix (Thermo Fisher Scientific) on the LightCycler 480 System (Roche) with the following primers (5’ to 3’): BIRC3: AAGCTACCTCTCAGCCTACTTT and CCACTGTTTTCTGTACCCGGA, CXCL8: TTTTGGCAGGATGTCTAAGTA and ACAAGATCTCGACCCAGTTTTC, TNFα: CTCTTCTGCCTGCCTACCTTT and ATGGGGCTACAGGCTTGTCACTTT, HIV-1 unspliced RNA: GCGACGAAGACCTCCTCAAG and GAGGTGGGTTGCTTTGATAGAGA, and GAPDH: CAAGATCATCAGCAATGCCT and AGGGATGATGTTCTGGAGAG. mRNA levels were quantified by calculating ∆∆Ct. Target transcript Ct values were normalized to the Ct value of the housekeeping gene GAPDH followed by calculating fold change to untreated or empty vector treated cells.

**Live-Cell Imaging**

U2OS-53PB1-GFP and U2OS-NBS1-GFP cells were imaged using the IncuCyte S3 Live-Cell Analysis Instrument (Sartorius). Mean Fluorescence Intensity (MFI) was calculated using the Sartorius software. For higher resolution, U2OS-53PB1-GFP and U2OS-NBS1-GFP cells were imaged using the LSM 900. Foci per cell and foci size were analyzed using ImageJ.

**Immunofluorescence**

Cells were plated in 6- or 24-well tissue culture plates (Greiner) and allowed to adhere overnight, then infected with VLP (equal protein expression), rAAV-2.5 (1.4 × 10⁸ copies/well), or etoposide (Sigma). U2OS cells were permeabilized with 0.5% Triton X-100 in PBS at 4°C for 5 min and fixed in 4% PFA for 20 min, MDM cells were permeabilized with 0.1% Saponin (Fisher) in PBS at 4°C for 15 min and fixed in 4% PFA for 15 min. Cells were washed, incubated with blocking buffer (3% BSA, 0.05% Tween 20, and 0.04 NaNO₃ in PBS for U2OS cells or 3% FBS in 0.1% Saponin for MDM cells) for 30 min. Cells were probed with appropriate primary antibodies (anti-γH2A.x Ser139, anti-53BP1, or anti-RelA(p65) [Cell Signaling], anti-GFP [Takara], and anti-MRE11 [Novous]) and then washed and probed with Alexa Fluor-conjugated secondary antibodies (Life Technologies). Nuclei were stained with diamidino-2-phenylindole (DAPI; Life Technologies). Images were acquired on the LSM 980.
Western Blots
Protein was collected from cells as previously described (28). Membranes were blocked in
intercept blocking buffer (Li-COR Biosciences). Immunoblotting was performed using mouse anti-
FLAG M2 (Sigma-Aldrich), rabbit anti-Actin (Bethyl), or mouse anti-DCAF1 (Proteintech) for 1 hr.
Blots were incubated with secondary antibodies IRDye 800CW anti-Rabbit and IRDye 680RD
anti-Mouse (Li-COR Biosciences) for 1 hr and then visualized using the Li-COR Odyssey M (Li-
COR Biosciences).

HIV-1 ∆Env Infection
MDMs were plated in 96-well tissue culture plates (Greiner) and allowed to adhere overnight.
MDMs underwent spinfection with HIV-1 ∆Env pseudotyped with VSV-G at 1200× g for 90 min at
37°C. Infection was assessed 48 hours after infection via qRT-PCR and Flow Cytometry.

Flow Cytometry
Isolated monocytes and infected MDMs were lifted from tissue culture plates using accutase
(STEM CELL). Cells were stained for CD14-FITC, CD45-APC, or CD16-APC (STEM CELL) for
30 min at 4°C, washed with PBS, fixed in 4% PFA for 15 min, permeabilized with 0.1% Triton X-
100 in PBS at 4°C for 15 min, then washed with PBS. Cells were probed for HIV-1 core antigen-
FITC KC57 (Beckman Coulter) for 1 hr at 4°C then washed with PBS and resuspended in FACS
buffer (5% FBS in PBS). Events were assessed by flow cytometry on the AttuneNxt (Thermo
Fisher Scientific). At least 10,000 events were collected per run. Data was analyzed using FlowJo
software.

Statistical Analyses
All statistical analyses were performed using GraphPad Prism 9.

AUTHOR CONTRIBUTIONS
Conceptualization, C.S. and O.I.F; methodology, C.S.; writing original draft C.S and O.I.F; writing,
review and editing C.S. and O.I.F; visualization C.S. and O.I.F.; supervision and funding
acquisition O.I.F. All authors have read and agreed to the published version of the manuscript.

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COMPETING INTERESTS

The author(s) declare(s) they have no competing interests.
REFERENCES


Figure 1: HIV-1 Vpr induces DNA damage and alters cellular transcription independent of cell cycle arrest and CRL4A\textsuperscript{DCAF1} engagement. (A) Comet assay of U2OS cells infected with rAAV expressing 3xFLAG-tagged Vpr WT, H71R, Q65R, untreated (negative control), or 50uM etoposide (positive control). Percent tail DNA was quantified at 24 hours post infection (hpi) using the OpenComet plug-in for the ImageJ software. Each circle represents one cell. N=3, one
representative experiment shown. (B) Representative immunofluorescence images of U2OS cells infected under the same conditions as Fig. 1A; γH2A.x (magenta) and nuclei stained with DAPI (blue). Images were taken at 63x magnification. N=3, one representative experiment shown. EV, empty vector. (C) RNA-seq of U2OS cells at 36 hpi. Heat map displays Log_{2} fold changes of upregulated genes in red and downregulated genes in blue. Each column represents a biological replicate, n=3 (D) Venn diagram of the top 100 upregulated and top 100 downregulated genes among Vpr WT, S79A, H71R, and Q65R. E) Dot plot of the 68 conserved differentially expressed genes among Vpr WT, S79A, and H71R. BIRC3 and CXCL8 are highlighted as the two most upregulated genes (Log_{2} fold change) under all conditions. (F) Gene ontology and (G) TRRUST analysis of the 30 upregulated genes performed with Metascape software. Asterisk indicate statistical significance compared to untreated control, as determined by one-way ANOVA test (NS, nonsignificant; *** P< 0.0004, **** P< 0.0001). Related to Figures S1 and S2.
Figure 2: HIV-1 Vpr activates RelA and promotes NF-κB transcription independent of cell cycle arrest and CRL4A\textsuperscript{DCAF1} engagement. (A) qRT-PCR validation of upregulated NF-κB target genes BIRC3 and CXCL8. Normalized expression (\(\Delta\Delta Ct\)) was calculated by normalizing to GAPDH followed by calculating fold change to untreated or empty vector treated cells. Cells treated under the same conditions as Fig. 1C. n=3, one representative experiment shown. (B) Representative immunofluorescence images of U2OS cells infected under the same conditions as Fig. 1A; RelA (red) and nuclei stained with DAPI (blue). Images were taken at 63x magnification. n=3, one representative experiment shown. Asterisk indicate statistical significance compared to untreated control, as determined by one-way ANOVA test (NS, nonsignificant; *** P< 0.0003, **** P< 0.0001).
Figure 3: Vpr-induced DNA damage activates ATM signaling independent of cell cycle arrest and CRL4A\(^{\text{DCAF1}}\) engagement. (A) Live-cell imaging of U2OS cells stably expressing NBS1-GFP or 53PB1-GFP. Cells were infected under the same conditions as Fig. 1A and representative images were taken at 33 hpi at 63x magnification. n=3, one representative experiment shown. (B) Representative images of co-localization of DNA damage sensors MRE11 (magenta) and NBS1 (yellow) in U2OS NBS1-GFP or (C) DNA damage transducers yH2A.x (magenta) and 53PB1 (yellow) in U2OS 53PB1-GFP cells infected under the same conditions as Fig. 1A. Representative images taken at 24 hpi at 63x magnification. n=3, one representative experiment shown. Related to Figure S3.
Figure 4: Vpr upregulates NF-κB transcription dependent on NEMO, yet independent of cell cycle arrest and CRL4A
DCAF1 engagement. qRT-PCR of BIRC3 and CXCL8 in the presence or absence of Nemo-Binding Inhibitor peptide (NBD) at 36 hpi. U2OS cells were pretreated with 100uM of NBD for 2 hours prior to infection with rAAV expressing 3xFLAG-tagged Vpr WT, H71R, Q65R, empty vector (negative control). Normalized expression to GAPDH. Asterisk indicate statistical significance compared to untreated control, as determined by one-way ANOVA test (NS, nonsignificant; **** P< 0.0001).
Figure 5: Vpr-induced DNA damage activates ATM-NEMO signaling and NF-κB transcription in primary MDMs early during infection. (A) Experimental schematic depicting the isolation and differentiation of primary human monocyte-derived macrophages (MDMs) from peripheral blood mononuclear cells (PBMCs) followed by delivery of Vpr via Virus-like Particles (VLPs) or HIV-1 infection. Infected MDMs were assayed for induction of DNA damage, activation of DDR signaling, and the upregulation of NF-κB target genes in the context of NEMO inhibitor. (B) Comet assay of MDMs treated with VLPs packaging 3xFLAG-tagged Vpr WT, H71R, Q65R, empty VLPs (negative control), or 25μM Mitomycin C (positive control) for 8 hours. Comet assay analysis was performed as in Fig. 1A. n=2, one representative experiment shown. (C) Representative immunofluorescence images of MDMs infected with VLPs as in Fig. 5B and treated or not with Nemo-Binding Inhibitor peptide (NBD); γH2A.x (magenta) and nuclei stained with DAPI (blue). Images were taken at 100x magnification. n=3, one representative experiment shown. (D) qRT-PCR for CXCL8 of MDMs treated with NBD as in Fig. 5C with TNFα as positive control. n=3, one representative experiment shown. (E) qRT-PCR for CXCL8 of MDMs infected with HIV-1 ΔEnv for 8, 16, 24, and 48 hpi from four separate donors. Normalized expression to GAPDH. Asterisk indicate statistical significance compared to untreated control, as determined by one-way ANOVA test (NS, nonsignificant; * P<0.03, ** P < 0.005, **** P< 0.0001). Related to Figure S4.
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