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# Vpu-Deficient HIV Strains Stimulate Innate Immune Signaling Responses in Target Cells

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**Acute virus infection induces a cell-intrinsic innate immune response comprising our first line of immunity to limit virus replication and spread, but viruses have developed strategies to overcome these defenses. HIV-1 is a major public health problem; however, the virus-host interactions that regulate innate immune defenses against HIV-1 are not fully defined. We have recently identified the viral protein Vpu to be a key determinant responsible for HIV-1 targeting and degradation of interferon regulatory factor 3 (IRF3), a central transcription factor driving host cell innate immunity. IRF3 plays a major role in pathogen recognition receptor (PRR) signaling of innate immunity to drive the expression of type I interferon (IFN) and interferon-stimulated genes (ISGs), including a variety of HIV restriction factors, that serve to limit viral replication directly and/or program adaptive immunity. Here we interrogate the cellular responses to target cell infection with Vpu-deficient HIV-1 strains. Remarkably, in the absence of Vpu, HIV-1 triggers a potent intracellular innate immune response that suppresses infection. Thus, HIV-1 can be recognized by PRRs within the host cell to trigger an innate immune response, and this response is unmasked only in the absence of Vpu. Vpu modulation of IRF3 therefore prevents virus induction of specific innate defense programs that could otherwise limit infection. These observations show that HIV-1 can indeed be recognized as a pathogen in infected cells and provide a novel and effective platform for defining the native innate immune programs of target cells of HIV-1 infection.**

Timely and appropriate recognition of virus infection is essential for both the suppression of infection and programming of downstream immune responses. Host cells are able to recognize specific motifs within viral products as non-self- or pathogen-associated molecular patterns (PAMPs) by utilizing cellular factors, termed pattern recognition receptors (PRRs), for virus sensing (50, 52). Viral genomic RNA, DNA, and replication intermediates represent PAMPs that are sensed by several families of nucleic acid sensors, including Toll-like receptors (TLRs), RIG-I-like receptors (RLRs), as well as several classes of DNA sensors (50, 52). Once a viral PAMP is engaged by the appropriate PRR within a mammalian cell, an innate intracellular immune response is triggered in order to suppress viral replication and spread (50, 52).

Many PRRs signal downstream in a cascade that requires interferon regulatory factor 3 (IRF3), activation leading to the production of alpha/beta interferon (IFN-α/β), and expression of IRF3-dependent gene products (22, 52). IFN can then drive both autocrine and paracrine signaling programs to generate an antiviral response in the infected cell and surrounding tissue that induces hundreds of interferon-stimulated genes (ISGs) (52). ISG products have either direct antiviral or immune modulator actions that serve to limit virus infection (50, 52). A central strategy of viral evasion of host immunity is to disrupt a variety of innate immune signaling responses (i.e., disruption of PRR signaling) or inhibition of ISG functions (29). Viral control of IRF-3 activation is a strategy utilized by members of divergent viral genera to prevent the earliest innate immune responses. This allows the virus to avoid the effects of IFN-α/β, proinflammatory cytokines, and other IRF3-responsive gene products that otherwise enhance the immune response and limit infection (29).

HIV-1 is a human retrovirus that has evolved several sophisticated mechanisms to modulate intracellular innate immune effectors and restriction factors (9, 25, 36). While many known anti-HIV restriction factors display basal levels of expression in resting, noninfected cells, these genes are also induced in response to the IRF3 activation and/or IFN signaling that occurs during virus infection. IRF3 is important for the induction of innate immunity in T cells and macrophages, to drive the expression of IFN and ISGs, including a variety of ISGs that directly affect HIV-1, as well as to program downstream adaptive immunity (1, 24, 36, 40, 51). HIV-1-infected peripheral blood mononuclear cells (PBMCs) and T cell lines exhibit a limited spectrum of ISG expression and little if any IFN production (5, 17, 37, 48). This suggests that either acute HIV-1 infection fails to engage PRR signaling or viral programs are antagonizing these processes. Indeed, we and others have shown that productive infection of T cells by HIV-1 is accompanied by the specific targeted proteolysis of IRF3 that occurs through a virus-directed mechanism resulting in suppression of innate immune defenses (10, 38). These studies revealed that IRF3 activation drives an innate immune response that is highly deleterious to productive HIV-1 infection, suggesting that targeted viral antagonism of IRF3 by HIV-1 may provide an additional level of viral control of the innate immune system. Indeed, we have recently demonstrated (see the companion paper [8]) that the HIV-1 protein Vpu plays an important role in HIV-1 innate immune regulation by targeting and relocalizing IRF3 to the lysosomal compartment for proteolysis. Here we demonstrate that a

**Received** 17 February 2012 **Accepted** 19 May 2012
**Published ahead of print** 30 May 2012
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doi:10.1128/JVI.00424-12

functional outcome of infection of target cells in the absence of Vpu expression is a robust induction of innate immune signaling and antiviral gene expression that limits HIV infection. We show that induction of the innate immune response by HIV-1 occurs in an IRF3-dependent but IPS-1- and MyD88-independent mechanism. Thus, the absence of Vpu reveals the full context of innate immune signaling in host cells during acute HIV-1 infection.

## MATERIALS AND METHODS

**Cell culture and transfections.** All cells were grown under standard conditions as described previously (10). Jurkat R5 (obtained from M. Katze), THP-1, and primary macrophages were cultured in RPMI medium supplemented with 10% fetal bovine serum (FBS), L-glutamine, and antibiotics. PBMCs were isolated as described previously (10), and macrophages were cultured further using plastic adherence, as described elsewhere (51). Isolation of T cells from healthy vaginal mucosa was carried out as previously described in detail (20, 21) and were harvested from routinely discarded tissues from vaginal-repair surgeries performed in adult women at three medical centers in Seattle, WA. THP-1 cells were differentiated by exposure to 100 nM phorbol myristate acetate (PMA; Sigma) overnight. Tzm-bl cells were cultured in Dulbecco modified Eagle medium supplemented with 10% FBS, L-glutamine, and antibiotics.

**Stable knockdown cell lines.** Knockdown THP-1 cells for IRF3, IPS-1, and MyD88 genes were stably generated using Sigma Mission lentiviral particles. Cells were generated according to the manufacturer's suggested protocol and tested for knockdown by immunoblot analysis of protein levels compared to those in control non-targeting-vector (NTV) cells.

**Viral stocks and infection.** HIV-$1_{LAI}$ was propagated using standard procedures as described previously (10). YU2 point mutant proviral constructs have been described previously (42), and Vpu mutants of JR-CSF were generated via site-directed mutagenesis, as described in the companion paper (8). Generation of point mutant proviral clones was accomplished using a QuikChange mutagenesis kit (Stratagene) following the manufacturer's recommended protocol, optimizing for the large size of the plasmids. The following primers were used: JR-CSF A/C Forward (5′-GCAGTAAGTAGTGCATGTACTGCAACCTTTACAAATATTAGCAATAGTAGC-3′) and Reverse (5′-GCTACTATTGCTAATATTTGTAAAGGTTGCAGTACATGCACTACTTACTGC-3′); HIV-1 strains JR-CSF and YU2 and Vpu-deficient proviral clones were transfected into 293T cells as described previously to generate infectious virus (20, 21). Mock infections represent treatment with conditioned medium, and infections with virus were performed by adding virus to culture medium, with washing performed prior to harvest except where otherwise indicated. The titers of all HIV-1 strains were determined on Tzm-bl cells to determine the concentration of infectious virus. Sendai virus (SeV) strain Cantell was obtained from Charles River Laboratories.

**Cell treatments and fractionation.** Synthetic hepatitis C virus (HCV) PAMP RNA was generated as previously described and delivered into cells using a *Trans*IT kit (Mirus) according to the manufacturer's instructions (47). Nuclear and cytoplasmic fractionation was performed using standard methods previously described (28).

**Immunoblot analysis, coimmunoprecipitation, and immunofluorescence imaging.** SDS-PAGE and immunoblot analysis were performed using standard procedures as described previously (10). The following antibodies were used in the study: rabbit (Rb) anti-ISG56 (G. Sen), mouse (M) anti-p24, goat (Gt) anti-beta-actin (Santa Cruz), Rb total anti-IRF3 (a gift from Michael David), Rb anti-IRF3-p (Cell Signaling), M total anti-IRF-3 (19), Rb anti-lamin B (Abcam), M antitubulin (Sigma), Rb anti-HIV-$1_{NL4-3}$ Vpu, Rb anti-human IPS-1 (Alexis), and Rb anti-human MyD88 (Abcam). For immunoblot detection, the appropriate horseradish peroxidase-conjugated secondary antibody was used (Jackson ImmunoResearch Laboratories), followed by treatment of the membrane with ECL-plus reagent (Roche) and imaging on X-ray film. Densitometry was performed using ImageJ software (NIH) on unsaturated blots.

**Statistical analysis.** Differences between groups were analyzed for statistical significance by the Student *t* test.

**Targeted genomics analysis.** RNA was extracted using an RNeasy minikit (Qiagen) according to the manufacturer's instructions. Two hundred nanograms of total RNA was amplified and labeled using an Illumina TotalPrep RNA amplification kit (Ambion). cRNA quality was assessed by capillary electrophoresis on an Agilent 2100 bioanalyzer. Expression levels of mRNA transcripts were assessed by human-HT12 (version 4) expression BeadChips (Illumina). Hybridization was carried out according to the manufacturer's instructions.

**Data preprocessing.** Bead summary data were output from Illumina's BeadStudio software without background correction, as this has previously been shown to have detrimental effects (15). Data preprocessing, including a variance-stabilizing transformation (26) and robust-spline normalization, was applied as implemented in the lumi package of R (14). Data were prefiltered for probes that were not expressed (detection *P* value, $\leq 0.05$) in at least one group ($n = 4$/group), leaving a set of 17,993 probes for subsequent analysis. Data were further filtered on the basis of an experimentally derived list of virus-induced known ISGs and anti-HIV-1 molecules (Table 1) (7, 53). An additional filter for 74 annotated NF-κB-responsive genes was used to determine specificity of response (Table 1).

**Differential expression analysis.** A Bayesian statistical framework that was developed by Pierre Baldi and Tony Long called Cyber-T was used to test for the effect of infected (JR-CSF, JR-CSF A/C) versus mock-infected samples on gene expression (27). We used a false discovery rate (FDR) Benjamini-Hochberg method to control for multiple testing (3). Probes selected for further analysis had an FDR-adjusted *P* value of $\leq 0.05$. A $\log_2$-fold change was calculated as the difference between infected and mock-infected samples. All statistical testing for gene expression was performed in FlexArray, a front-end wrapper for R (4). To compare samples from infected and mock-infected groups, we clustered the expression profiles for a select gene set using unsupervised hierarchical clustering with Euclidean average linkage clustering with both gene and sample leaf ordering as implemented in the TMEV program (45, 46).

The following reagents were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: Tzm-bl cells were from John C. Kappes, Xiaoyun Wu, and Tranzyme Inc., monoclonal antibody to HIV-1 p24 (AG3.0) was from Jonathan Allan, and HIV-$1_{NL4-3}$ Vpu antiserum was from Klaus Strebel.

## RESULTS

**Vpu-deficient HIV-1 strains stimulate the production of ISGs upon infection of target cells.** Vpu-deficient strains of HIV-1 such as $HIV_{YU2}$ are unable to downmodulate IRF3-dependent signaling or drive relocalization of IRF3 to the lysosomal compartment (8). To evaluate the role of Vpu in regulating IRF3-dependent signaling of innate immunity during HIV-1 infection, we examined the response of cells acutely infected with HIV-$1_{JR-CSF}$ (Vpu-positive) or HIV-$1_{YU2}$ (Vpu-negative) virus strains. Infection of differentiated THP-1 cells and R5-permissive Jurkat cells with HIV-$1_{JR-CSF}$ failed to induce innate immune signaling responses marked by induction of expression of ISG56 (Fig. 1A and B, respectively), an IRF3 target gene (18). Conversely, HIV-$1_{YU2}$ infection triggered the robust induction of ISG56 production in both cell types (Fig. 1A and B). Moreover, HIV-$1_{YU2}$ induced the innate immune signaling of ISG56 production upon infection of primary macrophages (Fig. 1C), and importantly, *ex vivo* infection of cultures of T cells harvested from human vaginal epithelium samples by HIV-$1_{YU2}$ induced an innate immune response characterized by ISG expression, whereas infection by HIV-$1_{JR-CSF}$ did not induce this response (Fig. 1D). To control for the effects of other genetic differences between the HIV-$1_{YU2}$ and HIV-$1_{JR-CSF}$

**TABLE 1** Innate immune genes/ISGs and NF-κB genes utilized for targeted genomic analysis

| Gene | | |
|---|---|---|
| Innate immune/ISGs[a] | STAT2 | IL-18RAP |
| APOBEC3B[b] | STAT3 | IL-1A |
| APOBEC3C | STING | IL-1R1 |
| APOBEC3F[b] | TLR3 | IL-6 |
| APOBEC3G[b] | TLR7 | IL6R |
| BST2[b] (tetherin) | TLR9 | IL-8 |
| DDX58 (RIGI) | TMEM173 | NFKB2 |
| GIP2 | TNFSF10 | NFKB1B |
| IFI16 | TNFSF13B | PIK3AP1 |
| IFI27 | TRIM14 | PIK3C2A |
| IFI35 | TRIM22 | PIK3CB |
| IFI44 | TRIM34 | PIK3CG |
| IFI6 | TRIM5[b] | PIK3R1 |
| IFIH1 (MDA5) | TRIM56 | PIK3R2 |
| IFIT1 (ISG56) | | PIK4CA |
| IFIT2 | NF-κB[c] | RELA |
| IFIT3 | CASP5 | RELB |
| IFIT5 | CASP7 | RELL2 |
| IFITM1 | CASP9 | TNF |
| IFNA1 | CASS4 | TNFAIP1 |
| IFN7A | CCL1 | TNFAIP2 |
| IFNB1 | CCL14 | TNFAIP3 |
| IFNE | CCL2 | TNFAIP6 |
| IFNG | CCL20 | TNFAIP8 |
| IFRG28 | CCL3 | TNFAIP8L3 |
| IRF1 | CCL3L1 | TNFRSF10A |
| IRF3 | CCL3L3 | TNFRSF10B |
| IRF5 | CCL4L1 | TNFRSF12A |
| IRF7 | CCL4L2 | TNFRSF14 |
| IRF8 | CCL5 | TNFRSF19 |
| ISG15[b] | CCL7 | TNFRSF1A |
| ISG20[b] | CCL8 | TNFRSF1B |
| ISGF3G | CXCL9 | TNFRSF21 |
| MX1 | CXCR5 | TNFRSF9 |
| MX2 | IKBKE | TNFSF10 |
| MyD88 | IKBKG | TNFSF13B |
| NOD27 | IL-10 | TNFSF14 |
| NLRC5 | IL-10RA | TNFSF15 |
| OAS1 | IL-10RB | TNFSF4 |
| OAS3 | IL-11RA | TNFSF9 |
| OASL | IL-13RA1 | TRAF1 |
| PRKR | IL-17RA | TRAF3 |
| RSAD2[b] (viperin) | IL-17RD | TRAF3IP2 |
| RTP4 | IL-18BP | TRAF6 |
| STAT1 | IL-18R1 | TRAF7 |

[a] All genes have been experimentally determined to be induced in response to virus infection or have been reported as having direct anti-HIV-1 properties. Additional nomenclature for gene names is shown in parentheses.
[b] Known anti-HIV-1 factors.
[c] NF-κB target genes used for analysis of specificity. IL-10, interleukin-10; TNF, tumor necrosis factor.

strains, we took advantage of congenic HIV-$1_{JR\text{-}CSF}$ and HIV-$1_{JR\text{-}CSF\ A/C}$ mutant viruses for direct comparison of their innate immune stimulatory capacity. The mutant HIV-$1_{JR\text{-}CSF}$ containing an A/C transversion in the Vpu start codon triggered a robust innate immune response in THP-1 cells in a dose-dependent manner (Fig. 1E) which greatly exceeded the response to the wild-type (wt) HIV-1 strain. It is important to note that we have previously shown, as indicated in the companion paper (8), that these Vpu-deficient proviruses' ability to block innate immune signaling can be rescued by providing Vpu in *trans*. Additionally, when Vpu is ectopically expressed in producer cells along with Vpu-deficient provirus, the resulting virus produced is robustly stimulatory for innate immune induction (data not shown). These observations reveal that T cells and macrophages are fully competent to recognize HIV-1 through a pathogen-sensing pathway(s) that directs the innate antiviral response against infection but this response is blocked by HIV-1 in a Vpu-dependent manner.

**IRF3-dependent but IPS-1- and MyD88-independent induction of ISGs in response to Vpu-deficient HIV-1 infection.** The inability of HIV-$1_{YU2}$ to produce Vpu and suppress IRF3 provides a novel model system to examine the features of natural innate immune triggering during HIV-1 infection. We therefore assessed the role of canonical IRF3-dependent PRR pathways in viral recognition and innate immune signaling during acute infection of THP-1 cells. Infection with HIV-$1_{YU2}$ induced IRF3 phosphorylation, similar to that induced by infection with SeV (visible as laddering and slower-migrating IRF3 bands), but infection with HIV-$1_{JR\text{-}CSF}$ did not induce this response (Fig. 2A). Moreover, infection by HIV-$1_{YU2}$ resulted in the nuclear accumulation of phospho-IRF3 and concomitant expression of ISG56 (Fig. 2B and C). To assess the requirement for IRF3 and the possible roles of RLR- and MyD88-dependent PRR signaling pathways in this response, we examined signaling in THP-1 cells stably expressing a nontargeting control short hairpin RNA (shRNA) or shRNA targeting IRF3, IPS-1, or MyD88, respectively. Each cell line exhibited stable knockdown of target gene expression compared to

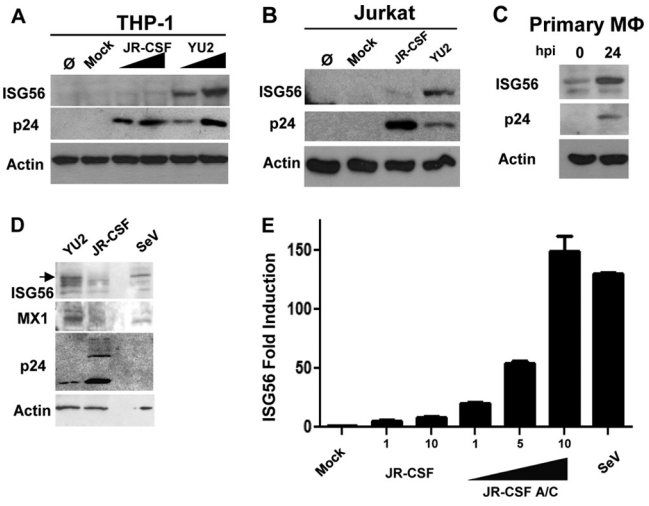


**FIG 1** Vpu-deficient HIV-1 strains stimulate innate immune signaling. (A) PMA-differentiated THP-1 cells were infected for 24 h with a multiplicity of infection of 0.5 or 1 of wild-type JR-CSF or YU2, mock treated, or untreated (Ø) and then analyzed by immunoblotting for ISG56, p24, and actin as a loading control. (B and C) Jurkat (R5 positive) and PBMC-derived macrophages (Mϕ) tested as described for panel A with virus at a multiplicity of infection of 1. (D) ISG56, MX1, HIV-1 p24, and actin protein levels in vaginal mucosal T cells infected *ex vivo* with SeV, HIV-$1_{JR\text{-}CSF}$, or HIV-$1_{YU2}$. Virus was added to cultures and allowed to infect for 24 h, after which extracts were prepared and subjected to immunoblot analysis. (E) Differentiated THP-1 cells were challenged with increasing multiplicities of infection of JR-CSF or Vpu-deficient JR-CSF A/C, with SeV challenge used as a control. Protein lysates were blotted as described for panels A to C and quantified; fold induction above that for mock-treated cells is shown. Representative immunoblots of at least 3 experiments are shown.

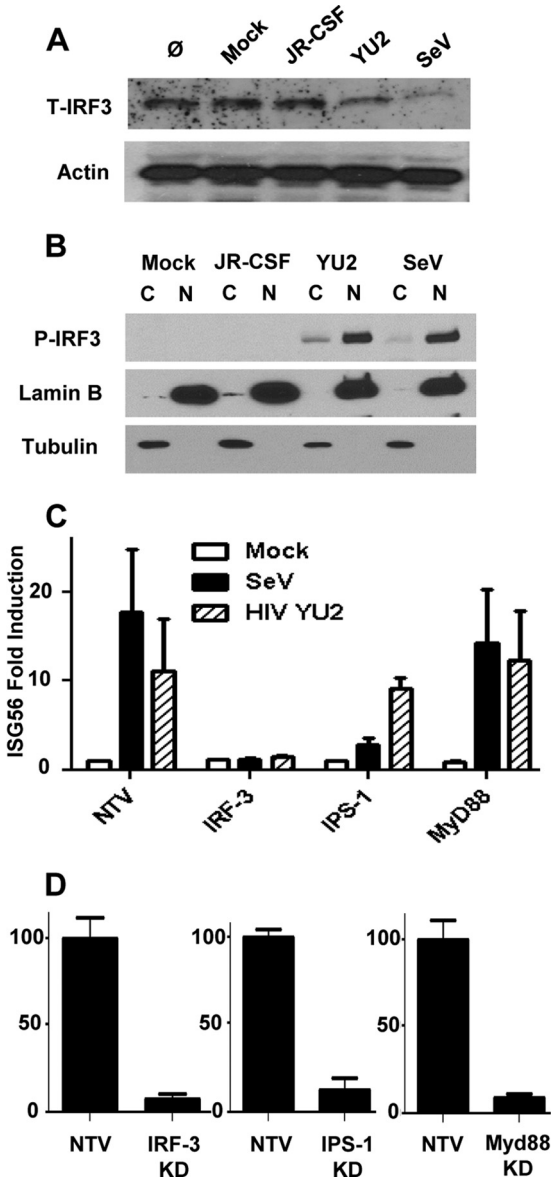


**FIG 2** Vpu-deficient HIV-1 strains stimulate an IRF3-dependent ISG response. (A) PMA-differentiated THP-1 cells were either infected with JR-CSF, YU2, or SeV, mock treated, or untreated (Ø) for 8 h and harvested for immunoblot analysis. Cell lysates were probed for total IRF3 (T-IRF3) and beta-actin as a loading control (multiplicity of infection = 1). (B) PMA-differentiated THP-1 cells were either mock treated or infected with YU2 or SeV for 8 h. Cells were fractionated into cytoplasmic (C) or nuclear (N) compartments and analyzed by immunoblotting for phosphorylated IRF3 (P-IRF3). Lamin B and tubulin mark loading of the nuclear and cytoplasmic fractions, respectively. (C) PMA-differentiated THP-1-knockdown cell lines for IRF3, IPS-1, or MyD88 or a nontargeting control were infected with SeV or HIV-$1_{YU2}$ or mock treated. Cells were treated and analyzed as described for Fig. 1E. (D) Comparison of non-targeting-vector (NTV) and knockdown (KD) cell protein levels for IRF3, IPS-1, and MyD88. Cells were harvested and immunoblotted for the targeted protein. Quantification is shown. Error bars are SDs.

nontargeting controls (Fig. 2D). Compared to the control cells, IRF3-knockdown cells no longer induced ISG56 expression in response to HIV-$1_{YU2}$ or SeV infection (Fig. 2C). However, IPS-1-knockdown cells and MyD88-knockdown cells both retained their response and induced ISG56 expression when infected with HIV-$1_{YU2}$. As expected, MyD88-knockdown cells also responded to SeV, a specific RLR agonist (28), but this response was ablated in IPS-1-knockdown cells (Fig. 2C). Thus, we conclude that virus recognition and innate immune signaling of HIV-1 infection occur through an IRF3-dependent but IPS-1- and MyD88-independent cellular pathway(s).

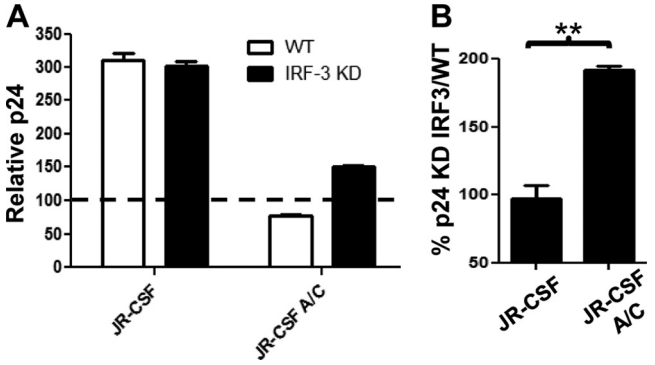


**FIG 3** Partial rescue of Vpu-deficient HIV-1 by knockdown of IRF3. Wild-type or IRF3-knockdown (KD) PMA-differentiated THP-1 cells were infected with JR-CSF or JR-CSF A/C at a multiplicity of infection of 1 for 4 h and then washed to remove free virus. Samples were harvested for immunoblotting of p24 and actin levels as a loading control at 8 and 24 h of infection. (A) Normalized 8-h p24 levels were set to 100 for each virus and compared to the 24-h values. (B) Percent increase of viral p24 values at 24 h postinfection, comparing virus growth in IRF3-knockdown cells and wt. **, $P < 0.005$. Mean values with SDs are plotted.

**Rescue of Vpu-deficient HIV-1 by knockdown of IRF3.** While Vpu-negative HIV-1 is replication competent (42), we observed a reduction in viral protein expression in cells infected with Vpu-negative HIV-1 relative to those infected with congenic wt virus, and this reduction associated with the stimulation of the host cell innate immune response (see Fig. 1D as an example). We hypothesized that the loss of IRF3 regulation by Vpu-negative viruses may impart cellular defenses that suppress HIV-1, similar to the outcome of expressing constitutively active IRF3 (10). Of note is the fact that HIV-$1_{JR\text{-}CSF}$ directs the rapid degradation of IRF3 during acute infection (10), and accordingly, we found that it replicated (measured by p24 accumulation at 24 h postinfection [hpi] compared to input measured at 8 hpi) to similar levels in both control and IRF3-knockdown THP-1 cells over the first 24 h of infection (Fig. 3A). In contrast, Vpu-negative HIV-$1_{JR\text{-}CSF\ A/C}$ replication was reduced in control cells compared to wt virus replication, but replication was partially rescued during acute infection in IRF3-knockdown cells (Fig. 4A and B). This resulted in a significant increase in viral growth of the HIV-$1_{JR\text{-}CSF\ A/C}$ mutant in the IRF3-knockdown cells, with no change observed for the wt strain (Fig. 4B). Importantly, these experiments focused only on the first round of HIV-1 infection/replication, therefore avoiding any contribution of tetherin/BST2 in suppressing virus production at the level of viral egress. Thus, HIV-1 triggering of IRF3-dependent innate immune defenses restricts viral replication fitness, wherein Vpu mediates a blockade of IRF3 signaling to support efficient HIV-1 replication.

**Vpu-deficient HIV strains stimulate the induced expression of many anti-HIV ISGs.** The lysosomal targeting and degradation of IRF3 by Vpu might alter IRF3-dependent innate immune responses within the infected cell, thus providing a permissive environment to support acute HIV-1 infection (see the companion

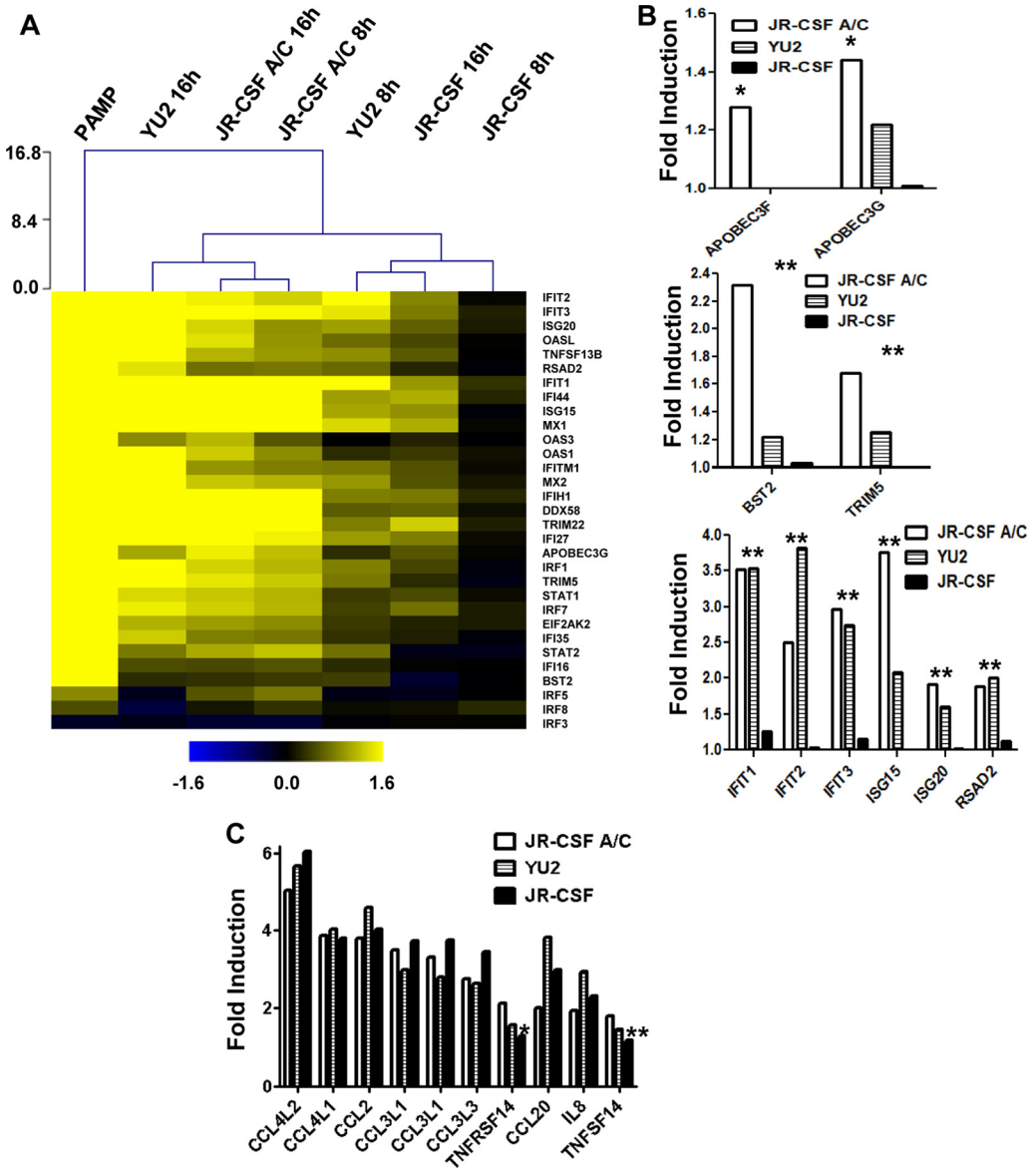


**FIG 4** Vpu-deficient HIV-1 stimulates the specific expression of a wide range of innate antiviral immune modulators. JR-CSF, Vpu-deficient JR-CSF A/C, and YU2 virus strains were used to infect PMA-differentiated THP-1 cells for 8 or 16 h. Synthetic HCV PAMP RNA was transfected as a positive control for IRF3-dependent innate immune stimulation. RNA was isolated and subjected to microarray analyses targeted at an experimentally derived set of ISGs and innate immune-associated genes. (A) Heat map cluster of selected significantly regulated innate immune and ISGs in $\log_2$ scale. (B) Fold induction of known anti-HIV-1 ISGs and restriction factors during HIV-1 infection with or without Vpu at 8 h postinfection. Mean of four biological replicates. *, $P < 0.0005$; **, $P < 0.000001$ (FDR-adjusted $P$ values). Results for all JR-CSF samples were not significant, with $P$ values of >0.05. (C) Fold induction of known NF-κB-responsive genes during HIV-1 infection with wt or Vpu-deficient strains at 16 h postinfection. Mean of four biological replicates. Results for all samples were significant, with FDR-adjusted $P$ values of <0.000001, with the exception of JR-CSF samples TNFRSF14 (*, $P < 0.0025$) and TNFSF14 (**, not significant).

paper [8]). To address this idea, we performed targeted genomics analyses of innate immune gene expression at 8 or 16 h posttreatment to compare the responses of mock-infected cells to those of cells infected with congenic wt HIV-$1_{JR\text{-}CSF}$ or HIV-$1_{JR\text{-}CSF\ A/C}$ as well as HIV-$1_{YU2}$ during an acute infection. As a positive control for innate immune induction by IRF3-dependent PAMP signaling, we assessed similar cells that were transfected with a well-characterized synthetic PAMP motif encoded within the RNA genome of hepatitis C virus (47). As shown in Fig. 4A, while THP-1 cells responded to PAMP RNA to induce the expression of innate immune response genes and ISGs, acute infection of THP-1 cells with HIV-$1_{JR\text{-}CSF}$ failed to induce a response at 8 h postinfection and induced only a very weak response at 16 h postinfection. Remarkably however, infection of THP-1 cells with HIV-$1_{JR\text{-}CSF\ A/C}$ or HIV-$1_{YU2}$ lacking Vpu expression resulted in a marked induction of innate immune response gene expression at 8 h postinfection and a highly robust response by 16 h postinfection. By comparison, the innate immune response induced by HIV-$1_{JR\text{-}CSF}$ at 16 h postinfection was substantially weaker than that of congenic HIV-$1_{JR\text{-}CSF\ A/C}$ occurring within 8 h postinfection (Fig. 4A). The majority of the HIV-1-induced genes encoded known antiviral factors and immune signaling molecules (PKR, MX1, STAT1)

(44) and, importantly, included many ISGs known to be HIV-1 restriction factors, including tetherin/BST2, viperin, ISG15, ISG20, TRIM5, and APOBEC3G (Fig. 4B) (16, 36, 39, 43). Surprisingly, we did not detect the expression of IFN-β in either the PAMP-treated cells or Vpu-deficient HIV-1-infected cells, possibly reflecting the possibility that IFN-β induction occurred temporally outside our sampling time points or that it was present below our limit of detection.

Our previous work has shown that lab strains of HIV-1 have no effect toward suppressing the NF-κB signaling capacity of infected cells (10). To determine if the differential ISG induction profile was specific for IRF3 signaling of the Vpu-deficient viral strains, we compared these results with the induction profile of known NF-κB-regulated genes, by analyzing the gene induction profile of over 70 annotated NF-κB-responsive genes (Table 1) from the same infection samples for HIV-$1_{JR\text{-}CSF}$, HIV-$1_{JR\text{-}CSF\ A/C}$, or HIV-$1_{YU2}$. Indeed, we found that several known NF-κB-dependent genes were upregulated during infection with wt HIV-$1_{JR\text{-}CSF}$ and Vpu-deficient HIV-1 strains (Fig. 4C). To compare these results directly, we examined the 10 most upregulated NF-κB-dependent genes from the HIV-$1_{JR\text{-}CSF\ A/C}$ infection and compared the expression induced by all three viral strains at 16 h postinfection (Fig. 4C). We found that all 10 genes were significantly upregulated in response to infection by all three viruses. With the exception of TNFSF14, the NF-κB-responsive genes were induced to similar levels. Thus, deletion of Vpu releases a specific IRF3 blockade imposed by HIV-1, without alteration of virus-induced NF-κB programs, to reveal an endogenous innate immune signaling program induced by HIV-1 infection. IRF3 suppression by Vpu therefore serves an important role to prevent the specific induction of innate immune gene expression, including the expression of well-characterized anti-HIV-1 restriction factors. These results define the Vpu-IRF3 interaction as a regulatory node that governs the innate immune response of the host cell to HIV-1 infection.

## DISCUSSION

HIV-1 rapidly induces the specific degradation of IRF3 in infected cells during acute infection of T cells and myeloid cells *in vitro* and within mucosal T cells *ex vivo* (10, 38). Our studies (here and in the companion paper [8]) reveal a novel role for Vpu in the control of innate antiviral immunity in HIV-1-infected cells. Our genetic and biochemical evidence suggests that Vpu is both necessary and sufficient for IRF3 degradation by HIV, in a lysosomal process similar to that previously described for tetherin/BST2 and CD4 downmodulation (12, 13, 23, 33, 35). Here we addressed the functional outcome of infection of HIV-1 target cells in the absence of this potent Vpu-directed antagonism of the innate immune signaling response. We found a robust and potent induction of IRF3-dependent innate signaling and downstream production of ISGs when target cells were infected with Vpu-deficient viruses compared to wt Vpu-producing HIV-1 strains. This innate immune activation is retained in a number of different HIV-1 target cells, including primary macrophages, and in *ex vivo* mucosal T cell cultures (Fig. 1). Our direct comparison of congenic Vpu-positive and -deficient HIV-1 strains uncovered a 5- to 10-fold increase in ISG induction by infection with the Vpu-deficient strain (Fig. 1E), showing that HIV-1 can truly be recognized by PRRs to induce innate immune responses from host cells.

We also show that IRF3 phosphorylation and nuclear accumulation occur in host cells in response to infection with Vpu-deficient HIV-1 strains. IRF3 activation is downstream of pathogen sensing from a number of PRRs, including the RLR family, several TLRs, as well as several less well characterized responses to non-self-products, including cytoplasmic DNA (50, 52). Interestingly, we found that IRF3 activation and subsequent production of IRF3-dependent ISGs were unaffected by shRNA ablation of IPS-1 or MyD88, the essential adaptor molecules required for RLR and many TLR signaling responses, respectively. This suggests that the upstream recognition event of HIV-1 infection in these target cells is not reliant on RIG-I, MDA5, TLR7, or TLR9, all of which have been implicated in recognition of HIV-1 in different cellular contexts (2, 6, 31, 34, 49). Thus, innate immune signaling within T cells or macrophages appears to be programmed differentially from that in plasmacytoid dendritic cells, which specifically utilize TLRs for HIV-1 recognition in an IRF3-independent program (2, 31, 34). Our working model of IRF3 regulation by HIV-1 suggests that Vpu antagonizes IRF3 later in the viral life cycle, perhaps as replication intermediates serving as PAMPs accumulate and trigger signaling. We note that recent reports provide evidence of innate immune activation in other contexts that is consistent with this idea (11, 54). We do observe production of Vpu at times concurrent with this innate immune signaling that occurs in specific cell types; it is possible, however, that these processes reflect an additional early event of virus recognition not directly tied to the Vpu targeting of IRF3 that is still uncovered by the absence of Vpu. This process of PAMP triggering of innate immune signaling would be in contrast to host cell sensing of viral capsids, where incoming virions are sensed in an AP-1- and NF-κB-dependent mechanism (41).

IRF3 activation is deleterious to HIV-1 infection and stimulates a robust and widely antiviral state within infected cells (9, 50, 52). By interacting with IRF3 and targeting it to the lysosome, Vpu facilitates IRF3 proteolysis and prevents the expression of genes involved in innate immune defenses against HIV-1, including type I IFN, ISGs, and direct IRF3 target genes that mediate antiviral actions. Among the genes responsive to IRF3 are known HIV-1 restriction factors, including APOBEC3G, tetherin, ISG15, and others. Indeed, we show that HIV-1 suppression of IRF3 serves to enhance cell permissiveness for infection by relieving innate immune restriction of virus replication and cell spread during the critical stage of acute infection. It is important to note that our study has separated the Vpu-dependent IRF3 phenotypes from the known role of Vpu in antagonizing tetherin. Whenever possible, we utilized in our experiments cell types that are known to be deficient in tetherin expression (i.e., 293T cells), and when utilizing cells know to express tetherin (i.e., THP-1 cells), we have focused on evaluating only the early effects of Vpu deficiency and innate immune signaling responses dependent on IRF3 in order to avoid the confounding influences of tetherin-dependent late-egress phenotypes on HIV-1 production. For this reason, we are not able to determine the effects of IRF3 activation on HIV-1 spread in the current study. We find a global induction of ISGs by Vpu-deficient HIV-1 strains but find no regulation of NF-κB target genes, again underscoring the specific IRF3 activation in the absence of Vpu antagonism.

Our study is the first to reveal additional pathogen sensing of HIV-1 independent of RLR and MyD88 signaling, which drives IRF3-responsive gene expression within acutely infected immune cells from mucosal tissue (Fig. 1D). This natural response does not

require overcoming additional HIV-1 infection blockades, as has been reported for the cryptic sensing of HIV-1 in dendritic cells (32), but is instead blocked downstream by antagonism of IRF3 signaling. IRF3 signaling is essential to promote cell expression of proinflammatory and immunomodulatory cytokines and chemokines from the site of infection that are required for effective adaptive immune responses (30), and therefore, IRF3 regulation by HIV-1 would contribute to early immune dysfunction in HIV-1-infected patients. This early permissive environment may aid in seeding the initial infection, but in the absence of Vpu control, innate immune signaling may provide the necessary signals for effective control of the acute infection. Further characterization and study of innate immune responses against Vpu-deficient viruses may provide a platform for understanding the innate signatures necessary for HIV-1 suppression and enhancement of vaccine and adjuvant design.

## ACKNOWLEDGMENTS

We thank Nadezda Andreeva and David Weston for technical assistance. We also thank Hilario Ramos, Mehul Suthar, and Arjun Rustagi for helpful conversation and review.

This work was supported by grants from the U.S. Public Health Service, including BMGF CAVD 38645 and NIH/NIAID U01 AI068618. This work was also supported by funds from the state of Washington, NIH grants DA024563 (to M.G.), AI078768 (to B.P.D.), and HD051455 (to F.H.), and the Burroughs-Wellcome Fund (to M.G.).

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