THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 289, NO. 31, pp. 21716–21726, August 1, 2014

HIV-1 Infection Induces Interleukin-1β Production via TLR8 Protein-dependent and NLRP3 Inflammasome Mechanisms in Human Monocytes*

Received for publication, March 23, 2014, and in revised form, June 14, 2014 Published, JBC Papers in Press, June 17, 2014, DOI 10.1074/jbc.M114.566620

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Background: Inflammation is associated with HIV-1 pathogenesis and the progression of AIDS.
Results: HIV-1 induces pro-IL-1β expression through Toll-like receptor 8 and caspase-1 activation through nucleotide-binding domain, leucine-rich repeat containing protein 3 (NLRP3) inflammasome.
Conclusion: Both TLR8 and NLRP3 are required for HIV-1-induced IL-1β production in monocytes.
Significance: Knowing the mechanism of HIV-1-induced IL-1β is important for understanding systematic inflammation in HIV-1 patients.

The induction of inflammatory cytokines such as IL-1β is associated with the progression of human immunodeficiency virus, type 1 (HIV-1) disease or AIDS. Unlike most inflammatory cytokines that are regulated by NF-κB at the transcriptional level, production of mature IL-1β also depends on inflammasome activation. The mechanism by which HIV-1 induces pro-IL-1β expression and activates inflammasomes to cleave pro-IL-1β into its bioactive form is not clearly defined. We report here that HIV-1 infection in human monocytes efficiently induced IL-1β expression and inflammasome activation. Toll-like receptor 8 (TLR8) was required for inducing pro-IL-1β expression, whereas the NLRP3 inflammasome was required for IL-1β maturation and release. Furthermore, the lysosomal protease cathepsin B and HIV-1 induced production of reactive oxygen species were critical for HIV-induced inflammasome activation and IL-1β production. HIV-1 entry, reverse transcription, and integration were all required for both pro-IL-1β expression and inflammasome activation. Finally, we show that HIV-1-derived RNA was sufficient to induce both pro-IL-1β expression and inflammasome activation. We conclude that HIV-1 infection induced the expression of pro-IL-1β via TLR8-mediated mechanisms and activated caspase-1 through the NLRP3 inflammasome to cleave pro-IL-1β into bioactive IL-1β. These findings help to elucidate mechanisms of HIV-1 disease progression and identify novel targets for treating HIV-1 induced inflammation and immune activation.

Human immunodeficiency virus, type 1 (HIV-1)[2] disease progression is characterized by persistent inflammation and chronic immune activation, which has been proposed to contribute to the development of AIDS (1, 2). However, the precise mechanism by which HIV-1 causes inflammation and immune activation remains poorly understood. The microbial translocation from the gut to the blood circulation because of the loss of mucosal integrity during HIV-1 disease progression has been proposed to be a possible contributing factor (3). A recent report has also shown that repeated treatment with a TLR7/8 agonist alone can induce AIDS-like disease, including elevated proinflammatory cytokines and lymphoid organ disruption (4).

Pathogen-associated molecular patterns are recognized by specific receptors of the host innate immune system, including Toll-like receptors (TLRs), C-type lectin receptors, retinoic acid-inducible gene I-like receptors, and nucleotide-binding domain, leucine-rich repeat-containing (NLR) proteins (5–7). The HIV-1 genomic RNA has been reported to activate TLR7 or TLR8 in different cell types (8–12). It is not clear whether other TLRs, retinoic acid-inducible gene I-like receptors, or NLRs are also activated by HIV-1 infection.

IL-1β plays a pivotal role in driving inflammation by binding to the IL-1 receptor to induce more proinflammatory cytokines. Its production is tightly controlled by two steps: induction of *pro-IL-1β* gene expression and caspase-1-mediated pro-IL-1β cleavage (13). Inflammasome complexes regulate the cleavage of pro-IL-1β by assembling into a multicomponent protein platform, which leads to the activation of pro-caspase 1 (14). Several inflammasome complexes have been described to date, defined by the presence of the NLR proteins NLRP1, NLRP3, NLRC4, NLRP6, and NLRP12 and the pyrin and HIN200 domain-containing protein AIM2 (15). The NLRP3 inflammasome is the most extensively studied and seems to respond to a number of stimuli of diverse physiochemical

* This work was supported, in whole or in part, by National Institute of Health Grants AI077454, AI080432, and AI095097 (to L. S.) and AI029564, AI057157, and CA156330 (to J. P. T.).
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[2] The abbreviations used are: HIV-1, human immunodeficiency virus, type 1; TLR, Toll-like receptor; NLR, nucleotide-binding domain, leucine-rich repeat; ROS, reactive oxygen species; PBMC, peripheral blood mononuclear cell; APDC, (2R,4R)-4-aminopyrrolidine-2,4-dicarboxylic acid; NVP, nevirapine; Z-VAD-fmk, benzyloxycarbonyl-VAD-fluoromethyl ketone; FLICA, fluorescent labeled inhibitor of caspase-1; dpi, day(s) post-infection; sCD4, soluble CD4; hpi, hour(s) post-infection; ssRNA, single-stranded RNA; qPCR, quantitative PCR; T20, enfuvirtide; AZT, zidovudine; ASC, apoptosis-associated, speck-like protein containing a CARD.

natures (16–21). Several types of viruses, including influenza A virus, dengue virus, hepatitis C virus, adenovirus, varicella zoster virus, encephalomyocarditis virus, vesicular stomatitis virus, myxoma virus, and respiratory syncytial virus have been found to induce IL-1β through the NLRP3 inflammasome (22–31). Interestingly, HIV-1 infection has been reported to induce *NLRP3* mRNA expression in monocyte-derived dendritic cells (32), and an SNP at the 3′ UTR of the NLRP3 gene is associated with susceptibility to HIV-1 infection (33). However, it is not clear whether HIV-1 infection leads to activation of the NLRP3 inflammasome.

In this report, we found that HIV-1 infection induced IL-1β production and caspase-1 activation in human monocytes. Using the siRNA approach, we showed that TLR8, but not TLR7, was required for the induction of pro-IL-1β expression and that the NLRP3 inflammasome was required for IL-1β maturation and release. We found that reactive oxygen species (ROS) were increased in the HIV-1-infected monocytic cells, which was also critical for HIV-induced IL-1β production. Cathepsin B, a lysosomal protease that has been reported to be an activator of NLRP3 (34, 35), was also important for HIV-1-induced IL-1β production. HIV-1 entry, reverse transcription, and integration were all required for both pro-IL-1β expression and caspase-1 activation. Finally, we found that HIV-1 derived RNA was sufficient to induce both pro-IL-1β expression and caspase-1 activation. We conclude that HIV-1 infection induced IL-1β production by induction of pro-IL-1β through a TLR8-dependent mechanism and activation of the NLRP3 inflammasome involving ROS and cathepsin B induction.

EXPERIMENTAL PROCEDURES

Cell Culture—The human monocytic cell line THP-1 was maintained in RPMI 1640 medium (Invitrogen) containing 10% FBS and 1% penicillin/streptomycin. THP-1 cells containing scramble shRNA or shRNA for *NLRP3*, *ASC*, *NLRC4* (36, 37), *MyD88* mutants, *MyD88*, *TRAF6* mutants, *TRAF6* (38), *TLR7*, and *TLR8* were cultured under the same conditions as THP-1, as described previously. Human peripheral blood mononuclear cells (PBMCs) were isolated from the buffy coat by Ficoll-Paque Plus and maintained in RPMI 1640 medium containing 10% FBS, 1% penicillin/streptomycin, and 1% nonessential amino acids. Primary human monocytes were isolated from PBMCs by negative selection using the human monocyte enrichment kit from Stemcell Technologies and were maintained in Iscove's modified Dulbecco's medium containing 10% FBS, 1% penicillin/streptomycin, and 1% nonessential amino acids. Primary human macrophages were differentiated from monocytes by culturing in medium containing 20 ng/ml recombinant human monocyte colony-stimulating factor (PeproTech, Rock Hill, NJ) for 7 days. All cells were maintained at 37 °C in 5% carbon dioxide.

Reagents and Antibodies—Z-VAD-fmk, Ac-YVAD-CHO, (2R,4R)-4-aminopyrrolidine-2,4-dicarboxylic acid (APDC) and CA-074 Me were from Enzo Life Sciences (Farmingdale, NY). Chloroquine, ssRNA40, and ssRNA41 were from Invivogen (San Diego, CA). Soluble CD4, enfuvirtide (T20), nevirapine (NVP), zidovudine (AZT), raltegravir, and amprenavir were from the National Institutes of Health AIDS Research and Reference Reagent Program. Antibodies used for immunoblotting included anti-IL-1β (catalog no. 2022, Cell Signaling Technology, Danvers, MA) and anti-caspase-1 p10 (catalog no. sc-515, Santa Cruz Biotechnology, Santa Cruz, CA).

Preparation of the shRNA Plasmid and Cell Lines for TLR Knockdown—The human 7 SK RNA polymerase III promoter and the shRNA sequence for human *TLR7* and *TLR8* were amplified by PCR from plasmid psiRNA-TLR7, psiRNA-TLR8 from Invivogen and inserted into lentiviral vector FG12 by XhoI and XbaI. Lentiviruses were made by cotransfection of lentiviral vector, VSVG, and ΔNRF into 293T cells. THP-1 cells were infected by lentiviruses containing shRNA for *TLR7* or *TLR8* by spinoculation (5500 rpm and 25 °C for 3 h) to generate the TLR7 or TLR8 knockdown cell line. Transduced cells were sorted by the GFP marker.

Virus Propagation—293T cells were transfected with the HIV-R3A proviral plasmid, and the cell-free supernatant was collected and filtered with a 0.45-μM filter. HIV-1 p24 was measured by p24 ELISA. SupT1 cells were infected by 293T-derived viruses, and cell-free supernatants were collected and filtered on different days after infection. PBMCs were cultured in medium containing 5 μg/ml phytohemagglutinin and 20 units/ml IL-2 for 3 days and then infected with 293T-derived viruses for 3 h. PBMCs were maintained in medium containing 20 units/ml IL-2, and cell-free supernatants were collected and filtered on different days after infection. HIV p24 was measured for SupT1- and PBMC-derived viral stocks, and supernatants containing more than 100 ng/ml of p24 were used. The infectious unit was determined by infecting the Magi-R5 reporter cell line (39). The infectivity of the viral stock was about 1×10^4 infectious units/ng p24.

HIV-1 Infection of Human Primary Cells and Cell Lines—4×10^5 THP-1 cells were incubated with 20 ng of p24 (SupT1-derived HIV-1 stocks, multiplicity of infection of 0.5) plus 8 μg/ml of Polybrene for 10 min at room temperature, and then spinoculation was performed at $900 \times g$ at 37 °C for 3 h. 4×10^5 human primary monocytes were incubated with PBMC-derived HIV-1 stocks (20 ng of p24, multiplicity of infection of 0.5) as above. Cells were then washed three times and divided into 96-well plates with 10^5 cells/well in triplicate wells. Human macrophages were cultured in 96-well plates with 10^5 cells/well and infected with PBMC-derived HIV-1 (10 ng of p24, multiplicity of infection of 1) plus 8 μg/ml of Polybrene for 3 h. Cells were then washed three times with medium. For pharmacological assessments, cells were treated with Z-VAD-fmk (25 or 50 μM), Ac-YVAD-CHO (25 or 50 μM), APDC (100 μM), CA-074 Me (25 μM), T20 (10 μg/ml), NVP (2 μM), AZT (5 μM), raltegravir (10 μM), and amprenavir (10 μM) for 1 h before infection and then cultured in the presence of those drugs. For blocking viral entry, viruses were incubated with sCD4 (10 μg/ml) at 37 °C for 30 min before infection.

Human Monocyte and Macrophage Transfection—10^7 of fresh isolated primary monocytes or primary macrophages differentiated by 20 ng/ml of M-CSF were nucleofected with 30 pmol of control siRNA, siRNA for *NLRP3*, or siRNA for *ASC*. The nucleofected cells were cultured for 48 h before infection. All siRNAs used were from Ambion.

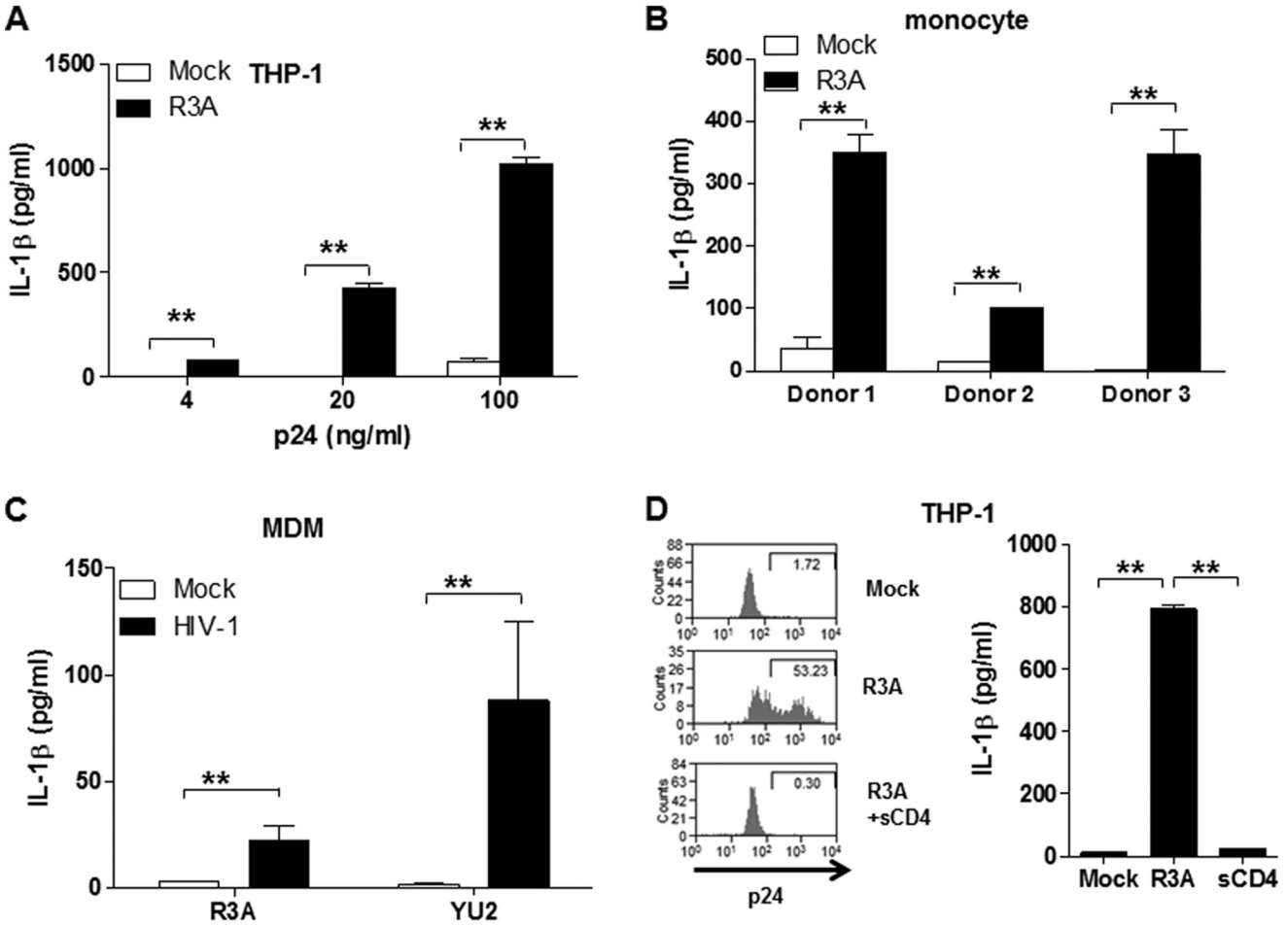

FIGURE 1. **HIV-1 induces IL-1β production in human monocytic cells.** *A*, THP-1 cells were infected with different doses of HIV-R3A or mock stock, and IL-1β in the supernatant was measured at 3 dpi. *B*, human primary monocytes from different donors were infected with mock or HIV-R3A, and IL-1β in the culture supernatant was measured at 1 dpi. *C*, primary macrophages differentiated from monocytes (*MDM*) were mock-infected or infected with HIV-R3A or HIV-YU2, and IL-1β in the culture supernatant was measured at 3 dpi. *D*, THP-1 cells were mock-infected or infected with HIV-R3A in the presence of sCD4. IL-1β in the supernatant was measured. Data are mean ± S.E. ($n = 3$). **, $p < 0.01$.

Real-time PCR—Real-time PCR was performed to detect *NLRP3*, *ASC*, and 18 S rRNA expression by TaqMan gene expression assays (Hs00918082_m1, Hs00203118_m1, and Hs99999901_s1) (Applied Biosystems, Carlsbad, CA) and *TLR7*, *TLR8*, *IL-1β*, and *GAPDH* expression by SYBR Green and the following primers: *TLR7*, 5′-AAACTCCTTGGGGCTAGATG-3′ (forward) and 5′-AGGGTGAGGTTCGTGGTGTT-3′ (reverse); *TLR8*, 5′-CTGTGAGTTATGCGCCGAAGA-3′ (forward) and 5′-TGGTGCTGTACATTGGGGTTG-3′ (reverse); *IL-1β*, 5′-ACAGTGGCAATGAGGATGAC-3′ (forward) and 5′-CCATGGCCACAACAACTGA-3′ (reverse); and *GAPDH*, 5′-ATGTTCGTCATGGGTGTGAA-3′ (forward) and 5′-GTCTTCTGGGTGGCAGTGAT-3′ (reverse).

ELISA—Cell-free supernatants were analyzed for IL-1β expression using an IL-1β ELISA kit (BD Biosciences) according to the instructions of the manufacturer.

ROS Detection—THP-1 cells were infected with HIV-1 and, at different time points after infection, cells were loaded with 10 μM fluoroprobe dihydrorhodamine 123 (AnaSpec, Fremont, CA) for 30 min and washed twice with Hanks' balanced salt solution containing calcium and magnesium but no phenol red. Cells were fixed with 10% formalin, and the mean fluorescence intensity was determined using a CyAn ADP flow cytometer (DAKO, Carpinteria, CA).

Detection of Caspase-1 Activation—THP-1 cells were infected with HIV-1 and loaded with caspase-1 FLICA (Immunochemistry Technologies, Bloomington, MN) at 3 dpi according to the instructions of the manufacturer. Caspase-1-positive cells were determined by a CyAn ADP flow cytometer (DAKO).

Statistics—Data are presented as the mean ± S.E. Data were analyzed for statistical significance by two-tailed Student's *t* test in Excel (Microsoft). In all cases, $p < 0.05$ was considered to be statistically significant.

RESULTS

HIV-1 Infection Induces IL-1β Production in human Monocytic Cells—To test whether HIV-1 infection of monocytes/macrophages can induce IL-1β production, we used the CXCR4/CCR5 dual tropic HIV-R3A virus, which infects both T cells and macrophages (40, 41). HIV-R3A induced a significant increase of IL-1β in THP-1 (a human monocytic cell line) in a dose-dependent manner (Fig. 1*A*). To confirm this in primary human cells, we infected enriched primary monocytes from human PBMCs (>80% CD14+). At 24 hpi, IL-1β in the super-

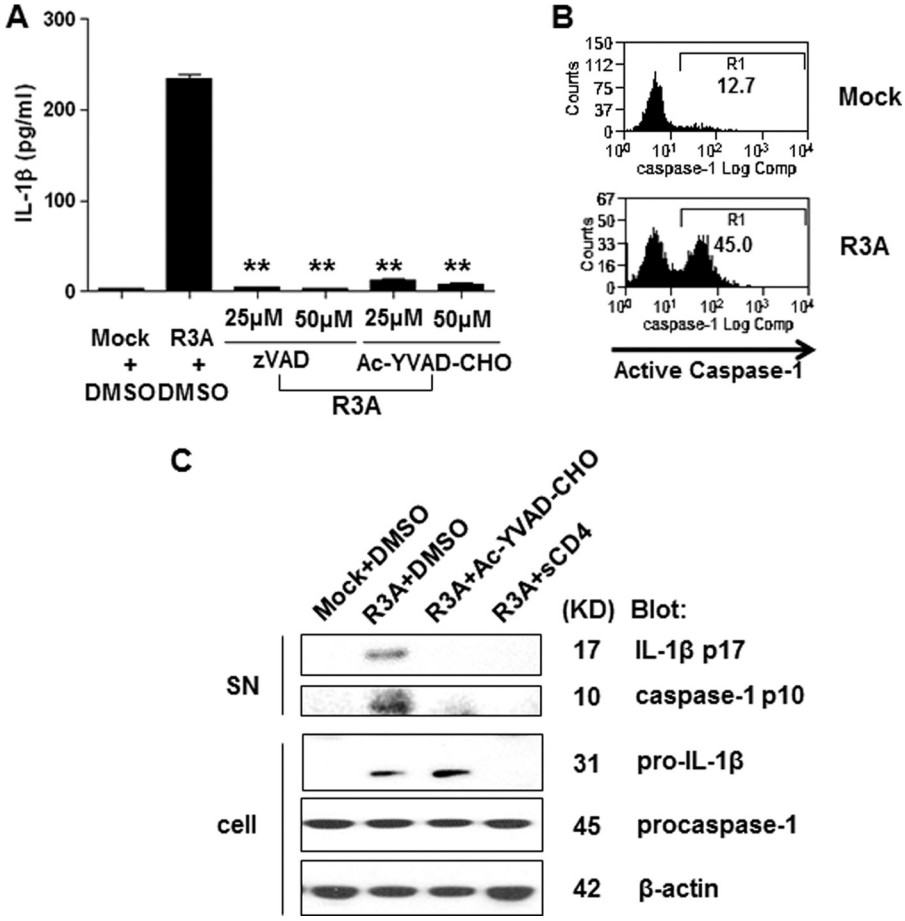

FIGURE 2. **HIV-1 induces pro-IL-1β expression and caspase-1 activation to promote IL-1β production.** *A*, THP-1 cells were mock-infected or infected with HIV-R3A in the absence or presence of the pan-caspase inhibitor Z-VAD-fmk (*zVAD*, 25 or 50 μM) and the caspase-1-specific inhibitor Ac-YVAD-CHO (25 μM or 50 μM). IL-1β in the supernatant was measured. Data are mean ± S.E. ($n = 3$). Statistical analysis was performed between the HIV-1 group and the HIV-1 plus Z-VAD-fmk or Ac-YVAD-CHO group. **, $p < 0.01$. *DMSO*, dimethyl sulfoxide. *B*, activation of caspase-1 in HIV-R3A-infected THP-1 cells was determined by FLICA staining. *C*, immunoblotting was performed to determine activated caspase-1 (p10) and mature IL-1β (p17) in supernatants (*SN*) and pro-IL-1β and pro-caspase-1 in cell lysates (*cell*) from mock- or HIV-R3A-infected THP-1 cells. β-actin was used as the loading control. The effect of AC-YVAD-CHO and soluble CD4 on caspase-1 activation and IL-1β production was also determined. The results are representative of two independent experiments.

natant was significantly increased in HIV-1-infected monocytes (Fig. 1*B*) from three different human blood donors. When monocyte-derived macrophages were tested, HIV-R3A infection also significantly induced IL-1β production (Fig. 1*C*). A CCR5-tropic and macrophage-tropic HIV-1 strain, YU2, was also able to induce IL-1β production in primary macrophages (Fig. 1*C*). We used soluble CD4 (sCD4) to block HIV-R3A infection of THP-1 cells (Fig. 1*D*). The data showed that blockage of HIV-R3A infection by sCD4 significantly inhibited IL-1β production (Fig. 1*D*). Therefore, we conclude that HIV-1 infection of monocytes/macrophages efficiently induced production of IL-1β.

HIV-1 Infection Induces Pro-IL-1β Expression and Caspase-1 Activation to Promote IL-1β Production—Production of mature IL-1β requires two signals. One activates the expression of pro-IL-1β, and the other activates the inflammasome and interleukin-1 converting enzyme caspase-1, which cleaves pro-IL-1β into mature IL-1β (13). IL-1β production induced by HIV-R3A was attenuated dramatically by a pan-caspase inhibitor, Z-VAD-fmk, and by the caspase-1-specific inhibitor Ac-YVAD-CHO (Fig. 2*A*). We also measured the level of active caspase-1 in HIV-R3A-infected THP-1 cells by flow cytometer with the fluorescent labeled caspase-1 inhibitor (FLICA). HIV-R3A infection significantly increased the percentage of cells with activated caspase-1 (Fig. 2*B*). These data indicate that HIV-R3A infection activated caspase-1 to promote IL-1β production. In the cell lysate, we detected an increase of pro-IL-1β in HIV-R3A-infected THP-1 cells (Fig. 2*C*), indicating that HIV-1 infection induced expression of pro-IL-1β. In addition, infected cells treated with Ac-YVAD-CHO accumulated more pro-IL-1β in the cytoplasm (Fig. 2*D*), indicating that Ac-YVAD-CHO did not affect pro-IL-1β induction but inhibited the activation of caspase 1 to generate mature IL-1β. Consistently, Ac-YVAD-CHO did not affect HIV-R3A infection of THP-1 cells (data not shown). Furthermore, we detected the active caspase-1 p10 and mature IL-1β p17 in the supernatant from HIV-R3A-infected THP-1 cells but not from mock cells (Fig. 2*D*). Importantly, blockage of HIV-R3A infection of THP-1 with sCD4 abolished both pro-IL-1β induction and caspase-1 activation (Fig. 2*D*). We conclude that HIV-1 infection of monocytes induces pro-IL-1β expression and caspase-1 activation to promote IL-1β production.

TLR8 Is Required for HIV-1-induced Pro-IL-1β Expression—HIV-1 RNA is reported to activate TLR7 in plasmacytoid den-

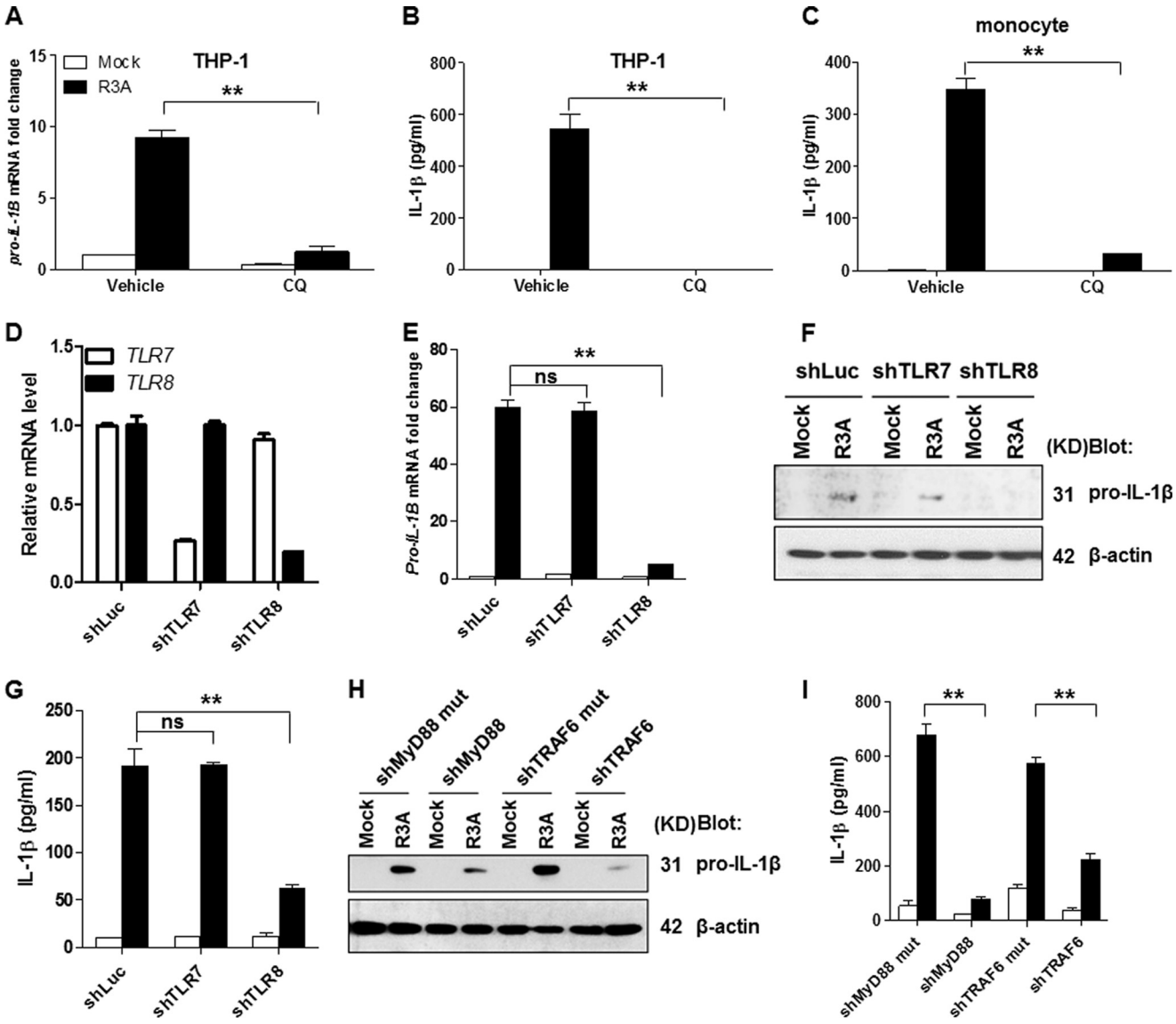

FIGURE 3. **TLR8 is required for HIV-induced pro-IL-1β expression.** *A* and *B*, THP-1 cells were mock-infected or infected with HIV-R3A in the absence or presence of chloroquine (*CQ*). qPCR analysis of *pro-IL-1β* mRNA (*A*) and ELISA analysis of supernatant IL-1β (*B*) were performed. *C*, primary monocytes were mock-infected or infected with HIV-R3A in the absence or presence of chloroquine, and IL-1β in the supernatant was measured. *D*, qPCR analysis of the *TLR7* or *TLR8* mRNA level in THP-1 cells containing shRNA for *luciferase*, *TLR7*, or *TLR8*. *E–G*, THP-1 cells stably transduced with lentivirus-based shRNA for *luciferase* (*shLuc*), *TLR7*, or *TLR8* were mock-infected or infected with HIV-R3A. *Pro-IL-1β* mRNA was measured by qPCR (*E*), pro-IL-1β protein in the cell lysate was measured by Western blot analysis (*F*), and IL-1β in the supernatant was measured by ELISA (*G*). *H* and *I*, THP-1 cell lines expressing shRNA for *MyD88* or *TRAF6* (and their control mutant shRNA, *mut*) were mock-infected or infected with HIV-R3A. Pro-IL-1β protein in the cell lysate (*H*) and IL-1β in the supernatant (*I*) were assessed. Data are mean ± S.E. ($n = 3$). **, $p < 0.01$; *n.s.*, not significant; *KD*, kilodalton. The results are representative of two independent experiments.

dritic cell (10). However, the ssRNA40 RNA derived from HIV-1 has also been reported to promote TNFα production via TLR8, but not TLR7, in macrophages (9). We investigated whether TLR7 or TLR8 was required for the induction of pro-IL-1β. Preventing acidification of the endosome by chloroquine inhibits the function of endosomal TLRs (42, 43). Chloroquine blocked HIV-1- induced pro-IL-1β expression (Fig. 3*A*) and IL-1β production (Fig. 3, *B* and *C*). To define the role of TLR7 and TLR8 in HIV-1-induced pro-IL-1β expression, shRNA was used to knock down their expression in THP-1 cells (Fig. 3*D*). Interestingly, knockdown of *TLR8* abolished pro-IL-1β induction in HIV-1-infected THP-1 cells, whereas knockdown of *TLR7* did not change pro-IL-1β induction (Fig. 3, *E* and *F*). Consistently, mature IL-1β from supernatants was also reduced dramatically in *TLR8*, but not *TLR7*, knockdown cells (Fig. 3*G*). Furthermore, we found that knockdown of downstream MyD88 and TRAF6 also dramatically attenuated pro-IL-1β expression (Fig. 3*H*) and IL-1β production (Fig. 3*I*) induced by HIV-R3A infection. In conclusion, the TLR8/MyD88/TRAF6 pathway is required for HIV-1-induced pro-IL-1β expression in human monocytic cells.

The NLRP3 Inflammasome Is Required for HIV-1-induced IL-1β Maturation and Release—To determine whether the NLRP3 inflammasome is involved in HIV-1-induced IL-1β production, we utilized shRNA to inhibit *NLRP3* or *ASC* gene expression to assess the contribution of NLRP3 or ASC (36, 37). Control THP-1 and knockdown cell lines were infected with mock or HIV-R3A. HIV-R3A induced a significant increase of

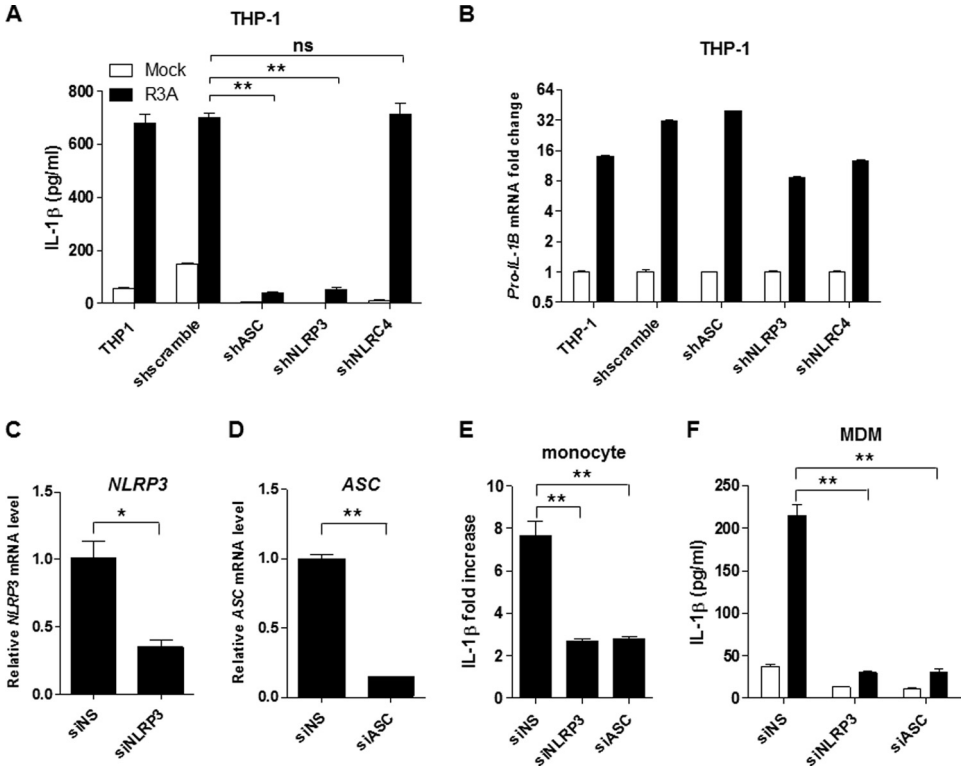

FIGURE 4. **The NLRP3-ASC inflammasome is required for HIV-induced IL-1β production.** *A* and *B*, control THP-1 cells or THP-1 cells expressing scramble shRNA or shRNA for *ASC*, *NLRP3*, and *NLRC4* were mock-infected or infected with HIV-R3A. IL-1β in the supernatant (*A*) and *pro-IL-1β* mRNA in the cell (*B*) were assessed. *C* and *D*, human primary monocytes were nucleofected with scramble siRNA, siRNA for *NLRP3*, or siRNA for *ASC*, and mRNAs of *NLRP3* (*C*) or *ASC* (*D*) were measured by qPCR. *E* and *F*, human primary monocytes (*E*) or macrophages (*F*) were nucleofected with scramble siRNA, siRNA for *NLRP3*, or siRNA for *ASC*. 48 h after nucleofection, cells were mock-infected or infected with HIV-R3A. IL-1β in the supernatant was measured. Data are mean ± S.E. ($n = 3$). **, $p < 0.01$; *n.s.*, not significant; *MDM*, monocyte-derived macrophages. The results are representative of three independent experiments.

IL-1β in parental THP-1 cells and cells transduced with scrambled control shRNA. As expected, a reported NLRP3 agonist, R848 (18), failed to induce IL-1β production in THP1 cells transduced with shRNA targeting *NLRP3* or *ASC* (data not shown). HIV-R3A also failed to induce IL-1β production in cells with suppressed expression of NLRP3 or ASC (Fig. 4*A*). However, inhibition of NLRC4 expression (>75%, data not shown) did not affect IL-1β production induced by HIV-1 infection (Fig. 4*A*). Furthermore, HIV-1 infection still induced expression of pro-IL-1β in cells with suppressed expression of NLRP3, ASC, or NLRC4 (Fig. 4*B*), suggesting that the NLRP3-ASC inflammasome is not involved in HIV-1-induced pro-IL-1β expression. Therefore, HIV-1 infection activated the NLRP3 inflammasome to induce IL-1β maturation and release.

To confirm that *NLRP3* and *ASC* are required in HIV-R3A-induced IL-1β production in primary monocytes, we transfected primary monocytes purified from human PBMC with siRNA targeting *NLRP3* and *ASC*. A scrambled siRNA was also included as a control. The transfection efficiency of primary monocytes was greater than 90% (data not shown). These cells were infected with HIV-R3A, and IL-1β was measured in supernatants collected at 24 hpi. Knockdown of *NLRP3* and *ASC* in primary monocytes was confirmed by quantitative PCR (Fig. 4, *C* and *D*). The induction of IL-1β by R848 stimulation was also attenuated significantly in primary monocytes transfected with siRNA targeting *NLRP3* and *ASC*, indicating that NLRP3 and ASC were functionally knocked down (data not shown). As shown above, HIV-1 infection induced a significant increase of IL-1β in monocytes transfected with control siRNA. In monocytes transfected with the siRNA for *NLRP3* or *ASC*, IL-1β induction by HIV-1 infection was attenuated significantly (Fig. 4*E*). Consistently, we also found that knockdown of *NLRP3* or *ASC* in primary macrophages abolished HIV-1-induced IL-1β production (Fig. 4*F*). These data suggest that HIV-1 induces IL-1β production via the NLRP3 inflammasome in primary monocytes/macrophages.

NLRP3 Inflammasome Activation in Responses to HIV-1 Is Dependent on ROS Production and Cathepsin B—The induction of ROS is often critical for inflammasome activation (44, 45). To test whether HIV-R3A infection induced ROS generation, we loaded THP-1 cells with the ROS dye dihydrorhodamine 123 at different time points post-infection. ROS levels increased over time during HIV-1 infection and reached a peak at 48 hpi (Fig. 5*A*). We showed that ROS was essential for HIV-1-induced IL-1β production because IL-1β secretion was totally inhibited by APDC, an NADPH oxidase inhibitor, in both THP-1 cells (Fig. 5*B*) and primary monocytes (Fig. 5*C*). A specific lysosomal cysteine protease, cathepsin B, has been reported to be associated with NLRP3 inflammasome activation and IL-1β production. Utilizing the cathepsin B-specific inhibitor CA-074-Me, we observed a significant attenuation in IL-1β release after HIV-R3A infection in both THP-1 cells (Fig. 5*D*) and primary monocytes (Fig. 5*E*). However, both ROS and cathepsin B inhibitors failed to inhibit HIV-R3A-induced pro-IL-1β expression (Fig. 5*F*). However, they inhibited caspase-1 activation (Fig. 5*G*). Therefore, HIV-1 induced ROS production

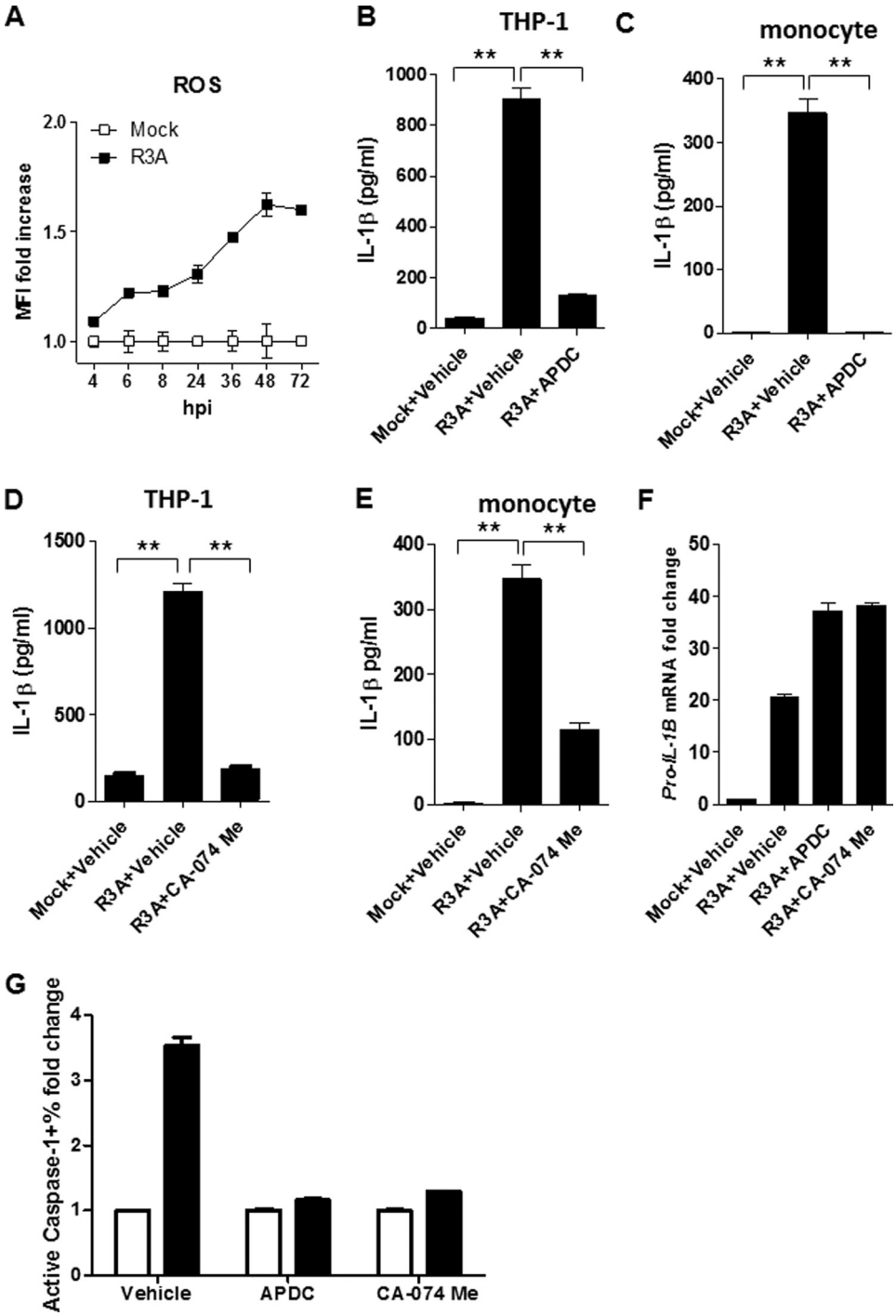

FIGURE 5. **ROS and cathepsin B are required for HIV-1-induced IL-1β production.** *A*, ROS production was determined by flow cytometry using the fluoroprobe dihydrorhodamine 123 in mock- or HIV-R3A-infected THP-1 cells at different hpi. Data are presented as increasing fold of mean fluorescence intensity (*MFI*) compared with mock. Data are mean ± S.E. ($n = 3$). *B* and *C*, ROS are critical for HIV-1-induced IL-1β production. ELISA was performed for IL-1β in culture supernatant from mock- or HIV-R3A-infected THP-1 cells (*B*) or primary monocytes (*C*) in the absence or presence of the ROS inhibitor APDC. *D* and *E*, cathepsin B is critical for HIV-1-induced IL-1β production. ELISA was performed for IL-1β in culture supernatant from mock- or HIV-R3A-infected THP-1 cells (*D*) or primary monocytes (*E*) in the absence or presence of the cathepsin B inhibitor CA-074 Me. *F*, qPCR analysis of *pro-IL-1β* mRNA in mock- or HIV-R3A-infected THP-1 cells in the absence or presence of APDC or CA-074 Me. *G*, activation of caspase-1 in HIV-R3A-infected THP-1 cells was determined by FLICA staining in the absence or presence of APDC or CA-074 Me. Data are presented as fold increase compared with mock infection. Data are mean ± S.E. ($n = 3$). **, $p < 0.01$. The results are representative of three experiments.

and cathepsin B activity are required for NLRP3 inflammasome activation.

Productive Infection Is Required for HIV-1-induced Pro-IL-1β Expression and NLRP3 Inflammasome Activation—HIV-R3A-induced IL-1β production was not significantly detectable until 48 hpi (Fig. 6, *A* and *B*). Consistently, pro-IL-1β expression and caspase-1 activation started to be detectable at 48 hpi (Fig. 6*B*). By HIV-1 p24 staining, we found that about 50% of cells were positive at 48 hpi (data not shown). The kinetics of viral replication and pro-IL-1β/IL-1β production were similar, implying

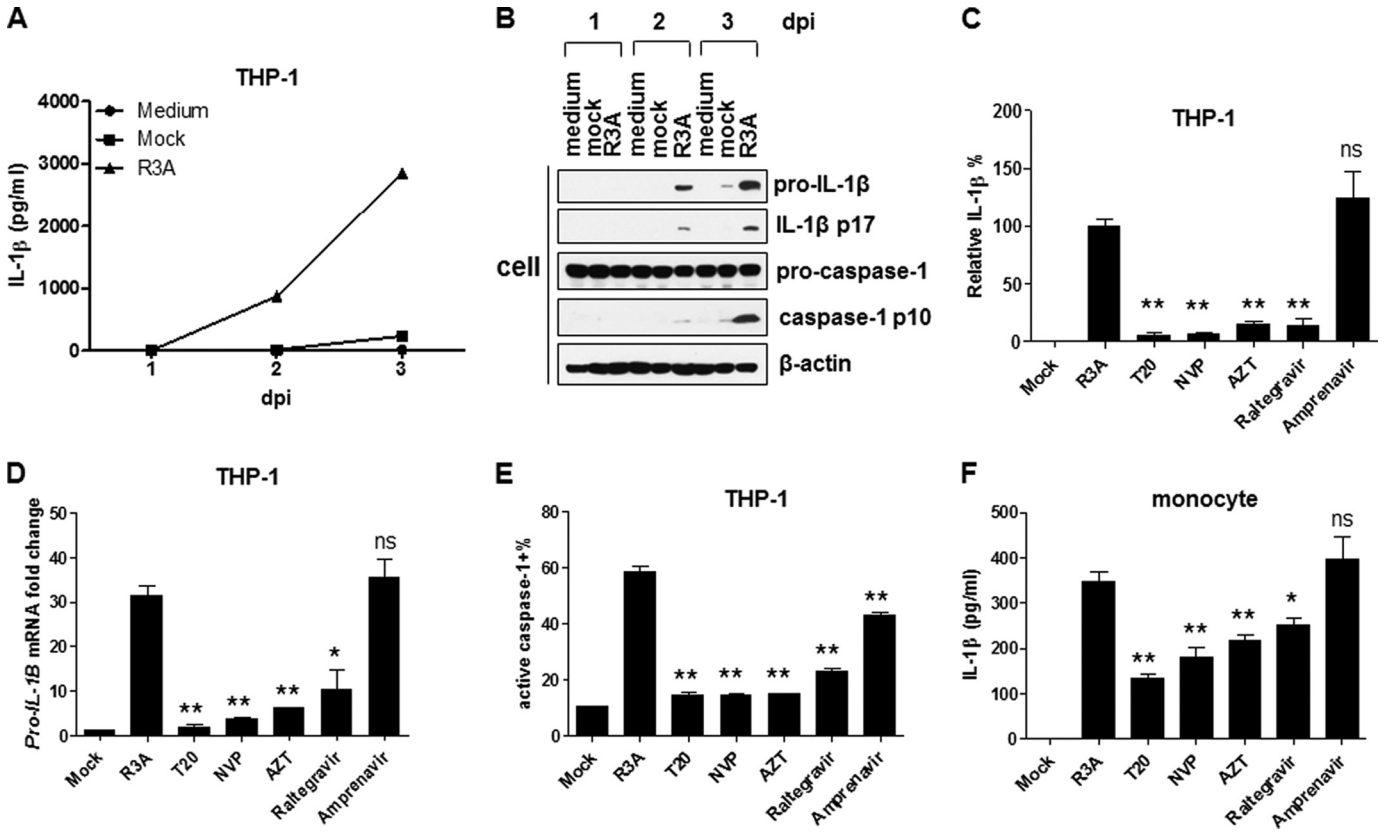

FIGURE 6. **HIV-1-productive infection is required for inflammasome activation.** *A*, THP-1 cells were mock-infected or infected with HIV-R3A. IL-1β in the cell free supernatant was assessed from 1–3 dpi. *B*, THP-1 cells were mock-infected or infected with HIV-R3A. Pro-IL-1β, mature IL-1β (p17), pro-caspase-1, and caspase-1 (p10) in cell lysates from 1–3 dpi were determined by Western blot analysis. β-actin was used as the loading control. *C–E*, THP-1 cells were mock-infected or infected with HIV-R3A in the absence or presence of various HIV-1 inhibitors (T20, NVP, AZT, raltegravir, or amprenavir). IL-1β from supernatant was measured by ELISA (*C*), *pro-IL-1*β mRNA was measured by qPCR (*D*), and caspase-1 activation was determined by FLICA staining (*E*). *F*, primary monocytes were mock-infected or infected with HIV-R3A in the absence or presence of various HIV-1 inhibitors (T20, NVP, AZT, raltegravir, or amprenavir). IL-1β from supernatant was measured by ELISA. Data are mean $\pm$ S.E. Statistical analysis was performed between HIV-1 and each HIV-1 plus virus inhibitor group ($n = 3$). **, $p < 0.01$; *n.s.*, not significant. The results are representative of two experiments.

that virus-productive infection may be important for HIV-1-induced pro-IL-1β expression and NLRP3-ASC-caspase-1 activation. To assess which step of viral replication is important for HIV-1-induced pro-IL-1β expression and inflammasome activation, we used T20 to block HIV-1 fusion, NVP and AZT to block HIV-1 reverse transcription, raltegravir to block HIV-1 integration, and amprenavir to block HIV-1 protease activity for viral polyprotein cleavage and viral particle maturation. All inhibitors, except the protease inhibitor amprenavir, significantly attenuated IL-1β release (Fig. 6*C*) and pro-IL-1β induction (Fig. 6*D*) in HIV-R3A-infected THP-1 cells. We also assessed caspase-1 activation by FLICA staining in the presence of HIV-1 inhibitors. Although T20, NVP, AZT, and raltegravir reduced the activity of caspase-1 to the level of mock infection, amprenavir only slightly reduced caspase-1 activation (Fig. 6*E*). Consistently, T20, NVP, AZT, and raltegravir, but not amprenavir, significantly attenuated HIV-R3A induced IL-1β production in primary monocytes (Fig. 6*F*). These data indicate that post-integration products, including HIV-1 RNA or proteins, are required for pro-IL-1β expression and NLRP3 inflammasome activation.

HIV-1 RNA Is Sufficient to Induce Pro-IL-1β *Expression and NLRP3 Inflammasome Activation*—TLR8, a sensor of single-stranded RNA, is critical for HIV-1 induced pro-IL-1β expression (Fig. 3). ssRNA40 is a 20-mer, U-rich, single-stranded RNA derived from the HIV-1 long terminal repeat U5 region, which has been reported to be a TLR8 agonist. ssRNA41 is derived from ssRNA40 by replacement of all U nucleotides with adenosines and is used as a negative control for ssRNA40 (46). We transfected the HIV-1-derived single-stranded RNA, ssRNA40, or the control ssRNA41 RNA into THP-1 cells. Compared with the control, ssRNA40 induced dramatic production of pro-IL-1β, active caspase-1 p10, and cleaved IL-1β p17 (Fig. 7*A*), indicating that HIV-1-derived RNA is sufficient for both pro-IL-1β expression and caspase-1 activation. Furthermore, ssRNA40-induced caspase-1 activation and IL-1β release were abolished in *NLRP3* and *ASC* knockdown THP-1 cells (Fig. 7, *B* and *C*). However, ssRNA40-induced pro-IL-1β expression was not affected in THP-1 cells with attenuated *NLRP3* and *ASC* expression (Fig. 7*D*). Even though the ssRNA40 RNA was transfected into the cytosol, its induction of pro-IL-1β was still dependent on TLR8 (Fig. 7*E*), which is consistent with the data in Fig. 3. Our data suggest that HIV-1 RNAs produced after the integration of the HIV-1 proviral genome can induce pro-IL-1β expression and activate the NLRP3 inflammasome.

DISCUSSION

HIV-1 disease progression is associated with elevated levels of inflammatory cytokines such as IL-1β. However, the mech-

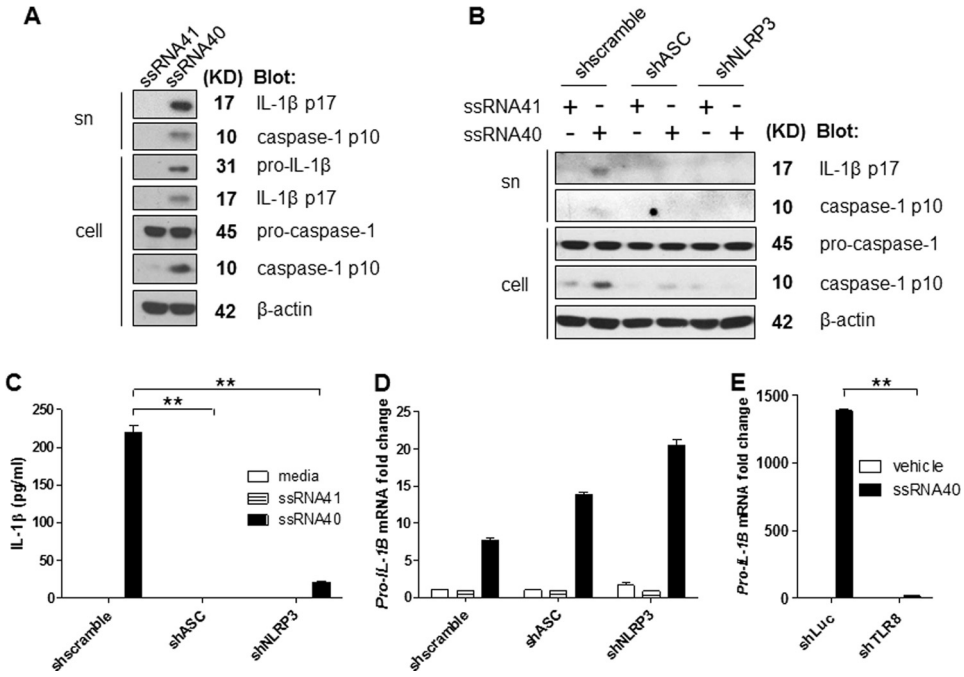

FIGURE 7. **HIV-1-derived RNA is sufficient for pro-IL-1β expression and NLRP3 inflammasome activation.** *A*, THP-1 cells were transfected with HIV-1-derived single-stranded RNA ssRNA40 or control RNA ssRNA41. Pro-IL-1β, mature IL-1β (p17), pro-caspase-1, and caspase-1 (p10) were detected in cell lysates (*cell*) and mature IL-1β (p17) and caspase-1 (p10) were detected in the supernatant (*sn*) by Western blot analysis. *KD*, kilodalton. *B–D*, THP-1 cells expressing scramble shRNA or shRNA for *ASC* or *NLRP3* were transfected with ssRNA41 or ssRNA40. *B*, IL-1β process and caspase-1 activation were determined by Western blot analysis. IL-1β in the supernatant (*C*) and *pro-IL-1β* mRNA in the cell (*D*) were also assessed by ELISA and qPCR. *E*, THP-1 cells expressing shRNA for *luciferase* or *TLR8* were transfected by vehicle or ssRNA40. *Pro-IL-1β* mRNA in the cell was determined by qPCR. Data are mean ± S.E. ($n = 3$). The results are representative of two experiments.

anism by which HIV-1 infection leads to IL-1β production is not clear. IL-1β production requires both the induction of *pro-IL-1β* gene expression and activation of the inflammasome complex (13). We report here that HIV-1 infection induced expression of pro-IL-1β through TLR8-dependent mechanisms and IL-1β maturation via NLRP3 inflammasome-dependent mechanisms in human monocytic cells. Interestingly, HIV-1 replication steps, including entry, reverse transcription, and integration, but not virion maturation, were all required. We report that HIV-1-induced ROS and cathepsin B were required for the activation of the NLRP3 inflammasome. It has been reported previously that there are no differences in the IL-1β pattern in HIV-1-infected *versus* non-infected monocytes (47–49). The discrepancy is probably due to the different HIV-1 strains or the lower multiplicity of infection used in previous experiments.

During influenza virus infection, viral genomic RNA is endocytosed to activate TLR7 in the endosome of monocytes, which induces *pro-IL-1β* gene expression (25). In this study, soluble CD4 completely abrogated both the induction of pro-IL-1β expression and caspase-1 activation by HIV-1. We discovered that HIV-1 infection induced pro-IL-1β expression via TLR8, but not TLR7, in monocytes, although both TLR7 and TLR8 can be activated by HIV-1 infection (8–12). As expected, inhibitors of endosome acidification, which can disrupt endosomal TLR function, also blocked HIV-induced pro-IL-1β expression.

The NLRP3 inflammasome is activated by a number of different stimuli, but the molecular mechanisms are undefined (15). It is not clear how HIV-1 infection activates the NLRP3 inflammasome, although cathepsin B activity and induction of ROS were required. We show that HIV-1-productive infection was required for both pro-IL-1β expression and inflammasome activation, suggesting a novel mechanism for inflammasome activation and IL-1β production. We quantified the total HIV-1 RNA in the cell and found that the cathepsin B inhibitor CA-074 Me did not affect HIV-1 RNA accumulation, whereas chloroquine and the ROS inhibitor APDC only slightly reduced HIV-1 RNA expression, thus excluding the possibility that the inhibition of pro-IL-1β by chloroquine and inhibition of NLRP3 inflammasome activation and IL-1β production by APDC or CA-074 Me is due to the inhibition of HIV-1 replication. Specific HIV-1-derived RNAs and/or HIV-1-encoded proteins may contribute to inflammasome activation. For the influenza virus, activation of the NLRP3 inflammasome requires the virus-encoded M2 protein, a proton-selective ion channel protein (25). Of interest is the HIV-1 accessory protein Vpu, whose transmembrane domain also acts as an ion channel (50). Expression of each HIV-1 encoded ORF alone in THP1 cells and primary macrophages showed no significant effect on inflammasome activation.[3] However, transfection of HIV-1-derived single-stranded RNA into THP-1 cells induced significant caspase-1 activation and IL-1β production. It seems that HIV-1 activates NLRP3 through its RNA but not the ion channel protein Vpu. It is possible that HIV-1 RNA accumulation triggered ROS production and cathepsin B release from the lysosome, which contribute to NLRP3 inflammasome activation. It will be interesting to define the specific HIV-1 RNA

[3] Guo, H., and Su, L., unpublished data.

sequences and the receptors required for HIV-1-induced NLRP3 inflammasome activation in future experiments.

Consistent with our findings, HIV-1 disease progression is associated with activated macrophages and increased levels of IL-1β (51), which likely lead to immune dysregulation and CD4 T cell depletion. Therefore, reducing the IL-1β level or activity *in vivo* is likely beneficial to HIV-1 patients. This hypothesis will be tested in the humanized mouse models of HIV pathogenesis (52–54) or in the simian immunodeficiency virus-infected rhesus monkey models (55–58) using IL-1β-neutralizing antibodies.

Acknowledgments—We thank Liqun Chi for technical support and the members of the Su laboratory for discussions.

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