Epigenetic Regulation of Tumor Necrosis Factor α (TNFα) Release in Human Macrophages by HIV-1 Single-stranded RNA (ssRNA) Is Dependent on TLR8 Signaling*

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Background: TLR7/TLR8 recognize viral ssRNA, but in human macrophages recognition of HIV-1 ssRNA is not known.
Results: In human macrophages, HIV-1 ssRNA promotes TNFα release and requires endocytosis through TLR8, MyD88, and histone modification.
Conclusion: TLR8 recognizes HIV-1 ssRNA and promotes TNFα release through chromatin remodeling.
Significance: Targeting macrophage TLR8 signaling may serve as a novel therapeutic strategy to modify HIV infection.

Human macrophages at mucosal sites are essential targets for acute HIV infection. During the chronic phase of infection, they are persistent reservoirs for the AIDS virus. HIV virions gain entry into macrophages following ligation of surface CD4-CR5 co-receptors, which leads to the release of two copies of HIV ssRNA. These events lead to reverse transcription and viral replication initiation. Toll-like receptors TLR7 and TLR8 recognize specific intracellular viral ssRNA sequences, but in human alveolar macrophages, their individual roles in TLR-mediated HIV ssRNA recognition are unclear. In the current study, HIV-1 ssRNA induced TNFα release in a dose-dependent manner in adherent human macrophages expressing both intracellular TLR7 and TLR8. This response was reduced by inhibiting either endocytosis (50 μM dynasore) or endosomal acidification (1 μg/ml chloroquine). Either MYD88 or TLR8 gene knockdown with relevant siRNA reduced HIV-1 ssRNA-mediated TNFα release, but silencing TLR7 had no effect on this response. Furthermore, HIV-1 ssRNA induced histone 4 acetylation at the TNFα promoter as well as trimethylation of histone 3 at lysine 4, whereas TLR8 gene knockdown reduced these effects. Taken together in human macrophages, TLR8 binds and internalizes HIV ssRNA, leading to endosomal acidification, chromatin remodeling, and increases in TNFα release. Drugs targeting macrophage TLR8-linked signaling pathways may modulate the innate immune response to acute HIV infection by reducing viral replication.

In the alveolar airspace, the most abundant immune cells found in this compartment are alveolar macrophages (AM).2 These cells represent critical effector cells in the innate immune response to infectious challenge in the lungs (1). Toll-like receptors (TLRs) on AM function as pattern recognition receptors to induce responses to pathogens (2). At least 13 TLRs have been discovered thus far, each with degrees of specificity for various endogenous and exogenous ligands (3). TLRs can be broadly divided into two categories: cell membrane TLRs (TLR1, TLR2, TLR4, TLR5, TLR6, and TLR11) and nucleic acid-sensing TLRs (TLR3, TLR7, TLR8, and murine TLR13), which localize to intracellular vesicles including the endoplasmic reticulum, endosomes, and lysosomes (4).

TLRs function to induce antiviral defenses in different cell types, and either TLR7 or TLR8 recognizes HIV-1-derived ligands to induce innate immune responses (5). In vivo HIV-1 infection occurs as a consequence of TLR7 activation on primary dendritic cells (pDCs) (6). In addition, the ability of HIV-1 to infect AM and monocyte-derived macrophages in vitro is well established, and it may play a role in viral pathogenesis (7, 8). Uridine-rich oligonucleotides derived from HIV-1 RNA activate TLR7/8 in human and mice macrophages and induce innate immune responses (5, 9, 10). However, in human AM, it is unknown whether endosomal TLR7 or TLR8 recognize HIV-1 ssRNA.

Transcription factor activation and chromatin remodeling are both modulators of gene expression. Transcription factor control of gene promoters is dependent on kinase signaling that enables transcription factor accessibility and binding to target sites (11), whereas chromatin remodeling occurs as a consequence of histone covalent modification modulating transcription factor activity (12). For example, post-translational modifi-

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2 The abbreviations used are: AM, alveolar macrophage(s); TLR, Toll-like receptor; pDC, primary dendritic cell; BLP, bacteria lipoprotein; PMA, phorbol myristic acid; MTA, 5′-deoxy-5′-methylthioadenosine; PE, phycocerythrin; CBP, CREB-binding protein; CREB, CAMP-responsive element-binding protein.
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EXPERIMENTAL PROCEDURES

Reagents—ssRNA 40/LyoVec and ssRNA 41/LyoVec were purchased from InvivoGen (San Diego, CA). Dynasore, chloroquine, bacteria lipoprotein (BLP), protease inhibitor mixture, phorbol myristic acid (PMA), curcumin, 5′-deoxy-5′-methylthioadenosine (MTA), and lipid A were purchased from Sigma. Furthermore, bacterial toxins induce histone modifications in HeLa cells (18). We show here in human macrophages that ligation of HIV-1 ssRNA by endosomal TLR8 induces TNFα release through a MyD88-dependent signaling pathway. Furthermore, we show that HIV-1 ssRNA mediates TLR8 linked pathway signaling activation through post-translational histone modification followed by increase in transcription factor binding to TNFα promoters. These results suggest that drug targeting macrophage TLR8 signaling pathways and enzymes/proteins involved in inducing epigenetic changes may provide a novel approach to deciphering HIV pathogenesis and to correct dysregulated innate immune responses to HIV infection.

Human Alveolar Macrophages—To determine the clinical relevance of the study, select experiments were carried out using human AM. Recruited healthy subjects had no active pulmonary disease and normal spirometry. They were confirmed to be HIV seronegative by ELISA and had no known risk factors for HIV infection. Using standard techniques, bronchoalveolar lavage was performed to obtain lung immune cells (19). All procedures were performed on consenting adults following protocols approved by the Beth Israel Deaconess Medical Center Institutional Review Board and Committee for Clinical Investigations. Cells were separated from the pooled bronchoalveolar lavage fluid, and AM were isolated (20). AM were isolated by adherence to culture plates, yielded cells that were >98% viable as determined by trypan blue dye exclusion, and demonstrated >95% positive nonspecific esterase staining.

Macrophage Cell Lines—Macrophages were differentiated from human promonocytic THP-1 (American Type Culture Collection (ATCC)). THP-1 cells were harvested during exponential growth phase, washed, and then incubated in complete medium (RPMI 1640 containing 10% heat-inactivated fetal calf serum (FCS), 2 mm glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin). To induce macrophage differentiation, THP-1 cells were incubated with 100 nm PMA at 37°C in a humidified atmosphere containing 5% CO2 for 24 h. Adherent cells were then washed three times with PBS (to remove PMA) and incubated in complete medium (without PMA) for an additional 24 h prior to use. THP-1 differentiation to macrophages was confirmed by CD11b expression and enhanced granularity by flow cytometry.

Western Blot Analyses—Western blotting was performed as described (21). Briefly, adherent human macrophages were treated with an indicated dose of HIV-1 ssRNA and washed twice with ice-cold PBS (pH 7.4). Cells were lysed in lysis buffer containing 25 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, and protease inhibitor mixture (Sigma) and placed on ice for 20 min. Cells were harvested by scraping followed by centrifugation at 4°C for 15 min at 14,000 rpm. Equal amounts of cell lysates were subjected to SDS-PAGE and Western blot analysis with designated antibodies and detected by the enhanced chemiluminescence (ECL) detection system (Amersham Biosciences). Resolved bands were quantified by densitometry (Amersham Biosciences).

Flow Cytometry Analysis—Cell intracellular surface expression of TLRs was determined by an Epics XL flow cytometer (Beckman Coulter) with laser power of 5.76 milliwatts. The instrument was calibrated before each measurement with standardized fluorescent particles (Immunotech; AMAC, Inc. Westbrook, ME). Fluorescent signals of the cells were measured simultaneously with three photomultiplier tubes and optical filters and expressed as the mean of the log fluorescence intensity of the cell population within a gate. Macrophages were spun and resuspended in 1 ml of 20 μg/ml lyssolecithin in 1% paraformaldehyde and incubated at room temperature for 2 min. Cells were then pelleted, resuspended in 2 ml of cold absolute methanol, and incubated on ice for 15 min. Cells were again pelleted and resuspended in 1 ml of 0.1% Nonidet P-40 and incubated on ice for 5 min. Pelleted cells were resuspended in PBS followed by incubation with an anti-TLR7 or TLR8 PE-conjugated antibody or isotype control and incubated at room temperature for 15 min. One ml of PBS was added to each tube, pelleted again, resuspended in 1 ml of PBS, and analyzed by flow cytometry. Data are expressed as a mean relative fluorescence units and the percentage of cells staining positive. Isotype primary conjugated antibodies served as a negative control. Samples contained a minimum of 5000 cells and were analyzed in triplicate.

ELISA—After cell stimulation, supernatants were collected, centrifuged to remove cellular debris, and assayed immediately or stored at −80°C until assayed. Cytokine measurements were performed using commercially available ELISA kits following the manufacturer’s instructions, and absorbance was measured at 450 nm on a Bio-Tek kinetic ELISA reader (Bio-Tek Instruments, Winooski, VT). The detection limit for TNFα was 4.4 pg/ml. All measurements were performed in duplicate, and mean values of four measurements were used for statistical analysis.

Targeted Gene Silencing (RNAi) in Macrophages—To determine the specific TLRs that engaged HIV-derived ssRNA, tar-
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FIGURE 1. Dose-dependent effects of HIV-1 ssRNA in human macrophages on TNFα release. A and B, human macrophages THP-1 (A) and or human alveolar macrophages (B) were incubated with different doses of HIV-1 ssRNA for 24 h. Cell-free supernatants were analyzed for TNFα by ELISA. Data shown are mean ± S.E. of three independent experiments done in triplicate (A) or mean ± S.E. from four independent experiments in triplicate (B). *, p < 0.05 when compared with control (cont). Control = ssRNA with uridine replaced with adenine. BLP = 10 μg/ml.

| HIV-ssRNA µg/ml | TNFα release (pg/ml) | Cont | 0.1 | 1.0 | 2.0 | 5.0 | BLP |
|-----------------|----------------------|------|-----|-----|-----|-----|-----|
| 0               |                      | 600  | 4000| 5000| 6000| 7000| 8000|

Quantitative Real-time PCR—To quantify the amount of DNA from specific regions of TNFα promoter, quantitative real-time PCR was used. In addition, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) promoter was used as internal control. The DNA sample isolated from ChIP assay was subjected to four PCR reactions for the four sets of primers and probes to analyze the four regions of the TNFα promoter. The primer-probe combinations for TNFα 1, 2, 3, and 4 have been previously described (22). The real-time PCR reaction (total 25 μl) contained 3 μl of DNA, 12.5 μl of 2X SYBR Green master mix, 300 nM of each primer, and ultrapure water. The real-time PCR procedure was: 2 min at 50 °C and 10 min at 95 °C followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C, using an ABI Prism 7000 sequence detection system (Applied Biosystems). Data were normalized to the input DNA and presented as -fold change relative to DNA from untreated cells.

Statistical Analysis—Group comparisons were performed using Student’s t test (two-way sample test) or one-way analysis of variance. Calculations were performed with the StatView (SAS Institute, Inc; Cary, NC) and INSTAT2 (GraphPad Software; San Diego, CA) software packages. Results are given as mean ± S.E. Statistical significance was accepted for p < 0.05.

RESULTS

Dose-dependent Effects of HIV-1 ssRNA on TNFα Release in Human Macrophages—HIV-1 induces increase in TNFα release in pDCs via TLR7 (6). Furthermore, in pDC HIV-1, long terminal uridine-enriched repeats also bind and activate TLR7/8 (5). We assessed the ability of uridine-rich HIV-1 ssRNA to stimulate TNFα release in human macrophages. HIV-1 ssRNA induced TNFα release in a dose-dependent manner in a PMA differentiated macrophage cell line, THP-1 (Fig. 1A). TNFα release began at 0.1 μg/ml, reached a maximum level at 2 μg/ml HIV-1 ssRNA, and declined with further increase in dose. These biphasic effects are specific because replacement of uridine with adenine failed to induce TNFα release (Fig. 1A). Furthermore, as a positive control, activation
of TLR2 with 10 μg/ml BLP increased TNFα release (Fig. 1A). Importantly, the same dose-dependent release was observed in clinically relevant AM obtained from healthy subjects (Fig. 1B). These results suggest that increase in TNFα release by HIV-1 ssRNA may contribute to systemic innate immune activation associated with HIV-1 disease progression.

**Dependence of HIV-1 ssRNA-induced TNFα Release in Macrophages on Endocytosis and Endosomal Acidification—**TLR7/8 expression is restricted to the luminal aspect of the endosomal membranes. Therefore, HIV-1 ssRNA must undergo either endocytosis or autophagy for it to activate TLR7/8 (23). In pDC, an HIV-1 long terminal enriched with uridine repeats activated an innate immune response via endosomal TLR7/8 (5). Furthermore, HIV-1 endocytosis activated plasmacytoid cells via Toll-like receptor-RNA interactions (6). We investigated the role of endocytosis in HIV-1 ssRNA induction of TNFα release in macrophages in the presence or absence of an inhibitor of guanosine triphosphate (GTPase) dynamin (dynasore). This inhibitor suppressed endocytosis through suppression of TLR4-mediated MyD88-independent pathway signaling (24, 25). Pretreatment of human macrophages with dynasore significantly reduced HIV-1 ssRNA-mediated TNFα release in THP-1 cells by ~50% (Fig. 2A) and in clinically relevant AM by ~45% (Fig. 2B), suggesting that in human macrophages, HIV-1 ssRNA-mediated TNFα release requires endocytosis and is dependent on GTPase dynamin.

Next, to characterize further the induction of TNFα by HIV-1 ssRNA in macrophages, we determined whether endosomal acidification is required. Chloroquine was used because it abolishes intracellular TLRs signaling through inhibition of lysosomal acidification (26). Pretreatment of human macrophages with chloroquine inhibited HIV-1 ssRNA-mediated TNFα release in THP-1 cells by 61% (Fig. 2A) and in AM by 83% (Fig. 2B), suggesting that in human macrophages, HIV-1 ssRNA-mediated TNFα release requires endosomal acidification. However, chloroquine did not inhibit the release of TNFα by phorbol ester (PMA) (data not shown). Taken together, these data demonstrate in macrophages that HIV-1 ssRNA elicits TNFα release through endocytosis, resulting in TLR7/8-induced endosomal acidification.

**Dependence of TNFα Release on TLR8 Activation by HIV-1 ssRNA in Human Macrophages—**Enriched uridine HIV-1 long terminal repeats activate TLR7/8 in murine dendritic cells and macrophages (5). Furthermore, plasmacytoid dendritic cells recognize HIV-1 ssRNA through TLR7, leading to the production of pro-inflammatory cytokines (27). We determined which of these endosomal TLRs recognize HIV-1 ssRNA in human macrophages. Flow cytometric analysis showed that TLR7 and TLR8 are both expressed intracellularly in THP-1 (Fig. 3A) and in primary AM (Fig. 3B). Accordingly, we next determined which of these subtypes engages HIV-1 ssRNA to stimulate TNFα release in human macrophages. TNFα release by HIV-1 ssRNA was comparable in the presence or absence of targeted functional gene silencing of either TLR7 or TLR8 using RNAi methodology. TLR7 protein expression fell by ~70% in human THP-1 (Fig. 3C, left panel). Furthermore, in another group of cells, siRNA gene silencing reduced TLR8 protein expression by ~75% (Fig. 3C, right panel). In TLR7 silenced and nonsilenced THP-1 cells, HIV-1 ssRNA induced comparable increase in TNFα release (Fig. 3D). In contrast, in human macrophages, TNFα release induced by HIV-1 ssRNA in TLR8 silenced cells was markedly reduced when compared with that in the nonsilencing control (Fig. 3E). This difference between the effects of HIV-1 ssRNA demonstrates that HIV-1 ssRNA selectively activates TLR8. Similar amounts of TNFα were released in both cell populations transfected with either TLR7 or TLR8 siRNA in the presence of 10 μg/ml BLP (TLR2 agonist), suggesting that the functions of other TLRs were not compromised (Fig. 3, D and E). Taken together, induction of increase in TNFα release by HIV-1 ssRNA in human macrophages is dependent on TLR8 activation rather than TLR7.

**HIV-1 ssRNA-induced MyD88-dependent Signaling in Macrophages Mediates TNFα Release—**TLR8 mediates TNFα gene transcription through a cascade of signaling events. MyD88 is an adaptor protein that is recruited to mediate TLR8 signaling events leading to the release of cytokines such as TNFα (28). To determine in human macrophages its involvement in TLR8-linked signaling, TNFα release was measured in the presence or absence of targeted MYD88 gene silencing. A reduced MyD88 protein level of about 60% was demonstrated in TLR7 cells after MYD88 siRNA gene silencing (Fig. 4A). Stimulation of these cells with HIV-1 ssRNA in the presence of nonsilencing siRNA dose-dependently increased TNFα release. Similarly, cells transfected with MYD88 siRNA also elicited such a response, but the magnitudes of increase in
release were reduced when compared with those in nonsilenced cells (Fig. 4B). Similarly, TNFα release induced by TLR2 activation with 10 μg/ml BLP was diminished in the MYD88 silenced cells. Taken together, in macrophages, TLR8 activation by HIV-1 ssRNA increases TNFα release through a MyD88-dependent signaling pathway.

Histone 4 Acetylation by HIV-1 ssRNA Increases TNFα Promoter Activity—Receptor-mediated signaling leading to histone covalent modification of chromatin structure plays an important role in determining transcription factor activity (11, 12). Acetylation of histone 4 generally enhances access to DNA, leading to gene expression activation (29). To determine whether HIV-1 ssRNA-induced increases in TNFα release are associated with an increase in histone acetylation at the TNFα promoter, a ChIP assay was performed. DNA-protein complexes cross-linked with formaldehyde from unstimulated or HIV-1 ssRNA-stimulated macrophages were immunoprecipitated with an antibody against ACH4 or bovine serum albumin (BSA). DNA isolated from the immunoprecipitates was analyzed by real-time PCR. Each sample was subjected to four PCR reactions encompassing four regions of the TNFα promoter designated TNF1-TNF4 (16) (Table 1). Histone H4 acetylation levels in unstimulated cells were higher in the TNF2 region than in other promoter regions. In contrast, in the presence of HIV-1 ssRNA, histone H4 acetylation levels were higher in TNF1 and TNF2, regions that are more proximal to the transcription start site (Fig. 5, A and B). Taken together, the selective increase in H4AC acetylation levels by HIV-1 ssRNA suggests that epigenetic factors might contribute substantially to increased TNFα gene expression in human macrophages.

Histone acetyltransferase modulates gene expression by catalyzing targeted acetylation of the ε-amino group of lysine residues on histones and nonhistone proteins (30). p300/CREB-binding protein (p300/CBP) is a protein with intrinsic histone acetyltransferase activity, and it can be specifically inhibited by curcumin (31). Pretreatment of AM with curcumin dose-dependently (0.1–1.0 μM) inhibited HIV-1 ssRNA-induced TNFα release (Fig. 5C). Furthermore, HIV-1 ssRNA dose-dependently increased p300/CBP protein expression, whereas curcumin suppressed this response (Fig. 5, D and E). Taken together, these data suggest that HIV-1 ssRNA induces increase in p300/CBP expression and mediates increase in TNFα expression via histone 4 acetylation.

HIV-1 ssRNA-induced H3 Lysine 4 Trimethylation with Concomitant Inhibition of H3 Lysine 27 Trimethylation Mediates TNFα Release—Human pDC activation leads to increase in inflammatory gene expression through post-translational histone modification (32). One histone change associated with

FIGURE 3. Dependence of TNFα release in human macrophages on TLR8 activation by HIV-1 ssRNA. A and B, intracellular expression of TLR7 and TLR8 in macrophages. THP-1 (A) and AM (B) were incubated with PE-conjugated anti-TLR7 or anti-TLR8 or isotype control antibody, and intracellular expression was determined by flow cytometry. Representative profiles were similar in three independent experiments (n = 3 subjects for AM). C–E, functional silencing of human TLR8 leads to marked diminution of TNFα release in human macrophages. C, flow cytometry analysis of human TLR7/8 after gene silencing with the use of TLR7 or TLR8 siRNA and siRNA or isotype control. A representative flow cytometry tracing shows representative results from one experiment repeated independently in three experiments. N.S., nonsilencing. D and E, THP-1 cells were pretreated with either TLR7 siRNA (D) or TLR8 siRNA (E) or nonsilencing control. Cells were differentiated with phorbol ester, challenged with HIV-1 ssRNA and incubated for 24 h. Cell free supernatant was assayed for TNFα by ELISA. Results are representative of three independent experiments performed in triplicate; *, p < 0.05 when compared with nonsilencing siRNA control. BLP served as positive control. US, unstimulated.
transcription activation is histone H3 lysine 4 trimethylation (16), and H3 lysine 27 trimethylation (H3K27me3) is associated with transcription repression (15). To determine whether HIV-1 ssRNA-induced increase in TNFα release is associated with such a modification at the TNFα promoter, a ChIP assay was performed with an antibody to H3K4me3 (anti-H3K4me3) or with anti-H3K27me3. ChIP assay analysis revealed that HIV-1 ssRNA increased trimethylation of histone 3 methylation at lysine 4 levels in the TNF1 and TNF2 regions when compared with unstimulated cells (Fig. 6A), but with a concomitant decreased trimethylation of histone 3 at lysine 27 levels in the TNF1 and TNF2 regions when compared with unstimulated cells (Fig. 6B). Furthermore, no promoter sequences were detected by omitting this antibody from the immunoprecipitation reaction (data not shown).

Next, to determine whether in macrophages methylation is critical for TNFα release in response to HIV-1 ssRNA, histone methyltransferase activity was inhibited with MTA. MTA inhibits lipopolysaccharide-mediated TNFα release in THP-1 cells (16). Cells were pretreated with different doses (0.1–1.0 mM) of MTA for 1 h followed by HIV-ssRNA stimulation for 24 h. Cell viability was essentially unchanged by MTA because trypan blue dye exclusion was always greater than 90%. MTA in 1 mM of MTA for 1 h followed by HIV-ssRNA stimulation for 24 h. Cell viability was essentially unchanged by MTA because trypan blue dye exclusion was always greater than 90%. MTA in 1 mM (Fig. 6A). Furthermore, the same trend was observed when H4Ac was analyzed (Fig. 7B). Taken together, HIV-1 ssRNA-induced increase in TNFα release is dependent on increase in activating histone (H3K4) with a concomitant decrease in suppressive histone (H3K27).

**Dependence of Chromatin Remodeling at TNFα Promoter on HIV-1 ssRNA-induced TLR8 Activation**—To determine in human macrophages whether engagement of HIV-1 ssRNA with TLR8 is associated with increased histone H3 lysine 4 trimethylation or histone 4 acetylation at the TNFα promoter, ChIP analysis was performed in the presence and absence of targeted functional TLR8 gene silencing. Trimethylation of histone lysine 4 was robust in cells transfected with nonsilencing control, but targeted gene silencing of TLR8 showed reduced trimethylation of histone at lysine 4 (Fig. 7A). Furthermore, the same trend was observed when H4Ac was analyzed (Fig. 7A). Taken together, HIV-1 ssRNA-induced TNFα expression is dependent on TLR8-mediated chromatin remodeling.

**DISCUSSION**

In this study, we demonstrated that in human macrophages, increases in pro-inflammatory cytokine TNFα release occur through selective recognition by endosomal TLR8 of uridine-rich HIV-1 ssRNA oligonucleotides. Their engagement with TLR8 depends on dynamin-mediated endocytosis and endosomal acidification and MD88-dependent signaling pathway activation. This sequence of TLR8-mediated responses was also demonstrated in clinically relevant primary human alveolar macrophages. Taken together, these findings indicate that increase in TNFα release induced by HIV-1 ssRNA interaction
with endosomal TLR8 may contribute to systemic immune activation and HIV-1 disease progression.

TLRs induce innate and adaptive immune responses as a consequence of recognition of nonself motifs expressed by viruses and bacteria (33). TLR7/8 are endosomally restricted and induce inflammatory cytokine release through their interaction with a uridine-rich ssRNA sequence within the HIV-1 long terminal repeat (5). TLR7 on human pDC also recognize and bind HIV-1 ssRNA to induce immune responses (6). In the current study, we found that although human macrophages express both TLR7 and TLR8, uridine-rich HIV-1-derived ssRNA selectively stimulated TNFα release through TLR8 and MyD88 signaling pathway activation. This selectivity was evident in human macrophages because only gene silencing of TLR8 suppressed HIV-1 ssRNA-induced TNFα release. The role of an endosomal localized TLR in inducing this response is in agreement with the inhibitory effects of chloroquine (endosomal acidification blocker) and dynasore (dynamin-mediated endocytosis blocker) on HIV-1 ssRNA-induced increase in TNFα release. Further indication of TLR8 involvement is supported by our finding that a substitution of HIV-1 ssRNA uridine-rich fragment with one containing adenines in place of uridine obviated TLR8 activation. These findings are relevant because they were replicated in clinically relevant alveolar macrophages. Taken together, HIV-1 ssRNA oligonucleotides trigger through TLR8- and MyD88-dependent signaling pathway activation of TNFα release. Promotion of this response in a clinical setting may have therapeutic value in suppressing HIV-1 pathogenesis and disease progression.

Inducible gene expression is now understood to be controlled by transcription factor activation and chromatin remodeling (11, 12). Transcription factor control of gene promoters is dependent on kinase signaling, enabling transcription factor accessibility and binding to target sites, whereas chromatin remodeling occurs as a consequence of histone covalent modification modulating transcription factor activity (11). We assessed the involvement of chromatin remodeling by TLR8 activation on TNFα gene expression. This was done through characterizing post-translational modifications of histone acetylation and methylation because such modifications affect agonist-mediated gene induction (34, 35). In addition, histone H3 (Ser-10) phosphorylation correlates with immediate early gene induction of c-fos, c-jun, and MKP-1 (36, 37). Histone acetylation is linked with transcriptional activation, whereas deacetylation is related to its repression (34, 38). Histone acetylation increases gene expression by increasing either transcrip-
factor promoter accessibility or signaling competence for nucleosome remodeling (38). In our study, we showed in human macrophages that chromatin remodeling occurs as a consequence of HIV-1 ssRNA-induced TLR8 activation, leading to TNFα release. These results are consistent with those in peripheral blood mononuclear cells and mouse macrophages in which dependence of TLR4-induced increase in pro-inflammatory cytokine release is dependent on chromatin remodeling (39). In addition to the modifications described by Tsaprouni et al. (39), we found that histone 3 lysine 4 (H3K4me3) methylation was also markedly increased, with concomitant decrease in histone 3 lysine 27 (H3K27me3) methylation. Taken together, chromatin remodeling plays a critical role in human macrophages in mediating through TLR8 activation TNFα release.

In conclusion, in alveolar macrophages, HIV-1 ssRNA uridine-rich oligonucleotide-induced TLR8 activation leads to TNFα release through MyD88-dependent signaling pathway activation. MyD88 activation by TLR8 is dependent on endosomal uptake of HIV-1 ssRNA and acidification. At the gene level, TNFα release occurs subsequent to chromatin remodeling resulting from histone acetylation at H4 and methylation at histone 3 lysine 4 (H3K4me3), leading to increased transcription factor accessibility to the TNFα promoter. A better understanding of epigenetics during HIV infection could be an important potential source of novel therapeutic targets for suppressing HIV pathogenesis.

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