TLR8 Activates HIV from Latently Infected Cells of Myeloid-Monocytic Origin Directly via the MAPK Pathway and from Latently Infected CD4+ T Cells Indirectly via TNF-α

Erika Schlaepfer and Roberto F. Speck

We previously showed that the TLR7/8 agonist, R-848, activated HIV from cells of myeloid-monocytic origin. In this work, we show that this effect was solely due to triggering TLR8 and that NF-κB was involved in the TLR8-mediated activation of HIV from latently infected cells of myeloid-monocytic origin. Inhibition of Erk1/2 or p38α resulted in attenuation of TLR8-mediated activation of NF-κB. Western blots confirmed that TLR8 triggering activated Erk1/2 and p38α but, surprisingly, not JNK. Although the Erk1/2 inhibitors resulted in a less attenuated TLR8-mediated NF-κB response than did p38α inhibitors, they had a more pronounced effect on blocking TLR8-mediated HIV replication, indicating that other transcription factors controlled by Erk1/2 are involved in TLR8-mediated HIV activation from latently infected cells. TNF-α, which was secreted subsequent to TLR8 triggering, contributed to the activation of HIV from the latently infected cells in an autocrine manner, revealing a bimodal mechanism by which the effect of TLR8 triggering can be sustained. We also found that TNF-α secreted by myeloid dendritic cells acted in a paracrine manner in the activation of HIV from neighboring latently infected CD4+ T cells, which do not express TLR8. Notably, monocytes from highly active antiretroviral therapy-treated HIV+ patients with suppressed HIV RNA showed a robust TNF-α secretion in response to TLR8 agonists, pointing to a functional TLR8 signaling axis in HIV infection. Thus, triggering TLR8 represents a very promising strategy for attacking the silent HIV from its reservoir in HIV+ patients treated successfully with highly active antiretroviral therapy. The Journal of Immunology, 2011, 186: 4314–4324.

Highly active antiretroviral therapy (HAART) has significantly decreased HIV-related morbidity and mortality in Europe and the United States. Nevertheless, HAART does not eradicate the latent reservoir of HIV. Consequently, treatment strategies aimed at eradicating HIV from HIV-infected individuals remain an unresolved medical issue of paramount importance.

Latently infected cells harbor HIV in the host chromosome without any or only minimal HIV transcription and, thus, no or only minimal translation into HIV proteins. Resting memory CD4+ T cells serve as the major reservoir for latent HIV (1). The significance of other reservoirs, including monocytes, microglia, macrophages, and hematopoietic stem cells, is less well defined (2). Based on the estimated half-life of latently HIV-infected CD4+ T cells ~44 mo, one speculates that it would take over 60 y of HAART for HIV-infected patients to be cured if there is no residual low-level replication (3).

Eradication strategies rely on the induction of HIV transcription by any means, with subsequent death of the previously latently infected cells by virally caused cytopathic effects or recognition by HIV-specific CD8+ T cells (1). In the current paradigm, the ongoing HAART prevents spreading of infection, which may result from the virions released by the previously latently infected cells. Various strategies to eradicate HIV have been tested in phase I clinical trials. Patients whose HIV RNA levels were suppressed with conventional or even intensified HAART were treated with IL-2, OKT3 (an Ab against CD3), or histone deacetylase (HDAC) inhibitors (4–6). The rather uniform T cell activation by IL-2 or OKT3 was also supposed to reach the latently infected CD4+ T cells, which subsequently would die off as the result of aforementioned mechanisms. HDACs are recruited to the highly conserved initiator region of the HIV promoter. They favor the condensation of the chromatin and, thus, may promote HIV latency. The inhibition of HDACs will favor induction of HIV. However, the latent reservoirs were only partially reduced in those clinical trials, as exemplified by rapid viral rebound whenever HAART was interrupted (4). More promising are novel compounds, such as the T cell cytokine IL-7; the phorbol-ester prostratin, which acts on NF-κB via protein kinase C; inhibitors selective for HDACs, which maintain long terminal repeat (LTR) repression (7); or compounds, such as hexamethylbisacetamide, which may activate HIV transcription in the absence of the trans-activator of transcription, TAT (1).

We recently reported that triggering TLR7/8 causes two opposite effects on HIV replication: on the one hand, it suppresses HIV replication in acute ex vivo-infected lymphoid tissue; on the other
hand, it stimulates the release of HIV virions from latently infected cells (8, 9). TLRs belong to the family of pattern recognition receptors, which are indispensable for fighting microorganisms by recognizing conserved microbial motifs and subsequent complex priming of the innate immune system. The signaling cascades of endosomal TLR3, 7, and 9 have been well studied by means of murine models and murine cell work. In contrast, the signaling cascade of TLR8 is poorly understood. Studies examining TLR8 are scarce (10). Notably, TLR8 triggering does not work in mouse models; in humans, it is expressed selectively on monocytes and myeloid dendritic cells (MDCs) (11), cell types that are difficult to work with. Because TLR7 and 8 share similar properties (i.e., endosomal location and recognition of ssRNA of viruses), their signaling pathways are assumed to be similar. However, they differ in their distinct cell subset-specific expression patterns: TLR7 is expressed on B cells and plasmacytoid dendritic cells (DCs), whereas TLR8 is expressed on monocytes and MDCs (11).

As a result, triggering TLR7 preferentially results in the release of IFN-α, whereas triggering TLR8 results mainly in the release of IL-12p70 and TNF-α (12, 13).

The critical roles of pattern recognition receptors are also appreciated for intragenic manipulation. In particular, they enhance endogenous immune defenses, improve immunogenicity of vaccines, and provide a potential therapeutic target in diseases caused by exaggerated TLR signaling activity (14, 15).

Thus, TLR7/8 triggering is an intriguing therapeutic option for activation of latently HIV-infected cells. However, for a targeted use of TLR7/8 agonists, we need a more detailed understanding of their molecular mechanism(s) resulting in activation of HIV, the involved pathways, and the indirect effects on neighboring latently HIV-infected cells. Thus, the aim of this study was to dissect the effects and the potential of TLR7 and TLR8 triggering for activating HIV from latently infected cells and to elucidate the participating signaling pathways.

Materials and Methods
Specimen collection and isolation of culture of PBMCs
Blood was obtained from six HIV+ patients at the University Hospital of Zurich. Inclusion criterion was successful antiretroviral treatment with HIV RNA ≤50 copies/ml for ≥1 y (CD4+ T cell count: median, 435/μl; range: 180–908/μl). Patients were selected according to good clinical practice. Buffy coats from HIV+ individuals were obtained from the local blood donation center in Zurich. Human PBMCs were isolated by Ficoll (Axis-Shield PoC AS, Norway) gradient centrifugation. Monocytes, isolated by using CD14 microbeads, were cultured in RPMI 1640 medium (BioWhittaker), supplemented with 10% FCS and 2 mM L-glutamine (BioWhittaker), and 1% penicillin/streptomycin (Invitrogen Life Technologies), and 30 U/ml recombinant human IL-2 (BioWhittaker), and 10 U/ml recombinant human IL-12p70 and TNF-α (BioWhittaker), supplemented with 10% FCS and 2 mM L-glutamine (BioWhittaker), supplemented with 10% AB serum and 5% FCS.

Cells and plasmids
U1 cells (16), OM-10 cells (17), J-Lat–Tat–GFP clone A2(35) were cultured in RPMI 1640 (BioWhittaker), supplemented with 10% FCS and 2 mM L-glutamine (BioWhittaker), and 1% penicillin/streptomycin (Invitrogen Life Technologies), and 30 U/ml recombinant human IL-2 (BioWhittaker), and 10 U/ml recombinant human IL-12p70 and TNF-α (BioWhittaker), supplemented with 10% FCS and 2 mM L-glutamine (BioWhittaker), supplemented with 10% AB serum and 5% FCS.

Reagents and cell-culture experiments
The TLR7, 7/8, and 8 agonists, 3M-001, 3M-002, and R-848 reagents, were used at 3 μM, unless stated otherwise. TNF-α (Immunotools) was used at 10 ng/ml. The concentrations used of the following subsequent compounds are indicated in the legends or in the figures: TNF-α neutralizing Ab (nAb) (Remicade; ImClone, Lieden, Netherlands), an agonist of TLR4, came from R&D Systems; and the inhibitor PD98059 (Invivogen), p38 inhibitor SB203580 (Calbiochem), NF-κB inhibitor Bay 11-7082 (Calbiochem), JNK inhibitor 1 (JNKI1; Alexis Biochemicals), NFAT inhibitor cyclosporin (LC Laboratories), and PISK inhibitor LY-294 (Sigma). The cell-culture experiments were performed in triplicate in 96-well round-bottom plates with 1 × 10^5 cells/ml. Cell-culture supernatant was harvested at 2 and 5 d after adding the compound for quantifying the p24 Ag; medium was partially replenished by substituting 50 μl medium containing 3M-002. In the assays examining the impact of TNF-α, nAb against TNF-α was added simultaneously with the TLR8 agonist. For interassay comparisons, we expressed the p24 Ag values over time as the area under the curve.

Quantitative PCR for measuring MyD88, TLR7 and 8, and HIV mRNA
We quantified TLR7 and 8 and MyD88 mRNA using commercially available primers and probes (Assays-on-demand; Applied Biosystems); reactions were performed as described previously (23). For each sample, the mean normalized gene expression was determined with the software application Q-Gene (calculation procedure 2 for mean normalized gene expression) (24).

We quantified HIV unspliced mRNA, according to the method described by Kaiser et al. (25), using the forward primer ts5′′gag (5′-CAAGAGGCT-ATGCAATGTTGAAAAGA-3′) and the reverse primer sskc (5′-TACTAG-TAGTTCTCTCTCTAGTCCTCTC-3′). The TaqMan probe used was m199q (5′-TGACGCTTCCCATGTAGTGTT-3′), as described by Althaus et al. (26). The PCR product mapped to the unspliced region of Gag.

HIV p24 Ag ELISA
A HIV+ patient was used, essentially as described (27).

Western blots
For measuring NF-κB p65, cells were lysed using NE-PER Nuclear and Cytoplasmatic Extraction Reagents (Pierce Biotechnology), according to the manufacturer’s instructions. Equal amounts of nuclear extracts were loaded on a precast 10% polyacrylamide gel (Invitrogen) and resolved by SDS-PAGE. Proteins were transferred onto nitrocellulose membranes (Whatman), and nonspecific sites were blocked with blocking buffer (5% nonfat dry milk and 0.1% Tween 20 in PBS). NF-κB p65 was detected with a monoclonal anti-p65 Ab (Abcam) for 1 h at room temperature. After three washes, membranes were incubated with a goat anti-rabbit secondary Ab conjugated to HRP (Abcam) for 1 h at room temperature. After three washes, membranes were incubated with a goat anti-rabbit secondary Ab conjugated to HRP (Abcam) for 1 h at room temperature. After three washes, membranes were incubated with a goat anti-rabbit secondary Ab conjugated to HRP (Abcam) for 1 h at room temperature. After three washes, membranes were incubated with a goat anti-rabbit secondary Ab conjugated to HRP (Abcam) for 1 h at room temperature.

To assess the effects of TLR8 triggering on the transcriptome in U1 cells, we used the TLR8 triggering array carvicoarray (29). The TLR8 triggering array carvicoarray (29) was used for gene expression analysis using luminometry and a second, independent method. The TLR8 triggering array carvicoarray (29) was used for gene expression analysis using luminometry and a second, independent method.

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TLR signaling pathway PCR array
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Cytokine measurements

Human cytokine protein levels were measured using a multiplexed particle-based flow cytometric cytokine assay (28). Cytokine kits were purchased from BioRad (Ismaning, Germany). The procedures closely followed the manufacturer’s instructions. The analysis was conducted using a conventional flow cytometer (Guava EasyCyte Plus, Millipore, Zug, Switzerland).

Statistics

For statistical analysis, we used the statistical software provided by GraphPad Prism (Version 5). The statistical test used for a given experiment is indicated in the figure legends. We defined a significance level of \( p < 0.05 \) as relevant.

Results

Triggering TLR8 results in activation of HIV from latently infected cells of myeloid-monocytic origin

We previously showed that the TLR7/8 agonist R-848 activates HIV production in latently infected U1 and OM-10 cells (8), which are of myeloid-monocytic origin. In this study, we examined the potential of agonists stimulating TLR7 and/or 8 for the activation of HIV in U1 and OM-10 cells, as well as in latently infected T cell lines. We observed a strong release of HIV particles when U1 cells were exposed to the TLR7/8- or TLR8-specific agonist R-848 and 3M-002, respectively (Fig. 1A). In contrast, the TLR7-specific agonist 3M-001 did not show any effect. OM-10 cells were less responsive to the TLR agonists than were U1 cells. Stimulation by the TLR agonists did not result in any release of HIV from the latently infected T cell lines J1.1 and ACH2, whereas exogenous TNF-\( \alpha \) treatment as a positive control resulted in \( \sim 2.5- \) and 25-fold inductions of HIV production in those cells, respectively. Thus, TLR8 triggering is key for activation of HIV in latently infected cells of myeloid-monocytic origin; it has no direct effect on latently infected T cells.

We did not find any greater frequency of p24 Ag\(^+\) cells when adding 10 \( \mu \)M 3M-002 to U1 cells compared with 3 \( \mu \)M 3M-002 (Fig. 1B). Thus, we preferentially used 3 \( \mu \)M in experiments lasting a couple of days and 10 \( \mu \)M in experiments lasting <1 d.

Cells of myeloid-monocytoid origin constitutively express a high amount of MyD88 but not of TLR7 or 8

To elucidate the distinct responses observed in the various cell lines to the TLR8-specific agonist 3M-002, we examined the TLR8-expression pattern of the cell lines studied. Surprisingly, U1 cells and OM-10 cells expressed little or no TLR7 and 8 mRNA (Fig. 2A). Thus, we investigated whether triggering TLR8 results in its own upregulation. Indeed, we found that exposure of U1 cells to the TLR8 agonist led to upregulation of TLR7, peaking at 6 h later, and TLR8 increasing for \( \geq 40 \) h (Fig. 2B, 2C, left panels); we found similar results when looking at MDMs (E. Schlaepfer and R.F. Speck, manuscript in preparation). The TLR7-specific agonist had no effect on the expression of either TLR (Fig. 2B, 2C, right panels). MyD88, the key adaptor molecule of the TLR7/8 signaling pathway, was very prominent in all cell lines investigated.

Silencing TLR8 or expression of a dominant negative mutant of MyD88 results in loss of TLR8-induced activation of HIV in U1 cells

To prove that the TLR8 agonist acts through the TLR8 signaling-transduction pathway, we generated U1 cell lines genetically complemented with shRNA against TLR8 or U1-DN/MyD88. Successful silencing of TLR8 mRNA by shRNA was confirmed by quantitative PCR (qPCR); no TLR8 mRNA was detected (data not shown). Monoclonal cell lines transduced with shRNA against TLR8 were unresponsive to TLR8 triggering, as assessed by the lack of HIV production (Fig. 3A). Control cells transduced with scrambled shRNA reacted similarly to TLR8 triggering, as did the

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**FIGURE 1.** TLR8 triggering results in activation of HIV from the latently infected U1 cells. A. The latently infected myeloid-monocytic cell lines U1 or OM-10, as well as the latently infected T-cell lines ACH-2 or J1.1, were treated with R-848 (TLR7/8 agonist), 3M-001 (TLR7 agonist), 3M-002 (TLR8 agonist), or TNF-\( \alpha \); control cultures were not treated. Cell culture supernatants were harvested over 1 wk, and virus production was assessed by measuring the p24 Ag concentration in the supernatants. For interassay comparisons, the area under the curve of p24 Ag concentrations over time was calculated. HIV activation is presented as the fold induction of p24 production relative to that in untreated cultures. Data are shown as scatter plots with mean and SEM. B, U1 cells were stimulated for 24 h with 3 or 10 \( \mu \)mol 3M-002; HIV activation was assessed by quantifying the frequency of p24 Ag\(^+\) cells by staining for intracellular p24 and subsequent acquisition on a flow cytometer.
parental U1 cells. Similar to cells with TLR8 knockdown, U1-DN/MyD88 lost their ability to activate HIV (Fig. 3B) or to upregulate TLR response genes subsequent to TLR8 triggering (Supplemental Table I). Thus, 3M-002–mediated activation of HIV occurs through TLR8 and the adaptor molecule MyD88.

**TLR8 triggering in U1 cells results in p38-dependent activation of NF-κB**

TLR triggering, in general, culminates in the activation of NF-κB, which is considered a key transcription factor for HIV activation. As a first step, we investigated whether inhibiting NF-κB results in inhibition of TLR8-mediated induction of HIV. Indeed, adding the NF-κB inhibitor Bay to U1 cells clearly inhibited TLR8-mediated HIV activation, as quantified by the frequency of p24 Ag+ cells (Fig. 4A). We verified the role of NF-κB in TLR8 responses uncoupled of HIV using Western blot (Fig. 4B). We found a clear TLR8-mediated activation of NF-κB p65. Because NF-κB activation also occurs by the MAPK pathway (29) that is otherwise activated by various TLRs, we assessed the TLR8-mediated phosphorylation status of NF-κB, when inhibiting Erk1/2 or p38α. Blocking p38 activation resulted in a lack of TLR8-mediated NF-κB p65 phosphorylation, whereas blocking Erk1/2 had a smaller effect.

**TLR8 triggering in U1 cells leads to phosphorylation of distinct nonreceptor-associated kinases**

The above-mentioned analysis revealed that activation of NF-κB is influenced by the MAPK pathway. We wanted to corroborate the
data obtained with the Erk1/2 and p38 inhibitors by examining the TLR8-mediated phosphorylation of distinct members of the MAPK pathway. Using Western blots, we found phosphorylation of p38 and Erk1/2 in U1 cells after stimulation with the TLR8 agonist 3M-002 (Fig. 5, upper and middle panels, respectively). In contrast, JNK was not phosphorylated in U1 cells subsequent to TLR8 triggering, whereas it was phosphorylated when cells were stimulated for 1 h with the potent JNK activator anisomycin (30).

Activation of HIV in U1 cells is blocked by MAPK-pathway inhibitors

To address the functional significance of the TLR8-mediated activation of the MAPK pathway on activation of HIV in U1 cells, we used various MAPK signaling inhibitors: the MEK1 inhibitor PD98059 (31) prevented significantly TLR8-mediated HIV activation in U1 cells, as assessed by quantifying virus production by p24 Ag ELISA ($p = 0.0006$, unpaired $t$ test) (Fig. 6A). The phosphorylation of Erk1/2 in response to TLR8 and the PD98059-mediated inhibition of TLR8-triggered HIV activation point to the key role of Erk1/2 in the transduction pathway of TLR8. The p38 inhibitor SB203580 partially blocked HIV production in U1 cells (not significant). We did not observe any decrease in TLR8-mediated HIV activation when inhibiting the JNK pathway by the JNKII inhibitor, the transcription factor NFAT by cyclosporin A (CyA), or the PI3K pathway by Ly294; all of these pathways have been shown to be implicated in TLR8 signaling or activation of HIV (Fig. 6A–C). The lack of JNK-mediated inhibition is consistent with the lack of JNK phosphorylation (Fig. 5). None of the signaling inhibitors affected cell viability at the concentrations used, as verified by trypan blue staining (data not shown). Thus, phosphorylation of Erk1/2 and p38 is central to TLR8-mediated activation of HIV in U1 cells, which subsequently activate NF-kB.

The functional activity of various HIV gene products, in particular Vif, is also dependent upon phosphorylation (32, 33). Thus, we examined whether HIV mRNA is affected by the concurrent treatment of PD98059 or SB203580 together with 3M-002. If 3M-002’s effects on HIV replication rely primarily on the activation of TLR8.

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**FIGURE 3.** HIV is not activated in U1 cells silenced for TLR8 or in U1-DN/MyD88 after TLR8 triggering. Various monoclonal cell lines of U1 cells silenced for TLR8 or genetically complemented with scrambled shRNA to TLR8 or parental U1 cells (A) or U1-DN/MyD88 (B) were stimulated with 3M-002 (10 μM), and cell culture supernatants were analyzed for p24 Ag concentrations after 4 d by ELISA. Statistical analysis was done using the nonparametric Kruskal–Wallis test, followed by the Dunn multiple-comparison test (A) and the Mann–Whitney $U$ test (B). The different symbols indicate distinct cell clones; each symbol represents one individual experiment.

**FIGURE 4.** NF-κB inhibition results in loss of TLR8-mediated activation of HIV. A, The NF-κB inhibitor (BAY) was added to U1 cells just before the TLR8 agonist 3M-002, and the effects on HIV activation were analyzed 24 h later by staining for intracellular p24 Ag, followed by flow cytometric analysis. Data were compared using unpaired $t$ test ($n = 4$). B, TLR8 results in activation of NF-κB. In addition, the signaling inhibitors PD98059 and/or SB203580 were added to U1 cells just before the TLR8 agonist 3M-002, and cells were analyzed for NF-κB p65 activation after 30 and 90 min by Western blots. We used β-actin as a loading control. One representative example of three is shown.
To gain insight into the TLR8-dependent modifications, we profiled U1 cells, as well as MDMs, treated with the TLR8 agonist using a TLR signaling pathway PCR array (Supplemental Tables I and II; Gene Expression Omnibus accession numbers GSE26469 and GSE26470 [http://www.ncbi.nlm.nih.gov/geo/]). The TLR8 agonist, 3M-002, resulted in the upregulation of a large number of TLR-dependent genes in U1 cells; as expected, rather all genes, which were upregulated in the parental U1 cells, were substantially downregulated or not modified in U1-DN/MyD88. Similarly to U1 cells, macrophages showed upregulation of a number of TLR-dependent genes; not very surprisingly, the pattern of upregulated genes differed between U1 cells and MDMs, which is consistent with cell-specific responses to TLR8 triggering.

Neutralizing TNF-α results in a decrease in HIV activation from U1 cells

TNF-α is a very potent inducer of HIV transcription, which acts via activation of the transcription factor NF-κB; multiple NF-κB sites are located within the LTR of HIV (34). We wondered to what extent the TLR8-mediated increase in TNF-α contributed to the activation of HIV. Neutralization of TNF-α by infliximab significantly inhibited TLR8-mediated activation of HIV by ∼50% (inhibition was significant at p < 0.05, with the exception of the lowest concentration of infliximab [1 µg/ml] tested; one-way ANOVA, followed by the Bonferroni multiple comparison) (Fig. 6D). Thus, TLR8-mediated activation of HIV is only partially due to the autocrine or paracrine activity of TNF-α.

TLR8-mediated activation of MoMDCs leads to activation and release of latently infected T cells

With the exception of CD4+ T regulatory cells, T cells do not react to TLR8 agonists (21). However, memory CD4+ cells are an important latent reservoir for HIV. Thus, we hypothesized that TLR8-mediated activation of MoMDCs might indirectly activate HIV from latently infected T cells. To address this hypothesis, we used J-Lat cells, which were generated by transduction of Jurkat cells with an HIV vector encoding an LTR-driven GFP. Quiescent J-Lat cells usually express a low level of GFP; however, their activation results in a substantial increase in GFP expression (35). J-Lat cells cocultured with MoMDCs showed only a subtle increase in GFP expression at increasing ratios of MoMDCs/J-Lat cells (i.e., no direct effect of unstimulated MoMDCs on HIV activation) (Fig. 7). However, when we added the TLR8 agonist to this coculture, we saw a marked increase in the percentage of GFP+ J-Lat cells, which was dependent on the number of MoMDCs, indicating that TLR8-mediated activation of MoMDCs results in the activation of HIV from latently infected T cells. Notably, J-Lat cells alone did not react to the addition of the TLR8 agonist 3M-002 (data not shown). We found that this effect was partially dependent upon TNF-α, because its neutralization with infliximab resulted in a substantial reduction in the frequency of GFP+ cells (Fig. 7B). Indeed, MoMDCs secreted a substantial amount of TNF-α in response to the TLR8 agonist 3M-002 (Fig. 7C). Using the signaling inhibitors against Erk1/2 and p38-MAPK, we found that the Erk1/2 pathway is also critically involved in the activation of HIV from latently infected T cells, to a great degree unquestionably through the secretion of TNF-α (Fig. 7B, 7C). To dissect whether the signaling inhibitors acted on MoMDCs and J-Lat cells or only on MoMDCs, we investigated whether they blocked TNF-α-mediated HIV activation from J-Lat cells. However, they had no effect (Fig. 7D), indicating that their effect is limited to blocking the TLR8 pathway in MoMDCs.

In addition, we examined the potential of TLR8 agonists to activate even a small number of latently infected cells by adding...
a decreasing number of J-Lat cells to a constant number of Jurkat cells and MoMDCs. We found that HIV was activated from J-Lat cells when as few as 10 cells were added to 10^5 Jurkat cells and 10^3 MoMDCs (Fig. 7E).

Finally, we examined TLR8 mRNA expression levels in monocytes from HIV+ patients successfully treated with HAART and HIV^- individuals. We found an equal expression of TLR8 (Fig. 7F). Notably, monocytes from HIV+ patients secreted similar amounts of TNF^- as did those from HIV^- individuals (Fig. 7G). Thus, patients successfully treated with HAART showed similar responsiveness to TLR8 agonist as did HIV^- volunteers.

Discussion
In this work, we investigated the molecular mechanisms that result in activation of HIV from latently infected monocytoid-like cell lines subsequent to TLR8-specific triggering. Our two main findings are that TLR8 triggering activates HIV in latently infected cells of myeloid-monocytic origin primarily via the MAPK pathway and that this pathway stimulates secretion of TNF^- which subsequently activates HIV in latently infected CD4 T cells. Thus, TLR8 agonists might be promising compounds for eradicating HIV.

The challenge of eradicating HIV is its silent behavior, with no or only minimal HIV mRNA transcription in the latently infected cells (36). The very small number of latently infected cells renders the study of the molecular aspects of latency with primary cells extremely difficult. Therefore, latently infected cell lines are often used to model latent HIV infection (e.g., the U1 cell line as a representative of latently infected cells of myeloid-monocytic origin) (16). The U1 cell line contains two integrated HIV proviruses, both with mutations in tat that explain the severely impaired viral replication (37).

We previously showed that TLR7/8 triggering results in activation of silent HIV in U1 cells (8). In the current study, we found that U1 cells responded with activation of HIV to TLR7/8 triggering primarily to the TLR8-specific agonist 3M-002, to a lesser degree to the TLR7/8 agonist R-848, and virtually not at all to the TLR7-specific agonist 3M-001. We verified the activity of the compounds in separate experiments with HEK 293 cells cotransfected with plasmids encoding the corresponding TLRs and an NF^-kB–driven reporter gene (data not shown). Similar to U1 cells, OM-10 cells showed TLR7/8–mediated HIV activation; however, it was less pronounced. The OM-10 cell line also contains HIV in...
FIGURE 7. TLR8-triggered MoMDCs produce TNF-α via activation of Erk1/2 and p38α, which, in turn, promotes the activation of HIV in latently infected CD4+ T cell lines. A, MoMDCs were cocultured in various ratios with the latently infected Jurkat cell line, J-Lat A2, and then were stimulated with 3M-002. One day later, the frequency of GFP+ cells was analyzed by flow cytometry. Squares represent cocultures stimulated with 3M-002, and circles represent mock-treated cocultures (n = 5). Supplemental Fig. 2 shows a representative example of how we analyzed these coculture experiments by flow cytometry. B, The signaling inhibitor PD98059 or SB203580 or infliximab was added to cocultures of MoMDCs and J-Lat A2 cells at a ratio of 400:105 just before the TLR8 agonist 3M-002 was added, and cells were analyzed as described in A (n = 3). C, A total of 10^5 MoMDCs/100 μl was stimulated in triplicates for 24 and 48 h (n = 3; each experiment with a different donor). PD98059 and SB203580 were added 30 min prior to the TLR8 agonist 3M-002. D, J-Lat cells were treated with TNF-α alone or in the presence of the MEK1 or p38-MAPK inhibitors and analyzed as described above (n = 3). E, Decreasing numbers of J-Lat cells were added to 10^5 Jurkat cells and 10^3 MoMDCs; 3M-002 was subsequently added to these cocultures, and GFP+ cells were analyzed 1 d later. The dashed line indicates the theoretical number of J-Lat cells present in the triple coculture, and the solid line represents the data measured (n = 3). F, Monocytes from successfully treated HIV+ patients and HIV-2 individuals were analyzed for TLR8 mRNA expression level 1 d after culturing. G, Monocytes from successfully treated patients and from HIV- volunteers were stimulated with 3M-002 at 3 μM overnight; TNF-α was measured by ELISA in the culture supernatants 24 h after stimulation. Statistical analyses of the experiments in B and D were done using repeated-measures ANOVA, followed by the Bonferroni multiple comparison test. The unpaired t test was used for the experiments in G and F.
a silent manner; it was originally derived from acutely HIV-infected cell clones from the promyelocytic HL-60 cell line (17). In contrast, the latently infected T cell lines, ACH-2 and J1.1, did not react to TLR7 and/or 8 agonists. The TLR7/8-expression patterns of those cell lines were puzzling: at baseline, all cell lines showed no or very low levels of mRNA expression of TLR7/8. A time-course analysis of TLRs’ mRNA expression revealed that the TLR8 agonist induced its own receptor, as well as that of TLR7. Thus, the initial upregulation of TLR8 might be critical to generate a TLR8 signal. Cross-regulation and modulation of TLRs by TLR agonists are known mechanisms for fine-tuning inflammatory responses (38). Notably, U1-DN/MyD88 did not respond with any HIV activation or upregulation of TLR-dependent genes (Supplemental Table I) in response to TLR8 triggering. Thus, 3M-002 is selectively triggering the TLR8-MyD88 pathway in U1 cells. The lack of any response to TLR7 agonist might be due to the lack of cofactors critical for TLR7 signaling in our cell lines used; indeed, CD14 was recently shown to act as a coreceptor for TLR7 and 9 (39).

In general, TLR signaling pathways encompass the ligation of a TLR by its specific TLR agonist, the transduction via the adapter protein MyD88, and subsequently, the activation of NF-κB, IRF, or MAPK pathways (40). However, the TLR8 signaling pathway is poorly defined because TLR8 is only expressed in short-lived cells, such as monocytes or DCs, and TLR8 is nonfunctional in mice. Thus, we explored the TLR8 signaling cascade in U1 cells in detail and tested its significance for activation of HIV. Notably, HIV has at least two NF-κB sites in the LTR (41); activation of NF-κB is considered a key factor in driving HIV transcription (42, 43). Thus, we wondered whether the TLR8-mediated activation of HIV operates via NF-κB; indeed, inhibition of NF-κB activation resulted in decreased TLR8-mediated HIV activation. We corroborated the TLR8-mediated activation of NF-κB, uncoupled of HIV infection, using Western blotting and gene-array experiments. U1 cells and MDMs showed a similar upregulation of mRNA expression in response to 3M-002, implying that data obtained in U1 cells can be extrapolated to primary cells.

Various pathways culminate in NF-κB activation; in particular, the MAPK pathway, by activation of Erk1/2 and p38α, may contribute to it (44). The MAPK pathway is also a key transduction pathway for other TLR agonists (40). Thus, we investigated whether inhibiting the MAPK pathway had any effect on NF-κB activation. Indeed, experiments using inhibitors of Erk1/2 and p38α revealed that NF-κB activation was substantially dependent upon activation of p38α and, to a lesser extent, activation of Erk1/2 (Fig. 4B).

In a next step, we verified whether TLR8 triggering results in the direct activation of the MAPK pathway; strikingly, we found that it resulted in phosphorylation of Erk1/2 and p38α but not JNK. The lack of phosphorylation of JNK in our system is surprising, because previous studies reported JNK phosphorylation in CD34-derived DCs stimulated by 3M-002 (10) and in MoMDCs stimulated by R-848 (45). The most likely explanations are the different cellular setup and agonists used. Irrespective of this difference, TLR8 agonists revealed similar transduction pathways as those induced by other TLR agonists (46). As expected based on the aforementioned results, inhibition of Erk1/2 and p38α resulted in complete or partial inhibition of TLR8-mediated HIV activation. Considering that the Erk1/2 inhibitor had less effect on the attenuation of TLR8-mediated NF-κB activation than did the p38α inhibitor, while more prominently inhibiting TLR8-mediated HIV replication, we assumed that other transcription factors critical for HIV replication are affected by inhibition of the Erk1/2 signaling pathway. Consistent with the lack of detectable phosphorylation of JNK after TLR8 triggering, the JNK signaling inhibitor used had no effect. Notably, CyA, which blocks NFAT, had no effect on TLR8-mediated HIV transcription, indicating that this pathway is not involved in the activation of HIV in U1 cells. NFAT is proposed to be a key component for activating HIV transcription in latently infected cells (47).

Although we clearly showed that the inhibitors used substantially attenuated NF-κB activation and, thus, blocked TLR8-mediated HIV activation, they may also act on the phosphorylation of HIV gene products, in particular Vif. Notably, phosphorylation of Vif is important for its activity and, thus, for HIV infectivity (33). The strong reduction of unspliced HIV mRNA, the production of which was induced by TLR8 triggering when adding the Erk1/2 and p38α inhibitors, corroborated our data that the TLR8-mediated activation of the MAPK pathway is essential for HIV activation from latently infected cells. However, we cannot exclude that TLR8-activated kinases are also implicated in HIV activation at later steps in the HIV-replication cycle.

Notably, U1 cells secreted a number of cytokines that promote mainly a Th1 response in reaction to 3M-002. TNF-α, which was secreted within 6 h, mainly activates NF-κB. Blocking TNF-α resulted in partial inhibition of TLR8-mediated activation of HIV. This downstream effect hints at a bimodal TLR8-mediated activation of NF-κB (i.e., initial direct TLR8-mediated activation of NF-κB, followed by secretion of TNF-α, which again leads to the activation of NF-κB). This activation of NF-κB induced by consecutive pathways will promote sustained immune activation (48). The MAPK pathway was also central for TNF-α secretion, because the MEK1 inhibitor or the p38 MAPK inhibitor blocked the TLR8-mediated secretion of TNF-α; this again underscores the key role of the MAPK pathway in the TLR8-mediated response.

We wondered whether TLR8-activated MoMDCs might indirectly lead to activation of latently infected CD4+ T cells and, thus, to a switch from the latent state to a productive infection. Indeed, this was the case; coculture of MoMDCs with J-Lat cells, a Jurkat cell line that was generated by transduction with an HIV vector encoding an LTR-driven GFP, at a ratio of 1:2.5 resulted in activation of HIV. Notably, adding the TLR8 agonist 3M-002 led to HIV activation, even when as few as 10 J-Lat cells were added to 105 uninfected parental Jurkat cells and to 103 MoMDCs. J-Lat cells alone were unresponsive to the TLR8 agonist 3M-002 (data not shown). Because DCs account for only 0.5% of WBCs in the lymphoid tissue and the peripheral blood, TLR8 agonists might be promising compounds for purging the HIV reservoir. We also observed that this effect was substantially attenuated or even completely abrogated when blocking the Erk1/2 or p38α pathway or TNF-α, respectively. Notably, treatment with TNF-α alone resulted in a substantial increase in LTR-driven GFP expression in J-Lat cells; adding the signaling inhibitors in this setting did not affect TNF-α-mediated HIV activation, indicating that their effect in the coculture relies primarily on the blocking of the TLR8 pathway in MoMDCs. Thus, TLR8 acts primarily through activation of the MAPK pathway in MoMDCs, which, in turn, results in the secretion of TNF-α, also promoting the activation of HIV in other latently infected cells.

HIV-associated immune activation might lead to tolerance induction vis-à-vis TLR8 agonists. HIV-associated immune activation may even persist in HAART-treated HIV+ patients with suppressed HIV RNA (49). However, there is no tolerance induction vis-à-vis TLR8 agonists, because we found a similar responsiveness when stimulating PBMCs from HIV-infected patients with suppressed HIV RNA compared with those from HIV− volunteers. Thus, TLR8 agonists may be highly active in treated HIV-infected patients.

Although our in vitro data suggested that TLR8 agonists are promising compounds for activating HIV from the latent reservoir, sustained triggering of TLR8 might be detrimental for the lymphoid
system (50). Conceptually, we believe that compounds, and, in particular, TLR8 agonists, acting on the latent reservoir should be given in cycles, because a longer-term administration of any such compound might be too toxic. Furthermore, activation of the latent reservoir must be accompanied by HAART for preventing spreading infection; otherwise, the latent reservoir will be replenished immediately.

Notably, other TLR agonists, such as TLR2, 4, and 9 agonists, may be beneficial for activation of latently infected cells (51–53). Combination of TLR agonists that target distinct cellular lymphoid subsets, based on their TLR-expression pattern, might even be superior for eradicating latently infected cells to one TLR agonist alone. Although we focused our work on a potential latent HIV reservoir in cells of myelo-monoctyic origin, their significance as a latent HIV reservoir remains controversial. Notably, HIV DNA is clearly detected in monocytes; however, they remain in circulation for only up to 3 d, raising the question of whether precursor cells are at the origin of latently infected monocytes (2, 54, 55). An argument that speaks in favor of true latency in macrophages is the in vivo HIV reactivation of infected macrophages in response to opportunistic infections (1, 56). Because clinical specimens from tissue are difficult to obtain, and the cell substrate that we used might not have recapitulated the in vivo behavior of resident tissue cells (57), the exact role for various cell subsets of the myeloid-monoctyic lineage as an HIV reservoir remain unanswered. In any case, cells of the myeloid-monoctyic lineage contribute to the persistent HIV reservoir in patients treated successfully with HAART, thus, the concept that we are proposing may be adopted to purging HIV, irrespective of true latency or low-level replication. All cell subsets of myelo-monoctyic origin, including monocytes, DCs, and microglial cells, as well as CD34+ cells, show a strong reaction in response to TLR7/8 agonists.

In summary, our data showed that TLR8 triggering culminated in the activation of NF-κB, resulting in the transcription of HIV in latently infected cells of myelo-monoctyic origin, and it induced the secretion of TNF-α, which activates latent HIV in CD4+ T cells. Thus, TLR8 agonists, in combination with HAART, are intriguing compounds for purging HIV from its latent reservoirs and sanctuary sites.

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Disclosures

The authors have no financial conflicts of interest.

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