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TLR7/8 Triggering Exerts Opposing Effects in Acute versus Latent HIV Infection

Erika Schlaepfer,* Annette Audigé,* Helene Joller,† and Roberto F. Speck2*

TLRs trigger innate immunity by recognizing conserved motifs of microorganisms. Recently, ssRNAs from HIV and influenza virus were shown to trigger TLR7 and 8. Thus, we hypothesized that HIV ssRNA, by triggering TLR7/8, affects HIV pathogenesis. Indeed, HIV ssRNA rendered human lymphoid tissue of tonsillar origin or PBMC barely permissive to HIV replication. The synthetic compound R-848, which also triggers TLR7/8, showed similar anti-HIV activity. Loss of R-848's activity in lymphoid tissue depleted of B cells suggested a role for B cells in innate immunity. TLR7/8 triggering appears to exert antiviral effects through soluble factors: conditioned medium reduced HIV replication in indicator cells. Although a number of cytokines and chemokines were increased upon adding R-848 to lymphoid tissue, blocking those cytokines/chemokines (i.e., IFN-γ, MIP-1α, -1β, RANTES, and stromal cell-derived factor-1) did not result in the reversal of R-848's anti-HIV activity. Thus, the nature of this soluble factor(s) remains unknown. Unlike lymphoid tissue acutely infected with HIV, triggering latently infected promonocytic cells induced the release of HIV virions. The anti-HIV effects of triggering TLR7/8 may inhibit rapid killing, while pro-HIV effects may guarantee a certain replication level. Compounds triggering TLR7/8 may be attractive drug candidates to purge latent HIV while preventing new infections. The Journal of Immunology, 2006, 176: 2888–2895.

Materials and Methods

Cells, cell lines, and reagents

PBMC from random donors were isolated by Ficoll–Hypaque density gradient centrifugation (Nycormed) and cultured at 10 × 10^6 cells/ml in RPMI 1640 (BioWhittaker) with 10% (by volume) FCS (PAA Laboratories), 100 U/ml penicillin and 100 μg/ml streptomycin (P/S; Invitrogen Life Technologies), 2 mM glutamine (Invitrogen Life Technologies), and 10 μU/ml IL-2 (National Institutes of Health AIDS Repository). For generating monocyte-derived macrophages (MDM), we isolated monocytes using CD14 microbeads (Miltenyi Biotec). Subsequently, 5 × 10^6 monocytes/well of a 96-well plate were kept in RPMI 1640 (BE 12-702F; BioWhittaker) with 5% FCS, 5% human AB serum (H1513, Sigma-Aldrich), 1% glutamine, and 1% penicillin-streptomycin for 1 wk until differentiation into MDM. A2.01 cells expressing CD4 (A2.01/CD4^+) were cultivated in the same medium as PBMC. U1 and OM10, for investigating the effects of TLR7/8 signaling on latent HIV infection.

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Biologie de Lille, Université E0364 1, Lille, France)) at 100 μg/ml, peptide glycan from Streptococcus aureus (Fluka) at 10 μg/ml and LPS (Escherichia coli K-235; Sigma-Aldrich) at 20 μg/ml. Sequences of phosphothioate-modified ssRNA (i.e., ssRNA40, 41, and 42) were identical to those published by Heil et al. (6); in particular the RNA40 is a 20 mer identical to the US region from 108 to 127; RNA41 and 42 are 20 mer where all U or G nucleotides were replaced with adenosine, respectively (IBA). For complexing ssRNA, we used the cationic lipid N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium methylsulfate (DOTAP) (Roche) as described by Heil et al. (6). Neutralizing Abs(nAbs) against the Nlleoxygen)propyl]-N,N,N-trimethylammonium methylsulfate (DOTAP) (Roche) as neutralizing Abs(nAbs) against the N

Measurement of metabolic activity

The WST-1 kit (Roche Diagnostics) was used according to the manufacturer's instructions.

Lymphoid tissue

Tonsils from otherwise healthy adult patients were obtained from the Department of the Ear, Nose, and Throat Surgery at the University Hospital of Zurich (Zurich, Switzerland) within 5 h after tonsillectomy. The use of tonsils was approved by the local ethical committee of the USZ. HLAC were prepared by transferring minced tissue into a cell strainer (70 μm; Falcon; BD Biosciences) and grinding the tissue through the sieve with a syringe plunger. Erythrocytes were lysed with ACK cell-lysing buffer (BioWhittaker). Lymphoid cells were cultured at a concentration of 10^7 cells/ml in RPMI 1640 containing 15% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml fungizone, 2 mM l-glutamine, 1 mM sodium pyruvate, and 1% nonessential amino acids. Viability was assessed by trypan blue exclusion.

Purification of cellular subsets from HLAC and PBMC

We depleted specific cellular subsets from PBMC with Abs coupled to MACS beads, according to the manufacturer's instructions (CD14^+; CD19^+; and BDCA-4 Microbeads; Miltenyi Biotech).

Viruses

Viral stocks were obtained by calcium phosphate-mediated transfection (Promega) of 293T cells with pNL4-3, pYU-2, pJR-CSF, and p89.6 (National Institutes of Health AIDS Repository) or p49.5. Virus was harvested 48 h after transfection, filtered (0.22 μm), and frozen at –80°C.

HIV p24 capsid Ag (p24) ELISA

A twin-site sandwich ELISA was performed essentially as described (15). Briefly, a polyclonal Ab was adsorbed to a solid phase to capture p24 Ag from a detergent lysate of virions. Bound p24 was visualized with an alkaline phosphatase-conjugated anti-p24 mAb and luminescent detection system. A twin-site sandwich ELISA was performed essentially as described (15). Briefly, a polyclonal Ab was adsorbed to a solid phase to capture p24 Ag from a detergent lysate of virions. Bound p24 was visualized with an alkaline phosphatase-conjugated anti-p24 mAb and luminescent detection system.

Infectivity assays

All infectivity assays with HLAC or PBMC were performed in triplicate in 96-well round-bottom plates. In most assays, R-848 was added 2 days before HIV infection and was partially replenished by substituting 50 μl of medium containing 1 μg/ml R-848 twice a week over the observation period. Cultures were exposed to HIV with p24 Ag in the range of 1–3 ng/sample for 4–6 h. Subsequently, the cultures were washed three times and resuspended in 200 μl of fresh medium containing R-848. In a subset of experiments, R-848 was added at either 2 days before HIV infection, immediately upon infection, or 4 days after infection. Supernatants were tested for p24. In the assays examining the impact of selective cytokines, NaB against either IFN-α, γ, MIP-1α, -β, RANTES, or SDF-1 was added simultaneously with R-848 and partially replenished twice a week. For interdonor comparisons, we expressed the p24 values over time as the area under the curve (AUC) representing HIV replication. We calculated the percent inhibition of replication by any given drug and compared it to the percent expressing the AUC of treated cultures as a percentage of an untreated, infected control culture and then subtracting the percent of AUCcontrol samples from 100% (16).

In the infectivity assays examining R-848’s ability to stimulate the B cell line, Namalwa, and its ability to affect HIV replication when subsequently cocultured with T cells, Namalwa cells at a density of 5 × 10^6 cells/well of a 96-well plate were pretreated with R-848 for 2 days. Subsequently, the same number of A2.01/CD4^+ cells was added to the B cells. Cocultures were infected with NL4-3 for 6 h and thereafter washed three times; HIV replication was quantified by p24 Ag in the supernatant at day 5.

Cytokine ELISA

IL-6, -8, -12, TNF-α, RANTES, MIP-1α, and -β were quantified by enzyme immunoassays (R&D Systems), according to the manufacturer’s instructions. IFN-α and -γ were quantified by enzyme immunoassays from Bender MedSystems Diagnostics and HyCult Biotechnology, respectively.

Immunostaining and flow cytometry

Cells were simultaneously stained with mAbs (BD Biosciences) against the cell surface markers CD4-phycocerythrin, CD8-FITC, or CD4^+; CD8^−; and CXCR4-Cy or CCR5-allophycocyanin together. For staining B cells, we used the mAb CD20. For staining PDC, we used the lineage mixture 1, CD123-PE, HLA-DR-PerCP and CD1c-allophycocyanin. Flow cytometry was performed on a FACScalibur (BD Biosciences), and data were analyzed with CellQuest or FlowJo software (Tree Star).

Cell-based fusion assay

We used a cell-based fusion assay with HeLa cells expressing gp140 from LAI and the HIV transactivator tat (HeLa-Env/LAI) (17) and HeLa cells expressing CD4 and the LTR-driven lacZ gene (HeLa SX CCR5) (18). Fusion of HeLa-Env/LAI cells and HeLa SX CCR5 results in transcription of the lacZ gene. The extent of fusion was quantified either by assaying β-galactosidase activity in cell lysates (E2000; Promega) or by histochemical staining of cells for β-galactosidase activity (Roche Molecular Biochemicals), according to the manufacturer’s instructions. HeLa-Env/LAI cells were treated with R-848 at 1 μg/ml or the fusion inhibitor T20 at 50 μg/ml for 1 h (19). Subsequently, a similar number of HeLa SX CCR5 cells were added to allow fusion to take place. β-Galactosidase activity was assessed 12 h later.

Pseudotype virus preparation and challenge of cells with pseudotype viruses

To prepare HIV pseudotype virus packaged by vesicular stomatitis virus (VSV) Env, an HIV proviral construct encoding a luciferase reporter gene (pNL4-3.lacZ Env, R5’) was cotransfected with a VSV Env expression vector in 293T cells as described (20). To determine permissiveness to entry by pseudotype viruses, HLAC were pretreated with R-848 for 2 days and exposed to pseudotype virus for 6 h. For increased sensitivity of the read-out, in six of eight experiments, PHA (Sigma-Aldrich) at 5 μg/ml was added 1 day after infection. PHA acts by immune activation of cells (21) and thereby cooperates with Tat-driven LTR activity resulting in the increased expression of the reporter gene. Luciferase expression was quantified with the luciferase assay system from Promega.

Quantitative PCR (qPCR) for measuring TLR7 and 8 mRNA in various cellular subsets

qPCR was performed as described (22). In brief, for measuring TLR7 and 8 mRNA, we used commercially available primers and probes (Assays-on-demand; Applied Biosystems). Hydroxymethyl bilane synthase (Gen-Bank X04217) was used as a housekeeping gene and designed as a 3’ minus groove binder probe (23). Data generated by real-time qPCR were analyzed by determining the mean normalized gene expression for every sample with the software application Q-Gene (calculation procedure 2 for mean normalized gene expression) (24).

Results

Triggering TLR7/8 blocks HIV replication in acutely infected HLAC and PBMC

Untreated tonsillar HLAC and IL-2-cultured PBMC uniformly showed vigorous viral replication when exposed to HIV as quantified by p24 Ag in the supernatant (Fig. 1A). Pretreatment of PBMC or HLAC with 10 μg/ml HIV ssRNA 40 complexed to DOTAP or 1 μg/ml R-848 for 1–2 days rendered the cell cultures virtually nonpermissive to HIV (Fig. 1, B and C). ssRNA not complexed or the control ssRNAs 41 or 42 had no antiviral activity (Fig. 1B and data not shown). To compare interindividual experiments, we calculated the percent inhibition of HIV replication over time as compared with untreated HIV-infected cultures. We observed a decrease of HIV replication of >75%, irrespective of HIV coreceptor selectivity (i.e., infection with the primary CCR5-tropic (R5) isolate JR-CSF, the dual-tropic strain 89.6, or the laboratory strains 49.5 (R5) and the CXCR4-tropic (X4) NL4-3).
Anti-HIV activity is specific for TLR7/8 triggering and is not matched by triggering other TLR

We wondered whether triggering other TLR would also block HIV replication in HLAC. Peptidoglycan (TLR2), poly(I:C) (TLR3), LPS (TLR4), and flagellin (TLR5) had only modest anti-HIV activities when HLAC were exposed to the R5-tropic strain 49.5 (Fig. 1D). Poly(I:C) and flagellin also inhibited the X4-tropic strain. However, peptidoglycan and LPS showed no effect. Thus, the robust anti-HIV activity is specific for TLR7/8 and not a uniform phenomenon of TLR triggering.

TLR7/8 triggering causes no adverse metabolic effects

To exclude a cytopathic effect of TLR7/8 triggering that could explain the observed anti-HIV activity, we examined the effects of

FIGURE 1. Robust anti-HIV activity is observed upon triggering of TLR7/8 by HIV ssRNA or R-848. A, Human HLAC or PBMC show robust viral replication, irrespective of HIV's coreceptor selectivity (upper panel, PMBC infected with the CCR5-tropic strain 49.5; lower panel, HLAC infected with the CXCR4-tropic strain NL4-3); C, untreated; D, R-848-treated lymphoid tissue). B, Human PBMC were treated with ssRNA40, -41, or -42 complexed to the cationic lipid DOTAP and subsequently infected with NL4-3 (n = 3). HIV replication was monitored twice weekly by quantifying p24 in the supernatant. C, HLAC of tonsillar origin (■) or PBMC (□) were treated with R-848 and subsequently infected with HIV strains with distinct coreceptor selectivity (HLAC infected with 49.5 (n = 12), NL4-3 (n = 15), JR-CSF (n = 2), or 89.6 (n = 2); PBMC infected with 49.5 (n = 5) or NL4-3 (n = 4). 49.5 and JR-CSF are CCR5-tropic, NL4-3 is CXCR4-tropic and 89.6 dual-tropic). D, HLAC were treated with peptidoglycan (TLR2), poly(I:C) (TLR3), LPS (TLR4), or flagellin (TLR5), and subsequently infected with 49.5 or NL4-3 (n = 6 for poly(I:C), n = 2 for peptidoglycan, LPS and flagellin). B–D, Data are represented as HIV replication over time after R-848 treatment as related to untreated and infected control culture. Data are presented as mean ± SD.

FIGURE 2. B cells are key players in the anti-HIV effect after TLR7/8 triggering. A and B, PBMC were either depleted of CD19⁺ B cells (A), BDCA4⁺ PDC (B), or monocytes (C) and were subsequently infected with NL4-3 (Wilcoxon signed rank test (Prism4)). D, R-848 was tested for its anti-HIV activity in the T cell line A2.01/CD4⁺ (for simplicity reasons in the graph termed A2.01) alone or when cocultured with the B cell line Namalwa (Friedman test followed by paired t test).
Hydrogenases in viable cells. On day 7, no differences in metabolic activity were observed between cultures either treated with R-848 or not (mean ± SEM of untreated vs R-848-treated, respectively, 0.27 ± 0.04 and 0.28 ± 0.07, n = 6; NS, Wilcoxon signed rank test).

**B cells are key to the anti-HIV activity of TLR7/8 triggering**

We wondered what cellular population from primary lymphoid tissue mediates the TLR7/8-dependent anti-HIV effects. For this purpose, we selectively depleted B cells, monocytes, or PDC from PBMC. As measured by flow cytometry, the numbers of these cells were reduced by >95%. Depleting PDC or monocytes did not reverse the observed anti-HIV effects; however, depleting B cells did (Fig. 2, A–C).

Anti-HIV activity was also observed in cocultures of B cell and T cell lines. HIV replication was strikingly reduced when the B cell line, Namalwa, was pretreated with R-848, cocultured with the T cell line A2.01/CD4<sup>+</sup>, and subsequently infected with NL4-3 (Fig. 2D).

**Soluble factors may mediate the anti-HIV activity of triggering TLR7/8**

To explore whether soluble factors were responsible for the anti-HIV effects observed, HeLa CD4<sup>-</sup>/R5 cells were infected with the R5-tropic HIV strain 49.5 and subsequently treated with R-848- conditioned medium from HLAC; indeed, this conditioned medium inhibited HIV replication substantially (Fig. 3A). R-848 added directly to HeLa-CD4/R5 cells after infection had no antiviral activity.

**Triggering TLR7/8 stimulates diverse cytokines**

To determine the effects of triggering TLR7/8 on the cellular microenvironment, the cytokine expression patterns in HLAC were examined at days 1 and 2 after R-848 treatment (Table I). The cytokine profile changed substantially: increases were noted in the levels of secretion of the proinflammatory cytokines IL-6 and TNF-α, of the innate immunity marker IFN-α, and of Th1 cytokines IFN-γ and IL-12. Levels of the chemotactic cytokines RANTES and MIP-1α and -1β were also increased.

**R-848’s anti-HIV activity is neither inhibited by neutralizing Abs against the IFN-α receptor nor against IFN-γ, MIP-1α, MIP-1β, RANTES, or SDF-1**

To explore whether the anti-HIV effects by triggering TLR7/8 is due to the release of IFN-α, IFN-γ, MIP-1α, -1β, RANTES, or SDF-1, we added nAb against the IFN-α receptor or those cytokines/chemokines. Blocking of the IFN-α receptor site or the cytokines/chemokines did not reverse the effects subsequent to

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**Table I. Analysis of cytokines after triggering TLR7/8 in HLAC**

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>At Day 1 After Triggering TLR7/8</th>
<th>At Day 2 After Triggering TLR7/8</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>After triggering TLR7/8</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>191; 81; 53</td>
<td>306; 75; 79</td>
</tr>
<tr>
<td>IL-6</td>
<td>3,637; 2,834; 1,595</td>
<td>7,298; 7,168; 3,994</td>
</tr>
<tr>
<td>IL-8</td>
<td>14,813; 14,127; 15,245</td>
<td>13,599; 13,515; 9,427</td>
</tr>
<tr>
<td>IL-12</td>
<td>2; 0.1&lt;sup&gt;a&lt;/sup&gt;; 3.6</td>
<td>23; 0.1&lt;sup&gt;b&lt;/sup&gt;; 11</td>
</tr>
<tr>
<td>TNF-α</td>
<td>74; 49; 7</td>
<td>226; 106; 67</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>131; 52; 37</td>
<td>221; 135; 128</td>
</tr>
<tr>
<td>MIP-1β</td>
<td>275; 85; 25</td>
<td>479; 286; 257</td>
</tr>
<tr>
<td>RANTES</td>
<td>134; 32; 14</td>
<td>646; 186; 146</td>
</tr>
</tbody>
</table>

<sup>a</sup> The numbers correspond to the three experiments performed to quantify various cytokines in response to TLR7/8 triggering (picograms per milliliter).

<sup>b</sup> Lower limit of detection is 0.1 pg/ml; thus, for calculating fold induction, we used the lower detection limit whenever cytokines were not detectable.
TLR7/8 triggering (Fig. 3B). As expected, blocking IFN-α by adding nAb against its receptor, without triggering TLR7/8 simultaneously, resulted in increased HIV replication; a similar but less prominent stimulation of HIV replication was observed when nAb against RANTES was added (Fig. 3C).

**Triggering TLR7/8 blocks HIV after entry and before integration**

We next sought to identify the step at which TLR7/8 triggering interferes with the HIV replication cycle. First, we investigated the expression level of the viral receptor complex of the cell surface molecules CD4 and either CCR5 or CXCR4. Untreated cultures contained 2.7 ± 0.8% of CD4⁺/CCR5⁺ cells, while R-848-treated cultures had 0.7 ± 0.4% of CD4⁺/CCR5⁺ cells (mean ± STD; n = 4). The corresponding values for CXCR4 are 93.1 ± 2.7% CD4⁺/CXCR4⁺ cells in untreated, and 91 ± 3.1% CD4⁺/ CXCR4⁺ cells in R-848-treated HLAC.

Next, we looked at the fusion of HIV and the cell membrane with a cell-cell fusion-based assay. As determined either histochemically or by measuring β-galactosidase activity in cell lysates, R-848 did not affect syncytium formation (Fig. 4, A and B). The fusion inhibitor Fuzeon was used as a positive control for blocking syncytium formation.

Replication-deficient reporter viruses encoding luciferase and pseudotyped with the VSV envelope (Env) permitted us to assess the postentry events of HIV. Cultures treated with R-848 displayed substantially fewer light units than untreated cultures (Fig. 4). Variability in these experiments is inherent in working with primary tissue; in particular, primary tissue may differ in activation status and host-genetic factors.

**Dose- and time-dependent inhibition of HIV replication**

In most experiments, R-848 levels were maintained in the culture medium throughout the entire observation period. However, we wondered whether R-848’s anti-HIV effect relies on an altered microenvironment or direct viral inhibition. To answer this question, we compared the results of adding R-848 at different time points. Adding R-848 only before or after exposing HLAC to HIV as well as continuous treatment inhibited viral replication (Fig. 5A). In contrast, R-848 added 4 days after HIV infection tended to be somewhat less effective. Our results indicate that an altered microenvironment is critical for the anti-HIV effects observed.

Based on published work (5, 13, 14), we usually tested cultures with R-848 at 1 µg/ml to investigate its effects on HIV replication. Titrating R-848 from 10–0.01 µg/ml revealed a clear dose dependency: R-848 gradually lost its anti-HIV activity at concentrations < 0.1 µg/ml (Fig. 5B).

**FIGURE 4.** TLR7/8 triggering interferes with HIV replication after virus-cell fusion but before integration. A and B, To assess whether R-848 affects virus entry, including fusion, HeLa-ENV/LAI cells expressing tat and HIV envelope from the HIV strain LAI (HeLa 243) were treated with R-848 or Fuzeon for 1 h and subsequently mixed with HeLa cells expressing CD4 and lacZ under the control of HIV-LTR (HeLa SX CCR5). Fusion between these two cell lines was assessed by (A) histochemical staining and (B) lysing the cells and quantifying β-galactosidase activity. Fuzeon served as a positive control. One representative experiment is shown (n = 2). C, TLR7/8 triggering by R-848 interferes with postentry steps of the HIV replication cycle. VSV-pseudotyped HIV viruses with a luciferase reporter gene were used to study the impact of R-848 in a “one replication round” assay on HIV replication in HLAC. The lines indicate that the samples are from the same tonsil (n = 10; Wilcoxon signed rank test).

**FIGURE 5.** Time- and dose-dependent suppression of HIV replication of triggering TLR7/8 by R-848. A, R-848 was maintained at a constant level throughout the entire experiment (all time) or given only before, immediately after, or 4 days after infection with the CXCR4-tropic strain NL4-3 (n = 5). Experiments with the R5-tropic strain 49.5 yielded similar results (data not shown). B, Tonsillar HLAC were treated with different concentrations of R-848 before infection with 49.5 (▪; n = 3) or NL4-3 (■; n = 3). R-848 levels were maintained throughout the culture. At 10 µg/ml, R-848 appears to have cytopathic effects, and thus we did not further investigate the effect of R-848 at this concentration.
TLR7/8 triggering culminates in the activation of NF-κB (6). Because the HIV LTR contains NF-κB transcription sites, activation of NF-κB results in increased LTR-dependent transcription and increased HIV replication (25). Thus, we wondered whether TLR7/8 triggering reactivates latent HIV infection. We examined this question in the promonocytic latently infected cell lines U1 and OM10 (26–29). Both cell lines express TLR7 and 8 as quantified by qPCR (Fig. 6A). Primary cells of myeloid-monocytic origin such as monocytes and MDM have even higher levels of TLR7/8 mRNA levels (Fig. 6A).

In both cell lines, R-848 induced the production of substantial amounts of virus as measured by either p24 in the supernatant or staining for intracellular p24 over time (Fig. 6, B–E). No HIV induction was observed when R-848 was added to the latently infected T cell line ACH-2. ACH-2 cells show virtually no expression of TLR7 and no expression of TLR8; therefore, the lack of induction is not surprising and provides evidence for R-848’s selective signaling through these receptors. Thus, in contrast to the indirect anti-HIV activity when triggering TLR7/8 in lymphoid tissue, the pro-HIV activity observed is a direct consequence of triggering latently infected cells.

**Discussion**

In this work, we explored the effects of triggering TLR7/8 on HIV replication. We found that triggering TLR7/8 by ssRNA or R-848 greatly reduces the ability of lymphoid tissue to support HIV infection. We also discovered an unexpected role for B cells in this antiviral defense. The site of “anti-HIV” action seems to be after fusion but before DNA integration into the host genome. Finally, we showed that triggering TLR7/8 directly activates latent virus in monocyted-like cells and may enable other drugs to be more effective in eradicating HIV from patients.

HIV ssRNA rendered human primary lymphoid tissue barely permissive to HIV replication. The guanosine (G)- and uridine (U)-rich ssRNA oligonucleotides, ssRNA40, derived from HIV (6), which induced the release of cytokines, was the most potent ssRNA. In contrast, the ssRNA41 and ssRNA42, in which all U’s or G’s were replaced with adenosine, were not or were only marginally anti-HIV. Consistent with published work, only ssRNA complexed to cationic lipids, which facilitate its cellular uptake, was anti-HIV active (6).

R-848 also triggers TLR7/8, and for practical reasons, we used it instead of ssRNA in the subsequent experiments (6). R-848 reduced HIV replication in a manner similar to HIV ssRNA. The antiviral state observed was independent of the origin of the lymphoid tissue or coreceptor preference of the virus. We have previously demonstrated that triggering TLR9 was also anti-HIV active (30). In contrast, we found that other TLR ligands, such as peptidoglycan (TLR2), poly(I:C) (TLR3), LPS (TLR4), or flagellin (TLR5), had only modest anti-HIV effects at best. Thus, triggering TLR7/8 or 9 unfavorably changes the microenvironment for HIV replication; the failure to induce an antiviral state after stimulation with other TLR ligands points to specific anti-HIV effects by triggering TLR7/8 or 9 and rules out a common biologic property of TLR triggering.

We wondered what cellular subset was responsible for the antiviral state induced. PDC are the most potent IFN-α-producing cells (31), and IFN-α has strong antiviral activity (32). However, depleting PDC from lymphoid tissue did not reverse the anti-HIV effects observed. Similarly, depleting monocytes did not change the anti-HIV activity of TLR7/8 triggering. In contrast, depleting B cells resulted in significant loss of anti-HIV activity. The critical
role of B cells was corroborated by data showing that treatment of the B cell line, Namalwa, with R-848 prevented HIV replication in CD4+ T cell lines. These data unequivocally indicate that B cells have an unexpected role in the innate immunity for constraining viral infection. The variability in these experiments may be explained by differences in genetic host factors. Alternatively, we have to consider that the induction of antiviral effects observed requires the cooperative action of more than one cell type which shows distinct responsiveness to TLR7/8 triggering. For example, a two-step recognition mechanism by TLR involving epithelial cells and DC is critical for the induction of effector T cells in a mouse model infected by HSV (33). Certainly, additional studies of this intriguing observation are warranted.

The antiviral activity is, at least partially, due to soluble factors. R-848-conditioned medium from HLAC decreased HIV replication in indicator cells and induced an array of cytokines. Some of these cytokines, such as IFN-α, have antiviral activity. Others, such as TNF-α and IL-6, stimulate HIV activity (22, 34). Thus, it is highly unlikely that one antiviral cytokine is responsible for these effects. Indeed, we previously showed that rIFN-α only modestly affected HIV replication in this experimental setting and, thus, was insufficient to explain the striking effects of TLR7/8 triggering (30). Moreover, nAb against the IFN-α receptor or IFN-γ, MIP-1α, -β, RANTES, or SDF-1 did not reverse the observed anti-HIV effect. Therefore, the sum of cytokine changes or a yet unidentified factor is likely responsible for the potent anti-HIV activity observed.

Triggering TLR7/8 blocks HIV infection after entry. In particular, TLR7/8 triggering resulted in a consistent reduction of the expression of HIV coreceptors CCR5 and CXCR4 on CD4+ T cells. Because the basal expression level of CCR5 is low, its relative reduction was more pronounced than for CXCR4. Chemokine receptor expression level, and in particular expression of CCR5, is a determinant of HIV infectability (35). To further explore the block in HIV replication, we used VSV Env-pseudotyped HIV strains encoding a luciferase-reporter gene. VSV Env-pseudotyped viruses circumvent the HIV entry step. We found that luciferase expression was decreased when HLAC were pretreated with R-848. Thus, while reduced CCR5 expression may contribute to R-848’s anti-HIV activity, the experiment using VSV-pseudotype viruses clearly demonstrate that TLR7/8 triggering interferes with HIV replication after virus-cell fusion but before viral integration into the host genome. We excluded a nonspecific blocking of the fusion process by R-848 as a mechanism for the antiviral state using a cell-cell fusion-based assay.

To more carefully examine R-848’s potency, we titrated it by its anti-HIV activity. Although there was a dose-dependent loss of R-848’s anti-HIV activity, a marked decrease in its activity was only observed when it was used at <0.1 μg/ml. The lowest concentration at which R-848 displays its anti-HIV activity is similar to the concentration at which it induces the release of cytokines (36). In animal models, 0.1 μg/ml R-848 protected against established infection with Herpes simplex (37) and enhanced clearance of Mycobacterium bovis (38). Thus, taking into account the wide therapeutic activity it displays in vivo against HIV, R-848 likely displays anti-HIV effects in vivo at similar concentrations.

R-848 added before HIV infection was only slightly less active against HIV than when it was added throughout the entire culture period. Thus, triggering TLR7/8 seems to induce an antiviral state that remains even after limited exposure of tissue to R-848. In contrast, triggering TLR7/8 with R-848 4 days after HIV infection resulted in only modest anti-HIV effects. The subsequent observation period may be too short to appreciate the full biologic effects of TLR7/8 activity, or key cells responding to R-848 may have lost their reactivity over time in this in vitro setting. Alternatively, we speculate that triggering TLR7/8 of HIV-infected cells of the myeloid-macrophage lineage activates HIV and thus masks the anti-HIV effects.

To further explore the effects on viral latency, we used the latently infected cell lines U1 and OM10. These cell lines express TLR7 and to a lesser extent TLR8. Triggering TLR7/8 resulted in a dramatic increase of HIV replication. Monocytes and MDM have a higher expression of TLR7 and 8 than the U1 or OM-10 cells; thus, it is highly likely that TLR7/8 triggering of latently infected primary cells of monocyte-macrophage origin will result in the increased release of HIV in vivo. Thus, TLR7/8 triggering may have a dichotomous effect in HIV infection: it may prevent infection of CD4+ T cells while it activates HIV replication in cells of the myeloid-macrophage lineage. Although at first seeming contradictory, these two opposite activities may be beneficial to HIV. They may block overwhelming infection and destruction of the host while simultaneously guaranteeing continuous HIV replication from cells of the myeloid-macrophage lineage. Notably, cells of the myeloid-macrophage lineage are less susceptible to the direct cytopathic effects of HIV.

In conclusion, TLR7/8 triggering inhibits HIV replication in unfragmented lymphoid tissue. In contrast, it induces the release of HIV virions in latently infected cells. These surprisingly different actions may play a critical role in acute and chronic HIV infections. In acute HIV infection, the antiviral state induced by TLR7/8 may restrict overwhelming HIV replication until the generation of an adaptive immune response. In chronic HIV infection, the anti-HIV effect of triggering TLR7/8 may still restrict overwhelming replication, whereas the pro-HIV effect guarantees a certain level of replication that does not kill the host too rapidly. Thus, HIV is able to exploit TLR7/8 triggering for its own purposes. However, the dichotomous effects of TLR7/8 signaling may be of therapeutic value. It may be possible to purge latently infected cells while preventing new infections (39). Notably, TLR signaling links the innate and adaptive immune response (40). Thus, similar to protocols investigating GM-CSF (41), we propose to exploit immune response modifiers targeting TLR7/8 in HIV-infected patients (42).
References


