Anti-HIV Activity Mediated by Natural Killer and CD8+ Cells after Toll-Like Receptor 7/8 Triggering

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Abstract

We previously found that triggering TLR7/8 either by single stranded HIV RNA or synthetic compounds induced changes in the lymphoid microenvironment unfavorable to HIV. In this study, we used selective TLR7 and 8 agonists to dissect their contribution to the anti-HIV effects. While triggering TLR7 inhibited efficiently HIV replication in lymphoid suspension cells from tonsillar origin, its effect was inconsistent in peripheral blood mononuclear cells (PBMC). In contrast, triggering TLR8 showed a very prominent and overall very consistent effect in PBMC and tonsillar lymphoid suspension cells. Depletion of dendritic cells (DC), natural killer cells (NK) and CD8+ T-cells from PBMC resulted in the reversal of TLR8 induced anti-HIV effects. Especially noteworthy, depletion of either NK or CD8+ T-cells alone was only partially effective. We interpret these findings that DC are the initiator of complex changes in the microenvironment that culminates in the anti-HIV active NK and CD8+ effector cells. The near lack of NK and the low number of CD8+ T-cells in tonsillar lymphoid suspension cells may explain the lower TLR8 agonist's anti-HIV effects in that tissue. However, additional cell-type specific differences must exist since the TLR7 agonists had a very strong inhibitory effect in tonsillar lymphoid suspension cells. Separation of effector from the CD4+ target cells did not abolish the anti-HIV effects pointing to the critical role of soluble factors. Triggering TLR7 or 8 were accompanied by major changes in the cytokine milieu; however, it appeared that not a single soluble factor could be assigned for the potent effects. These results delineate the complex effects of triggering TLR7/8 for an efficient antiviral defense. While the ultimate mechanism(s) remains unknown, the potent effects described may have therapeutic value for treating chronic viral diseases. Notably, HIV replication is blocked by TLR triggering before HIV integrates into the host chromosome which would prevent the establishment or maintenance of the latent reservoir.

Introduction

Pattern recognition receptors act as sentinels against microorganisms [1]. They recognize conserved motifs of microorganisms and trigger distinct signaling pathways that result in coordinated cellular changes critical to the immediate innate defense and the generation of adaptive immune response.

Toll-like receptors (TLR) are an essential family of pattern recognition receptors [1]. They can be grouped by their preferences for structural motifs. TLR3, 7, 8, and 9 are implicated in anti-viral defense [1,2]. TLR3 recognizes double-stranded viral RNA [3], TLR7 and 8 recognize single-stranded viral RNA [4,5] and TLR9 unmethylated DNA rich in cytosine-guanosine motifs from bacteria and viruses [6]. TLR3 is expressed on myeloid dendritic cells (MDC), TLR7 and 9 on plasmacytoid dendritic cells (PDC), and TLR8 on MDC and monocytes [7,8]. While the transduction pathways of individual TLRs are well described [9], the overall effects of TLR triggering on the lympho-reticular system and on viral diseases remain largely unknown.

Compounds triggering distinct TLR may be of great value for targeted immunomodulation of viral diseases, including HIV [10]. The complex changes from triggering TLR may be more beneficial than administration of single cytokines. In fact, human studies examining treatments with distinct cytokines have rather been discouraging [11]. A detailed mechanistic examination is certainly required for the targeted use of compounds triggering TLR.

We previously reported that the TLR7/8 agonist R-848 or HIV ssRNA has immediate anti-HIV activities [12]. Furthermore, TLR7/8 agonists which were given as vaccine adjuvants in concert with HIV proteins were able to impact on the magnitude and quality of anti-HIV CD8+ T-cell responses [13,14]. Thus, triggering TLR7/8 stimulates the innate as well as the adaptive immune response; this unique property makes them, at least theoretically, superior to cytokines, which are conventionally given as single agent and either act on the innate or the adaptive arm of the immune system. Thus, triggering TLR7/8 may represent an ideal therapeutic strategy for immunomodulation stimulating multiple arms of the immune response.

In the previous work, we examined the synthetic compound R-848, which triggers TLR7 and 8. Since TLR7 is preferentially expressed on PDC [8], the major producer of IFN-α, TLR7 triggering is believed to be a key factor in the overall anti-viral response. However, IFN-α did not have a major role in the anti-HIV activity that we observed since its neutralization did not reverse R-848’s anti-HIV effect. To differentiate the individual contributions of triggering TLR7 and 8 to the anti-HIV activity requires agonists that specifically and efficiently trigger the
corresponding TLR. Because TLR8 does not function in mice [15], this pathway has been less well studied, and its biologic significance is largely unknown.

In the present study, we used agonists that specifically trigger each pathway to characterize the individual contributions of TLR7 and 8 signaling to the overall anti-HIV effect. Our findings suggest novel strategies for anti-HIV therapies.

**Results**

**Anti-HIV activity of triggering TLR7 and/or 8 depends on the origin of lymphoid tissue**

We first determined the optimal working concentration of the TLR7 and/or 8 agonists; briefly, these agonists were added to tonsillar lymphoid suspensions cells in a range of 0.1–30 μmol; the tissue was infected two days later. HIV replication was monitored by measuring viral capsid p24 antigen (p24 Ag) in the supernatant. We also assessed the dose–dependent effects on cell viability of these TLR agonists. We found that the optimal working concentration was 3 μmol (Fig. 1A–C) which had a strong inhibitory effect on HIV replication while it had no negative effect on cell viability.

The HIV strains, NL4-3 (X4-tropic) and 49.5 (R5-tropic) we used replicates vigorously in untreated tonsillar lymphoid suspension cells and PBMC [12]. In tissue-matched tonsillar lymphoid suspension samples, triggering TLR7 or 7/8 resulted in substantial inhibition of NL4-3 replication, i.e., 3M-001: 64% (50.2 to 77.2) (median inhibition (25% to 75 percentile); R-848: 73.5% (70.2 to 81.5)) (Fig. 1 D). Although less effective, triggering TLR8 still yielded an inhibition with a median of 46.9% (40.8 to 56.2). In contrast, in PBMC, triggering TLR8 resulted in substantially higher anti-HIV activity than triggering TLR7 (median inhibition 3M-001: 2.5% (~54 to 60.2) vs 79.5% (63.3 to 89)) (Fig. 1E). Not unexpected, the TLR7/8 agonist R-848 again had high levels of anti-HIV activity (median inhibition: 79.1% (87.1 to 69.3)). In other experiments, we verified that the changes in the microenvironment induced by TLR7 or 8 agonists had similar anti-HIV effects on a number of HIV strains with distinct co-receptor selectivity (anti-HIV activity subsequent to 3M-001 in tonsillar lymphoid suspension cells infected with JR-CSF: 96.67% (96.42 to 96.72), 49.5: 93.5% (95.4 to 97.34), 89.6: 81% (75.6 to 86.4); anti-HIV activity subsequent to 3M-002: JR-CSF: 94.9% (94.7 to 95.2), 49.5: 91.4% (93.6 to 95.9), 89.6: 65% (73.7 to 82.5); n = 2; 49.5 and JR-CSF are R5-tropic strains, 89.6 is a dual-tropic strain).

The origin of the lymphoid tissue may be important for the distinct biologic effects observed. Indeed the cellular composition differed significantly between PBMC and tonsillar lymphoid suspension cells (Table 1). Most prominent was the nearly complete absence of NK and monocytes in tonsillar lymphoid suspension cell cultures. The percentage of CD4+, CD8+ and B-cells displayed also marked differences depending on the origin of the tissue. In contrast, the fraction of PDC and MDC was similar in PBMC and tonsillar lymphoid suspension cells.

**DC are critical for the anti-HIV effects observed**

TLR7 and 8 show cell-specific expression with TLR7 expressed on B-cells and PDC, and TLR8 on monocytes and MDC [7,8]. Based on their sentinel role and the expression of TLR8, we assumed that MDC are the most likely target cells responsible for the dramatic HIV inhibitory effects. Indeed when DC and precursor DC were depleted from PBMC, HIV replication increased 12-fold and 27-fold, respectively.

We observed striking differences of cytokine released in the media between unfractonated PBMC and PBMC lacking DC indicating the successful depletion of DC (Table 2). The striking differences observed is explained by the lack of DC which produce cytokines [16] as well as by the lack of DC which will activate down-stream effector cells such as NK cells and CD8+ T-cells [17,18]. Furthermore, there were differences in the cytokine release in response to the distinct TLR7 and/or 7/8 agonists tested. These results reflects the subset specific ability of DC to respond to TLR agonists [8] and underscores the importance of DC within the complex network of innate immunity. In line with data with R-848 [19], the potency of pure TLR8 agonists to activate monocyte-derived myeloid dendritic cells (MoMDC) was illustrated by their characteristic histological changes (Fig. 2B) and the marked increase of the maturation/activation markers, CD90, 83 and 86 one day after exposure to the TLR8 agonist 3M-002 (Fig. 2C–E). MoMDC which have been activated by TLR7 and/or 8 agonists show substantial induction of a large number of cytokines (Table 3).

**NK and CD8 T cells are the main effector cells for the anti-HIV activity after triggering TLR7 and/or 8**

We wanted to assess the role of CD8+ T-cells and NK cells for their significance in the TLR7/8’s HIV inhibitory activity. For this purpose, we depleted these cell subsets by using microbeads, added the TLR7 and/or 8 agonists and infected the cells two days later. We found that depleting CD8+ T-cells reversed the TLR8 and/or 7/8’s anti-HIV effects minimally, that depleting NK cells were more efficient in that sense, but strikingly depleting both simultaneously resulted in a nearly complete reversal of the anti-HIV effects (Fig. 3).

TLR8 and/or 7/8 resulted in activation of CD8+ T-cells and NK cells, as assessed by expression of the activation marker CD69, by intracellular staining for IFN-γ and TNF-α, by measuring CD107a, and by their cytotoxic activity against the NK-responsive cell line K562 (Fig. 4). CD107a is a granular protein and is exposed at the cellular surface during degranulation of cytokines and of cytotoxic proteins such as perforin and granzyme [20]. Staining for CD107, thus, quantifies the cumulative cytolytic activity of a distinct cell population.

To characterize in depth the NK phenotype leading to the anti-HIV activity, we quantified a large number of NK-specific cell surface markers known for as activating or inhibitory receptors by flow-cytometry. In particular, we hypothesized that TLR7/8 triggering will favor NK to express activating and less inhibitory receptors. However, the expression of the activating NK receptor, NKp2D, was significantly lower subsequent to the exposure of PBMC to R-848 than in the control (control vs R-848: median (minimum–maximum) % of cells expressing NKp2D 2.9 (2.4–3.4) vs 1.42 (1.04–1.50), n = 5, p = 0.007, paired T-test). The levels of expression of other inhibitory (CD94, KIR2DL2, KIR3DL1, ILT2) and activating receptors (NKp30, NKp46, NKp44, DNAM-1) on NK cells were similar irrespective of triggering TLR7/8 (data not shown).

In contrast, triggering TLR7 and/or 7/8 strikingly up-regulated TRAIL (control vs 3M-001: 3M-002: R-848: median (minimum–maximum) % of cells expressing TRAIL: 7.2 (3.8–14.2) vs 54 (34.3–75.2), 65 (44–73.3), 64 (37.9–79.4); ANOVA P = 0.003, P<0.05, <0.01 and <0.01, respectively).

**Soluble factors mediate anti-HIV effects after triggering of TLR7 and/or 8**

When effector cells were separated from target cells by transwells, HIV replication was efficiently inhibited. This finding clearly indicates that soluble factors have a potent anti-HIV effect (Fig. 5).
Figure 1. Triggering TLR7 and/or 8 results in strong anti-HIV activity that varied with the origin of human lymphatic tissue. Dose-dependent blocking of HIV by the compounds tested (■ = 3M-001, ▲ = 3M-002, ● = R-848) when exposing tonsillar lymphoid suspension cells to (A) the R5-tropic strain 49.5 (n = 2) or (B) the X4-tropic strain NL4-3 (n = 6 for 3M-001 and 3M-002 and for data points 3, 10, 30 μm, otherwise n = 2). (C) No toxic effects were noted in lymphoid tissue at 3 μm of any of the TLR agonists, as measured by the WST-1 assay (n = 6). Anti-HIV activities (D) in tonsillar lymphoid suspension cells and (E) in PBMC. Data obtained with R-848 were partially published before [12]. Data were first analyzed by one-way analysis of variance and if tested significantly different, by Bonferroni’s multiple comparison test.

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Levels of a large number of cytokines and chemokines were increased after triggering TLR7 and/or 8 (Table 4). Most impressively, IFN-α increased more than 100-fold after triggering either TLR7 and/or 8. The TLR agonists 3M-002 and R-848, which trigger TLR8, caused a more pronounced release of IL-12 and TNF-α than 3M-001. Furthermore, we observed moderate increases of the β-chemokines. Some of those cytokines are known to have anti-HIV activities.

To assess the significance of the individual cytokines on HIV replication, we have used commercially available neutralizing antibodies to block these cytokines, and followed mainly the providers’ instructions in regard to the concentration; in any case we used always concentrations at the upper limit feasible (see material and method section). We have formal proof that the neutralization of IFN-α and TNF-α was effective: the efficacy of the IFN-α antibody is shown by the fact that adding this antibody resulted in higher HIV replication in control cultures (Fig. 5B). The TNF-α blocking antibody showed complete suppression of recombinant TNF-α in a transient-transfection assay using a NFκB reporter assay (data not shown). Furthermore, there was a tendency that blocking IL-12, -15 and -18 was also effective in the reversal of TLR7-mediated anti-HIV effects. In any case, neutralization of individual cytokines or of combinations of diverse cytokines was not sufficient to reverse the anti-HIV effects after triggering TLR8 or 7/8 (Table 5). In contrast, the inhibitory activity in cultures treated with the TLR7 agonist was partially reversed when adding nAb against the IFN-α, pointing to the known anti-viral role IFN-α has in many settings. To conclude, the enigma concerning the soluble factor responsible for the potent TLR7/8 agonists’ anti-HIV effect remains.

HIV replication is blocked after entry but before integration

For simplicity, HIV replication can be divided into entry, post-entry, and post-integration steps. Using a cell-cell-based fusion

Table 1. Differential cell count in PBMC and tonsillar lymphoid suspension cells in % (avg±std; n = 4).

|                | CD4+ cells | CD8+ cells | CD19+ cells (B-cells) | CD14+ cells (monocytes) | CD56+ cells (NK) | MDC | PDC |
|----------------|------------|------------|-----------------------|------------------------|------------------|-----|-----|
| PBMC           | 37.2±7.5   | 19.0±7.5   | 13.3±4.4              | 6.3±2.4                | 10.6±3.0         | 0.5±0.4 | 0.8±0.7 |
| Tonsillar Lymphoid suspension cells | 19.5±1.5 | 3.8±1.1 | 67.0±7.2 | 0.6±0.27 | 0.3±0.07 | 0.5±0.09 | 1.0±0.58 |

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Figure 2. DC are critical in the generation of the microenvironment unfavorable for HIV replication. (A) Depletion of DC and DC precursors resulted in reversal of HIV inhibition (n = 2) despite triggering TLR8 and/or 7/8. Represented is the fold increase of HIV replication in PBMC depleted of DC as compared to matched samples. (B) The TLR agonists 3M-002 and R-848 which trigger TLR8 and 7/8 respectively resulted in clumping and activation of MoMDC. This was not the case with 3M-001, which triggers solely TLR7. (C–E) Triggering TLR8 or 7/8 resulted in marked activation of MoMDC as quantified by the cell-surface expression of CD80, CD83, and CD86 (n = 4). Data were first analyzed by one-way analysis of variance and if tested significantly different, by Bonferroni’s multiple comparison test.

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In an assay where one cell line expresses CD4 and CCR5, and another cell line expresses CCR5-tropic gp120, we have not observed any non-specific blocking of HIV fusion from the compounds examined (Fig. 6A). Notably, HeLa cells do not express TLR7 or 8.

Expression level of the HIV receptor complex is required for HIV to enter a cell. Indeed, we found that triggering TLR8 and/or TLR7/8 resulted in a decrease in the percentage of CD4+ cells expressing CCR5 while the percentage of CD4+ cells expressing CXCR4 was unchanged (Fig. 6B). The median intensity of CCR5 was not affected by triggering TLR7 and/or 8 (data not shown). The decrease of CCR5 may be explained by the increased release of β-chemokines. However, X4-tropic HIV strains were inhibited to the same extent as R5-tropic strains. Thus, other anti-HIV active mechanisms must be blocking HIV replication. Additionally, we used a lentiviral based reporter virus pseudotyped with the envelope from vesicular stomatitis virus. This virus uses an entirely different entry mechanism than HIV and was inhibited to the same extent as HIV when the tissue was treated with compounds triggering either TLR7 and/or TLR7/8 (Fig. 6D).

We further wanted to substantiate the above-mentioned findings using an experimental set-up that examines whether the TLR7/8 agonists induced effects inhibits HIV replication at the level of transcription or later in the HIV replication cycle. For this purpose, we infected isolated CD4+ T-cells with HIV using spinoculation [21] and added 12 h later the fusion inhibitor enfurvitide which prevents spreading infection. Spinoculation was used to increase the number of HIV infected CD4+ T-cells which

Table 2. Measurement of cytokines (pg/ml) one day after triggering TLR7 and/or 8 in unfractionated PBMC and PBMC without DC (n = 2).

| Cytokine | Condition | Control | 3M-001 | 3M-002 | R-848 |
|----------|-----------|---------|--------|--------|-------|
| IFN-α    | Unfractionated PBMC | <8; 9.6 | 3624; 9590 | 1057; 499 | 1989; 4250 |
|          | PBMC without DC       | <8; <8  | 8; 53.8 | 9.6; 17.2 | 9.6; 37.2 |
| IL-12 (p40) | Unfractionated PBMC | 32; 19  | 1564; 1168 | 13608; 5777 | 12784; 5591 |
|          | PBMC without DC       | 101; 190 | 146; 56 | 185; 264 | 129; 190 |
| IL-15    | Unfractionated PBMC   | <0.1; 1 | 0.59; 0.46 | 0.42; 0.21 | 0.52; 0.3 |
|          | PBMC without DC       | <0.1; 1 | 0.1; 1 | 0.1; 1 | 0.1; 1 |
| IL-18    | Unfractionated PBMC   | 37; 16  | 46; 24 | 615; 590 | 253; 333 |
|          | PBMC without DC       | <0.1; 1 | 1; 1.8 | 6.1; 24.1 | 6.1; 15.4 |
| TNF-α    | Unfractionated PBMC   | <0.9; 0.9 | 56; 101 | 37659; 33474 | 40332; 20478 |
|          | PBMC without DC       | <0.1; 1.3 | 1; 1.8 | 6.1; 24.1 | 6.1; 15.4 |

All data presented in this table were measured by bead assay as described in the Material and Method section.
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Table 3. Induction of cytokines (pg/ml) two days after triggering TLR7 and/or 8 in MoMDC; indicated are the average±std fold induction# and the individual absolute concentrations of cytokines measured in the supernatant [pg/ml] †.

| Cytokine | Condition | Control | 3M-001 | 3M-002 | R-848 |
|----------|-----------|---------|--------|--------|-------|
| IFN-γ    | 0, 0.2, 0 | 0, 0.2, 0.4 | 0.4, 0.8, 0 | 0.2, 0 | 0 |
| IL-6     | 139, 150, 163, 100 | 1.1±0.3 | 168±97 | 163±291 |
| IL-8     | 33558, 2708, 1117, 2199 | 1.1±0.7 | 73±73 | 15±13 |
| IL-12    | 63, 299, 61, 85 | 1.8±1.5 | 1.5±1.1 | 1.5±0.8 |
| TNF-α    | 4.6, 0.2, 3.4, 10 | 4.0±3.3 | 511±913 | 99±124 |
| MIP-1α   | 45, 12, 45, 12 | 1.1±0.8 | 91±86 | 9.0±12 |
| MIP-1β   | 609, 9.2, 49, 72 | 1.0±0.5 | 14±8.0 | 7.8±7.1 |
| RANTES   | 8, 2.2, 8, 2.2 | 1.1±0.5 | 33.1±28.4 | 14.26±16.62 |

# induction can not be calculated since denominator is 0.
†: All data presented in this table were measured by enzyme immunoassay as described in the Material and Method section.
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will increase the sensitivity of the read out. Thereafter, we co-cultured these infected CD4+ T-cells with autologous PBMC devoid of CD4+ T-cells which had been previously exposed to the TLR7/8 agonists. In this setup, the resulting HIV production was solely due to the ongoing HIV transcription of the CD4+ T-cells being already infected. If the changes in the microenvironment induced had any effect on HIV replication after integration, we would see a clear decrease of resulting HIV replication in samples treated with the TLR agonists. However, this was not the case (Fig. 6E), indicating that they display their inhibitory activity prior to integration of HIV into the host chromosome.

Discussion

We previously showed that triggering TLR7/8 causes changes to the lymphoid microenvironment that are unfavorable for HIV replication [12]. Here we sought to identify the individual contributions of TLR7 and 8 to the overall anti-HIV activity and to gain insights into the mechanisms of how TLR trigger antiviral defenses. We found that (i) the TLR7- or 8-mediated anti-HIV activity depended on the origin of the lymphoid tissue, (ii) dendritic cells are indispensable for the anti-HIV effect, (iii) the main effector cells for the anti-HIV activity were NK-cells and CD8+ T-cells, and iv) soluble factors mediated the anti-HIV effects and were not reversed when the antiviral active cytokines IFN-α, -γ, or TNF-α were neutralized.

The TLR7/8 agonist R-848 shows strong anti-HIV activity in PBMC and lymphoid suspension cells from tonsils [12]. In this study, we used two agonists which selectively trigger either TLR7 or 8 [22]. Their potency depended strongly on the origin of lymphoid tissue. Triggering TLR7 was primarily responsible for the anti-HIV activity in tonsillar lymphoid suspension cells, but it had only limited and inconsistent effects in PBMC. In contrast, triggering TLR8 had strong anti-HIV activity in PBMC; it had also consistent but somewhat less anti-HIV activity in tonsillar lymphoid suspension cells. Because of limited availability of tonsillar tissue, we focused in most experiments on PBMC for elucidating the anti-HIV mechanism(s) of TLR7 and/or 8 triggering.

Based on the sentinel function and the expression of TLR8, we speculated that MDC initiate the complex changes in PBMC that are eventually unfavorable for HIV replication. Indeed, when we depleted DC and DC precursors from PBMC, the anti-HIV effect from TLR7/8 triggering was mostly lost. Not surprisingly, depletion of these cell subsets from untreated cultures resulted in modest increases of HIV replication, pointing to HIV’s ability to trigger TLR directly [3,23,24] and thus to initiate an anti-viral defense program, although less potently than the TLR agonists examined. The potent antiviral defense program initiated by TLR8 or 7/8 was also reflected when exposing MoMDC to the TLR agonists: they showed cell clumping, up-regulation of the cell surface marker CD80, CD83 and CD86 as well as pronounced secretion of a number of cytokines. Triggering solely TLR7 had no visible effect on MoMDC.
Previously, we reported that depletion of B cells partially reversed the anti-HIV activity of R-848 [12]. Strikingly, when we depleted NK and CD8⁺ T-cells prior to adding the TLR8 or 7/8 agonists to PBMC, we observed a rather complete reversal of the anti-HIV effects; depleting either NK or CD8⁺ T-cells showed only partial inhibition, suggesting that there is an additive effect when stimulating NK and CD8⁺ T-cells or there is an eminent cross-talk between these cell-populations necessary for this striking anti-HIV effect. Triggering TLR7 in PBMC depleted from NK and CD8⁺ T-cells showed inconsistent effects which reflect its overall inconsistent anti-HIV effects. Since NK and CD8⁺ T-cells are the key effector cells for the TLR8-mediated HIV inhibitory activity, their low or even absent numbers in tonsillar lymphoid suspension cultures may explain the lower TLR8 agonist’s anti-HIV effects in that tissue as compared to PBMC. The robust HIV inhibitory activity in tonsillar lymphoid tissue and the rather poor one in PBMC in response to TLR7 triggering speak in favor of additional tissue type specific mechanism(s) beyond NK and CD8⁺ T-cells which may explain the differences between TLR7 and TLR8 agonists. Notably, while we have not assessed functional differences, the percentages of DC are similar in the lymphoid tissue studied.

The potent cell activation subsequent to triggering TLR8 or TLR7/8 is reflected by an up-regulation of intracellular IFN-γ and TNF-α and increased cytolytic activity of NK and CD8⁺ T-cells. Surprisingly, levels of expression of inhibitory (CD94, KIR2DL2, KIR3DL1, ILT2) and activating receptors (NKp30, NKp46, NKp44, DNAM-1) on NK cells were similar between the controls and PBMC treated with TLR agonists (data not shown). Since NK and CD8⁺ T-cells do not respond directly to TLR7/8 agonists [25], their activation has to occur either by cell-cell contact with a TLR7/8-responsive cell or by soluble factors. The most likely candidates are IL-12, -15, and -18, which are released from DC upon their activation. However, blocking the individual cytokines or all of them with neutralizing antibodies did not reverse the anti-HIV activity and neither prevented the cellular activation of NK and CD8⁺ T-cells that is consistent with a recently published study [26]. Thus, we assume that cell-cell contact and/or a variety of soluble redundant factors will ultimately result in the activation of these effector cells. Indeed, the significance of bi-directional interactions between DC and NK cells has been convincingly demonstrated for an efficient immune defense [17,18].

![Figure 4. Triggering TLR8 or 7/8 results in strong activation of NK and CD8⁺ T-cells.](https://doi:10.1371/journal.pone.0001999.g004)
So how do NK and CD8+ T-cells block HIV infection? To answer this question, we used trans-well experiments, in which HIV-infected CD4+ T-cells were separated from PBMC devoid of CD4+ T-cells. We found that separating the effector from the target cells did not abrogate the anti-HIV effects, thus implicating soluble factors in the process. Indeed, triggering TLR7 and/or 8 resulted in prominent changes in the cytokine profile. However, we were not able to assign the anti-HIV effect to one distinct

Figure 5. Soluble factors are critical to the anti-HIV effects after triggering TLR8 and 7/8. (A) Isolated CD4+ T-cells of PBMC were infected with HIV overnight. Transwells were added with the purpose to separate the HIV infected CD4+ T-cells by a semi-permeable membrane (indicated by the dashed line) from autologous uninfected PBMC devoid of CD4+ T-cells. TLR agonists were added to the culture medium as indicated in the figure. Infected PBMC where the TLR agonists were added to the insert served as positive control for the TLRs’ efficient antiviral activity. Notably, CD4+ T-cells do not express TLR7 and 8 and thus do not react to the TLR agonists added. In this setup any inhibitory HIV activity observed when HIV infected CD4+ T-cells were separated by a semi-permeable membrane from the PBMC (devoid of CD4+ T-cells) must be due to factors secreted by the PBMC into the medium, which then diffuse through the semi-permeable membrane (n = 3). This setup with separated cells was compared to PBMC which have been treated identically. Control indicates PBMC infected with HIV. B) Blocking the IFN-α/β receptor (IFN-α/β R) does not reverse the TLR7/8 triggering mediated inhibition of HIV. Neutralizing antibodies to IFN-α/β R was added at 10 μg/ml 30 minutes prior to R-848 to PBMC which was infected two days later with HIV. All values were indicated as relative to the matched control which was arbitrarily set to 1.

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Table 4. Induction of cytokines one day after triggering TLR7 and/or 8 in PBMC; indicated in each box upper line are the average ± std fold induction # and lower line the corresponding absolute concentrations of cytokines measured in the supernatant [pg/ml] (n = 3) ¶.

|                | control | 3M-001 | 3M-002 | R-848  |
|----------------|---------|--------|--------|--------|
| IFN-α ¶        | 46, 0.1, 0.1* | 1520±1931# | 427±738# | 1544±1680# |
| IFN-γ ¶        | 1000, 370, 84† | 64, 128, 0.1 | 562, 334, 128 |
| IL-6 ¶         | 191, 81, 53 | 0.8±0.2 | 1.6±1.0 | 1.3±0.4 |
| MIP-1α ¶       | 185, 50, 41 | 507, 50, 79 | 306, 75, 79 |
| MIP-1β ¶       | 3638, 2834, 1555 | 2.7±0.6 | 1.7±0.2 | 2.4±0.3 |
| RANTES ¶       | 3638, 2834, 1555 | 7534, 7960, 5174 | 5731, 5464, 2301 | 7298, 7168, 3994 |
| IL-8 ¶         | 14813, 14127, 15245 | 1.0±0.2 | 0.9±0.1 | 0.8±0.2 |
| MIP-1α ¶       | 17587, 15029, 11342 | 15529, 12319, 11717 | 13599, 13515, 9427 |
| MIP-1β ¶       | 185, 50, 41 | 507, 50, 79 | 306, 75, 79 |
| RANTES ¶       | 3638, 2834, 1555 | 7534, 7960, 5174 | 5731, 5464, 2301 | 7298, 7168, 3994 |
| IL-12 (p40) *  | 131, 52, 37 | 3.0±1.7 | 1.8±0.4 | 2.6±0.9 |
| TNF-α †        | 275, 84, 25 | 5.9±6.2 | 3.0±1.9 | 5.1±4.5 |
| RANTES ¶       | 344, 299, 327 | 394, 208, 129 | 479, 286,257 |
| IL-12 (p40) *  | 134, 32, 14 | 7.6±4.1 | 3.0±0.3 | 7.0±3.0 |
| TNF-α †        | 685, 938, 529 | 4.6±2.1 | 43.6±15.0 | 21.9±9.0 |
| RANTES ¶       | 685, 938, 529 | 3331, 1803, 3069 | 1710, 943, 1366 |
| IL-12 (p40) *  | 1122, 1449, 1357 | 1122, 1449, 1357 | 30779, 21380, 22470 | 17560, 14698, 8372 |

* ¶ IFN-α, IFN-γ, IL-6, IL-8, MIP-1α, MIP-1β, RANTES were measured by enzyme immunoassays, IL-12(p40) and TNF-α were measured with a bead assay (see material and method section).

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cytokine even though we neutralized a large number of soluble factors either alone or in combination. The lack of any reversal of the antiviral activity subsequent to triggering TLR8 or TLR7/8 when IFN-α was neutralized is most puzzling and speaks in favor of very potent unknown factor(s) inhibiting HIV. In contrast, TRAIL showed only minor changes of activating and/or inhibiting receptors (see above) and thus, we do not assume that they play a major role. In contrast, TRAIL showed a prominent up-regulation on NK cells. This increased TRAIL expression is reminiscent of a recent report describing the TLR7 triggering dependent generation of killer PDC expressing TRAIL, able to induce apoptosis of HIV-infected SupT1 cells [27]. The potential effects of TRAIL may be masked by the potent inhibitory anti-HIV effects of soluble factors in our experimental set-up that may explain the lack of any reversal of anti-HIV activity when neutralizing TRAIL (data not shown). The potent HIV-inhibition by soluble factors is reminiscent of the long-sought CD8+ T-cell antiviral factor, CAF [28].

TLR7/8 triggering appears to inhibit HIV before its integration into the host-genome. Downregulation of CCR5 after release of β-chemokines may contribute to the anti-HIV activity when challenging the lymphoid tissue with CCR5-tropic strains. However, other anti-HIV mechanism(s) must be involved. Downregulation of CCR5 after triggering TLR7. We assume that TLR7 triggering activates DC which in turn releases IL-12, -15 and -18 critical for activating downstream effector cells such as NK or CD8+ T-cells.

Table 5. Neutralization of cytokines had no impact on anti-HIV activity subsequent to triggering TLR8 and/or 7/8; indicated are the percent inhibition of anti-HIV activity (median (25 to 75% percentile).

| nAb to | 3M-001 | 3M-002 | R-848 |
|--------|--------|--------|-------|
|        | +      | +      | +     |
| IFN-γ; (n = 4) | 26.52 | 5.195 | 87.28 | 89.8 | 81.9 | 76.39 |
|         | (13.92 to 55.05) | (−33.89 to 43.42) | (80.8 to 92.15) | (66.68 to 93.46) | (73.03 to 90.22) | (69.78 to 86.03) |
| IFN-αR; (n = 6) | 14.08 | 67.4# | 83.75 | 90.42 | 91.15 |
|         | (−5.85 to 48.7) | (11.54 to 82.79) | (68.43 to 91.81) | (77.7 to 97) | (69.49 to 91.81) | (76.16 to 95.41) |
| IFN-αR/IL-12; (n = 3) | 7.16 | 3.43 | 89.65 | 68.86 | 94.63 | 80.24 |
|        | (−18.66 to 12.42) | (−17.64 to 14.28) | (77.85 to 93.29) | (−17.63 to 96.64) | (93.46 to 98.47) | (51.99 to 96.38) |
| IL-12; (n = 6) | 39.22 | 59.58 | 79.85 | 84.6 | 78.02 | 82.77 |
|        | (−35.8 to 65.03) | (12.13 to 71.96) | (66.53 to 88.95) | (72.83 to 92.96) | (74.74 to 93.71) | (79.58 to 94.72) |
| IL-15; (n = 19) | 57.3 | 59.17 | 77.1 | 80.5 | 77.3 | 78.4 |
|        | (−0.7 to 62.3) | (−74.5 to 71.8) | (70.3 to 81.8) | (67.7 to 85.2) | (70 to 83.9) | (69 to 83) |
| IL-12/IL-15/IL-18; (n = 6) | 39.22 | 63.46 | 78.95 | 81.14 | 78.02 | 81.16 |
|        | (−35.8 to 65.03) | (21.24 to 76.5) | (66.53 to 88.95) | (67.77 to 93.14) | (74.74 to 93.71) | (75.65 to 94.72) |
| IL-12/IL-15; (n = 6) | 39.22 | 66.94 | 78.95 | 87.51 | 78.02 | 86.27 |
|        | (−35.8 to 65.03) | (7.645 to 80.42) | (66.53 to 88.95) | (80.7 to 94.21) | (74.74 to 93.71) | (80.19 to 95.8) |
| IL-12/IL-18; (n = 6) | 39.22 | 63.58 | 78.95 | 77.92 | 78.02 | 79.87 |
|        | (−35.8 to 65.03) | (17.71 to 73.26) | (66.53 to 88.95) | (54.59 to 92.16) | (74.74 to 93.71) | (69.7 to 93.25) |
| IL-15/IL-18; (n = 6) | 39.22 | 59.17 | 78.95 | 77.4 | 78.02 | 72.14 |
|        | (−35.8 to 65.03) | (2.365 to 66.42) | (66.53 to 88.95) | (51.38 to 92.02) | (74.74 to 93.71) | (52.17 to 91.98) |
| IL-18; (n = 6) | 39.22 | 64.95 | 78.95 | 80.56 | 78.02 | 78.81 |
|        | (−35.8 to 65.03) | (2.75 to 79.42) | (66.53 to 88.95) | (63.29 to 93.67) | (74.74 to 93.71) | (66.42 to 94.34) |
| MIP-1α; (n = 4) | 43.91 | 38.39 | 87.28 | 86.37 | 81.9 | 81.83 |
|        | (13.91 to 72.56) | (14.07 to 66.64) | (80.8 to 92.15) | (81.85 to 91.57) | (73.03 to 90.22) | (69.36 to 89.27) |
| MIP-1β; (n = 4–6) | 26.39 | 36.17 | 89.79 | 74.64 | 81.9 | 65.26 |
|        | (13.91 to 54.92) | (−18.75 to 64.47) | (80.8 to 93.78) | (−12.02 to 88.91) | (73.03 to 90.22) | (49.54 to 82.02) |
| RANTES; (n = 4) | 26.39 | 10.73 | 87.28 | 87.4 | 81.9 | 77.94 |
|        | (13.91 to 54.56) | (−125.1 to 66.67) | (80.8 to 92.15) | (69.96 to 91.5) | (73.03 to 90.22) | (83.78 to 90.21) |
| SDF-1; (n = 4–6) | 35.56 | −8.055 | 87.28 | 89.64 | 81.9 | 79.11 |
|        | (13.91 to 55.4) | (−144.1 to 48.93) | (80.8 to 92.15) | (66.33 to 93.3) | (73.03 to 90.22) | (59.21 to 88.18) |

P = 0.012 (paired T-test)
doi:10.1371/journal.pone.0001999.t005

When IFN-α was neutralized is most puzzling and speaks in favor of very potent unknown factor(s) inhibiting HIV. In contrast, TRAIL showed only minor changes of activating and/or inhibiting receptors (see above) and thus, we do not assume that they play a major role. In contrast, TRAIL showed a prominent up-regulation on NK cells. This increased TRAIL expression is reminiscent of a recent report describing the TLR7 triggering dependent generation of killer PDC expressing TRAIL, able to induce apoptosis of HIV-infected SupT1 cells [27]. The potential effects of TRAIL may be masked by the potent inhibitory anti-HIV effects of soluble factors in our experimental set-up that may explain the lack of any reversal of anti-HIV activity when neutralizing TRAIL (data not shown). The potent HIV-inhibition by soluble factors is reminiscent of the long-sought CD8+ T-cell antiviral factor, CAF [28].

Triggering TLR7/8 appears to inhibit HIV before its integration into the host-genome. Downregulation of CCR5 after release of β-chemokines may contribute to the anti-HIV activity when challenging the lymphoid tissue with CCR5-tropic strains. However, other anti-HIV mechanism(s) must be involved. Downregulation of CCR5 was not complete, and more importantly, X4-tropic strains were also inhibited. We excluded an unspecific effect on the fusion process of the TLR agonists as examined by a cell-cell fusion assay. Furthermore, we used replication-incompetent viruses pseudotyped with VSV envelope, which enter cells by an entirely different mechanism(s) than HIV. We observed
Figure 6. Triggering TLR8 and/or 7/8 blocks HIV replication after HIV entry but before integration. (A) Unspecific inhibitory effects of the compounds triggering TLR7 and/or 8 on HIV entry was examined with a cell-cell-based fusion assay, in which one cell line expresses the HIV-gp120 and an LTR-driven lacZ gene (HeLa SX CCR5) and the other cell line expresses the HIV receptor complex (CD4 and CCR5) and Tat (HeLa 243). Fusion of these cell lines will result in Tat driven LTR lacZ gene expression which we quantified histochemically by staining for β-galactosidase activity (n = 2). (B and C) Percentage of C4a+ T-cells expressing CCR5 and CXCR4 was quantified by flow-cytometry two days after triggering TLR7 and/or 8 of PBMC.
a similar inhibition of the reporter gene in tissue exposed to TLR agonists, also suggesting that these agonists act after entry but before integration/transcription. To substantiate this finding, we investigated whether the TLR agonists induced changes influence HIV replication at the level of transcription: for that purpose CD4+ T-cells were infected overnight and spreading infection was inhibited by adding the fusion inhibitor enfuvirtide. The infected CD4+ T-cells were subsequently co-cultured with previously TLR7/8 agonist stimulated autologous PBMC devoid of CD4+ T-cells. Thus, resulting HIV output from this co-culture reflects exclusively HIV transcription from HIV-infected CD4+ T-cells. In this setup, the TLR agonists had no inhibitory activity as compared to the control excluding any inhibitory action at the transcriptional level.

In summary, we propose a new model, in which triggering TLR7 and/or 8 of DC activates NK cells and CD8+ T-cells. These cells, in turn, induce the release of soluble factors that result in a microenvironment unfavorable to HIV infection. Since B cells express TLR7, they may be activated directly or indirectly by the corresponding trigger, but in any case, they have less anti-HIV activity than NK and CD8+ T-cells. The inability to identify a specific soluble factor is characteristic of the vast array of changes induced by triggering pattern recognition receptors and points to the poorly understood redundancy of the innate immune responses. As outlined in the introduction, we would like to reiterate the fact that compounds triggering TLR7 and/or 8 also strengthen the adaptive immune responses. Thus, these compounds may combine by acting on diverse arms of the immune response optimal properties as immunomodulatory drugs in HIV infection.

**Materials and Methods**

**Primary lymphoid tissue, cell lines, separation of cells, and generation of monocyte-derived myeloid dendritic cells**

PBMC obtained from the blood bank (Stiftung Zürcher Blutspendenstelle SRK, Zürich, Switzerland) were prepared by Ficoll Hypaque and cultured in RPMI (BioWhittaker, Verviers, Belgium) supplemented with 10% fetal calf serum (PAA Laboratories, Vienna, Austria), 10 U/ml IL-2 (National Institutes of Health, AIDS Repository), 2 mM L-glutamine (Invitrogen Life Technologies, Paisley, Scotland), and 1% penicillin/streptomycin (Invitrogen Life Technologies). We were authorized by the Ethical Committee of the University Hospital Zurich to receive tonsillar tissue from the Department of Otorhinolaryngology, University Hospital of Zurich. Tonsillar tissue was minced and subsequently put into a cell strainer (70 μm; BD Biosciences, San Jose, CA) where the tissue was grinded through the sieve with a syringe plunger. Erythrocytes were lysed (70°C) where the tissue was grinded through the sieve with a syringe plunger. Erythrocytes were lysed (70°C).

**Reagents and antibodies for neutralization of cytokines**

The TLR ligands 3M-001, 3M-002, and R-848 were generously provided by 3M Pharmaceuticals (St. Paul, Minnesota). The following antibodies were used: mAb anti-human interferon-α/β receptor chain 2 at 10 μg/ml (clone MMHAR-2, PBL Biomedical Laboratories, purchased from Alexis, Lausen, Switzerland), anti-human IL-18 at 100 ng/ml (clone 125-2H; MBL Medical & Biological Laboratories), anti-human IL-15 at 300 ng/ml (clone 54359; R&D), anti-human IL-12 at 20 ng/ml (ab9992, Abcam, Cambridge, UK), anti-human MIP-1α at 4 μg/ml (ap10361 Abcam), anti-human MIP-1β at 3 μg/ml (#PP1051, Acris, Hildenhausen, Germany), anti-human Rantes at 7 μg/ml (ab9679, Abcam), anti-human IFN-γ at 100 ng/ml (ab9657, Abcam) and anti-human SDF-1α at 5 μg/ml (#PP1067, Acris, Hildenhausen, Germany).

**Colometric viability assay**

The Colorimetric Cell Viability WST-1 kit (Roche, Mannheim, Germany, Cat. No 1 664 807) is based on the metabolism of the tetrazolium salts to water-soluble, orange formazan dyes by dehydrogenases present in viable cells. The WST-1 kit was used according to the instruction of the provider.

**HIV strains**

We used the prototype HIV strains NL4-3, 49.5, JR-CSF and 89.6; all were proviral DNAs. To generate viral stocks, 293 T cells were transfected with proviral DNA by polyethyleneimine (PEI), and supernatants were harvested 2 days later, passed through a 0.22-μm filter, and stored at −80°C until use.
Transwell experiments

To determine if cell-cell contact or soluble factors are involved in the anti-HIV effects after TLR triggering, HIV-infected CD4+ T-cells were cultured at the bottom of a well of a six-well plate, and the PBMC devoid of CD4+ T-cells in the insert. As controls served untreated PBMC infected with HIV as well as PBMC infected similarly as the CD4+ T-cells which were placed at the bottom of wells and the TLR agonists added to the inserts. Inserts with a pore size of 4 μm were purchased from Millipore. The compounds triggering TLR were added to the medium. CD4+ T-cells do not respond to TLR7/8 since they have no TLR7/8. Thus, any observed effects must be due to soluble factors released from the treated PBMC devoid of CD4+ T-cells.

Cytokine measurement

In the initial experiments, IL-6 (D6050), -8 (D8050), -12 (D1200), TNF-α (HSTA50), RANTES (DRN00B), MIP-1α (DMA00), and -β (DMB00) were quantified by enzyme immunoassays (Quantikine ELISA, R&D Systems), according to the manufacturer’s instructions, IFN-α (Bender Med Systems BMS216) and -γ (HyCult Biotechnology, Hbx human Interferon gamma, HK0308) were quantified by enzyme immunoassays from Bender MedSystems Diagnostics and HyCult Biotechnology, respectively. We then changed to multiplexed particle-based flow cytometric cytokine assay for measuring cytokines [32]. Bioplex cytokine kits were purchased from BioRad (Esming, Germany). The procedures closely followed the manufacturer’s instructions. The analysis was conducted using a conventional flow cytometer (FC500 MPL, BeckmanCoulter, Nyon, Switzerland). For each data set, we have indicated what method was used.

Cell-based fusion assay

A cell-based fusion assay consisted of HeLa cells that express gp140 from LAI and the HIV transactivator tat (HeLa-Env/LAI) [33] and HeLa cells that express CD4 and the LTR-driven lacZ gene (HeLa SX CCR5) [34]. Fusion of HeLa-Env/LAI cells and HeLa SX CCR5 results in transcription of the lacZ gene. The extent of fusion was quantified by assaying β-galactosidase activity in cell lysates (E2000; Promega, Wallisellen, Switzerland) 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 the various compounds and the HIV fusion inhibitor enfuvirtide [35]. 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.Luc.R’ E’) was cotransfected with a VSV Env expression vector in 293T cells as described [36]. To determine permissiveness to entry by pseudotype viruses, PBMC cultures were pre-treated with R-848 for 1 day, exposed to pseudotype virus overnight, and cultured for additional 2 days before analysis. Luciferase expression was quantified with the luciferase assay system from Promega.

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Author Contributions
Conceived and designed the experiments: RS ES. Performed the experiments: ES. Analyzed the data: RS ES. Wrote the paper: RS.

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