HIV-1-neutrophil interactions trigger neutrophil activation and Toll-like receptor expression

Running title: HIV-1 and activation of neutrophils.

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Abbreviations:

PRRs: Pattern recognition receptors; TLRs: Toll-like receptors; ROS: Reactive oxygen species; NETs: Neutrophils extracellular traps.
Abstract

Although neutrophils are the first line of host defense against infection and express a wide number of pattern recognition receptors (PRRs), the function of these PRRs, including Toll-like receptors (TLRs), in HIV-1 infection remains unclear. TLRs play an important role in innate immunity, and while their involvement in viral immune-pathogenesis was recently proposed, little is known about their expression and function during the neutrophil response to HIV-1 exposure. Here, we have shown that freshly isolated human neutrophils from healthy donors exhibited altered TLR expression, which may affect their function, after being challenged with HIV-1, alone or in the presence of TLR agonists. TLRs may promote neutrophil activation, pro-inflammatory cytokine secretion, and the production of reactive oxygen species (ROS). To our knowledge, this study is the first demonstration of functional TLR expression on neutrophils in response to HIV-1 treatment, suggesting a possible neutrophil/HIV-1 interaction through TLRs. Although additional studies are required to confirm the function of TLRs in neutrophils, our data clearly suggest that they play a role in the regulation of innate immunity by neutrophils, which could be engaged in HIV-1 pathogenesis or host defense.

Keywords

Neutrophils, TLRs, PAMPs, HIV-1, cytokines
Introduction

HIV-1 infection disturbs the effectiveness of the human immune system, leading to the development of AIDS, which is a major public health problem worldwide (1). Although both the adaptive and innate immune responses play significant roles in HIV-1 disease progression, the role of the innate immune response to HIV-1 infection has become clearer in recent years following the discovery of pattern recognition receptors (PRRs) (2). Among these receptors, the Toll-like receptors (TLRs) TLR3, TLR7/TLR8 and TLR9 are involved in recognition of viral components such as dsRNA, ssRNA and DNA CpG motifs, respectively (3). TLRs play important roles in influenza, hepatitis C virus (HCV), hepatitis B virus, herpes simplex virus 2, West Nile virus and Dengue virus infections (4, 5). The role of TLRs in HIV-1 infection remains unclear, but certain viral products are known to activate HIV-1 replication through TLR stimulation (6, 7). The innate immune system, to which neutrophils belong, is highly specialized in its capacity to recognize foreign pathogens through PRRs. Indeed, neutrophils mediate the early response to infection and amplify the inflammatory response (8). Previously was reported that neutrophils express all TLRs, with the exception of TLR3 (9). The activation of TLRs in neutrophils induces pro-inflammatory cytokine production, modulates co-stimulatory molecules such as CD62L (L-selectin) and CD11b/CD18 (10-12), regulates the chemotactic response (13), and induces the production of reactive oxygen species (ROS) (10, 14).

During HIV-1 infection, the absolute neutrophil count in peripheral blood progressively declines, and chemotactic and bactericidal activities are impaired (15, 16). In addition, the expression of adhesion molecules such as
CD11a/LFA-1 and CD62L is modulated in individuals infected with HIV-1, but only after the onset of AIDS (17, 18). This finding suggests an activated neutrophil phenotype that is accompanied by functional alteration. Indeed, Bowers et al. reported that neutrophils from HIV-1-infected individuals suppress T cell function through the production of programmed death ligand 1 (PD-L1) and ROS, suggesting that HIV-1 infection induces immunosuppressive activity via neutrophils with an inhibitory effect on T cell function (19, 20). However, the mechanism through which neutrophils are activated in response to HIV-1 remains unclear. Although functional TLRs are expressed on neutrophils and promote the production of ROS and neutrophil extracellular traps (NETs) in an attempt to eliminate HIV-1 (21), no report has described the function of TLRs in neutrophils challenged with HIV-1.

Furthermore, their role in inducing an innate response, as indicated by pro-inflammatory cytokine secretion and ROS production, remains unknown. Here, we focused on determining whether the in vitro challenge of neutrophils with HIV-1 could induce their activation by modulating the expression or function of TLRs. We found that HIV-1, alone and in the presence of TLR agonists, modulates both the expression and function of specific TLRs, such as TLR2, TLR4, TLR7 and TLR8; activates neutrophils (i.e., regulates the expression of CD62L and CD11b); promotes the secretion of pro-inflammatory cytokines; and alters ROS production. Although additional studies are required to confirm the relationship between neutrophil, TLRs and HIV-1, our data suggest that neutrophils respond to the virus and thus participate in the antiviral host response to HIV-1 or disease progression.

**Materials and methods**
Ethics statement

This study was designed and performed according to the Declaration of Helsinki and was approved by the Ethics Committee of the Universidad de Antioquia.

Study population

Eight HIV-1-seronegative healthy individuals who voluntarily agreed to participate in this study were enrolled after providing written informed consent. Any volunteers reporting a recent infection or pregnancy were excluded. Furthermore, the donors declared that they were nonsmokers and were taking no medication. The individuals presented a regular leukocyte count (4,500-11,000 cells/mm$^3$) and a normal differential leukocyte count.

Sampling and neutrophil purification

Blood samples were collected by venipuncture in tubes containing ACD (citric acid, citrate, and dextrose) anticoagulant (BD Pharmingen, San Diego, CA, USA). Neutrophils were purified as previously reported (22) by dextran sedimentation and centrifugation in a Ficoll-Hypaque gradient (Sigma-Aldrich, St. Louis, MO, USA). Ficoll-Hypaque-purified neutrophils were resuspended in RPMI-1640 enriched with 10% fetal bovine serum (FBS). The purity of the neutrophil preparation was over 96%, and viability was approximately 95%, even after 8 h in culture.

Preparation of virus stocks
A high viral titer of HIV-1 was collected from the H9-HTLV-IIIcc cell line (chronically HIV-1-infected; NIH AIDS Research & Reference Reagent Program) within 7 days of culture in RPMI, as previously described (23).

**Monoclonal antibodies**

Monoclonal antibodies specific to CD11b conjugated with PE-Cy5 and to CD62L conjugated with FITC were obtained from BD Biosciences (Pharmingen, San Jose, CA). Phycoerythrin-conjugated monoclonal antibodies to TLR2 (clone TL2.1) and TLR4 (clone HTA125) were obtained from eBioscience (San Diego, CA, USA). FcR blocking reagent was obtained from Miltenyi Biotec (Auburn, CA, USA). Appropriate isotype-matched antibodies were used as controls.

**In vitro HIV-1 stimulation**

Immediately after purification, 2.5x10^5 neutrophils were plated onto 96-well plates in 250 µl of RPMI-1640 medium supplemented with 10% FBS at 37°C and 5% CO₂. Neutrophils were stimulated with HIV-1 H9-HTLV-IIIcc (0.5-5 ng/ml HIV-1 p24), and supernatant obtained from H9 uninfected cells was used as a mock. To assess the role of HIV-1 on TLR expression, neutrophils were challenged with HIV-1 and/or TLR ligands, such as TLR2, (Pam₃CSK₄, 20 ng/ml), TLR4 (LPS, 0.1 ng/ml) or TLR7/8 (R848, 1 μg/ml). All agonists were obtained from InvivoGen (San Diego, CA, USA). After 3 h of stimulation, the neutrophils were harvested and assayed for TLR2, TLR4, TLR7 and TLR8 mRNA by real-time PCR (23). Alternatively, the neutrophils were incubated for 8 h after stimulation (according to viability) and then stained with monoclonal
antibodies against surface molecules (FITC anti-CD62L, PE-Cy5 anti-CD11b, PE anti-TLR2 or PE anti-TLR4) to determine protein expression.

**Flow cytometry analysis**

Flow cytometry was used to evaluate the effect of HIV-1 challenge on the expression of TLR2, TLR4, CD62L and CD11b by neutrophils, as described previously (6). In brief, freshly isolated neutrophils were surface-stained with the appropriate antibodies for 25 min; data were acquired immediately using a FACScan II™ flow cytometer (BD Biosciences, San Jose, CA, USA). The acquired events were analyzed using FACSDiva software version 6.1.2 (BD Biosciences, San Jose, CA, USA). The results are expressed as the means of fluorescence intensities (MFIs) of the overall cell sub-population after subtraction of the isotype control.

**RNA isolation, cDNA synthesis and quantitative real-time PCR for TLR mRNAs**

TLR2, TLR4, TLR7 and TLR8 mRNA expression was quantified as described previously (6, 23). For total RNA preparation, an RNeasy Mini Isolation Kit was used (Qiagen, Valencia, CA, USA). cDNA was synthesized using a RevertAid Minus First Strand cDNA Synthesis Kit (Thermo Scientific, Wilmington, DE, USA) according to the manufacturer’s instructions. The primers used are listed in Table 1. The relative expression of each target gene was normalized to the housekeeping gene GAPDH (ΔCt) and is reported as the relative units of transcript.

**Enzyme-linked immunosorbent assay (ELISA)**
TNF-α and IL-6 levels in cell culture supernatant were determined with human ELISA sets (BD Biosciences, San Jose, CA, USA), according to the manufacturer’s instructions.

**Quantification of ROS**

ROS production was quantified using dihydrorhodamine 123 (Invitrogen, San Diego, CA, USA), according to the manufacturer’s recommendations, in neutrophils challenged with HIV-1 and with or without TLR agonists. In brief, commercial dihydrorhodamine was diluted 1:10 in PBS, and neutrophils were then incubated with 10 μl of the dilution in each well (96-well plate) for 8 h at 37°C. After incubation, the cells were harvested, washed with PBS, centrifuged for 5 min at 1800 rpm, and resuspended in PBS. Acquisition was performed immediately using a FACSCan II™ flow cytometer. Analyses were performed using FACSDiva software.

**Statistical analyses**

The data were plotted and analyzed using Prism 5.0 software (Graph Pad Software, CA, USA). All results represent at least five independent experiments. The Wilcoxon test was used to assess significance. Values of p<0.05 (*) and p<0.01 (**) were considered significant.
Results

**HIV-1 challenge increases expression of TLR4 and TLR7 but decreases that of TLR2 in human neutrophils**

To determine the effect of HIV-1 on TLR expression, human neutrophils were challenged with HIV-1, and TLR2, TLR4, TLR7 and TLR8 mRNA levels were determined by real-time PCR. In addition, neutrophils were stimulated with specific TLR agonists. We observed a significant decrease in TLR2 mRNA upon stimulation with HIV-1 (p=0.008) compared with control supernatant (obtained from uninfected H9 cells; Fig. 1a). In contrast, HIV-1 challenge increased TLR4 (p=0.016) and TLR7 (p=0.022) expression (Fig. 1b and c) compared with the mock-treated cells. Consistent with the mRNA studies, HIV treatment induced a significant decrease in TLR2 protein expression (p=0.031) but a significant increase in TLR4 protein expression (p=0.078) compared with the mock-treated cells (Fig. 1e and f). In addition, we observed a significant increase in the expression of mannose receptor (MR) mRNA compared with the mock-treated cells (Online resource 2a). No significant changes in RIG-I or MDA-5 mRNA levels were observed in human neutrophils that were challenged with HIV-1 (Online resource 2b and c). In summary, our in vitro studies show that HIV-1 modulates the expression of TLR2 and TLR4 at both the mRNA and protein levels.

**HIV-1 promotes neutrophil activation and leads to pro-inflammatory cytokine secretion and a decrease in ROS**

Because HIV-1 treatment can modulate TLR expression, we asked whether HIV-1 challenge promotes neutrophil activation, induces cytokine expression
and alters ROS production. As shown in Figure 2a and 2b, treatment with HIV-1 resulted in significant down-regulation of CD62L (p=0.004), but up-regulated CD11b expression (p=0.078) compared with the mock-treated cells, suggesting that HIV-1 promotes neutrophil activation. Moreover, challenge with HIV-1 induced a significant increase in the secretion of both IL-6 (p=0.001) and TNF-α (p=0.016) compared with the mock-treated cells (Fig. 2c). However, HIV-1 significantly reduced basal ROS production (p=0.047) compared with the mock-treated cells (Fig. 2e). The same effect was observed when neutrophils were challenged with X4-tropic or R5-tropic HIV-1 variants (online resource 1 and 3), verifying the result observed with HIV-1 obtained from H9 HTLVIII cells. Together, these results show that HIV-1, independent of tropism, activates neutrophils and alters their response, including ROS production.

We next evaluated which HIV-1 components have an effect on neutrophil activation or function. Neutrophils were stimulated with Tat (10 μg/ml), Rev (5 μg/ml), gp160 (0.1 μg/ml) or gp41 (0.5 μg/ml) in the presence of polymyxin B (50 μg/ml) and incubated at 37ºC for 8 h. Polymyxin B was used to prevent endotoxin contamination from recombinant proteins purified by chromatography and to act as an LPS inhibitor. Both Rev and gp160 induced a significant increase in IL-6 secretion compared with non-stimulated cells (Online resource 3c). These results suggest that both the viral envelope protein gp160 and the regulatory protein Rev alter the expression of proinflammatory cytokines in neutrophils.

**Effects of HIV-1 and TLR agonists on TLR expression in neutrophils**
To evaluate whether HIV challenge modulates TLR expression upon stimulation by a specific agonist, neutrophils were stimulated with only TLR agonist or with TLR agonist and HIV-1. A significant down-regulation of TLR4 and TLR8 mRNA (p=0.023 and p=0.039, respectively) was observed when the neutrophils were co-stimulated by HIV-1 and the respective TLR agonist compared with stimulation by the TLR agonist alone (Fig. 3b and d). Furthermore, there was a significant decrease in TLR4 protein expression (p=0.008) in response to co-stimulation with HIV-1 and LPS (Fig. 3f), but no changes were detected in neutrophil expression of TLR2 at the protein level (Fig. 3e).

Co-stimulation by HIV-1 and specific TLR agonists promotes crosstalk between TLRs

Recent studies highlight the importance of the combinatorial activation of TLRs (24, 25), suggesting that crosstalk between different TLRs may regulate the inflammatory response (26). To evaluate whether HIV challenge modulates TLR expression in primary human neutrophils upon stimulation by specific and non-specific agonists, neutrophils were treated with different TLR agonists in the presence of HIV-1. Neutrophils stimulated with either Pam\textsubscript{2}CSK\textsubscript{4} or R848 in the presence of HIV-1 down-regulated TLR4 mRNA expression (p=0.039 and p=0.008, respectively) (Fig. 3b). Although no effect on TLR2 and TLR7 mRNA levels was observed (data not shown), the level of TLR2 decreased significantly upon co-stimulation with HIV-1 and R848 (Fig. 3e). In contrast, challenge with HIV-1 promoted the up-regulation of TLR8 mRNA in the presence of LPS and Pam\textsubscript{2}CSK4 (Fig. 3d). These results suggest that crosstalk between TLRs may indeed occur in neutrophils.
Recently, stimulation with TLR7 and TLR9 was shown to dramatically up-regulate the expression of RIG-I in plasmacytoid dendritic cells (27). Following this strategy, we extended our study to evaluate the effect of TLRs on other PRRs, such as RIG-I, MDA-5 and mannose receptor (MR) to determine whether a relationship exists among these PRRs. We co-stimulated neutrophils with HIV-1 and TLR2-, TLR4- or TLR7/TLR8-specific agonists and evaluated the RIG-I, MDA5 and MR mRNA levels. Interestingly, in the presence of Pam2CSK4 and R848, HIV-1 significantly up-regulated the expression of RIG-I mRNA (Online resource. 4b). The combination of HIV-1 with Pam2CSK4 also significantly increased the expression of MDA5 mRNA (Online resource 4c). Finally, the combination of Pam2CSK4, LPS and R848 in the presence of HIV-1 had no effect on MR mRNA level (Online resource 4a). Thus, HIV-1 can modulate RIG-I and MDA5 mRNA levels through TLR activation, suggesting a possible synergistic effect between different PRRs on HIV1-challenged neutrophils.

**Stimulation with specific TLR agonists in the presence of HIV-1 leads to neutrophil activation, cytokine secretion and ROS production**

The simultaneous engagement of different TLRs through a specific agonist synergistically enhances cytokine, chemokine, and nitric oxide production in monocytes and macrophages (28-30). After observing that the combinatorial activation of select TLRs in the presence of HIV-1 leads to expression of another TLR, we examined whether this effect facilitates neutrophil activation, pro-inflammatory cytokine secretion and ROS production under the treatment conditions used in our original experiments. Interestingly, a lower CD62L expression level was observed in neutrophils that were co-stimulated with HIV-1
and Pam$_2$CSK$_4$ or R848 compared with the respective TLR agonist alone (Fig. 4a). Conversely, no significant changes in CD11b expression were observed (Fig. 4b). Pro-inflammatory cytokine secretion was then assessed by ELISA. As shown in Figure 4c, the inclusion of HIV-1 led to a significant down-regulation of IL-6 production in the presence of LPS, but no such effect was observed with Pam$_2$CSK$_4$ or R848. There was also a significant decrease in TNF-α secretion in response to HIV-1 stimulation and treatment with LPS (p=0.016) or R848 (p=0.003), but not with Pam$_2$CSK$_4$ (Fig. 4d).

Finally, ROS production was significantly induced in neutrophils in the presence of HIV-1 and either Pam$_2$CSK$_4$ or LPS compared with stimulation with TLR2 or TLR4 agonist alone (p=0.022 and p=0.016, respectively; Fig. 4e). Taken together, our findings show that neutrophils challenged simultaneously with HIV-1 and TLR agonists were activated and produced a proportional amount of pro-inflammatory cytokines and ROS.
Discussion

Neutrophils are the first cells to arrive at the site of an infection, where they recognize pathogenic agents through PRRs such as TLR family members and contribute to the inflammatory response (2). However, although TLR expression has been described in neutrophils (9, 10), the modulation of their mRNA and protein levels and their effects on neutrophil activation upon stimulation with HIV-1 have not been documented. For the first time, we provide a broad analysis of TLR regulation by HIV-1 in the presence or absence of TLR agonists. Our results clearly show that HIV-1 strongly down- or up-regulates the expression of TLRs (including 2, 4, and 7) at the mRNA level and regulates the level of TLR2 and TLR4 protein in neutrophils, as was previously reported for other cell types, using HIV-1 and other viral components (31-33). In subjects with chronic HIV-1 infection, an increase in TLR2, TLR3, TLR4, TLR6, TLR7 and TLR8 mRNA has also been reported (34). Furthermore, these authors reported a correlation between TLR expression - especially for TLR6 and TLR7 - and HIV RNA load in plasma. Our results are consistent with a modulation of TLR expression by HIV-1 in monocytes (23). Because the activation of TLR4 promotes HIV-1 replication in epithelial cells through the activation of NF-κB (35) and in myeloid dendritic cells through the down-regulation of TLR2 (36), we hypothesize that HIV-1 developed strategies to evade the TLR-dependent innate response or to regulate TLR expression to increase viral release, as reported for other viruses (37). Here, we observed that the HIV-1-induced activation of the TLR signal on neutrophils enhanced the production of pro-inflammatory cytokines, such as IL-6 and TNF-α. In agreement with our results, the *in vitro* stimulation of PBMCs with LPS and with HIV-1 has been shown to
increase pro-inflammatory cytokine production (32). Furthermore, HIV-1 causes an imbalance in the production of pro-inflammatory cytokines, including IL-1β, IL-6, IL-8 and TNF-α, thereby contributing to HIV-1 pathogenesis (38, 39).

One of the main neutrophil mechanisms for pathogen control is the production of ROS (40). However, little is known about the effect of ROS production in the face of viral infection. Influenza virus induces ROS production, which is associated with lung damage (41). However, the administration of ROS inhibitors reduces mortality in influenza virus-infected mice (42). ROS production is also induced by HCV and is considered a marker of chronic HCV infection (43). The production of ROS has been described in cases of HIV-1 infection (44, 45), and in vitro stimulation of neutrophils with the HIV-1 Nef protein induces a rapid increase in ROS production (46). These results suggest that different components of HIV-1 can activate neutrophils and promote ROS production. Moreover, a relationship between TLRs and ROS production has been demonstrated (47, 48). József et al. showed that TLR9 activation by CpG-ODN induces an increase in peroxynitrite, a ROS intermediate (49), but the synergistic function of TLR agonists and HIV-1 has not been reported. We observed that stimulation with Pam2CSK4 or LPS in the presence of HIV-1 increased the production of ROS compared with agonist alone. This finding is consistent with a recently published study that describes an increase in ROS production by HIV-1 through TLR7/8, which may act as a defense mechanism during HIV-1 infection (50). These results provide evidence that HIV-1 enhances ROS production through TLRs, which could play a dual role in HIV-1 infection, either by aiding opportunistic pathogens or by increasing epithelial damage to promote microbial translocation, a common characteristic of HIV-1.
infection. Interestingly, we have also found that neutrophils from HIV-1-exposed seronegative (HESN) individuals exhibited reduced expression of TLR4 and TLR9 and that these neutrophils, when stimulated with HIV-1, TLR2 and TLR4 agonists, produced significantly lower levels of ROS compared with HIV-1-infected individuals (51). This finding is interesting because the neutrophils from HIV-1-infected patients suppress the function of T cells via the ROS pathway (19). Furthermore, ROS production appears to be the main mechanism of neutrophil-mediated T cell suppression (52). In summary, these results suggest that HIV-1 modulates not only the expression of TLRs but also the activation and function of these receptors by inducing neutrophil activation, although it remains unknown whether the recognition of HIV-1 by TLRs promotes a protective immune response or contributes to pathogenesis. Additional studies are needed to reveal the true role of neutrophils during HIV-1 infection. However, treatment of PBMCs with TLR3, TLR7, TLR8 or TLR9 agonist prior to infection with HIV-1 significantly reduces infection (53).

Human neutrophil activation is characterized by changes in the expression of adhesion molecules, such as CD62L and CD11b (54). Here, we showed that stimulation by HIV-1 alone or with TLR agonists alters the expression of CD62L and CD11b. These results are consistent with previously reported data showing that neutrophils from HIV-1-infected patients exhibit decreased CD62L expression and increased CD11b expression during the acute phase of infection (18, 44, 55). Similar regulation has also been reported during infection by other viruses, such as influenza A (37) and respiratory syncytial virus (56). Furthermore, TLR2 and TLR4 induce changes in CD11b and CD62L expression in CD14+ monocytes from patients with coronary artery disease after
whole blood stimulation by specific TLR agonists (57), suggesting a connection between the TLR response pattern and clinical characteristics.

Recently, cross-regulation between different PRRs was demonstrated to lead to a synergistic, additive or antagonistic response (26, 58). In murine and human dendritic cells, synergy between TLR3 or TLR4 with TLR7/8 and TLR9 has also been described, leading to the transcription of IL-12 and IL-23 (25). Cross-regulation between TLR4 and TLR2 promotes interaction between different cell populations, including between neutrophils and alveolar macrophages and between neutrophils and endothelial cells, thus increasing the overall inflammatory response (59). This cross-regulation occurs not only between TLRs but also between different types of PRRs (24). Consequently, interactions between various PRRs may be required to ensure an effective innate immune response (60). Here, we have extended the analysis of synergy to stimulation with HIV-1 combined with TLR2, TLR4 and TLR7/8 agonists, which led to a decrease in the expression of TLR4; when combined with TLR2 or TLR4, synergistic TLR8 expression was observed. Similarly, synergistic CD62L and CD11b expression, pro-inflammatory cytokine (IL-6 and TNF-α) secretion and ROS production were observed when human neutrophils were stimulated with TLR agonists combined with HIV-1, indicating that HIV-1 can amplify neutrophil activation via the stimulation provided by TLR agonists. In agreement with our results, studies performed in DC cells have reported that certain TLR agonist combinations can induce synergistic secretion of pro-inflammatory cytokines (25, 61). Together, these findings suggest that HIV-1 infection induces neutrophil activation via different TLR pathways. This activation could lead to immunosuppressive activity against T cell function; accumulating evidence
supports the role played by neutrophils in the negative regulation of T cell function through the production of ROS (8, 52). A novel mechanism mediated by crosstalk between different TLRs likely participates in the neutrophil response to HIV-1 infection. A better understanding of this mechanism will help us to unravel the pathogenesis of HIV-1.

In conclusion, the present study reveals three major findings: i) TLR expression is altered in response to HIV-1; ii) neutrophil activation and cytokine production is triggered in response to HIV-1 or TLR agonists; and iii) ROS production is regulated by stimulation with HIV-1, alone or in combination with a TLR agonists. This finding is particularly noteworthy because TLRs and their signaling pathways are emerging as novel therapeutic targets.
Competing interests

None of the authors has any potential financial conflict of interest related to this manuscript.

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**Figure legends**

**Fig. 1 HIV-1 modulates the expression of TLR2, TLR4 and TLR7.**

Neutrophils (2x10^5) purified from healthy donors (n=8) were exposed *in vitro* to 5 ng/ml of HIV-1 collected from H9 HTLVIII (chronically infected) supernatant or H9 (mock) supernatant as a control and were stimulated with the following specific TLR agonists: Pam2CSK4 (20 ng/μl) for TLR2, LPS (0.1 ng/μl) for TLR4, or R848 (1 μg/ml) for TLR7 and TLR8. Stimulations were performed for 3 h prior to determination of TLR2 (a), TLR4 (b), TLR7 (c) and TLR8 (d) mRNA expression by real time RT-PCR. The median and range of each transcript's relative expression versus that of a housekeeping gene transcript are shown.
Stimulations were performed for 8 h prior to determining TLR2 (e) and TLR4 (f) expression by flow cytometry. The data are presented as the mean fluorescence intensity (MFI) of each specific TLR. Comparisons were performed using the Wilcoxon test. The level of significance was p<0.05 (*) and p<0.01 (**).

**Fig. 2 HIV-1 modulates the activation and function of neutrophils.**
Neutrophils (2.5x10^5) obtained from healthy donors (n=8) were stimulated with 5 ng/ml of HIV-1. H9 cell line supernatant was used as a control. After 8 h of stimulation, the expression of CD62L (a) and CD11b (b) was assessed by flow cytometry, the production of IL-6 (c) and TNF-α (d) was determined by ELISA, and the production of ROS (e) was assessed by flow cytometry (presented as the percentage of neutrophils producing ROS). Comparisons were performed using the Wilcoxon test. Median and range are shown. The level of significance was p<0.05 (*) and p<0.01 (**).

**Fig. 3 Cross-regulation between TLRs is altered in neutrophils stimulated with HIV-1.** A total of 2.5x10^5 neutrophils obtained from healthy donors (n=8) were co-stimulated with specific agonists for each TLR evaluated (20 ng/μl of Pam2CSK4 for TLR2; 0.1 ng/μl of LPS to TLR4 or 1 μg/μl of R848 for TLR7/TLR8) and 5 ng/ml of HIV-1. Expression of TLR2 (a), TLR4 (b), TLR7 (c) and TLR8 (d) mRNA was evaluated 3 h post-stimulation by real time RT-PCR. The median and range of each transcript versus a housekeeping gene transcript are shown. The expression of TLR2 (e) and TLR4 (f) protein in neutrophils was examined by flow cytometry after 8 h in culture and are reported as the mean fluorescence intensity (MFI). Comparisons were performed
using the Wilcoxon test. Median and range are shown. The level of significance was $p<0.05$ (*) and $p<0.01$ (**).

**Fig. 4** Treatment of neutrophils with HIV-1 modulates their functionality through the stimulation of TLR2, TLR4, TLR7 and TLR8. Neutrophils (2.5x10$^5$) purified from healthy donors ($n=8$) were stimulated with TLR agonists (20 ng/μl Pam2CSK4; 0.1 ng/μl LPS or 1 μg/μl R848) and were simultaneously exposed to HIV-1 (5 ng/ml p24) for 8 h. Expression of the adhesion molecules CD62L (a) and CD11b (b) on neutrophils was measured by flow cytometry and is reported as the mean fluorescence intensity (MFI). Production of IL-6 (c) and TNF-α (d) was assessed by ELISA, and ROS (e) production was assessed by flow cytometry. Comparisons were performed using the Wilcoxon test. The median and range are shown. The level of significance was $p<0.05$ (*) and $p<0.01$ (**).

**Online resource 2** MR mRNA expression is altered in neutrophils stimulated *in vitro* with HIV-1. Neutrophils (2.5x10$^5$) obtained from healthy donors ($n=8$) were stimulated with 5 ng/ml of HIV-1. For each stimulus condition, H9 supernatant was used as a control. The expression of MR (a), RIG-I (b) and MDA-5 (c) mRNA was evaluated after 3 h in culture by real time RT-PCR. The median and range of the relative transcript level of these genes versus a housekeeping gene are presented. Comparisons were performed using the Wilcoxon test. The level of significance was $p<0.05$ (*) and $p<0.01$ (**).

**Online resource 3** The functional activation of neutrophils by HIV-1 is independent of viral tropism. Neutrophils (2.5x10$^5$) purified from healthy donors ($n=5$) were exposed to HIV-1 at a concentration of 2 ng/μl (p24 virus
obtained from the supernatant of 293T cells transfected with the plasmid pNL4-3 to produce R5 tropic virus) or were transfected with pNL4-3Δenv + SRα to produce X4 tropic virus. Untransfected 293T supernatant was used as a control. IL-6 secretion was quantified by ELISA (a). In addition, ROS production (b) was measured by flow cytometry and is reported as the percentage of neutrophils producing ROS. Neutrophils were exposed in vitro to the recombinant viral proteins Rev (5 μg/ml), Tat (10 μg/ml), gp160 (0.1 μg/μl) and gp41 (0.5 μg/μl) for 8 h while being treated with 50 μg/ml polymyxin B. The production of IL-6 was quantified by ELISA and is reported in pg/ml (c). Comparisons were performed using the Wilcoxon test. The median and range are shown. The level of significance was p<0.05 (*) and p<0.01 (**).

**Online resource 4** Stimulation of neutrophils by HIV-1 alters the RIG-I and MDA-5 mRNA levels under TLR agonist stimulation. A total of 2.5x10^5 neutrophils were stimulated simultaneously with TLR agonists (20 ng/μl Pam2CSK4, 0.1 ng/μl LPS or 1 μg/μl R848) and HIV-1 (5 ng/ml p24). The mRNA expression levels of MR (a), RIG-I (b) and MDA-5 (c) were evaluated by real time RT-PCR after 3 h of stimulation. The median and range of relative transcript expression versus a housekeeping gene are shown. Comparisons were performed using the Wilcoxon test. The level of significance was p<0.05 (*) and p<0.01 (**).