HIV-1-exposed seronegative individuals show alteration in TLR expression and pro-inflammatory cytokine production *ex vivo*: an innate immune-quiescence status?

Juan C. Hernandez[1], St Laurent George 3rd[2] and Silvio Urcuqui-Inchima[3*].

[1]INFETARE, Facultad de Medicina, Universidad Cooperativa de Colombia, Medellin, Colombia.

[2]St Laurent Institute, 317 New Boston St, Woburn, MA 01801, USA

[3]Grupo Inmunovirología, Facultad de Medicina, Universidad de Antioquia, UdeA, Calle 70 No. 52-21, Medellín, Colombia

Running title: PRR and cytokine modulation in HESNs

Abstract

Pattern-recognition receptors (PRRs) are involved in direct recognition of viruses, promoting cellular activation and the production of pro-inflammatory cytokines. However despite the reduced systemic immune activation described in HIV-1-exposed seronegatives (HESNs), few studies have focused on determining the relationship between PRR expression and cytokine production. We have aimed here to evaluate the expression level of PRRs and cytokines in HESNs, HIV-1-patients and healthy donors. Basal PRR expression levels in PBMCs, dendritic cells (DCs) and monocytes, and plasma cytokine levels as well as the PRR ligand-induced cytokine productions were determined by flow cytometry, qPCR and ELISA. Higher TLR2/4 expression in DCs and monocytes from HESNs were observed. Nevertheless, TLR4/8, NOD2 and RIG-I mRNA levels were lower in PBMCs from HESNs than HIV-1-infected patients. Comparable IL-1β, IL-18 and TNF-α mRNA levels were observed among the groups examined; however, at the protein level, production of IL-1β, IL-6 and IL-10 was significantly lower in plasma from HESNs than from HIV-1-infected patients. Our results suggest that exposure to HIV-1 without infection could be associated with reduced basal pro-inflammatory responses. Further studies are required to define the cell subsets responsible for these

differences and the role of PRRs on protection against HIV-1 infection.

Keywords: HIV-1; HIV-1-exposed seronegative individuals; Pattern-recognition receptors; pro-inflammatory cytokines.

Introduction

HIV-1 has currently infected over 34 million people globally [1]. Nonetheless, there is heterogeneity in susceptibility and individuals who have been repeatedly exposed to HIV-1 but remain seronegative (HESNs), have been described in multiple settings [2-5]. This particular population elicits the existence of immunological mechanisms conferring natural resistance to HIV-1 infection.

On the other hand, pattern-recognition receptors (PRRs) are involved in the initiation of the inflammatory response against invading pathogens, including cytokine production, expression of co-stimulatory molecules, and migration of dendritic cells (DCs) into lymphoid tissues, culminating with the establishment of adaptive immunity [6,7]. Several families of PRRs have been described, including Toll-like receptors (TLRs), NOD-like receptors (NLRs) and RNA helicase-like receptors (RLRs), among others. However, few reports describe a possible role for innate immunity receptors on the modulation of progression/susceptibility to HIV-1 infection, either *in vitro* or *in vivo* [8-10].

Previously we showed that HIV-1 alters the expression and function of TLR2/TLR4 both *in vivo* and *in vitro*, in monocytes and DCs [8,9]. Furthermore, an anti-viral activity through TLRs, such as TLR3, TLR7/8, and TLR9 has been reported [11]. Although a polymorphism in the *tlr3* gene was associated with protection against HIV-1 infection [12], few studies have reported the influence of TLRs on HIV-1 resistance observed in HESNs [10,13,14]. A better understanding the innate immunity to HIV-1, especially among protected HESNs individuals, will offer a new opportunity to develop new therapies to fight HIV-1, including vaccines or microbicides [15]. Increased immune activation is strongly correlated with HIV-1 disease progression, and recent studies have shown that it can also represent a risk factor of acquiring HIV-1 [4,16-22]. Therefore, it is very important to evaluate the innate immunity and inflammatory responses in HESNs individuals at the baseline level so as to better understand their role in natural resistance to HIV-1 infection. Interestingly, higher expression of TLR3/TLR7, and of the transcriptional factor NF-κB was recently demonstrated in cervical mononuclear cells from HESNs [13]. Furthermore, the authors described lower levels of IL-1β, IL-8 and RANTES. Moreover, the polymorphism rs3775291 (Leu412Phe) in the *tlr*3 gene was associated with HIV-1 protection among HESNs intravenous drug users [12,23].

Based on these findings, it has become urgent to determine the innate factors involved in the establishment of HIV-1 productive infection. Therefore, our aim was to investigate the basal innate immunity status and the effects of engagement of PPRs as well as the profiles of cytokine production in innate immune cellular subsets from a cohort of HESNs individuals, comparing these findings with data obtained from HIV-1-infected patients and healthy donors. Our results indicate higher expression of TLR2 and TLR4 on DCs and monocytes, but at the same time a decreased production of cytokines. These results may contribute to a better understanding of immune-pathogenesis and the mechanisms of natural resistance associated with HIV-1 infection.

Material and Methods

Ethics statement. Following the ethical guidelines, all participants gave written informed consent before enrollment in the study. Study protocols were reviewed and approved by the institutional Research Ethics Boards at the Universidad de Antioquia-Colombia, and were conducted according to the Declaration of Helsinki.

Study population and diagnostics. Eleven HESNs were enrolled. All of them were confirmed to be HIV-1-seronegative using the standard test. Inclusion criteria included a history of multiple unprotected anal/vaginal penetrative sexual intercourses, more than five anal/vaginal intercourses with an HIV-1-infected individual during the previous six months, or an average of two weekly anal/vaginal intercourses for over four months within the last two years, and having had a negative HIV-1/2 ELISA test within one month before sampling [24,25]. All HESNs were considered highly exposed, according to the viral load of their HIV-1-infected partners (23,500-109,147 copies/ml). None of the HESNs individuals had a history of intravenous drug use. Whenever possible, the HIV-1-infected partner was also recruited and the serological status was confirmed by western blot. Thirty-one HIV-1-infected patients, without active infections in the past 6 months, were included in the study. Viral load, CD4+ T-cell counts and the highly active antiretroviral therapy (HAART) status are presented in Table 1. Thirteen HIV-1-seronegative healthy donors without known risk factor of HIV-1 infection and without illness during the past three months were included in the study as controls; they were volunteers from the general population. Patients with cancer, individuals with low hemoglobin values (less than 8 g/dl), and pregnant women were excluded. The HIV-1 loads and the CD4+ T-cell counts were performed as we previously reported [24,25].

Specimen collection and *in vitro* stimulation. Fresh whole-blood sample collection and PBMC isolation were performed as previously reported [12,23]. PBMC enumeration and viability were assessed by trypan blue exclusion. PBMC ($2x10^5$) were incubated for 20 h with RPMI-1640 medium alone (negative control) or stimulated with TLR agonists: TLR2/TLR6, 10 nM palmitoyl-2-cysteine-serine-lysine-4 (Pam_2CSK_4),

TLR2/TLR1, 10 nM palmitoyl-3-cysteine-serine-lysine-4 (Pam_3CSK_4), TLR4, 10 ng/ml ultrapure LPS from *Escherichia coli* 0127:B8, TLR9, 10 μM CpG-B, TLR7/8, 5 μM R848 or TLR3, 5 μg/ml poly(I:C) at 37°C in 5% CO_2 (all ligands were from Invivogen, San Diego, CA). The TLR agonists and their concentration were used as in previous studies to induce pro-inflammatory cytokine production [8,9]. Three or 16 h of incubation were selected to maximize RNA and protein yield for targeted immune outputs, respectively. After incubation, cell pellets and supernatants were harvested by centrifugation at 2,000 rpm for 5 min. Cell pellets used for RNA isolation and culture supernatants were cryo-preserved at -80°C to be later thawed for cytokine quantification. For *ex vivo* TLR expression assays, fresh PBMCs were used immediately after purification.

Monoclonal antibodies. The following monoclonal antibodies were obtained from BD Biosciences: Lin1 FITC (CD3, CD14, CD16, CD19, CD20, and CD56 cocktail), and CD123 PE-Cy5 and CD11c PE-Cy5. TLR2 (clone TLR2.1), and TLR4 (clone HTA125) were phycoerythrin conjugates (eBiosciences, San Diego, CA). Anti-BDCA-2 FITC and FcR blocking reagents were from Miltenyi Biotec, Auburn, CA. Conjugated isotype-control antibodies served as controls.

Flow cytometry analysis. Flow cytometry was used to evaluate TLR expression in monocytes, NK cells, myeloid-DCs (mDCs), and plasmacytoid-DCs (pDCs), following the protocol reported previously [8,9]. Logical gating was used to identify monocyte (CD14+), pDC (BDCA-2+/$CD123^{high}$), mDC (Lin1-/$CD11c^{high}$) and NK cell ($CD56^{bright}$; $CD56^{low}$; and CD16+CD56-) populations. For every case, at least 100,000 events were acquired and gated as PBMCs based on forward-scatter and side-scatter properties. The events acquired were analyzed using the FACSDiva software. TLR expression is expressed as the mean fluorescence intensity (MFI) of the overall cell subpopulation; fluoscence minus one controls were used.

RNA extraction, reverse transcription and qPCR. An optimized quantitative real-time PCR (qPCR) assay was used to quantify the mRNA, as previously described [8,9,26]. A list of primer sequences used to detect immune and housekeeping gene mRNAs is presented in Supporting information 1.

Cytokine quantification. Concentrations (pg mL^{-1}) of IL-1β, IL-6, IL-10 and TNF-α in plasma and PBMC culture supernatants were determined using a commercial ELISA assay (BD Biosciences) according to the manufacturer's instructions. All samples were run in duplicate. Cytokine concentration was calculated from a standard curve of the corresponding recombinant human cytokine.

Statistical analysis. Data were plotted and analyzed using the Prism 5.0 software (Graph Pad Software, CA). All the results of the *in vitro* assays represent at least three independent experiments. Normality was considered by the Shapiro-Wilks and the Levene's test was used to evaluate the equality of variances. The Kruskal-Wallis test were used to assess the statistical significance of TLR expression or function in the different groups, when more than three independent data sets were available. As *post hoc* Dunn's post-tests were done when significant differences were observed in Kruskal-Wallis test, in order to correct for multiple

comparisons problem. Values of $p<0.05$ (*) were considered significant and values of $p<0.01$ (**) were considered highly significant.

Results

HESNs present higher percentage and absolute number of DCs in peripheral blood

The demographics of the individuals examined are presented in Table 1. To gain insight in the role that DCs could play during HIV-1 infection, quantification of the cellular subpopulations was established to assess the possible differences among subject groups. Interestingly, HESNs have significantly higher amounts of mDCs/pDCs in the peripheral blood, compared to both HIV-1-infected patients and healthy donors (Fig. 1). Furthermore, in agreement with previous studies [27], the amount of CD16+CD56- NK cells was significantly higher in HIV-1-infected patients, compared to HESNs and healthy donors, while no statistically significant differences were observed between other NK cell subpopulations or monocytes, between the groups (data not shown).

DCs and monocytes from HESNs express high levels of TLR2 and TLR4

Recently, we have reported altered expression of TLR2 and TLR4 in mDCs, pDCs and monocytes from HIV-1-infected patients [8,9]. Here, we aim to explore the expression of these TLRs on DCs, monocytes and NK cells from HESNs, HIV-1-infected individuals and healthy donors. No significant changes in either TLR expression was observed in NK cell subsets, as previously reported [28], when comparing all the participants in the study (data not shown). However, TLR2 expression was significantly higher in monocytes, mDCs and pDCs of HESNs individuals compared to HIV-1-infected patients and healthy donors (Fig. 2A). Regarding TLR4, a significantly higher expression level was observed in monocytes, mDCs and pDCs of HESNs compared to healthy donors (Fig. 2B). In conclusion, our findings suggest important changes in TLR2 and TLR4 expression on antigen-presenting cells (APCs) from HESNs, which could be involved in the inflammatory response.

HESNs have significantly lower mRNA levels of TLR2, TLR4, TLR8, NOD2, RIG-I and the pro-inflammatory cytokines TNF-α, IL-1β and IL-18

Subsequently, we examined whether the TLR mRNA levels are linked to the higher TLR2 and TLR4 protein expression observed by flow cytometry in HESNs. Other PRRs and several cytokines were also included in the study to obtain a general overview of the expression pattern and activation of the innate immunity receptors. To perform these experiments, total RNA obtained from PBMCs and purified monocytes from HESNs, HIV-1-infected patients, and healthy donors were analyzed by qRT-PCR using specific primers

(Supporting information). Concerning TLR mRNAs, significantly lower expression of TLR4 and TLR8 was observed in PBMCs from HESNs, compared to HIV-1-infected patients; however no differences were observed when compared to healthy donors (Fig. 3). In addition to a change in the expression profile of some TLRs, a significantly higher level of expression of NOD2 in PBMCs from HIV-1 patients versus those from HESNs was also visible (Supporting information 2). When the pro-inflammatory cytokine mRNAs were quantified, a significant decrease in the expression of the IL-1β, IL-18 and TNF-α mRNAs was observed in HESNs as compared to only HIV-1-infected patients (Fig. 4). Based on these results, we propose a possible functional link between the modulation of PRR expression and the inflammatory status.

Since by flow cytometry a significant increase in the expression of TLR2 and TLR4 in monocytes was observed, these results were further verified using purified monocytes. Monocytes were therefore purified using magnetic beads (CD14 MicroBeads® Miltenyi Biotechs) followed by adherence to culture plates. The mRNA level of TLRs, NLRs and RLRs and of pro-inflammatory cytokines, such as IL-1β, IL-6, IL-18 and TNF-α was then quantified. However, no significant changes were observed between the study groups, in the expression of either PRRs or pro-inflammatory cytokines. For DCs, it was not possible to measure the mRNA levels, due to the low frequency of mDCs and pDCs in peripheral blood. We then quantified the levels of pro-inflammatory cytokines in the plasma of HESNs, HIV-1-infected patients and healthy donors, so as to complete our description of the recognition and activation of the innate immunity receptors. Interestingly, HESNs and healthy donors showed significantly lower levels of IL-1β, IL-6, and of the anti-inflammatory cytokine IL-10 compared to HIV-1-infected patients (Fig. 5). These findings suggest that HESNs individuals present a lower inflammatory response that might due at least in part to alterations in PRR expression on APC.

Monocytes from HESNs increase IL-6 production in response to TLR2 and TLR9 ligands

To determine whether TLR triggering promotes cytokine expression, the *in vitro* production of IL-1β, IL-6, IL-10, and TNF-α in response to TLR triggering with specific agonists, in cells obtained from HESNs, HIV-1-infected patients and healthy donors was examined. For these experiments, PBMCs and purified monocytes were stimulated with TLR agonists. After 20 h of culture, the cytokines released were measured in the supernatants by ELISA. Based on the *ex vivo* results, lower TLR activation in cells from HESNs, compared to both HIV-1-infected patients and healthy donors was expected. Indeed, the results shown a low production of IL-1β, in response to TLR2, TLR3 and TLR9 agonists in PBMCs from HESNs, compared to those from healthy donors (Figure 6A); however, no statistical significance were noted for IL-1β production and other cytokines such as IL-6 and IL-10 (Data not shown). As observed in Figure 6B, a statistically significant increase in the accumulation of IL-6 was observed in monocytes from HIV-1-infected patients triggered with Pam_2CSK_4 compared to healthy controls. This was also observed in monocytes from HESNs and from HIV-1-infected patients triggered with CpG-B, when compared to healthy controls (Figure 6B). No statistical

differences were noted for IL-10 (Figure 6C) or IL-1β and TNF-α (data no shown) in response to TLR activation, among HIV-1-infected patients and HESNs individuals enrolled in this study.

Discussion

The role of innate immunity receptors has not been widely studied in HESNs individuals, even though this can lead to the description of important facts for a comprehensive understanding of natural resistance to HIV-1 infection. Current studies have reported an association between HIV-1 resistance and genetic polymorphisms in innate receptors including TLR2, TLR4, TLR9, and particularly TLR3, which in European HESNs individuals, has been linked to higher expression of immune activation factors [12,29,30]. Similarly, other reports suggest that immune activation and inflammation could be protective, reducing susceptibility to HIV-1 infection. Nevertheless, most of these studies have focused on adaptive immunity, including CD4+ and CD8+ T-cells [31-33]. Here, we evaluated the expression level and function of a wide range of PRRs, including TLRs, NLRs and RLRs, in primary cells (monocytes, DCs, and NK cells) obtained from HIV-1-infected patients, HESNs and healthy donors.

As previously reported [34,35], our initial analysis indicated that HESNs present a significant increase in mDC and pDC counts compared to HIV-1-infected patients and healthy donors. Since the main function of these cells is to present antigens to T cells, they act as link between the innate and adaptive immune systems and help to fight against infection. In contrast to previous reports [34,36], no differences were observed in NK cell counts, among the groups of donors studied here. However, when the NK cell subpopulations were evaluated, an increase in an unusual NK cell subset (CD56-/CD16+) was observed, in agreement with previous studies [27]. Similarly, we show that HESNs exhibit higher levels of CD4+ T-cells compared to HIV-1-infected patients, as previously reported [36]. The cellular subset alterations observed in HIV-1-infected patients, have been associated with impaired immune response after HIV-1 infection itself; however, this could be possibly related to the natural resistance to HIV-1 infection exhibited by HESNs individuals, as previously reported [14,37,38]

The PRRs trigger the innate immune response, and the TLRs are the best-characterized. Since previous studies have shown that TLR expression is altered within the context of HIV-1 infection [8,9], in this study we focused on the expression of TLRs on APCs from HESNs, HIV-1-infected patients and healthy donors. Notably, TLR2 and TLR4 expression in monocytes and DCs was significantly higher in HESNs compared to HIV-1-infected patients. In contrast, low transcriptional levels for both TLRs were observed in PBMCs from HESNs individuals, indicating a negative-feedback regulation on protein-mRNA balance. Songok et al. reported similar results in the HESNs of female sex workers for TLR2, TLR4, and TLR8 using microarray analysis [39]. To achieve a general overview of the innate immunity recognition system, other PRRs were

also analyzed in the present study. PBMCs from HESNs express low levels of mRNAs for TLR4, TLR8, and NOD2, compared to PBMCs from HIV-1-infected patients. However, no significant differences were observed in the transcription levels of the PRRs evaluated using purified monocytes, which is comparable to what was previously reported for TLR4 mRNA in HESNs and healthy donors [10]. In addition, Wu et al., reported a global down-regulation of genetic networks including TLR4, TLR8, IL-1β, and IL-8, using *Illumina* microarray in purified monocytes from HIV-1-progressor patients, compared to HIV-1-infected patients who control viral replication (long-term non-progressor) [40]. Even though HESNs have higher amount DCs than healthy donors, and higher surface expression of TLR2 and TLR4 on monocytes and DCs, there is no difference in mRNA expression. Since we observed higher expression of NOD2 in HIV-1-infected patients, we suggested that this could explain, at least in part, the susceptibility to HIV-1 infection. NOD2 is constitutively expressed in genital mucosa and up-regulated after microbial stimuli [41]; furthermore, it was previously reported that NOD2 activation favors DCs susceptibility to HIV-1 infection and promotes *cis*-infection of CD4+ T-cells [42]. Consequently, a lower level of NOD2 at entry sites (such as in mucosal membranes for example) will decrease the ability of DCs to promote HIV-1 infection, resulting in resistance to infection as observed in HESNs.

Following the recognition of specific agonists by PRRs, different signaling pathways are activated, promoting the expression of both pro- and anti-inflammatory cytokines. HESNs exhibited lower mRNA levels of pro-inflammatory cytokines, such as TNF-α, IL-1β, and IL-18, which are potent regulators of inflammation. Based on our observation showing differences in the mRNA cytokine pattern in PBMCs from HESNs and HIV-1-infected patients, we expanded our comparison evaluating cytokine production in the plasma. Lower concentration of IL-1β, as well as of IL-6 was observed in the plasma from HESNs, compared to HIV-1-infected patients, as previously reported in a cohort of Kenyan HESNs individuals [19]. In addition, prior studies have shown elevated levels of IL-18, IL-1β, IL-2, IL-6, IL-7, IL-8, IL-15, MCP-1, TNF-α, and IFN-γ in HIV-1-infected patients compared to uninfected controls [43,44]. This could be associated with the immune-quiescence status previously proposed for HESNs, suggesting an association between low inflammation markers and HIV-1 protection [4,16-22,45]. Moreover, our results have shown a low concentration of IL-10 in HESNs, compared to HIV-1-infected patients, suggesting an immunological imbalance, as previously reported in HIV-1-infected patients [46-48]. Taken together, our results suggest that HESNs present a lower inflammatory response, which could be due to altered PRR expression on APC. Thus, and as was previously proposed by other authors [13], our current evidence suggests that a balance in cytokine production may contribute to maintaining innate immune-quiescence and may play an important role in the control of excessive immune activation and inflammation that could favor susceptibility to HIV-1 infection.

Finally, we show that TLR stimulation results in similar cytokine production in HESNs, HIV-1-infected patients, and healthy donors. However, other authors have reported in HESNs a link between higher response

to TLR ligand stimulation and production of IL-1β, IL-6, TNF-a, and CCL3 release, compared to healthy donors [10]. The authors suggest that these receptors could influence the induction of a strong adaptive antiviral immune response. Even high immune activation reported in HIV-1-infected patients has no effect on the cellular response to TLR ligands, as previously reported [47]. Despite higher expression of TLR2 and TLR4 on monocytes and DCs from HESNs, they do not secrete more cytokines such as IL-1β, IL-6 and IL-10 than healthy donors. Based on the modulation of PRR expression observed in HESNs, we propose a differential cytokine response after PRR stimulation; however, functional studies were performed on total PBMCs because a mixed-cell culture more accurately reflects what might happen *in vivo*. Nevertheless, the expression pattern of PRRs varies between cell types, and analyses of purified cells are required to improve our understanding of the dynamics of the innate immune response in HESNs.

Together, our *in vitro* and *ex vivo* results suggest that HIV-1 exposure without infection is not associated with inflammatory responses, compared to HIV-1-infected patients. Despite the study limitations, *e.g.* a low number of patients enrolled, it will be important to examine the mechanisms involved in the natural resistance to HIV-1 infection, as well as to define the cell subsets responsible for these differences, in order to propose immune-prophylactic/therapeutic approaches. In conclusion, we show that HESNs have a distinct pattern of PRR expression and cytokine production compared to those from HIV-1-infected patients and healthy donors, and this could contribute to the immune quiescence phenotype observed in HESNs individuals. We consider that a low innate immune response against HIV-1 challenge, including pro-inflammatory cytokines, could be involved in protection against HIV-1 infection, by preventing infiltration of activated target cells.

References

1. HIV/AIDS JUNPo, (UNAIDS) (2013) 2013 UNAIDS Report on the global AIDS epidemic. Switzerland: Joint United Nations Programme on HIV/AIDS (UNAIDS).

2. Fowke KR, Nagelkerke NJ, Kimani J, Simonsen JN, Anzala AO, Bwayo JJ, et al. Resistance to HIV-1 infection among persistently seronegative prostitutes in Nairobi, Kenya. Lancet. 1996; 348: 1347-1351.

3. Makedonas G, Bruneau J, Alary M, Tsoukas CM, Lowndes CM, Lamothe F, et al. Comparison of HIV-specific CD8 T-cell responses among uninfected individuals exposed to HIV parenterally and mucosally. Aids. 2005; 19: 251-259.

4. Begaud E, Chartier L, Marechal V, Ipero J, Leal J, Versmisse P, et al. Reduced CD4 T cell activation and in vitro susceptibility to HIV-1 infection in exposed uninfected Central Africans. Retrovirology. 2006; 3: 35.

5. Horton RE, McLaren PJ, Fowke K, Kimani J, Ball TB Cohorts for the study of HIV-1-exposed but uninfected individuals: benefits and limitations. J Infect Dis. 2010; 202 Suppl 3: S377-381.

6. Palsson-McDermott EM, O'Neill LA Building an immune system from nine domains. Biochem Soc Trans. 2007; 35: 1437-1444.

7. Krieg AM CpG motifs in bacterial DNA and their immune effects. Annu Rev Immunol. 2002; 20: 709-760.

8. Hernandez JC, Arteaga J, Paul S, Kumar A, Latz E, Urcuqui-Inchima S Up-regulation of TLR2 and TLR4 in dendritic cells in response to HIV type 1 and coinfection with opportunistic pathogens. AIDS Res Hum Retroviruses. 2011; 27: 1099-1109.

9. Hernandez JC, Stevenson M, Latz E, Urcuqui-Inchima S HIV type 1 infection up-regulates TLR2 and TLR4 expression and function in vivo and in vitro. AIDS Res Hum Retroviruses. 2012; 28: 1313-1328.

10. Biasin M, Piacentini L, Lo Caputo S, Naddeo V, Pierotti P, Borelli M, et al. TLR activation pathways in HIV-1-exposed seronegative individuals. J Immunol. 2010; 184: 2710-2717.

11. Barton GM Viral recognition by Toll-like receptors. Semin Immunol. 2007; 19: 33-40.

12. Sironi M, Biasin M, Cagliani R, Forni D, De Luca M, Saulle I, et al. A common polymorphism in TLR3 confers natural resistance to HIV-1 infection. J Immunol. 2012; 188: 818-823.

13. Yao XD, Omange RW, Henrick BM, Lester RT, Kimani J, Ball TB, et al. Acting locally: innate mucosal immunity in resistance to HIV-1 infection in Kenyan commercial sex workers. Mucosal Immunol. 2013.

14. Hernandez JC, Giraldo DM, Paul S, Urcuqui-Inchima S Involvement of neutrophil hyporesponse and the role of toll-like receptors in human immunodeficiency virus 1 protection. PLoS One. 2015; 10: e0119844.

15. Berkley S, Bertram K, Delfraissy JF, Draghia-Akli R, Fauci A, Hallenbeck C, et al. The 2010 scientific strategic plan of the Global HIV Vaccine Enterprise. Nat Med. 2010; 16: 981-989.

16. McLaren PJ, Ball TB, Wachihi C, Jaoko W, Kelvin DJ, Danesh A, et al. HIV-exposed seronegative commercial sex workers show a quiescent phenotype in the CD4+ T cell compartment and reduced expression of HIV-dependent host factors. J Infect Dis. 2010; 202 Suppl 3: S339-344.

17. Koning FA, Otto SA, Hazenberg MD, Dekker L, Prins M, Miedema F, et al. Low-level CD4+ T cell activation is associated with low susceptibility to HIV-1 infection. J Immunol. 2005; 175: 6117-6122.

18. Card CM, McLaren PJ, Wachihi C, Kimani J, Plummer FA, Fowke KR Decreased immune activation in resistance to HIV-1 infection is associated with an elevated frequency of CD4(+)CD25(+)FOXP3(+) regulatory T cells. J Infect Dis. 2009; 199: 1318-1322.

19. Lajoie J, Juno J, Burgener A, Rahman S, Mogk K, Wachihi C, et al. A distinct cytokine and chemokine profile at the genital mucosa is associated with HIV-1 protection among HIV-exposed seronegative commercial sex workers. Mucosal Immunol. 2012; 5: 277-287.

20. Card CM, Ball TB, Fowke KR Immune Quiescence: a model of protection against HIV infection. Retrovirology. 2013; 10: 141.

21. Jennes W, Evertse D, Borget MY, Vuylsteke B, Maurice C, Nkengasong JN, et al. Suppressed cellular alloimmune responses in HIV-exposed seronegative female sex workers. Clin Exp Immunol. 2006; 143: 435-444.

22. Chege D, Chai Y, Huibner S, Kain T, Wachihi C, Kimani M, et al. Blunted IL17/IL22 and pro-inflammatory cytokine responses in the genital tract and blood of HIV-exposed, seronegative female sex workers in Kenya. PLoS One. 2012; 7: e43670.

23. Huik K, Avi R, Pauskar M, Kallas E, Jogeda EL, Karki T, et al. Association between TLR3 rs3775291 and resistance to HIV among highly exposed Caucasian intravenous drug users. Infect Genet Evol. 2013; 20: 78-82.

24. Goh WC, Markee J, Akridge RE, Meldorf M, Musey L, Karchmer T, et al. Protection against human immunodeficiency virus type 1 infection in persons with repeated exposure: evidence for T cell immunity in the absence of inherited CCR5 coreceptor defects. J Infect Dis. 1999; 179: 548-557.

25. Zapata W, Rodriguez B, Weber J, Estrada H, Quinones-Mateu ME, Zimermman PA, et al. Increased levels of human beta-defensins mRNA in sexually HIV-1 exposed but uninfected individuals. Curr HIV Res. 2008; 6: 531-538.

26. Torres S, Hernandez JC, Giraldo D, Arboleda M, Rojas M, Smit JM, et al. Differential expression of Toll-like receptors in dendritic cells of patients with dengue during early and late acute phases of the disease. PLoS Negl Trop Dis. 2013; 7: e2060.

27. Mavilio D, Lombardo G, Benjamin J, Kim D, Follman D, Marcenaro E, et al. Characterization of CD56-/CD16+ natural killer (NK) cells: a highly dysfunctional NK subset expanded in HIV-infected viremic individuals. Proc Natl Acad Sci U S A. 2005; 102: 2886-2891.

28. Jost S, Altfeld M Evasion from NK cell-mediated immune responses by HIV-1. Microbes Infect. 2012; 14: 904-915.

29. Freguja R, Gianesin K, Del Bianco P, Malacrida S, Rampon O, Zanchetta M, et al. Polymorphisms of innate immunity genes influence disease progression in HIV-1-infected children. Aids. 2012; 26: 765-768.

30. Pine SO, McElrath MJ, Bochud PY Polymorphisms in toll-like receptor 4 and toll-like receptor 9 influence viral load in a seroincident cohort of HIV-1-infected individuals. Aids. 2009; 23: 2387-2395.

31. Biasin M, Caputo SL, Speciale L, Colombo F, Racioppi L, Zagliani A, et al. Mucosal and systemic immune activation is present in human immunodeficiency virus-exposed seronegative women. J Infect Dis. 2000; 182: 1365-1374.

32. Jennes W, Sawadogo S, Koblavi-Deme S, Vuylsteke B, Maurice C, Roels TH, et al. Cellular human immunodeficiency virus (HIV)-protective factors: a comparison of HIV-exposed seronegative female sex workers and female blood donors in Abidjan, Cote d'Ivoire. J Infect Dis. 2003; 187: 206-214.

33. Tran HK, Chartier L, Troung LX, Nguyen NN, Fontanet A, Barre-Sinoussi FE, et al. Systemic immune activation in HIV-1-exposed uninfected Vietnamese intravascular drug users. AIDS Res Hum Retroviruses. 2006; 22: 255-261.

34. Montoya CJ, Velilla PA, Chougnet C, Landay AL, Rugeles MT Increased IFN-gamma production by NK and CD3+/CD56+ cells in sexually HIV-1-exposed but uninfected individuals. Clin Immunol. 2006; 120: 138-146.

35. Kaushik S, Teque F, Patel M, Fujimura SH, Schmidt B, Levy JA Plasmacytoid dendritic cell number and responses to Toll-like receptor 7 and 9 agonists vary in HIV Type 1-infected individuals in relation to clinical state. AIDS Res Hum Retroviruses. 2013; 29: 501-510.

36. Singla A, Jacobs R, Schmidt R, Wanchu A, Arora S Increased Activity of NK Cells and Plasmacytoid Dendritic Cells in HIV-Exposed Seronegative (ESN) Individuals. World Journal of AIDS. 2012; 2: 6-16.

365 37. Taborda NA, Hernandez JC, Lajoie J, Juno JA, Kimani J, Rugeles MT, et al. Low Expression of
366 Activation and Inhibitory Molecules on NK Cells and CD4 T Cells Is Associated with Viral Control. AIDS
367 Res Hum Retroviruses. 2015; [Epub ahead of print].

368 38. Tomescu C, Seaton KE, Smith P, Taylor M, Tomaras GD, Metzger DS, et al. Innate activation of MDC
369 and NK cells in high-risk HIV-1-exposed seronegative IV-drug users who share needles when compared with
370 low-risk nonsharing IV-drug user controls. J Acquir Immune Defic Syndr. 2015; 68: 264-273.

371 39. Songok EM, Luo M, Liang B, McLaren P, Kaefer N, Apidi W, et al. Microarray analysis of HIV resistant
372 female sex workers reveal a gene expression signature pattern reminiscent of a lowered immune activation
373 state. PLoS One. 2012; 7: e30048.

374 40. Wu JQ, Sasse TR, Wolkenstein G, Conceicao V, Saksena MM, Soedjono M, et al. Transcriptome analysis
375 of primary monocytes shows global down-regulation of genetic networks in HIV viremic patients versus
376 long-term non-progressors. Virology. 2013; 435: 308-319.

377 41. Ghosh M, Shen Z, Fahey JV, Crist SG, Patel M, Smith JM, et al. Pathogen recognition in the human
378 female reproductive tract: expression of intracellular cytosolic sensors NOD1, NOD2, RIG-1, and MDA5 and
379 response to HIV-1 and Neisseria gonorrhea. Am J Reprod Immunol. 2013; 69: 41-51.

380 42. Cote SC, Plante A, Tardif MR, Tremblay MJ Dectin-1/TLR2 and NOD2 agonists render dendritic cells
381 susceptible to infection by X4-using HIV-1 and promote cis-infection of CD4(+) T cells. PLoS One. 2013; 8:
382 e67735.

383 43. Gay C, Dibben O, Anderson JA, Stacey A, Mayo AJ, Norris PJ, et al. Cross-sectional detection of acute
384 HIV infection: timing of transmission, inflammation and antiretroviral therapy. PLoS One. 2011; 6: e19617.

385 44. Haissman JM, Vestergaard LS, Sembuche S, Erikstrup C, Mmbando B, Mtullu S, et al. Plasma cytokine
386 levels in Tanzanian HIV-1-infected adults and the effect of antiretroviral treatment. J Acquir Immune Defic
387 Syndr. 2009; 52: 493-497.

388 45. Novis CL, Archin NM, Buzon MJ, Verdin E, Round JL, Lichterfeld M, et al. Reactivation of latent HIV-1
389 in central memory CD4(+) T cells through TLR-1/2 stimulation. Retrovirology. 2013; 10: 119.

390 46. Taborda NA, Catano JC, Delgado JC, Rugeles MT, Montoya CJ Higher SLPI expression, lower immune
391 activation, and increased frequency of immune cells in a cohort of Colombian HIV-1 controllers. J Acquir
392 Immune Defic Syndr. 2012; 60: 12-19.

393 47. Yonkers NL, Rodriguez B, Asaad R, Lederman MM, Anthony DD Systemic immune activation in HIV
394 infection is associated with decreased MDC responsiveness to TLR ligand and inability to activate naive CD4
395 T-cells. PLoS One. 2011; 6: e23884.

396 48. Keating SM, Golub ET, Nowicki M, Young M, Anastos K, Crystal H, et al. The effect of HIV infection
397 and HAART on inflammatory biomarkers in a population-based cohort of women. Aids. 2011; 25: 1823-
398 1832.

399

400 **Competing interests**

401 The authors have no financial conflict of interest.

402

Authors' contributions

JCH and SUI were responsible for the conception and design of the project, as well as the acquisition, analysis and interpretation of the data. The authors read and approved the final manuscript.

Acknowledgments

The authors thank the patients and control individuals who participated in this study and the collaboration of the personnel from the institutions where the patients were recruited. We acknowledge the help of Anne-Lise Haenni for critically reading and reviewing of the manuscript. The authors also acknowledge the technical assistance of Diana Giraldo, Lanie Ruiz and Maria Patricia García.

Sources of financial

This study was funded by COLCIENCIAS, grant number 111549326099, and Universidad de Antioquia (estrategia de sostenibilidad, 2014-2015). The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Table 1. Demographic features of enrolled HIV-1-infected patients, HIV-1-exposed seronegative donors, and healthy donors

	HIV-1-infected patients n= 31	**HIV-1-exposed seronegative donors (HESNs) n= 11**	**Healthy donors n= 13**
Age, years Median (Range)	38 (28-47)	38 (26-50)	28 (21-41)
Male : Female	23 : 8	3 : 8	6 : 7
Viral load in RNA copies/ml plasma Median (Range)	63700 (1261-109147)	N/A	N/A
With HAART[a] : Without HAART	21 : 10	N/A	N/A
CD4+ T-cell count cells/mm^3 peripheral blood[b] Median (Range)	304 (14-948)	997 (977-1433)	710 (617-1143)

[a]Patients undergoing HAART treatment were using combinations of nucleoside reverse transcriptase inhibitors (abacavir, lamivudine, didanosine, stavudine, and zidovudine), non-nucleoside reverse

transcriptase inhibitors (efavirenz and nevirapine), and protease inhibitors (lopinavir, fosamprenavir, amprenavir, nelfinavir, and saquinavir). [b]CD4+ T cell counts below 200 cells/µl were receiving fluconazol, acliclovir, and TMS as prophylactic drugs. HAART: highly active antiretroviral therapy; N/A: not applicable.

Figure Legends

Figure 1. Dendritic cell counts in healthy donors, HIV-1-infected patients and HESNs individuals. Myeloid dendritic cells (mDCs), defined as Lin1- $CD11c^{high}$ and plasmacytoid dendritic cells (pDCs), defined as BDCA-2+ $CD123^{high}$, were quantified from peripheral whole blood by flow cytometry, and using the total and differential leukocytes counts. Relative (percentage) and absolute numbers (number of cells per mm^3 peripheral blood) are shown. Comparisons were made by Kruskal-Wallis tests and Dunn's post-tests. The level of significance was $p<0.05$ (*), $p<0.01$ (**) and $p<0.001$ (***); median with ranges are shown. Healthy donors (n=13), HIV-1(+) (n=31) and HESNs (n=11).

Figure 2. Higher TLR2 and TLR4 expression in monocytes and DCs from HESNs individuals. Mononuclear cells were gated according to physical characteristics, excluding dead cells. TLR2 (A) and TLR4 (B) expression in monocytes ($CD14^+$), mDCs ($Lin1^-$ $CD11c^{high}$) and pDCs ($BDCA2^+$ $CD123^{high}$) from total PBMCs were measured by flow cytometry. Data are presented as overall MFI, after substracting the isotype-staining background. The MFI of TLR2 and TLR4 in monocytes, mDCs, and pDCs were plotted for each group: healthy donors (n=13), HIV-1-infected patients (n=31), and HESNs (n=11). Comparisons were performed using the Kruskal-Wallis tests and Dunn's post-tests. The level of significance was $p<0.05$ (*) and $p<0.01$ (**). Median with ranges are shown.

Figure 3. HESNs express lower TLR4 and TLR8 mRNA levels than HIV-1-infected patients. The mRNA levels of several TLRs were measured by using qRT-PCR, and normalized with the housekeeping genes β-actin, GAPDH and β2-microglobulin. Relative units of transcripts versus average of housekeeping gene transcripts are shown as median and range. Comparisons were performed by the Kruskal-Wallis tests and Dunn's post-tests. The level of significance was $p<0.05$ (*); median with ranges are shown. Healthy donors (n=13), HIV-1-infected patients (n=31) and HESNs (n=11).

Figure 4. HESNs express lower levels of the pro-inflammatory cytokines, like IL-1β, IL18, and TNF-α than HIV-1-infected patients. The mRNA of pro-inflammatory cytokines was measured using qRT-PCR and normalized with the housekeeping genes β-actin, GAPDH and β2-microglobulin. Relative

451 units of transcripts versus average of housekeeping gene transcripts are shown as median and range.
452 Comparisons were performed by the Kruskal-Wallis tests and Dunn's post-tests. The level of significance
453 was $p<0.05$ (*) and $p<0.01$ (**); median with ranges are shown. Healthy donors (n=13), HIV-1-infected
454 patients (n=31) and HESNs (n=11).

455 **Figure 5. HESNs express lower plasma levels of the pro-inflammatory cytokines such as IL-1β and**
456 **IL-6, and the anti-inflammatory cytokine like IL-10 than HIV-1-infected patients.** Plasma levels of
457 IL-1β, IL-6, IL-10 and TNF-α were quantified by ELISA. Comparisons were performed using the
458 Kruskal-Wallis tests and Dunn's post-tests. The level of significance was $p<0.05$ (*) and $p<0.01$ (**);
459 median with ranges are shown. Healthy donors (n=13), HIV-1-infected patients (n=31) and HESNs
460 (n=11).

461

462 **Figure 6. Cytokine release in PBMCs and Monocytes treated with TLR agonists.** IL-1β, IL-6, and IL-10
463 production was quantified by ELISA in the supernatants of PBMCs and purified monocyte cultures stimulated
464 with TLR2 agonists (10 nM Pam_2CSK_4 or 10 nM Pam_3CSK_4), the TLR4 agonist (10 ng/ml LPS), TLR3
465 agonist (5 μg/ml poly(I:C)), TLR7/8 agonist (5 μM R848) and TLR9 agonist (10 μM CpG-B), for 18 h at
466 37ºC and 5% CO_2. Comparisons were performed using the Kruskal-Wallis tests and Dunn's post-tests. The
467 level of significance was $p<0.05$ (*); median with ranges are shown.

468

469 **Supporting information 2. HESNs express lower NOD2 mRNA levels than HIV-1-infected patients.** The
470 mRNA of several NOD-like receptors and RIG-I-like receptors were measured using qRT-PCR and
471 normalized with the housekeeping genes β-actin, GAPDH, and β2-microglobulin. Relative units of transcripts
472 versus average of housekeeping gene transcripts are shown as median and range. Comparisons were
473 performed by the Kruskal-Wallis tests and Dunn's post-tests. The level of significance was $p<0.05$ (*).
474 Median with ranges are shown. Healthy donors (n=13), HIV-1-infected patients (n=31) and HESNs (n=11).