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Sex differences in the TLR-mediated response of pDCs to HIV-1 are associated with higher immune activation in infected women

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Abstract

Manifestations of viral infections can differ between women and men¹, and significant sex differences have been described in the course of HIV-1 disease. HIV-1-infected women tend to have lower viral load levels early in HIV-1 infection, but progress faster to AIDS for a given viral load than men^{2–7}. Here we demonstrate substantial sex differences in the response of plasmacytoid dendritic cells (pDCs) to HIV-1. pDCs derived from women produce significantly more interferon- α (IFN- α) in response to HIV-1-encoded TLR7 ligands than pDCs derived from men, resulting in stronger secondary activation of CD8+ T cells. In line with these *in vitro* studies, treatment-naïve chronically HIV-1-infected women had significantly higher levels of CD8+ T cell activation than men after adjusting for viral load. These data show that sex differences in TLR-mediated activation of pDCs can account for higher immune activation in women compared to men at a given HIV-1 viral load, and provide a mechanism by which the same level of viral replication might result in faster HIV-1 disease progression in women compared to men. Modulation of the TLR7 pathway in pDCs may therefore represent a novel approach to reduce HIV-1-associated pathology.

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A.M. and J.J.C. conducted the *in vitro* experiments, data analysis and contributed to manuscript preparation; H.K.S., T.F.W. and R.J.L. also conducted the *in vitro* experiments; E.S.C., R.J.B., O.L., and D.M. contributed to the statistical analysis and interpretation of the data; A.M., J.J.C., R.J.B., G.A., H.S. and M.A. participated in the planning of the experiments; S.K. and M.C. conducted the genetic polymorphism experiments; S.B. helped with the enrollment of study subjects, R.B.P. and G.K.R. provided the data for ACTG 384; J.D.L. provided the AT-2 virus and vesicle controls used in the *in vitro* experiments; and M.A. planned the studies, prepared the manuscript and supervised the project.

According to UNAIDS, almost half of all HIV-1-infected individuals worldwide are women. Studies comparing the course of HIV-1 infection between women and men have demonstrated significant sex differences in the manifestations of HIV-1 disease. While HIV-1-infected women present with lower viral load early in HIV-1 infection, women with the same HIV-1 viral load as men have a 1.6-fold higher risk of developing AIDS^{2–7}. The mechanisms underlying these significant sex differences in the manifestation of HIV-1 disease are not understood.

There is increasing consensus that the level of immune activation in HIV-1-infected subjects is a strong independent predictor for HIV-1 disease progression^{8–16}. Plasmacytoid dendritic cells (pDCs) play a central role in this HIV-1-induced activation of the immune system, as they can sense HIV-1 ssRNA via Toll-like receptor (TLR)^{17–20}. Interestingly, PBMCs derived from women have been shown to produce significantly more IFN- α in response to the synthetic TLR7 ligand Imiquimod than PBMCs derived from men²¹. We therefore reasoned that sex differences in HIV-1-induced immune activation might be responsible for the observed differences in HIV-1 disease, and investigated differences in cytokine production by PBMC in response to HIV-1 between men and women, and their consequences for T cell activation.

Intracellular cytokine staining (ICS) using multiparameter flow cytometry was performed to quantify the percentage of pDCs producing IFN- α or TNF- α after stimulation with HIV-1-derived TLR7/8 ligands, TLR9 ligand ODN2216 (CpG-A), or inactivated HIV-1 virus (AT-2 virus) (Fig. 1). A significantly higher percentage of pDCs derived from women produced IFN- α in response to HIV-1-derived TLR ligands or AT-2 virus as compared to pDCs derived from men (Fig. 1a–c). In line with previous reports²¹ we detected no significant sex difference in the percentage of IFN- α -producing pDCs in response to the TLR9 ligand (Fig. 1a,b). In contrast to the significant differences in IFN- α production by pDCs, the mean percent of pDCs responding with TNF- α -production to stimulation with HIV-1-derived TLR7/8 ligands, while slightly higher, did not significantly differ between women and men (Fig. 1d,e), suggesting that the differences in cytokine production that we observed were mediated by a signaling event downstream of TLR7. The sex differences in IFN- α -producing pDCs were not due to differences between the studied men and women in race/ethnicity, the frequencies of described polymorphisms within the genes encoding for TLR7, MyD88 and IRF7, or pDC numbers (data not shown). Furthermore, neither HIV-1-induced cytokine production by mDCs nor monocytes differed between women and men ($P > 0.4$ for all comparisons). In summary, these data demonstrate significant sex differences in the IFN- α pDC response to HIV-1-encoded TLR7/8 ligands and inactivated HIV-1.

Progesterin has recently been demonstrated to modulate pDC function *in vitro*²², suggesting that pDC function may be modulated by sex hormones. In our study, IFN- α production by pDCs in response to HIV-1-encoded TLR7/8 ligands did not differ between women who reported the use of oral contraceptives and those who did not ($p = 0.79$). However, plasma progesterone levels were significantly correlated to the percentage of IFN- α ⁺ pDCs following stimulation with the HIV-1-derived TLR7/8 ligand GagRNA₁₁₆₆ (Fig. 2, $R = 0.57$, $P = 0.02$). Furthermore, there was a trend towards lower percentages of IFN- α -producing pDCs in response to TLR7/8 ligands in post-menopausal women compared to pre-menopausal women (mean 16.9% vs. 27.6%, $P = 0.08$, data not shown), overall suggesting that sex hormone levels can modulate the ability of pDCs to produce IFN- α in response to TLR7 stimulation.

IFN- α is a central cytokine in activating an antiviral immune response and higher circulating levels of IFN- α are an important prognostic indicator for HIV-1 clinical progression^{8,23–25}. Furthermore, IFN- α has been shown *in vitro* to induce up-regulation of CD38 on CD8⁺ T cells²⁶, a strong independent predictor of HIV-1 disease progression^{8–10}. In line with this observation, CD8⁺ T cells significantly up-regulated CD38-expression following stimulation

of PBMC with HIV-1-derived TLR7/8 ligands or AT-2 virus *in vitro* ($P < 0.001$, Fig. 3a). Furthermore, the expression of CD38 on CD8⁺ T cells following incubation with HIV-1-encoded TLR7/8 ligands and AT-2 virus was higher in women compared to men (Fig. 3b). This *in vitro* activation of CD8⁺ T cells was at least partially mediated by IFN- α (Fig. 3c,d). Based on these data demonstrating sex differences in HIV-1-induced immune activation *in vitro*, we hypothesized that treatment-naïve, chronically HIV-1-infected women will display higher levels of CD8⁺ T cell activation than men with comparable HIV-1 viral loads.

To test this hypothesis, *ex vivo* T cell activation was quantified on pre-treatment samples from chronically HIV-1-infected subjects enrolled into the ACTG 384 study, including 109 treatment naïve women and 514 treatment naïve men^{27,28}. In ACTG 384, women had higher CD4⁺ T cell counts (340 vs. 273 mm⁻³, $P = 0.002$) and lower HIV-1 RNA (4.7 vs. 5.0 log₁₀ copies ml⁻¹, $P < 0.001$) at enrollment, prior to initiation of antiretroviral therapy²⁷. In line with previous reports, the level of immune activation, defined by the percentage of CD38⁺HLA-DR⁺CD8⁺ T cells, was significantly associated with HIV-1 viral load at baseline ($R = 0.24$, $P < 0.001$), but did not significantly differ between women and men at baseline. However, after adjusting for baseline HIV-1 viral load, the percentage of CD38⁺HLA-DR⁺CD8⁺ T cells, but not CD38⁺HLA-DR⁺CD4⁺ T cells, was significantly higher in women compared to men ($P = 0.28$ for CD4⁺ T cells, Fig. 4a; and $P = 0.006$ for CD8⁺ T cells, Fig. 4b). When race/ethnicity or intravenous drug use were included into the model, CD8⁺ T cell activation remained significantly higher in women compared to men adjusting for HIV-1 viral load. The higher CD8⁺ T cell activation in women compared to men (average of 4.6% more CD38⁺HLA-DR⁺CD8⁺ T cells) corresponds to the effect of approximately one log₁₀ higher HIV-1 RNA. These *in vivo* data are consistent with the *in vitro* studies demonstrating significant differences in immune activation mediated by sex differences in the pDC response to HIV-1 ssRNA-encoded TLR7 ligands, and provide for the first time a mechanism by which the same HIV-1 viral load can result in higher immune activation and faster progression to AIDS^{3,29} in women compared to men.

Chronic T cell activation is a hallmark of progressing HIV-1 disease in humans and SIV disease in rhesus macaques, and the expression of immune activation markers such as HLA-DR or CD38 on CD8⁺ T cells has been demonstrated to independently predict the rate of HIV-1 disease progression^{8–10,30,31}. The stimulation of the innate immune system and in particular, the chronic production of IFN- α , has been suggested to play a crucial role in driving this generalized immune activation and disease progression in HIV-1-infected individuals^{16,18,23,24,32–34}. Furthermore, IFN- α has been suggested to be a key mediator for SIV pathogenesis in rhesus macaques^{35,36}, and pDCs from rhesus macaques, similar to the results in humans, produce large amounts of IFN- α when stimulated with SIV or HIV-1. In contrast, sooty mangabeys, the natural host of SIV that do not progress to AIDS³⁷, have significantly lower levels of T cell activation in chronic SIV infection, and pDCs of sooty mangabeys have been reported to produce less IFN- α in response to SIV³⁵. These data suggest that persistent IFN- α production by pDCs in response to SIV or HIV-1 is a central factor in mediating virus-induced immune activation, and resulting pathogenesis in chronic infection.

Significant differences in the manifestations of HIV-1 disease have been previously reported between females and males, with women experiencing a significantly increased risk of developing AIDS compared to men for the same level of HIV-1 replication. Here we demonstrate for the first time that treatment-naïve, chronically HIV-1-infected women also have significantly higher levels of CD8⁺ T cell activation compared to men for the same HIV-1 viral load. In an attempt to elucidate a mechanism underlying this sex difference in the level of immune activation induced by the same amount of HIV-1 RNA, we demonstrate that pDCs from women produce significantly higher levels of pro-inflammatory IFN- α in response to HIV-1 than pDCs from men, and that these higher levels of IFN- α secretion can lead to higher

levels of CD8⁺ T cell activation. These data are consistent with a model in which higher TLR7-mediated responsiveness to HIV-1 RNA in women contributes to a more rapid HIV-1 disease progression in the presence of persistent chronic viral replication.

In conclusion, we demonstrate sex differences in the IFN- α production by pDCs following stimulation with HIV-1 ssRNA that translate into enhanced CD8⁺ T cell activation *in vitro* and *in vivo* for the same amount of HIV-1 RNA in women compared to men. This enhanced immune activation in women can explain the previously reported clinical observation that women have a higher risk for HIV-1 disease progression than men during chronic infection at a given HIV-1 viral load. Modulation of the TLR7 pathways in pDCs could therefore represent a novel approach to reduce HIV-1-associated pathogenesis, and might have implications that go beyond HIV-1 infection, as differential susceptibility to several RNA viruses have been described for men and women¹, and autoimmune diseases that show sex differences in their incidence, such as systemic lupus erythematosus, have also been demonstrated to involve the TLR7/8 pathway^{38,39}.

Material and Methods

Study subjects

ACTG 384 recruited 980 antiretroviral naïve HIV-1-infected subjects²⁸. A subset of subjects (514 men and 109 women) followed at USA sites underwent comprehensive immunologic assessments which included the analysis of CD4⁺ and CD8⁺ T cell activation by flow cytometry. These included 287 white non-hispanic individuals, 240 black non-hispanic individuals, and 96 other. 578 of the 623 study subjects never reported use of intravenous drugs, and 48 reported previous or current use of intravenous drugs. Furthermore, samples from 63 HIV-1-negative subjects enrolled at Massachusetts General Hospital (MGH) were included in this study. Pre-menopausal women in the study were less than 40 years old, while the average age of post-menopausal women was 60 years. When available, information on use of oral contraceptives containing sex hormones were collected, and 43% of pre-menopausal women reported the use of oral contraceptives. All subjects gave informed consent for participation in these studies. The study was approved by the Partners Institutional Review Board (IRB) and the ACTG 384 study was approved by local IRBs.

Analysis of polymorphisms within TLR7, MyD88 and IRF7

The coding regions of MyD88 and IRF7 genes were amplified from genomic DNA using primers listed in Supplementary Table 1. In addition, the genomic sequence flanking the TLR7 SNPs rs179008 and rs2302267 that have been implicated in HIV-1 and HCV disease progression, were also amplified (see Supplementary Table 1 for primer sequences). Briefly, 10–20 ng of genomic DNA was amplified in 10 μ l reactions (200nm dNTPs, 1.5 mM MgCl₂, 0.2 μ M of each primer). A modified stepdown program (annealing temperatures given in Supplementary Table 1) was used for amplification of IRF7 and MyD88 in order to ensure precise amplification. TLR7 SNPs were amplified at an annealing temperature of 55 °C. PCR products were subjected to direct bidirectional cycle sequencing using BigDye® terminator v 1.1 and run on an ABI3730 sequencer.

Measurement of cytokine production on the single cell level using flow cytometry

One and a half million PBMC ml⁻¹ were stimulated with 15 μ g ml⁻¹ ssRNA [sequences HIV-1 ssRNA: Gag_{RNA1166} (UUGUUAAGUGUUUCAUUGU) and Gp160_{RNA2093} (UUUUUGCUGUACUUUCUAUA)] complexed with DOTAP (Roche), 1 μ g ml⁻¹ CL097 (Invivogen), or 5 μ M ODN2216 (Invivogen). Cells were also stimulated with aldrithiol-2 inactivated HIV-1 virus (AT-2, MN strain, lot P4097) and microvesicle controls (Ves, lot P4079; AIDS and Cancer Virus Program, SAIC Frederick, Inc, NCI Frederick). Unstimulated

cells served as negative controls. $5 \mu\text{g ml}^{-1}$ Brefeldin A (Sigma) was immediately added to each tube following the addition of TLR ligands in order to inhibit cellular cytokine release. Intracellular cytokine content of monocytes, mDCs, and pDCs was determined after 20 hours of incubation with the respective TLR ligands, as described previously²⁰. All samples were acquired on an LSR II (BD Biosciences). The percentage of cytokine producing pDCs, mDCs, and monocytes was determined by subsequent analysis using Flow Jo software (Treestar, Inc). An average of 92% of $\text{lin}^{\text{neg}}\text{CD11c}^{\text{neg}}\text{CD123}^{\text{bright}}\text{IFN-}\alpha^+$ pDCs also expressed CD303 (BDCA-2) (data not shown).

Measurement of *in vitro* T cell activation in HIV-1 negative men and women

Flow cytometric quantification of $\text{CD38}^{\text{high}}\text{CD4}^+$ and $\text{CD38}^{\text{high}}\text{CD8}^+$ T cells was performed on freshly isolated PBMCs from healthy HIV-1 negative controls following stimulation with HIV-1-derived ssRNA ligands, Vesicle control and AT-2 virus. For the *in vitro* IFN- α blocking assays, PBMC from HIV-1 negative individuals were pre-treated with antibodies directed against the IFN- α receptors 1/2 (anti-IFNAR1/2; Abcam and PBL Interferonsource) for 1 h at 37°C . PBMC were then stimulated with a TLR7/8 ligand (CL097 at $1 \mu\text{g ml}^{-1}$) or with media alone for 20 h. Furthermore, PBMC were stimulated *in vitro* with increasing amounts of recombinant human IFN- α ($1\text{--}100 \text{ ng ml}^{-1}$) for 20 h and T cell activation was measured as described above.

Measurement of *ex vivo* T cell activation in HIV-1-infected men and women enrolled into ACTG 384

Flow cytometric quantification of CD4^+ and CD8^+ T cell activation was performed using freshly isolated PBMCs from study participants at baseline, prior to the initiation of antiretroviral therapy, according to established ACTG protocols. Activated CD4^+ and CD8^+ T cells were defined by their expression of either CD4 or CD8, and the expression of CD38 and HLA-DR. Results are reported as the percentage of $\text{CD38}^+\text{HLA-DR}^+\text{CD4}^+$ T cells or percentage of $\text{CD38}^+\text{HLA-DR}^+\text{CD8}^+$ T cells.

Statistical Analysis

For the immunologic assays on HIV-1 negative individuals, two tailed student's *t*-tests and Wilcoxon Rank tests (Mann Whitney or Wilcoxon signed rank test for paired samples) were used to determine statistically significant differences. For ACTG study, continuous outcomes were compared between men and women with Wilcoxon Rank-Sum tests. Rank-based (Spearman) correlations were used to assess correspondence between responses. Multiple regression models were used to assess the association between activation percent and sex adjusting for HIV-1 viral load. All statistical tests were two-sided exploratory without adjustments for multiple testing at the 5% nominal level of significance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Sex differences in IFN- α production of pDC following TLR7/8 stimulation with HIV-1-derived ligands. **(a)** Representative flow plots demonstrating the IFN- α production by CD123⁺ pDCs derived from women (upper panel) and by CD123⁺ pDCs derived from men (lower panel) for unstimulated controls (left column), and following 20 h of stimulation with the HIV-1 derived TLR7/8 ligand Gag_{RNA1166} (middle column) or the TLR9 ligand ODN2216 (right column) are shown. The percentage of IFN- α producing pDCs is shown in the upper right corner of each plot. **(b)** The percentage of IFN- α ⁺ pDCs in men (shown in black, $n = 20$) as compared to women (shown in red, $n = 23$) following stimulation with the HIV-1-derived ssRNAs, Gag_{RNA1166} and Gp160_{RNA2093}, or the TLR9 ligand ODN2216 are shown. **(c)** The percentage of IFN- α ⁺ pDCs from men (shown in black, $n = 19$) and women (shown in red, $n = 22$) following

stimulation with AT-2 inactivated HIV-1 virus (AT-2) or vesicle control (Ves) is shown. **(d)** Representative flow plots demonstrating the TNF- α production by CD123⁺ pDCs are shown. The percentage of TNF- α producing pDCs is shown in the upper right corner of each plot. **(e)** The percentage of TNF- α ⁺ pDCs in men (shown in black, $n = 20$) and women (shown in red, $n = 23$) following stimulation with the HIV-1-derived ssRNAs, Gag_{RNA1166} and Gp160_{RNA2093}, or ODN2216 are shown. All horizontal bars represent mean value for that sample group.

Figure 2.

Impact of sex hormone levels on the IFN-α production by pDCs in response to TLR7/8 agonists. Plasma progesterone levels measured in 16 pre-menopausal women were significantly correlated to the percentage of IFN-α⁺ pDCs in response to stimulation with the HIV-1 derived TLR7/8 ligand Gag_{RNA1166}.

Figure 3.

Sex differences in CD38 expression on CD8⁺ T cells following stimulation with HIV-1-derived TLR7/8 ligands is dependent on IFN- α . (a) The percent CD38^{high} CD8⁺ T cells following 20 h of stimulation with HIV-1-derived TLR7/8 ligands (Gp160_{RNA2093} and Gag_{RNA1166}), inactive control ssRNA (Gag_{RNA1166A}), AT-2 virus, or vesicle control for all individuals is shown. (b) Differences in the percent CD38^{high} CD8⁺ T cells from men (shown in black) and from women (shown in red) following 20 h of stimulation of PBMCs with the HIV-1 derived TLR7/8 ligands, Gp160_{RNA2093} and Gag_{RNA1166}, inactive control ssRNA (Gag_{RNA1166A}), AT-2 virus, or vesicle control is shown. (c) The percentage of CD38^{high}CD8⁺ T cells above background in response to the TLR7 ligand CL097 was set to 100%, and the remaining activation in the presence of blocking antibodies against IFN- α receptors 1 and 2 is shown in the right column (CL097 + anti-IFNAR1/2). (d) Dose dependent increase in T cell activation as represented by percent of CD38^{high}CD8⁺ T cells was observed following stimulation of PBMCs from healthy donors with increasing amounts of human IFN- α ranging from 0–100 ng ml⁻¹. All horizontal bars represent mean value for that sample group.

Figure 4.

Sex differences in the CD4⁺ and CD8⁺ T cell activation of HIV-1-infected, treatment-naïve individuals. The association between sex and the percentage of CD38⁺HLA-DR⁺CD4⁺ (a) or CD38⁺HLA-DR⁺CD8⁺ (b) T cells adjusting for HIV-1 viral load is shown. HIV-1 RNA load measured at baseline in women (open red symbol) and men (closed blue symbol) are correlated to CD4⁺ and CD8⁺ T cell activation (correlation for women shown with red dotted line and for men with blue solid line).