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Sex differences in the Toll-like receptor–mediated response of plasmacytoid dendritic cells to HIV-1

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Manifestations of viral infections can differ between women and men[1], and marked sex differences have been described in the course of HIV-1 disease. HIV-1–infected women tend to have lower viral loads early in HIV-1 infection but progress faster to AIDS for a given viral load than men[2–7]. Here we show substantial sex differences in the response of plasmacytoid dendritic cells (pDCs) to HIV-1. pDCs derived from women produce markedly more interferon-α (IFN-α) in response to HIV-1–encoded Toll-like receptor 7 (TLR7) ligands than pDCs derived from men, resulting in stronger secondary activation of $CD8^+$ T cells. In line with these *in vitro* studies, treatment-naive women chronically infected with HIV-1 had considerably 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 may 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 new approach to reduce HIV-1–associated pathology.

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 considerable sex differences in the manifestations of HIV-1 disease. Whereas 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 major sex differences in the manifestation of HIV-1 disease are not understood.

There is increasing consensus that the degree of immune activation in HIV-1–infected subjects is a strong independent predictor for HIV-1 disease progression[8–16]. pDCs have a central role in this HIV-1–induced activation of the immune system, as they can sense HIV-1 single-stranded RNA via TLR7 (refs. 17–20). Notably, peripheral blood mononuclear cells (PBMCs) derived from women have been shown to produce markedly more IFN-α in response to the synthetic TLR7 ligand imiquimod than PBMCs derived from men[21]. 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 PBMCs in response to HIV-1 between men and women, as well as the consequences for T cell activation.

We used intracellular cytokine staining (ICS) by multiparameter flow cytometry to quantify the percentage of pDCs producing IFN-α or tumor necrosis factor-α (TNF-α) after stimulation with HIV-1–derived TLR7 and TLR8 (TLR7/8) ligands, TLR9 ligand ODN2216 (CpG-A) or aldrithiol-2–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 reports[21], 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 differences in IFN-α production by pDCs, the mean percentage of pDCs responding with TNF-α-production to stimulation with HIV-1–derived TLR7/8 ligands, although 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 or ethnicity, the frequencies of described polymorphisms within the genes encoding TLR7, myeloid differentiation factor-88 (MyD88) and interferon regulatory factor-7 (IRF-7) or pDC numbers (data not shown). Furthermore, neither HIV-1–induced cytokine production by myeloid dendritic cells (mDCs) nor that by monocytes differed between women and men ($P > 0.4$ for all comparisons). In summary, these data demonstrate major sex differences in the IFN-α pDC response to HIV-1–encoded TLR7/8 ligands and inactivated HIV-1.

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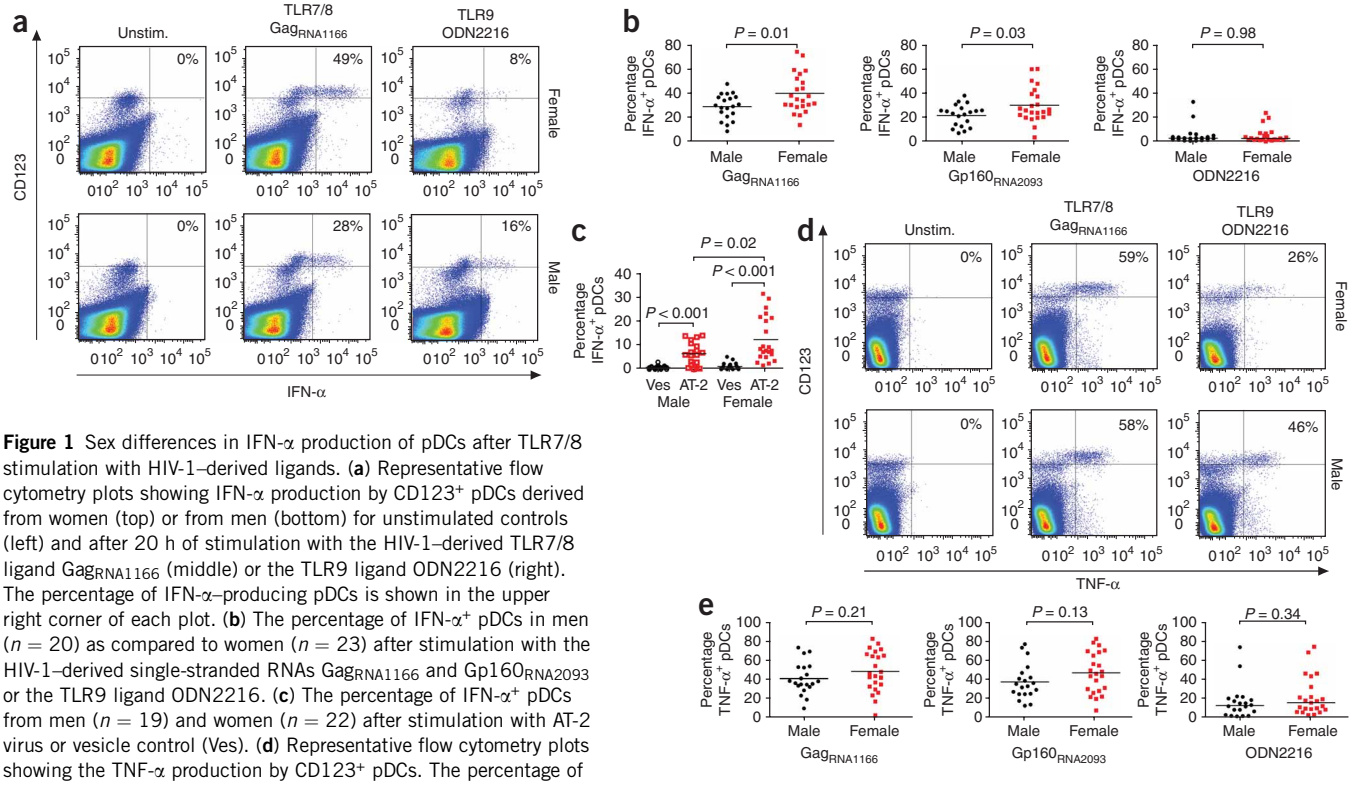

Figure 1 Sex differences in IFN-α production of pDCs after TLR7/8 stimulation with HIV-1–derived ligands. (**a**) Representative flow cytometry plots showing IFN-α production by CD123$^+$ pDCs derived from women (top) or from men (bottom) for unstimulated controls (left) and after 20 h of stimulation with the HIV-1–derived TLR7/8 ligand Gag$_{RNA1166}$ (middle) or the TLR9 ligand ODN2216 (right). 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 ($n = 20$) as compared to women ($n = 23$) after stimulation with the HIV-1–derived single-stranded RNAs Gag$_{RNA1166}$ and Gp160$_{RNA2093}$ or the TLR9 ligand ODN2216. (**c**) The percentage of IFN-α$^+$ pDCs from men ($n = 19$) and women ($n = 22$) after stimulation with AT-2 virus or vesicle control (Ves). (**d**) Representative flow cytometry plots showing the TNF-α production by CD123$^+$ pDCs. 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 ($n = 20$) and women ($n = 23$) after stimulation with the HIV-1–derived single-stranded RNAs Gag$_{RNA1166}$ and Gp160$_{RNA2093}$ or ODN2216. All horizontal bars represent mean value for that sample group.

Progestin has recently been shown 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 TLR7/8ligands did not differ between women who reported the use of oral contraceptives and those who did not ($P = 0.79$, data not shown). However, plasma progesterone concentrations significantly correlated with the percentage of IFN-α$^+$ pDCs after stimulation with the HIV-1–derived TLR7/8 ligand Gag$_{RNA1166}$ (**Fig. 2**, $R = 0.57$, $P = 0.02$). Furthermore, there was a trend toward lower percentages of IFN-α–producing pDCs in response to TLR7/8 ligands in postmenopausal women compared to premenopausal women (mean 16.9% versus 27.6%, $P = 0.08$, data not shown), overall suggesting that sex hormone abundance 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 high circulating amounts of IFN-α are a major prognostic indicator for HIV-1 clinical progression[8,23–25]. Furthermore, IFN-α has been shown *in vitro* to induce upregulation of CD38 on CD8$^+$ T cells[26], a strong independent predictor of HIV-1 disease progression[8–10]. In line with these observations, CD8$^+$ T cells significantly upregulated CD38 expression after stimulation of PBMCs 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 after 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**). On the basis of these data demonstrating sex differences in HIV-1–induced immune activation *in vitro*, we hypothesized that treatment-naive, chronically HIV-1–infected women will show higher levels of CD8$^+$ T cell activation than men with comparable HIV-1 viral loads.

To test this hypothesis, we quantified *ex vivo* T cell activation on pretreatment samples from chronically HIV-1–infected subjects enrolled into the AIDS Clinical Trials Group (ACTG) 384 study, including 109 treatment-naive women and 514 treatment-naive men[27,28]. In ACTG 384, women had higher CD4$^+$ T cell counts (340 versus 273 cells per mm^3, $P = 0.002$) and lower plasma HIV-1 RNA copy numbers (4.7 versus 5.0 $\log_{10}$ copies per ml, $P < 0.001$) at enrollment, before initiation of antiretroviral therapy[27]. In line with previous reports, the degree of immune activation, defined by the percentage of CD38$^+$ human leukocyte antigen (HLA)-DR$^+$CD8$^+$

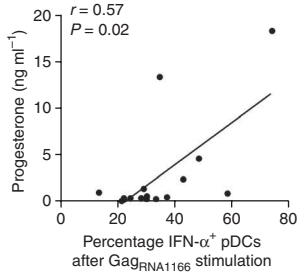

Figure 2 Impact of sex hormone abundance on the IFN-α production by pDCs in response to TLR7/8 agonists. Plasma progesterone concentration measured in 16 premenopausal women significantly correlated with the percentage of IFN-α$^+$ pDCs in response to stimulation with the HIV-1–derived TLR7/8 ligand Gag$_{RNA1166}$.

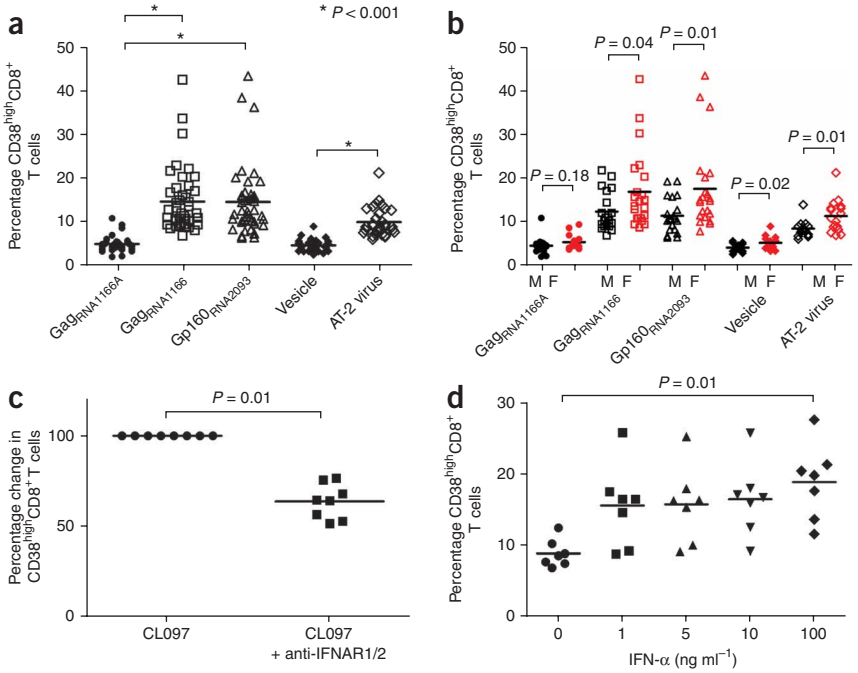

Figure 3 Sex differences in CD38 expression on CD8+ T cells after stimulation with HIV-1–derived TLR7/8 ligands is dependent on IFN-α. (**a**) The percentage of $CD38^{high}CD8^+$ T cells after 20 h of stimulation with HIV-1–derived TLR7/8 ligands ($Gp160_{RNA2093}$ and $Gag_{RNA1166}$), inactive control single-stranded RNA ($Gag_{RNA1166A}$), AT-2 virus or vesicle control for all individuals. (**b**) Differences in the percentage of $CD38^{high}CD8^+$ T cells from men and from women after 20 h of stimulation of PBMCs with the HIV-1–derived TLR7/8 ligands $Gp160_{RNA2093}$ and $GagRNA_{1166}$, inactive control single-stranded RNA ($Gag_{RNA1166A}$), AT-2 virus or vesicle control. (**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 the percentage of $CD38^{high}CD8^+$ T cells after stimulation of PBMCs from healthy donors with increasing amounts of human IFN-α ranging from 0 to 100 ng ml^{-1}. All horizontal bars represent mean value for that sample group.

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 (**Fig. 4**). However, after adjusting for baseline HIV-1 viral load, the percentage of $CD38^+HLA\text{-}DR^+CD8^+$ T cells, but not $CD38^+HLA\text{-}DR^+CD4^+$ 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 ethnicity or intravenous drug use were included into the model, $CD8^+$ T cell activation remained significantly higher in women compared to men after adjusting for HIV-1 viral load ($P = 0.004$, data not shown). The higher $CD8^+$ T cell activation in women compared to men (an average of 4.6% more $CD38^+HLA\text{-}DR^+CD8^+$ T cells) corresponds to the effect of approximately one $\log_{10}$ higher plasma HIV-1 RNA copy numbers (**Fig. 4b**). These *in vivo* data are consistent with the *in vitro* studies shown above (**Fig. 1b**) in which significant differences in immune activation mediated by sex differences in the pDC response to HIV-1 single-stranded RNA–encoded TLR7 ligands were observed and provide 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.

The expression of immune activation markers such as HLA-DR or CD38 on $CD8^+$ T cells has been shown 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 have 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], where pDCs from rhesus macaques, similarly to pDCs in humans, produce large amounts of IFN-α when stimulated with SIV or HIV-1. In contrast, sooty mangabeys, the natural hosts of SIV that do not progress to AIDS[37], have markedly 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 SIV[35]. 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 the resulting pathogenesis in chronic infection.

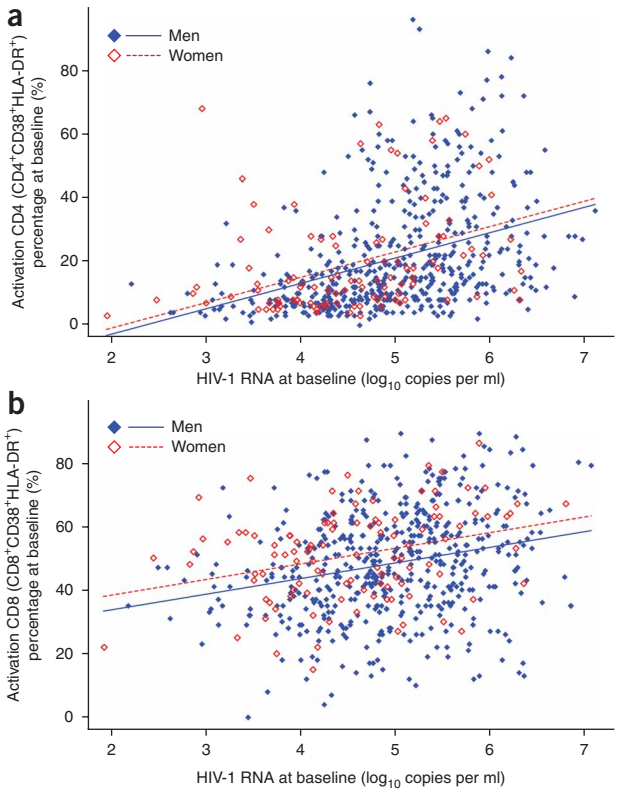

Figure 4 Sex differences in the $CD4^+$ and $CD8^+$ T cell activation of HIV-1–infected, treatment-naive individuals. The association between sex and the percentage of $CD38^+HLA\text{-}DR^+CD4^+$ (**a**) or $CD38^+HLA\text{-}DR^+CD8^+$ (**b**) T cells, adjusting for HIV-1 viral load. HIV-1 RNA load measured at baseline in women and men are correlated to $CD4^+$ and $CD8^+$ T cell activation (correlation are shown by lines).

Major differences in the manifestations of HIV-1 disease have been previously reported between females and males, with women experiencing a substantially increased risk of developing AIDS compared to men for the same level of HIV-1 replication[2–7]. Here we show that treatment-naive women chronically infected with HIV-1 also have markedly higher levels of $CD8^+$ T cell activation compared to men with 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 similar amounts of HIV-1 RNA, we show that pDCs from women produce considerably higher amounts 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 after stimulation with HIV-1 single-stranded RNA 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 then men during chronic infection at a given HIV-1 viral load. Modulation of the TLR7 pathways in pDCs could therefore represent a unique 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 women[1], and autoimmune diseases that show sex differences in their incidence, such as systemic lupus erythematosus, have also been shown to involve the TLR7 and TLR8 pathway[38,39].

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemedicine/.

Note: Supplementary information is available on the Nature Medicine website.

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AUTHOR CONTRIBUTIONS

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., L.O. 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.

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ONLINE METHODS

Study subjects. The ACTG 384 study recruited 980 antiretroviral-naive HIV-1–infected subjects[28]. A subset of subjects (514 men and 109 women) followed at US 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 individuals with other backgrounds including Hispanics and those of Asian descent (all demographic information is as self-identified by the subjects). 578 of the 623 study subjects reported never using 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 were included in this study. Premenopausal women in the study were less than 40 years old, whereas the average age of postmenopausal women was 60 years. When available, we collected information on the use of oral contraceptives containing sex hormones, and 43% of premenopausal 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, and the ACTG 384 study was approved by local institutional review boards.

Analysis of polymorphisms within the genes encoding Toll-like receptor 7, myeloid differentiation factor-88 and interferon regulatory factor-7. We amplified the coding regions of the genes encoding MyD88 and IRF7 from genomic DNA with the primers listed in **Supplementary Table 1**. In addition, we also amplified the genomic sequences flanking the TLR7 single nucleotide polymorphisms rs179008 and rs2302267 that have been implicated in HIV-1 and hepatitis C virus disease progression (see **Supplementary Table 1** for primer sequences). Briefly, we amplified 10–20 ng of genomic DNA in 10-μl reactions (200 nm dNTPs, 1.5 mM $MgCl_2$ and 0.2 μM of each primer). We used a modified step-down program (annealing temperatures given in **Supplementary Table 1**) for amplification of the genes encoding IRF7 and MyD88 to ensure precise amplification. We amplified TLR7 single nucleotide polymorphisms at an annealing temperature of 55 °C. We subjected PCR products to direct bidirectional cycle sequencing using Bigdye terminator v. 1.1 run on an ABI3730 sequencer.

Measurement of single-cell cytokine production by flow cytometry. We stimulated 1.5×10^6 PBMCs per ml with 15 μg ml^{-1} single-stranded RNA (sequences of HIV-1 single-stranded RNA, $Gag_{RNA1166}$ (5′-UUGUUAAGUGUU UCAAUUGU-3′) and $Gp160_{RNA2093}$ (5′-UUUUUGCUGUACUUUCUAUA-3′)) complexed with DOTAP transfection reagent (Roche), 1 μg ml^{-1} CL097 (Invivogen) or 5 μM ODN2216 (Invivogen). We also stimulated cells with aldrithiol-2–inactivated HIV-1 virus (AT-2, MN strain, lot P4097) and microvesicle controls (lot P4079; AIDS and Cancer Virus Program, Science Applications International Corporation Frederick, Inc., National Cancer Institute, Frederick). Unstimulated cells served as negative controls. We immediately added 5 μg ml^{-1} brefeldin A (Sigma) to each tube after adding TLR ligands to inhibit cellular cytokine release. We determined the intracellular cytokine content of monocytes, mDCs and pDCs after 20 h of incubation with the respective TLR ligands, as described previously[20]. We acquired all samples on an LSR II flow cytometer (BD Biosciences). We determined the percentage of cytokine-producing pDCs, mDCs and monocytes by subsequent analysis with FlowJo software (Treestar, Inc.). An average of 92% of $lin^{neg}CD11c^{neg}CD123^{bright}IFN\text{-}\alpha^+$ pDCs also expressed CD303 (blood dendritic cell antigen-2) (data not shown).

Measurement of *in vitro* T cell activation in HIV-1–negative men and women. We performed flow cytometric quantification of $CD38^{high}CD4^+$ and $CD38^{high}CD8^+$ T cells on freshly isolated PBMCs from healthy HIV-1–negative control individuals after stimulation with HIV-1–derived single-stranded RNA ligands, vesicle control and AT-2 virus. For the *in vitro* IFN-α blocking assays, we pretreated PBMCs from HIV-1–negative individuals with antibodies directed against IFN-α receptor 1 and IFN-α receptor 2 (Abcam and PBL Interferonsource, respectively) for 1 h at 37 °C. We then stimulated PBMCs with a TLR7/8 ligand (CL097 at 1 μg ml^{-1}) or with medium alone for 20 h. Furthermore, we stimulated PBMCs *in vitro* with increasing amounts of recombinant human IFN-α (1–100 ng ml^{-1}) for 20 h, and we measured T cell activation as described above.

Measurement of *ex vivo* T cell activation in HIV-1–infected men and women enrolled in ACTG 384. We quantified $CD4^+$ and $CD8^+$ T cell activation by flow cytometry of freshly isolated PBMCs from study participants at baseline, before the initiation of antiretroviral therapy, according to established ACTG protocols. We defined activated $CD4^+$ and $CD8^+$ T cells by their expression of either CD4 or CD8 and their expression of CD38 and HLA-DR. Results are reported as the percentage of $CD38^+HLA\text{-}DR^+CD4^+$ T cells or percentage of $CD38^+HLA\text{-}DR^+CD8^+$ T cells.

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