Reducing IRF-1 to Levels Observed in HESN Subjects Limits HIV Replication, But Not the Extent of Host Immune Activation

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Cells from women who are epidemiologically deemed resistant to HIV infection exhibit a 40–60% reduction in endogenous IRF-1 (interferon regulatory factor-1), an essential regulator of host antiviral immunity and the early HIV replication. This study examined the functional consequences of reducing endogenous IRF-1 on HIV-1 replication and immune response to HIV in natural HIV target cells. IRF-1 knockdown was achieved in ex vivo CD4+ T cells and monocytes with siRNA. IRF-1 level was assessed using flow cytometry, prior to infection with HIV-Bal, HIV-IIIB, or HIV-VSV-G. Transactivation of HIV long terminal repeats was assessed by p24 secretion (ELISA) and Gag expression (reverse transcription–polymerase chain reaction (RT–PCR)). The expression of IRF-1–regulated antiviral genes was quantitated with RT–PCR. A modest 20–40% reduction in endogenous IRF-1 was achieved in >87% of ex vivo–derived peripheral CD4+ T cells and monocytes, resulted in >90% reduction in the transactivation of the HIV-1 genes (Gag, p24) and, hence, HIV replication. Curiously, these HIV-resistant women demonstrated normal immune responses, nor an increased susceptibility to other infection. Similarly, modest IRF-1 knockdown had limited impact on the magnitude of HIV-1–elicited activation of IRF-1–regulated host immunologic genes but resulted in lessened duration of these responses. These data suggest that early expression of HIV-1 genes requires a higher IRF-1 level, compared to the host antiviral genes. Together, these provide one key mechanism underlying the natural resistance against HIV infection and further suggest that modest IRF-1 reduction could effectively limit productive HIV infection yet remain sufficient to activate a robust but transient immune response.

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Introduction

HIV-1 transmission via genital mucosal surfaces is an inefficient process with an estimated risk of 3–50 events per 10,000 sexual exposures or <1% per unprotected sexual exposure. The local and systemic establishment of HIV-1 infection is contingent upon the viral fitness,1 the availability of shared host factor, and the efficacy of antiviral immunity.2,3 Delineation of molecular events occurring at early stages of infection and determination of the critical events for successful HIV-1 transmission may help develop better prevention measures. However, examining the early events of successful, natural HIV infection in human subjects is technically challenging.

Fortunately, not everyone exposed to HIV-1 become infected. In all investigated HIV-exposed cohorts, there are ~10–15% of these HIV-exposed individuals who remain seronegative (HIV-exposed seronegative (HESN)).4 Studies of molecular events that may be involved in hindering the establishment of HIV infection can be performed in HESN,5–7 and the findings will help identifying genetic and immune correlates of protection.8–25 Our earlier work identified interferon (IFN) regulatory factor-1 (IRF-1) to be a genetic and functional correlate of protection against HIV-1 acquisition in a highly HIV-exposed commercial sex worker cohort in Nairobi, Kenya26–28; these HESN women can be defined as epidemiologically “resistant” to HIV infection.29,30 IRFs are a family of transcriptional regulators found in all principle metazoan groups including simple organisms such as sea sponges.31 IRF genes are thought to have coevolved with Rel/NF-κB genes, which together play important roles in regulating host immunity.32 IRF-1, the first IRF identified, functions as a transcription regulator33 by binding to an IFN-stimulated response element (ISRE), found in numerous genes controlling immune responses and cellular apoptosis.34,35,36 IRF-1 expression is expressed at low basal level in most cell types and can be induced by specific cytokine/chemokines and by viral infection. It was recently shown to be upregulated in CD4+ T cells,35,36 monocyte-derived dendritic cells, and monocyte-derived macrophages37,38 by in vitro HIV infection. In addition to the antiviral role, IRF-1/NF-κB are essential facilitators of the early transactivation of HIV-1 genome.39 Deleting the ISRE39 or NF-κB site36 in the HIV LTR (long terminal repeats) results in a virus with reduced replicative capacity, directly pointing to a role for IRF-1 in regulating HIV replication.

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Polymorphisms in the IRF-1 gene are associated with disease progression in hepatitis C infection and with altered susceptibility to HIV infection. Several linked IRF-1 polymorphisms were found to associate with reduced susceptibility to HIV-infection but not disease progression. These polymorphisms were also functionally linked with reduced endogenous IRF-1 expression and a reduced responsiveness to exogenous IFN-γ signaling. Importantly, they also correlated with the decreased, transient transactivation of the HIV-1 LTR. However, not all HESN subjects have these protective IRF-1 polymorphisms; yet, the majority of HESN women who can be epidemiologically defined as relatively resistant to HIV infection in studies from Nairobi, Kenya have reduced endogenous IRF-1 expression (Figure 1a, P < 0.001) that may be regulated through epigenetic mechanisms. In vitro studies have shown that the complete knockdown of IRF-1 in Jurkat T-cell lines reduced HIV-1 transactivation, emphasizing the absolute requirement for IRF-1 in HIV replication. However, it is unknown if a modest reduction of IRF-1 expression, as observed in vivo in HESN women, could limit HIV replication and, importantly, how this reduction would affect IRF-1-regulated IFN-stimulated genes (ISGs), the antiviral immune responses.

Results

Endogenous IRF-1 protein level was assessed in the HESN subjects, women who exhibit natural resistance to HIV-1 acquisition (Figure 1a). Although not all HESN female sex workers (FSWs) have the IRF-1 protective polymorphisms (at least one), which is strongly associated with reduced IRF-1 expression, most peripheral blood mononuclear cell (PBMC) samples from the HESN FSWs analyzed showed reduced IRF-1 expression, in comparison to the HIV-seronegative, non-HESN FSW controls (P values <0.01), suggesting that mechanisms other than IRF-1 genetic polymorphism are responsible for the reduced IRF-1 expression, and that reduced IRF-1 expression may have a critical role in the resistance phenotype against HIV-1 acquisition in these HESN FSWs.

We then determined if transiently reducing endogenous IRF-1 expression in ex vivo PBMCs would limit HIV replication. A complete knockdown of gene expression in primary cells remains a technical challenge and a complete IRF-1 knockout may not be desirable, as IRF-1 regulates cell viability. However, transiently altering IRF-1 expression in primary cells is technically feasible and may be more biologically relevant, reflecting the level of IRF-1 expression observed in most HESN women. Here, partial IRF-1 reduction was achieved in ex vivo CD4+ T cells and monocytes using IRF-1–specific siRNA. A significant reduction of endogenous IRF-1 protein could be detected by flow cytometry at 8 hours posttransfection (Figure 1b). The efficiency of siRNA uptake was monitored with fluorescence (Alexa 647)-tagged siRNA spiked into the nontagged siRNA. In unstimulated ex vivo PBMCs, IRF-1 protein expression was reduced in ~25–40% of total PBMCs (Figure 1b), and similar frequency of PBMCs demonstrated the uptake of siRNA (positive for Alexa 647, Figure 1c), perhaps due to the preferential transfection of T cells with the T-cell–specific Nucleofector solution. To determine the half-time of IRF-1 knockdown, CD4+ T cells and CD14+ monocytes transfected with siRNA specific for IRF-1 were stained for IRF-1 expression at 18, 42, and 66 hours posttransfection (Figure 2). No further reduction in IRF-1 expression level was observed past 18 hours in transfected cells and the half-time of transient IRF-1 knockdown was ~42 hours posttransfection in both cell types (Figure 2) and was accounted for in later experimental design.

Moreover, greater than 90% of the enriched CD4+ T cells and CD14+ monocytes could be successfully transfected with siRNA using human T-cell Nucelofector™ solution and

![Figure 1 Endogenous IRF-1 expression in ex vivo, unstimulated peripheral blood mononuclear cells (PBMCs), and the uptake of IRF-1–specific siRNA by PBMCs.](image)

(a) Endogenous IRF-1 protein level was assessed in unstimulated PBMCs (2 × 10^6 cells, n = 10 per group) from healthy HIV-seronegative Kenyan female sex workers (FSWs) (not HIV-resistant controls, ▲) using intracellular staining assay with antibody specific for IRF-1 (C-20; Santa Cruz Biotechnologies) and flow cytometry. Resistance to HIV-1 infection was defined epidemiologically; the FSWs must be active in sex trade with frequent exposures to HIV+ clients but remained seronegative for HIV-1 for >7 years. Blood genomic DNA of the same PBMC samples was typed for protective IRF-1 genotype (the 619A, 179 microsatellite, and 6516G). FSWs with one or more of these protective polymorphisms were found to associate with resistance phenotype to HIV-1 infection. (HIV-resistant FSWs with at least one protective IRF-1 genotype, ▲; without any protective IRF-1 genotype, ○). Analysis of variance test was used in the statistical analysis shown in the figure. (b) siRNA specific for IRF-1 (10 nmol/l, 90% untagged, 10% tagged with Alexa 647) or a negative control siRNA tagged with Alexa 647 (10 nmol/l) were used in transfecting ex vivo unstimulated PBMCs (5 × 10^6 cells) from healthy local donors. Endogenous IRF-1 expression was examined at 18 hours posttransfection using flow cytometry (n = 4). (c) The uptake of siRNA by PBMCs was assessed by the uptake of Alexa 647-tagged siRNA (n = 4). FITC, fluorescein isothiocyanate; IRF, interferon regulatory factor; MFI, mean florescent intensity. **P-values <0.001.
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Figure 2. A time course: Transient knockdown of endogenous IRF-1 expression. (a) Ex vivo unstimulated CD4+ T cells (5 x 10^6 cells) and (b) monocytes (3 x 10^6 cells) from healthy local blood donors were transfected with an IRF-1–specific siRNA (10 nmol/l) or a negative control siRNA (10 nmol/l). At 6 hours posttransfection, monocytes were stimulated with low dose of phorbol 12-myristate 13-acetate (1 ng/ml) and ionomycin (50 ng/ml). % Cell count (t = 18 hours) was observed in IRF-1 siRNA–treated cells compared to the control. FITC, fluorescein isothiocyanate; IRF, interferon regulatory factor.

Figure 3. Ex vivo unstimulated CD4+ T cells or monocytes were transfected with a type 5 adenovirus (Ad5), containing a luciferase reporter gene. IRF-1 knockdown did not affect the transactivation of the Ad5 promoter, which contains no ISRE. Together, these data indicate that a mere 38% decrease in endogenous IRF-1 could drastically impair HIV-1 LTR transactivation in unstimulated primary CD4+ T cells and thus, HIV replication. It further supports the hypothesis that naturally reduced IRF-1 expression observed in vivo may, at least partly, be accountable for reduced susceptibility to HIV infection observed in these HESN women.

IRF-1 binds to the ISRE at the promoter of numerous antiviral ISGs and also regulates many other ISGs indirectly, not through direct binding to the promoter. We previously showed that HESN women who exhibit naturally reduced IRF-1 have normal immune function. Here, we show that the modest reduction of IRF-1 with siRNA had no marked impact on host immunological genes (Figure 5). Stimulation of ex vivo CD4+ T cells, transfected with either IRF-1–specific or control siRNA, using exogenous IPN-γ, led to comparable increases in mRNA expression of key IRF-1–regulated genes (either directly or indirectly), STAT1α (~25-fold), IFN-γ (~35-fold), and TNF-α (~3-folds) (4 hours posttransfection, Figure 5b). Similarly, IRF-1–mediated direct suppression of the IL-4 gene was not affected by IRF-1 knockdown (Figure 5).

IRF-1 plays a critical role in innate antiviral response and the...
functional response of STAT1, IFN-γ, and TNF-α to IFN-signaling signifies the cell’s proper antiviral potential. Hence, these ex vivo observations suggest that modest reduction in endogenous IRF-1 level, such as that observed in these HESN women, does not affect host cellular IFN responsiveness, the key antiviral innate function.

Early and persistent induction of IRF-1 and IRF-1–regulated ISGs were recently shown as a viral strategy used by HIV to enhance replication in dendritic cells. Interestingly, our earlier work found IRF-1 induction to be transient but robust in cells from these HESN women, compared to a prolonged IRF-1 response in susceptible controls. Recent work has also shown that innate IFN-stimulated antiviral responses are likely critical in limiting the establishment of HIV infection. Here, we examined how transient IRF-1 reduction in the CD4+ T cells from the HIV-S FSWs would affect the kinetics of the expression of key antiviral genes, IFN-γ, STAT1, and TNF-α. The kinetics of HIV-1–elicited activation of primary unstimulated CD4+ T cells differed from that of CD3/CD28–elicited activation. The transactivation of HIV-1 LTR could be detected as early as 42 hours postinfection (60 hours after siRNA transfection), evident by detectable luciferase activity during the infection by HIV-1 VSV-G and detectable Gag mRNA expression (RT–qPCR) during the infection by HIV-1 BaL and HIV-1 IIIB. To evaluate the impact of siRNA–mediated IRF-1 knockdown on early host antiviral responses, elicited by HIV-1 infection, the mRNA level of IRF-1, IFN-γ, STAT1, and TNF-α were assessed at 22 and 42 hours posttransfection that is 40 and 60 hours posttransfection with IRF-1–specific or negative control siRNA. As expected, IRF-1–specific siRNA limited the induction of endogenous IRF-1 elicited by HIV-1 IIIB (Figure 6a) or HIV-BaL (Figure 6b) infection of CD4+ T cells at both 22 and 42 hours postinfection, regardless of viral tropism. At 22 hours postinfection, only a
two- to four-fold increase in IRF-1 transcript was induced in siRNA-transfected cells compared to a much more robust increase in controls (11- to 14-fold). However, the limited IRF-1 induction mediated by IRF-1 siRNA had no significant effect on HIV-induced transactivation of host immune genes (Figures 5 and 6); both IFN-γ and TNF-α transcripts were similarly upregulated in both control and IRF-1 siRNA groups, at 22 hours postinfection, irrespective of strain (Figure 6). HIV-IIIB infection increased IFN-γ transcripts by ~25-fold, TNF-α transcripts by 42-fold, and STAT1 transcripts by 15-fold. Although significantly less IFN-γ (~22-fold increase) and TNF-α (~12-fold increase) transcripts were induced by HIV-BaL, the magnitude of responses was similar for both the control and IRF-1 siRNA groups. We noted that prolonged IRF-1 induction might be required to sustain the HIV-induced cytokine upregulation. In the IRF-1 siRNA treatment group, IFN-γ and TNF-α mRNA levels dropped by ~65% between 22 and 42 hours postinfection while the levels of both persisted in the siRNA control groups, again irrespective of viral strain (Figure 6). There was an unexpected decline (~45%) in the

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![Graphical data]
This study provides the key mechanism of how a previously discussed host IRF-1, induced by HIV infection; while a lower threshold of innate antiviral response was observed in the control siRNA group (data not shown). Together, these observations highlighted the importance of increasing IRF-1 expression during early HIV-1 infection to sustain the ongoing antiviral response. All in all, these data suggest that transactivation of HIV-1 LTR requires a higher and perhaps sustained level of the continuous upregulation of antiviral genes, IFN-γ, TNF-α, and STAT1, was significant noticeably at 42 hours postinfection (Figure 6). It is plausible that early IRF-1 inhibition effectively limits HIV replication and thus the immune activation was dampened by the absence of viral replication at 42 hours postinfection. Our earlier kinetic study using exogenous IFN-γ as stimulus (in place of HIV-1 infection) led to similar observation that CD4+ T cells, transfected with IRF-1–specific siRNA, had robust but transient IFN response (i.e., increased IFN-γ, IL-12RB1, and TNF-α expression). Equally robust and persisting IFN response was observed in the control siRNA group (data not shown). Together, these observations highlighted the importance of increasing IRF-1 expression during early HIV-1 infection to sustain the ongoing antiviral response. All in all, these data suggest that transactivation of the HIV-1 LTR requires a higher and perhaps sustained level of host IRF-1, induced by HIV infection; while a lower threshold is sufficient for transactivating the host immune genes, regulated by IRF-1.

**Discussion**

This study provides the key mechanism of how a previously identified genetic correlate impacts HIV-1 infection in primary human cells and, additionally, a deeper understanding of how IRF-1 expression specifically acts on human antiviral ISGs (either directly or indirectly) and HIV regulation. The implications of these findings are that a moderate reduction of IRF-1 expression, such as that naturally observed in some HESN women, could be achieved using siRNA or other therapeutic modalities specific for IRF-1 is sufficient to effectively limit initial HIV replication, and perhaps, to also curtail the prolonged immune activation, while perhaps maintaining innate antiviral responses. This study echoes a recent in vivo study, showing the importance of IFN responses in protecting against simian immunodeficiency virus infection in rhesus macaques. Together, these studies emphasize the need to achieve a balance between the antiviral effects of inflammation and the generation of activated CD4+ T-cell targets for HIV infection. Further insight comes from observations that the strict regulation of IFN antiviral responses is observed in nonpathogenic simian immunodeficiency virus infection, and that transient antiviral responses could limit immune activation and thus, systemic dissemination of simian immunodeficiency virus. Here, we showed that early transactivation of HIV-1 genes and persistent host IFN antiviral response requires upregulation of IRF-1 expression (Figure 6) but the transactivation host IFN antiviral genes were not affected by the modest 38% reduction in endogenous IRF-1 level (Figures 5 and 6). Together, these prompt crucial cautions in studying host–viral interactions; the regulation of the magnitude and duration of gene expression is mostly different in transformed cell lines versus primary human cells and has often been overlooked. Although complete knockdown of cellular IRF-1 expression has been shown in Jurkat T-cell line to inhibit the HIV-1 replication, this is the first study to demonstrate that partial knockdown of IRF-1 expression is achievable in unstimulated primary CD4+ T-cell groups (Figures 1 and 2) and is sufficient to restrain early HIV-1 replication (Figure 4). Furthermore, as IRF-1 has been implicated in several biologic processes, this is the first report to show that the modestly reduced IRF-1 expression in nontransformed CD4+ T cells had no effects on the initiation of innate antiviral response but shortened the duration of the responses (Figure 6). As virus employs host factors for its replication, the possibility of host and viral genes requiring different amounts of shared factors begs the needs to carefully define the physiological and functional levels of the host factors involved.

In addition to its role in transactivating HIV-1 LTR, IRF-1 also plays a key role in regulating host immune activation, by suppressing the differentiation of regulatory T cells (Treg). Although modest reduction in IRF-1 expression had no significant impacts on the baseline host antiviral gene expression (Figure 5a), reduced IRF-1 level may affect the frequency of Treg. HESN women exhibit baseline immunological quiescence and the HESN phenotype is associated with increased Treg frequency. It remains to be tested whether modest reduction in IRF-1 expression could have significant effects on the differentiation of Treg, contributing to the immunological quiescence, an unfavorable environment for the early stage of HIV replication.

**Figure 4 Effects of IRF-1 knockdown on the transactivation of HIV-1 LTR in ex vivo infected CD4+ T cells and monocytes.** (a) Unstimulated, primary CD4+ T cells (n = 7) and (b,c) monocytes from HIV-S female sex worker (FSW) controls were transfected with either siRNA specific for IRF-1 (10 nmol/l) or a control siRNA (10 nmol/l). At 6 hours posttransfection, monocytes were either (b) cultured in media alone (n = 9) or (c) treated with low dose of PMA (1 ng/ml) and ionomycin (50 ng/ml) (n = 9). At 18 hours posttransfection, these cells were either infected with HIV-IIIB or mock infected. Transactivation of HIV LTR was measured via the enzymatic activity of an integrated luciferase reporter gene (y-axis) at 76 hours postinfection. (d,e) Unstimulated, primary CD4+ T cells (4 × 10^5 cells) from non-HESN (HIV-exposed seronegative), HIV-S FSWs, transfected with either IRF-1 (10 nmol/l) or control siRNA (10 nmol/l) were also infected with HIV-Bal, HIV-IIIB, and HIV_vivo. At 96 hours postinfection, (d) HIV-1 Gag RNA expression in CD4+ T cells was assessed using quantitative reverse transcription–polymerase chain reaction, normalized to cellular 18S rRNA level (an internal reference), and (e) secreted p24 in CD4+ T-cell culture supernatants was assayed using ELISA (n = 6, per group). The threshold of p24 detection is 0.05 ng/ml; the gray dashed line represents 0.1 ng/ml. (f) HIV-IIIB–infected CD4+ T cells of HIV-S, non-HESN FSWs were stained for intracellular p24 and IRF-1 (Ab26109 antibody) at 42 hours postinfection, after 6 hours of Golgi Plug incubation to inhibit the secretion of p24 (n = 9). (g) Ex vivo CD4+ T cells from HIV-S non-HESN FSWs, transfected with IRF-1–specific (10 nmol/l) or control siRNA (10 nmol/l) were infected with either HIV-1 or adenovirus type 5 (Ad5), as in (a) (n = 6). Fold changes in luciferase activity were calculated against mock infection, as shown in y-axis. **P < 0.0005, ***P < 0.005. FITC, fluorescein isothiocyanate; IRF, interferon regulatory factor; n.s., not significant; RLU, relative light unit.
In summary, restricting HIV replication and activating innate host antiviral defenses during HIV transmission may be sufficient to thwart systemic HIV infection, and, here, we demonstrated a potential tool to achieve these activities simultaneously. A modest reduction in IRF-1 limited HIV-1 replication, prevented a prolonged antiviral inflammatory response, but allowed for the adequate induction of antiviral responses. However, it is unlikely that reducing endogenous IRF-1 alone would completely protect against HIV acquisition. In conjunction with other therapeutic modalities, like a...
microbicide, or perhaps approaches using other inducers of innate antiviral immunity and/or vaccine-induced adaptive immunity, regulating IRF-1 expression could be a novel component to a successful HIV intervention strategy.

Materials and methods

Ethics. The study was approved by the ethics review committees of the University of Manitoba, Canada and the Kenyatta National Hospital, Kenya. Informed written consents from the human volunteers were obtained in this study, following the declaration of Helsinki protocols.

Study subjects. Volunteers with natural resistance to HIV infection (HIV-R, HESN) and HIV-susceptible seronegative volunteers (HIV-S) were chosen randomly from subsets of a well-characterized cohort of FSWs at the Pumwani district of Nairobi, Kenya. These women maintained high-risk sexual behavior (17.5 ± 7.2 clients and 1.6 ± 2.3 regular partners/week) with known HIV-infected clients. HIV-R and HIV-S FSWs were HIV negative (by ELISA and DNA/RNA testing) for >7 years and <2 years of follow-up, respectively. The HIV-S volunteers were enrolled within 2-year time and were seronegative when the blood samples were taken. Samples from healthy local blood donors, not participated in sex work, were obtained from student and staff volunteers of various genetic backgrounds at the University of Manitoba. The endogenous level of IRF-1 protein was assessed in the FSWs and local blood donors. All blood donors were females, with age ranging from 22 to 38. IRF-1 genotyping was performed in the FSW samples as previous study by PCR sequencing.28,42

Cell culture. PBMCs were isolated by Ficoll–Hypaque density gradient centrifugation, frozen, and shipped to University of Manitoba where experiments were performed. Upon thawing (cell viability > 90%), PBMCs were cultured immediately in RPMI 1640 (supplemented with 10% fetal bovine serum, 100 unit/ml of penicillin, and 100 µg/ml of streptomycin) for 3 hours at 37 °C and 5% CO₂. The PBMCs were then stimulated with IFN-γ (10 ng/ml; Sigma, Oakville, ON, Canada) and flow cytometry. There was no significant difference in IFN-1 expression level between the Kenyan FSWs and the local blood donors. All blood donors were females, with age ranging from 22 to 38. IRF-1 genotyping was performed in the FSW samples as previous study by PCR sequencing.28,42

Transfection and siRNA. Predesigned siRNA specific for IRF-1 gene at two different regions (Hs_IRF1_1 SI00034083 and Hs_IRF1_2 SI00034090; Qiagen, Toronto, ON, Canada) and the matching scrambled siRNA control with similar G/C content and Alexa 647 tag (SI03650325; Qiagen, Toronto, ON, Canada) was used in transfection. The efficiency of siRNA uptake was monitored with fluorescence (Alexa 647)-tagged siRNA (1 nmol/l), mixed in with the not tagged, functional siRNA, and scored as percentage of Alexa 647⁺ cells. Nonstimulated primary CD4⁺ T cells and primary CD14⁺ monocytes were transfected using Nucleofector Amaxa technology (Nucleofector II Device; Lonza, Walkersville, TX) and the associated transfection kits: Human T cell Nucleofector Kit (Program V-024) and Human Monocyte Nucleofector Kit (Program Y-001), following the manufacturer’s protocols.

Viruses. The VSV-G–pseudotyped virus, HIV-1VSV-G, was produced by cotransfection of HEK293T cells with plasmid SPCMV-VSV-G and plasmid pNL-Bru/E-luc. Construction of the plasmids is detailed in previous studies.43 Plasmid pNL-Bru/E-luc consists of pNL4.3 HIV-1 backbone with an inactivated Env gene and the Nef gene replaced by a firefly luciferase reporter gene under the control of the HIV-1 LTR. Plasmid SPCMV-VSV-G encodes vesicular stomatitis virus envelope G-protein (VSV-G). Viral supernatant was collected at 48 hours after cotransfection. Reverse transcriptase assays were used to determine viral titer, as described.43 HIV-IIIB and HIV-Bal were obtained from Dr. R. Gallo through the AIDS Research and Reference Reagent Program. HIV-IIIB and HIV-Bal were grown in phytohemagglutinin-stimulated PBMCs from HIV-1-seronegative local blood donors. The cells were cultured for 10 days before the culture supernatant was harvested and frozen at −80 °C. TCID₅₀ was calculated by the method of Reed and Muench.44 Adenovirus type 5 (Ad5, Ad-CMV-luciferase) was purchased from Vector BioLabs. The protocol of infecting ex vivo CD4⁺ T cells with Ad5 was similar to HIV-1 infection.

HIV-1 infection. For HIV-1 infection assay, CD4⁺ T cells or CD14⁺ monocytes (10⁶ cells/well of a 96-well plate) were incubated with HIV-1VSV-G (MOI: 0.1). HIV-IIIB (MOI: 1.0), or HIV-Bal (MOI: 1.0) in 100 µl volume (supplemented with 4 µg/ml of polybrene, Sigma, Oakville, ON, Canada) and centrifuged at 37 °C, 1,000 g for 2 hours. After virus absorption, the cells were washed once, resuspended in RPMI 1640 containing 10% fetal calf serum and antibiotics, and incubated at 37 °C for 4 days. Culture supernatants and cells were harvested at 22, 42, and 96 hours postinfection, as indicated. The infection conditions and time points were optimized for infection, high viability, and optimum gene expression. For luciferase assay, cells were resuspended in 30 µl cell lysis buffer (Promega, Madison, WI), vortexed, and centrifuged to remove cellular debris. Whole cell lysate was then collected and stored at −80 °C. Luciferase activity in the cell lysate was measured using Luciferase Assay System (Promega, Madison, WI), following the manufacturer’s protocol. Luciferase activity (measured in relative light units) was read on a microplate luminometer. The background luciferase activity in mock infection was just above the sensitivity of the assay. Culture supernatants were assayed for p24 antigen as previously described.43 HIV-1 p24 hybridoma 183-H12-5G (NIH AIDS Research and Reference Reagent Program, cat. no. 1513) was used as plate-coating antibody. Rabbit polyclonal antibody specific for HIV-IIIB p24
gag (Advanced Biotechnologies Inc., Eldersburg, MD) was used as a secondary capture antibody. HIV-1 p24 standard was produced by infecting SupT cell line with HIV-IIIB and titrated against a purchased p24 standard (HIV-1 p24 Antigen Capture Assay Kit, AIDS Vaccine Program, NCI-Frederick Cancer Research and Development Center). All three viruses—HIV-1-IIIB, HIV-1-BaL, and HIV-1_VSVG—elicited comparable amounts of p24 production, 96 hours postinfection. p24 production in mock-infected samples was below the sensitivity (0.05 ng/ml) of the assay and was graphed as 0.05 ng/ml.

**Flow cytometry.** For surface staining, 3 × 10^5 cells (per tube) were washed once with wash buffer (2% fetal bovine serum in phosphate-buffered saline, 1 mM oligo ethylene diamine tetraacetic acid) and incubated with antibodies specific for CD4 (clone RM4-5, V450), CD3 (clone SK7, V500), or CD14 (clone M5E2, Pacific Blue) in 100 µl volume on ice for 45 minutes. Cells were then washed, fixed with 1% paraformaldehyde (Sigma, Oakville, ON, Canada), and analyzed on BD LSRII Flow Cytometer. Intracellular staining was done using BD Cytofix/Cytopermit Kit (BD Biosciences, Mississauga, ON, Canada). Briefly, 3 × 10^5 cells (per tube), washed once with wash buffer, were fixed for 20 minutes on ice, washed twice with Perm/Wash Buffer, and then stained with primary antibody (specificity: IRF-1 (Clone C-20; Santa Cruz Biotechnology; ab26109, http://www.abcam.com) or rabbit IgG control (Jackson ImmunoResearch Laboratories, West Grove, PA)). Allophycocyanin-conjugated goat anti-rabbit antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) was used to visualize primary antibody binding. Stained cells were analyzed using BD LSRII Flow Cytometer. Only optimized antibody concentrations were used to generate results presented in the study.

**Quantitative RT–PCR.** Total RNA was prepared as described previously, using Trizol (Sigma, Oakville, ON, Canada) and RNEasy MinElute Cleanup Kit (Qiagen, Toronto, ON, Canada). RNA was treated with RNase-free DNase I prior to reverse transcription (Qiagen, Toronto, ON, Canada). Resulting cDNA was evaluated in qPCR with specific primer sets for IRF-1, IFN-γ, TNF-α, STAT1, IL-4, HDAC2, GAPDH, IL-12p35, and 18S rRNA (sequences available upon request). Annealing temperature for all primer sets was 60 °C. All qPCR were performed with SYBR-Green qPCR Master Mix (Qiagen, Toronto, ON, Canada). All primer sets used in the study were tested for amplification efficiencies and the results were similar. Average threshold cycle (CT) from duplicate wells (with covariance less than 10%) was determined and standardized with the 18S rRNA internal control (input control) and normalized to untreated or uninfected, culture media (CM) alone culture condition (as a reference) using comparative ∆ΔCT program (LightCycler 480 Real-Time PCR System; Roche Applied Science, Laval, QC, Canada).

**Data analysis.** Statistical analyses were performed with Graph Pad Prism 6.0 (San Diego, CA). Normality tests were performed for each sample set. Data sets assuming Gaussian distribution were analyzed using parametric statistical tests; non-Gaussian distributed sample sets were analyzed using nonparametric statistical tests. Unpaired t-test was used to determine whether mean/median values differed significantly between two groups of sample sets. One-way analysis of variance was used when more than two groups of data sets were involved in the analysis.

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1. Joseph, SB and Swanson, R (2014). HIV/AIDS. A fitness bottleneck in HIV-1 transmission. Science 345: 136–137.
2. Siervo, A, Su, RC, Plummer, FA and Ball, TB (2014). Interferon responses in HIV infection: from protection to disease. AIDS Rev 16: 43–51.
3. Pasay, PM (2011). Innate immune response: role in HIV-1 infection. Viruses 3: 1179–1203.
4. Shearer, G and Clerici, M (2010). Historical perspective on HIV-exposed seronegative individuals: has nature done the experiment for us? J Infect Dis 202 (suppl. 3): S329–S332.
5. McLaren, PJ, Ball, TB, Wachihi, C, Joako, W, Kelvin, DJ, Danesh, A et al. (2010). 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 202 (suppl. 3): S339–S344.
6. Songok, EM, Luo, M, Liang, B, Mclaren, P, Kaferl, N, Apidi, W et al. (2012). Microarray analysis of HIV resistant female sex workers reveal a gene expression signature pattern reminiscent of a lowered immune activation state. PLoS One 7: e30048.
7. Songok, EM, Osero, B, McKinnon, L, Rono, MK, Apidi, W, Matey, EJ et al. (2010). CD28 diphopyridiphosphate IV (CD28/DPP1V) is highly expressed in peripheral blood of HIV-1 uninfected female sex workers. Viro J 7: 343.
8. Guerrini, FR, Lo Caputo, S, Gori, A, Bandera, A, Mazzotta, F, Uglietti, A et al. (2011). Underrepresentation of the inhibitory KIR3DL1 molecule and the KIR3DL1+/BW4+ complex in HIV exposed seronegative individuals. J Infect Dis 203: 1235–1239.
9. Tomesucu, C, Abdulhashq, S and Montarer, LJ (2011). Evidence for the innate immune response as a correlate of protection in human immunodeficiency virus (HIV)-1 highly exposed seronegative subjects (HESN). Clin Exp Immunol 164: 158–169.
10. Mahotra, R, Hu, L, Song, W, Brill, I, Mulenga, J, Allen, S et al. (2011). Association of chemokine receptor gene (CCR2/CCRS) haplotypes with acquisition and control of HIV-1 infection in Zambians. Retrovirology 8: 22.
11. Schellenberg, JJJ, Links, MG, Hill, JE, Dumonceaux, TJ, Kimani, J, Jacko, W et al. (2011). Molecular definition of vaginal microbiota in East African commercial sex workers. Appl Environ Microbiol 77: 4696–4704.
12. Ghaddiay, H, Keynan, Y, Kimani, J, Kimani, M, Ball, TB, Plummer, FA et al. (2012). Altered dendritic cell-natural killer interaction in Kenyan sex workers resistant to HIV-1 infection. AIDS 26: 429–436.
13. Sironi, M, Bisin, M, Caggiani, R, Forini, D, De Luca, M, Säule, I et al. (2012). A common polymorphism in TLR3 confers natural resistance to HIV-1 infection. J Immunol 188: 818–823.
14. Lajoie, J, Juno, J, Burgener, A, Rahman, S, Mogk, K, Wachihi, C et al. (2012). 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 5: 277–287.
15. Habegger de Sorrentino, A, Sinchi, JL, Marinc, K, Lopez, R and Illoovich, E (2013). KIR-HLA-A and B alleles of the Bw4 epitope against HIV infection in discordant heterosexual couples in Chaco Argentina. Immunology 140: 273–279.
16. P Attacciarri, L, Murante, PM, Kahele, EM, Bolton, MJ, Dettow, J, Lingappa, JR et al. (2013). Differential regulatory T cell activity in HIV type 1-exposed seronegative individuals. AIDS Res Hum Retroviruses 29: 1321–1329.
17. Zapata, W, Aguilar-Jiménez, W, Piñeda-Trujillo, N, Rojas, W, Estrada, H and Rugeles, MT (2013). Influence of CCR5 and CCR2 genetic variants in the resistance/susceptibility representation of the inhibitory KIR3DL1 molecule and the KIR3DL1+/BW4+ complex in HIV infected individuals: has nature done the experiment for us? J Infect Dis 202 (suppl. 3): S329–S332.
18. Zapata, W, Aguilar-Jiménez, W, Piñeda-Trujillo, N, Rojas, W, Estrada, H and Rugeles, MT (2013). Influence of CCR5 and CCR2 genetic variants in the resistance/susceptibility representation of the inhibitory KIR3DL1 molecule and the KIR3DL1+/BW4+ complex in HIV infected individuals: has nature done the experiment for us? J Infect Dis 202 (suppl. 3): S329–S332.
18. Card, CM, Ball, TB and Fowke, KR (2013). Immune quiescence: a model of protection against HIV infection. Virology 440: 141.

19. Alejandri-Gomez, W, Zapata, C, Caniz, A and Rugueles, MT (2013). High transcript levels of vitamin D receptor are correlated with higher mRNA expression of human beta defensins and IL-10 in mucosa of HIV-1-exposed seronegative individuals. PLoS One 8: e82177.

20. Shen, R and Smith, PD (2014). Mucosal correlates of protection in HIV-1-exposed seronegative persons. Am J Reprod Immunol 72: 219–227.

21. Li, H, Liu, TJ and Hong, ZH (2014). Gene polymorphisms in CCR5, CCR2, SDF1 and RANTES among Chinese Han population with HIV-1 infection. J Infect Genet Evol 24: 99–104.

22. Van Raemdonck, G, Zegels, G, Coen, E, Vuytsteke, B, Jenne, W and Van Oostade, X (2014). Increased Serpin A6 levels in the cervicovaginal fluid of HIV-1-exposed seronegatives suggest that a subtle balance between serine proteases and their inhibitors may determine susceptibility to HIV-1 infection. Virology 453-459: 11–21.

23. Sironi, M, Biasini, M, Grundi, F, Cagnassi, R, Saulie, I, Forni, D et al. (2014). A regulatory polymorphism in HAVCR2 modulates susceptibility to HIV-1 infection. PLoS One 9: e83442.

24. Lima, JF, Oliveira, LM, Pereira, NZ, Mitsunari, GE, Duarte, AJ and Sato, MN (2014). Virology 65: 532–540.

25. Su, RC, Sivro, A, Kimani, J, Jaoko, W, Plummer, FA and Ball, TB (2011). Epigenetic control of HIV-1 expression in highly exposed but uninfected Kenyan blood donors. J Leukoc Biol 89: 1181–1189.

26. Horton, RE, McLaren, PJ, Fowke, K, Kimani, J and Ball, TB (2010). Cohorts for the study of HIV in serodiscordant couples from Colombia. AIDS Res Hum Retroviruses 26: 353–354.

27. Ji, H, Ball, TB, Ao, Z, Kimani, J and Plummer, FA (2010). Reduced HIV-1 long terminal repeat transcription in subjects with protective interferon regulatory factor-1 genotype: a potential mechanism mediating resistance to infection by HIV-1. Scand J Infect Dis 42: 369–374.

28. Ball, TB, Ji, H, Kimani, J, McLean, R, Marin, C, Hill, A et al. (2007). Polymorphisms in IRF-1 associated with resistance to HIV-1 infection in highly exposed but uninfected Kenyan sex workers. AIDS 21: 1091–1101.

29. Horton, RE, McLaren, PJ, Fowke, K, Kimani, J and Ball, TB (2010). Cohorts for the study of HIV-1-exposed but uninfected individuals: benefits and limitations. J Infect Dis 202 (Suppl 3): S327–S331.

30. Cohen, J (2009). AIDS vaccine research: HIV natural resistance field finally overcomes the belief in its impotence. Blood 113: 2649–2657.

31. Nehyba, J, Hrdlicková, R and Bose, HR (2009). Dynamic evolution of immune system regulators: the history of the interferon regulatory factor family. Annu Rev Immunol 27: 20065–20069.

32. Ozato, K, Tailor, P and Kubota, T (2007). The interferon regulatory factor family in host defense: a complex web of host defenses. Eur J Immunol 37: 1073–1085.

33. Masi, G, Remoli, AL, Sgarbanti, M, Perrotti, E, Fragale, A and Battistini, A (2012). HIV-1, elements downstream of the human immunodeficiency virus type 1 long terminal repeat are required for efficient viral gene transcription. J Mol Biol 415: 167–177.

34. Wietzke-Braun, P, Maouzi, AB, Mänhardt, LB, Bickeböller, H, Ramadori, G and Mihm, S (2008). siRNA stabilization prolongs gene knockdown in primary T lymphocytes. Eur J Immunol 38: 2821–2825.

35. Ow, LF, Naid-Dreyer, CA, Pott, GB, Zapo, J, Saavedra, MT, Kim, SH et al. (2008). Reduced IL-32 controls cytokine and HIV-1 production. J Immunol 181: 557–565.

36. Nature Preceding (2013) HIV-1 coreceptor usage, transmission, and disease progression. Curr HIV Res 1: 217–227.

37. Yao, X, Yiotaki, F, Vlahovic, T, Eto, Y, Naranjis, S, Mizuguchi, H et al. (2010). Adenovirus vector covalently conjugated to polylysine glyceral with a cancer-specific promoter suppresses the tumor growth through systemic administration. Biol Pharm Bull 33: 1073–1076.

38. Hong, LH, Sin, H, Chatterjee-Kishore, M, Hatzimisourou, I, Devenish, RJ, Stark, G et al. (2002). Isolation and characterization of a human STAT1 gene regulatory element. In: Cell 119: 718–726.

39. Saldanha, SM (2003). HIV-1 coreceptor usage, transmission, and disease progression. Curr HIV Res 1: 461–479.

40. Saldanha, CA, Thomas, KE, Cady, MJ and Vogel, SN (2000). Impaired IFN-gamma production in IFN regulatory factor-1 knockout mice during ectodermic development is secondary to a loss of both IL-12 and IL-12 receptor expression. J Immunol 165: 3970–3977.

41. Elser, B, Lohoff, M, Kock, S, Gais, M, Kirchhoff, S, Kramer, P et al. (2002). IFN-gamma receptors II-4 expression by interferon regulatory factors 1 and 2 in mouse macrophages. J Immunol 161: 4811–4857.

42. Saldanha, CA, Thomas, KE, Cady, MJ and Vogel, SN (2000). Impaired IFN-gamma production in IFN regulatory factor-1 knockout mice during ectodermic development is secondary to a loss of both IL-12 and IL-12 receptor expression. J Immunol 165: 3970–3977.

43. Telfer, A (2014). HIV: the mixed blessing of interferon. Nature 511: 537–538.

44. Sandler, NG, Bosinger, SE, Estes, JD, Zhu, YT, Tharp, G, Korbitz, E et al. (2014). Type I interferon responses in mesenchymal scabs prevent SIV infection and slow disease progression. Nature 511: 801–805.

45. Jacobson, B, Mayav, V, Targat, B, Livotto, AS, Kunkel, D, Petitjean, G et al. (2009). Nonpathogenic SIV infection of African green monkeys induces a strong but rapidly controlled type I IFN response. J Virol 83: 7108–7119.

46. Schoggins, JW, Wilson, SJ, Panis, M, Murphy, MY, Jones, CT, Bieniasz, P et al. (2015). A diverse range of gene products are effectors of the type I interferon antiviral response. Nature 524: 471–485.

47. Telfer, A (2014). HIV: the mixed blessing of interferon. Nature 511: 537–538.

48. Sandler, NG, Bosinger, SE, Estes, JD, Zhu, YT, Tharp, G, Korbitz, E et al. (2014). Type I interferon responses in mesenchymal scabs prevent SIV infection and slow disease progression. Nature 511: 801–805.

49. Schoggins, JW, Wilson, SJ, Panis, M, Murphy, MY, Jones, CT, Bieniasz, P et al. (2015). A diverse range of gene products are effectors of the type I interferon antiviral response. Nature 524: 471–485.