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.

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, Kenya[26–28]; 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 regulator[33] by binding to an IFN-stimulated response element (ISRE), found in numerous genes controlling immune responses and cellular apoptosis.[2,31,34] 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 macrophages[37,38] 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.[3,27] Deleting the ISRE[39] or NF-κB site[36] 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[40] and with altered susceptibility to HIV infection.[28] Several linked IRF-1 polymorphisms were found to associate with reduced susceptibility to HIV-infection,[27,28] but not disease progression.[41] These polymorphisms were also functionally linked with reduced endogenous IRF-1 expression and a reduced responsiveness to exogenous IFN-γ signaling.[28] Importantly, they also correlated with the decreased, transient transactivation of the HIV-1 LTR.[27] 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.[26] *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.[36] 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,[28] 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,[26] and that reduced IRF-1 expression may have a critical role in the resistance phenotype against HIV-1 acquisition in these HESN FSWs.[27,28]

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.[46,47] However, transiently altering IRF-1 expression in primary cells is technically feasible[48,49] 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
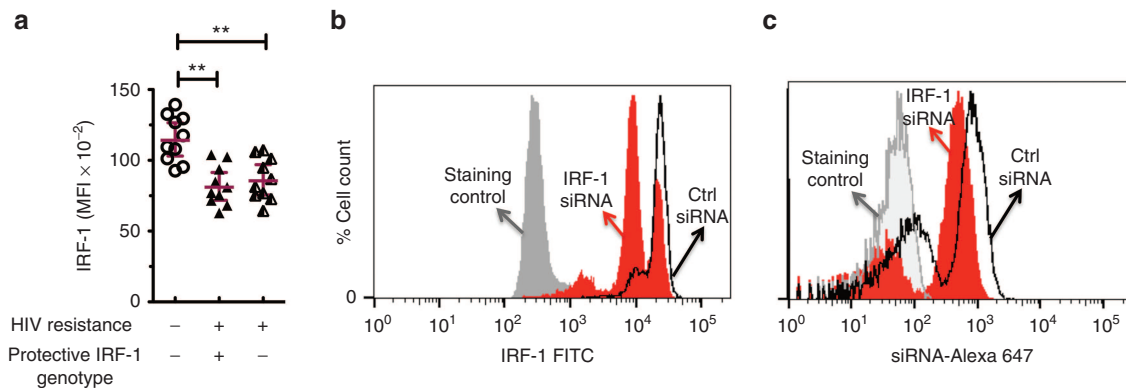

Figure 1 Endogenous IRF-1 expression in *ex vivo*, unstimulated peripheral blood mononuclear cells (PBMCs), and the uptake of IRF-1–specific siRNA by PBMCs. (**a**) Endogenous IRF-1 protein level was assessed in unstimulated PBMCs (2×10^6 cells, $n = 10$ per group) from healthy HIV-seronegative Kenyan 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 associated with the 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.
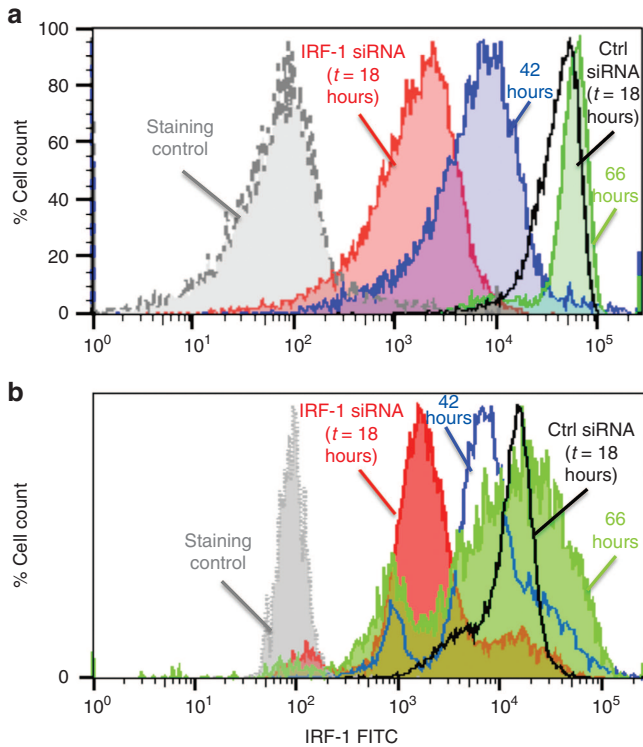
Figure 2 A time course: Transient knockdown of endogenous IRF-1 expression. (**a**) *Ex vivo* unstimulated CD4+ T cells (5×10^6 cells) and (**b**) monocytes (3×10^6 cells) from healthy local blood donors were transfected with a IRF-1–specific siRNA (10 nmol/l) or a negative control siRNA (10 nmol/l). At 6 hours posttransfection, monocytes were stimulated with low dose of phorbol 12-myristate 13-acetate (1 ng/ml) and ionomycin (50 ng/ml). At 18, 42, and 66 hours posttransfection, intracellular IRF-1 expression was examined using intracellular staining (IRF-1–specific Ab26109 Ab) and flow cytometry. Representative histograms were shown ($n = 4$). FITC, fluorescein isothiocyanate; IRF, interferon regulatory factor.

human monocyte Nucleofection solution, respectively (**Figures 2** and **3**). The effectiveness of IRF-1 knockdown was, hence, measured at 18 hours posttransfection (**Figure 3**). The efficiency of knocking down IRF-1 protein expression ranged from 25 to 55% in CD4+ T cells (**Figure 3a**: mean value: 38%, $n = 9$) and 30–60% in unstimulated monocytes (**Figure 3b**: mean value: 44%, $n = 6$). To examine whether cellular stimuli would affect the efficiency of IRF-1 knockdown in monocytes, at 6 hours posttransfection, monocytes were treated with low-dose mitogen (phorbol 12-myristate 13-acetate: 1 ng/ml and ionomycin: 50 ng/ml). Cell viability dropped to <50%, if transfected cells (with IRF-1 siRNA or control) were stimulated prior to 6 hours posttransfection, suggesting a minimum of 6 hours recovery from transfection was required. Low-dose stimulation of CD14+ monocytes consistently increased the endogenous IRF-1 expression level but maintained the efficiency of IRF-1 knockdown (**Figure 3c**: 60–80%, mean value: 75%, $n = 8$). The reduction of endogenous IRF-1 expression in CD4+ T cells and monocytes were within the range of that observed in HESN women (**Figure 1a**, 35–60%). Transfection of CD4+ T and CD14+ monocytes from healthy local blood donors with the scrambled siRNA control showed no effect on IRF-1, HDAC2, GAPDH, IL-12p35, IFN-γ, tumor necrosis

factor-α (TNF-α), STAT1α, IL-4 mRNA, or 18s rRNA levels in quantitative reverse transcription–polymerase chain reaction (RT–qPCR) assays.

To examine the effects of IRF-1 knockdown after viral entry, transfected cells and controls were infected with HIV$_{VSV-G}$ (vesicular stomatitis virus G glycoprotein pseudotyped, multiplicity of infection (MOI): 0.1) at 18 hours posttransfection (**Figure 4**). HIV$_{VSV-G}$ consists of an LTR-dependent luciferase gene, replacing the HIV-1 *Nef* (43) and is capable of entering cells independent of normal CD4/gp120 attachment. At 76 hours following HIV$_{VSV-G}$ infection, a threefold increase in LTR-driven luciferase activity was observed in CD4+ T cells transfected with IRF-1–specific siRNA, compared to a 84-fold increase with control (**Figure 4a**). A similar reduction of HIV LTR–driven luciferase activity (≥90%) after IRF-1 knockdown was observed in *ex vivo* unstimulated (**Figure 4b**) and stimulated monocytes (**Figure 4c**). These data clearly demonstrate that transactivation of LTR in HIV$_{VSV-G}$ depends on the IRF-1 expression, and that knocking down IRF-1 by as little as 38% markedly inhibits HIV-1 LTR–driven transcription. We further examined the dependence of high IRF-1 for transactivation of the HIV-1 LTR in replication-competent HIV-1 virus, unstimulated CD4+ T cells treated with IRF-1–specific siRNA were infecting with X4- and R5-tropic laboratory isolates (HIV-IIIB and HIV-BaL (MOI: 1.0)).[50] At 96 hours postinfection, a significant reduction in transactivation of the HIV-1 LTR, measured by Gag RNA transcripts (**Figure 4d**) and p24 secretion (by ≥85%, **Figure 4e**), was observed in IRF-1 siRNA–treated cells, compared to control siRNA. Furthermore, flow cytometric analyses confirmed that less of the IRF-1 siRNA–transfected cells (with reduced endogenous IRF-1 level) were also positive for HIV-1 p24 (**Figure 4f**: 4%), compared to the control siRNA–transfected cells (**Figure 4f**: 20%). To insure that these results were specific to HIV-1, we again infected the transfected CD4+ T cells 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[51] (**Figure 4g**). 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.[26,28]

IRF-1 binds to the ISRE at the promoter of numerous antiviral ISGs[31,33,46] 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.[5] 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 IFN-γ, led to comparable increases in mRNA expression of key IRF-1–regulated genes (either directly or indirectly)[52–54]: STAT1α (~25-fold), IFN-γ (~35-fold), and TNF-α (~5-fold) (4 hours poststimulation, **Figure 5b**). Similarly, IRF-1–mediated direct suppression of the IL-4 gene[55] was not affected by IRF-1 knockdown (**Figure 5**). IRF-1 plays a critical role in innate antiviral response and the
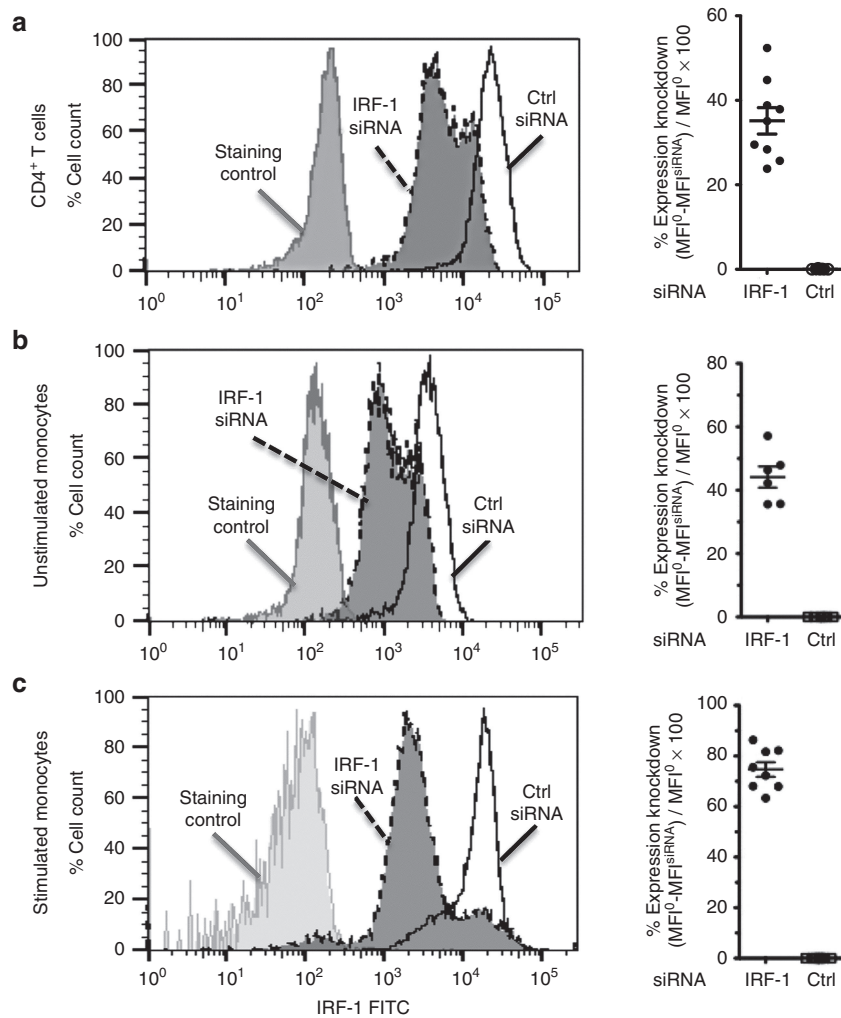
Figure 3 The efficiency of siRNA-mediated knockdown of endogenous IRF-1 expression in *ex vivo* primary CD4+ T cells and monocytes of HIV-S female sex worker (FSW) controls. Knockdown of endogenous IRF-1 expression was performed in *ex vivo* (**a**) unstimulated CD4+ T cells (*n* = 9), (**b**) unstimulated (*n* = 6), and (**c**) stimulated CD14+ monocytes (*n* = 8) from HIV negative, non-HESN (HIV-exposed seronegative), FSW controls (HIV-S). IRF-1–specific siRNA (10 nmol/l) or a scrambled control siRNA (10 nmol/l) was used in transfection (Amaxa Nucleofection). Endogenous IRF-1 protein level was assessed at 18 hours posttransfection using flow cytometry (IRF-1–specific Ab26109 Ab). Representative histograms were shown. The efficiency of IRF-1 knockdown in CD4+ T cells and monocytes were calculated using IRF-1 mean florescent intensities (MFIs): [(MFI^o (of control siRNA) – MFI^{siRNA} (of IRF-1–specific siRNA))/MFI^o] is shown on the y-axis. FITC, fluorescein isothiocyanate; IRF, interferon regulatory factor.

functional response of STAT1α, IFN-γ, and TNF-α to IFN-signaling signifies the cell's proper antiviral potential.[56,57] 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.[37] 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.[26] Recent work has also shown that innate IFN-stimulated antiviral responses are likely critical in limiting the establishment of HIV infection.[58] 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, STAT1α, and TNF-α were assessed at 22 and 42 hours postinfection 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-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
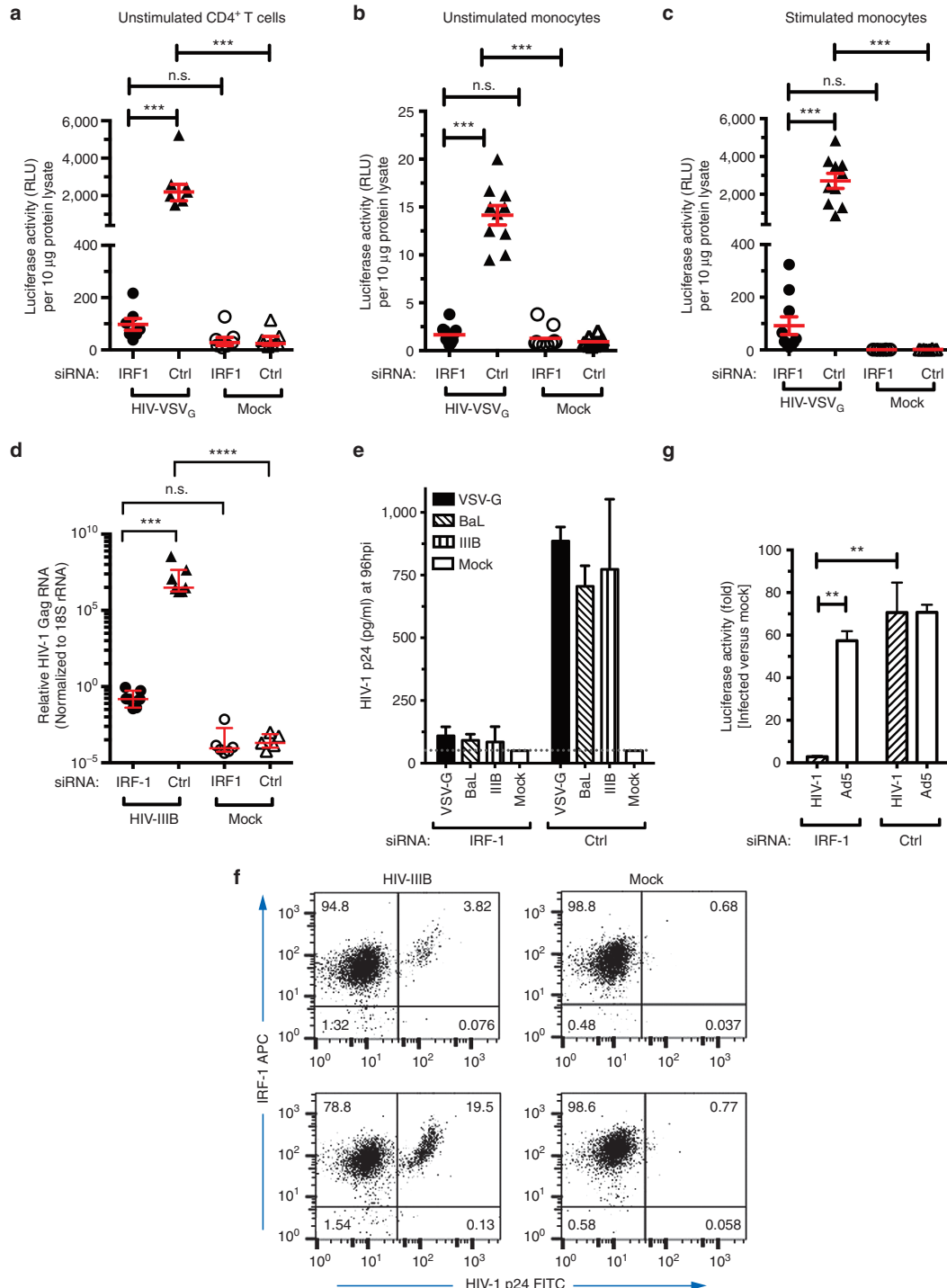
TNF-α response induced by HIV-IIIB in the control siRNA group (**Figure 6a**). While the induction of TNF-α was much larger in HIV-IIIB at 22 hours postinfection, the TNF-α mRNA level at 42 hours postinfection (~20-fold increase) was not significantly different from that induced by HIV-BaL (~18-fold increase), suggesting differential regulatory mechanisms for TNF-α induction by HIV-BaL and HIV-IIIB. Interestingly, at 42 hours postinfection (i.e., 60 hours posttransfection), the endogenous IRF-1 level was no longer inhibited by the IRF-1–specific siRNA (**Figure 2**, the half-time of IRF-1 knockdown was ~42 hours). But the impact of IRF-1–specific siRNA on 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[28] 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 implication 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.[59,60] 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.[61,62] 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,[36] this is the first study to demonstrate that partial knockdown of IRF-1 expression is achievable in unstimulated primary CD4$^+$ T cells (**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,[34,54,63,64] 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).[45,65] 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.[18] 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$_{VSV-G}$ 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 (4×10^6 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$_{VSV-G}$. 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 ($n = 2$). (**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$_{VSV-G}$ 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.
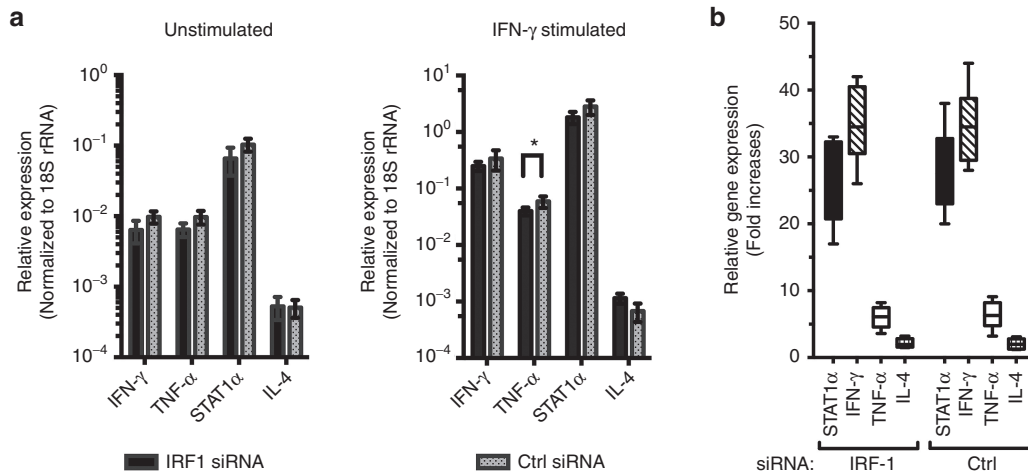
Figure 5 Expression of IRF-1–regulated genes in IFN-γ–stimulated *ex vivo* CD4⁺ T cells, transfected with IRF-1 siRNA. Primary CD4⁺ T cells (3×10^6 cells) from HIV-S female sex workers were transfected with siRNA specific for either IRF-1 (10 nmol/l) or a control siRNA (10 nmol/l). At 18 hours posttransfection, these cells were stimulated with exogenous IFN-γ (10 ng/ml). At 4 hours poststimulation, expression of STAT1α, IFN-γ, TNF-α, and IL-4 mRNA were examined using quantitative reverse transcription–polymerase chain reaction. (**a**) Relative mRNA levels of STAT1α, IFN-γ, TNF-α, and IL-4, normalized to 18S rRNA (an internal reference) level, were plotted in log scale. (**b**) Fold changes in gene expression were calculated against mock stimulation after normalization with 18S rRNA using ΔΔCt analysis program, as shown in y-axis ($n = 8$ per group, $*P < 0.05$). IFN, interferon; IRF, interferon regulatory factor; IL, interleukin; TNF, tumor necrosis factor.
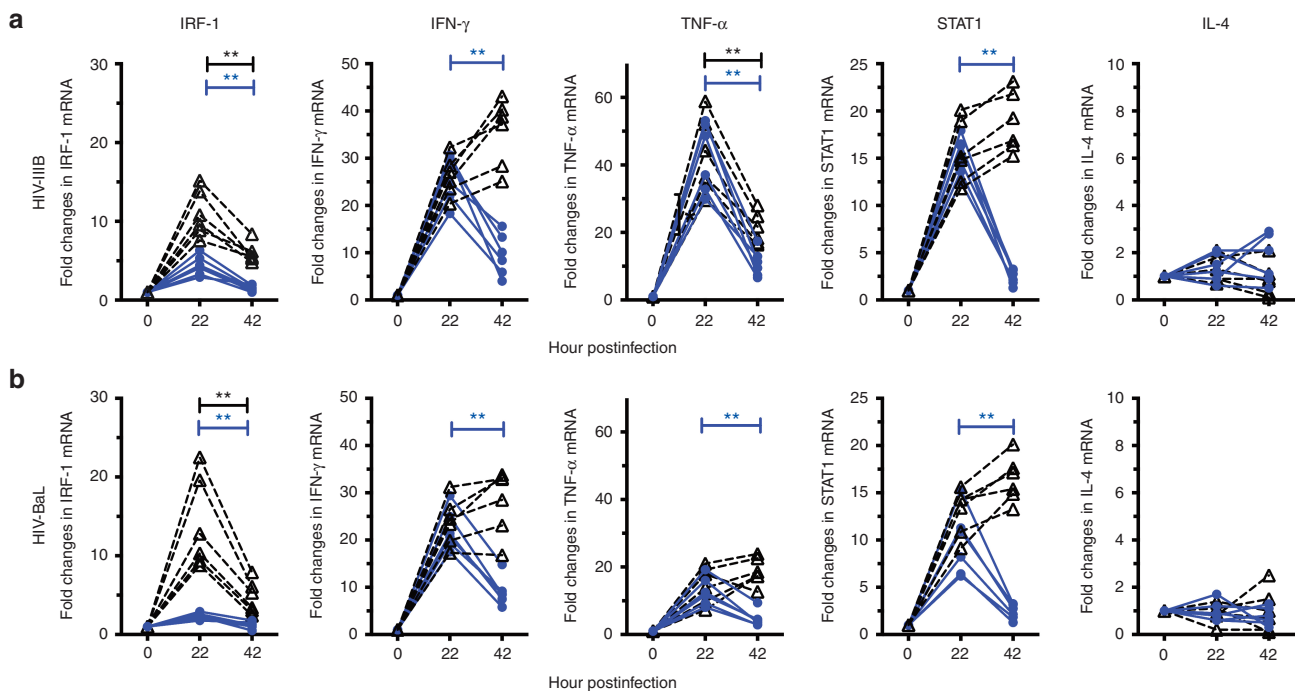

Figure 6 Altered kinetics of antiviral responsive genes to HIV infection in IRF-1 knockdown cells. Unstimulated, primary CD4⁺ T cells (4×10^6 cells) from healthy HIV-S, female sex worker controls were transfected with either IRF-1 (blue, solid circles, 10 nmol/l) or control (black, open triangles) siRNA (10 nmol/l). At 18 hours posttransfection, these cells were infected with HIV-IIIB or HIV-BaL. At 22 and 42 hours postinfection by (**a**) HIV-IIIB or (**b**) HIV-BaL, the mRNA transcript levels of immunologic genes, IRF-1, TNF-α, IFN-γ, STAT1, and IL-4, were examined using quantitative reverse transcription–polymerase chain reaction ($n = 6$). IFN-γ, interferon-γ; IL-4, interleukin-4; IRF-1, interferon regulatory factor-1; TNF-α, tumor necrosis factor-α. $**P$-values <0.001.

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 stuff 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 with intracellular staining (IRF-1–specific antibody clone C-20 from Santa Cruz Biotechnologies, Dallas, TX) and flow cytometry. There was no significant difference in IRF-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]

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% CO_2. The PBMCs were then stimulated with IFN-γ (10 ng/ml; Sigma, Oakville, ON, Canada) for the indicated time. PBMCs from healthy local donors were isolated using the same protocol and used freshly. Resting CD4+ T cells were enriched using StemSep Human Naïve CD4+ T Cell Enrichment Kit (STEMCELL Technologies, Vancouver, BC, Canada), following the manufacturer's protocol. The enriched population was CD4+ when assessed on flow cytometer (>95% CD3+ CD4+). Untouched CD14+ monocytes were enriched using Monocyte Isolation Kit II (Miltenyi Biotec, San Diego, CA) with two magnetic column purification following the manufacturer's protocol. Consistently, >87% of the enriched population stained positive for CD14.

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-1$_{VSV-G}$, was produced by cotransfection of HEK293T cells with plasmid SVCMV-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 SVCMV-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-L (Sigma, Oakville, ON, Canada) 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. $TCID_{50}$ 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 (10^6 cells/well of a 96-well plate) were incubated with HIV-1$_{VSV-G}$ (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.[45] HIV-1 p24 hybridoma 183-H12-5C (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$_{VSV-G}$—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 mmol/l ethylenediaminetetraacetic 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/Cytoperm 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 ΔΔCt 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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