TRAF6 and IRF7 Control HIV Replication in Macrophages

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Abstract

The innate immune system recognizes virus infection and evokes antiviral responses which include producing type I interferons (IFNs). The induction of IFN provides a crucial mechanism of antiviral defense by upregulating interferon-stimulated genes (ISGs) that restrict viral replication. ISGs inhibit the replication of many viruses by acting at different steps of their viral cycle. Specifically, IFN treatment prior to in vitro human immunodeficiency virus (HIV) infection stops or significantly delays HIV-1 production indicating that potent inhibitory factors are generated. We report that HIV-1 infection of primary human macrophages decreases tumor necrosis factor receptor-associated factor 6 (TRAF6) and virus-induced signaling adaptor (VISA) expression, which are both components of the IFN signaling pathway controlling viral replication. Knocking down the expression of TRAF6 in macrophages increased HIV-1 replication and augmented the expression of IRF7 but not IRF3. Suppressing VISA had no impact on viral replication. Overexpression of IRF7 resulted in enhanced viral replication while knocking down IRF7 expression in macrophages significantly reduced viral output. These findings are the first demonstration that TRAF6 can regulate HIV-1 production and furthermore that expression of IRF7 promotes HIV-1 replication.

Introduction

Infection by RNA viruses, such as HIV-1, initiates antiviral innate immune responses by inducing type I IFNs [1,2]. The treatment of primary human macrophages in vitro with type I IFN prior to HIV-1 infection inhibits virus replication, indicating that potent inhibitory factors are present [3,4]. However, pre-treatment is not a viable clinical option and, ultimately, innate immune responses in vivo fail to completely protect the human host even though genes integral to host defense are expressed. This may be due to the deregulation by HIV-1 of the signaling events necessary for induction of an appropriate innate immune response mediated by IFN or that HIV-1 replication outpaces these defenses. Type I IFNs display diverse biological effects that restrict virus replication by upregulating the expression of numerous genes (ISGs) [5–13]. For example, eukaryotic translation initiation factor 2-alpha kinase (eIF2AK2, also known as PKR), oligoadenylate synthetase 1 (OAS1) and interferon-stimulated gene 15 (ISG15) are known to be anti-HIV ISGs [14–16]. However, HIV-1 circumvents the protective effects of IFN and may even upregulate certain ISGs to its benefit [17]. Recently, Smith and collaborators identified ISGs expressed in inguinal lymph nodes that were positively associated with HIV-1 viral replication [18]. Moreover, it has been shown that the level of Type I IFN correlates with AIDS pathogenesis [19].

Production of IFN is induced by two major receptor systems for detecting RNA viruses: the toll-like receptors (TLRs) and cytoplasmic retinoic acid-inducible gene 1 (RIG-I)-like helicases (RLHs) [20,21]. The adaptor molecule TRAF6 has been shown to be involved in the TLR signaling pathway and activates IRF7, IRF3 and nuclear factor kappa B (NFkB) [22,23]. Furthermore, the RLH signaling pathway involves RNA helicase RIG-I and melanoma differentiation associated protein-5 (MDA5) [24,25] that interact with VISA through a caspase recruitment domain (CARD/CARD) interaction [26–29]. VISA induces I-kappaB kinase (IKK) and TANK binding kinase 1 (TBK1) which are responsible for the activation of IFN-regulatory factors (IRF3 and IRF7) through phosphorylation and consequently the production of type 1 IFN [30,31]. IFN production is cell type and stimuli specific and our aim was to ascertain the phenotype in the process of HIV-1 infection of primary human macrophages.

The goal of this study was to identify factors belonging to the interferon pathway that are altered during HIV-1 infection which contribute to the modulation of viral replication. We identified genes involved in the IFN signaling pathway that were impacted by HIV-1 infection of human primary macrophages. Our model is highly relevant to HIV-1 infection since macrophages are among the first cell types infected during transmission of HIV-1. In addition, macrophages infection by SIV is similar to that of CD4+ T cells in the acute phase of SIV infection [32]. We demonstrated that IFNα2 pre-treated macrophages, infected or not with HIV-1, modulated genes that were involved in the transcriptional regulation of the IFN pathway. Among them, HIV-1 downreg-
ulated TRAF6 and VISA gene expression. The downregulation of TRAF6 in macrophages infected with HIV-1 resulted in enhanced viral replication. Suppression of TRAF6 resulted in increased expression of IRF7. Overexpression of IRF7 lead to enhanced viral expression and suppression of IRF7 resulted in diminished viral output. The characterization of antiviral innate immunity genes modulated by HIV-1 infection provides a greater understanding of the mechanisms that may be used to combat the virus and improve antiviral treatments.

Results

Type I IFNα2 inhibits HIV-1 replication in primary macrophages

Kornbluth and collaborators demonstrated that macrophages treated 18 hours with 1000 IU/ml of IFNα2 prior to HIV-1 NL4-3BaLenv strain infection inhibited viral production [3]. To ascertain the stage at which HIV-1 replication was curtailed in the IFNα2 pre-treated macrophages, we monitored viral replication by quantifying TAT spliced message expression by qRT-PCR, a marker of productive viral transcription distinct from incoming viral genomic RNA. Treatment of infected cells with a non-nucleoside reverse transcriptase inhibitor, efavirenz, blocked all steps of infection after entry and prevented the appearance of TAT spliced message, demonstrating specificity for new viral production (data not shown). TAT spliced message could be detected as early as 2 hours post-infection, remained stable until 8 hours, and increased over time thereafter in macrophages infected with HIV-1. It is possible that initial TAT spliced detection could be due to viral nucleic content that’s encapsidated within virions. Still, kinetic was greatly delayed in IFNα2 pre-treated macrophages infected with HIV-1, where initial detection was observed at 120 hours and subsequently increased over time (Fig. 1). Similarly, HIV-1 p24 antigen could be detected in cellular supernatant in untreated samples at day 7 after infection. For the purpose of this study, we used the early TAT spliced message detection since the HIV-1 p24 can only be detected from day 7 in our model.

To determine the effect of HIV-1 on the IFN response and uncover potential effectors of HIV-1 replication, gene expression analysis in HIV-infected and non-infected IFNα2-treated macrophages was compared. This strategy enabled the identification of interferon-induced genes that were differentially modulated during HIV-1 infection.

HIV-1 modulates TRAF6 and VISA gene expression in IFNα2 pre-treated macrophages

To identify specific genes involved in Type I IFN signaling modulated by HIV-1 infection, gene expression in IFNα2 pre-treated macrophages infected or not with HIV-1 was measured at 2, 4, 8, and 24 hours post-infection using high-density oligonucleotide microarrays (U133 Plus 2.0 arrays). Identification of the most highly modulated genes was ascertained by applying the short time series analysis framework of Shah and Corbeil with a linear kernel [33]. The application of linear kernel in conjunction with the framework results in a configuration that effectively scores the genes based on averaged differences between consecutive measurements scaled by their respective means, and this, over all the time points in the two conditions tested. We then compared the results obtained to untreated and uninfected control cultures. The most significantly up or downregulated genes were then identified as the ones obtaining extreme (respectively high and low) scores. This analysis demonstrated significant differences in IFNα2 pre-treated macrophages infected with HIV-1 when compared to the IFNα2 pre-treated macrophages only. Analysis of the top 500 differentially expressed genes (maximum scores of comparison) between IFNα2 treated macrophages that were either infected or not with HIV-1 through all time points was performed using

![Figure 1. Type I IFNα2 inhibits HIV-1 replication in primary macrophages. A) Gene expression of TAT spliced at 2, 4, 8, 24, 120 and 168 hours post-infection of macrophages. The macrophages were incubated in the absence or presence of IFNα2 (1000 UI/ml for 18 hours). The expression level was calculated by qRT-PCR assessments of triplicates and normalized to the level of 18 S. Results shown are a representative of 2 independent experiments. A.U. corresponds to arbitrary units. B) Cell-free culture supernatants were collected at 24, 72, 120 and 168 hours post-infection and analyzed for p24 content. Results shown represent 2 combined independent experiments. doi:10.1371/journal.pone.0028125.g001](https://www.plosone.org/doi/10.1371/journal.pone.0028125.g001)
tested the expression of experiments described (see material and methods section). We
ment. Altogether we used 15 independent donors to perform the pre-treated macrophages, we evaluated independent donors, two genes occurred at 8 hours post-infection (Fig. 2A). To validate array analysis, the greatest differences in gene expression for these dynamics of their expression. As shown by the oligonucleotide IFN-related genes were unaffected by the presence of HIV-1 after the HIV-1 antiviral factors, such as APOBEC3G, APOBEC3A and TRIM22, were upregulated in IFNα2 pre-treated macrophages compared to untreated control. However, most of these IFN-related genes were unaffected by the presence of HIV-1 after the IFNα2 treatment.

Our intent was to identify genes related to interferon that were inhibited by HIV-1. Table 3 shows candidate interferon-related genes from the top 500 genes that were modulated significantly when HIV-1 was present. These genes represent potential regulators of interferon and innate immunity pathways that are modulated by HIV-1 infection in macrophages. We were specifically interested in VISA and TRAF6 since these two factors represent key control points in the regulation of the IRFs pathway, ISGs production and innate immunity, and expression of these two genes was reduced when HIV-1 was present. Therefore, we examined TRAF6 and VISA gene expression over time to assess the dynamics of their expression. As shown by the oligonucleotide array analysis, the greatest differences in gene expression for these two genes occurred at 8 hours post-infection (Fig. 2A). To validate the modulation of HIV-1 on host gene expression of the IFNα2 pre-treated macrophages, we evaluated independent donors, under the same conditions as the oligonucleotide array experiment. Altogether we used 15 independent donors to perform the experiments described (see material and methods section). We tested the expression of TRAF6 and VISA, using qRT-PCR (Fig. 2B). TRAF6 and VISA gene expression were upregulated in IFNα2 pre-treated macrophages but downregulated after HIV-1 infection.

**Table 1.** Ten most significant functional categories associated with genes differentially modulated in IFNα2 pre-treated macrophages infected or not with HIV-1.

| Function                  | p-value | No. of genes |
|---------------------------|---------|--------------|
| Phosphoprotein            | 1.9E-08 | 126          |
| Alternative splicing      | 9.1E-05 | 127          |
| Nucleus                   | 3.3E-04 | 90           |
| RNA-binding               | 1.3E-03 | 19           |
| DNA-binding               | 2.2E-03 | 47           |
| Chromosomal rearrangement | 3.0E-03 | 12           |
| Transcription             | 5.4E-03 | 46           |
| Transcription regulation  | 6.6E-03 | 45           |
| Peroxidase                | 6.9E-03 | 4            |
| Cytoskeleton              | 7.7E-03 | 14           |

Functional categories were obtained using the DAVID Bioinformatics tool. Selection of the ten most significant functional categories out of 32 functions involved the top 500 genes differentially modulated in IFNα2 pre-treated macrophages infected or not with HIV-1.
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We examined two genes was reduced when HIV-1 was present. Therefore, we examined TRAF6 and VISA gene expression over time to assess the dynamics of their expression. As shown by the oligonucleotide array analysis, the greatest differences in gene expression for these two genes occurred at 8 hours post-infection (Fig. 2A). To validate the modulation of HIV-1 on host gene expression of the IFNα2 pre-treated macrophages, we evaluated independent donors, under the same conditions as the oligonucleotide array experiment. Altogether we used 15 independent donors to perform the experiments described (see material and methods section). We tested the expression of TRAF6 and VISA, using qRT-PCR (Fig. 2B). TRAF6 and VISA gene expression were upregulated in IFNα2 pre-treated macrophages but downregulated after HIV-1 infection.

**Table 2.** Peak expression and time point of known genes associated in IFN response that were modulated in IFNα2 pre-treated macrophages compared to untreated control.

| IFN-related genes | Reference Sequence | Peak expression (Fold induction) | Time (hours) |
|-------------------|--------------------|----------------------------------|--------------|
| APOBEC3A          | NM_145699          | 1267                             | 2            |
| APOBEC3G          | NM_021822          | 3                                | 2            |
| EIF2AK2           | NM_002759          | 3                                | 4            |
| G1P2 (ISG15)      | NM_005101          | 47                               | 2            |
| G1P3              | NM_002038          | 10                               | 24           |
| IF27              | NM_005332          | 1131                             | 4            |
| IFH4L             | NM_006820          | 1182                             | 2            |
| IFIT2             | NM_001547          | 74                               | 2            |
| IFIT3             | NM_001549          | 32                               | 8            |
| IFITM1            | NM_003641          | 707                              | 4            |
| IFITM3            | NM_021034          | 27                               | 8            |
| IRF1              | NM_002198          | 3                                | 8            |
| IRF7              | NM_001572          | 12                               | 2            |
| ISG29             | NM_002201          | 702                              | 2            |
| MX1               | NM_002462          | 197                              | 8            |
| MX2               | NM_002463          | 24                               | 2            |
| OAS1              | NM_016816          | 33                               | 2            |
| OAS2              | NM_016817          | 41                               | 2            |
| OASL              | NM_003733          | 446                              | 2            |
| TRIM22            | NM_006074          | 11                               | 2            |

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at 8 hours. The downregulation of TRAF6 and VISA after HIV-1 infection of IFNα2 pre-treated macrophages suggested a possible role for these factors in the regulation of HIV-1 expression.

**TRAF6 but not VISA controls the level of expression of IRF7 and HIV-1 replication**

To analyze the impact of TRAF6 and VISA in the context of HIV-1 infection, we suppressed both genes independently using siRNA. Gene expression inhibition levels of 65% and 61% were obtained for TRAF6 and VISA respectively as compared to a non-targeted siRNA negative control (Fig. 3A). To further decipher the role of TRAF6 in the IFN antiviral response, we evaluated HIV-1 replication, 24 hours after infection of IFNα2 pre-treated or not macrophages. HIV-1 replication was significantly upregulated in macrophages with knockdown expression of TRAF6 as compared to the control pre-treated macrophages independently of IFN treatment (Fig. 3B). This is the first report that TRAF6 can function to limit HIV-1 replication. No significant differences were detected in macrophages treated with VISA siRNA when compared to the negative control siRNA irrespective of the presence of IFN pretreatment (Fig. 3B).

In this transduction pathway, TRAF6 activates IRF3 and IRF7 to promote their transcriptional functions [22,23,30,31]. Therefore, we evaluated the expression of IRF3 and IRF7 genes in this context. As shown in figure 3C and D, upregulation of HIV-1 replication in macrophages with knockdown of TRAF6 expression seen in figure 3B is accompanied by significant overexpression of IRF7 compared to the HIV-1 infected negative control (Fig 3C). No significant difference in the expression of IRF7 in HIV-1 infected macrophages with knockdown of VISA expression was

![Image](image-url)
observed. Conversely, IRF3 was downregulated in HIV-1 infected TRAF6 knockdown macrophage, while no significant difference in the expression of IRF3 in the VISA siRNA treated targets was found (Fig. 3D). This suggested that VISA was not responsible for the observed increase in viral replication. It is possible that alternative effectors can substitute for its function or that its modulation has no bearing on HIV-1 production.

To validate our finding at the protein expression level, we performed western blotting for both IRF3 and IRF7. Levels of these proteins in lysates of TRAF6 knockdown or control siRNA treated (200 nM) macrophages from three independent donors were normalized using β-actin. As seen in figure 3E, IRF3 is downregulated (0.83 fold, corresponding to a 17% reduction) and IRF7 is upregulated (3.59 fold) in TRAF6 knockdown macrophages compared to control siRNA. The protein data confirmed our gene expression results and suggested that the regulation of HIV-1 replication by TRAF6 involved IRF7.

IRF7 downregulation decreases HIV-1 replication

To elucidate if IRF7 directly alters HIV-1 replication, we first evaluated the effect of downregulation of IRF7 expression using siRNA. Macrophages were transfected with specific siRNAs targeted against IRF7 or silencer negative control. As shown in figure 4, transfection of specific IRF7 siRNA (50 nM) significantly reduced IRF7 mRNA (55% reduction) (Fig. 4A) and protein (42% reduction) (Fig. 4B) expression. The inhibition of IRF7 resulted in a comparable downregulation of HIV-1 replication (51% reduction) (Fig. 4C).

IRF7 overexpression increases HIV-1 replication

To confirm that IRF7 promoted HIV-1 replication, IRF7 was overexpressed in macrophages followed by infection with HIV-1 at a multiplicity of infection (MOI) of 0.002. While protein levels expression were only increased modestly (2 fold) by transfection of an IRF7 expressing vector (Fig. 5A) HIV-1 replication was markedly upregulated (14 fold) indicating enhanced viral replication when IRF7 is present in sufficient quantity (Fig. 5B). This occurred despite IFNα/β expression in the presence or absence of IRF7 overexpression (data not shown). This is the first demonstration that links IRF7 to an increased HIV-1 replication.

Discussion

Our investigation demonstrated the capability of HIV-1 infection to alter critical early events involved in appropriate induction of the IFN response. This ability emphasizes the importance of the IFN signaling pathways in controlling HIV-1 replication and suggests that HIV-1 has evolved mechanisms to compromise this innate host immune response to favor its propagation. We present evidence that TRAF6, important for transcriptional regulation of the interferon pathway, is affected early in the process of HIV-1 infection of primary human

Table 3. Peak expression and time point of candidate genes associated with IFN response that were modulated in IFNα2 pre-treated macrophages compared to untreated control and modulated by HIV-1.

| IFN-related genes | Reference Sequence | Peak expression IFNα2/CTRL (Fold induction) | Time (hours) | Peak expression IFNα2/HIV infection (Fold induction) | Time (hours) |
|-------------------|--------------------|------------------------------------------|--------------|-------------------------------------------------|--------------|
| BIRC3             | NM_001165          | 7                                        | 2            | 3.5                                              | 2            |
| CCL5              | NM_002985          | 8                                        | 2            | 4                                                 | 8            |
| CD38              | NM_001775          | 39                                       | 4            | 13                                                | 16           |
| CEBPD             | NM_005195          | 2                                        | 8            | 1                                                 | 16           |
| CH3L1             | NM_001276          | 2                                        | 2            | 1                                                 | 2            |
| CXCL10            | NM_001565          | 124                                      | 2            | 31                                                | 2            |
| CXCL11            | NM_005409          | 118                                      | 2            | 39                                                | 8            |
| CXCL9             | NM_002416          | 28                                       | 2            | 9                                                 | 16           |
| EHF               | NM_012153          | 4                                        | 8            | 1                                                 | 16           |
| HMGA1             | NM_145899          | 2                                        | 8            | 1                                                 | 24           |
| LILRB2            | NM_005874          | 16                                       | 8            | 8                                                 | 2            |
| NCOA3             | NM_181659          | 2                                        | 2            | 4                                                 | 4            |
| OGT               | NM_181632          | 5                                        | 8            | 4                                                 | 2            |
| PIGR              | NM_002644          | 3                                        | 8            | 1.5                                               | 8            |
| PIP5K2A           | NM_005028          | 3                                        | 8            | 1.5                                               | 8            |
| SAMHD1            | NM_015474          | 2                                        | 8            | 1                                                 | 8            |
| TAP2              | NM_000544          | 16                                       | 2            | 8                                                 | 2            |
| TCF4              | NM_003199          | 2                                        | 2            | 0.7                                               | 16           |
| TRAF6             | NM_004620          | 2                                        | 8            | 1                                                 | 8            |
| TXNIP             | NM_006472          | 2                                        | 8            | 1                                                 | 16           |
| VISA              | NM_020746          | 2                                        | 8            | 1                                                 | 8            |

Selection of the top 500 most modulated genes when comparing the IFNα2 pre-treated macrophages infected or not with HIV-1. Of these, 55 were related to interferon as a keyword. From these 55, 21 were upregulated by interferon as compared to untreated control (peak expression and time point) but modulated differentially when HIV-1 was added. Those genes may represent interferon effectors that are modulated by HIV-1 infection during the interferon response.

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macrophages. Knockdown of the antiviral factor TRAF6 resulted in a significant augmentation in HIV-1 replication, as assessed by the expression of TAT spliced message.

TRAF6 has been shown to be important for control of replication of other viruses. Konno and collaborators reported that the absence of TRAF6 in TRAF6−/− mouse embryonic fibroblasts, (MEF cells) resulted in enhanced replication of RNA viruses such as Newcastle disease virus and encephalomyocarditis virus [23]. Similarly, TRAF6 has been shown to be critical for IFNα and β induction in response to vesicular stomatitis virus infection and intracellular double-stranded RNA, poly (I:C) [35]. All of these studies confirm that TRAF6 is induced as part of the normal innate immune response against viruses and our findings indicate it plays a similar role in curtailing HIV-1 replication. Interestingly, our results also suggest that HIV-1 has devised strategies to circumvent this inhibition. HIV-1 infection reduced TRAF6 gene expression with the resulting enhanced viral replication. Despite being upregulated by interferon and repressed by HIV-1 infection, VISA, another important mediator of innate immunity, did not have any impact on HIV-1 replication in our study. Instead, HIV-1 replication was similar to that of the control. There may be compensatory mechanisms circumventing the ablation or reduction of VISA expression during HIV-1 infection.

TRAF6 is implicated in the activation of the transcription factors IRF3 and IRF7 [22,36]. TRAF6 polyubiquitinates IRF7, a post-translational modification necessary for the IFN production as demonstrated in fibroblasts [22,36]. Both transcription factors are key regulators of IFN production [37–44] and have essential roles in the activation of antiviral immunity [44–46]. Certain viruses have evolved mechanisms to overcome their effect such as the Thogoto virus ML protein that reportedly inhibits the activation of IRF7 [47]. In addition, vesicular stomatitis virus was more efficient at infecting fibroblasts when IRF7 was ablated [48]. In contrast, IRF7 increased human papillomavirus (HPV) type 8 late promoter activity via direct binding to viral DNA and IRF3 induced strong HPV8 suppression in primary keratinocytes. This is consistent with our study where IRF7 promoted HIV-1 replication [49]. The knockdown of TRAF6 induced the expression of IRF7 but decreased the expression of IRF3 at both the gene and protein levels.

Overexpression of IRF7 resulted in a significant increase in HIV-1 replication in primary human macrophages. Moreover, inhibition of IRF7 brought about a concomitant decrease of viral output. Our results show that when TRAF6 is reduced, IRF7 is overproduced and contributes to enhanced viral replication. Recently, a study by Smith and collaborators identified host genes expressed in inguinal lymph nodes that were associated determinants of HIV-1 viral load [18]. Moreover, in primate models, higher levels of type 1 interferon characterize the pathogenic state [19]. Consistent with our results with IRF7, these analyses reflect an antiviral host response mediated by the interferon pathway that is associated with higher viral load rather than inhibition of HIV-1.
replication, suggesting that HIV-1 subverts the innate immune response to its own benefits. IRF7 could contribute to enhanced HIV-1 replication by several potential, non-exclusive mechanisms. IRF7 could facilitate Long Terminal Repeat (LTR) driven expression of the virus and/or favor transcriptional activation of cellular genes that could contribute to increased viral output. However, on its own IRF7 is not capable of enhancing the activity of a LTR-luciferase reporter in primary human macrophages (Figure S1). Thus, if IRF7 modulates the HIV-1 LTR, it may require interaction with another protein. In opposition, IRF1, a positive control in this setting, could readily enhance LTR-driven replication. Presumably, IRF7 could facilitate the translocation

Figure 3. TRAF6 but not VISA controls the level of expression of IRF7 and HIV replication. Primary human macrophages were subjected to siRNA knockdown for TRAF6 and VISA. The concentration of siRNA used was 200 nM. A) Percentages of inhibition of TRAF6 and VISA in the presence of specific siRNA targeting both gene individually [n = 3] compared to a control non-targeted siRNA [n = 3]. Level of expression was measured by qRT-PCR B) Macrophages were all infected with HIV-1 with and without an IFNα2 pretreatment, and treated with specific siRNA. The level of TAT spliced message measured 24 hours post-infection in TRAF6 and VISA conditions were compared to the negative control siRNA. C) Level of IRF7 and D) Level of IRF3 message expression in the absence of IFNα2 when TRAF6 and VISA are repressed by siRNA compared to the negative control siRNA. The qRT-PCR results were normalized to the level of 18S. An * denotes a significant difference (P<0.05, one sample t-test) compared to the negative control siRNA. Each symbol in a group represents one independent donor. E) Western blot for IRF3 and IRF7, 24 hours post-infection in macrophages with TRAF6 suppression compared to siRNA control. The densitometry of the bands was normalized to that of β-actin and shows a decrease in protein level of IRF3 and an increase of IRF7. The ratio is shown next to the respective panels. Results shown are representative of 3 independent experiments.

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and activity of NFkB resulting in the promotion of HIV-1 replication since NFkB is a potent activator of LTR-driven expression of the virus. Although demonstrated in Jurkat T cells, IRF7 could, in a situation analogous to IRF1, interact with NFkB to promote this LTR-driven transcription [50,51]. Preliminary chromatin immunoprecipitation experiments (ChIP on chip monitoring 19,000 genes) using HIV-infected primary macrophages indicate enrichment for the promoter regions of three genes that stimulate the NFkB pathway, coactivator-associated arginine methyl transferase 1 (CARM1), B-cell CLL/lymphoma 10 (BCL10) and solute carrier family 20 member (SLC20A1) [52–54]. These interesting candidates warrant

Figure 4. IRF7 expression modulates HIV-1 replication in primary macrophages. A) Level of IRF7 gene expression in macrophages treated with siRNA targeting IRF7 (50 nM) \( [n = 3] \) compared to a control non-targeted siRNA \( [n = 3] \) resulted in a 55% reduction in IRF7 gene expression. The qRT-PCR results were normalized to the level of 18 S. B) Western blot for IRF7 protein treated as in panel A shows a decrease of 42% for IRF7 protein. The densitometry of the bands was normalized to that of \( \beta \)-actin. Results shown are representative of 2 independent experiments. C) Level of TAT spliced expression following inhibition (51%) by IRF7 as compared to control. The qRT-PCR results were normalized to the level of 18 S. An * denotes a significant difference \( (P<0.001, \) one sample \( t \)-test) compared to the negative control siRNA. Each symbol in a group represents one independent donor.

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Figure 5. IRF7 overexpression increases HIV-1 replication in primary macrophages. A) Western blot for IRF7 protein, 24 hours post-transfection of macrophages overexpressing IRF7 showed a 2 fold increase compared to empty vector control. The densitometry of the bands was normalized to that of \( \beta \)-actin. The ratio is shown next to the respective panels. Results shown are representative of 2 independent experiments. B) TAT spliced expression was augmented 14 fold in primary human macrophages overexpressing IRF7 compared to empty vector control. The qRT-PCR results were normalized to the level of 18 S. An * denotes a significant difference \( (P<0.05, \) one sample \( t \)-test) compared to the empty vector control. Each symbol in a group represents one independent donor.

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additional investigation to determine if they promote HIV-1 replication.

Alternatively, the increase in IRF7 expression together with the reduction in IRF3 expression might favour the formation of IRF7 homodimers instead of IRF3 homodimers or IRF3/IRF7 heterodimers. IRF3 and IRF7 require dimerization among other post-translational modifications to act as transcription factors. This shift in the transcription factor complex composition would induce the activation of a distinct set of genes some of which could contribute to HIV-1 replication. There is evidence in paramyxovirus infection of lymphocyte B-cells that the relative ratio of IRF3/IRF7 contributed to differential expression of IFNα2-related genes [55].

In conclusion, we demonstrated that TRAF6 is an important factor involved in the replication of HIV-1 in primary human macrophages. HIV-1 infection downregulated TRAF6 expression. In the absence of TRAF6, IRF7 is overproduced and contributes to enhanced HIV-1 replication. It is important to elucidate how HIV-1 interferes with this innate immunity in order to favour its own replication.

Methods

Cells

Monocytes were recovered from whole blood of healthy human donors by negative selection with RosetteSep™ human monocyte enrichment cocktail (Stem Cell Technologies Inc, Vancouver, BC), according to the manufacturer’s instructions. A total of 15 independent donors were utilized for the experiments described in this manuscript. Monocytes to be differentiated into macrophages were plated into T25 flasks in RPMI 1640 supplemented with 10% human serum (Wisent Inc., Québec, QC) and 25 ng/ml of monocyte/macrophage colony-stimulating factor (M-CSF) (Genscript Corp, Piscataway, NJ) for differentiation experiments and subsequent validation by real-time quantitative RT-PCR (qRT-PCR). qRT-PCR quantification, knockdown assays and western blot assays used monocytes plated in RPMI 1640 with 10% fetal bovine serum (Invitrogen Canada, Burlington, ON) and 25 ng/ml of monocyte/macrophage colony-stimulating factor (M-CSF) (Genscript Corp, Piscataway, NJ) for differentiation into macrophages. Cell-surface expression of CD14 was characterized by using fluorescein isothiocyanate (FITC)-conjugated mAb (clone MEM-18) (Cedarlane Laboratories Limited, Burlington, ON) after allowing monocytes to differentiate for 5 days. By this criterion, the cells were >97% monocytes differentiated macrophages (MDMs) as assessed by flow-cytometry evaluation by using EPICS XL (Beckman Coulter, Fullerton, CA) (data not shown).

Production of virus stocks

Virus stocks were produced by the transfection of 293T cells using the calcium phosphate co-precipitation method [56]. The infectious molecular clone used in this study was pNL4-3BaLenv. The pNL4-3BaLenv vector was generated by replacing the env gene of the T-tropic HIV-1 strain, NL4-3, with that of the macrophage-tropic HIV-1 BaL strain, thus resulting in an infectious molecular clone with macrophages-tropic properties [57] (pNL4-3BaLenv) was kindly provided by M. J. Tremblay, Laval University, Québec, QC). Supernatants from transfected cells were clarified by filtration through 0.22 micron cellulose acetate syringe filter and ultracentrifuged. Viruses were purified with the OptiPrep velocity gradient method (Axis-Shield PoC, Oslo, Norway). This methodology precludes having secreted products such as cytokines in the viral preparations. The 50% tissue culture infectious dose (TCID 50) of HIV-1 stock was calculated by using the nonparametric methods of Spearman-Karber [58], T2M-BL cell line was used to standardize every viral stock used in this study.

Treatment and infection of macrophages

After allowing monocytes to differentiate for 5 days, purified primary macrophages were treated 18 hours with 1000 IU/ml of IFNα2 (PBL, Biomedical Laboratories, Piscataway, NJ), which represents the lowest concentration achieving maximum inhibition of HIV-1 NL4-3BaLenv strain replication in these cells [3]. Pre-treated macrophages (18 hours) were infected with the NL4-3BaLenv strain of HIV-1 at a MOI of 0.002 (physiological dose). Uninfected and untreated cells were used as controls. Aliquots of cells (3 × 10⁵ cells) were taken at 2, 4, 8, and 24 hours after infection, lysed in 1 ml of TRIzol (Invitrogen Canada, Burlington, ON) and stored at −80°C.

RNA isolation, labeling, and array hybridization

Total RNA from the same donor was isolated for each condition and time point using the TRIzol method according to the manufacturer’s instructions (Invitrogen Canada, Burlington, ON) and then digested with deoxyribonuclease to remove any contaminating genomic DNA (Turbo DNase-free, Ambion, Applied Biosystems Canada, Streetsville, ON). RNA quality and quantity was assessed using an Agilent Technologies 2100 bioanalyzer and RNA 6000 Nano LabChip kit (Agilent Technologies Canada, Mississauga, ON). RNA integrity numbers (RIN), which estimate the integrity of total RNA samples, ranged from intact (RIN 10) to degraded (RIN 2). RIN were above eight for all samples in our experiments. Total RNA (100 ng) was converted to complementary DNA (cDNA), which was amplified and transcribed to produce biotinylated cRNA using the Two-Cycle cDNA synthesis kit (Affymetrix, Santa Clara, CA). Fragmented cRNA (15 μg) were hybridized to Affymetrix Human Genome U133 Plus 2.0 arrays (Affymetrix, Santa Clara, CA) for 16 h at 45°C with constant rotation at 60 rpm. The arrays were washed and stained with streptavidin-phycocerythrin ( Molecular Probes, Eugene, OR) and biotinylated goat anti-streptavidin (Vector Laboratories, Burlingame, CA) using the Affymetrix Fluidics Station 450 (protocol EukGE-WS2v5.450), then read using the Affymetrix GeneChip Scanner 3000.

Oligonucleotide array gene expression analysis

GeneChip Robust Multi-array Average (GCRMA) was the procedure used to normalize the data obtained from the oligonucleotide array analysis [59]. Identification of the most differentially expressed genes between IFNα2 pre-treated macrophages infected or not with HIV-1 was done using the short time series analysis framework of Shah and Corbeil [33]. This framework generalizes the Hilbert-Schmidt Independence Criterion (HSIC) based framework of Song and collaborators [60] to the short time-series setting by utilizing tensor analysis techniques resulting in a generic analysis tool that allows both identification of most differentially expressed genes and patterns of interest in gene behavior (such as upregulation by IFNα2 with subsequent suppression by HIV-1). We incorporated a linear kernel in the framework, and obtain a ranking criterion, which is analogous to the classical mean-difference based criterion for static gene expression data [33,61]. The criterion is based on averaging the differences between consecutive measurements scaled by their respective means, over all the time points. The framework also allows identification of highly non-monotonic variations in gene behavior. The most differentially expressed genes were then extracted using an empirically determined threshold over the ranking scores. These genes were then analyzed with respect to their functionalities and ontological classification using the DAVID analysis software to determine significant functionalities associated with HIV-1 infection after IFNα2 treatment of the cells.
Furthermore, the top 500 differentially expressed genes between IFNα2 macrophages that were either infected with HIV-1 or non-infected were analyzed to determine which genes had an association with the keyword interferon using Chilbot software [62].

### qRT-PCR examination of gene expression

cDNA from independent donor cultures was generated from 200 ng of total RNA using a random primer hexamer following the instructions for Superscript II (Invitrogen Canada, Burlington, ON). Primers were designed using Primer Express 2.0 (Applied Biosystems Canada, Streetsville, ON) and their sequences are presented in Table 4. Amplicons were detected in most cases using the AmpliFhir UniPrimer amplification and detection system (Chemicon International, Temecula, CA) except for TAT spliced message, a marker of HIV replication, which was detected using the TaqMan system (Applied Biosystems Canada, Streetsville, ON), which allows for greater sensitivity and can detect infection as early as 2 hours post-inoculation. Forward primers used in the AmpliFhir UniPrimer system contained an additional 5’ Z sequence (ACTGAACT/GACGGTACA) that is not included in Table 4. Equal amounts of cDNA (20 ng) were run in triplicate and amplified using the AmpliFhir UniPrimer in a 15 μl reaction containing 7.5 μl of 2X Universal PCR Master Mix (Applied Biosystems Canada, Streetsville, ON), 10 nM of Z-tailed forward primer, 100 nM of reverse primer, 100 nM of AmpliFhir Uniprimer fluorescein probe (Chemicon International, Temecula, CA) and 5 μl of DNA target [63]. Moreover, no-template controls were used. The mixture was incubated at 50°C for 2 min, at 95°C for 4 min, then cycled at 95°C for 15 sec and at 55°C for 40 sec, 55 times using the Applied Biosystems 7900HT Sequence Detection System. The amplifications using the Taqman system were run in a 15 μl reaction containing 7.5 μl of 2X Universal PCR Master Mix, 200 nM of forward primer, 200 nM of reverse primer, 250 nM of Taqman probe and 5 μl of DNA target. The mixture was incubated at 50°C for 2 min, at 95°C for 10 min, then cycled 40 times at 95°C for 15 sec and at 60°C for 1 min. Amplification efficiencies were validated and normalized to ribosomal 18 S and quantity of target gene (arbitrary units) was calculated according to a standard curve. The standard curve consists of different dilution of a RNA sample that generates data points in the linear portion of the PCR amplification.

### Enzyme-linked immunosorbent assay (ELISA)

Macrophages were infected with HIV-1 at a MOI of 0.002 for 2 hours at 37°C. Next, the virus-cell mixture was washed with PBS to remove unbound virus. The p24 content was determined using a sensitive in-house double-antibody sandwich ELISA specific for the viral p24 protein. In this test, the 183-H12-5C and 31-90-25 antibodies are used in combination to quantify p24 levels. Virus production was estimated by measuring p24 levels in cell free culture supernatants.

### Knockdown assays

Macrophages from healthy donors were transfected with pre-designed siRNA SMARTpool for TRAF6 (M-004712-00; Dharmacon, Lafayette, CO), VISA (L-024237-00; Dharmacon, Lafayette, CO) or IRF7 (L-011810-00; Dharmacon, Lafayette, CO) with the Oligofectamine Transfection Reagent according to the manufacturer’s instructions (Invitrogen Canada, Burlington, ON). SMARTpool technology combines four siRNAs that target different mRNA regions. Silencer Negative control siRNAs (AM4635; Ambion, Applied Biosystems Canada, Streetsville, ON), used as control do not target any human gene product. The best gene expression inhibition levels of 63%, 61% and 55% were obtained for TRAF6, VISA and IRF7, respectively, 48 hours post-transfection (see Figure S2 for TRAF6 results). The transfected macrophages were then infected with the NL4-3BaLow strain of HIV-1 at an MOI of 0.002 for 24 hours. In the case of IFNα2 pre-treatment, 30 hours post-transfection the macrophages were treated for 18 hours before the infection.

### Protein assays

Immunoblot assays were performed on total cell lysates of independent donors. Proteins were isolated for each condition using total extract buffer (Tris-base pH 6.8, SDS 20%, mercaptoethanol and glycerol). Cell extracts (30 μg per lane) were resolved by SDS-PAGE on 4–20% gels (BIO-RAD Laboratories Canada Ltd, Mississauga, ON) and transferred on PVDF membranes. The indicated antibodies against IRF7 (H-246; Santa Cruz Biotechnology, Santa Cruz, CA), IRF5 (S-12; BD-Pharmingen, Oakville, ON), TRAF6 (H-274; Santa Cruz Biotechnology, Santa Cruz, CA) and β-actin (A-15; SIGMA, Saint-Louis, MO) were visualized by alkaline phosphatase-based enhanced chemiluminescence. The densitometry of the bands was compared after normalization with β-actin.

### Overexpression assays

Macrophages (15×10⁶ cells) from healthy donors were transfected with 9 μg of pcDNA3-IRF7-myc (Kindly provided by J. Hiscott, McGill University, Montréal, QC) or pcDNA3 (empty vector control) with the Lipofectamine Transfection Reagent according to the manufacturer’s instructions (Invitrogen Canada, Burlington, ON). We obtained the best gene overexpression after 24 hours of transfection, after which, the transfected macrophages were infected with the NL4-3BaLow strain of HIV-1 at a MOI of 0.002 for 24 hours.

### Microarray data accession number

Microarray results have been deposited in Gene Expression Omnibus database under accession number GSE30536.

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**Table 4.** Sequence of Oligonucleotide Primers used in Real-Time qRT-PCR Gene Expression Analysis.

| Primera | Nucleotide Sequence |
|---------|---------------------|
| TRAF6-F | AAGGATGCGAGTTCACAATGT |
| TRAF6-R | TTTTCCACAGATTTCATGCA |
| VISA-F | ACTTCTAGGAGCCCCAATT |
| VISA-R | CTTGTCGCGAGATCATCACT |
| IRF7-F | CGACATCGAGTGCTTCCTATG |
| IRF7-R | ACTGGTCTCTAGGGCCG |
| IRF3-F | TCTGTACCCGGAGAACATCTG |
| IRF3-R | CAACACCTGTATTGCTAACTCAT |
| TAT Spliceda | CCTAAATCCGTCCTGACCATG |
| TAT Splicedb | GGAGTTCGGGTCTTTGATAGAG |
| TAT Spliced-probe | AAAGGCTTAGGCAATTC |

aTAT Spliced was detected using Taqman.
bTAT Spliced-probe was in 5’-3’ orientation, R (reverse) primer sequence is reverse complemented.
Statistical analysis
Analysis was performed by paired t-test to compare two population means in the case of two samples that are correlated (same donor) or one sample t-test to compare the sample mean to the population mean (control). P values of less than 0.05 were considered to be statistically significant.

Ethics statement
Comité d'éthique de la recherche du CHUQ approved the study and written informed consent was provided by study participants.

Supporting Information
Figure S1 Transactivation of the HIV-1 LTR by IRF1, IRF3 and IRF7. Expression vector for IRFs and pBlue_5’ LTR LUC were co-transfected in primary macrophages. Luciferase activity (RLU) was measured at 24 h post-transfection. Results are the mean of two separate experiments. (TIF)

References
1. Beutler B, Hoebe K, Du X, Ulevitch RJ (2003) How we detect microbes and respond to them: the Toll-like receptors and their transducers. J Leukoc Biol 74: 479–495.
2. Takeda K, Akira S (2005) Toll receptors and pathogen resistance. Cell Microbiol 5: 145–155.
3. Kornfeld RS, Oh PS, Munis JR, Cleveland PH, Richman DD (1988) Interferons and bacterial lipopolysaccharide protect macrophages from productive infection by human immunodeficiency virus in vitro. J Exp Med 169: 1137–1151.
4. Meylan PR, Guatelli JC, Munis JR, Richman DD, Kornfeld RS (1993) Mechanisms for the inhibition of HIV replication by interferons-alpha, -beta, and -gamma in primary human macrophages. Virology 193: 138–148.
5. Pestka S, Langer JA, Zoon KC, Samuel CE (1987) Interferons and their actions. Annu Rev Biochem 56: 727–777.
6. Samuel CE (1988) Mechanisms of the antiviral action of interferons. Prog Nucleic Acid Res Mol Biol 35: 27–148.
7. Stark GR, Kerr IM, Williams BR, Silverman RH, Schreiber RD (1998) How cells respond to interferons. Annu Rev Biochem 67: 227–264.
8. Barr SD, Smiley JR, Bushman FD (2008) The interferon response inhibits HIV particle production by induction of TRIM22. PLoS Pathog 4: e1000087.
9. Bishop KN, Holmes RK, Malim MH (2006) Antiviral potency of APOBEC proteins does not correlate with cytidine deamination. J Virol 80: 8450–8458.
10. Jouvenet N, Neil SJ, Zhadina M, Zang T, Kratovac Z, et al. (2009) Broad-spectrum inhibition of retroviral and filoviral particle release by tetherin. J Virol 83: 1837–1844.
11. Neil SJ, Zang T, Biensassy PD (2008) Tetherin inhibits retrovirus release and is antagonized by HIV-1 Vpu. Nature 451: 425–430.
12. Peng G, Lei KJ, Jin W, Greenswell-Wild T, Wahl SM (2006) Induction of APOBEC3 family proteins, a defensive maneuver underlying interferon-induced anti-HIV-1 activity. J Exp Med 203: 41–46.
13. Stremlau M, Owens CM, Perron MJ, Kiesling M, Antiasier P, et al. (2004) The cytoplasmic body component TRIMEAlpha restricts HIV-1 infection in Old World monkeys. Nature 427: 848–853.
14. Kunim MS, Farzadegan H, Margolick JB, Vlahov D, Pitta PM (1995) Identification of human immunodeficiency virus primary isolates resistant to interferon-alpha and correlation of prevalence to disease progression. J Infect Dis 171: 822–829.
15. Muto NF, Marinard-Mari C, Adelson ME, Suhadolnik RJ (1999) Inhibition of particle production by induction of MDA5 and LGP2 in antiviral immune. J Immunol 173: 2851–2858.
16. Schroder HC, Ugarkovic D, Merz H, Kuchino Y, Okamoto T, et al. (1990) AIDS progression is associated with the emergence of IL-17-producing cells early after simian immunodeficiency virus infection. J Immunol 144: 984–992.
17. Izmailova E, Bertley FM, Huang Q, Makori N, Miller CJ, et al. (2003) HIV-1 Tat reprograms immature dendritic cells to express chemoattractants for activated T cells and macrophages. Nat Med 9: 191–197.
18. Smith AJ, Li Q, Wiertz SL, Schacker TW, Riffy CS, et al. (2010) Host genes associated with HIV-1 replication in lymphatic tissue. J Immunol 185: 5417–5424.
19. Campillo-Gimenez L, Cunmut MC, Fay M, Kared H, Monceaux V, et al. (2010) AIDS progression is associated with the emergence of IL-17-producing cells upon infection of macrophages knockdown with TRAF6 siRNAs. J Immunol 138: 9358–9367.

Figure S2 Western blot for TRAF6 24 hours post-infection of macrophages knockdown with 50, 100, 200 and 500 nM of TRAF6 siRNAs. β-actin was used as a normalizer for input. 200 nM of siRNA were used since a better percentage of inhibition in TRAF6 knockdown experiments were obtained at this concentration with respect to the protein level. (TIF)

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Author Contributions
Conceived and designed the experiments: M. Sirois LR JC. Performed the experiments: M. Sirois RA. Analyzed the data: M. Sirois LR M. Shah CHW JE JC. Wrote the paper: M. Sirois CHW JE JC.
37. Lin R, Heybroeck C, Piña PM, Hiscott J (1998) Virus-dependent phosphorylation of the IRF-3 transcription factor regulates nuclear translocation, transactivation potential, and proteasome-mediated degradation. Mol Cell Biol 18: 2986-2996.
38. Marie I, Durbim JE, Levy DE (1998) Differential viral induction of distinct interferon-alpha genes by positive feedback through interferon regulatory factor-7. EMBO J 17: 6660-6669.
39. Sato M, Hata N, Asagiri M, Nakaya T, Taniuchi T, et al. (1998) Positive feedback regulation of type I IFN genes by the IFN-inducible transcription factor IRF-7. FEBS Lett 441: 106-110.
40. Sato M, Tanaka N, Hata N, Oda E, Taniuchi T (1998) Involvement of the IRF family transcription factor IRF-3 in virus-induced activation of the IFN-beta gene. FEBS Lett 425: 112-116.
41. Wahelet MG, Lin CH, Parikh BS, Ronco LV, Howley PM, et al. (1998) Virus infection induces the assembly of coordinately activated transcription factors on the IFN-beta enhancer in vivo. Mol Cell 1: 507-518.
42. Weaver BK, Kumar KP, Reich NC (1998) Interferon regulatory factor 3 and CREB-binding protein/p300 are subunits of double-stranded RNA-activated transcription factor DRAF1. Mol Cell Biol 18: 1539-1568.
43. Yoneyama M, Suhrara W, Fukahara Y, Fukuda M, Nishida E, et al. (1998) Direct triggering of the type I interferon system by virus infection: activation of a transcription factor complex containing IRF-3 and CBP/p300. EMBO J 17: 1087-1095.
44. Ning S, Pagano JS, Barber GN (2011) IRF7: activation, regulation, modification and function. Genes Immun.
45. Honda K, Yama H, Nejish H, Asagiri M, Sato M, et al. (2005) IRF-7 is the master regulator of type I interferon-dependent immune responses. Nature 434: 772-777.
46. Sato M, Saemori H, Hata N, Asagiri M, Ogasawara K, et al. (2000) Distinct and essential roles of transcription factors IRF-3 and IRF-7 in response to viruses for IFN-alpha/beta gene induction. Immunity 13: 539-548.
47. Buettner N, Vög C, Martinez-Sobrido L, Weber F, Waiblinger Z, et al. (2010) Thogoto virus ML protein is a potent inhibitor of the interferon regulatory factor-7 transcription factor. J Gen Virol 91: 220-227.
48. Colina R, Costa-Mattioli M, Dowling RJ, Jaramillo M, Tai LH, et al. (2008) Translational control of the innate immune response through IRF-7. Nature 452: 325-328.
49. Oldik M, Tolzmann L, Wirrswski A, Podgsorka MJ, Silling S, et al. (2011) Differential regulation of human papillomavirus type 8 by interferon regulatory factors 3 and 7. J Virol 85: 178-188.
50. Sgarbanti M, Borsetti A, Mosco C, Belloch MC, Ridel R, et al. (2002) Modulation of human immunodeficiency virus 1 replication by interferon regulatory factors. J Exp Med 195: 1359-1370.
51. Sgarbanti M, Remoli AL, Marsili G, Ridel R, Borsetti A, et al. (2008) IRF-1 is required for full NF-kappaB transcriptional activity at the human immunodeficiency virus type 1 long terminal repeat enhancer. J Virol 82: 3632-3641.
52. Covic M, Hassa PO, Saccani S, Baerki C, Meier N, et al. (2005) Arginine methyltransferase CARM1 is a promoter-specific regulator of NF-kappaB-dependent gene expression. EMBO J 24: 83-96.
53. Ruland J, Duncan GS, Elia A, del Barco Barrantes I, Nguyen L, et al. (2001) Bel10 is a positive regulator of antigen receptor-induced activation of NF-kappaB and neural tube closure. Cell 104: 33-42.
54. Matuda A, Suzuki Y, Houda G, Muramatsu S, Matsuzaki O, et al. (2003) Large-scale identification and characterization of human genes that activate NF-kappaB and MAPK signaling pathways. Oncogene 22: 3307-3318.
55. Gerin J, Lin R, Hiscott J, Ciras A (2009) Differential regulation of human interferon A gene expression by interferon regulatory factors 3 and 7. Mol Cell Biol 29: 3435-3450.
56. Fortin JF, Gantt R, Lambertge G, Tremblay M (1997) Host-derived ICAM-1 glycoproteins incorporated on human immunodeficiency virus type 1 are biologically active and enhance viral infectivity. J Virol 71: 3588-3596.
57. Lambert AA, Gilbert C, Richard M, Beaudein AD, Tremblay MJ (2008) The C-type lectin surface receptor DCIR acts as a new attachment factor for HIV-1 in dendritic cells and contributes to trans- and cis-infection pathways. Blood 112: 1299-1307.
58. Richman DD, Johnson VA, Mayers DM, Shirasaka T, O'Brien MC, et al. (2001) In vitro evaluation of experimental agents for anti-HIV activity. Curr Protoc Immunol Chapter 12: Unit 12 19.
59. Wu Z, Irizarry RA (2005) Stochastic models inspired by hybridization theory for short oligonucleotide arrays. J Comput Biol 12: 882-893.
60. Song L, Bedo J, Borgwardt KM, Gretton A, Smola A (2007) Gene selection via the BAHSIC family of algorithms. Bioinformatics 23: i490-498.
61. Shah Mohak CJ (2009) A General Framework for Analyzing Data from Two Short Time-Series Microarray Experiments. IEEE/ACM Transactions on Computational Biology and Bioinformatics IEEE computer Society Digital Library. IEEE Computer Society, 8: 14–26.
62. Chen H, Sharp BM (2004) Content-rich biological network constructed by mining PubMed abstracts. BMC Bioinformatics 5: 147.
63. Nuovo GJ, Hohnan Rj, Nadkane GA, Nazarenko IA (1999) In situ amplification using universal energy transfer-labeled primers. J Histochem Cytochem 47: 273-289.