HIV infection of dendritic cells subverts the interferon induction pathway via IRF1 and inhibits Type 1 interferon production

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Abstract
Many viruses have developed mechanisms to evade the interferon (IFN) response. Here HIV-1 was shown to induce a distinct subset of IFN stimulated genes (ISGs) in monocyte derived dendritic cells, without detectable Type I or II IFN. These ISGs all contained an interferon regulatory factor (IRF)-1 binding site in their promoters and their expression was shown to be driven by IRF-1, indicating this subset was induced directly by viral infection via IRF-1. IRF-1 and 7 protein expression was enriched in HIV p24 antigen positive DCs. A HIV deletion mutant with the IRF-1 binding site deleted from the LTR showed reduced growth kinetics. Early and persistent induction of IRF-1 was coupled with sequential transient up-regulation of its two inhibitors, IRF-8, followed by IRF-2, suggesting a mechanism for IFN inhibition. HIV-1 mutants with Vpr deleted induced IFN demonstrating that Vpr is inhibitory. However HIV IFN inhibition was mediated by failure of IRF3 activation rather than by its degradation, as in T-cells. In contrast, herpes simplex virus type 2 (HSV2) markedly induced IFNβ and a broader range of ISGs to higher levels, supporting the hypothesis that HIV-1 specifically manipulates the induction of IFN and ISGs to enhance its non cytopathic replication in DCs.
INTRODUCTION

Langerhans cells and lamina propria DCs in the anogenital and cervical mucosa and the male foreskin are key target cells for sexual transmission of HIV-1[1-3] and probably facilitate access to CD4+ T-cells in the submucosa and lymph nodes, resulting in a productive infection and subsequent dissemination[4-7]. After HIV-1 binding to C-type lectin receptors (CLRs) on monocyte derived DCs (MDDC) the majority (>95%) of HIV-1 is endocytosed and subject to acid proteolytic digestion over 6-12 hours[8] or taken up in tetraspanin rich caves. A minority is transferred to CD4/CCR5 resulting in fusion of the virus envelope with the plasma membrane and de novo infection, apparent only at a later phase >24 hours post infection. After contact between DCs and T-cells, HIV-1 is transferred to the latter in two phases, firstly from ‘caves’ and then later from the cytosol[5, 9-12].

However, how HIV-1 manipulates DC biology to use the cell for viral transfer to T-cells without marked cytopathic effects is still unclear. Viruses often shape their intracellular environment through alterations to host cell gene transcription, protein translation and post-translational modification, often initiating these changes by signalling through cell surface receptors or at subsequent stages in their replication cycle[13, 14].

To determine the effects of HIV-1_BaL on the DC transcriptome, we have previously carried out rigorous microarray experiments using highly purified, high titre HIV-1_BaL virus stocks and purified recombinant gp120 in a single replication cycle over 48 hours. We showed that HIV-1_BaL induces changes in expression of several distinct gene clusters in two major groups in two transient and sequential phases, one group corresponding to HIV binding/entry and endocytosis over 6 hours post infection and the second group corresponding to the later stages of de novo replication (post reverse transcription) at 24-96 hours post infection[15]. A minor group of genes showed persistent up-regulated expression across both phases. In the second phase HIV induced partial maturation of DCs which leads to enhanced migration and T-cell stimulation[5] and also reduction in lysosomal enzyme expression and function[15]. In this study using a similar approach we show that a specific cluster of interferon stimulated genes (ISG) is up regulated in
response to HIV-1Bal, but there was no detectable type I or II interferon (IFN) induction. The majority of this subset showed the kinetics of the minor group of up-regulated genes. We demonstrate that this ISG subset can be driven by IRF-1 and that this is the case in HIV infection of DCs. In addition we show that deletion of the IRF-1/7 binding site from the HIV-1 LTR results in a virus with decreased growth kinetics indicating that HIV-1 induces IRF-1 and 7 expression early after infection of MDDCs in order to aid its own replication. Recent reports have indicated that the HIV-1 accessory proteins Vpr and Vif are required for the inhibition of an IFN response in T-cells via targeted degradation of constitutively expressed IRF-3, the major IFN inducing IRF [16, 17]. However in MDDCs HIV-1 infection had no effect on IRF-3 expression but it inhibited its activation and translocation to the nucleus. Here we show that Vpr but not Vif is required to inhibit the IFN response in MDDCs via an alternative mechanism to IRF-3 degradation.

Conversely, infection of DCs with HSV-2186, a virus known to productively infect MDDCs, resulted in IFN induction and increased expression of a broader range of ISGs to higher levels. Since ISGs potentially modulate effects on cell proliferation, activation, differentiation and survival as well as restricting viral replication, the nature of the ISG subset regulated is likely to have an important role in determination of the outcome of HIV trafficking in myeloid DCs, their infection and transfer to CD4+ lymphocytes.

Thus HIV induced defects in myeloid DCs probably allows the virus to obtain a toehold in the genital tract prior to the infiltration of other interferon secreting cells, in particular plasmacytoid DCs. Furthermore the induction of IRF1 in myeloid DCs in the genital tract might provide part of the explanation for reduced susceptibility to HIV infection of Kenyan prostitutes with certain IRF1 polymorphisms [18].
Materials and Methods

Preparation of Monocyte Derived Dendritic Cells
MDDCs were generated from CD14+ monocytes isolated from peripheral blood mononuclear cells (PBMC) using CD14 magnetic beads (Miltenyi Biotech, Gladbach, Germany) as described previously[5].

Viral Stock Preparation and Construction of HIV-1 Deletion Mutants
Purified high titre HIV-1_{BaL} stocks in the order of 5x10^{10} TCID_{50}/ml were produced using tangential filter concentration as described previously[5, 10, 19]. Virus content was determined by p24 gag ELISA (Beckman-Coulter, Hialeah, FL) and as 50% tissue culture infectious dose (TCID_{50}) values generated in TZM-BL cells (NIH AIDS Research and Reference Reagent Program, contributed by John Kappes and Xiaoyn Wu) measured by LTR β-galactosidase reporter gene expression after a single round of infection[20]. The endotoxin levels of these virus stocks were below the detectable limit of 0.005 U/ml or 0.0005ng/ml (Limulus amebocyte lysate assay; Sigma) and testing for residual TNF-α, IFNα, IFNβ and IFNγ by ELISA (R&D systems, CA) was negative. An IRF-1/7 binding site (or interferon stimulated response element (ISRE)) deletion mutant virus (pBaL_{ISRE(Mut)}) was constructed by replacing the ISRE with the 18bp Zeichner linker sequence[21], containing NdeI, XhoI, and SalI restriction sites. VSVG pseudotyped HIV-1 deletion mutants were produced by transfection of either NL43ΔVpr or NL43ΔVif plasmid constructs. NL43ΔVpr and NL43ΔVif were generated by the addition of stop codons into the protein open reading fame via site-directed mutagenesis using specific PCR primers. NL43ΔVpr was generated by the insertion of a stop codon at amino acid position 21 in the Vpr open reading frame. NL43ΔVif was generated by the insertion of a stop codon at amino acid position 18 in the Vif open reading frame. Overlapping open reading frames were not affected by these mutations. Stocks of HSV-2 (strain 186) were generated as described previously [22].
Treatment of cultured cells with HIV-1, HSV-2 and Sendai Virus
MDDCs were seeded at 1 x 10^6 cells/ml and treated with HIV-1_{BaL} at a MOI 10, 3 or 1; HSV-2_{186} at MOI 3; at day 6, or at day 2 with VSVG pseudotyped HIV-1 virus stocks. TZMBL were infected with Sendai Virus at 150HA units/ml

Microarray Hybridization and data analysis
Total RNA derived from HIV-1_{BaL} or HSV-2_{186} treated MDDCs was prepared for hybridisation to Human ResGen 8k (Australian Genome Research Facility, Melbourne, Australia) glass microarrays or to sentrix human 6 (version 2) expression chips (Illumina, San Diego, CA) as described previously[15].

Confirmation of Differential Gene Expression by Quantitative PCR
Total unamplified RNA was DNase I treated (Promega, Madison WI) and then reverse transcribed using oligo d(T) and superscript III (Invitrogen). The cDNA was then subject to QPCR using defined primers (Sigma) and SYBR Green (Invitrogen). The relative quantitation method (ΔΔC_T)[23] was used to evaluate the expression of selected genes with the glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) as an internal control and the normalizer for all data[24].

In silico promoter analysis
Ensembl and RefSeq gene identifiers were obtained for all differentially regulated genes and proximal promoter sequences were extracted using the UCSC genome browser (http://www.genome.ucsc.edu) and the Ensembl Genome Browser (http://www.ensembl.org). Sequence regions 3000 and 1500 bp immediately 5' upstream from the transcription start site and the 5' UTR was extracted. All differentially regulated genes were analysed for IFN signatures using the INTERFEROME database (http://www.interferome.org)[25]. The Java software, Toucan2 was used in the comparative promoter analysis. 20 genes that were not differentially regulated during this experiment and 20 random IFN regulated genes were chosen as controls for promoter analysis. The Transfac professional database (ver. 11.4)[26] was used to obtain the vertebrate transcription factor binding site (TFBS) matrices. The Toucan2 tool
MotifScanner (Gibbs sampler) was utilised in identifying potential TFBS in the sets of selected sequences. The prior (stringency level) for motif prediction was set to a value of either 0.05 or 0.1, and the human promoter set from the Eukaryotic Promoter database (http://www.epd.isb-sib.ch/) was chosen as a third order background model to determine over-represented TFBS. Toucan2 statistical tool was applied to the data obtained by MotifScanner to identify over-represented TFBS (those showing positive significance values) in the selected gene set [27].

**Transfection of 293T cells**
293-T cells were plated at 1.2x10^6 cells per well in a six well plate overnight (80% confluence). Cells were then transfected using Polyethyleneimine (PEI) as described previously [28] with 1, 5 or 10 μg of the IRF1 expressing plasmid CMVBL-IRF1-HNK (kindly provided by from Dr Angella Battistini, Istituto superiore di sanita, Rome, Italy) or empty vector. Cells were harvested at 24 and 48 hours post transfection and assayed for ISG expression by QPCR.

**Chromatin Immunoprecipitation Assay**
Chromatin Immunoprecipitation (ChIP) experiments were performed in MDDCs using a ChIP assay kit according to the manufacturer's instructions (Upstate Biotechnology, Lake Placid, NY) using an IRF-1 antibody (Santa Cruz Biotechnology Inc., sc-497x) as described previously [29].

**ELISA**
Levels of secreted IFNα, IFNβ and IFNγ from viral inoculum and MDDCs treated with HIV-1Bal, HSV-2186 or mock treated for 6-48 hours were determined by ELISA (R&D systems) according to the manufacturer’s instructions.

**Flow Cytometry**
PE conjugated p24 (clone KC57-RD1) and IgG1 mouse coulter clone monoclonal antibodies were obtained from Beckman Coulter (Fullerton, CA). Purified mouse polyclonal antibodies directed towards IRF2, IRF7 and IRF3 was obtained from AbD
Serotech (Kidlington, UK), BD (San Diego, CA) biosciences and Dr. Michael Gale (University Washington) respectively. Rabbit polyclonal antibodies directed towards IRF1 and IRF8 were obtained from Abcam (Cambridge MA). Alexa 488 conjugated goat anti mouse and FITC conjugated goat anti rabbit secondary antibodies were obtained from Invitrogen and Sigma Aldrich respectively. HIV-1BaL or mock treated MDDCs were fixed and permeablised in Cytofix/Cytoperm (BD). All antibody incubations were carried out in permwash buffer (1% human AB serum, 0.1% saponin, 0.1% sodium azide, made up in PBS). IgG isotype control antibodies were incubated with cells to control for nonspecific binding. Cells were then analyzed with a FACS-Canto flow cytometer (Becton Dickenson, San Jose, CA) and FlowJo software (Ashland, OR).

**Western Blot**

HIV-1BaL or mock treated MDDCs were lysed using Berman lysis buffer (SDS (0.1%), sodium deoxyxcholate (0.5% w/v), NP-40 (1% v/v) containing a Protease inhibitor cocktail (Roche), separated in a 12% poly-acrylamide gel by electrophoresis and transferred to poly-vinylidene difluoride (PVDF) membrane (Biorad). Membranes were blocked overnight (5% w/v skim milk, 0.1% v/v Tween and 1 x PBS), and probed with a 1/500 dilution of monoclonal IRF-1 or IRF-3 primary antibody (BD Pharmingen, Cell Signalling), followed by a secondary biotin conjugated α-mouse IgG antibody (Sigma-Aldrich). The antibody-reactive IRF proteins were visualised using the 5-Bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium liquid substrate system (BCIP/NBT; Sigma-Aldrich).
Results

HIV-1 treatment triggers the expression of a subset of IFN stimulated genes in MDDCs

cDNA microarrays were used to determine the genes differentially expressed in MDDCs in response to a purified high titre HIV-1_{BαL} stock compared to mock treatment at 6, 24 and 48 hours post treatment, initially with Human Resgen 8k arrays and then later checked with Illumina Sentrix Human 6 arrays. As previously reported, HIV uptake by DCs up-regulated expression of two major groups of genes in corresponding temporal phases: expression of 255 genes was transiently enhanced in the first phase (6 hours post exposure) and 385 in the second phase (48 hours post exposure) [15]. A third small group of approximately 30 genes bridged these two phases. 18 genes associated with IFN induction (ISGs) were shown to be up-regulated in their expression by viable HIV-1 (Table 1) and half formed part of this minor group, i.e. half of these genes were up regulated by 6 hours post virus treatment and all of them were up regulated at 48 hours with the magnitude of up-regulation of all being higher at this later time point. AT2 inactivated HIV-1 had little effect on ISGs; only Mx1 expression was consistently up-regulated at 6 to 48 hpi (data not shown).

10 genes from this list were chosen for confirmation of their differential expression by quantitative PCR (QPCR). In every case QPCR confirmed that the gene was up-regulated, although QPCR usually showed a larger magnitude of up-regulation than the corresponding microarray values. QPCR was also used to investigate the expression of 9 additional IFN associated genes that were not present on the microarrays (Table 1, bottom 9 rows), which were also shown to be up regulated in their expression. However no genes that encode Type I or II IFNs were increased in their expression as detected by microarray or QPCR (Table 1, top 3 rows).

HIV-1 Treatment of MDDCs Does not Trigger IFN Induction

In order to determine if the HIV-1_{BαL} treated MDDCs produced IFNs, cells were infected with virus for 6 to 72 hours. QPCR was used to determine the expression levels of mRNAs encoding all IFNα isoforms, IFNβ and IFNγ. In addition, secreted IFNα and β
levels were determined in infected supernatants using ELISA. HIV-1 treated MDDCs showed no up-regulation of any IFN genes (Figure 1 and data not shown) and no IFNα, β or γ proteins were detected in the supernatants or the HIV inoculum (data not shown).

**IFN Regulatory Factor Gene Expression in Response to HIV-1 Treatment of MDDCs**

Since three IRFs showed altered expression, we next used QPCR to determine the gene expression profiles of four other key IRF family members in MDDCs infected with HIV-1BaL for 6 to 48 hours (Table 2). By 6 hours the IRF-1, 7 and 8 genes were all increased in their expression compared to mock treated cells. Expression of IRF-1 steadily increased to 48 hours, whereas IRF-8 steadily decreased. IRF-7 expression rose at 24 hours and then remained constant at 48hpi. At 24 hours the IRF-2, 4 and 9 genes were also increased in their expression. IRF-2 gene expression was increased further at 48 hours, whereas that for IRF-4 remained constant. IRF-9 gene expression however was up-regulated to a lesser degree at 48 hours.

**HIV-1 Triggers the Expression of IRF-1, 2, 7 and 8 proteins in MDDCs**

Both microarrays and QPCR showed significantly increased gene expression of IRF-1 and IRF-7 at all time points (Tables 1 and 2). Given the previously identified role of the IRF-1 protein in HIV-1 infection of T-cells [30-32], and the concurrent up-regulation of gene expression of its inhibitors, IRF-2 (at later time points) and 8 (at earlier time points), we focused on this protein and its inhibitors and next determined if they were increased in their expression in response to HIV-1 treatment in DCs using western blot and flow cytometry (Figure 2). In addition we focused on the IRF-7 protein. The IRF-1 protein was up regulated by 6 hours post treatment and was still increased in its expression at 48 hours (Figure 2A and D). In addition, the IRF-8 protein was up regulated early after infection but not at later time points (no up regulation by 120 hours) and the IRF-2 protein was upregulated in the later stages of infection but not at early time points (Figure 2E) which closely matched the kinetics of the gene expression data. At 48 and 120 hours post infection where HIV-1 infectivity could be determined by p24 staining and flow cytometry (Figure 2B), the HIV-1 infected population showed a consistent 2-3 fold
increase in IRF-1 expression compared to bystander cells (Figure 2C). Similar results were also observed for IRF-2, 7 and 8 (Figure 2E). As the MOI of HIV-1 was sufficient for all DCs to be exposed to HIV which is then endocytosed and destroyed by 48 hours, these results suggest that although exposure to HIV-1 may be enough to trigger the expression of IRFs, productive infection has a much greater effect.

**Genes upregulated in response to HIV-1 in MDDCs contain a strong IFN signature and are enriched for IRF-1 binding sites in their promoters**

*In silico* promoter analysis was performed on the (microarray) up-regulated subset of 18 ISGs and compared to 18 genes selected randomly from those in the microarray data set which were not differentially regulated and also to 18 other ISGs. The up-regulated subset was found to be highly enriched (16/18) for statistically significant IRF-1 binding sites within their proximal promoters ($p= 4.17 \times 10^{-4}$ when prior=0.1) suggesting they form an IRF-1 regulated subset of ISGs that are induced during HIV-1 infection (Figure 3).

**IRF-1 is Able to Drive the Expression of HIV Stimulated ISG**

To confirm that IRF-1 is able to drive the expression of the HIV-1 induced ISGs we transfected 293T cells with the IRF-1 expression vector CMVBL-IRF1-HNK or with empty vector. IRF-1 was highly expressed in the IRF-1 transfected cells only and was able to drive the expression of all 17 HIV-1 stimulated ISGs tested in a dose dependent manner with the exception of IRF8. Some highly up regulated examples include ISG15, IFIT2, IFIT3 and RSAD2 (aka viperin). In addition, the expression of two ISGs (IFITM3 and IFITM5) that were not up regulated by HIV treatment in MDDCs was not affected by IRF1 transfection.

**IRF-1 Binds to the Promoters of HIV-1 Induced IFN regulatory genes in MDDCs**

To determine if IRF-1 binds to the promoters of HIV-1 induced IFN regulatory genes *in vivo* a ChIP assay was performed in MDDCs exposed to HIV-1 BaL. IRF-1 was demonstrated to bind to the promoters of two of the up-regulated ISGs that were chosen for validation (Figure 4), IRF-2 and IFIT5, using an IRF-1 specific antibody and PCR amplification of promoter regions identified *in silico* to contain IRF-1 binding sites, in
MDDCs treated with either HIV-1\textsubscript{BaL} (lane 2) or IFN\textsubscript{γ}, a known inducer of IRF-1 (lane 4).

**Deletion of the ISRE From the HIV LTR Region Results in Reduced Infectivity.**

HIV-1 contains an IFN stimulated response element (ISRE) in its LTR region with a known binding sequence for IRF-1/7. To determine if HIV-1 requires IRF-1 binding to this for efficient replication in MDDCs we next constructed a mutant virus with this ISRE deleted. This virus showed significantly reduced replication kinetics as compared to the parental wildtype (Figure 5A). In order to confirm that the virus with the ISRE deleted was less replication competent in DCs we titrated the ratio of plasmids encoding the wild type and mutant viruses and infected MDDCs with the progeny virions. The proportion of infected cells decreased as the ratio of HIV mutant plasmid increased compared to wild type (Figure 5B).

**Exposure of MDDCs to HSV-2 Leads to IFN Induction**

In order to determine if the block in (or lack of) IFN induction and the distinct pattern of ISGs and IRFs in DCs was virus specific we next exposed MDDCs to viable or UV inactivated HSV-2\textsubscript{186} for 30 minutes or 6 hours. Microarray analysis was conducted using the Illumina system to determine differential expression of genes including ISGs (Table 4). In addition IFN\textalpha and \textbeta levels from the infected cell supernatants were determined by ELISA (Figure 2B). No ISGs were differentially expressed in MDDCs in response to treatment with UV inactivated HSV-2\textsubscript{186} or with viable HSV-2\textsubscript{186} for 30 minutes. However 34 ISGs were up regulated in MDDCs treated with viable HSV-2\textsubscript{186} for 6 hours (including IFN\textbeta) and 20 with UV inactivated HSV-2\textsubscript{186}. 9 genes from the list were chosen for validation by QPCR, which confirmed up-regulation in every case, though in most cases QPCR indicated a much greater degree of up-regulation (Table 4). Although many of the detected up-regulated genes were also increased in their expression in MDDCs treated with HIV-1\textsubscript{BaL}, the fold change increases were far higher in HSV-2\textsubscript{186} treated cells. In contrast to cells treated with HIV-1\textsubscript{BaL} (where no IFN encoding genes were upregulated) the IFN\textbeta gene was markedly up regulated by 600 fold by QPCR. No IFN\textalpha was detected. This was confirmed by ELISA on tissue culture supernatants, which
detected the presence of IFNβ but not IFNα in HSV-2 infected MDDC supernatants (Figure 1), indicating induction of \textit{de novo} IFNs by viral infection. Neither IFNα nor β were detected in the viral inoculum used for infection (data not shown). Furthermore, the pattern of IRFs induced by HSV-2 was broader than HIV-1 and the early IRF-1/8 and late IRF-1/2 pairings were not observed (Table 2B).

**Suppression of IFN induction is mediated by Vpr and by inhibiting activation but not degradation of IRF3.**

Recently the HIV accessory proteins Vpr [16, 17] and Vif [17] have been shown to be key mediators in the suppression of IFN induction in T cells by inducing the degradation of IRF-3. To determine if these virally encoded proteins are also involved in suppression of IFN induction in MDDCs we prepared HIV-1 viral stocks with the Vpr, Vif or both Vpr and Vif genes deleted. As shown in Figure 6A, the Vpr single deletion mutant and the Vpr, Vif double deletion mutant induced IFNβ mRNA expression in MDDCs at both 48 and 96 hours. In contrast, no IFNβ was induced in response to wild type HIV-1 or to the Vif deletion mutant.

To examine the mechanism of inhibition of IFNB production, we next determined if IRF-3 was degraded in MDDCs infected with HIV-1 as in T-cells, or alternatively if its activation was impaired, as shown by failure to translocate to the nucleus. No down regulation of IRF-3 was demonstrated either by western blot or flow cytometry (Figure 6B). However IRF-3 did not translocate to the nucleus of MDDCs after HIV-1 infection in contrast to positive Sendai virus infected TZMBL cells [33] (Figure 6C).
Discussion

HIV-1 capture and infection by DCs induces changes in clusters of genes which represent either responses of the cell to the virus or viral manipulations of the intracellular environment. The latter assist in viral replication and, in the case of DCs, transfer to T-cells. We and others have found that the clusters of genes induced by HIV-1 in the major target cells T-cells, macrophages and DCs, differ substantially between each cell type, although there is greater similarity between DCs and macrophages [5, 15, 34-37] (and unpublished observations). As recently reported, expression of two major groups of genes were transiently and sequentially up-regulated in DCs by HIV, the first peaking at 6-24 and the second at 48 hpi, correlating with and probably due to the two phases of viral trafficking: the vesicular endosomal phase with declining intracellular HIV concentrations over 24 hours and de novo viral replication with HIV DNA appearing at 24 hpi and plateauing at 48-96 hpi[15]. A minor group of 30 genes with up-regulated expression extends across the two phases. In this study we have shown that HIV-1 induces a specific subset of ISGs originally identified by DNA microarray studies and confirmed and broadened by downstream analysis initially by QPCR and then by protein studies, including western blot and flow cytometry.

Induction of ISGs is a common feature of the innate response of many cells to viral infection but here there were unusual features: Firstly, up-regulation of more than half of the ISG subset was not transient but extended across the two phases of trafficking (i.e. part of the minor group) suggesting a sustained effect. Secondly, no type I or type II IFNs could be detected, either in the supernatant by ELISA or by extensive QPCR assays of all IFNα sub-types, as well as IFNβ and γ from infected cell lysates at serial time points. Thirdly, a specific subset of ISGs were induced (especially compared with HSV-2 infection), which contained an unusual pattern of up-regulation of key IRFs, particularly at early time points, consisting of IRF-1, 7 and 8 at 6 hpi and later IRF-1, 2, 7, 4 and 9. Induction of IRF-1 and 8 and later IRF-2 constitute an unusual pattern in that IRF-1 is much less well characterised than IRF-3 or 7 for IFN and ISG induction. IRF-2 and 8 can both inhibit IRF-1 mediated induction of transcription. IRF-2 competes with IRF-1 for its binding to the cellular promoter binding site [38, 39] whereas IRF-8 does not bind DNA.
but rather forms a complex with IRF-1 which inhibits its transcriptional activation activity by blocking protein:protein interactions [31].

Comparisons between the effect of HIV-1 and HSV-2 infection of DCs was conducted to determine if these effects were virus specific. HSV-2 was chosen because it had been shown in the laboratory to productively infect MDDCs and induce IFN production as also shown here[40]. Furthermore, there is a pathogenetic relationship between HIV-1 and HSV-2 in that both have been shown to co-exist in the same recurrent herpetic lesion[41]. Thus co-infection or adjacent infection of epidermal and perhaps dermal DCs in these lesions with these viruses is likely. In these studies highly purified viral preparations and similar multiplicities of each virus were used over a range of 1-10 per cell. In contrast to HIV-1, HSV-2 infection of DCs induced functional IFNβ protein in the infected DC supernatants and IFNβ transcripts were induced as early as 3 hpi. Furthermore, a much broader range of ISGs was induced and at a much higher level. IFN induced chemokines such as CXCL10 and 11[42, 43] were also more prominent. These findings of marked induction of a type I IFN in HSV-2 infected MDDCs build on those of Pollara et al., [40]. The unusual subset of ISGs and particularly the unusual pattern of IRFs induced by viable HIV-1 in DCs raises several questions. What is the mechanism of the induction of such ISGs and of the failure of induction of type I or II IFNs? Does this altered pattern of ISGs benefit the host, the virus or to both? What components of the virus and cellular signalling pathways does HIV-1 use to induce such an altered pattern? A clue to the mechanism of induction of the subset of ISGs induced by HIV-1 in DCs was shown by heavy and significant weighting towards those containing an IRF-1/7 binding site in their promoters, initially identified by our recently developed INTERFEROME database and associated bioinformatics tools and later confirmed by chromatin immunoprecipitation assays[25]. The ability of IRF1 to induce the expression of this specific ISG subset was shown after transfection of an IRF1 expression vector into a 293T cell line in the absence of any interferon induction. The only exception was IRF8 which is also induced early after infection similar to IRF,1 so this is not surprising. By analogy Vesicular Stomatitis Virus has recently been reported to induce the antiviral ISG viperin via IRF1 and in the
absence of type I interferons (as we show here in DCs). In contrast Newcastle Disease Virus induces viperin via a non IRF1 dependent pathway [44].

The early kinetics of induction of IRF-1, 2, 7 and 8 provide a clue to the role of the specific IRFs induced by HIV-1. The combination of IRF-1, 2 and 8 may negate any IFN inducing effects of IRF-1, as IRF-2 and 8 can both act to inhibit the transcriptional activity of IRF-1 and appear to be complimentary in their kinetics, one being induced early (IRF-8) and the other late (IRF-2). Furthermore, the LTR of most HIV-1 isolates has an IRF-1/7 binding site (the IFN stimulated response element, ISRE) adjacent to the U5 region between the NF-AT and SP-1 sites[45]. In T-cells Sgarbanti et al., have shown that IRF-1 and 2 can bind to this site and that IRF-1 also form complexes with the p65/50 NFkB heterodimer to bind to upstream sites of their target genes. They showed that early IRF-1 induction by HIV-1 in de novo infection of T-cells stimulates early HIV-1 transcription prior to Tat induction and also that IRF-2 is not inhibitory to this effect, in contrast to cellular gene promoters, rather it was IRF-8 that was responsible for the inhibitory effects on IRF-1 [31, 32, 46]. The failure of a cellular ISRE to substitute functionally for the HIV (LTR) ISRE suggests they are differentially regulated by IRF-1 and/or IRF-2/8[46]. Thus in DCs the IRF-1/2/8 combination might selectively stimulate the HIV-1 LTR via either the IRF-1 or NFkB binding site. In order to test the functional requirements of the HIV-1 IRF-1/7 binding site for viral replication we constructed an HIV-1 mutant with this site deleted. This resulted in a significant 20-30% decrease in infectivity in DCs by 120 hours post infection. Combination of wt and deletion mutant in various ratios further demonstrated and confirmed the lower infectivity of the mutant virus. This system may be especially important in DCs where de novo infection occurs at lower levels than in macrophages or T-cell partly because most virus binding occurs via C-type lectin receptors on the surface of DCs and is rapidly endocytosed and degraded by the endocytic pathway. IRF-1 may also provide early stimulation in DCs prior to later production of Tat as in T-cells. IRF-1 also appears to be driving the induction of the ISG subset observed in this study, explaining their induction without the detectible presence of IFN. A strong correlation between reduced susceptibility to HIV infection and IRF1 polymorphisms and consequent aberrant patterns of IRF1 induction in PBMCs by IFN-γ.
in Kenyan sex workers has been reported recently [47]. In particular transient rather than sustained IRF1 patterns were shown. If this is also occurring in the genital mucosal DCs after HIV exposure, it may contribute to the observed reduction in HIV acquisition.

The early and sustained induction of IRF-7 is also likely to be important. Its induction in the absence of IFNs suggests a parallel with dengue virus, which also induces IRF-7 in the absence of type 1 IFNs[48]. IRF-3 and 7 are the two most important inducers of type I IFNs in most cell types and are activated via interactions of viral RNA with Toll like receptors in the endosome or by cytosolic RIG-I like receptor pathways resulting in their phosphorylation, homo- or heterodimerization with IRF-3 and translocation to the nucleus. In myeloid DCs IRF-7 concentrations are constitutively low, perhaps explaining the need for up-regulation by HIV-1. However the reason for early induction of IRF-7 is not clear. Does IRF-7 also stimulate HIV replication? Furthermore, if IRF-7 is activated normally, why is there no IFN induction? In T-cells the HIV-1 accessory proteins Vpr and Vif have been shown to ubiquitinate IRF-3, which leads to it being targeted to the proteasome and degraded, resulting in the inhibition of type I interferon induction [16, 17]. Thus we pursued the hypothesis that HIV vpr and/or vif proteins may also be involved in the inhibition of IFN induction in DCs. We therefore constructed Vpr and Vif deletion mutants and showed that deletion of Vpr (but not Vif) did indeed result in a virus that had the ability to induce IFNβ in DCs. However we did not observe down-regulation of IRF-3 protein, as has been shown in T-cells [16, 17]. This strongly suggests that Vpr might be enhancing ubiquitination and degradation of other enzymes involved in phosphorylation and activation of IRF-3 and/or 7. As IRF3 did not translocate to the nucleus this indicates inhibition of its activation most probably by a failure of phosphorylation of key upstream enzymes such as TBK1 or IKKe.

Some of the HIV induced ISGs have intrinsic unique anti-viral actions such as MxA, OAS1-3 and ISG15, and may substitute for absent type I IFNs by restricting HIV-1 replication to low levels, as previously characterised[49-51]. The much higher levels and broader range of ISGs induced in DCs infected by HSV-2 are clearly a response to the very high levels of IFNβ induced via ISGF3/STAT pathways, not solely a response to
IRF-1. Although the biology of HIV and HSV infection of DCs is quite different they provide a reasonable comparison and contrast with the HIV-1 induced subset of ISGs. Interestingly, the subset of ISGs induced by HIV-1 in DCs is similar but not identical to that observed in macrophages where IRF-1 is similarly induced but IRF-2 and 8 were not prominent (data not shown). The pattern of antiviral ISGs also showed some differences. However, the pattern of ISGs induced in T-cells was more markedly different[15, 34-37] (and unpublished observations).

The direct stimulation of a unique ISG subset by HIV-1 contrasts with different patterns of ISG stimulated by other viruses and by HIV in other cell types (Maddocks et al., unpublished data)[44]. This suggests direct viral modulation to enable successful HIV-1 transfer to T-cells, their primary target cell, via viral synapses in the genital mucosa and other body sites. The IRF-1/2/8 combination may provide an early stimulus to the small inoculum of HIV-1 delivered into the cytoplasm of DCs while restricting viral replication through the antiviral ISGs to retain the integrity of the cell until it reaches the sub-mucosal T lymphocytes or lymph nodes. Here virus is transferred via the viral synapses. Now that this novel ISG and IFN pattern, distinct from that in T cells, has been identified, their mechanisms and role in maintaining the HIV-DC equilibrium can also be further investigated, focussing on IRF-1/2/8 and IRF-3/7 induction and activation and also inhibition of IFN induction. The patterns of ISG and IRF induction in DCs from patients with ‘resistant’ and ‘sensitive’ IRF1 genotypes also require urgent investigation.
Author contributions:

**Andrew Harman** prepared the manuscript and conducted the bulk of the experimental work with technical assistance from **Sarah Mercier**.

**Joey Lai** conducted all experiments involving HSV-2.

**Stuart Turville** prepared HIV deletion mutant virus stocks.

**Shamith Samarajiwa** conducted the *in silico* promoter analysis.

**Lachlan Gray** generated and provided the HIV-1 ISRE site deletion mutant.

**Melissa Churchill** provided academic input into the design of the IRF-1 binding site HIV-1 deletion mutant.

**Valerie Marsden** prepared all HSV-2 virus stocks and performed confocal microscopy.

**Kate Jones** constructed and provided the HIV-1 Vpr and Vif deletion mutants.

**Johnson Mak** provided academic input into the design of the HIV-1 Vpr and Vif deletion mutant studies.

**Heather Donaghy** conducted interferon ELISA assays and provided intellectual input.

**Paul Hertzog** provided academic input into the design of the *in silico* promoter analysis and ChIP assays.

**Tony Cunningham** conceived and supervised the study and jointly prepared the manuscript.

**Acknowledgments**

Chris Bye for his help with microarray analysis of cDNA microarrays.

This project was supported by a National Health and Medical Research Council (NHMRC) Program Grant. ID number 358399.
References


19. Chertova, E., et al., Envelope glycoprotein incorporation, not shedding of surface envelope glycoprotein (gp120/SU), Is the primary determinant of SU content of


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Table 1. Microarray and QPCR derived differential expression data for genes encoding ISGs in HIV-1 treated MDDCs. Day 6 MDDCs were treated with HIV-1(BaL) (MOI 10) and ISG expression was determined by QPCR or 8K cDNA microarrays. Differential expression data is presented for HIV-1 vs. mock treated cells at 6, 24 and 48 hours post treatment from four independent experiments. – indicates that no microarray data was available, / indicates that QPCR was not carried out to confirm the expression of this gene, * indicates that the expression of this gene was shown to be driven by IRF-1 in 293T cells (see Table 3).
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Table 2. Differential expression data for genes encoding key IRFs in HIV-1 and HSV-2 treated MDDCs. Day 6 MDDCs were mock treated or treated with HIV-1_{Bal} (MOI 10) or HSV-2_{186} (MOI 3) and IRF gene expression determined by QPCR. The mean fold change (FC) in expression of IRF genes in HIV-1 vs. mock treated cells at 6, 24 and 48 hours post treatment, and HSV-2 vs. mock treated cells for 3, 6 and 12 hours post treatment from 4 independent experiments as well as the associated standard errors (SE) are presented in the table.
Table 3. QPCR derived ISG gene expression data for 293T cells transfected with an IRF-1 expression vector. 293T cells were either untreated or transfected with 1, 5 and 10μg of the IRF1 expression vector CMVBL-IRF1-HNK or empty vector or treated with PEI transfection reagent only. ISG expression was determined by QPCR 48 hours post treatment. The data shown in italics represents genes that were not up regulated in their expression in response to IRF1 transfection. The values shown represent fold change in gene expression compared to untransfected cells. – indicates a fold change of less than 2.0.
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**Table 4. ISG expression in HSV-2 treated MDDCs.** Day 6 MDDCs were treated with HSV-2<sub>186</sub> (MOI 3) for 30 mins or 6 hours and ISG expression was determined by 48K Illumina microarrays or QPCR (6 hours only). Expression data from three independent experiments is presented for HSV-2 vs. mock treated cells. – indicates that QPCR was not carried to confirm the expression of this gene. Those genes with an asterisk next to their symbol were also detected to be increased in their expression in MDDCs exposed to HIV-1<sub>BaL</sub> (Table 1).
Figure Legends

Figure 1. HIV-1 and HSV-2 induction of IFNβ in MDDCs. Day 6 MDDCs were exposed to purified HIV-1_{BaL} or HSV-2_{186} at MOI 3 for 3 to 96 hours. A) IFNβ mRNA expression was determined by QPCR at 3, 6, 12 and 24 hours post infection. B) The level of IFNβ secreted into the supernatant was determined by ELISA at 6, 12, 24 and 48 hpi. The mean data from 3 experiments is shown with standard error bars.

Figure 2. IRF protein expression following exposure to HIV. Day 6 MDDCs were treated with HIV-1_{BaL} (MOI 3) or mock treated for 6 - 120 hours. A) IRF-1 intracellular expression levels were determined by flow cytometry; the isotype control is shown as filled curve, mock treated cells with a solid line and HIV-1 treated cells with a broken line. B) The percentage of HIV-1 infected cells was determined by flow cytometry using a PE conjugated p24 antibody and C) peak IRF-1 expression determined in p24- cells and p24+ cells separately (mean of five experiments shown with standard error bars). D) IRF-1 expression was also determined by Western blot. E) A - C were repeated for IRF2, IRF-7 and IRF-8. Representative data is shown from one out of three experiments.

Figure 3: In silico promoter analysis of identified up-regulated IFN associated genes in MDDCs. RefSeq IDs and promoters were extracted using the UCSC genome browser (www.genome.ucsc.edu) and sequences 3000 base pairs 5’ upstream from the transcription start site, plus the 5’ UTR were extracted. Transfac database (Wingender et al., 2001) was then used to obtain vertebrate transcription factor binding site (TFBS) matrices and the Toucan2 tool MotifScanner was used to detect potential TFBS in the sets of selected sequences. The prior (stringency level) was set to a value of 0.05. A small box indicates the location of identified potential IRF-1 binding sites.

Figure 4: IRF-1 Chromatin immunoprecipitation assay. Day 6 MDDCs were treated with HIV-1_{BaL} or IFNγ for 48 hours. A ChIP assay was then carried out using an IRF-1 antibody and QPCR primers directed towards IRF-2 or IFIT5 promoter sequences to determine IRF-1 binding. Lanes; 1) HIV-1 treated MDDCs no IRF-1 antibody, 2) HIV-1 treated MDDCs plus IRF-1 antibody, 3) IFNγ treated MDDCs no IRF-1 antibody, 4)
IFNγ treated MDDCs plus IRF-1 antibody, 5) cell input DNA, 6) PCR negative no DNA control, 7) ChIP reagents only negative control.

**Figure 5: Deletion of the IRF-1/7 binding site (IRF-1bs) from the HIV-1 LTR results in reduced infectivity.** Day 2 MDDCs were treated with VSVG pseudotyped HIV-1_{Bal-IRF-1bs} or HIV-1_{Bal wt} at MOI 1 for 6 to 134 hours either alone in combination at various ratios. A) The percentage of HIV-1 infected cells was determined by flow cytometry between 48 and 134 hours post infection. The mean data from three independent experiments are shown with standard error bars. There was a statistically significant difference between the rates of increase of the percentage of infected cells over time for HIV-1_{Bal wt} vs HIV-1_{Bal-IRF-1bs} (p=0.021 using a linear mixed effects model). The mean difference in these infection rates was 0.028% per hour. B) The greater the ratio of wild type compared to mutant plasmid transfected the greater the proportion of cells infected at 48 hours post infection. The mean data from three independent experiments are shown with standard error bars.

**Figure 6: Interferon induction in MDDCs by HIV viruses with Vpr but not Vif deleted from their genome.** A) Day 2 MDDCs were treated with VSVG pseudotyped HIV-1_{NLAD8ΔVpr}, HIV-1_{NLAD8ΔVif}, HIV-1_{NLAD8ΔVpr,Vif} or HIV-1_{NLAD8} at MOI 1 for 48 and 96 hours. A) IFNβ mRNA expression was determined by QPCR. The mean data from three independent donors is shown with standard error bars. B-D. Day 6 MDDCs were treated with HIV-1_{Bal} or mock treated for 24 and 48 hour. B) IRF-3 intracellular expression levels were determined by flow cytometry; the isotype control is shown as filled curve, mock treated cells are shown with a solid line and HIV-1 treated cells with a broken line. C) The percentage of HIV-1 infected cells was determined by flow cytometry using a PE conjugated p24 antibody and IRF-3 expression determined in p24- cells and p24+ cells separately (mean of five experiments shown with standard error bars). D -E) IRF-3 expression in response to HIV-1_{Bal} was also determined by Western blot in both MDDCs (D) and SupT1 cells (E). F) IRF3 cellular localisation was determined by confocal microscopy in mock and HIV-1 treated cells compared to Sendai virus treated TZMBL cells.
Figure 2

A  
6hr  48hr

MFI
IRF-1 FITC
IRF-1 FITC

C

MFI
all cells  P24-  P24+

B

P24-  P24+

SSC
80%  20%
P24-PE

D

Mock (6hr) HIV (6hr)  Mock (48hr) HIV (48hr)

IRF-1

GAPDH

E  
6hr  48hr  120hr

MFI
IRF-7 APC
IRF-7 APC

MFI
IRF-2 Alexa 488
IRF-2 Alexa 488
IRF-2 Alexa 488

MFI
IRF-8 Alexa 488
IRF-8 Alexa 488
IRF-8 Alexa 488

MFI
all cells  P24-  P24+

MFI
all cells  P24-  P24+

MFI
all cells  P24-  P24+
Figure 4

IRF2

IFIT5
Figure 5

A

Percentage MDDCs infected

- wt
- -IRF1 bs

Time post infection (hours)

B

% of cells infected

Ratio of Plasmids added (pBal wt : pBal-IRF1bs)
HIV infection of dendritic cells subverts the interferon induction pathway via IRF1 and inhibits type 1 interferon production

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