Influenza virus activates human immunodeficiency virus type-1 gene expression in human CD4-expressing T cells through an NF-κB-dependent pathway

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Abstract

Influenza virus infection can cause severe complications in human immunodeficiency virus type-1 (HIV-1)-infected individuals leading to an increased risk of complications and death compared to that seen in uninfected individuals. We assessed the capacity of influenza virus (Flu) to modulate transcription of the HIV-1 long terminal repeat (LTR) in human CD4+ T cells. We found that Flu is able to promote expression of both the transiently transfected and stably integrated HIV-1 LTR-driven reporter gene. Experiments performed with Arthrobacter-derived neuraminidase and ammonium chloride revealed that Flu-dependent activation of HIV-1 transcription required an intimate contact between Flu and the target cell and efficient entry of Flu inside human CD4+ T cells. Amplification of a Flu-specific mRNA by RT-PCR indicated that human T cells were indeed productively infected with Flu. Virus preparations rendered noninfectious after UV irradiation could no longer upregulate HIV-1 LTR activity. Furthermore, experiments conducted with wild type and NF-κB-mutated HIV-1 LTR-directed reporter vectors suggested that the positive action of Flu on HIV-1 LTR activity was mediated through the induction of NF-κB.

Our data show that fully competent Flu can lead to NF-κB-dependent activation of HIV-1 transcription in CD4+ T cells.

Keywords: HIV; Influenza virus; T cells; NF-κB

Introduction

Infection with influenza virus (Flu) is considered as one of the oldest and most common disease known to mankind. Influenza viruses are unique among respiratory viruses in regard to their seasonality and impact on the general population. They can cause explosive outbreaks of febrile respiratory illnesses across all groups and often lead to substantial mortality, particularly in aged and chronically ill persons. Flu-associated mortality is attributed to its high and rapid mutational rate, often producing new strains against which human beings have no immunity. Although Flu preferentially replicates in respiratory epithelial cells, other cell types such as monocytes, macrophages, and B and T lymphocytes are also susceptible to Flu infection [1–12]. Interestingly, the human immunodeficiency virus type 1 (HIV-1) can productively infect macrophages and CD4+ T cells. It is thus possible that Flu interacts with HIV-1-infected cells. Many lymphoid tissues, particularly mucosal-associated lymphoid tissues, line the airways in close association with epithelial cells and lymphoid cells have been reported to undergo clonal expansion and proliferation during inflammation accompanying Flu challenges. It should be noted that lymphoid tissues are also considered as a major reservoir for HIV-1 [13].

In immunocompromised persons, a fundamental feature of Flu is prolonged virus shedding [14]. For example, the shedding of Flu in an HIV-1-infected child persisted for at least 9 weeks and was associated with recurrent respiratory symptoms [15]. A previous work has revealed that
concurrent infection by Flu led to an increase in HIV-1 viral load [16], an observation that was however not confirmed by other reports [17,18]. It has been proposed that upregulation of HIV-1 replication is due to Flu-mediated alteration of the overall host immune response. For example, T-cell proliferation that is seen upon Flu infection might favor HIV-1 replication. It has also been postulated that activation of host transcription factors and secretion of certain cytokines following Flu infection might directly stimulate HIV-1 gene expression.

Given that CD4⁺ T cells act as a major reservoir for HIV-1 and Flu can potentially interact with this cell type in dually infected individuals, it is of prime importance to define whether Flu can affect HIV-1 transcription in CD4⁺ T lymphocytes. In the present work, we have made use of human T lymphoid cells containing either a transiently transfected or stably integrated luciferase reporter gene driven by the HIV-1 long terminal repeat (LTR). Our results suggest that Flu can infect established T cells and activate HIV-1 LTR-directed transcriptional activity. This Flu-mediated induction of HIV-1 transcription relied on the NF-κB transcription factor.

**Methods**

**Cell lines and reagents**

The human lymphoid T cell lines used in this study included Jurkat E6.1 [19] and 1G5 [20]. The latter cell line is derived from Jurkat E6.1 and is stably transfected with a construct harboring the luciferase gene driven by the LTR region from HIV-1SF-2. Both cell lines were maintained in RPMI 1640 culture medium supplemented with 10% fetal bovine serum (FBS). Madin–Darby canine kidney (MDCK) cells were cultured in MEM supplemented with 10% FBS. 293T cells express the simian virus 40 large T antigen and were cultured in MEM supplemented with 0.1% bovine serum albumin (GibcoBRL-Life Technologies), glucose (0.4%), and trypsin (2 μg/ml). The culture supernatant was collected after 48 h. Flu preparations were partially purified by low-speed centrifugation followed by filtration through a 0.45-μm pore filter. Virus stocks were titrated by a standard plaque assay as described elsewhere [29] and commonly ranged between 10⁶ and 10⁷ plaque forming unit (PFU) per ml. To achieve UV inactivation, Flu preparations were directly exposed to a UV lamp in opened culture dishes (providing an intensity of 100 μW/cm, the horizontal plane being defined by the bottom of the culture dish) at a distance of 0.7 m for 20 min in a laminar flow hood. Complete loss of virus infectivity was confirmed by standard plaque forming assay. Virus stocks were aliquoted and stored at −80°C until use.

**Exposure of cells to Flu and neuraminidase treatment**

To study the effect of Flu preparations on HIV-1 LTR activity, 1G5 or transfected Jurkat cells (1 × 10⁶/ml) were either left untreated or incubated with various Flu strains (either fully competent or UV inactivated) in serum-free medium at 37°C for 30 min. After extensive washing with phosphate-buffered saline (PBS), cells were resuspended and incubated in complete RPMI 1640 medium for the indicated periods of time. In some experiments, cells were pretreated with *Arthrobacter* derived NA (0.1 U/ml) at 37°C for 60 min before incubation with Flu. As a control, in some experiments, cells were also infected in parallel with NL4-3 virions (10 ng of p24) for 24 h. An 8-h treatment with the PTP inhibitor bpV[pic] (10 μM) was also included in experiments using transfected Jurkat cells.

**NH₄Cl inhibition assay**

1G5 cells (1 × 10⁶/ml) were either left untreated or incubated with A/England/427/88(H3N2) at a MOI of 1 at 4°C for 15 min to allow virus adsorption to the cell surface. Cells were then transferred to 37°C and treated immediately

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**Plasmids and transient transfection**

pNL4-3 is a full-length infectious molecular clone of HIV-1 [22]. The pLTRX-Luc plasmid, a kind gift from Dr. Schwartz (Unité d’Oncologie Virale, Institut Pasteur, Paris, France), contains a 722-bp *XhoI–HindIII* fragment from HIV-1LAI positioned upstream of the luciferase reporter gene [23]. We also used pLTR-Luc (HIV-1 LTR from strain HXB-2) and pmBLTR-Luc vectors (kindly provided by Dr. Calame, Columbia University, NY, USA). These molecular constructs contain the luciferase reporter gene under the control of wild-type (GGAAGCTTTCC) or NF-κB-binding site-mutated (CTCACCTTTC) HIV-LTR [24]. Jurkat cells were transiently transfected with pLTRX-Luc, pLTR-Luc, or pmBLTR-Luc using a previously described DEAE-Dextran transfection protocol [25].

**Production of virus stocks and UV inactivation**

The prototypic X4-tropic strain of HIV-1 NL4-3 was produced by transfecting 293T cells as described before [26,27]. The virus preparation was normalized for virion content by using a homemade p24 enzymatic test [28]. The following human Flu strains were used: A/England/427/88(H3N2), A/Sydney/05/97(H3N2), and A/Beijing/262/95(H1N1). Virus preparations were produced by incubating MDCK cells (90–95% confluent) with each virus strain at a 0.01 multiplicity of infection (MOI) at 37°C in serum-free MEM supplemented with 0.1% bovine serum albumin, (GibcoBRL-Life Technologies), glucose (0.4%), and trypsin (2 μg/ml). The culture supernatant was collected after 42 h. Flu preparations were partially purified by low-speed centrifugation followed by filtration through a 0.45-μm pore filter. Virus stocks were titrated by a standard plaque assay as described elsewhere [29] and commonly ranged between 10⁶ and 10⁷ plaque forming unit (PFU) per ml. To achieve UV inactivation, Flu preparations were directly exposed to a UV lamp in opened culture dishes (providing an intensity of 100 μW/cm, the horizontal plane being defined by the bottom of the culture dish) at a distance of 0.7 m for 20 min in a laminar flow hood. Complete loss of virus infectivity was confirmed by standard plaque forming assay. Virus stocks were aliquoted and stored at −80°C until use.
or 30 min later with NH₄Cl (2 mM). Cells were incubated for an additional 30 min, washed with ice-cold PBS, resuspended in complete culture medium, and left to incubate for 48 h. Finally, cells were lysed and luciferase activity was measured with a luminometer device as previously described [30].

RT-PCR detection of Flu HA3 mRNA and sequence analysis of PCR products

1G5 and Jurkat cells (4 × 10⁵) were either left untreated or infected with A/England/427/88(H3N2) at a MOI of 0.1 or 0.5 in a final volume of 400 µl of serum-free medium. Jurkat cells were also inoculated with UV-inactivated A/England/427/88(H3N2) at a MOI of 5. After extensive washing, cells were incubated in complete RPMI 1640 medium supplemented with 10% FBS at a concentration of 5 × 10⁵/ml at 37°C for 48 h. Next, cells were washed twice with ice-cold PBS. Total RNA from the pelleted cells was isolated with TRIzol reagent according to the manufacturer’s instructions (GibcoBRL). HA3 viral mRNA was specifically reverse-transcribed into DNA strand using Moloney-murine leukemia virus (M-MLV) reverse transcriptase (RT) and HA3 gene-specific antisense downstream primer AH3D11 (5’ GTTTCTCTGGTACATTCCGC 3’, positions 960–979). PCR amplification was performed using the same downstream primer and the upstream primer AH3A (5’ CAGATTGAAGTGACTAATGC 3’, positions 97–116). The PCR fragment was visualized and photographed on a 1.5% agarose gel. PCR products were purified using a commercial nucleic acid purification kit (NucleoSpin® from Clontech). Purified DNA fragments were subjected to sequence analysis using the BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, CA) and an ABI PRISM 377 DNA Sequencer according to the manufacturer’s instructions (Perkin-Elmer, CA).

Statistical analyses

Statistical analyses were carried out according to the methods outlined previously [31]. Means were compared using single factor ANOVAs. Statistically significant differences between means were determined using Dunnett’s test for comparing a control mean to each other group mean. When only two groups were considered, means were compared using Student’s t test. P values of less than 0.05 were deemed statistically significant.

Results

Flu upregulates HIV-1 LTR-driven reporter gene activity in both transiently and stably transfected human T cells

Our initial series of investigations was performed by transiently transfecting the parental Jurkat cell line with pLTRX-Luc prior to exposure to increasing doses of the Flu strain A/Beijing/262/95(H1N1). As shown in Fig. 1, treatment of such cells with Flu resulted in a dose-dependent increase in HIV-1 LTR-directed transcription reaching a peak at a MOI of 20. Our next objective was to monitor the possible effect of Flu on 1G5, a T lymphoid cell line that stably harbors a HIV-1 LTR-driven luciferase gene construct. It should be noted that the 1G5 reporter cell line expresses a very low basal level of LTR activity allowing the detection of weak changes in reporter gene expression [20]. Time course experiments demonstrated that HIV-1 LTR-dependent gene expression was increased in 1G5 cells after the addition of the Flu strain A/England/427/88(H3N2). Peak luciferase activity was reached at the 48-h time point following virus exposure (Fig. 2A). Incubation of 1G5 cells with increasing concentrations of Flu led to a dose-dependent augmentation of the stably integrated HIV-1 LTR construct (Fig. 2B). These experiments imply that Flu can activate expression of the transiently transfected and chromosomally integrated HIV-1 LTR-mediated reporter gene.

Given that Flu A causes the most serious illness in humans and displays a wide antigenic variability due to mutations in hemagglutinin (HA) and neuraminidase (NA) surface antigens (reviewed in Ref. [32]), two other strains of Flu A in addition to A/England/427/88(H3N2) were tested...
in 1G5 cells. As illustrated in Fig. 3, luciferase activity in 1G5 cells was induced by the three tested variants of Flu A.

An intimate contact between Flu and human T cells is necessary to achieve induction of HIV-1 transcription

Terminal sialic acid residues of various glycoproteins and glycolipids act as cellular receptors for Flu [33], and the removal of sialic acids from the cell surface by NA treatment has long been known to prevent Flu attachment [34]. Following the demonstration that Flu can upregulate HIV-1 LTR-dependent gene expression in human T cells, we next evaluated whether specific binding of Flu to target cells via interactions between viral HA envelope proteins and sialic acid residues was mandatory to obtain the observed effect. Hence, 1G5 cells were treated with *Arthrobacter*-derived NA before incubation with Flu A/England/427/88(H3N2). In this set of experiments, inoculation of 1G5 cells with HIV-1 was used as a positive control based on the concept that HIV-1 infectivity is increased upon NA treatment [35,36]. The Flu-mediated enhancement of HIV-1 LTR-dependent luciferase activity was abolished upon

![Fig. 2. Transcription of integrated HIV-1 LTR reporter vector is augmented by Flu virus in a dose- and time-dependent manner.](image-url)

(A) 1G5 cells (1 × 10^5) were either left untreated or exposed to Flu A/England/427/88(H3N2) (MOI: 2) in serum-free medium for 30 min. Cells were washed and incubated at increasing time lapse ranging from 8 to 96h before lysis to monitor luciferase activity with a microplate luminometer. Data are presented as fold induction of luciferase activity over untreated samples from the calculated means ± SD of four different lysed cell samples in the same experimental setting. These results are representative of three independent experiments. (B) 1G5 cells (1 × 10^5) were exposed to increasing doses of A/England/427/88(H3N2) in serum-free medium for 30 min. Cells were washed and incubated for 48 h before lysis. Luciferase activity was next monitored with a microplate luminometer. Results are presented as luciferase activity expressed in relative light units (RLU) from the calculated means ± SD of four different lysed cell samples in the same experimental setting. These data are representative of three independent experiments. The asterisks indicate significant differences between cells exposed to Flu and cells that were not exposed to Flu (P < 0.05). In panel A, the mean luciferase counts for cells that were not exposed to Flu were 3.2 RLU and standard deviations were equal or less than 10%.

![Fig. 3. Transcription of integrated HIV-1 LTR reporter plasmid is enhanced by different variants of Flu A. 1G5 cells (1 × 10^5) were either left untreated or exposed to A/England/427/88(H3N2), A/Beijing/262/95(H1N1), or A/Sydney/05/97(H3N2) (MOI: 2) in serum-free medium for 30 min. Cells were washed and incubated for 48 h before lysis. Luciferase activity was next monitored with a microplate luminometer. Results are expressed as luciferase activity (RLU) from the calculated means ± SD of quadruplicate-lysed cell samples in the same experimental setting. These data are representative of three independent experiments. The asterisks indicate significant differences between cells exposed to the indicated Flu strains and cells that were not exposed to Flu (P < 0.05).]
exposure of 1G5 cells to NA (Fig. 4). On the other hand, as expected, a similar treatment of 1G5 cells with NA before addition of HIV-1 in the absence of Flu resulted in an augmentation of LTR activity. These data indicate that the Flu-mediated induction of HIV-1 LTR-driven gene expression necessitates a close contact between Flu virus particles and the cell surface.

Activation of HIV-1 transcription requires an efficient entry of Flu

Having established that interactions between Flu and its natural attachment receptors (i.e., sialic acids) are crucial for enhancement of LTR reporter gene expression, we next assessed if this process also required uptake of Flu by human T cells. Entry of Flu into target cells occurs through endocytosis and fusion of the viral envelope with the endosomal membrane [37,38]. The acidic environment in endocytic organelles triggers conformational changes in envelope glycoproteins that initiate the membrane fusion event leading to viral penetration. As fusion is absolutely dependent upon exposure to an acid pH environment, weak bases such as ammonium chloride (NH₄Cl), which neutralizes acidic endocytic organelles, inhibit virus-mediated membrane fusion with the endosomal membrane and thus the pH-dependent entry process of Flu [38]. The importance of Flu uptake in activation of integrated HIV-1 LTR was tested by first incubating 1G5 cells with Flu A/England/427/88(H3N2) at 4°C to allow virus adsorption to the cell surface. Synchronized entry of Flu into such cells was achieved by a transfer to 37°C. Treatment with NH₄Cl was initiated either immediately or 30 min later with the lysosomotropic-alkalinizing agent NH₄Cl. Cells were washed and incubated for 48 h before lysis. Luciferase activity was next monitored with a microplate luminometer. Results are presented as luciferase activity expressed in relative light units (RLU) from the calculated means ± SD of quadruplicate-lysed cell samples in the same experimental setting. These data are representative of three independent experiments. The asterisks indicate significant differences between cells exposed to Flu and cells that were not exposed to Flu (P < 0.05).

Flu gene expression in T cells parallels the induction of HIV-1 transcription

Treatment of Flu preparations with long-wavelength UV light is known to eliminate virus infectivity by damaging
nucleic acids without affecting virus-encoded protein function such as NA and HA. RT-PCR was used to detect the mRNA specific for the viral HA type 3 in an attempt to test the susceptibility of Jurkat and 1G5 cells to infection with the Flu strain A/England/427/88(H3N2). As shown in Fig. 6, the mRNA specific for viral HA3 was detected in both cell lines and the intensity of the signal was increased when higher doses of Flu were used (lanes 4 and 5 for Jurkat cells and lanes 7 and 8 for 1G5 cells). The amplified fragment was absent following inoculation of Jurkat cells with UV-irradiated Flu at the highest tested MOI (lane 2). PCR amplification carried out in the absence of cDNA also resulted in the absence of any signal (lane 1). The PCR-amplified product obtained in this experiment was sequenced and was shown to match the HA cDNA sequence of the A/England/427/88(H3N2) strain (GenBank access number, AF180584) [39] (data not shown). Experiments performed with fully infectious and UV-inactivated Flu virus stocks demonstrate that infectivity of Flu is crucial to achieve induction of HIV-1 LTR-driven reporter gene activity (Fig. 6B). Thus, these results suggest that Flu gene expression in human T cells is concomitantly detected with the induction of HIV-1 transcription.

**Flu-mediated activation of HIV-1 LTR gene expression is NF-κB dependent**

The previous observation that some specific Flu proteins are capable of inducing NF-κB [40,41] prompted us to monitor the involvement of this transcription factor in Flu-induced activation of HIV-1 transcription. Jurkat cells were transiently transfected with pLTR-Luc and pmBLTR-Luc vectors. The latter molecular construct can no longer respond to NF-κB due to mutations in the two NF-κB-binding sites present in the LTR. As a positive control, cells were also treated with bpV[pic], a potent PTP inhibitor previously shown to lead to strong activation of HIV-1 transcription mainly through NF-κB [25]. Indeed, this activator demonstrated a powerful activation of the wild-type LTR construct, while lesser activation of luciferase gene expression was observed in pmBLTR-Luc-transfected cells (Fig. 7). The Flu-dependent enhancing effect on HIV-1 LTR-driven luciferase activity was totally abolished in Jurkat cells transfected with pmBLTR-Luc, thus suggesting that NF-κB is playing a pivotal role in the upregulatory effect of Flu in HIV-1 transcription.

**Discussion**

A previous study has revealed that expression of a single Flu protein, that is, the viral HA protein, strongly activates NF-κB upon transient transfection in human epithelial HeLa and 293 cells [41]. Moreover, NF-κB-dependent activation of HIV-1 transcription was seen following individual expression of Flu HA, matrix protein (MA), and nucleoprotein (NP) in human epithelial and T cells [40]. It should be stated that the latter findings were made following transient co-transfection of human A3.01 T lymphoid cells with a HIV-1 LTR reporter gene construct together with either HA, MA, or NP expression vector. In the present work, we demonstrate that HIV-1 transcriptional activity is indeed promoted following infection by fully competent Flu. Moreover, our experiments were carried out with two established human cell lines (i.e., Jurkat and 1G5) that are
different from the one used by Flory et al. [40]. We provide evidence that HIV-1 transcription is activated by fully infectious Flu in a dose- and time-dependent fashion. An important feature of the current study is the observation that Flu-mediated induction of HIV-1 LTR activity was not only seen in transiently transfected cells but also in Jurkat-derived cells containing chromosomally integrated HIV-1 LTR constructs. This last series of investigations is important considering that expression of integrated human retroviruses such as HIV-1 and human T-cell leukemia virus type 1 requires cellular factors different from those necessary for expression of transiently transfected plasmids of the same retroviruses [42–44]. Incubation of the two tested human T cell lines with Flu was not associated with cell lysis as monitored by trypan blue exclusion (data not shown). Other novel aspects of our study are that activation of HIV-1 transcription requires fully infectious Flu particles, an intimate contact with the cell surface, and efficient cellular entry. Finally, we demonstrate that Flu exerted its activating effect on HIV-1 transcription through induction of the NF-κB signal transduction pathway. These results are thus perfectly in line with previous reports [40,41]. Our in vitro data showing an NF-κB-mediated activation of HIV-1 transcription following Flu infection of human T cells harboring stably integrated HIV-1 LTR region reveal a new aspect of the complex interactions occurring between Flu and HIV-1.

Clinical studies have shown that immunocompromised HIV-1-infected persons are more likely to contract influenza [45]. Additionally, previous studies have revealed that Flu replicates for a longer time period, Flu-associated symptoms are prolonged, and the risk for complications is increased in HIV-1-infected patients compared to that seen in HIV-1-uninfected individuals [15,45–49]. Although a transient increase in HIV-1 load has been documented by Ho [16], most studies indicate that the use of Flu inactivated viruses for vaccination does not induce noticeable increases of HIV-1 viral load or loss of circulating CD4+ T cells [50–54]. Our observations that UV-inactivated Flu virus can no longer upregulate HIV-1 transcription in healthy T cells might help to explain the reported inability of Flu vaccines to affect viral load in HIV-1-infected individuals.

In light of the present data, it can be proposed that clinical studies should be undertaken to assess the effect of manipulating Flu replication in persons infected with HIV-1. However, a previous study indicated that neither HIV-1 viral load, clinical progression, nor CD4+ T cell count was affected upon Flu infection of HIV-1-positive individuals [55,56]. Furthermore, a study by Pinto et al. [57] has in fact shown that the supernatant of Flu-exposed peripheral blood lymphocytes can reduce HIV-1 replication, an effect that in vivo could counteract the positive modulation of HIV-1 replication caused by Flu infection observed in our study. In addition, the presence of Flu virus in some specific specialized areas in the upper respiratory tract might reduce the likelihood of a possible contact between Flu and cells harboring the HIV-1 genome under an integrated form. Randomized clinical studies are thus needed to provide additional useful information on the potential role of Flu as a cofactor in the setting of HIV-1 infection.

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