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The effect of dexamethasone on lentiviral vector infection is associated with importin α

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Abstract. Importin α (Imα) plays an important role during the shuttling of the HIV-1 preintegration complex (PIC) from the cytoplasm to the nucleus. Imα may bind to the glucocorticoid receptor (GR), which is localized to nucleus following hormone binding. However, it remains unclear whether the binding of dexamethasone (Dex) to GR affects the Imα redistribution and, thus, alters PIC import. In our study, 293T cells were transfected with the lentiviral vector (LV) carrying the luciferase (Luci) gene following Dex or RU486 pretreatment. The Luci activity (LucA) in the Dex or RU486 group was significantly higher compared to that in the control group (P≤0.01). The effects of Dex and RU486 were inhibited by the Imα inhibitor Bimax1 (P≤0.01), although the inhibitory effect of Bimax1 was alleviated by increasing the Dex dose. Furthermore, it was observed that the LucA in the 30-min Dex treatment group was lower compared to that in the 30-min Dex pretreatment group (P≤0.01). These results suggested that Dex may improve PIC import via increasing the cytoplasmic Imα levels. Kunming mice were transfected *in vivo* with the LV, either 30 min or 15 h following an intraperitoneal injection of Dex. The LucA in the liver of the 30-min group mice was significantly lower compared to that of the 15-h group mice (P≤0.01), suggesting that the effect of Dex on LV infection depends mainly on the suppression of immune and inflammatory responses *in vivo*. Taken together, our data indicated that the effect of Dex on LV infection may be associated with Imα, constituting a novel signaling pathway mediating the effects of Dex on HIV-1 infection.

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Introduction

The capsid of the lentiviral vector (LV) is disassembled shortly after LV is endocytosed into the cytoplasm and the retrotranscription process is initiated, producing a double-strand viral DNA. This double-strand viral DNA, together with matrix protein (MA), integrase (IN), HIV-1 viral protein R (Vpr) and cellular factors, form the preintegration complex (PIC). Subsequently, PIC binds to importin α (Imα) through the interaction of Imα with the nuclear location signal (NLS) located in MA and IN (1-4). Furthermore, Vpr was shown to increase the Imα affinity for NLS (5).

The Imα carrying PIC is then combined with importin β, forming the Imα/β heterodimer. The Imα/β interacts with a nucleoporin of phenylalanine-glycine repeats, leading to PIC import (6). Subsequently, PIC is disassembled in the nucleus through the binding of RanGTP to importin β, resulting in the separation of PIC from Imα. The double-strand viral DNA is then integrated into the target cell genome to achieve LV infection.

The glucocorticoid receptor (GR) is also combined with Imα and transported to the nucleus, even in the absence of glucocorticoid (3). GR has two NLSs and one nuclear export signal. The first NLS (NLS-1), which is located in the DNA-binding domain and is similar to the SV40 NLS, binds Imα. The second NLS (NLS-2), which is located in the ligand-binding domain, binds glucocorticoid. GR is localized to the cytoplasm in the absence of hormone and localizes to the nucleus following hormone binding (3). Since GR is able to bind Imα, GR retention in the nucleus may theoretically result in Imα redistribution, affecting PIC import.

In the cytoplasm, GR transports transcription factors to the nucleus and alters their activity (e.g., nuclear factor NF-κB), triggering gene transcription modulation (7). In the nucleus, GR may directly bind to a specific DNA sequence, referred to as glucocorticoid response element (GRE), which is a short sequence of DNA within the promoter of a gene that is able to bind the GR complex and regulate transcription. Depending on the cell line, glucocorticoids may differentially affect HIV-1 expression (8).

Despite the well-known effect of dexamethasone (Dex) on HIV-1 gene transcription, it has not thus far been determined whether the binding of Dex to GR affects the Imα redistribu-

tion. This study was designed to confirm the Imα redistribution induced by Dex through detecting the luciferase (Luci) activity (LucA) in cells transfected with the Luci reporter LV. The Imα redistribution affects LV infection, as well as other nucleophilic imports.

Materials and methods

Luci expression vector construct. The Luci gene was obtained from the pGl3 plasmid (Promega, Madison, WI, USA) by a polymerase chain reaction and cloned into pcDNA™6.2-GW/miR (Invitrogen, Carlsbad, CA, USA) to form pcDNA™6.2-GW/Luci. pcDNA6.2-GW/Luci was then recombined with pDONR™221 (Invitrogen) to generate a pDONR™Luci entry clone (BP recombination). The pDONR Luci entry clone was again recombined with pLenti6/V5-DEST™ (Invitrogen) to construct pLenti6/Luci (LP recombination). Subsequently, the products of the LP recombination were treated with proteinase K and then transformed into One Shot® Stbl3™ Chemically Competent *E. coli* to obtain pLenti6/Luci (Fig. 1). pLenti6/Luci was identified with nucleic acid electrophoresis and LucA assay.

LV preparation

Preparation of the DNA complex. A total of 9 μg of ViraPower™ Packaging Mix (Invitrogen) and 3 μg of pLenti6/Luci were added to 1.5 ml Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO, USA) without serum and mixed gently.

Preparation of the GenEscort III complex. A total of 36 μl GenEscort III (Wisegen Biotechnology Corp., Nanjing, China) were diluted in 1.5 ml DMEM without serum, mixed gently and incubated for 5 min at room temperature.

Preparation of the transfection complex. The DNA complex was added to the GenEscort III complex, mixed gently and incubated for 20 min at room temperature. At the same time, the 293T cells were resuspended in DMEM at a density of $1.2x10^6$ cells/ml. Subsequently, DNA-GenEscort III was added to a 10-cm tissue culture plate containing 5 ml DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 0.1 mM minimum essential medium (MEM) non-essential amino acids and 1 mM MEM sodium pyruvate. The 293T cell suspension (5 ml) was added to the plate and mixed by gentle rocking. Finally, the cells were incubated overnight at 37˚C in a CO_2 incubator. The following day, the medium containing DNA-GenEscort III was removed and replaced with complete culture medium. The supernatants were harvested 48-72 h after transfection, centrifuged at 1,000 x g for 5 min at 4˚C and stored at -80˚C. The LV was titered immediately prior to use. All the operations applied to Biosafety Level 2.

Cell culture and pretreatment. The 293T cells ($1x10^3$/well) were maintained in 96-well plates containing 200 μl DMEM (as described above) and each well was repeated 3 times. RU486 ($1x10^{-6}$ M; Sigma) was used for stimulating GR shuttling to the nucleus (9). Bimax1 ($2.5x10^{-8}$ M; Shanghai Science Peptide Biological Technology Co., Ltd, Shanghai, China) was used for inhibiting Imα (10,11). Pretreatment with Dex (Sigma) was classified into 0-, 5-, 15-, 30-, 60- and 120-min groups (12); the grouping for Dex treatment was the same as that for Dex pretreatment. The 30-min group of Dex pretreatment was again classified into two dose groups of $1x10^{-7}$ and $1x10^{-6}$ M (Dex-1 and Dex-2, respectively) (13). All the cells were used for LucA assay 72 h after pretreatment or treatment.

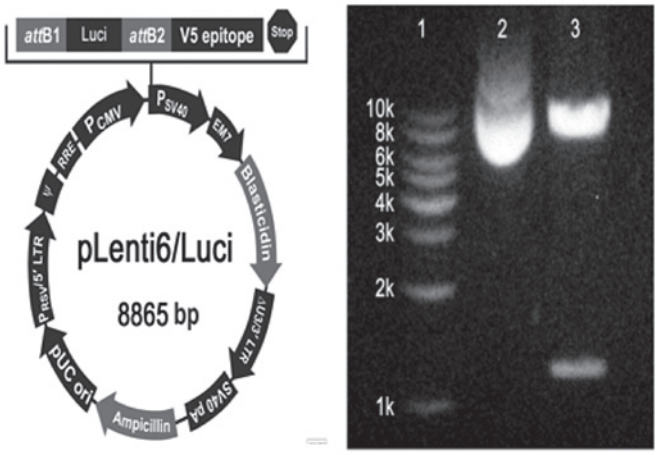

Figure 1. pLenti6/Luci map (left panel) and electrophoretogram (right panel). Lane 2, pLenti6/Luci; and lane 3, pLenti6/Luci digested by *Bln*I.

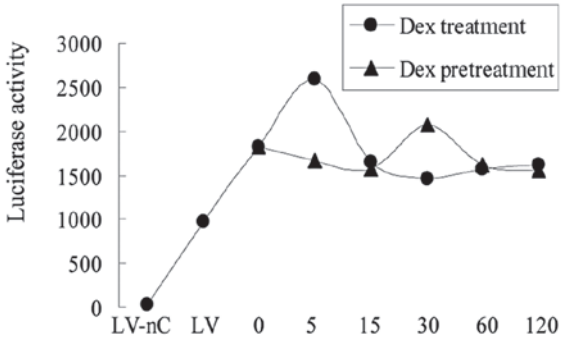

Figure 2. The effect of dexamethasone (Dex) on luciferase activity. The difference between the lentiviral vector (LV) and the 0-min group was P≤0.01. Upon Dex pretreatment, the difference between the 30- and the 15- or 60-min groups was P≤0.01. Upon Dex treatment, the difference between the 5- and the 0-, 15-, 30-, 60- or 120-min groups was P≤0.01. The difference between the 30-min Dex treatment and pretreatment groups was P≤0.01. LV-nC was used as a negative control.

Animals. An amount of 0.5 mg/10 g Dex and LV (titer=10^6) was administered by intraperitoneal injection (14). A total of 42 Kunming mice of clean grade (half of the animals were female, although the gender of the animal was not considered a significant factor) were randomly assigned into 7 groups as follows: LV-nC (LucA-negative control); LV-1C [20 μl LV/mouse, normal saline (NS) pretreatment control]; LV-2C (100 μl LV/mouse, NS pretreatment control); LV-1/P-1 (20 μl LV/mouse, 30-min Dex pretreatment); LV-2/P-1 (100 μl LV/mouse, 30-min Dex pretreatment); LV-1/P-2 (20 μl LV/mouse, 15-h Dex pretreatment); LV-2/P-2 (100 μl LV/mouse, 15-h Dex pretreatment). Seven days after the injection of LV, the mice were anaesthetized with 0.4% pentobarbital sodium and sacrificed and their livers were immediately excised. The mice were maintained and handled in accordance with the National and International Guiding Principles for Biomedical Research Involving Animals, as promulgated by the Society for the Study of Reproduction.

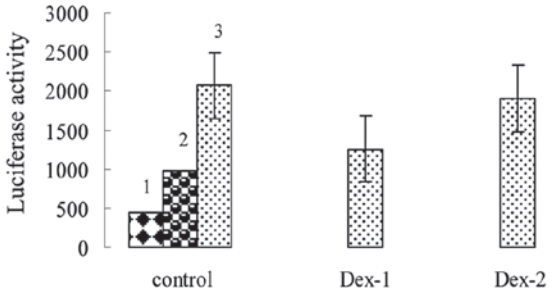

Figure 3. The effect of Bimax1 on importin α (Imα) was alleviated by Dex. Control 1: the cells were pretreated with Bimax1 30 min prior to LV transfection; control 2: the cells were transfected with LV alone; control 3: the cells were pretreated with Dex 30 min prior to LV transfection (difference between control 1 and control 2 and 3, P≤0.01); Dex-1 and Dex-2 group: the cells were pretreated by Bmax1, followed by the addition of $1x10^{-7}$ and $1x10^{-6}$ M Dex respectively, 30 min prior to LV transfection (difference between Dex-1 and Dex-2 groups, P≤0.01).

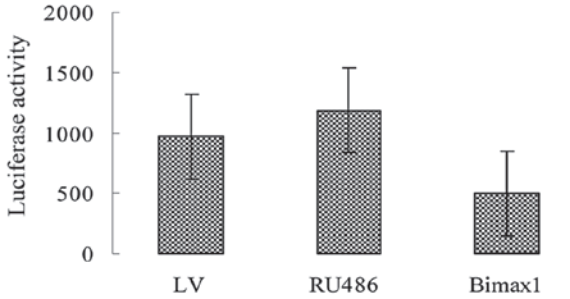

Figure 4. Effect of RU486 on importin α (Imα). RU486 group: the cells were pretreated with RU486 30 min prior to transfection; Bimax1 group: the cells were pretreated with RU486, followed by the addition of Bmax1 30 min prior to LV transfection (difference between RU486 and Bimax1 groups, P≤0.01).

LucA assay. The 293T cells and 0.2 g of mouse liver were lysed in cell culture lysis reagent (Promega) at -80˚C for 30 min and then immersed in 37˚C water bath for 5 min. Subsequently, the lysates were collected and centrifuged at 13,800 x g for 2 min at 4˚C to remove cell debris. The LucA in 100 μl of lysate was assessed by the addition of 100 μl Luci assay reagent (Promega). After 30 min of pre-incubation, the produced light was measured for 10 sec with the GloMax®-Multi Jr Single Tube Multimode Reader (Promega).

Statistical analysis. Data were analyzed for significant differences by the independent samples t-test. A P<0.05 was considered to indicate a statistically significant difference. The tests were performed with SPSS software, version 10.0 for Windows (SPSS, Inc., Chicago, IL, USA). All the values are presented as means ± standard deviation.

Results

Effect of Dex on LucA (Fig. 2). Considering that Dex results in GR retention in the nucleus, it may be hypothesized that cytoplasmic Imα is decreased due to the ability of GR to bind Imα, which suggests that Dex pretreatment may inhibit PIC import. To determine whether Dex affects PIC import, the 293T cells were pretreated with Dex. As a result, the LucA in the Dex pretreatment group was significantly higher compared to that in the LV alone group (P≤0.01). Furthermore, the LucA in the 30-min Dex pretreatment group was the highest, with a significant difference between the 30-min Dex pretreatment group and the 15- or the 60-min groups (P≤0.01). However, these results were not sufficient to determine whether cytoplasmic Imα is decreased even if Dex improves LV transcription. To verify that Dex improves LV transcription, the 293T cells were treated with Dex following transfection. The LucA in the Dex treatment groups was significantly higher compared to that in the LV alone group (P≤0.01), with the LucA in the 5-min Dex treatment group being the highest. Of note, the LucA in the 5-min Dex treatment group was higher compared to that in the 5-min Dex pretreatment group; however, the LucA in 30-min Dex treatment group was lower compared to that in the 30-min pretreatment group (P≤0.01), which suggests that pretreatment for 30 min with Dex improves PIC import as well as LV transcription, whereas treatment for 30 min with Dex only improves LV transcription.

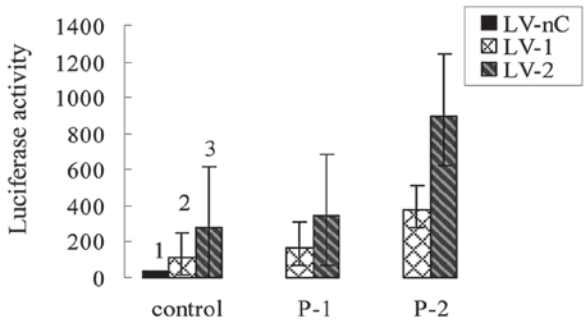

Figure 5. Effect of dexamethasone (Dex) on LucA *in vivo*. LV-1C: 20 μl (titer=10^6) LV/mouse, normal saline (NS) pretreatment; LV-2C: 100 μl (titer=10^6) LV/mouse, NS pretreatment; P-1: Dex pretreatment for 30 min; P-2: Dex pretreatment for 15 h (difference between LV-1C and LV-1/P-2 and between LV-2C and LV-2/P-2, P≤0.01; difference between LV-1C and LV-1/P-1 and between LV-2C and LV-2/P-1, P≥0.05). LV-nC, LucA-negative control.

Effect of Bimax1 on Imα alleviated by Dex (Fig. 3). The increased LucA may be partly attributed to the increasing levels of cytoplasmic Imα. Thus, LucA may be decreased by Bmax1 and the decreased LucA may be elevated through increasing the Dex dose. To verify the above, the 293T cells were pretreated with Bmax1, followed by the addition of Dex-1 and Dex-2 30 min prior to transfection. The LucA in the Bmax1 group was significantly lower compared to that in the LV alone group (P≤0.01), suggesting that Bimax1 was able to block PIC import through inhibiting Imα. In comparison with the Bmax1 group, the LucA in the Dex-1 and Dex-2 groups was significantly elevated (P≤0.01), with the LucA present in the Dex-2 group being higher compared to that in the Dex-1 group (P≤0.01). These results suggest that the elevated LucA may be attributed to the Imα increase mediated by Dex, as well as to the transcription enhanced by Dex.

Effect of RU486 on Imα (Fig. 4). To exclude the effect of transcription on LucA, Dex was replaced with RU486. The 293T cells, which were pretreated with RU486, followed by the addition of Bmax1 30 min prior to transfection, were transfected with LV. Compared to the RU486 alone group, the LucA in the Bmax1 group was significantly decreased (P≤0.01),

further confirming that the increased LucA was associated with the increasing cytoplasmic Imα levels.

Effect of Dex on LucA in vivo (Fig. 5). The mice were pretreated with Dex either 30 min or 15 h prior to LV transfection. The 30-min pretreatment aimed to increase cytoplasmic Imα levels, whereas the 15-h pretreatment aimed to suppress immune and inflammatory responses. Compared to the LV-1C and LV-2C groups, the LucA in the LV-1/P-1 and LV-2/P-1 groups, respectively, was not increased ($P \geq 0.05$), although the LucA in the LV-1/P-2 and LV-2/P-2 groups was significantly elevated ($P \leq 0.01$). These findings suggest that the efficiency of LV transfection depends mainly on suppressing immune and inflammatory responses *in vivo*.

Discussion

To the best of our knowledge, this study was the first to demonstrate that the effect of Dex on LV infection is associated with Imα, suggesting that Imα may be a novel pathway mediating the effects of Dex on HIV-1 infection.

Dex increases cytoplasmic Imα levels. Imα may bind to the NLS-1 of GR in the absence of gluocorticoid (3). Thus, the cytoplasm contains two types of Imα, namely Imα and Imα-GR. Following Dex pretreatment, Dex is combined with the NLS-2 of GR to form two new complexes, GR-GR-Dex and Imα-GR-Dex (15). Therefore, the cytoplasm contains Dex, GR, Imα, Imα-GR, Imα-GR-Dex and GR-GR-Dex, in a dynamic balance. Dex promotes GR import, thus resulting in the nuclear retention of GR (7,16-18). Subsequently, the GR-GR dimer changes into GR in order to bind GRE, triggering LV gene transcription modulation (19). This may be a mechanism through which Dex pretreatment leads to elevated LucA. Furthermore, the nuclear retention of GR-GR results in decreased cytoplasmic GR levels. According to the balance theory, cytoplasmic GR attenuation inhibits the binding of GR to Imα, resulting in increased cytoplasmic Imα levels and improving PIC import. This may be another mechanism underlying the association of elevated LucA with Dex pretreatment. In addition, Dex induces the downregulation of GR (20), which may also help increase cytoplasmic Imα levels.

Bimax1, a peptide with a NLS-like sequence exhibiting a high affinity for Imα (11), may block the import of the nucleus accumbens-associated protein 1 by the Imα pathway (10). In our study, Bimax1 impaired the PIC import that was induced by Dex, resulting in decreased LucA. However, increasing the Dex dose elevated LucA, which was attributed to promoting PIC import and LV gene transcription. RU486 binds to the co-activator pocket of GR (GR1), with domain swapping of the GR3 between the subunits of the GR dimer (21), inducing GR shuttling to the nucleus, without stimulating subsequent events (22). In the present study, Dex was replaced by RU486. LucA was elevated by RU486, but decreased by Bimax1, further suggesting that the change in cytoplasmic Imα levels affects LV infection.

Increased cytoplasmic Imα levels may also result from a change in the GR affinity for Imα. The conformational change of the GR D-loop and second helix was shown to confer a change in the GR affinity for Imα (23,24), which was, however, not investigated in the present study.

Increasing cytoplasmic Imα levels favor PIC shuttling to the nucleus. The interaction of Imα with IN and MA results in PIC import (2,25-27), which is followed by the integration of double-strand LV DNA into the target cell genome. Subsequently, the GR accumulated in the nucleus is combined with LV GRE to improve Luci transcription, resulting in increased LucA. Several previous experiments also confirmed that Dex improves LV gene transcription (28-30).

Of note, the inactivation of NLS within IN and MA was previously reported to inhibit viral infection (2,26). Considering that PIC import depends on Imα (31-33), the inactivation of NLS within Imα may also inhibit HIV-1 infection. The majority of the currently available drugs for the treatment of HIV-1 have focused on inhibiting viral entry, viral genome replication and virus-specific proteolysis. However, numerous viruses are able to exploit cellular kinases, facilitating subcellular targeting during infection to achieve drug resistance (34). Accordingly, blocking PIC import may suppress HIV-1 infection through the Imα pathway, although it remains unclear whether inhibiting Imα leads to HIV-1 drug resistance due to the HIV-1 accumulation in the cytoplasm.

In vivo, increasing LucA depends mainly on immunosuppression and anti-inflammation. Following intraperitoneal injection, LV must go through several processes to reach the target cell. During these processes, LV may be sequestered in bypass organs and destroyed by opsonization, immune and inflammatory responses, resulting in significantly impaired gene delivery. Dex is one of the most frequently used immunosuppressive and anti-inflammatory drugs. The binding of Dex to GR suppresses the transcription of numerous cytokines, adhesion molecules and proinflammatory genes via the NF-κB pathway (35). Vpr is a coactivator of GR and its effects, including suppressing IL-12 transcription in human monocytes and inducing the apoptosis of human $CD4^+$ T cells and thymocytes, are enhanced by the binding of Dex to GR (36-38). Megadose Dex may preserve lysosomal membrane integrity by rapid non-genomic effects and long-term receptor-dependent genomic events (14). In our study, it was demonstrated that Dex promotes LV infection via the Imα pathway.

In conclusion, LucA may be increased through improving PIC import and LV transcription. The improved PIC import by Dex is associated with Imα, suggesting that Imα may be a novel pathway mediating the effects of Dex on HIV-1 infection.

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