

*Full Length Research Paper*

# Human immunodeficiency virus (HIV)-1 Tat downregulates the phosphorylation of recepteur d'origine nantais (RON) receptor tyrosine kinase induced by macrophage-stimulating protein

T. Feng<sup>1,2</sup>, X. Shao<sup>1</sup>, J. Li<sup>1</sup>, L. Cheng<sup>1</sup>, M. Fang<sup>1</sup>, L. Wu<sup>1</sup> and N. Wu<sup>1\*</sup>

<sup>1</sup>State Key Laboratory for Diagnosis and Treatment of Infectious Diseases, Zhejiang University School of Medicine, Hangzhou, Zhejiang 310003, China.

<sup>2</sup>The First Affiliated Hospital of Soochow University, Suzhou, Jiangsu 215002, China.

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The aim of this study is to examine the effects of the human immunodeficiency virus (HIV)-1 Tat protein on the expression and phosphorylation of recepteur d'origine nantais (RON) receptor tyrosine kinase and the mechanisms involved. We first determined the expression levels of RON and macrophage-stimulating protein (MSP) were determined in the peripheral blood of HIV-positive patients and control subjects. The 293T cells were transfected with the pDR2-RON plasmid to establish the 293T-RON cell line. The pcDNA3.1 (+)-Tat-His plasmid was constructed to obtain recombinant Tat protein. The 293T-RON cells pretreated with MSP were co-cultured with the H9/HTLV-IIIIB cells or were stimulated with the Tat protein; then, the change of RON expression was determined. This study revealed higher expression of RON and relatively lower expression of MSP in HIV-infected patients than in the uninfected patients. We demonstrated that HIV-1 Tat down-regulated the expression and phosphorylation of RON in 293T-RON cells. Flow cytometry analysis of 293T-RON cells revealed that RON was expressed in 293T-RON cells. The 293T-RON cells co-cultured with the H9/HTLV-IIIIB cells RON expression did not change significantly, while stimulation with 500 ng/ml Tat considerably decreased RON expression in 293T-RON cells. These findings imply that Tat may play a role in modulating the function of RON, which is activated by MSP; this may provide a permissive environment for both HIV and other opportunistic microbes.

**Key words:** Human immunodeficiency virus (HIV)-1, Tat, receptor tyrosine kinase, macrophage-stimulating protein, inflammation regulation.

## INTRODUCTION

Concurrent infections with pathogens are common in acquired immunodeficiency syndrome (AIDS) patients. However, a huge portion of the immune network of cytokines in patients infected with the human immunodeficiency virus (HIV) is not well studied. The immune reconstitution inflammatory syndrome involves exuberant and dysregulated inflammatory responses in patients infected with HIV. This syndrome is manifested when the host immune system abruptly shifts from an anti-inflammatory and immunosuppressive state to a patho-

genic proinflammatory state as a result of rapid decrease in or removal of factors promoting immunosuppression or inhibiting inflammation (Sun and Singh, 2009). The clinical and biological bases of the potential associations between chronic oral inflammatory disorders, such as periodontal disease, and exacerbation of HIV viremia have received little attention (Gonzalez et al., 2009). In the process of inflammatory and anti-inflammatory responses, HIV along with its proteins can regulate many host factors to create a microenvironment suited to its reproduction.

In particular, the HIV-1 Tat protein is a regulatory protein that is essential for gene expression, replication, and infectivity of HIV-1 (Caputo et al., 2009; Kalantari et al.,

\*Corresponding author. Email: [flwnp@yahoo.com.cn](mailto:flwnp@yahoo.com.cn). Tel: +86-571-87236580. Fax: +86-571-87236582.

2008; Rayne et al., 2009). After Tat was found to act as a transcription factor, it has been demonstrated to possess other abilities as well. Intracellularly, Tat can deregulate the expression of several heterologous cellular genes. Several studies have revealed that extra-cellular Tat can stimulate the expression of immuno-regulatory cytokines such as tumor necrosis factor (TNF), interleukin (IL)-2, and transforming growth factor (TGF) (Apolloni et al., 2007; Guendel et al., 2009; Charnay et al., 2009). Extracellularly, Tat can contribute to the spreading of HIV-1 and immunosuppression of uninfected cells (Nappi et al., 2009). Meanwhile, there are evidence that exogenous Tat is involved in AIDS-associated pathologies such as Kaposi's sarcoma and HIV-associated dementia. These abilities together accelerate the progression of AIDS (Apolloni et al., 2007; Minghetti et al., 2004; Peruzzi, 2006).

Recepteur d'origine nantais (RON) is a receptor tyrosine kinase closely related to c-Met (Leonis et al., 2007). Macrophage-stimulating protein (MSP), a member of the plasminogen-related growth factor family, was identified as the ligand of RON (Wang et al., 1997). Activation of RON by MSP exerts dual functions on macrophages. The stimulatory activities include the induction of macrophage spreading, migration, and phagocytosis (Wang et al., 2002). Some recent studies using chromatin immunoprecipitation demonstrated that RON inhibits HIV transcription. RON was found to decrease the efficiency of transcription elongation by reducing the amount of RNA polymerase II associated with HIV-1 genomic sequences downstream of the transcriptional start site (Kalantari et al., 2008; Klatt et al., 2008). In addition, RON activated by MSP inhibits the production of proinflammatory mediators such as IL-12, TNF- $\alpha$ , and nitric oxide (NO) (Chen et al., 1998; Correll et al., 1997) and regulates the expression of genes associated with resolving inflammation, including the genes encoding scavenger receptor A, IL-1R $\alpha$ , and arginase. Macrophages from RON knockout mice produce elevated levels of NO in response to interferon- $\gamma$  and lipopolysaccharide (LPS); the mice exhibit increased inflammation, tissue damage, and death due to endotoxic shock upon LPS challenge (Wilson et al., 2008). In summary, some relationship exists among RON, MSP, inflammation, and virus infection.

To gain insights into the relationship between RON and HIV-related inflammation and regulation mechanisms, we constructed the pcDNA3.1 (+)/Tat and pDR2/RON plasmids and established a 293T cell line expressing RON (293T/RON). We determined the changes in the expression levels of RON, which is activated by MSP, in 293T/RON cells stimulated with HIV or the Tat protein.

## MATERIALS AND METHODS

### Materials

Roswell Park Memorial Institute medium (RPMI) 1640 and

Dulbecco's modified Eagle's medium (DMEM) were purchased from Invitrogen (Grand Island, NY, USA). Antibodies directed against  $\beta$ -actin and HIV-1 Tat were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA); rabbit anti-RON (5029) antibody and mouse anti-RON monoclonal antibodies (mAbs) (Zt/G4 and 2F2) were provided by Prof. Wang MH (Guin et al., 2010; Yao et al., 2006). All of these antibodies were used for the immunoblotting or immunocytochemistry experiments. The 6xHis-Tag fusion protein purification kit (Pierce Company, MN, USA), was used for the purification of the Tat protein. The Phospho-Tyrosine Monoclonal Antibody (P-Tyr-100) and cell lysis buffer were purchased from Cell Signaling Technology (Beverly, MA, USA). Recombinant MSP and MSP Quantikine Colorimetric Sandwich enzyme-linked immunosorbent assay (ELISA) kit were purchased from R and D systems (Minneapolis, MN, USA).

### Cell culture

The 293T human embryonic kidney cell line (American Type Culture Collection) was cultured in DMEM supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, and 2 mM L-glutamine (Invitrogen, MD, USA). The 293T/RON cells were generated by transfecting 293T cells with the pDR2-RON plasmid and selecting with Hygromycin B (Invitrogen, MD, USA) (Guin et al., 2010). The H9/HTLV-III B cell line (human T cell line infected with HIV III, American Type Culture Collection) was cultured in RPMI 1640 medium supplemented with 10% FCS, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, and 2 mM L-glutamine.

### Human subjects

A total of 141 HIV-1-infected individuals and 37 healthy subjects were enrolled in this study. Blood samples were evaluated for viral load, CD4+ T-cell count, and CD4+ T-cell percentage as part of routine clinical monitoring. Cells were marked with anti-CD4 mAb and analyzed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA). Data were analyzed using WINMDI software by J. Trotter (The Scripps Institute, USA). Of the 104 HIV-1-positive patients, 82 patients satisfied the World Health Organization (WHO) criteria for highly active antiretroviral therapy (HAART) initiation and were on a stable antiretroviral regimen; the remaining 22 patients were seropositive adults who did not satisfy the WHO criteria for HAART initiation. RON levels in the same blood samples were determined by double antibody sandwich ELISA using Zt/G4 and 2F2 mAbs against RON (Yao et al., 2006; Lu et al., 2008). Meanwhile, the level of MSP have been detect using ELISA test. For the analysis, we obtained the consent of the participants or their guardians in a manner consistent with the policies of the appropriate local institutions. HIV-1 infection was confirmed by a positive western blot reaction and AIDS was diagnosed according to the Centers for Disease Control classification system for HIV-1 infection. The controls were HIV-negative healthy volunteers who were age, sex, and ethnically matched with the pediatric and adult subjects with AIDS. A short history was obtained from all healthy participants to ensure that they had no infectious disease in the past 3 months.

### Determination of the viral load of HIV

The levels of HIV RNA in the samples from pediatric and adult AIDS patients were determined using a standardized reverse transcriptase-polymerase chain reaction (RT-PCR) assay (Amplicor HIV-1 Monitor, Roche Diagnostics, Indianapolis, IN, USA) according to the manufacturer's instructions. The detection limit in the plasma

**Table 1.** Serum levels of RON and MSP in HIV-positive patients.

HIV-1 infected group	Cases(n)	CD4 <sup>+</sup> T cell	Virus load ( $\times 10^5$ cps/ml)	RON (ng/ul)	MSP (ng/ul)
Untreated	22	282.41 $\pm$ 91.81	7.86 $\pm$ 0.00296	0.49 $\pm$ 0.135 <sup>#</sup>	0.97 $\pm$ 0.29 <sup>▲</sup>
Treated	82	313.47 $\pm$ 138.06	0.53 $\pm$ 0.00244 <sup>▲</sup>	0.77 $\pm$ 0.202 <sup>*</sup>	0.64 $\pm$ 0.39 <sup>*</sup>
Normal	37	768.30 $\pm$ 104.12		0.26 $\pm$ 0.36	2.08 $\pm$ 1.49

\* P < 0.01 (compared to the normal group); # P < 0.05 (compared to the untreated group); ▲ P < 0.01 (compared to the untreated group).

was 50 copies of HIV-1 per ml.

### Expression and purification of the Tat protein

The recombinant Tat protein was produced and purified as described previously. Briefly, a 6xHis tag was added to the gene fragment encoding the first 86 amino acids of the HIV-1 Tat protein; this tagged fragment was inserted into the pcDNA3.1(+) plasmid. Then, the recombinant plasmid was transfected into 293T cells using FuGENE 6 Transfection Reagent (Roche Diagnostics, Indianapolis, IN, USA). The 293T cells transfected with the Tat gene were selected with G418, the recombinant Tat protein was purified with the 6xHis Fusion Protein Purification kit. The purity and concentration of the recombinant protein were evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and the bicinchoninic acid (BCA) method.

### Treatment and processing of 293T/RON cells

The 293T/RON cells ( $1 \times 10^6$ ) were cultured in a 10 cm culture dish and were treated with 500 ng/ml of Tat for 24 h in the presence or absence of MSP (2 nM). In addition, the 293T/RON cells ( $1 \times 10^6$ ) were co-cultured with H9/HTLV-III B cells as a control. Cells were collected and lysed with a lysis buffer for 30 min at 4°C. The insoluble material was then removed by centrifugation at 8000  $\times$  g for 10 min at 4°C. The protein concentration in each cell lysate was determined using a BCA Protein Assay kit (Pierce, IL, USA) with bovine serum albumin as the standard.

### Immunofluorescence assay for RON detection

Cells were cultured in slides (Lab-Tek Chamber Slide System, Nalge Nunc International) and were treated as above. Cells were fixed with 4% paraformaldehyde at 4°C for 20 min and incubated with anti-RON mAb (2F2) at room temperature for 1 h. The cells were then further incubated with fluorescein isothiocyanate (FITC)-coupled secondary antibody (Santa Cruz Biotechnology, CA, USA) for 30 min. Fluorescence was detected using a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA), and photographs were obtained using a fluorescence microscope (Olympus IX81, Japan).

### Immunoprecipitation and western blotting

Immunoprecipitation and western immunoblotting was performed as follows: 20  $\mu$ g of cell lysate protein from each sample was mixed with 2 $\times$  SDS loading buffer containing dithiothreitol (DTT), was heated at 100°C for 10 min, and was then resolved by SDS-PAGE. Proteins were transferred to polyvinylidene difluoride (PVDF) membrane and blocked with 5% nonfat dry milk in phosphate-

buffered saline (PBS) with 0.02% v/v Tween-20. Anti-RON and anti-p65 or phospho-p65 polyclonal antibodies were used as the primary antibodies. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin G (IgG) was used as the secondary antibody. For detection of  $\beta$ -actin, membranes were stripped with 100 mM 2-mercaptoethanol, 62.5 mM Tris-HCl (pH 6.7), and 2% SDS for 30 min at 55°C and were reprobed with mouse anti- $\beta$ -actin antibody for 1 h at 25°C; the primary antibody was detected with HRP-conjugated goat anti-mouse secondary antibody. The phospho-RON (p-RON) level was determined by immunoprecipitation. After immunoprecipitation of cellular RON protein with Zt/G4 using protein A/G agarose beads (Santa Cruz Biotechnology, CA, USA), phosphorylation of RON was detected by western blot analysis using the P-Tyr-100. Protein-bound beads were washed 4 times with PBS, the protein was resuspended in 1 $\times$  SDS sample buffer (50 mM Tris-HCl [pH 6.8], 2% SDS, 0.1% bromophenol blue, 10% glycerol, and 100 mM DTT) and then analyzed by SDS-PAGE and western blotting.

### Statistical analysis

All experiments were performed in triplicate. The test results are expressed as the mean  $\pm$  standard deviation. Single-factor analysis of variance and linear correlation analysis were conducted using the statistical software, SPSS 10.0; P < 0.05 indicated a significant difference.

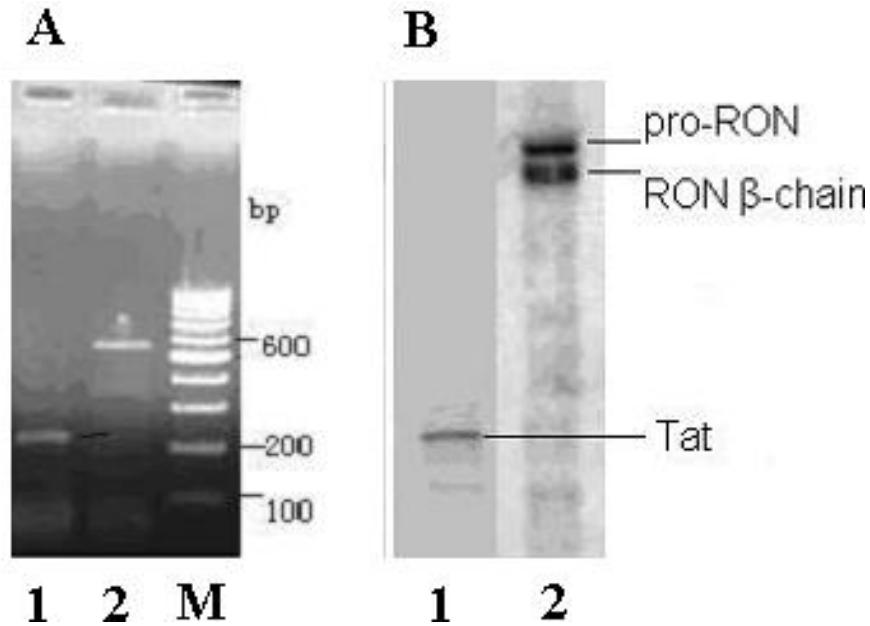
## RESULTS AND DISCUSSION

### RON and MSP levels in the serum of HIV-positive patients

The RON level was considerably higher in infected patients with HAART treatment than in infected patients without HAART treatment. The levels in both these groups were higher than the level in the normal group. In addition, the difference in the levels between them was significant. The MSP level was considerably higher in the normal patients than in the infected patients with HAART treatment and in those without HAART treatment. The level in both the infected groups was lower than the control level. The difference between them was significant (Table 1).

### Examination of the effects of the HIV-1 Tat protein on the expression and phosphorylation of RON receptor tyrosine kinase

We detected the expression of RON and Tat in



**Figure 1.** RT-PCR and immunoprecipitation for the detection of RON and Tat expression in 293T/RON and 293T/Tat-His cell lines; (A) The total RNA was isolated from 293T/RON and 293T/Tat-His cells using the TRIzol reagent (Invitrogen). RT-PCR was carried out using 2  $\mu$ g of total RNA with the Superscript kit (Invitrogen). Oligo-primers specific for the extracellular region of RON were used. The PCR products were electrophoresed in a 1% agarose gel. Lane 1: Tat (258 bp); Lane 2: RON (589 bp); Lane M: Marker; (B) the 293T/RON and 293T/Tat-His cells were lysed in a lysis buffer on ice for 30 min. The cellular protein was mixed and incubated with the antibody overnight with constant shaking. Cell lysates were analyzed by SDS-PAGE. Proteins transferred to the PVDF membranes were probed with the mAbs. Lane 1: Tat expression in 293T/Tat-His cells detected with antibody to HIV-1 Tat; Lane 2: RON expression in 293T/RON cells after photoshop.

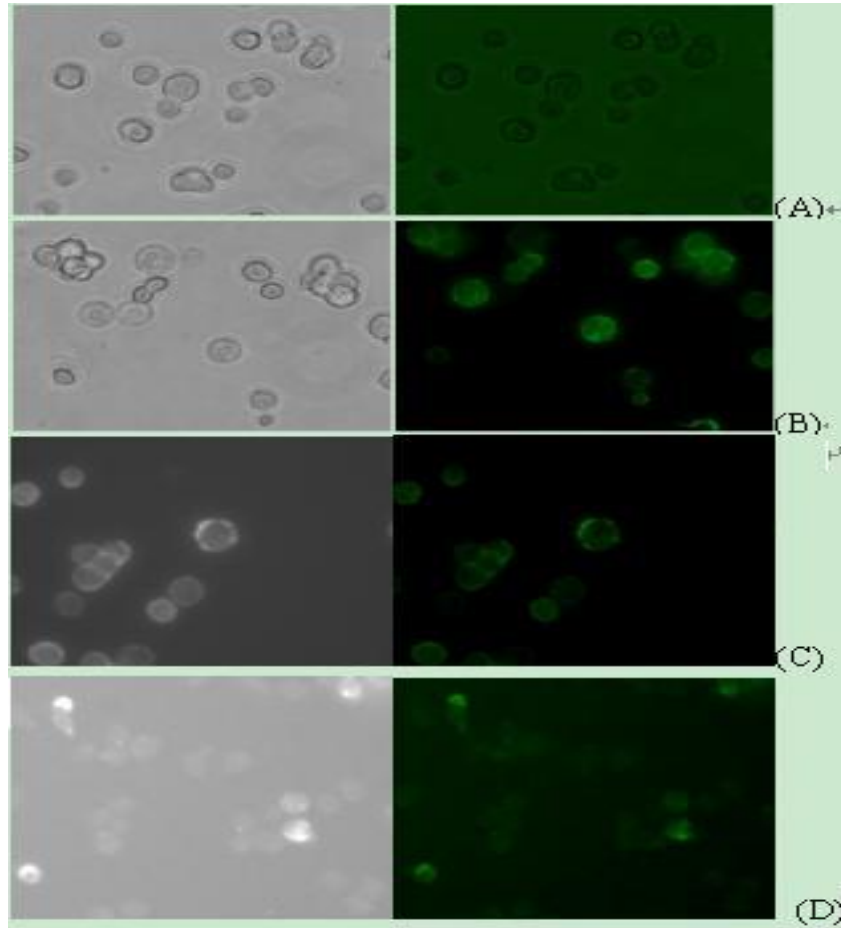
293T/RON and 293T/Tat cells (Figure 1). Cells were visualized with FITC-labeled antibody (2F2) for RON (green). The fluorescence intensities were determined using the LCS software associated with the Olympus IX81 microscope. The cellular location and distribution of RON were determined by image analysis. Immunofluorescence staining of RON revealed that RON was localized in the cyto-membrane. Flow cytometry (FCM) analysis of 293T/RON cells revealed that RON was expressed in 99.6% of the cells. Further, RON expression was unchanged in 293T/RON cells co-cultured with H9/HTLV-IIIB cells, while the expression was found to be considerably lower in 293T/RON cells stimulated with 500 ng/ml Tat, as determined by FCM (Figure 2). Western blot analysis revealed that the HIV-1 Tat protein can down-regulate RON expression as well as RON phosphorylation, which is induced by MSP.

Henderson et al. (2008) reported that RON initiates signaling pathways that negatively regulate HIV-1 transcription in monocytes/macrophages and that, HIV-1 suppresses RON function by decreasing protein levels in the brain to assure efficient replication. Furthermore, HIV-1 infection would compromise the ability of RON to protect against inflammation and consequent central

nervous system damage (Lee et al., 2004).

HIV-1 Tat is a potent transactivator of transcription and is essential for viral replication; it has been shown to activate the nuclear factor (NF)- $\kappa$ B signaling pathway to transactivate gene expression (Kalantari et al., 2008). Several studies have also shown that Tat represses the transcription of multiple genes such as those encoding major histocompatibility class I molecules. As an important component of HIV-1, Tat protein can regulate the inflammatory and cell signaling pathways in different ways. RON, MSP protein as the newly discovered immune factor, is involved in Tat-induced regulation of inflammatory response and cell signaling pathways such as the NF- $\kappa$ B signaling pathway. This aspect has not been well studied.

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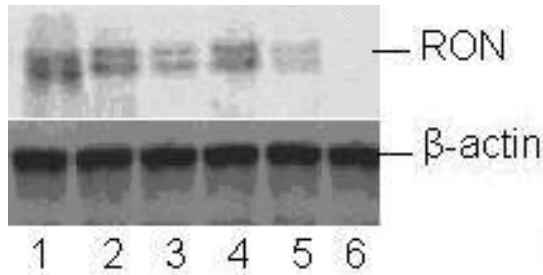


**Figure 2.** Immunofluorescence analysis of RON expression in 293T/RON cells stimulated with different factors. Cells were visualized with FITC-labeled antibody (2F2) against RON (green). The fluorescence intensities were determined using the LCS software associated with the Olympus IX81 microscope. At least 100 cells were analyzed for each test condition ( $n = 3$ ) from 2 separate experiments. The cellular location and distribution of RON were determined by image analysis; (A) Negative controls showed no staining (0%); (B) immunofluorescence staining of 293T/RON cells for RON revealed that RON was localized in the cell surface; FCM analysis of 293T/RON cells revealed that RON was expressed in 99.6% of the cells; (C) FCM analysis of 293T/RON cells co-cultured with the H9/HTLV-III B cells for 24 h revealed RON expression in 98.9% of the cells; (D) FCM analysis of 293T/RON cells stimulated with 500 ng/ml Tat for 24 h revealed RON expression in 56.9% of the cells.

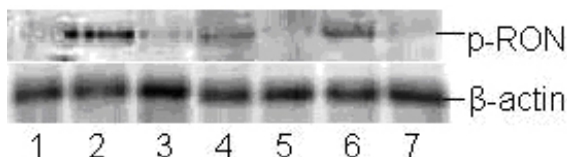
been well studied thus far (Lee et al., 2004). Considering the results of Henderson et al. (2008), we think that our results are reasonable. The samples we tested differed from their samples. In AIDS encephalitis, RON expression is very low. This is because at the end stage of AIDS, the immune system of patients collapses. However, our samples were obtained from patients who met the treatment standard, and their immune system could be partly reconstructed. The conclusion from our result is the same as that of Henderson et al. (2008). Some inhibition relationship must exist between RON and HIV-1 or the HIV-1 Tat protein.

Furthermore, the HIV-1 Tat protein is a key HIV-1

transactivator, which is essential for the control of HIV-1 gene transcription (Aksenov et al., 2009). HIV-infected cells release Tat, and extracellular Tat can then be taken up by uninfected cells (Charnay et al., 2009; Ensoli et al., 2009; Meredith et al., 2009). After Tat was found to act as a transcription factor, it was demonstrated to possess other abilities as well. Finally, evidence has been provided to prove that exogenous Tat is involved in AIDS-associated pathologies such as Kaposi's sarcoma and HIV-associated dementia (Huigen et al., 2004). These abilities together accelerate the progression of AIDS. Several studies have demonstrated that extracellular Tat can stimulate the expression of immunoregulatory



**Figure 3.** Western blot analysis for detection of RON expression in 293T/RON cells stimulated with different factors. 1, 293T /RON cells with no stimulation; 2, stimulation with  $10^5$  HIV particles for 24 h; 3, stimulation with 500 ng/ml Tat protein for 24 h; 4, stimulation with  $10^5$  HIV particles for 48 h; 5, stimulation with 500 ng/ml Tat protein for 48 h; 6. 293T cells with no stimulation (negative control).



**Figure 4.** Immunoprecipitation for the detection of p-RON in 293T/RON cells stimulated with different factors. 1, 293T /RON cells with no stimulation; 2, stimulation with MSP for 10 min; 3, stimulation with MSP for 10 min and 500 ng/ml Tat protein for 2 h; 4, stimulation with HIV-1 for 24 h; 5, stimulation with MSP for 10 min and 500 ng/ml Tat for 24 h; 6, stimulation with MSP for 10 min and HIV-1 for 24 h; 7, 293T cells with no stimulation (control).

cytokines such as TNF, IL-2, and TGF (Ehret et al., 2001; Sastry et al., 1990).

Deregulation of the expression of cellular genes causes abnormalities, which may be involved in AIDS pathogenesis and in the development of AIDS-associated disorders. MSP also inhibits LPS-induced production of inflammatory mediators, including inducible NO and prostaglandins. These suppressive effects are mediated by RON-transduced signals that block LPS-induced enzymatic cascades that activate NF- $\kappa$ B pathways. (Wang et al., 2002; Wang et al., 2003) Thus, MSP and RON are potential regulators that control macrophage activities during infection *in vivo*. Abnormal accumulation and activation of RON and MSP could play a critical role *in vivo* in the progression of immune deficiency.

In order to determine how the HIV-1 Tat protein affects the expression and functional changes of RON and MSP, we have successfully constructed the plasmids pcDNA3.1 (+)/Tat and pDR2/RON *in vitro* using genetic engineering methods (Figure 2). We used HIV-1 and HIV-1 Tat protein to stimulate the 293T/RON cells. The results showed that the HIV-1 Tat protein can down-regulate the expression of RON; the down-regulation effect over time was significant. However, the down-regulation effect of HIV-1 was

not obvious. This is reasonable because receptors for other HIV proteins are not present on the surface of 293T cells. Thus, these findings show that Tat, not other HIV-1 proteins, influences the expression of RON in 293T/RON cells. This is probably related with the membrane penetration activity of the Tat protein.

Further, we examined the mechanisms underlying the effects of Tat on the NF- $\kappa$ B signaling pathway, and we determined whether this regulates inflammatory gene activity in 293T-RON cells exposed to HIV-1 Tat proteins. Our results showed that the HIV-1 Tat protein can down-regulate the expression and phosphorylation of RON unexpended with NF- $\kappa$ B signaling pathway (Figure 3 and 4) and that Tat can abnormally activate a small number of 293T/RON cells. These findings imply that Tat may play a role in modulating the immune responses induced by other scatter factor, thus providing a permissive environment for HIV-1 infection. Further indepth mechanism studies are being designed to further explore this concept.

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#### Abbreviations

**AIDS**, Acquired immunodeficiency syndrome; **HIV**, human immunodeficiency virus; **RON**, Recepteur d'origine nantais; **MSP**, Macrophage-stimulating protein; **LPS**, lipopolysaccharide; **RPMI**, Roswell Park Memorial Institute medium; **DMEM**, Dulbecco's modified Eagle's medium; **ELISA**, enzyme-linked immunosorbent assay; **FCS**, fetal calf serum; **WHO**, World Health Organisation; **HAART**, highly active antiretroviral therapy; **RT-PCR**, reverse transcriptase-polymerase chain reaction; **SDS-PAGE**, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; **BCA**, bicinchoninic acid; **DTT**, dithiothreitol; **PVDF**, polyvinylidene difluoride; **HRP**, horseradish.

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