Influenza virus non-structural protein 1 inhibits the production of interferon β of alveolar epithelial cells upon the infection of influenza A H1N1

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Abstract. Influenza A affects a large population worldwide. Influenza virus evades immune responses via various mechanisms, including through the modification of the immune microenvironment. Influenza virus non-structural protein 1 (NS1) encoded by the virus genome inhibits type I interferon (IFN) signaling pathways, which is essential for viral clearance. However, the precise mechanisms of NS1-mediated immune suppression remain poorly understood. The results of the present study demonstrated that mice infected with NS1-expressing influenza A H1N1 virus had lower expression levels of IFNβ in the lung. In addition, it was revealed that the human alveolar epithelial A549 cell infected with influenza virus A H1N1 produced antiviral IFNβ. The production of IFNβ during infection was demonstrated to be a self-dependent autocrine process. A549 cells transfected with H1N1 NS1 lost the ability to produce IFNβ upon H1N1 infection or IFNβ stimulation. NS1 inhibited the expression of IFN receptors. Furthermore, NS1 inhibited the activation of signal transducers and activators of transcription (STAT)1 and STAT2, as well as the consequent IFNβ production. These data indicate that NS1 serves an important role during virus evasion by affecting the production of IFNβ via multiple mechanisms.

Introduction

Influenza viruses are a class of highly contagious pathogen affecting both human and animals, which result in seasonal epidemics and occasional pandemic worldwide. Regardless of the great achievements in the modern medicine, the therapeutic strategies for influenza virus infection are not very effective. Annually, influenza viruses cause infection in about 10% of the human population, and induce more than 250,000 cases of death (1). The reasons for this suboptimal situation are that influenza virus can evade the immune surveillance by antigenic shift and/or altering the microenvironment (2). Therefore, understanding the mechanisms of influenza virus-induced immune evasion is a key to improve the treatments and outcome of influenza virus infection.

Upon influenza virus infection, the innate immune system utilizes germline gene-encoded receptors called pattern recognition receptors (PRRs) to detect the conserved molecular patterns of viruses to initiate antiviral responses, the activation of which results in the production of type I interferon (IFN, mainly IFNα/β), pro-inflammatory cytokines and chemokines (3). Type I IFNs, especially IFNβ, are critical for the clearance of viruses. IFNβ binds its receptors called IFNα receptor (IFNAR1) and IFNAR2 to activate and phosphorylate Janus kinase (JAK)1 and tyrosine kinase (TYK)2. Phosphorylated JAK1 and TYK2 then in turn phosphorylate IFNAR1/2 at specific tyrosine residues in the intracellular part. This phosphorylation leads to the recruitment and the consequent phosphorylation of signal transducers and activators of transcription (STAT)1 and STAT2. A heterodimer formed by phosphorylated STAT1 and STAT2 recruits the IFN-regulatory factor (IFR)9 to generate the IFN-stimulated gene factor (ISGF)3, which results in the production of IFNβ (4). Thus, STAT1 and STAT2 are essential for the IFNβ-mediated autocrine expression of IFNβ and the consequent antiviral responses.

Many viruses have the abilities to increase their survival in the host by inhibiting the production of IFNs (5). The mechanisms of virus-induced inhibition of IFNs include disruption of IFN expression, interruption of IFN signaling pathways, and blockage of IFN-associated effectors. The non-structural protein 1 (NS1) of influenza A viruses inhibits the effects of IFN mainly via blocking the expression of IFNs to facilitate influenza virus evasion (6). To the best of our knowledge, NS1 downregulates the expression of IFNs by various mechanisms,
including: i) Inhibiting the virus detector retinoic acid-inducible gene (RIG-I), the initiator for antiviral responses, which leads to the production of IFNβ (7); ii) NS1 inhibits the activation of nuclear factor-xB (NF-xB) and IRF3 (8), which are required for the production of IFNs (9); and iii) NS1 binds cellular cleavage and polyadenylation specificity factor (CPSF)30 to account for the accumulation of unprocessed mRNAs in the nucleus, thus mRNA production including IFN mRNAs in the cytoplasm is then inhibited (10).

However, the inhibitory roles of NS1 against the IFN production have not been fully understood. It has been reported that NS1 of the laboratory-generated influenza A H1N1 PR/8/34 interacts with RIG-I to block the production of IFNβ (11). NS1 of the influenza A H5N1 was demonstrated to inhibit the activation of STAT1 and STAT2 to decrease the expression of IFNβ (12). However, whether NS1 of the influenza A H1N1 inhibits the production of IFNβ in human alveolar epithelial cells by affecting the phosphorylation of STAT1 and STAT2 and the loop of IFNβ-mediated autocrine process of IFNβ production remains elusive. Here, we report that NS1 inhibits the production of IFNβ in influenza A H1N1 infected alveolar epithelial cells by downregulating the production of IFNAR1 and 2, as well as inhibiting the activation of STAT1 and STAT2.

Materials and methods

Reagents. Anti-IFNβ neutralizing antibodies and the corresponding isotype control antibodies (goat IgG control) were purchased from R&D Systems (Minneapolis, MN, USA). Recombinant human IFNβ-a (rhIFNβ-a) was purchased from PBL Assay Science (Piscataway, NJ, USA). Antibodies for STAT1, p-STAT1, STAT-2, and p-STAT2 were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Fluorescein isothiocyanate (FITC)-conjugated anti-human IFNAR1 antibodies and matched isotype antibodies were from Clinisciences (Nanterre, France). FITC-conjugated anti-IFNAR2 antibodies and isotype antibodies were purchased from Sino Biological Inc. (Beijing, China). Anti-influenza NS1 and anti-β actin antibodies were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA).

Cells and cell culture. The human alveolar epithelial cell line A549 and the Madin-Darby canine kidney (MDCK) cell lines were purchased from American Type Culture Collection (Manassas, VA, USA). Cell lines A549 and MDCK were cultured in Dulbecco's Modified Eagle's medium (DMEM) and DMEM: Nutrient Mixture F12 (DMEM/F12, both from Thermo Fisher Scientific, Inc., Waltham, MA, USA), respectively, containing 10% fetal bovine serum, and 1X Penicillin-Streptomycin (both from Thermo Fisher Scientific, Inc.) in a humidified incubator with a 5% CO2 atmosphere at 37°C.

Virus and cells treated with viral infection. Influenza virus A/Nanjing/1110/2010 (H1N1, referred to as H1N1 below), which lacks NS1 expression, was isolated and cultured. We also isolated and cultured NS1-expressing influenza virus A/Nanjing/1108/2010 (H1N1, referred to as H1N1-NS1 below). The virus culture and storage procedures were approved by local institutions. Influenza virus A H1N1 was grown in 9-day-old embryonated chicken eggs. Virus allantoic fluid (VAF) was collected 48 h after inoculation, and then titrated with the standard haemagglutination tests (HA) and plaque assays in MDCK cells (13). A549 cells were cultured in 6-cm Petri dishes with the density of 5x10^4 per plate 12 h before viral infection. A549 cells were infected with H1N1 inocula in VAF at a multiplicity of infection of 1. Virus inocula were suctioned after 1 h of co-culture, and then 3 ml of serum-free DMEM medium was added into petri dishes following removing the virus inocula. Infected A549 cells were incubated at 37°C and 5% CO2 for the designed periods.

Virus-infected animal model. Animal experiment protocols were reviewed and approved by the Ethics Committee of Jinling Hospital, Nanjing University School of Medicine (Nanjing, China). Wild C57BL/6 mice (6- to 8-week-old) were purchased from (the Jackson Laboratory, Bar Harbor, ME, USA). Mice were maintained in a specific pathogen-free environment. Mice were infected intratracheally with 1x10^4 plaque forming units (PFU) of influenza A virus under isoflurane anesthesia. Lungs were collected 48 h post-infection for histological assessment staining (hematoxylin and eosin), flow cytometry, and real-time q-PCR analysis.

Transfection. Cells (2x10^5) were transfected with Lipofectamine LTX (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instruction. Briefly, cells were planted in 6-well plates 24 h prior to the transfection procedure. Plasmid DNA or empty vectors and transfection reagents were mixed in serum-free medium and incubated for 30 min at room temperature. Transfection complexes were added into the corresponding wells of culture plates.

Real-time q-PCR. A549 cells were harvested and used for RNA isolation using a commercial RNeasy mini kit (Qiagen, Valencia, CA, USA). Total RNA (2 µg) was reverse-transcribed to cDNA using an RT-PCR kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Real-time analysis for IFNβ, IFNAR1, IFNAR2, and control gene HPRT was performed by SYBR-Green Master mix (Thermo Fisher Scientific, Inc.) following the manufacturer's instructions. Relative mRNA expression values were normalized according to a housekeeping gene HPRT. The fold change of mRNA expression was calculated using the formula: 2^ΔΔCt of gene / ΔΔCt of HPRT. The specificity of each amplicon was checked by analyzing the corresponding melting curve. The sequences of primers used in real-time PCR were listed in Table I.

Western blot analysis. Whole-cell lysates were obtained in 30 µl of lysis buffer (1% Triton X-100, 0.5% Nonidet P-40, 10 mM Tris-HCl, 150 mM NaCl, pH 7.4, 1 mM EDTA, 1 mM EGTA, 0.2 mM phenylmethylsulfonyl fluoride). A total of 50 µg of protein was resolved in sodium dodecyl sulfate-polyacrylamide (SDS) gel electrophoresis and transferred to nitrocellulose membranes. Membranes were blocked in 5% skim milk for 1 h at room temperature. Then membranes were incubated with the primary antibodies at the optimized titrations at 4°C overnight. After incubation with horseradish
Table I. Primer sequences for quantitative real-time PCR.

| Target gene | Sequence (5′-3′) |
|-------------|------------------|
| Ifn-β       | F: TGGGAGGCTTGAATACGCTC | R: TCCTTGGCCTTCAGGTAATGC | | |
| Ifnar1      | F: CACTGACTGTATATTGTGAAAGGC | R: CACTCTACTGGAAGAGTTTAAGTG | | |
| Ifnar2      | F: ATTTCCTGCTCATTATCAT | R: ACTGAACACCTTGTTGTCC | | |
| HPRT        | F: GCCACCTTTGCTTTCTTGG | R: AAGCAGATGCGCCACAGA | | |

F, forward; R, reverse.

peroxidase-conjugated secondary antibodies, the bands were visualized by using enhancing chemiluminescence system (Amersham Biosciences, Pittsburg, PA, USA). Densitometry analysis was performed with Image J software.

Flow cytometry. Lungs were collected 48 h post-infection. Single cell suspensions were obtained by using a commercial lung cell isolation kit (Miltenyi Biotec, Inc., Auburn, CA, USA) according to the manufacturer's instructions. Leukocytes were stained with antibody-conjugated CD45-APC antibodies (eBioscience, San Diego, CA, USA).

Cells with different treatments were collected by trypsin digestion. Cells were stained with FITC-conjugated anti-human IFNAR1 or IFNAR2 antibodies at the designated dilutions. Corresponding isotype antibodies were used to indicate the background fluorescence. Cells were analyzed using a commercial ATTune Flow cytometer (Applied Biosystems, Foster City, CA, USA). The data were analyzed by Kaluza software 1.3 (Beckman Coulter, Brea, CA, USA).

Statistical analysis. Data were presented as mean ± SEM (standard error of the means). The difference between two groups was analyzed using a two-tailed student's t-test. The statistical significance between more than two groups was measured by ANOVA followed by Bonferroni's post hoc tests. Statistical analysis was performed by GraphPad Prism 5 Windows Edition (GraphPad Software, La Jolla, CA, USA). A P-value of less than 0.05 was considered statistically significant.

Results

Mice infected with H1N1-NS1 virus had fewer infiltrated neutrophils and decreased levels of IFNβ in the lung. First, we checked the effects of H1N1 and H1N1-NS1 viruses in a mouse influenza A infection model, respectively. Mice were intratracheally infected with influenza viruses as described. Forty-eight hours after infection, mice were sacrificed to collect lungs. As demonstrated in Fig. 1A, mice infected with N1N1 had infiltrated leukocytes in lungs, whereas mice treated with H1N1-NS1 viruses showed mild leukocyte infiltration, although histological damage was present. Then, we measured leukocyte infiltration by flow cytometry. The proportion and absolute number of CD45+ leukocytes in the lung were calculated. As shown in Fig. 1B, mice infected with H1N1-NS1 had decreased proportions and numbers of CD45+ leukocytes in the lung, compared to mice treated with H1N1, indicating an impaired leukocyte recruitment in the lung of mice infected with NS1-expressing H1N1 viruses. As IFNβ is a key IFN for the virus defense, we also measured the mRNA levels of IFNβ in the lung of infected mice (Fig. 1C). Not surprisingly, mice infected with H1N1-NS1 viruses had a lower level of IFNβ in the lung compared to mice treated with H1N1, suggesting NS1 protein inhibits the production of IFNβ during influenza A infection. These data show NS1 is an inhibitor for anti-virus immune responses.

A549 cells infected with H1N1 produce IFNβ via an IFNβ-dependent autocrine mechanism. Human alveolar epithelial cell line A549 was infected with H1N1 (1/64 HA units). Non-infected cells (vehicle-treated) were used as control cells. Forty-eight hours after infection, cells were thoroughly washed by PBS and collected. Purified RNA was obtained using a commercial RNA isolation kit as described in the Materials and methods. The levels of Ifnβ mRNA were measured by real-time PCR and normalized to the housekeeping gene HPRT. As shown in Fig. 2A, A549 cells infected with H1N1 had significantly increased levels of Ifnβ mRNA compared to non-infected cells. As it has been reported that IFNβ has autocrine effects on cells producing itself, we questioned whether the upregulation of Ifnβ mRNA was an autocrine process. An anti-human IFNβ neutralizing antibodies or matched isotype control antibodies were administered in culture media. Infected A549 cells treated with anti-IFNβ neutralizing antibodies (5 μg/ml) in the medium expressed significantly decreased levels of Ifnβ mRNA compared to infected cells incubated with isotype control antibodies (Fig. 2B), suggesting IFNβ itself is involved in this process. Then, we used recombinant human IFNβ (rhIFNβ1) supplementation (100 IU/ml for 15 min) in culture media. As demonstrated in Fig. 2C, rhIFNβ1a administration increased Ifnβ mRNA levels. These data indicate that cells infected with H1N1 produce IFNβ, and IFNβ acts with its receptors for further expression of IFNβ via an autocrine mechanism.

NS1 inhibits the production of IFNβ in A549 cells upon the infection of H1N1. NS1 of influenza A virus exerts anti-inflammatory effects to facilitate virus evasion by interfering the IFNβ signaling pathways (14). However, the precise mechanisms underlying NS1-interfered IFNβ signaling pathways have not been fully understood. We explored the effects of H1N1 NS1 on IFNβ production. A549 cells were infected with either H1N1 or H1N1-NS1 viruses. Not surprisingly, A549 cells infected with H1N1-NS1 lost the ability to produce Ifnβ mRNA (Fig. 3A). Then, we asked whether NS1 could inhibit IFNβ-mediated IFNβ production. A549 cells transfected with either empty vectors or those with H1N1 Ns1 complementary DNA were stimulated with IFNβ or vehicles. NS1-expressing A549 cells produced lower levels of Ifnβ mRNA compared to NS1-null A549 cells in responses to the treatment of rhIFNβ1a (Fig. 3B), suggesting NS1 inhibits the autocrine process of IFNβ expression.
NS1 inhibits the upregulation of IFNAR1 and IFNAR2 upon the infection of H1N1. Next, we explored mechanisms underlying NS1-mediated IFNβ inhibition. It is possible that NS1 affects many levels of IFNβ signaling pathways. We firstly examined the effect of NS1 on the receptors of IFNβ, which are IFNAR1 and IFNAR2. A549 cells were transfected with Ns1 vectors or empty vectors. Cells were infected with H1N1 24 h after transfection. Cells were then collected 48 h after the infection process. The expression of Ifnar1 and Ifnar2 were assessed by real-time PCR. As shown in Fig. 4A and B, in response to viral infection, both Ifnar1 and Ifnar2 mRNA levels were elevated in NS1-lacking cells, however NS1-expressing cells had decreased levels of mRNAs for these two genes. These data suggest the upregulation of receptors for IFNβ is part of antiviral immunity, and NS1 inhibits this upregulation to build a microenvironment that favors virus survival. The protein levels of IFNAR1 and IFNAR2 were measured using western blot analysis. As demonstrated in Fig. 4C, H1N1 infection increased the expression of IFNAR1 and IFNAR2, while NS1-expressing cells had lower levels of IFNAR1 and IFNAR2, suggesting NS1 inhibits the upregulation of IFNAR1 and IFNAR2 upon viral infection.

We also used flow cytometry to examine the surface expression of IFNAR1 and IFNAR2 of A549 cells, as surface receptors are functional. In parallel to the results of real-time q-PCR and western blot analysis, NS1-harboring A549 cells had a decreased proportion of IFNAR1- and IFNAR2-expressing cells upon the infection of H1N1 compared to cells lacking NS1 (Fig. 4D-F). We infected A549 cells with H1N1 or H1N1-NS1 viruses, and observed similar results as experiments using...
transfected cells (data not shown). These data indicate that NS1 inhibits the expression of IFNβ receptors at both mRNA and protein levels.

NS1 inhibit the phosphorylation of STAT1 and STAT2 to block the production of IFNβ. Then we examined the downstream molecules of IFNβ receptors. It has been well described that binding between receptors and type I IFNs activates and phosphorylates STAT1 and STAT2 to produce type I IFNs via an autocrine manner (15,16). We assessed the levels of phosphorylated STAT1 and STAT2 in NS1-null and -expressing A549 cells upon IFNβ incubation. A549 cells were transfected with vectors of Ns1 using the same strategies described above. Cells were incubated with or without the presence of rhIFNβ1a in media 24 h after transfection. By the end of incubation, cells were thoroughly washed and collected. Total proteins were obtained as described in Materials and methods. The interested proteins and the corresponding phosphorylated ones were checked by western blot analysis. The representative images of western blot analysis results were shown in Fig. 5. In the steady statuses, neither STAT1 nor STAT2 were activated. However, upon the administration of IFNβ in the medium,
NSI-null cells showed strong phosphorylation of STAT1 and STAT2. The strong phosphorylation of STAT1 and STAT2 was decreased in NS1-expressing A549 cells, suggesting NS1 is a negative regulator of the activation of STAT1 and STAT2.

Discussion

In this study, we demonstrated the effects of H1N1 NS1 on the secretion of IFNβ in A549 cells upon influenza A H1N1 infection by affecting the production of receptors for IFNβ and the activation of STAT1 and STAT2 that are essential for IFNβ secretion. The production of IFNβ is essential for anti-virus inflammation and clearance. The physiological process of producing IFNβ is a positive feedback loop via an autocrine mechanism. Upon viral infection, host cells detect the molecular patterns of virus by PRRs and initiate anti-virus responses including the production of IFNβ. IFNβ itself increases the surface expression of receptors, named IFNAR1 and IFNAR2. IFNβ then binds IFNAR1 and IFNAR2 to trigger the activation of receptors and the subsequent downstream molecules.
NS1 protein affects IFN production following production of IFN. We observed that NS1 protein downregulates the levels of IFNAR1 and IFNAR2 to inhibit the binding between IFN and receptors. We also observed that NS1-expressing cells present lower levels of p-STAT1 and p-STAT2 in the existence of IFNβ, which lead to the activation of IRF9 and the following production of IFNβ. These data explain that NS1 protein affects IFNβ production upon viral infection in the levels of ligand-receptor interaction and activation of transcription factors.

In agreement with previous publications, influenza A NS1 functions as an immune suppressor during responses against viruses with a structure-dependent manner (19). In a study using primary differentiated human tracheobronchial epithelial cells infected with influenza A virus bearing a truncated NS1 mutation (20), results showed lung epithelial cells infected with NS1 mutant virus had less viral replication and more type I IFN production, compared to cells infected with virus having functional NS1. Moreover, dendritic cells infected with NS1 mutant viruses demonstrated increased levels of activation and enhanced abilities to prime naïve T cells with the comparison to dendritic cells incubated with viruses having intact NS1. These data indicate NS1 has an important role to inhibit type I IFN production and dendritic cell activation to favor virus replication.

Type I IFNs are critical for anti-virus responses. Type I IFNs inhibit viral replication and invasion via the production of hundreds of genes including pro-inflammatory cytokines and chemokines that are required for the clearance of virus and virus-infected cells (21). Additionally, Type I IFNs promote the differentiation and maturation of dendritic cells, which present processed viral antigens along with the major histocompatibility complex to effective T cells. In addition, type I IFNs increase co-stimulatory molecule expression to facilitate the activation of virus-specific CD4+ and CD8+ T cells (21).

Influenza A NS1 protein is a series of peptide with the length between 215 and 237 amino acids, which composes of two mainly functional domains: N-terminal RNA-binding domain and C-terminal effector domain (19). NS1 protein favors virus evasion via various mechanisms, among which inhibiting the expression of IFNs is the most important one. It has been observed that NS1 inhibits the activation of IRF3 and NF-κB that are needed for the production of IFNs (8). RIG-1 recognizes and binds the PAMP in viral RNA, and subsequently activates the transcription for IFNs (22). NS1 can block the effects of RIG-1 in producing IFNβ (7). NS1 binds CPSF30 that is required for the 3′ end processing of pre-mRNA (23). As a result, unprocessed mRNAs accumulate in the nucleus, and the production of mRNAs in cytoplasm is inhibited, including mRNAs for IFNs (10). In the current study, for the first time, we extend the understanding of inhibitory roles of NS1 on IFN production. NS1 can inhibit the production of IFNAR1 and IFNAR2 that are required for the autocrine production of IFNs. NS1 also blocks the activation of STAT1 and STAT2, which are important transcription factors for the expression of IFNβ.

In conclusion, NS1 is an essential player during virus evasion by inhibiting the production of IFNβ in host cells. In the future, therapeutic strategies targeting NS1 will improve the outcome of influenza A H1N1 infection.

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