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Influenza A virus lacking the effector and C terminal domains of NS1 protein induces cytokines associated with high pathogenicity in mice

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Summary. – Non-structural NS1 protein of influenza A virus counters host antiviral defences by antagonizing the interferon response. The C-terminal effector domain suppresses the host response and is associated with the pathogenicity of the virus. To better understand the regulatory role of the C-terminal domain, we used reverse genetics system to generate NS1-truncated virus (NS80) and compared the cytokine profiles in the lungs of mice infected with the NS80 mutant and with the control virus A/WSN/33 (WSN). The NS80 virus was attenuated and the viral titer in the lungs was about 25 times lower than viral titer of control A/WSN/33. Mice infected with NS80 virus exhibited more severe clinical symptoms and 2 mice died 6 days post infection. NS80 virus activated retinoic-inducible gene (RIG)-1-like receptor signaling pathway more strongly than control WSN virus and mice infected with NS80 virus exhibited a greater abundance and more diverse cytokine profile. Infection with NS80 virus induced the expression of the following factors: pro-inflammatory cytokines (IL-1α, IL-1β, TNF-α, IL-16), interferons (IFN-α and IFN-ε), chemokines (CCL2, CCL11, CXCL1, CXCL5, CXCL10, CXCL11 and CXCL13), matrix metallopeptidase 9 (MMP-9), metallopeptidase inhibitor 1 (TIMP-1), macrophage colony-stimulating factor (M-CSF), and vascular cell adhesion protein 1 (VCAM-1). All these cytokines are associated with viral pathogenicity. Our data show that attenuation of the virus should not be directly linked with pathogenicity.

Keywords: influenza virus; NS1 protein; cytokines; interferon; pathogenicity

Introduction

Influenza A virus (IAV) belongs to the family *Orthomyxoviridae*. Its genome contains eight segments of negative-sense, single-stranded RNA, each embedded into ribonucleoproteins by multiple copies of the viral nucleoprotein and the viral RNA-dependent RNA polymerase. The approximately 13 kb genome encodes up to 18 proteins. The eighth segment encodes the non-proteins structural NS1 and NEP/NS2. The multifunctional NS1 protein antagonizes host antiviral responses and contributes to efficient viral replication during infection. The NS1 protein consists of a N-terminal RNA binding domain, a short linker, an effector domain, and a C-terminal 'tail' (Kerry *et al*., 2011; Ayllon *et al*., 2012). The RNA-binding domain binds in particular dsRNAs, allowing the virus to inhibit the α/β interferon response (Qian *et al*., 1995; Donelan *et al*., 2003). This domain interacts with the eukaryotic translation initiation factor 4G1, facilitating preferential translation of viral mRNA over host mRNAs (Aragón *et al*., 2000). A recent study shows that RNA-binding domain plays an essential role in the virus replication cycle, notably in

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Abbreviations: CPSF30 = polyadenylation specific factor 30; IFN = interferon; IRF = IFN regulatory factor; IAV = influenza A virus; M-CSF = macrophage colony-stimulating factor; MMP-9 = matrix metallopeptidase 9; NF-κB = nuclear factor-kappa beta; PAI-1 = plasminogen activator inhibitor type 1; p.i. = post-infection; PFU = plaque-forming units; RIG-1 = retinoic acid-inducible protein 1; TIM-1 = T cell immunoglobulin and mucin domain; TLR = toll-like receptor; TIMP-1 = metallopeptidase inhibitor 1; VCAM-1 = vascular cell adhesion protein 1

expression and translation of viral mRNAs (Trapp *et al*., 2018). The effector domain binds the cellular cleavage and polyadenylation specific factor 30 (CPSF30), which is essential for processing of the 3' end of cellular pre-mRNAs. Sequestration of CPSF30 blocks processing of interferon (IFN)-β and cytokine-independent antiviral pre-RNAs in the nucleus to form mature RNAs in the cytoplasm (Nemeroff *et al*., 1998). In addition, the sequestration of dsRNA and/or interaction with the RNA helicase RIG-1 prevents activation of the transcription factors IRF3, nuclear factor-kappa beta (NF-κB) and cJun/ATF2, and hence the synthesis of type I IFNs (Talon *et al*., 2000a; Wang *et al*., 2000; Smith *et al*., 2001; Ludwig *et al*., 2002). Shortening of the linker region of the avian H1N1 influenza virus NS1 protein increases its replication and pathogenicity in chickens (Trapp *et al*., 2014). The C-terminal region is essential for regulation of antiviral responses, may interact with PDZ-binding protein(s) and can modulate pathogenicity through alternative mechanisms (Jackson *et al*., 2008; Anastasina *et al*., 2015).

Previous studies have demonstrated that retinoic acid-inducible protein 1 (RIG-1) and toll-like receptors 3 and 7 (TLR3 and 7) play important role in the recognition of influenza viruses (Guo *et al*., 2007; Malathi *et al*., 2007; Mibayashi *et al*., 2007; Sabbah *et al*., 2009; Thomas *et al*., 2009). Signaling through both RIG-1 and TLR3 is important for IFN induction (Wu *et al*., 2015). Stimulation of RIG-1 activates specific pathway that leads to activation of NF-κB, which is crucial for the induction of inflammatory cytokines (Fitzgerald *et al*., 2003). Excessive inflammatory response together with viral virulence can increase disease severity (Kuiken *et al*., 2012).

It is still not easy to evaluate all possible functions of the NS1 protein since some functions of NS1 that influence virus pathogenicity and host range are strain- or cell-type-specific (Pu *et al*., 2010; Yan *et al*., 2017). In the present study, we have investigated the impact of the effector domain and C-terminus of NS1 protein on cytokine profile induced in infected Balb/c mice. The results provide novel insights into the pathology of influenza A infection and may have applications for the improvement of influenza vaccine and therapy.

Materials and Methods

Cells and viruses. MDCK (ATCC CCL34), Vero (ATCC CCL81) and A549 (ATCC CCL185) cells were grown in Dulbecco's modified Eagle medium (DMEM) containing 10% calf serum in a 5% CO_2 atmosphere at 37°C. Viruses prepared by reverse genetics system (WSN and NS80) were propagated in Vero cells.

Plasmid construction and generation of viruses. Virus with mutated NS1 protein was generated using the plasmid-based reverse genetics system, a kind gift of Dr. Y. Kawaoka (Hoffmann *et al*., 2000). The stop codon was introduced to NS1 sequence of pHW 188-NS plasmid at nucleotide position 263 by inverse PCR using back-to-back primers: NS80 forward 5'-CGATGAG GCACTTAAAATGACCTAAATGGCCTCTGTACCTGCGTCG-3' and reverse 5'-GATTCTTCTTTCAGAATCCGCTC-3' and Phusion Site-Directed Mutagenesis Kit (Molecular Biology). The nucleotide sequence of resulting construct was confirmed by sequencing. DNA was purified using Pure Yield™ Plasmid Maxiprep System (Promega). The viruses WSN and NS80 were prepared as previously described (Švančarová and Betáková, 2018). All viruses were sequenced to confirm the presence of wanted mutations.

Cytokine array . Female BALB/c mice ages 4–5 weeks were purchased from Faculty of Medicine, Masaryk University (Czech Republic). A total of 32 mice in two groups of 16 mice were anesthetized with Zoletil (50 mg/kg) and inoculated intranasally with 10^3 plaque-forming units (PFU) of WSN or NS80 virus (40 µl). Mice were monitored daily and humanely sacrificed at the experimental endpoint, which was defined as weight loss exceeding 25% of the original body weight. For the cytokine assay, 4 mice were sacrificed by cervical dislocation and the lungs were aseptically collected at 0, 2, 4 and 8 days post-infection (p.i.). Organ homogenates were pooled together, and aliquots were stored at -80°C. Lung tissue homogenates (100 µl) were lysed and the protein concentration was determined using the Pierce BCA Protein assay kit (Thermo Fisher Scientific, Illinois, USA). Chemokine expression in lung tissue lysates was assessed using the Proteome Profiler Mouse XL Cytokine array kit (R&D Systems, Minnesota, USA). Signal intensities on autoradiography films were quantified using Gene Tools software (Syngene). The expression levels of cytokines were normalized to the expression level of the reference spots. The assay was performed in duplicate to ensure reproducibility of the results.

Determination of virus titres. Cellular debris was removed from lung tissue samples by centrifuging at 160 x g for 10 min at 4°C and the supernatants were used for virus quantification. The viral titers are expressed as PFU/ml of lung homogenate in MDCK cells using a plaque assay as previously described (Svetlíkova *et al*., 2010). The results are expressed as the mean of two independent experiments.

Semi-quantitative RT-PCR. Total RNA from the lungs was extracted using SV Total RNA Isolation System (Promega, Wisconsin, USA). 400 ng/µl of RNA was reverse transcribed using random hexa-nucleotide primers and MuLV reverse transcriptase (Finnzyme, Thermo Fisher Scientific, Massachusetts, USA). Viral transcripts were detected by semi-quantitative PCR. The primers targeting β-actin, IFN-α, IFN-β, IFN-γ, IFN-λ, and RIG-1 were previously described (Švančarová *et al*. 2015). The sequences of other primers used in this study were as following: MDA5 forward 5'-GAGAGTGATGACGAGG CCAG-3' and reverse 5'- ACTGGGAAAGTGCGTAGGTG-3'; NF-κB forward 5'-GCA GGGTCACTCGATTTCAT-3' and reverse 5'-TCAA GACACTGCACCTGAGC-3'; IRF3 forward 5'-GTCCTCAGATCTG

GCTATTG-3' and reverse 5'-GCTTCAGTGGATTTTC TTGG-3'; IRF4 forward 5'-TCACTTGTTCGTGGAGCATC-3' and reverse 5'- TCTGG AGTCAGTGCTGATGG-3'; IRF7 forward 5'- CCACGGAAAATAGG GAAGAA-3' and reverse 5'-CATAGGGTTCCTCGTAAACA-3'; and IFN-ε forward 5'-CAGCAGCCTG TGAGTCCTCACCAG-3' and reverse 5'-GGTTTTCCTCCCAAATGC CCATAGTC-3'.

The intensity of the PCR products was determined using Gene Tools image analysis software (Syngene). β-actin was used as an internal control to normalize the expression of the target mRNA levels between different samples.

Statistical analyses. Statistical analysis was performed by comparing the data obtained from two independent experiments. Significant differences of the values between the controls group (WSN) and NS80 were calculated using the unpaired Student's *t*-test. P-values <0.05 were considered to be significant. Statistical analysis was performed using Graph-Pad Prism software (http://www.graphpad.com/quickcalcs/ttest1.cfm).

Ethics statement. All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at the Biomedical Research Center SAS, Institute of Virology. The animals were treated according to the European Union standards and the fundamental ethical principles, including animal welfare requirements, were respected. All of the animal experiments were evaluated and approved by the State Veterinary and Food Administration of the Slovak Republic, Permit Number: 4370/13-221 and 1204/11-221.

Results

Attenuation of NS80 virus

The viruses WSN (control virus) and NS80 (NS1-truncated virus) have been generated by using reverse genetics system. NS80 virus underwent about 20 passages on Vero cells and NS gene was sequenced to exclude reversion of NS80 to wild type. To prove the attenuation of NS80 virus, we examined the multiple-cycle growth kinetics of NS80. MDCK and A549 cells were infected at an MOI of 0.01 and the virus titer in the supernatants of infected cells was measured at different times post infection by the plaque assay. The NS80 virus was more attenuated in MDCK cells than in A549 cells (Fig. 1).

Mortality and virus replication

To determine whether the introduction of the deletion results in any changes in the mortality and virus replication *in vivo*, mice were intranasally inoculated with 10^3 PFU of the respective virus. Balb/c mice infected with control WSN virus did not show any signs and symptoms of illness and exhibited maximum weight loss up to 13% (Fig. 2a). The mice infected with NS80 virus were

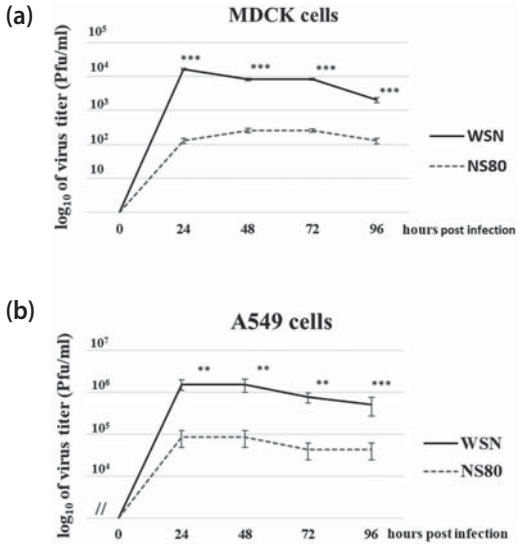

Fig. 1

Multi-step growth curves in MDCK and A549 cells
Confluent monolayers of Vero or A549 cells were infected with viruses at an MOI of 0.01 and incubated at 37°C. At different time points, virus titer in culture medium was determined by a plaque assay on Vero cells as described in Materials and Methods. The data shown represent the mean ± SD for three independent experiments. Statistically significant differences between WSN and NS80 are indicated as follows: $^{**}P < 0.001$ and $^{***}P < 0.0001$.

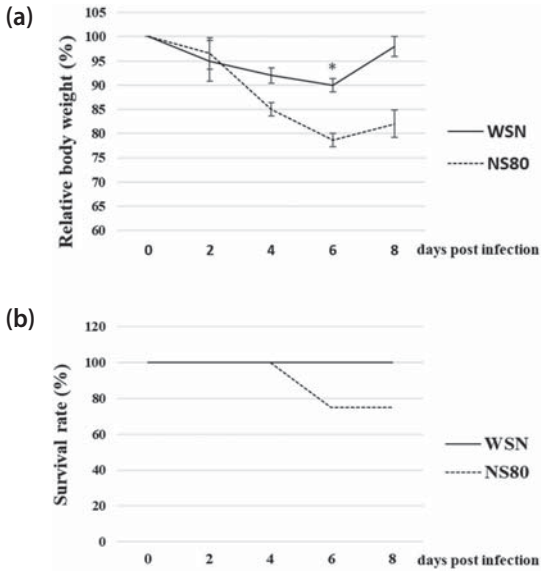

Fig. 2

Survival rate after infection with NS80 and WSN virus
BALB/c mice were infected with NS80 and control WSN viruses (10^3 PFU) as described in Materials and Methods. **(a)** Percentage body weight loss relative to the initial weights were recorded daily until 8 days p.i. Statistically significant differences between WSN and NS80 are indicated as follows: $^{*}P < 0.05$, $^{**}P < 0.001$ and $^{***}P < 0.0001$. **(b)** Survival rates (%) were calculated as the number of surviving animals / total number of animals x 100. n = 8 mice at each time point.

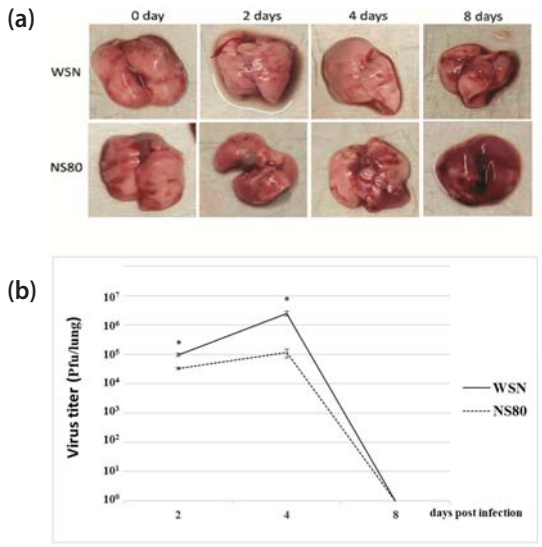

Fig. 3

Macroscopic changes and virus titer in the lungs
Mice were infected with NS80 and control WSN viruses as described in Materials and Methods. **(a)** The lungs were collected on days 2, 4, and 8 p.i. 0 day represents uninfected mice. **(b)** The virus titer was determined in the lung tissue homogenates as described in materials and methods. The values are the means of two independent experiments. Statistically significant differences between WSN and NS80 are indicated as *P <0.05.

lethargic and had bristled fur between 4–6 days p.i. They exhibited maximum weight loss of 22% and 2 mice even died (Fig. 2a,b). On the eighth day, the surviving mice did not show any signs and symptoms of illness.

Macroscopic changes in the lungs correlated with virus pathogenicity. Infection with viruses caused visible macroscopic changes in the lungs at 4 and 8 days p.i. (Fig. 3a). Other organs (such as heart, spleen, liver etc.) did not show visible signs of pathology. Naked-eye observation of lungs revealed that the most severe signs of damage were recognized in the mouse infected with NS80 virus at 8 days p.i. Minimal pathological changes were visible in the lungs infected with control WSN virus.

To ensure that the NS80 virus still remains phenotypically intact, the homogenates from the lungs were used for purification of RNA and NS gene was sequenced to confirm the presence of wanted mutations. The obtained data proved the stability of NS80 virus.

The virus titer was established in the lungs homogenates as described in materials and methods. Both viruses reached the maximal viral titer at 4 days p.i. and no virus was detected in the lungs at 8 days p.i. (Fig. 3b). In agreement with the results obtained *in vitro*, the NS80 virus was attenuated and the virus titer was about 25 times lower than the titer of control WSN virus.

Activation of RIG-1-like receptor signaling pathway

Since the NS1 protein suppresses the antiviral host defence by blocking the activation of IRF3, we investigated the induction of some genes important for the regulation of IFNs expression in response to viral infection. mRNA levels of selected genes in infected lungs were determined using semi-quantitative PCR assay at 0, 2, 4, and 8 days p.i. (Fig. 4a,b). NS80 virus activated RIG-1-like receptor signaling pathway more efficiently than control WSN virus. Higher expression of RIG-1 and MDA-5 mRNA resulted in higher expression of IRF3 and IRF7 mRNA in the lungs infected with NS80 virus at 4 and 8 days p.i. Consequently, the level of IFNs, especially IFN-α and IFN-ε, was effectively activated by NS80 virus. NS80 virus also induced higher expression of NF-κB and IRF4 mRNA than WSN virus.

Infection with NS80 virus significantly increased the expression of cytokines associated with pathogenicity

Different cytokine expression profiles were observed following infection with control WSN and NS80 viruses. The expression of 130 soluble mouse proteins in the lungs of mice infected with either virus were compared. Infection with WSN virus did not significantly influence the expression of the tested cytokines compared with uninfected mice (Fig. 5). On the other hand, infection with NS80 virus induced major changes in the expression of some cytokines. A significant decrease in the expression of IL-1α, TNF-α, CXCL10, CCL2, macrophage colony stimulating factor (M-CSF), and T cell immunoglobulin and mucin domain (TIM-1) was observed at 2, 4, and 8 days p.i. In contrast, an additional group of the cytokines, including IL-1β, IL-16, CXCL11, and vascular cell adhesion molecule 1 (VCAM-1) was highly induced by NS80 virus at 2 and 4 days p.i. Cytokines, CXCL5, plasminogen activator inhibitor type 1 (PAI-1) and matrix metallopeptidase (MMP)-9 were more abundant in the lungs of mice infected with NS80 virus, particularly at 2 days p.i. The increased level of chemokine CXCL13 was observed at 4 days p.i. The expression of these cytokines was significantly higher when compared to uninfected control at the same time points post-infection.

Discussion

It is believed that virus pathogenicity is directly proportional to the replication potential of the virus. It was already shown that deletion mutant NS80 was more attenuated on MDCK cells than mutants NS99 and NS124.

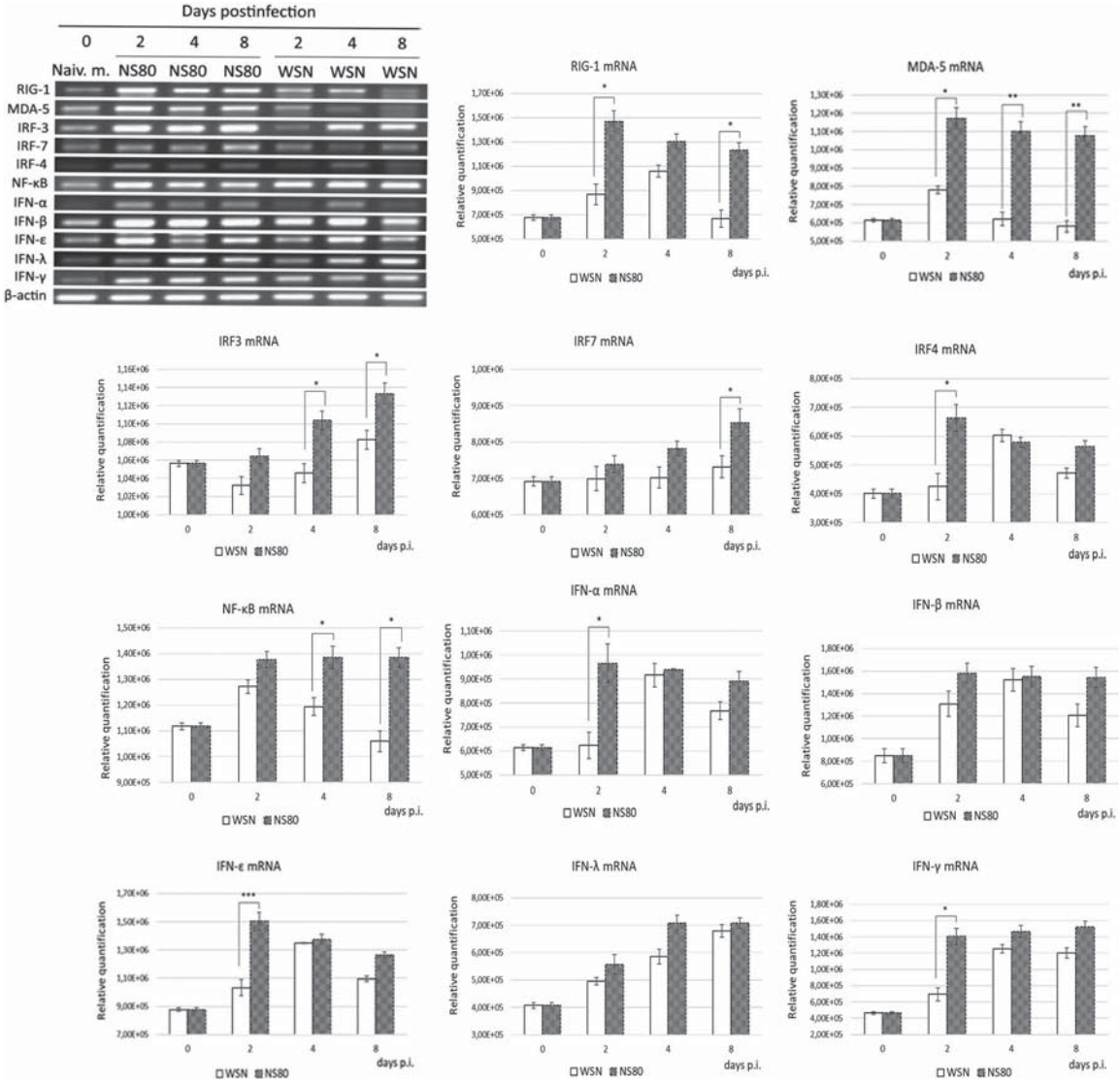

Fig. 4

Activation of RIG-1-like receptor signaling pathway

BALB/c mice were infected intranasally with NS80 and control WSN viruses (10^3 PFU). The lungs were collected at day 2, 4, and 8 p.i. The lung homogenates were used for assessment of mRNAs. The representative RT-PCR products **(a)** and quantitative relative levels of mRNAs were obtained **(b).** The expression values represent the mean of two separate experiments and are expressed as the mean ± SD. Statistically significant differences between WSN and NS80 are indicated as follows: $^{*}P < 0.05$; $^{**}P < 0.001$; $^{***}P < 0.0001$.

These viruses were much less virulent than the wild-type virus and replication in mice decreased with increasing length of the deletion (Egorov *et al*., 1998; Talon *et al*., 2000b; Ferko *et al*., 2004). We have shown that NS80 virus was attenuated on MDCK cells and only moderately attenuated on A549 cells. The viruses were grown and titrated in Vero cells. It was shown previously that Vero cells lack the capacity to produce their own IFN and are suitable for propagation of NS80 virus (Enemy and Morgan, 1979). The A549 cells produce a high level of MxA protein in response to IFN and that is why the virus titers are lower than in Vero cells (Files *et al*., 1998). The NS80 virus was also attenuated in Balb/c mice. In comparison with the control WSN virus, the viral titer was about 25 times lower in the lungs of mice infected with NS80 virus. Despite the fact that the replication of NS80 virus was much lower, the infected animals exhibited disease symptoms as lethargy, bristled fur, significant weight lost and 2 mice died at 6 days p.i. In order to verify our initial observation, we have performed several independent experiments and

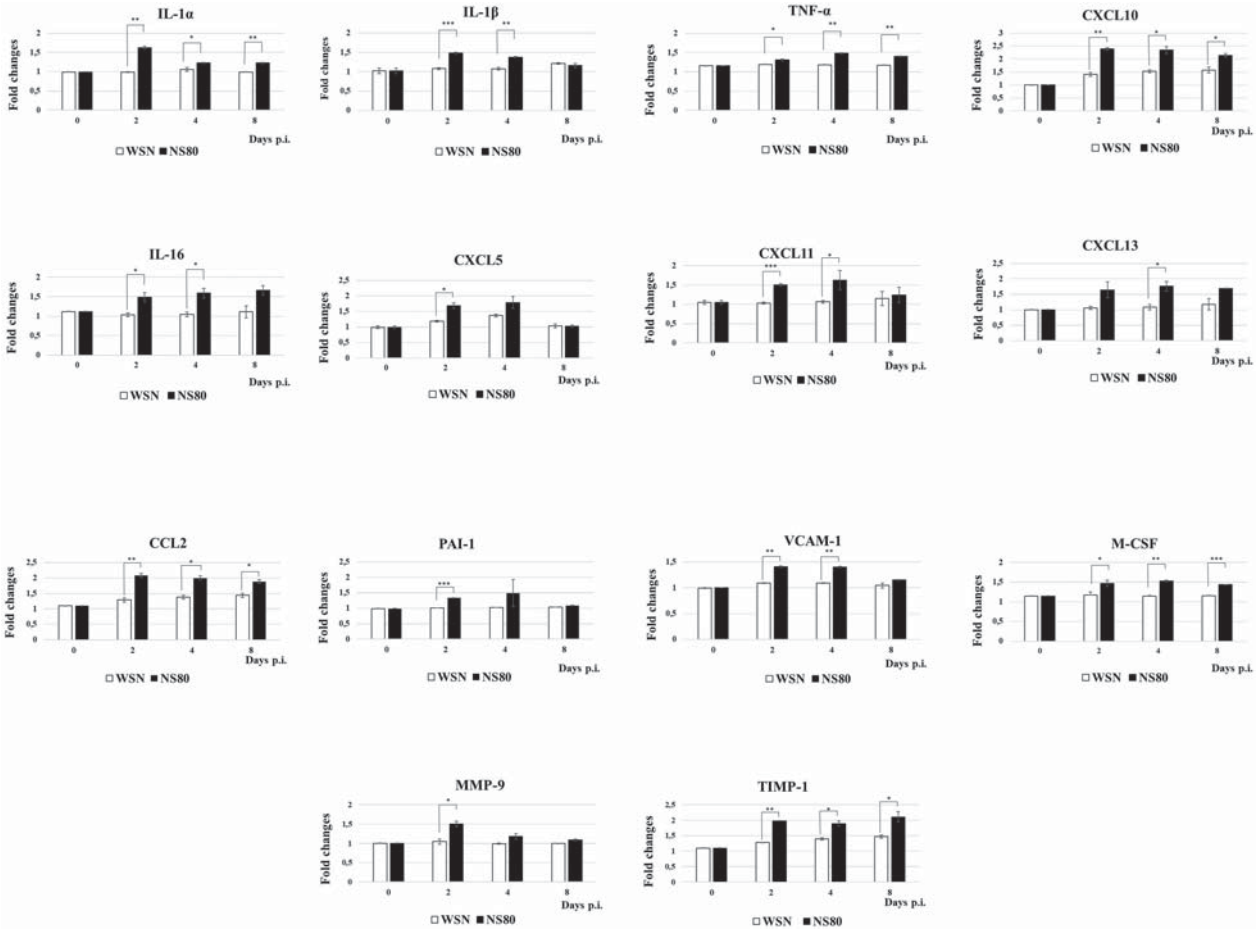

Fig. 5

Cytokines exhibiting increased expression in the lungs of the mice infected with NS80 virus

BALB/c mice were infected intranasally with NS80 and control WSN virus (10^3 PFU). The protein levels of cytokines were determined in the lungs harvested at 2, 4, and 8 days post infection. Day 0 represents uninfected mice. The value represents the mean of two separate experiments. Statistically significant differences between WSN and NS80 are indicated as follows: $^{*}P < 0.05$; $^{**}P < 0.001$; $^{***}P < 0.0001$.

used different amount of WSN and NS80 viruses. The mice infected with NS80 virus repeatedly showed disease symptoms and severe macroscopic changes of lungs (data not shown), in contrast with the mice infected with the same amount of control WSN virus.

The NS1 protein of influenza A virus regulates RIG-1-like receptor pathway and pathogenicity of the virus. It was not surprising that NS80 virus highly stimulated RIG-1 and MDA5 signaling cascades that led to higher activation of IRF3, IRF7, and NF-κB. IRF3 and IRF7 act as transcriptional factors for type I and type III IFNs (Fitzgerald *et al.*, 2003; Kotenko *et al.*, 2003). The levels of IFN-α/β mRNA increased immediately after infection with NS80 virus and stayed increased up to 8 days p.i. The importance of IFN-α/β and IFN-λ in response to influenza virus has been demonstrated in several contexts (Svetlikova *et al.*, 2010; Wu *et al.*, 2015). We have tested a newly described type I IFN, namely IFN-ε. IFN-ε is constitutively expressed in mucosal tissues, reproductive tissue and intestine and promotes clearance of viral infection in the lungs (Xi *et al.*, 2012). Our results suggest that IFN-ε plays an important role in controlling mucosal pathogens such as influenza virus.

The expression of IRF4 was also greatly induced by NS80 virus at 4 days p.i. IRF4 controls the magnitude of the $CD8^+$ T cell response to acute virus infection in a dose-dependent manner and even modest differences in IRF4 expression can dramatically influence the intensity and quality of the adaptive immune response (Nayar *et al.*, 2014). It would be worthwhile to further investigate how NS1 protein influences adaptive immune response.

Activation of RIG-1-like receptor pathway and production of IFNs influence the replication of NS80 virus. It is interesting that attenuated NS80 virus was more patho-

genic in Balb/c mice than control WSN virus. Comparison of cytokine profiles obtained from the lungs infected with NS80 and WSN viruses led as to suggestion that excessive immune response could be responsible for higher pathogenicity of NS80 virus. Activation of RIG-1-like receptor signaling pathway by NS80 virus resulted in induction of inflammatory cytokines, which are associated with higher pathogenicity of some viruses (de Jiong *et al*., 2006; Kash *et al*., 2006; LaGruta *et al*., 2007). The IFN-α and TNF-α can stimulate local inflammatory response and activate the chemokines and monokines that recruit monocytes, macrophages and neutrophils. Macrophages and monocytes newly recruited to the inflamed lungs can be subsequently infected by the virus (Baskin *et al*., 2009; Sanders *et al*., 2011). Induction of the chemokines CXCL5 and CXCL11 depended on the virus replication and levels of these chemokines went back to normal at 8 days p.i., when the viral titer was no more detectable in the lungs. On the other hand, the levels of cytokines CCL2, CXCL10, and CXCL13 were still very high after the virus clearance. Cytokine IL-16 and chemokines CCL2, CXCL11, CXCL13 are associated with disease severity after infection with avian and highly pathogenic human viruses (Cole *et al*., 1998; Qin *et al*., 1998; Huang *et al*., 2012; Davey *et al*., 2013; Betakova *et al*., 2017, Turianova *et al*., 2019). Overexpression of chemokines CCL2 and CXCL10 is linked with mortality (Lai *et al*., 2017; Turianova *et al*., 2019). Chemokine CXCL5 regulates neutrophil trafficking and activity. As a primary target of influenza virus, airway epithelial cells produce inflammatory cytokines upon infection (Huang *et al*., 2012). Changes in CXCL5 level have already been correlated with severity of influenza virus infection in mice and humans (Fu *et al*., 2016; Blackmore *et al*., 2017).

Li *et al*. (2013) have showed that M-CSF promotes the development of mature monocytes and tissue resident macrophages and M-CSF treated humanized mice exhibited an enhanced protection against influenza virus. The positive role of M-CSF was associated with increased level of pro-inflammatory cytokines (IL-6, TNF-α, and IL-1β). On the other hand, the increased level of TIMP-1, M-CSF, PAI-1, and MMP-9 correlated with inflammatory cytokine expression and lungs damage (Khuth *et al*., 2001; Hoffman *et al*., 2006). The overexpression of MMP-9 is associated with the damage of many organs and tissues and plays an essential role in the infection and in the host response to infection (Luplertlop *et al*., 2006; Muhammad *et al*., 2016). The MMP-9 a activates cycle that is one of the important mechanisms of multiple organ failure in severe influenza. A/PR/8/34 infection increased the levels of CCL2, MMP-9 and trypsin in serum and/or the lungs and heart (Takahashi *et al*., 2018).

Taken together, our results showed that virus NS80 influences NF-κB pathway leading to the upregulation of cytokines IL-16, CCL2, CCL11, CXCL-1, CXCL5, CXCL10, CXCL13, MMP-9, TIMP-1 and PAI-1. Excessively high activities of these cytokines are linked with tissue damage, spread of virus into other organs, and viral-induced diseases (Herold *et al*., 2015; Marro *et al*., 2016). Despite the lower viral titer, NS80 virus elicited a robust immune response, which worsened the severity of lung injury. Our results showed that attenuation of a virus should not be directly correlated with its pathogenicity.

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