Comparison of cytokine profiles induced by nonlethal and lethal doses of influenza A virus in mice

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Abstract. Influenza viruses are among the most common human pathogens and are responsible for causing extensive seasonal morbidity and mortality. To investigate the immunological factors associated with severe influenza infection, the immune responses in mice infected with nonlethal (LD0) doses of A/PR/8/34 (H1N1) influenza virus were compared with those of mice infected with a lethal dose (LD100) of the virus. The virus titer and activation of retinoic acid-inducible gene (RIG)-I-like receptor signaling pathways were similar in the mice infected with LD0 and LD100 at 2 days post-infection; however, mice infected with LD100 exhibited a greater abundance of cytokines and a more diverse cytokine profile. Infection with LD100 induced the expression of the following factors: Interleukins (ILs), IL-4, IL-7, IL-10, IL-11, IL-12p40, IL-13 and IL-15; inflammatory chemokines, C-C motif chemokine ligand (CCL)2, CCL3/4, CCL12, CCL17, CCL19; and lung injury-associated cytokines, leptin, leukaemia inhibitory factor, macrophage colony stimulating factor, pentraxin (PTX)2 and PTX3, WNT1-inducible-signaling pathway protein 1, matrix metallopeptidase (MMP)-2, MMP-3, proprotein convertase subtilisin/kexin type 9, and T cell immunoglobulin and mucin domain. Switching in macrophage polarization from M1 to M2 was evidenced by the increase in M2 markers, including arginase-1 (Arg1) and early growth response protein 2 (Egr2), in the lungs of mice infected with LD100. Since IL-12 and interferon-γ are the major T helper (Th1) cytokines, increased expression of interferon regulatory factor 4, IL-4, IL-10 and IL-13 promoted the differentiation of naive CD4+ T cells into Th2 cells. In conclusion, the present study identified key cytokines involved in the pathogenicity of influenza infection, and demonstrated that lethal influenza virus infection induces a mixed Th1/Th2 response.

Introduction

The influenza A virus is a major human pathogen. The severity of infection ranges from mild to severe and may even lead to death. Seasonal viruses can cause annual epidemics that result in 3-5 million cases of severe illness and 290,000-650,000 deaths (1).

In order to effectively diagnose and treat severe influenza virus infections, it is important to determine the infection status and the quality of the host immune response. Therefore, the identification of specific biomarkers that enable accurate diagnosis of the disease and have a prognostic value for predicting disease severity is required (2). Identifying biomarkers of influenza A infection is challenging, as many viral and cellular factors influence virulence, host response and pathogenicity of the virus. Hemagglutinin, neuraminidase, NS and polymerase PB1 and PB2 gene products serve a central role in determination of virulence (3-7). Abundant viral replication in the lungs and dissemination into non-respiratory tract tissues may result in increased pathogenicity and mortality (7,8). Both innate and adaptive immune responses are crucial for the control of influenza infection. The activity of innate and adaptive immune cells is coordinated by cytokines. However, an overactive or unbalanced immune response may result in the overproduction of cytokines that leads to severe inflammation, whereby an excessive number of neutrophils and mononuclear cells are recruited to the site of infection (9). Interleukin (IL)-6 and chemokines, C-C motif chemokine ligand (CCL)2, CCL4, C-X-C motif chemokine ligand (CXCL)8, CXCL9 and CXCL10, are associated with the pathogenicity of both avian (H5N1 and H7N9) and human (pdmH1N1 and H3N2) viruses (10-12). Chemokines, CCL2, CXCL8, CXCL9 and CXCL10, have been associated with mortality (13-17).

In present study, the cytokine expression profile in the lungs of mice infected with a nonlethal dose (LD0) of the A/PR/8/34 virus (H1N1) was analysed and compared with that of mice infected with a lethal dose (LD100) of the same virus. The aim of the present study was to identify the cytokines with altered expression patterns following infection with LD100 when
Materials and methods

Cells and viruses. MDCK (ATCC CCL-34) cells were grown in Dulbecco's modified Eagle's medium (Lonza) containing 10% fetal calf serum (HyClone Laboratories). Influenza virus A/PR/8/34 [H1N1] was cultured in 10-days-old fertile hen's eggs.

Female BALB/c mice (age, 4 weeks; body weight approximately 20 g) were purchased from the Faculty of Medicine, Masaryk University (Czech Republic). A total of 30 mice in two groups of 15 mice were anesthetized with Zoletil (5 mg/kg) intraperitoneal and inoculated intranasally with 10⁵ plaque-forming units (PFU; LD100) or 10⁷ PFU (LD0) of virus (40 µl). To ensure that the mice were fully anesthetized, the monitoring of rear foot reflexes was made before infection, and continual observation of respiratory pattern, mucous membrane colour and responsiveness to manipulations was made throughout the procedure. The mice were monitored daily and humanely sacrificed at the experimental endpoint. The criteria for euthanasia were established by using the total score for observation of possible animal distress. Weight loss exceeding 25% of the original body weight, decrease of appetite, weakness, shivering, depression and moribund state of animals were monitored twice per day. For the cytokine assay, all groups of 4 mice were sacrificed by cervical dislocation and the lungs were aseptically collected at 0, 2 and 4 days post-infection (p.i.). Experiment lasted for 8 days. Organ homogenates were pooled together, and aliquots were stored at -80°C.

Determination of virus titers. 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 were expressed as PFU/ml of lung homogenate in MDCK cells using a plaque assay as previously described (18). The results are expressed as the mean of two independent experiments.

Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR). Total RNA from the lungs was extracted using the SV Total RNA Isolation System (Promega). A total of 400 ng/µl RNA was reverse transcribed using random hexanucleotide primers and the MuLV reverse transcriptase (Finnzyme; Thermo Fisher Scientific). Viral transcripts were detected by semi-quantitative RT-PCR as previously described (18). The sequences of all additional primers used were as follows: Interferon regulatory factor (IRF)3, forward, 5'-ACC TG-3' and reverse, 5'-TCA TCA AGG TGG GAG CAT GG-3'; IRF7, forward, 5'-CCA CGG AAA ATA CTT GTT CGT GGA GCA TC-3' and reverse, 5'-TCT GGA GTC 5'-GCT TCA GTG GAT TTT CTT GG-3'; IRF4, forward, 5'-TCA CGG CTT TCG AT-3'; G-protein coupled receptor 18 (Gpr18), forward 5'-TGCCCATCGTACGGCAAA-3' and reverse, 5'-GTCGGAGATCTTCCAGGCAGG-3'. Formyl peptide receptor 2 (Fpr2) transcripts were detected by using primers as previously described (19).

The band 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 mRNA levels between different samples.

Cytokine array. Lung tissue homogenates (100 µl) were lysed and the protein concentration was determined using the Pierce BCA Protein assay kit (Thermo Fisher Scientific). Chemokine expression in lung tissue lysates was assessed using the Proteome Profiler Mouse XL Cytokine array kit (R&D Systems). Signal intensities on autoradiography films were quantified using Gene Tools software (Syngene). The expression levels of cytokines were normalized to the expression level of reference spots. The assay was performed in duplicate to ensure reproducibility of the results.

Statistical analysis. Statistical analysis was performed by comparing control group (noninfected mice) to infected mice or LD0 to LD100, respectively. Data were analyzed using the unpaired Student's t-test for data with two groups and ANOVA followed by Tukey's post hoc test was performed for data with ≥2 groups. P-values <0.05 were considered to be significant. Statistical analysis was performed using GraphPad Prism software (https://goodcalculators.com/one-way-anova-calculator/and http://www.graphpad.com/quickcalcs/ttest1.cfm).

Results

Mortality and virus replication. To determine the impact of viral infection on influenza-specific mortality, BALB/c mice were inoculated with either 10⁵ PFU or 10⁷ PFU of H1N1 virus. All animals rapidly developed severe signs of disease, including ruffled fur, huddling, lethargy and weight loss. Mice infected with LD0 exhibited body weight loss of up to 20% within 4 days of infection, followed by a gradual recovery by the end of the study (Fig. 1A). By contrast, animals infected with LD100 exhibited a >25% loss of body weight, and the aforementioned symptoms were more severe. These mice either died or were euthanized, and the death was recorded as infection-associated mortality (Fig. 1B).

The results demonstrated that macroscopic changes in the lungs were associated with virus pathogenicity. Naked-eye observations of the lungs revealed that the most severe signs of damage were identified in mice infected with LD100 at 4 days p.i. (Fig. 2A). Minimal pathological changes were observed in the lungs from mice in the LD0 group. In the same group, the maximum viral titer was observed at 2 days p.i. and these values were not altered over the subsequent 2 days (Fig. 2B). At 4 days p.i., the viral titer decreased and was undetectable at 8 days p.i. In LD100-infected mice, the viral titer increased up to 4 days p.i. The maximum viral titer in this group of mice was five times higher than mice in the LD0 group.

Activation of retinoic acid-inducible gene (RIG)-I-like receptor signaling pathway genes. The mRNA levels of
selected genes in the infected lungs were determined using a semi-quantitative PCR assay at days 0, 2 and 4 days p.i. As demonstrated in Fig. 3A and B, LD0 and LD100 doses of the virus induced similar expression alterations of RIG-I and Melanoma differentiation-associated protein 5 (MDA-5) mRNAs. LD100 induced significantly higher levels of RIG-I mRNA at 4 days p.i. No significant difference in IRF3 and NF-κB mRNA expression levels was observed. IRF7 mRNA levels were significantly increased in mice infected with lethal doses of the virus at 4 days p.i. (P<0.05). In addition, LD100 induced significant increases in IRF4 mRNA levels at 2 and 4 days p.i. (P<0.01 and P<0.05). Both viral doses induced similar expression alterations of interferon (IFN) mRNAs, except for IFN-β and IFN-ε. LD0 induced a significant decrease in the expression of vascular endothelial growth factor (VEGF) and osteopontin (OPN) was observed at 4 days p.i. An additional group of the cytokines, including CXCL5, fetuin A, myeloperoxidase (MPO), plasminogen activator inhibitor type 1 (PAI-1) and low density lipoprotein receptor (LDL-R) were highly induced by LD100 at 2 and 4 days p.i. The expression of these cytokines was significantly higher when compared to LD0 at the same time-points p.i.

**Cytokines induced by the lethal dose of virus only.** The most apparent differences between the cytokine levels induced by LD0 and LD100 doses of the virus are shown in Fig. 5. LD0 did not influence the expression of these cytokines, whereas LD100 increased their expression at 2 days p.i. These cytokines can be divided into the following three main groups of proteins: i) IFN-γ; ii) IL-4, IL-7, IL-10, IL-11, IL-12p40, IL-13, IL-15 and chemokines, CCL2, CCL3/4, CCL12, CCL17, CCL19; and iii) other immune response proteins, including leptin, leukaemia inhibitory factor (LIF), macrophage...
colony stimulating factor (M-CSF), pentraxin (PTX)2 and 3, WNT1-inducible-signaling pathway protein 1 (WISP-1), MMP-2, MMP-3, proprotein convertase subtilisin/kexin type 9 (PCSK9), T cell immunoglobulin and mucin domain (TIM-1).

Infection with a lethal dose of virus significantly increased the expression of M2 macrophage markers. Different transcriptional mRNA profiling in murine macrophages were observed in the lungs infected with with LD0 and LD100. Classically activated macrophages (M1) can be distinguished from alternatively activated macrophages (M2) by their relative expression of CD38, Gpr18, Fpr2 and Arg1, Egr2, respectively. As demonstrated in Fig. 6, no significant difference in CD38, Gpr18, and Fpr2 mRNA expression levels was observed at 2 and 4 days p.i. Arg1 and Egr2 mRNA levels were significantly increased in mice infected with lethal doses of the virus at 4 days p.i. These data indicate that infection with LD100 influences macrophage polarization in the lungs.

**Discussion**

Cytokines serve an important role in modulating the host immune response, clearing the virus, and healing any injury caused by the virus. Numerous factors influence the pathogenicity of the virus; thus, it is difficult to identify objective markers that can be used as an effective diagnostic tool and predict disease severity. It is hypothesized that virus...
pathogenicity is directly associated with the replication potential of the virus (7-11). Comparing the expression of cytokines induced by nonlethal and lethal doses of the virus may therefore identify the specific cytokines associated with high pathogenicity, which could be used as suitable biomarkers.

In the present study, the virus titer in mice infected with LD0 and LD100 was similar at 2 days p.i.; however, the cytokine expression profiles induced were different. We repeated this experiment several times and we obtained the same results. As it was expected, 100% mortality was observed within animals infected with LD100. In LD100-infected mice, the virus titer increased up to 4 days p.i. and none of these mice survived until 8 day p.i. Many factors as virus input, immune response etc. influenced the virus replication in the lungs. The most severe sign of damage was observed in the lungs of mice infected with LD100 at 4 days p.i. Unfortunately, we did not examine histological changes in the lungs after influenza infection. We would like to do it in the future.

No significant difference in the activation of RIG-I-like receptor signaling pathways was observed in the lungs of mice infected with nonlethal and lethal doses of influenza virus. Nevertheless, infection with LD100 induced the expression of a greater abundance and more diverse array of cytokines. The cytokine profile induced by LD100 was similar to the profiles previously observed in sera from patients with severe influenza infection and influenza virus-associated encephalopathy (11,12,20). IFN-γ, IL-4, IL-7, IL-10, IL-11, IL-12p40, IL-13 and IL-15 levels were increased only in the lungs of mice infected with a lethal dose of influenza. An interesting observation in our study was a switching in macrophage polar-

Figure 4. Cytokine expression in the lungs of mice infected with LD0 and LD100 doses of H1N1. Protein expression levels of cytokines in the lungs of infected mice harvested at 2 and 4 days p.i were determined. *P<0.05, **P<0.001 and ***P<0.0001 vs. mock; #P<0.05 and ##P<0.001, as indicated. LD0, 10^0 plaque-forming units; LD100, 10^3 plaque-forming units; H1N1, A/PR/8/34 virus; p.i., post-infection.
in the eradication of viral infection. M1 macrophages secrete chemokines, such as CXCL5, CXCL9 and CXCL10 (23). However, excessive pro-inflammatory responses can lead to uncontrolled tissue damage. Anti-inflammatory cytokines, such as IL-4, IL-10 and IL-13 are the main Th2 cytokines. In addition, increased IRF4 and IL-4 levels promote the differentiation of naïve CD4+ T cells into Th2 cells (24-28). Th2 lymphocytes release anti-inflammatory cytokines, IL-4, IL-5, IL-6, IL-10 and IL-13 (29). In the murine system, IL-10 downregulates the Th1 response (30). Subsequent activation of the complement system leads to neutrophil or eosinophil influx by IL-4 and IL-5 (31,32). In addition, Th2 cytokines, such IL-4 and IL-13, promote differentiation of M2 macrophages. M2 macrophages which produce high levels of IL-10 are generally thought to function as anti-inflammatory macrophages and serve key roles in the suppression of Th1 cell responses, wound healing and tissue repair (33-35). Overexpression of chemokines and the Th2 phenotype serve central roles in
influenza pathogenesis (36). The results of the present study therefore indicate that infection with LD100 invokes a mixed Th1/Th2 response.

In the current study, infection with LD100 induced overexpression of specific C-C motif chemokine ligand chemokines, which may contribute to disease pathogenicity and even death of the mice. Overexpression of chemokines CCL2, CCL17 and CCL19 is associated with the activity of M2 macrophages, and serves an important role in protective immune responses (37-39). CCL2 and CCL4 chemokines exert potent effects on the recruitment and degranulation of eosinophils and basophils, which may provide valuable insights into the immunopathogenesis of respiratory viral infections (40). CCL2 and CCL12 mediate acute lung injury induced by lethal influenza infection, as well as by the γ-herpesvirus or fungal (Aspergillus fumigatus) infections (41-45). However, the precise role of CCL19 in influenza infection remains unknown. CXCL2 and CCL3 chemokines mediate neutrophil infiltration during the early phase of infection and can serve an important role in the pathogenesis of the disease in the lungs (46,47).

In the current study, increased expression of CRP, LDL-R, MMP-9, MPO, PAI-1, OPN and VEGF correlated with increased inflammatory cytokine expression and lung tissue damage. These proteins are normally expressed during tissue damage or injury and serve important roles in tissue repair and lung immunity. Increased expression of these cytokines has been associated with the pathogenicity of virus infection. MPO-derived oxidants are pathogenic and can promote inflammation and tissue damage (48). Increased MMP-9 expression is associated with organ and tissue damage, and serves an essential role in infection and in the host response to infection (49). The MPP-9 cycle is an important mechanism underlying multiple organ failure during severe influenza infection. A previous study in mouse models demonstrated that H1N1 infection increased the levels of CCL2, MPP-9 and trypsin in serum and/or the lungs and heart (50).

In the present study, infection of mice with a lethal dose of the virus induced the expression of cytokines associated with severe lung injury and pathology. Increased expression of cytokines, including leptin, LIF, M-CSF, PTX2, PTX3, WISP-1, MMP-2, MMP-3, PCSK9 and TIM-1, correlated with extensive lung damage following lethal influenza infection. These proteins modulate infection response, inflammation and tissue repair. A previous study demonstrated that PTX2 reduces the severity of acute lung injury in an animal model and in humans and might be useful for a variety of disease therapy (51). In addition, the IL-10/CREB/WISP-1 signaling pathway has been shown to link innate immune activation to mucosal wound repair (52). MMP-2 and MMP-9 serve a protective role through pathogen clearance (53). However, increased expression of PCSK9 exacerbates multi-organ damage, and MMP3 contributes to the pathogenesis of acute respiratory distress syndrome (54,55). M-CSF promotes the differentiation and survival of macrophages, and preferentially induces anti-inflammatory M2 rather than pro-inflammatory M1 macrophages. Increased levels of C-MSF promote the development of a mixed Th1/Th2 immune response (56). However, it will be necessary to clarify the function of leptin, LIF, M-CSF, PTX2, PTX3, WISP-1, MMP-2, MMP-3, PCSK9 and TIM-1 in influenza pathogenicity. It is possible that these factors are released due to extensive tissue damage caused by viral replication, the increased expression of other cytokines or due to induction of a mixed Th1/Th2 immune response.

The balance between pro- and anti-inflammatory cytokines is essential for maintaining homeostasis in the respiratory system, and an imbalance has been implicated in the pathogenesis of granulomatous diseases and pulmonary fibrosis (57,58). By comparing the cytokine profiles obtained from the lungs of mice infected with nonlethal and lethal influenza doses, the authors of the present study hypothesize that lethal infection may induce a mixed Th1/Th2 response. A previous study demonstrated that Th2-dominated immune responses to influenza virus infection exacerbate lung tissue damage and delay viral clearance (59). In addition, the results of the current study indicate that immune cells, such as macrophages, eosinophils, neutrophils, monocytes, NK cells and basophils may serve a greater role in the pathogenesis of influenza infection than previously thought. However, the mechanisms by which Th1 and Th2 cells influence the inflammatory response during influenza virus infection require further investigation in future studies.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

TB conceived and designed the experiments, performed experiments, analyzed data and wrote the manuscript. LT and VL performed experiments and analyzed the data. DS performed experiments. AK analyzed data and performed the literature search.

Ethics approval and consent to participate

All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the 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 (approval nos. 4370/13-221 and 1204/11-221).

Patient consent for publication

Not applicable.
Competing interests

The authors declare that they have no competing interests.

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