Inducible Nitric Oxide Contributes to Viral Pathogenesis Following Highly Pathogenic Influenza Virus Infection in Mice

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Highly pathogenic influenza A viruses, including avian H5N1 viruses and the 1918 pandemic virus, cause severe respiratory disease in humans and animals. Virus infection is followed by intense pulmonary congestion due to an extensive influx of macrophages and neutrophils, which can release large quantities of reactive oxygen species potentially contributing to the pathogenesis of lung disease. Here, the role of nitric oxide (NO), a potent signaling molecule in inflammation, was evaluated following highly pathogenic influenza virus challenge in mice. We observed higher levels of NO in mice infected with H5N1 and 1918 viruses compared to a seasonal H1N1 virus. Mice deficient in inducible NO synthase (NOS2−/−) exhibited reduced morbidity, reduced mortality, and diminished cytokine production in lung tissue following H5N1 and 1918-virus challenge, compared with wild-type control mice. Furthermore, systemic treatment of mice with the NOS inhibitor NG-monomethyl-L-arginine delayed weight loss and death among 1918 virus infected mice compared to untreated control animals. This study demonstrates that NO contributes to the pathogenic outcome of H5N1 and 1918 viral infections in the mouse model.

Keywords. nitric oxide; H5N1; 1918; influenza.

The high mortality rates associated with H5N1 influenza virus infections in humans remains a public health concern. Since the reemergence of the H5N1 subtype in humans in 2003, there have been >600 documented cases of infection in humans worldwide, of which approximately 60% have been fatal [1]. Most H5N1 virus–infected patients initially present with clinical signs of influenza-like illness, such as fever and cough [2, 3]. In severe cases, a clinical course of progressive respiratory failure consistent with acute respiratory distress syndrome (ARDS) occurs. ARDS is a pulmonary condition that usually arises secondary to elevated levels of local inflammatory mediators that potentially complicate recovery [4–6]. H5N1 viruses are potent inducers of immune mediators, and elevated levels of serum cytokines among H5N1 virus–infected patients have been detected, which may contribute to the overall pathogenesis of the disease [7–9].

The H1N1 influenza virus of 1918 caused the worst influenza pandemic in recorded history [10]. Patients who died from infection during the 1918 pandemic had severe lung pathology marked by necrotizing bronchiolitis with intraalveolar edema, often characterized by extensive infiltration of polymorphonuclear neutrophils [11]. Similarly, in animal models of 1918 virus infection, ferret and mouse lungs exhibit necrotizing bronchiolitis and moderate-to-severe alveolitis with edema [12–14]. The histopathology associated with 1918 virus infection appears to be similar to that observed following H5N1 virus infection, which is characterized by severe alveolitis and a significant
increase in inflammatory infiltrates composed mainly of macrophages and neutrophils [2, 12, 14, 15]. These major effector cells of the innate immune response are capable of producing reactive oxygen and nitrogen species, including nitric oxide (NO), which is a powerful vasodilator and potent inflammatory cell chemoattractant [16, 17].

Reactive oxygen species may directly contribute to cell death in highly pathogenic influenza virus–infected lung tissue and exacerbate pathology caused by virus replication during acute H5N1 infection [18–20]. As one of many free-radical molecules produced by inflammatory cells during influenza virus infection, NO has been suggested to contribute to the disease process [21–24]. It is also notable in this context that lung disease in chronic asthmatics is often indicated by an increased NO concentration in exhaled breath and that patients can be treated by administration of steroids to reduce inflammation and thereby lower NO levels [25]. NO is produced by 3 different isoforms of the enzyme NO synthase (NOS), neuronal NOS (NOS1), inducible NOS (NOS2), and endothelial NOS (NOS3), which are expressed in different cell types. A previous study investigated the role of NO in laboratory-adapted influenza virus infection and showed that systemic inhibition of NO improves disease course in mice [23], but it remains unknown whether this reactive oxygen species plays an important pathogenic role in highly pathogenic influenza virus infections, such as those caused by the 1918 or H5N1 viruses. To understand potential nonviral causes of lung pathology and to identify potential therapeutic strategies, we studied the role of NO during highly pathogenic influenza virus infection in a small-animal model of influenza.

MATERIALS AND METHODS

Viruses
H1N1 influenza viruses used in the study, the reconstructed 1918 virus and the nonlethal seasonal A/Texas/36/91 (Tx/91) virus, were propagated in Madin-Darby canine kidney (MDCK) cells as described elsewhere [12]. A/Thailand/16/2004 virus (abbreviated H5N1) was propagated in 10-day-old embryonating hens’ eggs at 37°C for 24 hours. Pooled cell supernatant or allantoic fluid was clarified by centrifugation and titrated in a standard plaque assay, expressed as plaque-forming units (PFU). All experiments were performed in biosafety level 3 laboratories with enhancements (BSL3+) as outlined in the Biomedical Microbiological and Biomedical Laboratories manual [26].

Mouse Experiments
All animal research was conducted under the guidance of the Centers for Disease Control and Prevention’s Institutional Animal Care and Use Committee and in an Association for Assessment and Accreditation of Laboratory Animal Care International–accredited BSL3+ facility. Mouse 50% lethal doses (LD50) of viral stocks in 8–10-week-old female BALB/c mice (Jackson Laboratories, Bar Harbor, ME) were calculated as described elsewhere [12].

Mice (19–22 g) were inoculated intranasally with 100 PFU/50 µL of virus [27]. At the indicated times after inoculation, lungs (n = 3 per group) were removed and homogenized in 1 mL of phosphate-buffered saline (PBS) and titered for virus, cytokine levels, or NO amounts [28]. Homogenates were titrated for virus using a standard plaque assay on MDCK cells and expressed as the mean log10 PFU/mL. Statistical differences in virus titer between experimental groups were measured by the Student’s t test. Mice were observed and weighed daily for 14 days after inoculation. Mice survival data and mean time to death were analyzed using Kaplan-Meier and log-rank (Mantel-Cox) tests. Analysis of variance (ANOVA) was performed to evaluate differences in weight loss. For NO analysis, lung homogenates were microfiltered and assayed as described below.

NOS2-deficient (NOS2−/−; B6.129P2-NOS2 [tm1Lau]/J) and age-matched wild-type (WT) C57BL/6J mice were obtained from Jackson Laboratories. Eight NOS2−/− and 8 WT mice were inoculated intranasally with the 5 LD50 of 1918 or H5N1 virus. Three NOS2−/− mice and 3 WT mice were administered PBS to serve as controls. On day 5 after inoculation, 3 mice in each infection group were euthanized, and lungs were removed for virus titration and cytokine levels.

Treatment of Mice With the NOS Inhibitor NG-Monomethyl-L-Arginine (l-NMMA)
Ten BALB/c mice were treated with l-NMMA (Cayman Chemical, Ann Arbor, MI) following 5 LD50 1918 virus infection. l-NMMA is a nonselective competitive inhibitor of all 3 NOS isoforms [29], competing with the natural substrate, L-arginine. The K_i values for nNOS (rat), eNOS (human), and iNOS (murine) are approximately 0.18, 0.4, and 6 µM, respectively [30, 31]. Ten control mice were treated similarly with the inactive enantiomer D-NMMA (Cayman Chemical) or PBS. From days 3–7 after inoculation, mice received daily intraperitoneal injections of 2 mg/500 µL of l-NMMA or D-NMMA. Four mice per group were euthanized on day 4 after inoculation to measure virus replication in whole lungs. Uninfected mice (which received 50 µL of PBS intranasally) were similarly treated with l-NMMA or D-NMMA (4 mice per group) to control for the effects of drugs contributing to disease.

NO Analysis
In vivo, the NO molecule exists as either nitrate (NO3−) or nitrite (NO2−), and therefore the most accurate measurement of the NO concentration is to measure levels of both NO metabolites [32]. Total nitrate and nitrite (indicated as NO) in mouse lung tissue was measured in a colorimetric Griess
RESULTS

NOS2 Contributes to Morbidity and Mortality in Mice Infected With Highly Pathogenic 1918 and H5N1 Influenza Viruses

We began these studies with an assessment of physiologic NO levels and measurement of NO induction in mouse lungs following infection with H5N1, 1918, and the nonlethal Tx/91 (H1N1) influenza viruses. Three mice per group were euthanized on days 1, 3, 5, and 7 after inoculation, and lung homogenates were assayed for NO production (Figure 1A). During the first 5 days after inoculation, the levels of NO were generally similar among all infected groups. However, mice infected with the 1918 virus exhibited slightly higher NO levels through day 5 after inoculation. On day 7 after inoculation, a period that shortly preceded the death of the inoculated mice,

Cytokine and Chemokine Quantification

Clarified lung homogenates were analyzed using an enzyme-linked immunosorbent assay for the presence of mouse interleukin 6 (IL-6), interleukin 10 (IL-10), interleukin 12 (IL-12), and monocyte chemotactic protein 1 (MCP-1) in accordance with the manufacturer’s instructions (BD Biosciences, San Jose, CA). The BioPlex protein array system (Bio-Rad, Hercules, CA) was used to measure lung cytokines from NOS2−/− mice infected with 1918 or H5N1 virus, according to the manufacturer’s instructions. Statistical differences between groups were determined by the Student’s t test.

Isolation and Inoculation of Primary Lung Neutrophils

Primary lung neutrophils from 10 BALB/c mice were removed and cell suspensions prepared as described elsewhere [27]. Neutrophils were extracted from contaminating cells by positive selection (Ly6G/C+ beads) on magnetic columns (Miltenyi Biotech, Auburn, CA). Following elution from the separation column, neutrophils were washed twice with Roswell Park Memorial Institute medium (Gibco) containing 20% fetal calf serum and seeded at a cell density of 1 × 10⁶ cells in 12-well plates for 24 hours. Supernatants were sampled before infection and stored at −80°C for baseline NO levels. Neutrophils were exposed to the 1918 or Tx/91 virus at a multiplicity of infection of 2.0 in the presence of TPCK trypsin (1 μg/mL; Sigma Aldrich). Virus inoculum was kept in the wells, and supernatants were sampled 24 hours after inoculation to measure post-exposure NO production. Adjusted NO concentrations reflect pre-exposure titers subtracted from post-exposure titers.

Figure 1. Nitric oxide (NO) levels following infection with highly pathogenic influenza viruses. A, NO levels in lungs. BALB/c mice were infected with 100 plaque-forming units (PFU) of the influenza viruses shown. Three mice per time point were euthanized, and lungs were homogenized, processed, and analyzed for total NO concentration (shown as total μM of NO/40 μL homogenate). The mean NO level (±SD) in lungs of BALB/c mice infected with the 1918 virus exhibited slightly higher NO levels than those infected with H5N1 virus and 1918 virus infection groups, using the Student’s t test. *P < .05 between highly pathogenic (H5N1 and 1918) and seasonal Tx/91 virus–infected mice, using the Student’s t test. B, Virus replication in lungs of BALB/c mice following infection with 100 PFU of virus. Three lungs were titered for infectious virus by plaque assay, and differences between infection groups were analyzed using the Student’s t test. *P < .05 between H1N1 virus–infected mice and other infection groups. C, NO production from primary mouse lung–associated neutrophils. Following 24 hours of culture, supernatants were sampled for baseline levels of NO (μM/40 μL), and neutrophils were exposed to the indicated viruses (multiplicity of infection, 2). Values shown represent NO levels (μM/40 μL) from 3 individual experiments. *P < .02 between seasonal Tx/91 virus and 1918 virus infection groups, using the Student’s t test.
Nitric Oxide Exacerbates Influenza Virus Infection

NO levels among H5N1 virus– and 1918 virus–infected mice lungs were significantly higher than those among Tx/91 virus–infected mice. The kinetics of viral replication in the lungs was assessed to determine whether NO production in lungs was associated with viral replication. By day 5 after inoculation, all 3 viruses reached high titers (4.9–6.5 log$_{10}$ PFU/mL) in lung tissue, consistent with findings of earlier studies [27]. Virus replication was sustained and significantly greater in both H5N1 virus– and 1918 virus–infected groups through day 7 after inoculation, whereas Tx/91 virus was undetectable by this time ($P<.05$; Figure 2A).

Because of the heightened presence of neutrophils in the lungs of mice following infection with the lethal 1918 virus [27] and the known ability of these cells to produce an oxidative burst upon exposure to influenza virus [33], we measured the NO response of primary lung neutrophils to the 1918 virus. Total lung neutrophils were isolated from uninfected mouse lung tissue and exposed to either 1918 or Tx/91 virus (multiplicity of infection, 2). As shown in Figure 1C, the 1918 pandemic virus induced a stronger NO response from lung neutrophils as compared to seasonal Tx/91 virus–exposed cells. Our previous studies determined that primary mouse lung neutrophils do not support 1918, Tx/91, or even H5N1 virus replication in vitro (data not shown). However, the present results indicate that these innate immune cells detect and respond to a highly pathogenic influenza virus with an oxidative response that is stronger than the response to low-pathogenic influenza virus.

The high levels of NO observed among 1918 virus– and H5N1 virus–infected lungs further prompted us to investigate the role of NOS2 in highly pathogenic influenza virus infection. Here, NOS2$^{-/-}$ mice were challenged with H5N1 or 1918 virus, and weight loss, survival, and virus replication were measured and compared to findings in infected WT C57BL/6J mice (Figure 2). All of the infected NOS2$^{-/-}$ mice lost significantly less body weight on days 4–8 after inoculation ($P<.05$, by ANOVA), compared with infected WT mice, whereas control mice that received PBS in place of virus had an increase of approximately 4% in body weight over the observation period (Figure 2A). Although all NOS2$^{-/-}$ and WT mice eventually succumbed to both 1918 and H5N1 virus infection, NOS2$^{-/-}$ mice showed a significant delay in mean day of death, compared with infected WT mice (Figure 2B). The 1918 virus–infected NOS2$^{-/-}$ mice lived longer (mean time to death, 9 days) than 1918 virus–infected WT mice (mean time to death, 7.8 days; $P<.02$, by the log-rank Mantel-Cox test), and NOS2 deficiency also increased the mean time to death among H5N1 virus–infected mice (8.5 days for NOS2$^{-/-}$ mice and 7.5 days for WT mice; $P<.02$, by the log-rank Mantel-Cox test). The NOS2$^{-/-}$ and WT mice had comparable viral titers detected in the lungs on day 4 after inoculation (Figure 2C), indicating that the observed changes in mouse morbidity between mouse genotypes were not attributed to differences in virus replication.

**Systemically Inhibiting NOS Prolongs Survival and Reduces Morbidity in 1918 Virus–Infected Mice**

We sought to further investigate the role of NO in 1918 virus pathogenesis through systemic inhibition of all 3 isoforms of the NOS enzyme by pharmacological treatment with the compound L-NMMA. Mice were either given PBS or infected with 1918 virus intranasally and treated with L-NMMA or D-NMMA, the inactive stereoisomer of L-NMMA. As shown in Figure 3A, systemic inhibition of NOS with L-NMMA during 1918 virus infection resulted in a significant delay in weight loss. Morbidity in 1918 virus–infected NOS2$^{-/-}$ mice exhibited a delay in mortality following H5N1 virus or 1918 virus challenge. Eight wild-type C57BL/6J and 8 NOS2$^{-/-}$ mice were inoculated intranasally with 5 50% lethal doses of 1918 or H5N1 virus. A, Morbidity of infected and control mice are shown as mean percentage of original body weight. *$P<.05$ between infected NOS2$^{-/-}$ and WT mice on indicated days after inoculation, using analysis of variance. B, Mouse survival is shown, and survival data were analyzed using the Kaplan-Meier and Mantle-Cox methods to determine statistical significance of mean time to death (1918 NOS2$^{-/-}$, 9.0 days [*$P<.02$]; 1918/WT, 7.8 days; H5N1/NOS2$^{-/-}$, 8.5 days [*$P<.02$]; H5N1/WT, 7.5 days [$P<.02$]). C, Virus replication in the lungs. Three mice per group were euthanized on day 4 after inoculation, and lungs were titered for virus by standard plaque assay.
loss on days 4 and 6 after inoculation as compared to control animals (P < .05, by ANOVA), and the effect of this systemic NO inhibition on mouse morbidity could be observed as early as day 2 after inoculation. Furthermore, L-NMMA treatment had a significant impact on time to death among 1918 virus–infected mice as compared to control animals: 1918-infected mice treated with L-NMMA lived an average of 2 days longer than control mice (mean times to death, 9 days for 1918/L-NMMA [P < .001, by the log-rank Mantel-Cox test], 6.5 days for 1918/PBS, and 7.0 days for 1918/o-NMMA; Figure 3B). The enhanced survival of 1918 virus–infected mice following L-NMMA treatment, however, could not be attributed to a reduction in virus replication, as all mice exhibited similar levels of viral load in lung tissue at the peak of infection (Figure 3C).

In summary, consistent with results from experiments in NOS2−/− mice, systemic inhibition of NOS enzymes significantly reduced morbidity and mortality in 1918 virus–infected mice.

**Diminished Cytokine Production in the Lungs of Influenza Virus–Infected NOS2−/− and L-NMMA–Treated Mice**

It has been suggested that elevated levels of cytokines among H5N1 virus– and 1918 virus–infected animals may contribute to pulmonary disease and increased morbidity [27, 34–36]. Therefore, we determined whether H5N1 virus– and 1918 virus–mediated cytokine induction was altered in the infected lungs of NOS2−/− or L-NMMA–treated mice, compared with their respective control groups. We measured the induction of
cytokines that are critical for the recruitment and activation of immune cells and were shown previously to be produced at high concentrations in H5N1 virus– and 1918 virus–infected mouse lungs [27]. Compared with d-NMMA control mice, l-NMMA mice had significantly lower mean concentrations of IL-12 (2752 vs 4817 pg/mL; \( P < .001 \)) and MCP-1 (17.5 vs 34.4 pg/mL; \( P < .05 \)), whereas lung levels of IL-6 and IL-10 cytokines were generally similar among 1918 virus–infected mice (data not shown). We also determined whether cytokine production was altered in infected NOS2−/− mice. For both highly pathogenic 1918 and H5N1 virus infections, infected NOS2−/− mice exhibited significantly lower levels of interleukin 1α/β, IL-12, granulocyte colony-stimulating factor, interferon γ (IFN-γ), and macrophage inflammatory protein 1α/β in lung tissues on day 4 after inoculation (Figure 4A). Four additional cytokines, tumor necrosis factor α (TNF-α), IL-4, IL-6, and KC, were significantly reduced among H5N1 virus–infected NOS2−/− mice (Figure 4B) compared to WT mice. Taken together, these results suggest that NO contributes to proinflammatory cytokine production and its inhibition delays the death of H5N1 and 1918 infected mice.

**DISCUSSION**

The exceptional virulence and ability of the 1918 virus and viruses of the H5N1 subtype to induce severe lung pathology, particularly in young adults, suggests that host factors may contribute to the pathogenicity of these viruses. We previously demonstrated that macrophages and neutrophils accounted for the majority of increased cellularity in the lungs of H5N1 virus– and 1918 virus–infected mice [27, 28]. Because activated macrophages and neutrophils release NO, potentially contributing to the pathogenesis of lung disease, we evaluated the role of this signaling molecule following highly pathogenic influenza virus infection. NO induction appears to be a function of the severity of virus infection. We found that, in comparison to a low-pathogenic seasonal H1N1 isolate, the highly pathogenic H5N1 and 1918 viruses elicited greater NO production in the lungs of mice and from lung-derived neutrophils infected ex vivo with the 1918 virus. Therefore, NO induction occurred without the need for prior viral adaptation, which is usually required for human influenza viruses in mice. By blocking NO production pharmacologically or by using NOS2−/− mice, we observed a reduction in lung cytokine production, reduced morbidity, and delay in the time of death, compared with findings for infected control mice. The observed phenotypic changes in our l-NMMA–treated or NOS2−/− mice could not be attributed to differences in viral replication, since mice exhibited similar levels of viral load in lung tissue at the peak of virus replication. These results are in agreement with a recent study that showed that inhaled NO treatment had no effect on lung viral load, compared with the load in control mice that received compressed air [37].

Neutrophils and macrophages are found to be associated with severe lung disease following infection with highly pathogenic influenza viruses [27, 28]. Although H5N1 virus–infected lung specimens from human subjects are limited because of the lack of autopsy material, the pathology of nonhuman primates infected with H5N1 virus showed extensive lung inflammation with intraalveolar edema and hemorrhage containing numerous neutrophils and macrophages [38]. Similarly, experimental infection of mice with 1918 recombinant viruses resulted in relatively high numbers of neutrophils and alveolar macrophages in the lungs, and depletion of these cells was associated with decreased expression of cytokines [28, 39]. In 1918, histological observations of archived fixed tissues showed heavy infiltrations of white blood cells in influenza virus–infected lungs, including the identification of neutrophils in the absence of secondary bacterial infection [11,40,41]. Our ex vivo experiments revealed that mouse lung–associated neutrophils elicited a robust NO synthesis following 1918 virus infection. During 1918 or H5N1 virus infection, neutrophils leave the peripheral circulation, migrate into the interstitial airways, and, in the process of their transmigration to infected areas, become altered in their phenotype [42]. Pulmonary neutrophils exhibit an increase in antiapoptotic gene expression as compared to circulating neutrophils and exist in a primed state in which they are prone to increased production of reactive oxygen species, including NO [43]. Further experiments are needed to elucidate cellular changes such as surface protein expression and intracellular signaling of virus-exposed lung-associated neutrophils as compared to peripheral blood neutrophils.

NO can be an important element of host defense and is generally beneficial during normal homeostasis, as well as during mild-to-moderate infection, allowing for a heightened immune response and cellular migration across vascular epithelial barriers [44]. However, at the molecular level, NO has a mutagenic effect on nucleic acids of target cells when present in excess amounts, as is the case during viral pneumonia [45]. We observed higher NO levels in the lungs of mice infected with 1918 and H5N1 viruses on day 7 after inoculation, a time point that correlates with high numbers of total lung macrophages and neutrophils [27]. Conceivably, excessive accumulation of these inflammatory cells and subsequent release of cytokines and NO may contribute to lung tissue damage by causing vascular injury and destruction of the parenchymal cells. Further progression of lung injury may result in loss of functional alveolar surface area, inadequate gas exchange, lower respiration, and ultimately death. Deterioration of pulmonary function has been indicated by the need for hospital ventilator support in H5N1 virus–infected patients [46]. NO is generated by NOS2, an enzyme expressed in leukocytes,
including neutrophils and alveolar macrophages. Additionally, these cells are known to secrete the cytokines IFN-γ and TNF-α following influenza virus infection, which could further up-regulate the expression of NOS2, thereby increasing the local production of NO in lung tissue [35, 47, 48].

By using NOS2−/− mice, we demonstrated that inducible NO is important for increased proinflammatory cytokine production and impacts disease outcome following infection with 1918 and H5N1 viruses. The infected NOS2−/− and WT mice all exhibited similar lung virus titers, suggesting that the reduced morbidity and mortality of infected NOS2−/− mice was not due to inhibition of virus replication. While we did not examine the lung histopathology of infected NOS2−/− mice, we hypothesize that the decrease in early morbidity following H5N1 and 1918 viral infection and the delay in mean time to death of these mice are due to reduced lung consolidation and improved respiratory function. Previous studies have implicated a role for NO in the pathogenesis of influenza viruses in mice [23, 49]. In particular, Akaike et al demonstrated that L-NMMA treatment resulted in significant improvement in the survival rate after infection with a mouse-adapted H2N2 virus [23]. As we demonstrated here with the non-mouse-adapted 1918 virus, systemic NO inhibition with L-NMMA has a clearly beneficial effect on mouse morbidity and survival.

In summary, the lack of NO signaling through the use of NOS2−/− mice or by systemically blocking NO production pharmacologically revealed an important role for NO in the pathogenic outcome of H5N1 virus and 1918 virus infection in mice. Our studies presented here show that there may be a considerable therapeutic benefit in reducing NO levels in lungs infected with highly pathogenic influenza viruses. Although treatment with antiinflammatory drugs has been proposed as a therapeutic option for patients infected with H5N1 viruses, preclinical evaluation of steroids or anticytokine testing in H5N1 virus infection models shows little benefit of those treatments [35, 50]. Such previous attempts to combat the amplified immune response may be too broad in their effects on the immune system, and identification of more-targeted treatments, such as inhibition of NO, could be effective in reducing acute lung injury associated with these infections and, in combination with antiviral drug treatment, benefit patients.

Figure 4. Lung cytokine levels displaying a significant reduction between inducible nitric oxide (NO) synthase (NOS2)−deficient (NOS2−/−) and wild-type (WT) mice infected with H5N1 virus or 1918 virus (A) and H5N1 virus−infected mice only (B). Three mice per group were infected with 5 50% lethal doses of the viruses shown, and lungs were collected on day 4 after inoculation. Clarified lung homogenates were assayed individually by the BioPlex array system for the cytokines shown. * P < .05 and ** P < .005, by the Student’s t test. Abbreviations: G-CSF, granulocyte colony-stimulating factor; IFN-γ, interferon γ; IL-1α, interleukin 1α; IL-1β, interleukin 1β; IL-4, interleukin 4; IL-6, interleukin 6; IL-12(p40), interleukin 12(p40); IL-12(p70), interleukin 12(p70); MIP-1α, macrophage inflammatory protein 1α; MIP-1β, macrophage inflammatory protein 1β; TNF-α, tumor necrosis factor α.
Notes

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