The Journal of Infectious Diseases

MAJOR ARTICLE

1918 H1N1 Influenza Virus Replicates and Induces Proinflammatory Cytokine Responses in Extrarespiratory Tissues of Ferrets

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Background. The 1918 Spanish H1N1 influenza pandemic was the most severe recorded influenza pandemic with an estimated 20–50 million deaths worldwide. Even though it is known that influenza viruses can cause extrarespiratory tract complications—which are often severe or even fatal—the potential contribution of extrarespiratory tissues to the pathogenesis of 1918 H1N1 virus infection has not been studied comprehensively.

Methods. Here, we performed a time-course study in ferrets inoculated intranasally with 1918 H1N1 influenza virus, with special emphasis on the involvement of extrarespiratory tissues. Respiratory and extrarespiratory tissues were collected after inoculation for virological, histological, and immunological analysis.

Results. Infectious virus was detected at high titers in respiratory tissues and, at lower titers in most extrarespiratory tissues. Evidence for active virus replication, as indicated by the detection of nucleoprotein by immunohistochemistry, was observed in the respiratory tract, peripheral and central nervous system, and liver. Proinflammatory cytokines were up-regulated in respiratory tissues, olfactory bulb, spinal cord, liver, heart, and pancreas.

Conclusions. 1918 H1N1 virus spread to and induced cytokine responses in tissues outside the respiratory tract, which likely contributed to the severity of infection. Moreover, our data support the suggested link between 1918 H1N1 infection and central nervous system disease.

Keywords. influenza A virus; 1918 H1N1 virus; pathogenesis; CNS disease; extrarespiratory.

There is a large variation in the pathogenicity of different influenza A viruses in humans: Seasonal influenza A viruses generally cause mild disease while highly pathogenic avian influenza (HPAI) H5N1 virus causes severe, often fatal disease in humans. Whereas mild influenza A virus infections are usually limited to the upper respiratory tract, severe infections are characterized by spread to the lower respiratory tract and, in some cases, outside the respiratory tract such as to the central nervous system (CNS) and liver [1, 2]. Besides the extrarespiratory virus replication, systemic proinflammatory cytokines, which can be detected in the circulation of infected patients, or in extrarespiratory tissues of experimentally infected ferrets, are thought to play an important role in the pathogenesis of severe influenza virus infections [3–8]. The ability of influenza A virus to cause severe disease thus not only depends on involvement of the lower respiratory tract, but most likely also of extrarespiratory tissues.

Of all known influenza pandemics, the 1918 H1N1 Spanish influenza pandemic was the most severe, with an estimated 20–50 million deaths worldwide. Although a viral pneumonia, often complicated by secondary bacterial infections, was the main clinical finding, extrarespiratory complications—such as encephalitis lethargica—have been reported [9–11]. Reconstruction of the 1918 H1N1 virus showed that intrinsic features contained in the hemagglutinin (HA), neuraminidase (NA), polymerase, and nonstructural protein 1 (NS1) genes of the 1918 H1N1 virus contributed to pathogenicity of this virus in mice, ferrets, and macaques [12–16]. So far, these studies have mainly focused on virus replication in the respiratory tract, although virus has been detected in the heart and spleen of experimentally infected cynomolgus macaques [17]. However, the potential role of extrarespiratory involvement in the pathogenesis of 1918 H1N1 virus infection has not been studied comprehensively.

Here, we studied the pathogenesis of 1918 H1N1 virus infection in the ferret model at 1, 3, 5, and 7 days post–intranasal inoculation in the respiratory tract, nervous system, and other extrarespiratory tissues. We show that 1918 H1N1 virus

Received 9 November 2017; editorial decision 22 December 2017; accepted 8 January 2018; published online January 10, 2018.

Presented in part: Sixth Influenza Conference of the European Scientific Working Group on Influenza, Riga, Latvia, 10–13 September 2017.

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The Journal of Infectious Diseases® 2018;217:1237–46

DOI: 10.1093/infdis/jiy003

replicated efficiently in the upper and lower respiratory tract and spread to extrarespiratory tissues, including the nervous system and liver. In the respiratory tract, CNS, heart, liver, and pancreas, tumor necrosis factor alpha (TNF-α), interleukin 6 (IL-6), or interleukin 8 (IL-8) cytokines, implicated in influenza A virus pathogenesis [4–6], were up-regulated. Together, these data suggest that extrarespiratory tissues play a role in the pathogenesis of 1918 H1N1 virus infections.

METHODS

Ethics Statement

All animal experiments were approved by the Institutional Animal Care and Use Committee of Rocky Mountain Laboratories, National Institutes of Health, and carried out by certified staff in an Association for Assessment and Accreditation of Laboratory Animal Care international-accredited facility, according to the institution's guidelines for animal use, and followed the guidelines and basic principles in the United States Public Health Service Policy on Humane Care and Use of Laboratory Animals, and the Guide for the Care and Use of Laboratory Animals [18]. Sample inactivation was performed per Institutional Biosafety Committee-approved standard operating procedures for removal of specimens from high containment.

Experimental Inoculation of Ferrets

In total, 20 influenza A (H1N1 and H3N2 virus)–, influenza B–, and Aleutian disease–seronegative, 3-month-old female ferrets (*Mustela putorius furo*) were obtained (Triple F Farms, Gillet, Pennsylvania). All procedures were performed on ferrets anesthetized with ketamine (5–8 mg/kg), dexmedetomidine (0.05–0.08 mg/kg) and butorphanol (0.1–0.2 mg/kg); after the procedure, dexmedetomidine was reversed with atipamezole (0.15 mg/kg). Animals were randomly assigned to groups before inoculation. Four untreated ferrets were included in the control group and euthanized for use as uninfected controls. On day 0, 16 ferrets were inoculated intranasally with 10^6 50% tissue culture infectious dose ($TCID_{50}$) of 1918 H1N1 virus [17], divided between both nostrils (250 µL per nostril), and kept sedated for 10–15 minutes while on their backs. Nasal and pharyngeal swab samples and bodyweights were collected every other day. Ferrets were observed for clinical signs daily. At 1, 3, 5, and 7 days postinoculation (dpi), 1 group of 4 ferrets was euthanized by exsanguination under anesthesia, and tissues were collected for virological, pathological, and/or immunological analyses, including nasal turbinates (respiratory and olfactory mucosa), trachea, lungs, tonsil, adrenal gland, tracheobronchial lymph node, liver, spleen, kidney, heart, pancreas, duodenum, jejunum, trigeminal ganglion, olfactory bulb, cerebrum, cerebellum, cervical spinal cord, blood, and cerebrospinal fluid (CSF). CSF was collected from the cisterna magna via the foramen magnum. During necropsy, the percentage of the lungs affected by gross lesions was assessed by a board-certified veterinary pathologist.

Cells, Virus, and Virus Titrations

Madin-Darby Canine kidney (MDCK) cells were cultured in Eagle's minimum essential medium (Gibco) supplemented with 10% fetal calf serum, 50 IU/mL penicillin, 50 µg/mL streptomycin, 2 mM glutamine, 0.75 mg/mL sodium bicarbonate, and nonessential amino acids. The recombinant 1918 influenza virus was kindly provided by Dr Yoshihiro Kawaoka, University of Wisconsin–Madison, and propagated once in MDCK cells [17]. Virus titers ($TCID_{50}$) in nasal swab, pharyngeal swab, CSF, and homogenized tissue samples from inoculated ferrets were determined by endpoint titration on MDCK cells, as described elsewhere [19].

Pathology and Immunohistochemistry

All tissues collected in 10% neutral-buffered formalin during necropsy were fixed for ≥7 days. Tissues were embedded in paraffin, sectioned at 3 µm, and stained with hematoxylin-eosin for evaluation of histological lesions. For the detection of influenza virus by immunohistochemistry (IHC), tissues were stained with a monoclonal antibody against influenza A virus nucleoprotein (clone HB-65; ATCC) as a primary antibody, as described elsewhere [20]. The detection of nucleoprotein in the nucleus is indicative for active virus replication since it shows that nucleoprotein is translated and transported to the nucleus.

RNA Extraction and Complementary DNA Synthesis

RNA was extracted from 140 µL whole blood or CSF using the QiaAmp Viral RNA Mini Kit (Qiagen) according to the manufacturer's instructions; an additional AW1 wash step was added during the extraction of RNA from whole blood to remove potential inhibitory substances of RNA amplification.

For extraction of RNA from tissues, tissue samples were homogenized in Dulbecco's modified Eagle's medium and centrifuged to remove cell debris. Homogenate was then added to Trizol (Invitrogen) and RNA extraction was performed using a Direct-zol RNA Miniprep kit (Zymo Research) according to the manufacturer's instructions. For the detection of cytokine messenger RNA, complementary DNA was synthesized using SuperScript III reverse transcriptase (Invitrogen) and random primers.

Quantitative Polymerase Chain Reaction for Virus RNA and Cytokine Analysis

Influenza A virus RNA was detected in samples using a quantitative reverse-transcription polymerase chain reaction (qRT-PCR) assay targeting the M gene segment as described previously [21]. Messenger RNA for the proinflammatory cytokines IL-6, IL-8, and TNF-α, and the household genes glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and hypoxanthine-guanine phosphoribosyltransferase (HPRT)

were detected using the Universal PCR master mix (Applied Biosystems) as described [8, 22]. IL-6, IL-8, and TNF-α were chosen based on previous experiments, indicating that these proinflammatory cytokines are up-regulated in ferret tissue during influenza A virus infection [8]. Reactions were performed on a 7500 Real-Time PCR system (Applied Biosystems). Fold changes were calculated using the –ΔΔCt method. In brief, all cycle threshold (Ct) values were first corrected for reaction efficiency per gene as recommended by the manufacturer. Subsequently, normalization was performed using the mean Ct values of household genes (GAPDH and HPRT) as a loading control for every sample. All cytokine fold changes (ie, –ΔΔCt values) were given relative to the mean of mock-infected ferrets.

Figures were created using GraphPad Prism (version 7) and statistical analysis (Student *t* test) was performed using Microsoft Excel software (version 15).

RESULTS

Ferrets Inoculated With 1918 H1N1 Virus Develop Respiratory Disease

All ferrets inoculated intranasally with 10^6 $TCID_{50}$ of 1918 H1N1 virus showed signs of disease, starting on 2 dpi with sneezing and piloerection. Out of 4 ferrets euthanized at 7 dpi, 3 became lethargic during the course of infection (Table 1). Nasal discharge was observed in 2 of 4 ferrets euthanized at 7 dpi; 1 of those developed clear respiratory disease signs with increased respiration and a hunched posture. This ferret also lost a significant amount of body weight, indicative of severe disease (Figure 1A). On 1 dpi, distinct gross lesions, consisting of multifocal dark red areas, could already be observed in the lungs of all 4 ferrets at necropsy; these lesions were progressively larger in size during subsequent time points (Figure 1B).

1918 H1N1 Virus Replicates Efficiently and Induces IL-6, IL-8, and TNF-α in the Respiratory Tract

Nose and throat swabs were collected on 1, 3, 5, and 7 dpi from the 4 ferrets euthanized at 7 dpi. Virus shedding was higher from the throat than the nose and remained high between 1 and 5 dpi, then dropped rapidly between 5 and 7 dpi (Figure 2A).

Table 1. Clinical Signs in 1918 H1N1 Influenza Virus–Inoculated Ferrets Euthanized 7 Days Postinoculation

Clinical Sign	No./Total (Days Postinoculation)
Respiratory signs	
Sneezing	4/4 (2–7)
Nasal discharge	2/4 (5)
Increased respiration	1/4 (6)
Hunched posture	1/4 (6)
Systemic signs	
Piloerection	4/4 (2–5)
Lethargy	3/4 (2–7)
Ruffled fur	3/4 (6–7)

Data are presented as the number of ferrets that showed specific clinical signs (days postinoculation these clinical signs were observed).

On 1, 3, 5, and 7 days after inoculation with 1918 H1N1 virus, 4 ferrets were euthanized to monitor the progression of the infection. Samples of nasal turbinates, trachea, and lungs were analyzed for the presence of virus and histological lesions. In the nasal turbinates and trachea, virus titers peaked on 1 dpi and remained high through 5 dpi, then dropped on 7 dpi. In the lungs, titers peaked on 1 dpi and then steadily declined until the end of the experiment on 7 dpi (Figure 2B).

Histologically, all animals showed necrotizing and suppurative rhinitis from 1 dpi onward, which was mild at 1 dpi and moderate to severe at 3 and 5 dpi as characterized by epithelial necrosis with infiltration of neutrophils and macrophages. At 7 dpi, rhinitis was mild to moderate with evidence of regeneration of the epithelium. In the nasal turbinates, the majority of respiratory epithelial cells were influenza virus antigen positive by IHC on 1 dpi. In contrast, only few cells in the olfactory epithelium were influenza virus antigen positive. On 3 and 5 dpi, fewer respiratory epithelial cells contained virus antigen, and many of the positive cells were sloughed off and resided in the lumen of the nasal cavity. At 7 dpi, few epithelial cells contained virus antigen. In the olfactory epithelium, the number of influenza virus positive cells increased up to 5 dpi, and decreased by 7 dpi (Figure 2C and Table 2).

In the lower respiratory tract, there was a severe necrotizing suppurative bronchointerstitial pneumonia and bronchoadenitis characterized by necrosis of bronchial, bronchiolar, glandular, and alveolar epithelium and infiltration of moderate numbers of neutrophils, both viable and degenerated, and increased numbers of macrophages. Overall, histological lesions were associated with the presence of virus antigen throughout the respiratory tract and virus antigen was abundantly present at 1, 3, and 5 dpi, and occasionally at 7 dpi. In the trachea, few respiratory epithelial cells and submucosal glands were positive for virus antigen (data not shown). In the bronchus and bronchioles virus antigen was detected in the epithelial cells and submucosal glands, and in the alveoli virus antigen was detected multifocally in type I and type II pneumocytes (Figure 2C and Table 2).

RNA was extracted from the nasal turbinates and lungs on 1, 3, 5, and 7 dpi and analyzed for the expression of TNF-α, IL-6, and IL-8, proinflammatory cytokines that have been shown to play a role in influenza A virus pathogenesis. All 3 cytokines were up-regulated in the nasal turbinates and lungs in response to 1918 H1N1 virus infection. In the nasal turbinates, TNF-α and IL-6 were most prominently up-regulated, from day 1 and 3 onward, respectively. In the lungs, all 3 cytokines were up-regulated at 1 dpi with distinct expression from 3 dpi onward (Figure 3).

1918 H1N1 Virus Replicates in the Nervous System

To monitor whether 1918 H1N1 virus can enter and replicate in the nervous system, CSF and several nervous system tissues were collected during necropsy, including olfactory bulb, cerebrum, and spinal cord for virological and cytokine analysis.

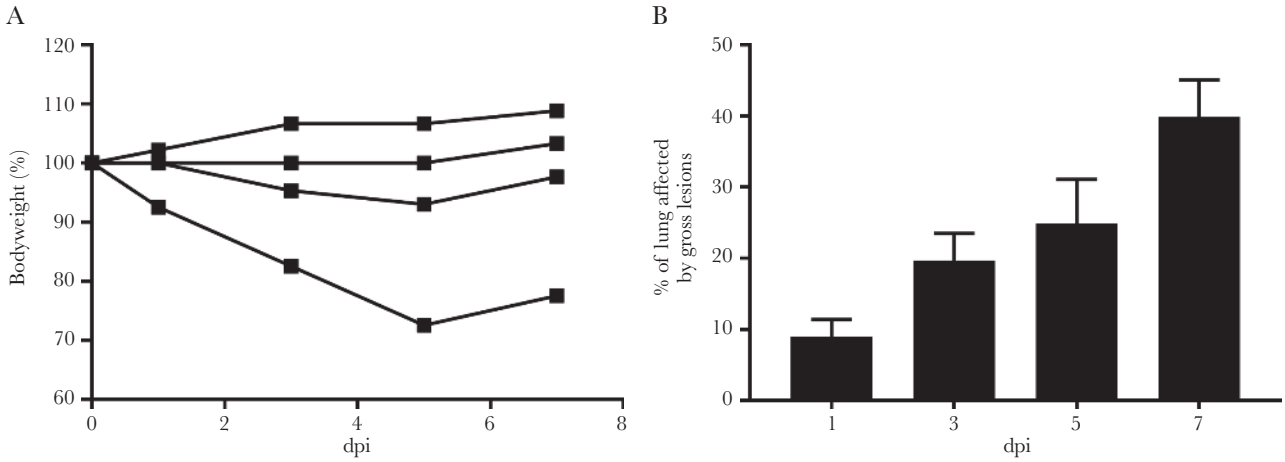

Figure 1. Bodyweight loss and gross lung lesions in 1918 H1N1 virus–inoculated ferrets. Ferrets were inoculated intranasally with 10^6 50% tissue culture infectious dose ($TCID_{50}$) of 1918 H1N1 influenza virus. *A*, Bodyweight of ferrets throughout the experiment was calculated as the percentage of start weight. Each line represents an individual ferret euthanized at 7 days postinoculation (dpi). *B*, Percentage of lung affected by gross lesions as observed at necropsy on 1, 3, 5, and 7 dpi. For each time point, average and standard deviation of 4 ferrets is shown.

Additionally, the trigeminal ganglion, brain stem, cerebellum, and from some ferrets, the pituitary gland were collected for histological analysis. Infectious virus was detected by virus titration in the olfactory bulb and cerebrum at early time points after inoculation; virus could not be detected in the spinal cord or CSF (Figure 4A). There were no histological lesions observed in the trigeminal ganglion or the CNS. By immunohistochemistry, influenza virus antigen in the olfactory bulb could only be detected in very few cells in 1 ferret at 1 dpi. In addition, virus antigen was detected in few cells in the pituitary gland of some ferrets at 1 dpi, brainstem at 1 and 5 dpi, and few neurons in the trigeminal nerve at 5 and 7 dpi (Figure 4B and Table 2). These observations indicate that 1918 influenza virus can enter and replicate within the nervous system.

RNA was extracted from the olfactory bulb, cerebrum, and spinal cord on 1, 3, 5, and 7 dpi and analyzed for the expression of TNF-α, IL-6, and IL-8. Overall, up-regulation of TNF-α and IL-6 and down-regulation of IL-8 could be detected in the olfactory bulb (Figure 4C). In the cerebrum, there was only down-regulation of TNF-α at 1 dpi, and in the spinal cord TNF-α, IL-6, and IL-8 were down-regulated from 1, 3, and 1 dpi onward respectively, with the exception that TNF-α was up-regulated at 5 dpi.

1918 H1N1 Virus Replicates in Extrarespiratory Tissues Besides the Nervous System

During necropsy, samples of the heart, liver, spleen, pancreas, duodenum, jejunum, colon, kidney, adrenal gland, and blood were collected for virological and histological analysis. In addition, heart, liver, and pancreas were collected for cytokine analysis. Similar to the detection of infectious 1918 H1N1 virus in the CNS, infectious virus was isolated from all other extrarespiratory tissues. Infectious virus was isolated more often early after inoculation (1 and 3 dpi) than at later time points, with only 1 pancreas positive in virus isolation by 7 dpi (Figure 5A). The systemic distribution of virus indicates that virus spread to these tissues via the circulation. However, viral RNA (vRNA) could not be detected by qRT-PCR in whole blood samples collected at the time of necropsy (data not shown).

No histological lesions or virus antigen could be detected in any of the collected tissues, except the liver. Virus antigen could be detected by IHC multifocally in the liver of 2 ferrets at 5 dpi. Virus antigen was predominantly detected in hepatocytes (Figure 5B and Table 2); this antigen expression was associated with multifocal hepatocyte necrosis and moderate infiltrates of neutrophils and macrophages. The absence of histological evidence of active virus replication in the majority of tissues, despite the isolation of infectious virus, could indicate that virus replication was very localized and/or inefficient.

Because the liver, heart, and pancreas have previously been reported to up-regulate proinflammatory cytokines during HPAI H5N1 infection in ferrets [8], RNA was extracted from these tissues on 1, 3, 5, and 7 dpi and analyzed for the expression of TNF-α, IL-6, and IL-8. All 3 cytokines were up-regulated in the liver (1 and 3 dpi) and the heart (1, 3, and 5 dpi); at 5 and 7 dpi, only TNF-α and IL-8 were up-regulated in the liver. In the pancreas, TNF-α was down-regulated at 1 and 7 dpi and IL-6 was up-regulated at 5 dpi (Figure 5C).

DISCUSSION

This study shows that extrarespiratory tissues are involved in pathogenesis of 1918 H1N1 virus infection in ferrets. Virus was isolated from almost all extrarespiratory tissues sampled, and active virus replication—evidenced by the detection of nucleoprotein in the nucleus by immunohistochemistry—occurred

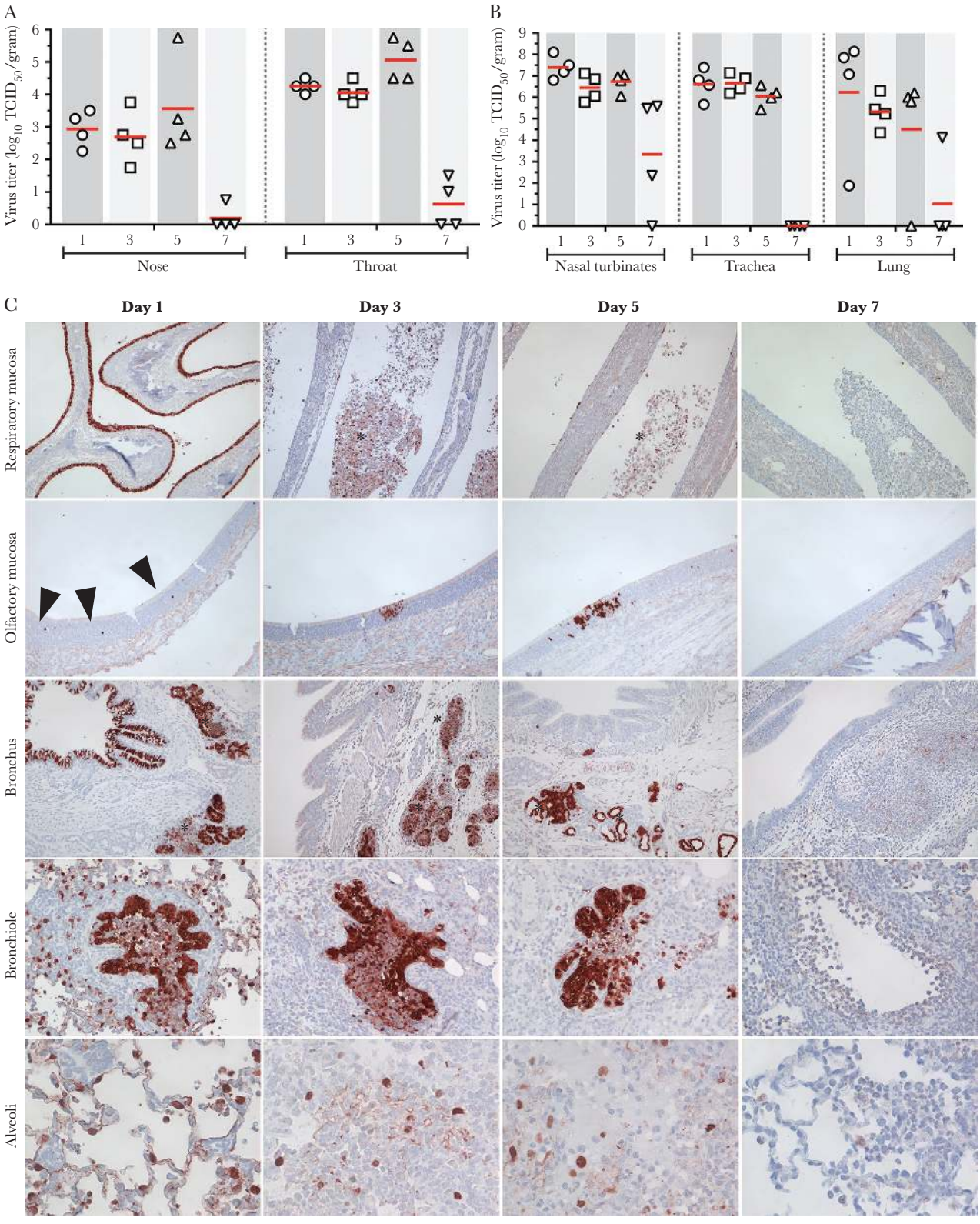

Figure 2. Virus shedding and replication in the respiratory tract of ferrets inoculated with 1918 influenza H1N1 virus. Ferrets were inoculated intranasally with 10^6 50% tissue culture infectious dose ($TCID_{50}$) of 1918 H1N1 influenza virus. *A*, Virus titers in nose and throat swabs collected at 1, 3, 5, and 7 days postinoculation (dpi) from 4 ferrets euthanized at 7 dpi. Horizontal lines represent geometric mean titers. *B*, Virus titers in the nasal turbinates, trachea, and lung at 1, 3, 5, and 7 dpi. Horizontal lines represent geometric mean titers. *C*, Detection of influenza A virus nucleoprotein in the epithelium of the nasal respiratory mucosa, olfactory mucosa, bronchus, bronchioles, and alveoli at 1, 3, 5, and 7 dpi. An asterisk (*) in the respiratory mucosa indicates the detection of influenza virus antigen in sloughed-off epithelial cells. An arrowhead in the olfactory mucosa indicates influenza virus antigen in individual cells. An asterisk in the bronchus indicates influenza virus antigen in the submucosal glands.

Table 2. Influenza Virus Nucleoprotein Antigen Detection by Immunohistochemistry in 1918 H1N1 Influenza Virus—Inoculated Ferrets in Different Tissues at 1, 3, 5, and 7 Days Post inoculation

	Day 1	Day 3	Day 5	Day 7
Respiratory tract				
Respiratory epithelium	4/4	4/4	4/4	2/4
Olfactory epithelium	4/4	4/4	4/4	2/4
Trachea	3/4[a]	3/4[a]	4/4[a]	2/4
Bronchus	3/4[a]	3/4[a]	4/4[a]	2/4[a]
Bronchioles	2/4	3/4	4/4	1/4
Alveolus	2/4	3/4	4/4	[b]
Nervous system				
Olfactory bulb	1/4	[b]	[b]	[b]
Cerebrum	[b]	[b]	[b]	[b]
Cerebellum	[b]	[b]	[b]	[b]
Brain stem	1/4	[b]	2/4	[b]
Spinal cord	[b]	[b]	[b]	[b]
Trigeminal nerve	[b]	[b]	3/4	1/4
Pituitary gland	3/4	[b,c]	[b,c]	[b,c]
Other tissues				
Heart	[b]	[b]	[b]	[b]
Liver	[b]	[b]	2/4	[b]
Spleen	[b]	[b]	[b]	[b]
Pancreas	[b]	[b]	[b]	[b]
Kidney	[b]	[b]	[b]	[b]
Adrenal gland	[b]	[b]	[b]	[b]

Data are presented as No./total days postinoculation.

[a]Includes replication in epithelial cells and submucosal glands.

[b]No antigen detected in any of the ferrets.

[c]Pituitary gland was not present in tissues collected from 2, 2, and 1 of 4 ferrets on days 3, 5, and 7 postinoculation, respectively.

in the nervous system, pituitary gland, and liver. In addition, proinflammatory cytokines were detected in respiratory as well as extrarespiratory tissues.

The detection of influenza virus antigen in the trigeminal ganglion and brainstem indicated that 1918 H1N1 influenza virus spreads to and replicates in the nervous system. In addition, 1918 influenza virus replication was detected in the pituitary gland, which is associated with the nervous system. In contrast, viral antigen was not detected in the nervous system of ferrets after intranasal or intranasal and intratracheal inoculation with pandemic 2009 H1N1 virus [21, 23]. In the current study virus replication was multifocal but limited and not associated with severe inflammatory lesions as observed in the CNS of HPAI H5N1 virus–infected ferrets [24–26]. Although the olfactory nerve is known to be a route of entry for influenza A viruses into the CNS in ferrets and humans, we showed that 1918 H1N1 virus entered the olfactory bulb, but did not replicate efficiently there. The presence of virus antigen in the trigeminal ganglion and brain stem of several ferrets suggests that 1918 H1N1 virus entered the CNS via sensory neurons of the trigeminal nerve, which innervate the nasal and oral cavity and converge into the trigeminal ganglion, which enters the brain stem. 1918 H1N1 virus infection has been linked to encephalitis and postencephalitis Parkinsonism in a minority of patients [9, 27]. Even though vRNA was not isolated from archival CNS tissues of fatal encephalitis cases from 1916 to 1920, our observation shows that 1918 H1N1 virus is able to spread to, replicate

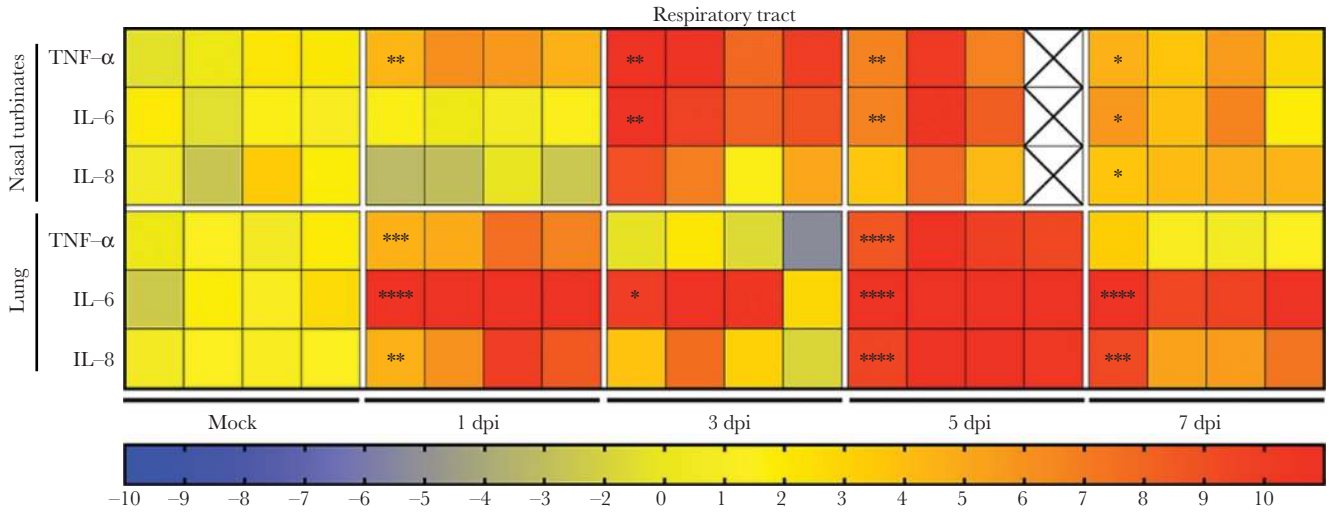

Figure 3. Induction of tumor necrosis factor alpha (TNF-α), interleukin 6 (IL-6), and interleukin 8 (IL-8) in 1918 H1N1 virus–inoculated ferrets. Ferrets were inoculated intranasally with 10^6 50% tissue culture infectious dose ($TCID_{50}$) of 1918 H1N1 influenza virus. Four ferrets were euthanized at each of the time points 1, 3, 5, and 7 days postinoculation (dpi), and tissue samples were collected for analysis of expression of proinflammatory cytokines. Log fold change expression (-ΔΔCt values) of TNF-α, IL-6, and IL-8 in the nasal turbinates and lungs of ferrets inoculated with 1918 H1N1 virus relative to the mean of the 4 Mock ferrets was calculated. Colors indicate up- or down-regulation of cytokines compared to the mean of Mock ferrets. Each square indicates expression levels in an individual ferret. Asterisks in the first square in a group indicate a statistically significant difference (*t* test) of the group of 4 ferrets as compared to the 4 Mock ferrets. **P* < .05; ***P* < .01; ****P* < .001; *****P* < .0001.

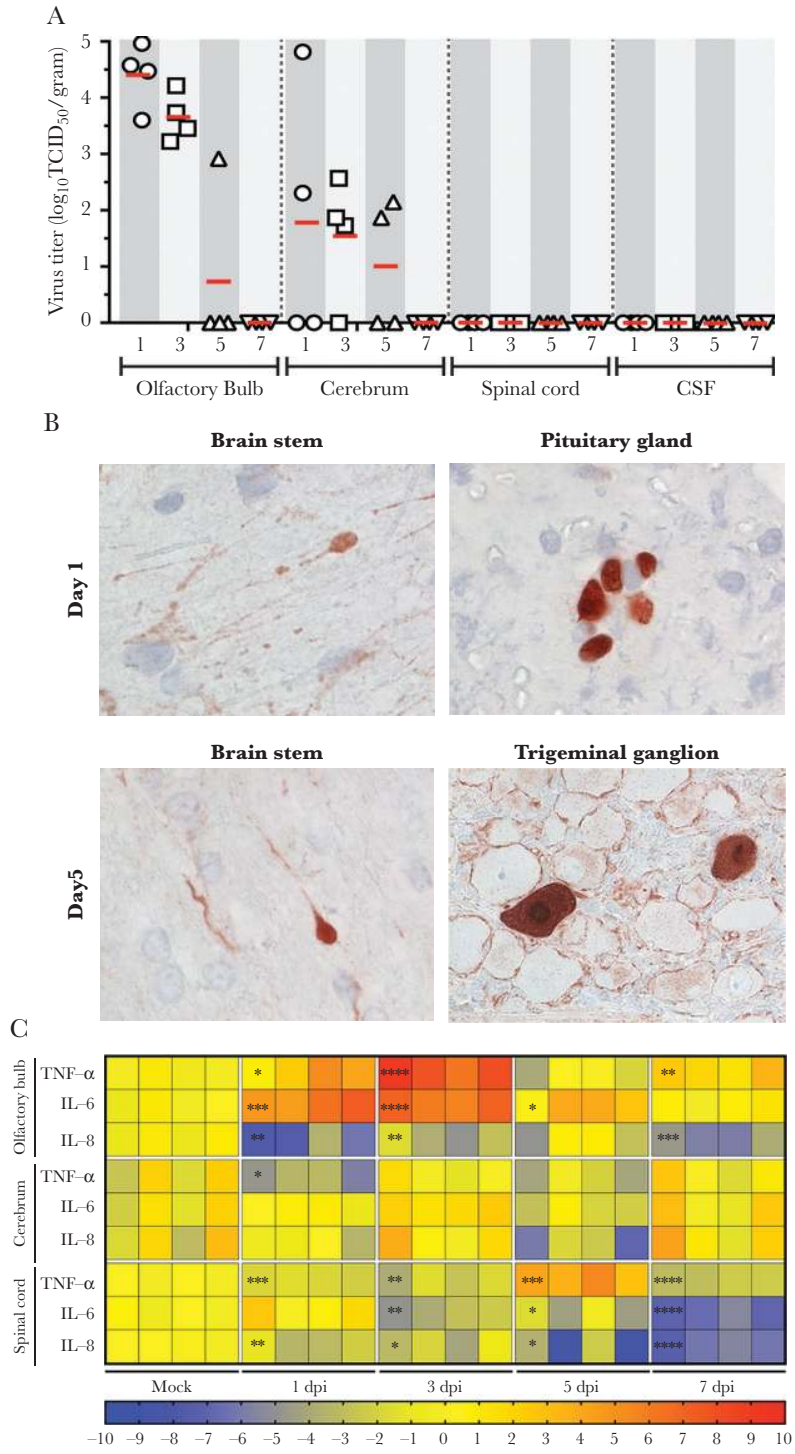

Figure 4. Virus titers, virus antigen, and cytokine expression in the nervous system of 1918 H1N1 virus–inoculated ferrets. Ferrets were inoculated intranasally with 10^6 50% tissue culture infectious dose ($TCID_{50}$) of 1918 H1N1 influenza virus. Four ferrets were euthanized at each of the time points 1, 3, 5, and 7 days postinoculation (dpi) and tissue samples were collected for analysis. *A*, Virus titers in olfactory bulb, cerebrum, spinal cord, and cerebrospinal fluid (CSF) at 1, 3, 5, and 7 dpi. Horizontal lines represent geometric mean titers. *B*, Detection of influenza virus nucleoprotein in the brain stem at 1 and 5 dpi, the pituitary gland at 1 dpi, and the trigeminal ganglion at 5 dpi (×1000 magnification). *C*, Fold change expression of tumor necrosis factor alpha (TNF-α), interleukin 6 (IL-6), and interleukin 8 (IL-8) in the olfactory bulb, cerebrum, and spinal cord of ferrets inoculated with 1918 H1N1 virus relative to the mean of 4 Mock ferrets was calculated. Colors indicate up- or down-regulation of cytokines compared to the mean of the Mock ferrets. Each square indicates expression levels in an individual ferret. Asterisks in the first square in a group indicate a statistically significant difference (*t* test) of the group of 4 ferrets as compared to the 4 Mock ferrets. *$P \leq .05$; **$P \leq .01$; ***$P \leq .001$; ****$P \leq .0001$.

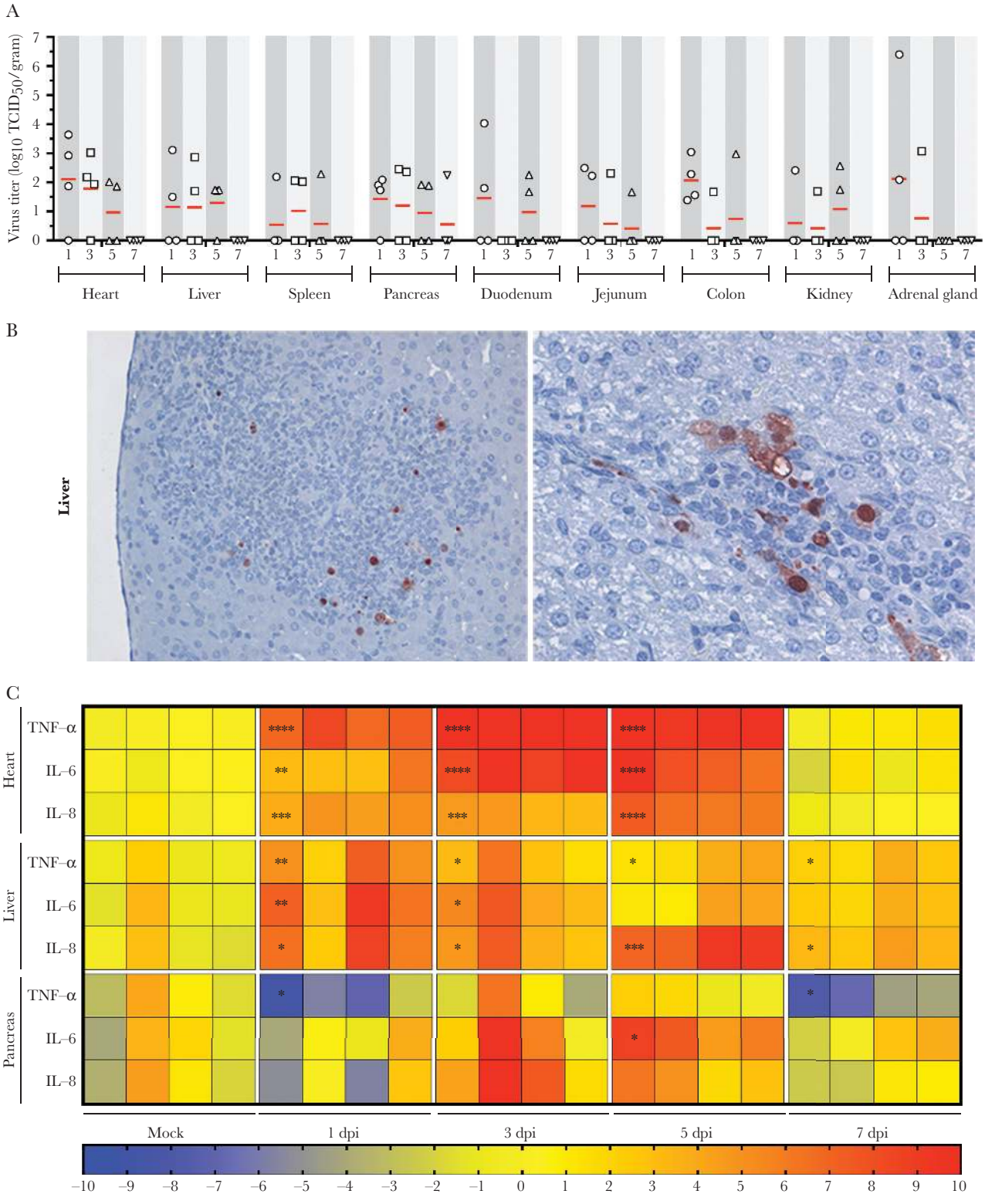

Figure 5. Virus titers, virus antigen, and cytokine expression in 1918 H1N1 influenza virus–inoculated ferrets. Ferrets were inoculated intranasally with 10^6 50% tissue culture infectious dose ($TCID_{50}$) of 1918 H1N1 influenza virus. Four ferrets were euthanized at each of the time points 1, 3, 5, and 7 days postinoculation (dpi) and tissue samples were collected for analysis. *A*, Virus titers in heart, liver, spleen, pancreas, duodenum, jejunum, colon, kidney, and adrenal gland at 1, 3, 5, and 7 dpi. *B*, Detection of influenza virus nucleoprotein in hepatocytes of the liver of 2 ferrets euthanized at 5 dpi (left panel: ×400 magnification; right panel: ×1000 magnification). *C*, Fold change expression of tumor necrosis factor alpha (TNF-α), interleukin 6 (IL-6), and interleukin 8 (IL-8) in the heart, liver, and pancreas of ferrets inoculated with 1918 H1N1 virus relative to the mean of 4 Mock ferrets was calculated. Colors indicate up- or down-regulation of cytokines relative to the mean the Mock ferrets. Each square indicates expression levels in an individual ferret. Asterisks in the first square in a group indicate a statistically significant difference (*t* test) of the group of 4 ferrets as compared to the 4 Mock ferrets. **P* ≤ .05; ***P* ≤ .01; ****P* ≤ .001; *****P* ≤ .0001.

in, and regulate the expression of cytokines in the nervous system, supporting the suggested link between 1918 H1N1 virus infections and CNS disease.

Besides the respiratory tract, infectious virus could be isolated from many extrarespiratory tissues. Virus has also been isolated from extrarespiratory tissues of HPAI H5N1 virus, but not pandemic 2009 H1N1 virus–inoculated ferrets, using the same inoculation route and dose [21, 25]. In the current study, active virus replication, as determined by nucleoprotein detection in the nucleus, could only be detected in the nervous system and liver. The fact that virus antigen could not be detected in the majority of tissues, suggests that virus replication within these tissues is inefficient or abortive, likely due to tissue-specific factors. In general, virus titers in extrarespiratory tissues peaked at 1 dpi, which coincides with the peak of virus replication in the respiratory tract, suggesting that virus in the respiratory tract spilled over to the blood resulting in hematogenous spread of virus. The fact that viral RNA could not be detected in whole blood suggests that virus titers in blood were below the detection limit of our assay, or that virus was spread intermittently via the hematogenous route and filtered out rapidly by the spleen [28].

Proinflammatory cytokines were up-regulated not only in the respiratory tract, but also in the olfactory bulb, spinal cord, liver, heart, and pancreas. This systemic cytokine response has recently also been described for infections with 2009 pandemic H1N1 virus and HPAI H5N1 virus [8]. Interestingly, both those viruses induced proinflammatory cytokines in the respiratory tract and CNS, but only H5N1 virus induced proinflammatory cytokine expression in the heart, liver, and pancreas, indicating that extrarespiratory cytokine responses after 1918 H1N1 virus inoculation mimic that observed after infection with HPAI H5N1 virus. The fact that different influenza A viruses induce proinflammatory cytokines in different organs suggests that these responses are both strain and tissue specific. As virus antigen or histological lesions were not detected in the majority of tissues in which cytokines were induced, it is likely that these were induced by parenchymal cells as described previously [8]. Whether these responses contribute to the pathogenicity of 1918 influenza virus has to be confirmed, but high levels of proinflammatory cytokines in the respiratory tract and circulation have been associated with more severe disease and a poor disease outcome in ferrets and patients [4–6, 29]. Moreover, TNF-α, IL-6, and IL-8 have been associated with the pathogenesis of extrarespiratory complications. For example, TNF-α expression in the heart is involved in the pathogenesis of viral myocarditis [30], and TNF-α and IL-6 expression in the CNS is associated with the development of seizures, one of the most common clinical signs of influenza virus–associated CNS disease.

Involvement of extrarespiratory tissues during 1918 H1N1 virus infections is more profuse than previously observed for 2009 H1N1 virus, but less extensive than observed for HPAI H5N1 virus [21, 23, 25, 31]. Active virus replication—detection of nucleoprotein within the nucleus—in extrarespiratory tissues has not previously been observed for pandemic 2009 H1N1 virus [23]. Extrarespiratory replication has been observed in ferrets inoculated with HPAI H5N1 virus, which was dependent on the presence of a multibasic cleavage site [25]. Although the 1918 H1N1 virus HA does not contain a multibasic cleavage site, it is not dependent on cleavage by trypsin for replication, suggesting that the cleavage of HA may be an important determinant of extrarespiratory virus replication.

Extrarespiratory replication of 1918 H1N1 virus and induction of proinflammatory cytokines most likely contributed to its ability to cause severe disease in a minority (but still substantial percentage) of infected individuals, resulting in 50 million deaths worldwide. In addition, our data support the link between 1918 H1N1 virus infection and CNS disease, observed during the 1918 Spanish influenza pandemic. Whether future pandemics will harbor similar features to 1918 H1N1 is unclear, but avian influenza viruses that are closely related to the 1918 H1N1 virus are still prevalent in wild birds [32]. Therefore, more research is needed to understand how influenza A virus can spread to and induce responses outside the respiratory tract and how this process contributes to its pathogenicity.

Notes

Acknowledgments. The authors thank Yoshihiro Kawaoka for providing the 1918 influenza virus; Ricki Feldmann, Kimberly Meade-White, and Tina Thomas for technical assistance; and Thijs Kuiken for useful discussions.

Financial support. This work was partially funded by the Intramural Research Program of the National Institute of Allergy and Infectious Diseases, National Institutes of Health, and by fellowships to D. v. R. from the Netherlands Organization for Scientific Research (contract number 91614115) and the Erasmus MC Foundation.

Potential conflicts of interest. All authors: No reported conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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