ORIGINAL ARTICLE

Extrapulmonary tissue responses in cynomolgus macaques (*Macaca fascicularis*) infected with highly pathogenic avian influenza A (H5N1) virus

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Abstract The mechanisms responsible for virulence of influenza viruses in humans remain poorly understood. A prevailing hypothesis is that the highly pathogenic virus isolates cause a severe cytokinemia precipitating acute respiratory distress syndrome and multiple organ dysfunction syndrome. Cynomolgus macaques (*Macaca fascicularis*) infected with a human highly pathogenic avian influenza (HPAI) H5N1 virus isolate (A/Vietnam/1203/ 2004) or reassortants of human influenza virus A/Texas/36/ 91 (H1N1) containing genes from the 1918 pandemic influenza A (H1N1) virus developed severe pneumonia within 24 h postinfection. However, virus spread beyond the lungs was only detected in the H5N1 group, and signs of extrapulmonary tissue reactions, including microglia activation and sustained up-regulation of inflammatory markers, most notably hypoxia inducible factor-1α (HIF-1α),

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were largely limited to this group. Extrapulmonary pathology may thus contribute to the morbidities induced by H5N1 viruses.

Introduction

The Spanish influenza pandemic of 1918–1919 was the most devastating outbreak of infectious disease in recorded history, with an estimated 20–50 million human deaths following an unusually severe and rapid clinical course [1, 2]. The molecular pathogenesis of this pandemic is still poorly understood, but a prevailing hypothesis is that the severe tissue necrosis, influx of inflammatory cells and profound vascular leakage in the lower respiratory tract precipitated an acute respiratory distress syndrome (ARDS) leading to multiple organ dysfunction syndrome (MODS). Similar mechanisms have been proposed for the clinical symptomatology and the pathology induced by the currently circulating avian H5N1 influenza A virus strains [3].

Reports of extrapulmonary distribution of highly pathogenic influenza viruses (HPIV) in humans are limited to a few recent reports of detection of viral antigen in or isolation of H5N1 virus from brain, CSF, intestine and fecal material from individuals who died with influenza virus-induced coma and diarrhea [1, 4–6]. Infection with influenza viruses is generally restricted to the respiratory tract in humans, although encephalopathy has been reported for seasonal influenza, particularly in children [7].

There appears to be wide disparity in results concerning extrapulmonary spread of avian H5N1 viruses and pathology in animal models. In mice, virus has been recovered from most extrapulmonary tissues examined, including brain, but viral titers were often low and inconsistent [8–10]. In cynomolgus macaques infected with the H5N1 virus-strain A/Hong Kong/156/97, virus replication was mainly confined to the respiratory tract [11, 12]. In contrast, in cats infected with H5N1-strain A/Vietnam/1194/2004, high titers of virus were re-isolated from most extrapulmonary tissues and organs regardless of infection route [13]. Similar results were obtained in ferrets with the virus strain A/Hong Kong/486/97, despite the latter strains having relatively low pathogenicity in mice [8, 10, 14]. These species differences may reflect variations in virus receptor distribution [15], variations in inflammatory and immune responses [16–19], or differences inherent to the H5N1 strains used, such as replication efficiency in host cells, interference with interferon responses [8, 10, 20, 21], or any combination of these variables.

Using a macaque model of influenza [22, 23] and employing a recent human isolate of H5N1 influenza A virus as well as reassortant H1N1 influenza A viruses containing genes encoding two (HA, NA) or four (HA, NA, NS1 + NEP) proteins of the 1918 Spanish influenza virus strain, we previously reported on pulmonary pathology [23]. We subsequently have sought to further address questions regarding extrapulmonary virus replication and inflammation-associated extrapulmonary effects caused directly or indirectly by these virulent viruses. In particular, we hypothesized that the transcription factor hypoxia inducible factor-1 (HIF-1) would be up-regulated in animals with severe pneumonia, either as a result of hypoxia or the inflammatory response. HIF-1 has emerged as a key regulatory molecule due to its responsiveness to both microenvironmental tissue conditions, such as hypoxia, and inflammatory mediators [24–28]. We found that the expression of HIF-1α appeared to reflect the extent and kinetics of lung inflammation following infection with these influenza viruses and was an indicator of extrapulmonary tissue reactions in the absence of frank pathology or virus replication.

Materials and methods

A detailed description of the experimental protocol, including animal sources, virus sources and the infection protocol has been published [23]. Briefly, 34 Chinese-origin *M. fascicularis* were assigned to five experimental groups matched for age, weight, and gender to the extent possible. Four groups of eight animals were inoculated through the combined intratracheal, intranasal, tonsillar, and conjunctival routes with a total of 10^7 pfu of either A/Vietnam/1203/2004 (H5N1) virus, A/Texas/36/91 (H1N1; hereafter designated A/Texas) virus or reassortants of this virus containing either two (HA, NA; hereafter designated HANA) or three (HA, NA, NS; hereafter designated HANANS) genes from influenza A/Brevig Mission/1/1918 virus (NA, NS) and A/South Carolina/1/1918 virus (HA). Influenza-virus-infected animals were housed in an ABSL-3 enhanced facility. The study was conducted in accordance with guidelines approved by the Institutional Biosafety and the Animal Care and Use Committees of the Battelle Memorial Institute, University of Washington and Colorado State University.

Two animals per group were euthanized at days 1, 2, 4, and 7 postinfection (pi). Two animals were used as mock-infected control animals and terminated at day 7 post-challenge. At necropsy, all tissues were examined grossly and harvested. Samples for histology and immunohistochemistry were fixed in 10% neutral-buffered formaldehyde for 48 h, followed by transfer into 70% methanol-free ethanol, and stored until processing. The tissues were routine embedded in paraffin and sectioned (4–5 μm). Hematoxylene- and eosin (HE)-stained sections

were examined on an Olympus BX41 microscope equipped with a Q-Color3 camera (Olympus) and corresponding computer software.

Immunohistochemistry (IHC) detection of influenza virus antigen in formaldehyde-fixed, paraffin-embedded tissues was carried out as described previously [22, 23], except that proteinase K was used for antigen retrieval. The H1N1 viruses were detected using the influenza virus nucleoprotein-specific monoclonal antibody M322211 (Fitzgerald Industries, Concord, MA, USA). Because this antibody appeared to cross-react poorly with the H5N1 nucleoprotein, a polyclonal rabbit antibody specific for H5N1 viruses (source: CDC) was used on tissues of the H5N1 group. Tissues from our previous studies were used as positive controls [22, 29]. Other controls included substitution of the influenza-virus-specific antibodies with monoclonal antibodies specific for various flaviviruses (West Nile virus, St. Louis encephalitis virus, bovine pestivirus) and omission of the primary or secondary antibodies.
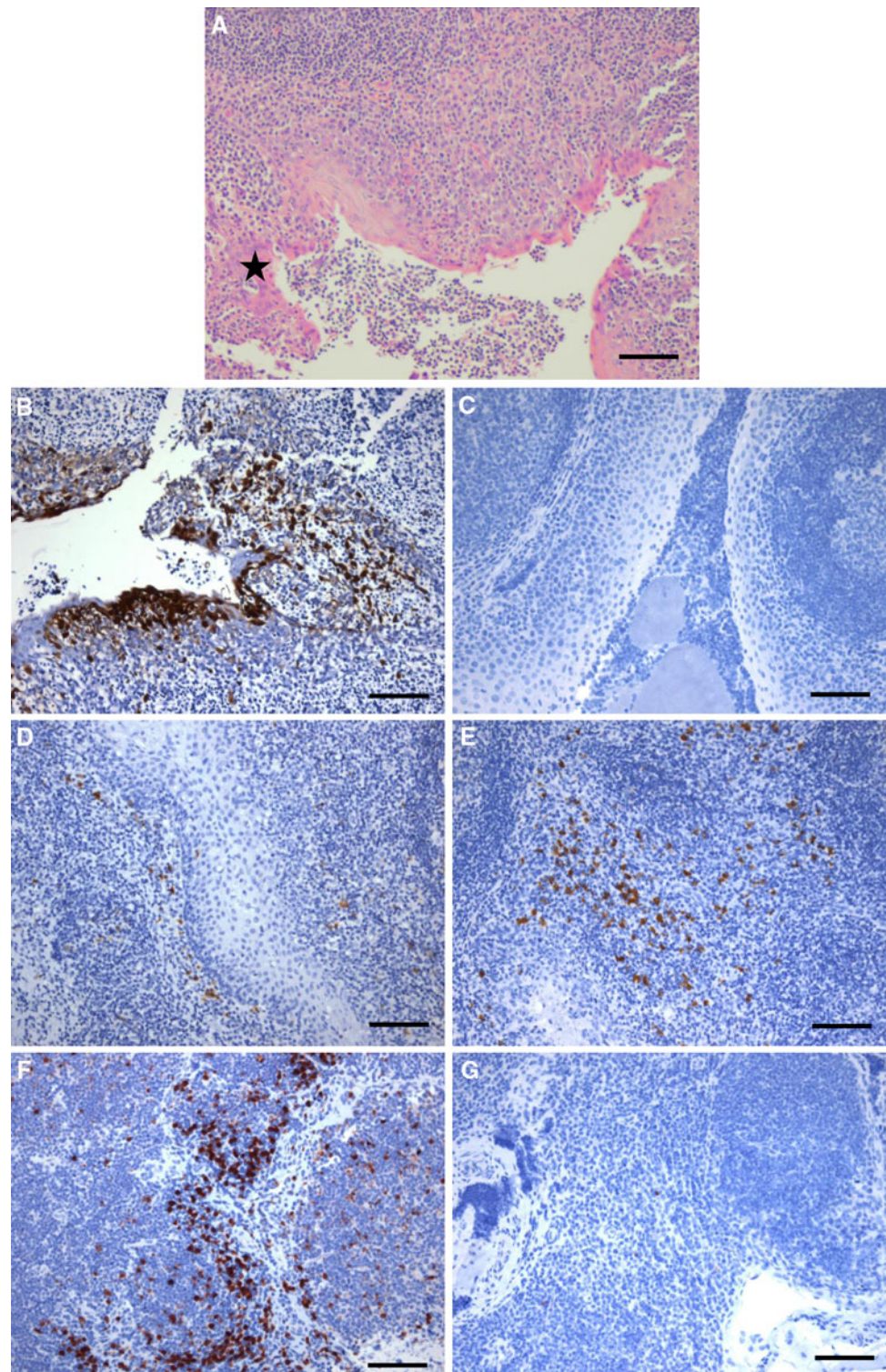
To characterize the inflammatory reactions in pulmonary and extrapulmonary tissues, sections from lungs [23], tracheobronchial and retropharyngeal lymph nodes, tonsils, spleen, heart, liver, right kidney, colon and ileum as well as several areas of the brain were immunolabeled for Mac387 (myelomonocytic lineage marker), CD83 (mature dendritic cells), HIF-1α, hemeoxygenase-1 (HO-1), cyclooxygenase-2 (COX-2), inducible nitric oxide syntase (NOS2), vascular endothelial growth factor-A (VEGF-A), phospho-p38α mitogen-activated protein kinase (phospho-p38α MAPK), and the apoptosis marker activated caspase-3. Brain sections were also labeled for glial fibrillar acidic protein (GFAP) to test for gliosis or loss of astrocytes, for microglial cells using the Iba-1 marker and for oligodendrocytes, using the transcription factor OLIG2 as a cell marker [30]. Sources of antibodies and immunolabeling protocols have been described in detail elsewhere [30, 31]. Positive control tissues included samples from inflammatory processes in lungs, intestine and brain of macaques, cattle, mice, guinea pigs, and cats [30–32]. The sections were counterstained with Meyer's hematoxylin (Scytek Laboratories; Logan, Utah), mounted with coverslips, and examined on an Olympus BX41 light microscope. The immunolabeling was scored semi-quantitatively on a scale of 0–6, based on the number of positive cells and the overall intensity of labeling, with 0 indicating no apparent labeling, 1 through 6 indicating increasing expression above normal levels, and a score of 6 denoting diffuse occurrence of positive cells with high-intensity labeling. For HIF-1α, the scores relate to nuclear expression (nuclear translocation) rather than cytoplasmic expression. In the instances of a marker for normal cellular constituents, such as microglia in the brain or dendritic cells in the lymphoid

tissues including bronchiole-associated lymphoid tissue (BALT), normal levels were given a score of 0. The scale therefore differs for each marker but is consistent for a particular phenotype, depending on the normal tissue levels of expression in naïve animals. Photomicrographs were acquired with an Olympus Q-Color 3 camera and associated computer software and are reproduced without manipulation.

Results

Cynomolgus macaques (*Macaca fascicularis*) infected with a human HPAI H5N1 virus isolate (A/Vietnam/1203/ 2004), or reassortants of human influenza virus A/Texas/ 36/91 (H1N1) containing genes from the 1918 pandemic influenza A (H1N1) virus developed severe pneumonia within 24 h postinfection, with inflammation peaking on days 2–4 pi in the 1918 reassortant groups, followed by resolution. In contrast, no cessation of inflammation was apparent in the H5N1 group [23]. Grossly, the most notable extrapulmonary changes occurred in the lymph nodes. The retropharyngeal and tracheobronchial lymph nodes appeared mildly to moderately enlarged after all of the influenza virus infections, most notably on days 4 and 7 pi. Early in the infections, there was variable edema of the lymph nodes, and by day 4 and 7 pi, the nodes in the HANA, HANANS and A/Texas groups were characterized microscopically by marked follicular and paracortical hypertrophy. In contrast, the follicles in the lymph nodes of H5N1-infected animals were involuting and appeared with variable degrees of hyalinization of the germinal centers, except at 7 days pi, where there were signs of follicular hypertrophy in the one surviving animal. Only in the H5N1-infected animals was there histological evidence of tonsil involvement, with frank neutrophilic and necrotizing tonsillitis on days 1 and 2 pi (Fig. 1a). By day 7, the inflammation was resolving. Notably, the histopathological changes in the lymph nodes and tonsils of the H5N1-infected animals correlated with the extent of virus infection as detected by IHC (exemplified by the tonsil in Fig. 1b). A few virus-infected cells were found in the tonsil and retropharyngeal lymph nodes of HANA-infected animals on day 1 pi (not shown). No virus-infected cells were detected in these tissues in any animal of the other groups. The number of CD83-positive cells in lymph nodes and tonsil either remained within normal levels or increased over time in the HANA, HANANS and A/Texas groups, while these cells decreased in frequency and intensity of labeling in the tissues of the H5N1 animals (Fig. 1d). The disappearance of CD83-positive cells from the lymphoid tissues of the H5N1-infected animals over time correlated temporally with a pronounced occurrence of apoptotic

Fig. 1 Tonsillitis in macaques infected with H5N1 virus. **a** Epithelial necrosis and loss (*star*) and infiltration of neutrophils amongst the normal lymphoid component of the tonsil and in the lumen. HE, *scale bar* 400 μm. **b** Virus infection in tonsillar epithelial cells, infiltrating leukocytes and underlying lymphoid tissue demonstrated by immunohistochemistry. **c** Absence of virus-antigen-positive cells in an A/Texas-infected animal (this image is representative of all groups other than H5N1). **d** Loss of CD83 positive cells 4 days pi with H5N1 virus. **e** Normal levels of CD83-positive dendritic cells in control tonsil, and representative for all other groups. **f** Pronounced apoptosis of cells, detected by IHC for activated caspase 3, in tonsil 4 days pi with H5N1 virus. **g** No apparent apoptosis in tonsils from any of the other groups. (**b–g**) IHC; *scale bars* 300 μm

cells, as reflected by expression of activated caspase 3 (Fig. 1f).

The spleen of the H5N1-infected animals presented with very pronounced accumulation of Mac387-positive cells, mainly neutrophils, in the red pulp, most notably on days 1 and 2 pi (Fig. 2) and then decreasing, but not completely disappearing, over the following 5 days. Small numbers of scattered virus-antigen-positive cells were detected in the spleen of H5N1-infected animals on days 1 and 2 pi, but not in any of the other groups, where the spleen
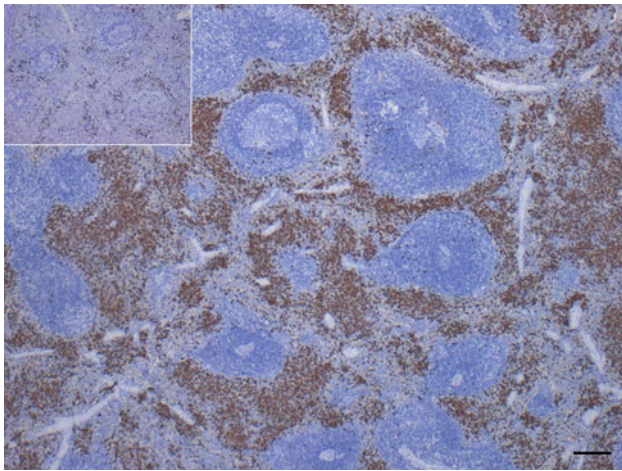
Fig. 2 Pronounced occurrence of Mac387-positive cells, mainly neutrophils, in the splenic red pulp of macaques 1–2 days after respiratory infection with H5N1 influenza virus. *Inset* Mac387-positive cells in spleen from a control animal. *Main* IHC, *scale bar* 500 μm

Fig. 3 Detection of influenza virus antigen in extrapulmonary tissues 2–4 days pi in H5N1-infected animals. **a** Viral antigen in the mesothelial cells of the meninges of a macaque infected with H5N1 virus 4 days earlier. *Inset* higher magnification of a virus-antigen-positive cell, *boxed* in the *main image*, to illustrate the nuclear labeling. **b** Nuclear labeling (*arrows*) for H5N1 virus in cells in the lamina propria of the colon of a macaque terminated on day 2 pi. The granular signal surrounding the positive nuclei may be cellular debris from infected cells or some cytoplasmic staining. **a, b** IHC, *scale bars* 20 μm

morphology tended to follow that of the lymph nodes, reflecting immune stimulation. The occurrence of CD83-positive cells also followed the pattern of the lymph nodes in these latter groups.

Virus-infected cells were detected in the mesothelial cells of the meninges of one H5N1-infected animal on day 4 pi, most pronounced over the occipital lobes (Fig. 3a), while neurons and glia cells were negative for virus antigen in all animals at all time points examined. Apart from the meninges, the only other extrapulmonary and non-lymphoid tissues with virus-antigen-positive signals were the colon (macrophage or dendritic-like cells in lamina propria; Fig. 3b) of one H5N1-infected animal on day 2 pi, and the abdominal mesothelium (mesothelial cells, most notably the lining associated with the kidney capsule) of one animal on day 4 pi. No extrapulmonary virus antigen was detected in any animal of any other group at any time point.

While HIF-1α, HO-1, NOS-2, phosphor-p38-MAPK and VEGF-A were markedly up-regulated in the lungs within 24 h pi in animals infected with either of the reassortants or with H5N1, and remained highly expressed in the latter group throughout the course of the study (Fig. 4, and data not shown), no consistent pattern was discernable in the expression of these inflammatory markers in the lymphoid tissues. Nevertheless, all five markers were markedly upregulated above control levels at one or more time points postinfection in all lymphoid tissues of all infection groups (data not shown).

Histologically, the only tissue outside the respiratory tract and lymphoid tissues with discernable changes on HE sections was the liver of animals in the H5N1 group, where
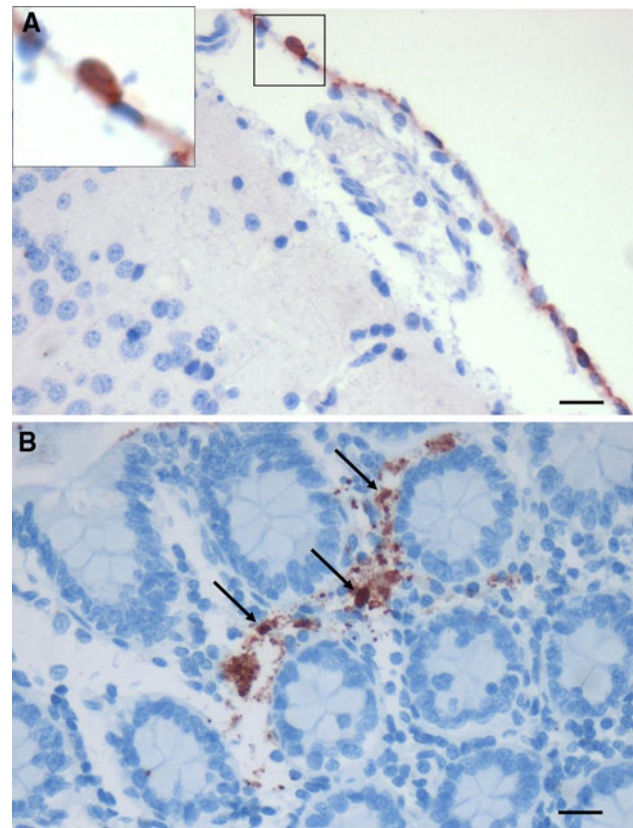
the Kupffer cells appeared mildly hypertrophic. This change was more apparent in sections stained for Mac387 and HIF-1α (Fig. 5a, c, and data not shown). Using these same phenotypic markers, it became evident that intravascular blood monocytes in many tissues and resident, interstitial macrophages in kidneys and heart of these animals were likely also activated, or alternatively, these tissues were infiltrated with a small but significant population of activated macrophages (Fig. 5e). A further indication of extrapulmonary activation of the macrophage lineage was seen in the brains of H5N1 infected animals, where a mild, but very notable hypertrophy, and possible increase in numbers, of microglial cells was apparent in sections labeled for the Iba-1 molecule (Fig. 6). Moreover, there were signs of astrogliosis in the H5N1-infected animals on days 4 and 7, as reflected by increased GFAP expression (data not shown). VEGF-A expression was more pronounced in the brain in this group from day 2 pi through
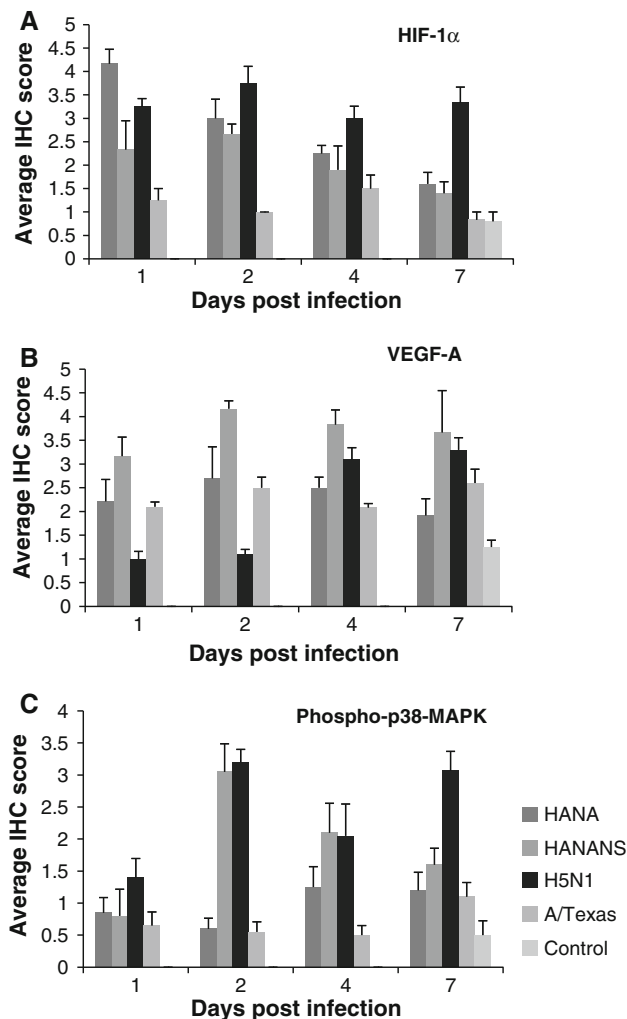
Fig. 4 a–e Semi-quantitative scores for expression of inflammatory markers in the lung of influenza-virus-infected cynomolgus macaques, judged on a scale of 0–6, taking into account the number of positive cells, intensity of labeling and, in the case of HIF-1α, nuclear localization. (**a**) HIF-1α, (**b**) VEGF-A, (**c**) phospho-p38α-MAPK. The data are based on the average score for 3–5 lung sections per animal, with standard errors of the mean shown

day 7, although on days 1 and 2, the expression in the other groups was also notable (data not shown).
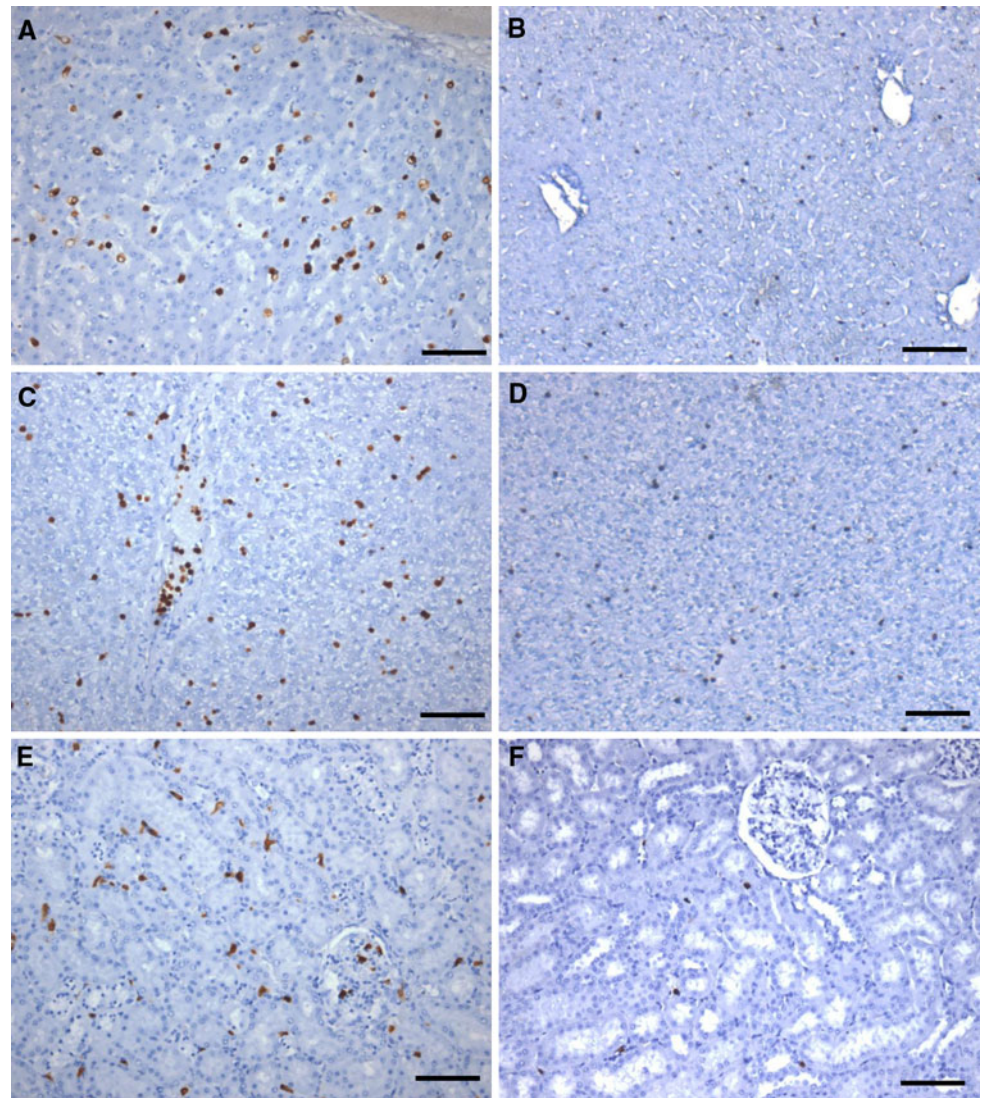
Discussion

We previously reported that H5N1 virus as well as reassortant H1N1 viruses containing the HA and NA genes from the 1918 pandemic influenza virus induce a severe pneumonia within 24 h of challenge, but that only the former virus gives rise to a sustained inflammatory response and massive parenchymal necrosis in the respiratory tract [23]. In this study, we show that this also applies to the tonsils, and it is accompanied by reactions in

extrapulmonary tissues consistent with a systemic cytokinemia. The 1918, HA and NA genes significantly increased the virulence of a seasonal human H1N1 virus of low pathogenicity but did not confer a virulence phenotype comparable to that of the H5N1 virus or the complete 1918 pandemic influenza virus [33], suggesting that other viral genes, such as PB1 [34], probably also play significant roles in the pathogenesis. Furthermore, only the H5N1 virus appeared to spread beyond the respiratory tract to lymphoid tissues, intestines and meninges. Previous studies with other H5N1 isolates and the fully reconstructed 1918 virus in macaques similarly found evidence of only very limited virus spread outside the respiratory tract [12, 33], whereas in cats and mice, H5N1 virus spread more widely [9, 13, 35]. Virus has also been isolated from or detected at extrapulmonary sites in human patients succumbing to H5N1 virus infection, including brain and intestine [4]. Thus, it would appear that the virus strain, timing postinfection, species, age and infection dose play important roles in determining virus spread beyond the respiratory tract, or at least the possibility of detecting it.

While overt pathology ascribable to the virus infection was not seen in any tissues outside the respiratory tract and associated lymphoid tissues by conventional histology, immunohistochemistry for inflammatory reactants suggested that the effect of at least the H5N1 virus infection was not limited to those tissues. Notably, severe hypoxia, as a result of the pronounced lung pathology [23], cytokinemia, or both may have given rise to extrapulmonary reactions. Both pathways may be regulated by HIF-1 [28]. HIF-1 was originally discovered as a biological O_2 sensor that enables the organism to adapt to hypoxia. Hypoxia is a reduction in the normal level of oxygen tension and occurs during acute and chronic pulmonary disease, vascular disease and cancer. Under hypoxic conditions, the rate of oxygen supply limits the rate of oxygen consumption, and aerobic metabolism is reduced. In cases of severe O_2 deficiency, the respiratory chain succumbs, and as a consequence, cellular death by necrosis or apoptosis may result. HIF-1 is a heterodimeric transcription factor composed of HIF-1α and HIF-1β subunits. Whereas HIF-1β is constitutively expressed, HIF-1α is targeted to ubiquitinylation by the von Hippel-Lindau tumor suppressor protein and is rapidly degraded in the proteasome. Under hypoxic conditions, hydroxylation of HIF-1α is blocked, which promotes protein stability and transactivation of HIF-1 [26]. Upon translocation to the nucleus, HIF-1 promotes the expression of genes encoding proteins that increase the cellular supply with oxygen and with energy-providing substrates [27]. HIF-1 initiates the defense against hypoxia at different levels, but in virtually all tissues, hypoxia induces the synthesis of proteins controlling local blood flow, notably VEGF and HO-1 [26, 27, 36].

Fig. 5 a, c, e Immunohistochemical detection of myeloid lineage cells and HIF-1α expression in liver and kidney of H5N1-virus-infected macaques compared to the other four groups. The *microphotos* are representative of expression throughout days 1–7 pi. **a** IHC for the myeloid cell marker Mac387 in liver reveals an increase in both numbers and size of macrophage-like cells including Kupffer cells on day 4 pi. **c** HIF-1α expression in intravascular and intrasinusoidal leukocytes in the liver of a H5N1-virus-infected macaque on day 1 pi. **e** Increased numbers of interstitial and intravascular Mac387-positive cells in the kidney of an H5N1 virus-infected animal, day 7 pi. **b, d, f** For each tissue and marker, similarly stained sections from a sham-infected animal and also representative of the other three virus-infected groups. *Scale bars* 200 μm

Several of the aforementioned agents downstream of HIF-1 are also potent inflammatory mediators, and recent studies have shown that HIF-1 plays a central role in stress responses beyond hypoxia (reviewed in [25, 28]). Many pro-inflammatory cytokines and reactive oxygen species (ROS) can activate HIF-1 even under normoxic conditions [25, 37], with subsequent HIF-1 induction of proteins that promote inflammation in a seemingly positive feedback loop of inflammation regulation (reviewed in [28, 38]). HIF-1 has also been shown to be essential for the regulation of the glycolytic capacity of myeloid cells. In the absence of HIF-1, the cellular ATP pool is drastically reduced, and this metabolic defect causes profound impairment of macrophage and neutrophil cell aggregation, motility, invasiveness, and antimicrobial activity [24].

In the context of severe pneumonia caused by infection with highly pathogenic influenza virus, HIF-1 induction by both tissue hypoxia, due to compromised respiratory capacity, and the cytokine cascade initiated by innate immune responses, notably IFN-α/β, would be possible [28, 39, 40]. Pulsoximetry was not carried out in the present study due to logistic constraints, and thus it is not possible to determine with certainty whether the marked up-regulation of HIF-1 expression was due to systemic hypoxia, the inflammatory cascade, or both. Regardless of the proximal cause, up-regulation of HIF-1 expression, notably nuclear expression, could then promote the amplification of the cytokine cascade, contributing to the systemic inflammation response syndrome (SIRS). We have demonstrated that H5N1-infection in macaques induces profound and sustained up-regulation of mRNA for IFNs and their downstream signaling molecules, as well as for IL-1, IL-6 and TNF-α, accompanied by very significant levels of circulating IL-6 and TNF-α proteins [23]. Furthermore, pronounced nuclear and cytoplasmic expression of HIF-1α was demonstrated by IHC in peripheral blood
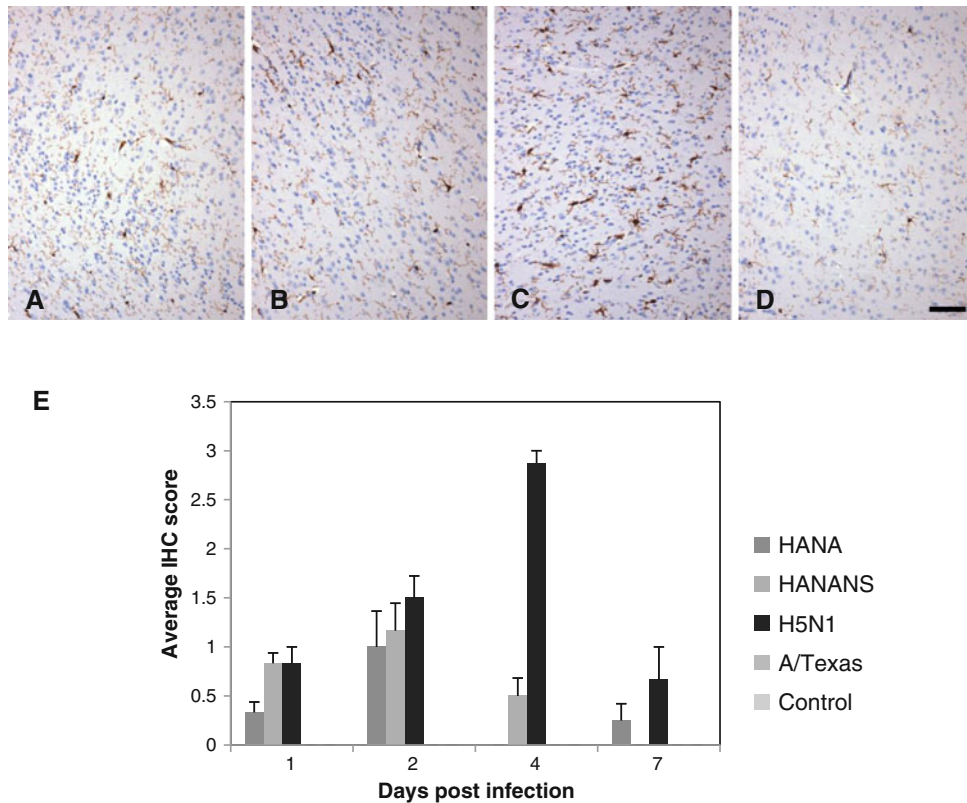
Fig. 6 a–d Demonstration of microglial cells (Iba-1-positive) in the brain (examples are all from the same region of the occipital lobe) of cynomolgus macaques at day 4 pi after infection with influenza viruses. **a** An animal infected with HANA reassortant virus. **b** An animal infected with HANANS reassortant virus. **c** An animal infected with H5N1 virus. **d** An animal infected with A/Texas virus. Expression in the sham-infected control animals was indistinguishable from that of the A/Texas-group (not shown). **a–d** IHC, *scale bar* 640 μm. **e** Semi-quantitative scores for expression of the Iba-1 marker, judged on a scale of 0 (normal level of expression) to 6, taking into account both number of positive cells and intensity of labeling. Three sections, from the parietal, temporal and occipital cortex, were examined for each animal and averaged for each time point and group. Standard errors of the mean are shown

leukocytes and tissue macrophages (described in this paper), and phospho-p38α-MAPK expression was up-regulated in both the respiratory tract and lymphoid tissues, lending support to the contention that increased cytokinemia is indeed induced during H5N1 infection, with systemic effects as a consequence. This interpretation is corroborated by findings by Lee et al. [3, 41], who also demonstrated that high cytokine levels in peripheral blood correlated with increased phospho-p38α-MAPK expression in H5N1 infection in human patients.

During SIRS and MODS, the brain appears to be a main target organ, with activation of glial cells, upregulation of NOS2, apoptosis and loss of neurons [7, 42]. The proximal inducers of this encephalopathy include TNF-α, type I IFNs, IL-1β and IL-6 [17, 43–45], all of which were highly up-regulated in animals with H5N1 virus infection throughout the 7-day observation period [23]. Neurological symptoms, other than 'depression', are difficult to monitor in macaques, and this, for logistic reasons, could not be done in this study. However, the finding of microglial activation, as evidenced by increased cell size and Iba-1

expression [30] and upregulation of VEGF-A expression in the neuropil and of HIF-1α expression in intravascular leukocytes and leukocytes in the interstitium of the choroid plexus of H5N1 infected animals, suggests that these animals might have experienced an encephalopathy, albeit relatively mild, that could have contributed to their constitutional symptoms and consequent high clinical scores [23].

Collectively, the data presented here suggest that extrapulmonary tissue responses are part of the pathophysiology of infections with highly pathogenic influenza viruses. This is in accord with recent clinical observations in human patients [46], where treatment for hypoxia improved survival rates. This would also appear to link in with the observed expression of HIF-1α, which in our study appeared to reflect the extent and kinetics of lung inflammation following infection of macaques with these various influenza viruses and, notably, was an indicator of extrapulmonary tissue reactions in the absence of frank pathology or virus replication. Thus, measurement of HIF-1 expression, e.g. in peripheral blood mononuclear

cells, might be used as a prognostic biomarker in severe respiratory infections.

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