THE PATHOGENESIS OF EXTRA-RESPIRATORY COMPLICATIONS OF INFLUENZA

YNZE JURRE SIEGERS

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Ynze Jurre Siegers

COLOFON

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“If I have seen further it is by standing on the shoulders of Giants”

Isaac Newton, 1675

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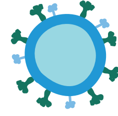
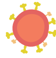

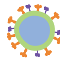

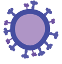

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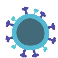
General Introduction



INFLUENZA A VIRUSES

Influenza A viruses —henceforth influenza viruses, are single stranded— negative-sense, and segmented RNA viruses of the family *Orthomyxoviridae* [1, 2]. Other members of the *Orthomyxovirida*e family include influenzaviruses B, C, and D; *Isavirus*; *Thogotovirus*, and *Quaranjavirus* [1, 2]. Further categorization of influenza viruses is based on the subtype of the surface glycoproteins hemagglutinin (HA) and neuraminidase (NA). To date, eighteen different HA subtypes (H1-H18) and eleven NA subtypes (N1-N11) are known [1, 3]. Influenza virus strains derive their names from their genus (type), species of isolation (humans excluded), geographical location, isolate number, year of isolation, and HA and NA subtype. For example, the first H5N1 virus isolate in 1996 from a goose in Guangdong China, is designated as A/Goose/Guangdong/1/1996(H5N1).

Reservoir and inter-species transmission

The natural reservoir of many antigenically diverse (H1-16 and N1-9) influenza viruses are wild birds, predominantly waterfowl (including ducks, geese and swans) and shorebirds (including gulls, terns and waders)[4, 5]. Influenza viruses of the subtypes H17N10 and H18N11 are only found in bats [6]. Occasionally, influenza viruses are transmitted from birds to other species, including but not limited to humans, pigs, poultry, horses, dogs, cats and marine mammals [7]. This cross-species transmission is often a dead-end without sustained transmission within this new host species. However, once an influenza virus is able to replicate and transmit efficiently in the new host, the virus could establish a novel lineage within that species.

Pandemics and epidemics in humans

Epidemics of an antigenically novel virus could jumpstart a pandemic in an immunologically naïve population [8]. Antigenically novel viruses can emerge through reassortment, which is the exchange of gene segments between two (different) viruses and may result in novel surface glycoproteins HA and/or NA, termed antigenic shift [8]. For example, a co-infection with a H1N1 and H3N2 virus could reassort into a H3N1 virus. After the initial pandemic event, "escape" mutations are acquired in antigenic sites over time, due to selection pressure by the host- and herd immunity [8-10]. This immune escape is called antigenic drift and responsible for seasonal influenza virus epidemics.

Since the year 1918, four major influenza pandemics have swept through the human population. Compared to seasonal epidemics, which have a mortality rate of <0.1% [11], pandemics have the potential for increased morbidity and mortality. The 1918 H1N1 pandemic, or 'Spanish flu', is the most lethal viral pandemic ever recorded. In 1918-1919, approximately one third of the global population was infected, and with a mortality rate of 2.5%, responsible for ~50 million deaths [11]. Initially it was thought that the 1918 H1N1 virus was completely derived from an avian source [11, 12]. However, more recent studies revealed that the virus was most likely a reassortment between avian and mammalian (swine

or human) viruses [13-15]. After the pandemic phase in 1918, the H1N1 virus caused seasonal epidemics for almost four decades. In 1957, the 1918 H1N1 virus was replaced by a reassorted virus containing the PB1, HA, and NA gene segments of an avian H2N2 virus [16-18]. The subsequent pandemic H2N2 virus, or 'Asian Flu', was responsible for ~2-3 million deaths worldwide and continued to circulate for 11 years [19]. In 1968, the H2N2 virus was replaced by a pandemic H3N2 virus, or 'Hong Kong Flu', resulting in the death of ~1 million humans worldwide[19, 20]. This H3N2 virus was a reassortment, containing the PB1 and HA gene of an avian H3N? virus and remains circulating in the human population until this day [17, 18, 21]. In 1977, an influenza virus genetically similar to the H1N1 virus circulating prior to 1957 re-emerged into the population and co-circulated with the H3N2 virus until 2009[22-24]. In 2009, the most recent pandemic influenza, the '2009 H1N1 Pandemic' virus emerged and replaced the 1977 H1N1 virus. This 2009 virus was a triple re-assortment between avian, human and swine influenza viruses [25, 26]. Compared to other pandemics, the 2009 H1N1 pandemic virus was relatively mild affecting mainly young adults with an estimated 150–578 thousand deaths worldwide [27]. Nevertheless, the 2009 pandemic was responsible for 3.4 times more years of life lost compared to seasonal epidemics[27] and co-circulates with H3N2 virus ever since.

Influenza A virus: genome structure and organization

Influenza viruses are enveloped viruses containing eight gene segments that carry about ~12-14 kB of genetic material [28]. The envelope, a lipid membrane derived from the host cell, contains the viral HA, NA and matrix-2 (M2) proteins (figure 1A) [1, 29]. Directly underneath the envelope, the viral matrix-1 (M1) protein coats the sub-surface and makes up the interior together with the nuclear export protein/non-structural protein 2 (NEP/NS2, figure 1A)[29, 30]. The core of the virion contains each of the eight gene segments and exists as a viral ribonucleoprotein (vRNP) complex (figure 1B) [29]. The vRNP complex consists of the polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2), polymerase acidic protein (PA), and nucleoprotein (NP) that encapsidates the viral RNA, (figure 1B) [29]. By using alternative splicing, ribosomal frameshifts, or alternative start codons, influenza viruses can expand their coding capacity (figure 1C) [31]. Some of these accessory proteins are not encoded by every influenza virus [31].

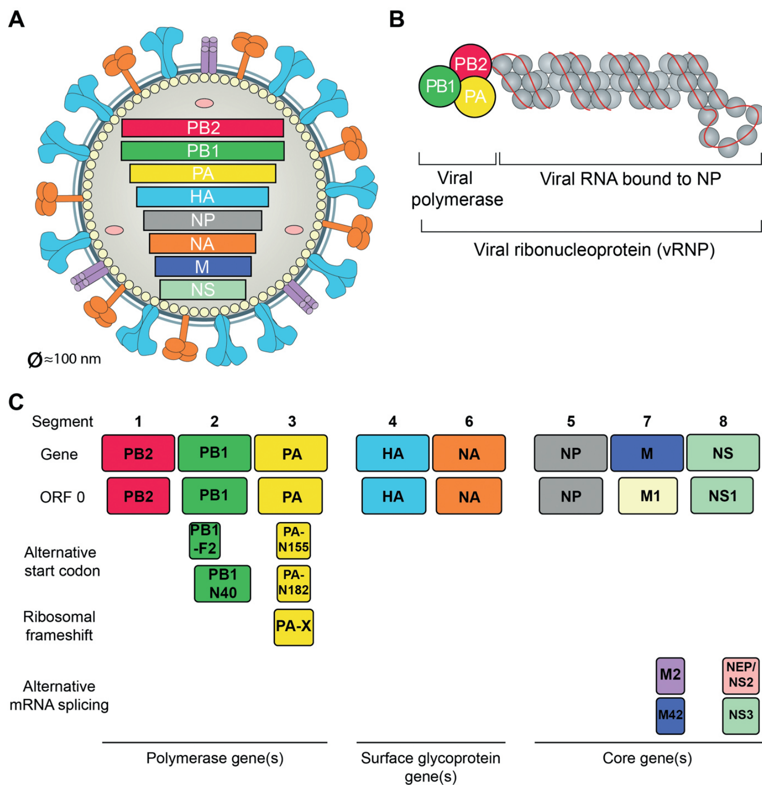


Figure 1. Influenza A virus structure and organization. Schematic representation of the influenza virion structure (A), viral ribonucleoprotein structure, and genome structure and organization (C). PB1; polymerase basic protein 1, PB2; polymerase basic protein 2, PA; polymerase acidic protein, HA; hemagglutinin protein, NA; neuraminidase protein, NP; nucleoprotein, M; matrix protein, NS; non-structural protein, NEP; nuclear export protein. Modified with permission from [71].

Influenza A virus: replication cycle

The full replication cycle of influenza viruses follows several orchestrated stages: (1) virus attachment to host cells, (2) internalization via endocytosis, (3) pH mediated fusion and uncoating of the virion, (4) nuclear import of the vRNPs, (5) transcription, (6) translation, (7) replication, (8) nuclear export of vRNPs, (9) packaging into new virions, and (10) budding and release (figure 2)[1]. In the first step, the HA attaches to sialic acids (SA) present on host cells (figure 2.1) [32]. After attachment, the virus is internalized via endocytosis (figure 2.2) [33, 34]. In the late endosome, the low pH triggers HA-mediated fusion between the viral and endosomal membrane resulting in the uncoating and releasing the vRNPs into the cytosol (figure 2.3) [33, 34]. The uncoating of the virus is dependent on the viral M2 protein which functions as a transmembrane proton ion-channel [35, 36]. The viral M42 protein is thought to act as a functionally alternative to M2 [37]. In the cytosol, the vRNPs which

contain nuclear localization signals, are imported into the nucleus via the host nuclear import machinery where the vRNP dissociates (figure 2.4) [38, 39].

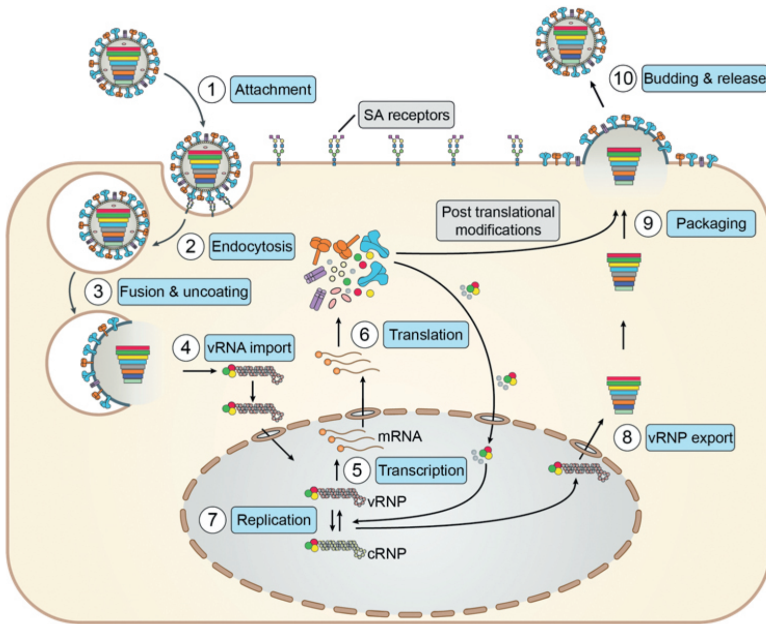


Figure 2. Influenza A virus replication cycle. Schematic representation of the influenza virus replication cycle. SA; sialic acids, vRNP; viral ribonucleoprotein, mRNA; messenger RNA, cRNP; complementary ribonucleoprotein. Modified with permission from [71].

Inside the nucleus, the influenza virus genome is transcribed and replicated by the RNA-dependent RNA polymerase (RdRp), a complex of the PB1, PB2, and PA proteins (figure 2.5, 2.7)[40, 41]. The PB1 protein is involved in initiation of replication and elongation of the RNA chain [42, 43]. The PB2 protein is involved in initiation of transcription by cap recognition and binding on host mRNAs [44-46]. Within the PA protein resides the endonuclease function, critical for cap-snatching [47, 48]. After cap-snatching, the RdRp starts transcribing viral RNA (vRNA) into messenger RNA (mRNA) [49]. These mRNAs are then transported out of the nucleus for translation on ribosomes in the cytosol and on the endoplasmic reticulum (ER) (figure 2.6) [50, 51]. After translation, some viral proteins return to the nucleus to catalyze the replication, transcription and nuclear export of the vRNPs and mRNAs (figure 2.7, 2.8) [51].

To counteract the host innate immune responses during replication, the NS1 protein contains interferon (IFN) antagonizing activity [52]. In addition to the interferon antagonizing activity of NS1, two additional proteins are generated via alternative splicing: NEP/NS2 and NS3. The NEP/NS2 protein is involved in the nuclear export of RNPs and regulation of

the RNA synthesis [53-56]. The NS3 protein is thought to be important for host switching of the virus [57]. The PB1 encodes two additional proteins, PB1-F2 and PB1-N40, through alternative start codons. The PB1-F2 protein is a proapoptotic protein and IFN antagonist that modulates innate immune responses [58-60]. PB1-N40 is thought to maintain the balance between PB1 and PB1-F2 expression but further research should verify this theory [61]. The protein generated from a ribosomal frameshift in the PA segment, PA-X, is involved in modulating the host immune response [62]. Through alternative start codons, two truncated PA proteins, namely PA-N155 and PA-N185, were identified. These truncated proteins are thought to play a role in the replication cycle, but the exact mechanism remains to be elucidated [63, 64].

The last steps of the influenza virus replication cycle involve virus assembly and release. Several viral proteins are involved in different stages of the assembly and budding process. The membrane bound proteins HA, NA, and M2 are translated by ribosomes on the ER and transported via the Golgi apparatus, where post translation modifications are made (e.g. glycosylation of HA and NA), to the cytosolic membrane [51, 65, 66]. In the nucleus, new vRNAs assemble into vRNPs and bind M1 proteins that are subsequently bound by NEP/NS2 proteins. These NEP/NS2 proteins contain nuclear export signals and escort the vRNPs to the cytoplasm (figure 2.8) [38, 67, and 68]. At the apical membrane, vRNPs are packaged into new virions under guidance of M1 proteins that have accumulated underneath the host cell membrane [69] (figure 2.9). Here, the M1 proteins initiates outward budding, possibly aided by the virus structural proteins, until the host cell membrane fuses at the basal surface via fission[70] (figure 2.10). The final steps involve NA-mediated cleavage of the virion from the host cell sialic acids [70] and cleavage of the immature HA, discussed into detail below.

Host and viral factors important for virus tropism

Influenza virus replication is dependent on the interplay of many host and viral factors. One of the key determinants of host range and cell- and tissue tropism is the ability and specificity of the virus to attach to a host cell [32]. Human influenza viruses attach preferably to glycans with a terminal SA linked to the underlying galactose by α-2,6 linkage whereas avian influenza viruses preferably attach to α-2,3 linked SAs[32, 72-77]. Epithelial cells in the upper respiratory tract of humans predominantly express α-2,6 linked SAs where in the lower respiratory tract both α-2,6 an α-2,3 linked SAs are present[32, 72-77]. Simply said, human adapted influenza viruses prefer α-2,6 SA present in the upper respiratory tract where avian influenza viruses prefer α-2,3 SA present in the lower respiratory tract. In addition to this, α-2,8 SA or even SA-independent attachment through C-type lectins such as DC-SIGN, L-SIGN, and MGL-1 have also been recently reported[78, 79]. Collectively, human and avian influenza viruses attach to and infect different parts of the human respiratory tract [80, 81].

The last step in the influenza virus replication cycle is achieved with activation of the immature-to-mature HA, essential for the infectivity of influenza virus progeny and a major determinant of pathogenicity [82-84]. During influenza virus replication, the HA is

synthesized as an 'immature' precursor protein HA0 [85, 86]. To become fusogenic, the HA0 protein has to be enzymatically cleaved into the 'mature' HA1 and HA2 proteins by host proteases. This cleavage site is located in a loop that projects from the HA surface and cleavage allows exposure of the fusion peptide [87]. Different influenza viruses have different amino acid sequences and loop conformations [88, 89], determining cleavability by different types of host proteases. This further contributes to the tissue tropism of the virus since presence of these proteases is restricted to certain tissues [90].

Avian influenza viruses are classified either as low- or high-pathogenic avian influenza viruses (LPAI or HPAI, respectively) based on their pathogenicity in chickens. Viruses that contain a mono basic amino acid at the cleavage site are classified as LPAI and replicate predominantly in the intestinal tract of mallards and black-headed gulls [90-92]. Like LPAI viruses, human influenza viruses contain a mono-basic cleave site and replicate predominantly the respiratory tract [87]. In vitro, LPAI viruses are cleaved by trypsin and trypsin-like proteases such as plasmin, tryptase Clara, and a chicken homologue of human factor X, a serine protease [84, 93-95]. Cleavage of the virus in the human respiratory tract likely happens by the proteases transmembrane protease serine S1 member 2 (TMPRSS2), human airway trypsin-like protease (HAT), and TMPRSS4 [96, 97] at the trans-Golgi network and plasma membrane [98]. However, in birds, proteases that cleave influenza viruses in the intestinal or respiratory tract remain to be identified. The chicken homologue to human TMPRSS2 demonstrated HA cleavage in vitro, thereby providing a glimpse into avian proteases capable of cleaving influenza viruses [99]. Another hypothesis argues the proteolytic cleavage of the HA by bacteria in the digestive tract of poultry [100].

Occasionally, in poultry farms ranging in size from backyard farming to large commercial and industrial farms, LPAI viruses evolve into HPAI that can trigger devastating outbreaks and spillovers to wild birds. In these poultry farms, HPAI viruses have only emerged from LPAI subtypes H5 and H7 [101]. Whether other HPAI virus subtypes could emerge at poultry farms remains to be elucidated. Evolution of LPAI into HPAI viruses happens through the addition of basic amino acids in the cleavage site, which create a multi basic cleavage site (MBCS) [101]. As a result, the cleavage site of a HA is cleaved by the subtilase-like protease furin, ubiquitously expressed in poultry and humans[87, 102-104], allowing systemic replication of the virus[105-107]. The exact mechanism(s) behind these insertions are not fully understood but could be the result of recombination, the RNA structure at the cleavage site, viral polymerase function, and possibly host factors [107, 108].

Severe disease and systemic infection of HPAI viruses is observed in many mammalians and certain bird species. However, it seems that HPAI H5N1 viruses isolated from humans are often more pathogenic in ferrets and mice, including systemic replication, than HPAI H5N1 viruses isolated from avian species [109-111]. Similar differences in pathogenicity of a HPAI virus are found between certain bird species (see section 'Pathogenesis in the natural reservoir & poultry'). This indicates that although a MBCS is important for systemic spread, it is not

the only factor. This could mean that furin is not completely conserved or ubiquitously expressed amongst wild bird species.

In addition to SA preference and MBCS presence, it is likely that other host and/or viral factors that influence immune responses or polymerase function are necessary for adaptation of the virus to a new environment. Whether that new environment is a different host species or a different organ system. Viral adaptation to such host factors could include importins [112, 113], essential for nuclear import of vRNPs or ANP32A proteins [114-116], important for functioning of the viral polymerase between avian and mammalian cells.

PATHOGENESIS

Pathogenesis in the natural reservoir & poultry

Wild aquatic birds are considered the natural reservoir of LPAI viruses that circulate in seasonal epidemics [5]. In these birds the virus mainly replicates in epithelial cells of the intestinal tract without obvious signs of disease or lesions [4, 91, 92, 117-122]. In contrast to wild birds, in poultry, the virus mainly replicates in the epithelial cells of the respiratory tract and is associated with limited signs of disease [123, 124]. However, in some poultry species, HPAI viruses of the subtype H5 and H7 can cause severe disease and up to 100% mortality [88, 125-129]. In wild birds, HPAI viruses are considered spillover events from poultry [130-132]. The pathogenicity of HPAI in wild birds is species specific and varies largely from severe and systemic disease in some wild aquatic birds [133, 134] to subclinical disease in others [135-138]. In some species of both wild birds and poultry, HPAI viruses can replicate systemically and associated with inflammation and necrosis [139-141]. Additionally, in some poultry species, systemic replication is associated with prominent replication in and damage to endothelial cells ultimately leading to edema and hemorrhage [139-141]. Collectively, the pathogenesis and disease severity in birds is dependent on the virus strain and bird species.

Pathogenesis in humans

Disease burden

Influenza A (and B) viruses are a major cause of human disease and seasonal influenza epidemics infect about 5-10% of adults and 20-30% of children globally[142, 143]. This is approximately ~1 billion cases per year, with 3-5 million hospitalizations, and 290-650 thousand deaths [142, 143]. In humans, only viruses of subtype H1N1, H2N2, and H3N2 have circulated extensively [144]. Besides human seasonal influenza viruses, zoonotic avian viruses occasionally transmit to humans and when they do, often cause severe disease. Since 2003 there have been 861 confirmed human cases with 455 deaths for avian H5N1 virus infection [145]. For infections with H7N9 virus, 1568 confirmed cases with 616 deaths [146]. Luckily, until this day, these zoonotic viruses are not transmitted efficiently between humans.

Clinical symptoms and primary complication

Human influenza viruses typically infect the upper respiratory tract and larger airways and the majority of infected individuals experience mild disease [147]. Symptoms of uncomplicated or mild influenza, —a tracheobronchitis—, are both local and systemic. Local symptoms include nasal obstruction, dry cough, and sore throat. Systemic symptoms include headache, confusion, chills, fever, myalgia, and anorexia. These symptoms are caused by local virus replication in respiratory epithelial cells and systemic release of pro-inflammatory cytokines and other immune mediators [1, 147-149].

Human influenza viruses are also able to cause severe disease. The primary complication of influenza virus infection is viral pneumonia. Viral pneumonia is caused by damage to the alveolar epithelium that consists of type I and type II pneumocytes, important for the gas exchange, and surfactant production, respectively [147]. Damage to these pneumocytes occurs through direct cytolytic effect of virus infection and indirect effect by the host immune system [150]. As a consequence, edema fluid, containing fibrin and erythrocytes, leaks into the alveolar lumina along with the accumulation of alveolar macrophages and other infiltrating leukocytes. Collectively, this has severe consequences for the gas exchange and therefor lung function, and could cause a potentially fatal respiratory dysfunction [147, 151].

Extra-respiratory tract complications

The clinical presentation of complicated influenza is diverse and can include organ systems other than the respiratory system. The most common and under-recognized extra-respiratory complications of influenza are central nervous system (CNS) and cardiac disease [152]. Influenza viruses have been linked to CNS disease since the 1918 H1N1 pandemic [153, 154] and observed during all subsequent pandemics [155-160]. During seasonal epidemics, sporadic detection of influenza virus in the CNS is observed [161-165]. In humans, CNS complications from influenza virus infections, or influenza-associated encephalopathy/ encephalitis (IAE) can occur with little or no involvement of the respiratory system [155, 161, 166, and 167]. In addition, influenza virus CNS invasion is observed for several strains and subtypes but seems to be more frequently observed for HPAI H5N1 viruses than pandemic viruses and even less for seasonal viruses [147, 168]. Whether this is a result of intrinsic differences between the viruses or a sampling bias remains to be investigated since neuroinvasiveness and replication efficiency of different influenza virus subtypes have never been investigated comprehensively.

The clinical presentation of IAE varies. The disease can range from mild febrile seizures and the acute onset of brain dysfunction (decreased consciousness, delirium) to more severe and potentially life-threatening conditions [152]. These include Reye's syndrome, Guillain-Barré syndrome, Kleine-Levin syndrome, encephalitis lethargica, post-encephalitic Parkinson's disease, acute necrotizing encephalopathy, myelitis, and (meningo)encephalitis [147, 152, 169, 170].

The incidence of IAE is difficult to calculate due to limited epidemiologic data, diagnostic challenges to detect virus in the CNS and/or CSF, and lack of a standardized case definition for IAE. However, increased recognition indicates that especially pediatric patients are at risk [152, 171-173]. The incidence of IAE in 1987-1988 in Sweden was estimated at 1.5 per 1000 hospitalized patients[174] and of symptomatic pandemic H1N1 2009 virus infections at 1.2 per 100 000 patients[172]. In hospitalized children, 6-19% of patients with pandemic 2009 H1N1 virus had neurologic disease [156, 172, 175, 176] compared to 1.9% of children over eight non-pandemic seasons [171, 177]. This indicates that there are large incidence differences between circulating strains and age distribution.

The seasonal overlap of peak incidences for both cardiovascular disease (CVD) and influenza has been observed for years and epidemiological investigations have indicated that influenza virus infection is a risk factor for CVD[152, 178-181]. These CVDs include heart failure, acute myocardial infarction, acute ischemic heart disease and myopericarditis [152]. Although absolute numbers on influenza-associated CVD are not available, a recent study observed that within the first seven days after laboratory confirmed influenza virus infection, hospitalization for acute myocardial infarction was six times higher, consistent with previous reports [180, 182]. Collectively, influenza-associated CNS and cardiac disease are underappreciated and could have a major impact on disease mortality and morbidity during seasonal epidemics and pandemics.

In addition to CNS and cardiovascular diseases, influenza viruses have been associated with other extra-respiratory tract complications. Musculoskeletal symptoms such as myalgia are often reported in influenza and in the more serious complication rhabdomyolysis, influenza virus is most often identified as causative agent [152, 183, and 184]. The most common ocular disease is conjunctivitis and can be the result of direct infection of the conjunctival epithelium [185]. Other eye diseases such as uveal effusion syndrome, optic neuritis, acute retinitis, and bleeding follicular conjunctivitis have also been reported [152, 185]. Renal complications of influenza such as acute kidney injury can occur in 18-66% of intensive care unit patient s[186-188]. However, whether renal complications of influenza are a direct consequence of virus infection, or an exacerbation of an underlying disease remains to be elucidated [152]. Influenza virus associated liver disease is historically rarely reported but more recent evidence suggests that it does occur [152, 189]. Interestingly, in 78% of fatal 2009 H1N1 virus cases, histopathologic evidence of centrizonal hemorrhagic necrosis was observed [190]. Although influenza virus is allegedly found in liver endothelial cells, sinusoidal epithelial cells, and Kupffer cells [189-194], influenza-associated liver disease is likely a result of the systemic immune response [189]. Hematological complications of influenza virus infection are rare and epidemiologic studies are scarce, occasionally contradictory, and speculative but include thromboembolic disease, thrombotic thrombocytopenic purpura, hemolytic-uremic syndrome, and hemophagocytic syndrome [152]. Endocrine complications of influenza such as diabetic ketoacidosis is occasionally reported but whether there is a true

association between influenza and endocrine complications or exacerbation of underlying disease, remains to be investigated[152].

The number of post mortem examinations and therefor available data about the pathogenesis of IAE in humans is limited. The pathogenesis of IAE is not completely understood but seems to include direct infection, local inflammation of neurons and systemic inflammation [195]. To investigate the pathogenesis of IAE, experimental infections of ferrets, mice and nonhuman primates are used [196]. Since ferrets are naturally susceptible to human and avian influenza viruses and clinical symptoms mimic human infections, pathogenesis and transmission studies on this species is preferred [196].

Sensory innervation of the respiratory tract

The respiratory tract is innervated by several branches of the peripheral nervous system that control all aspects of respiration from mucous secretion and vasoconstriction to sensation and cough reflexes [197]. The tropism of influenza virus in the respiratory tract are primarily epithelial cells of the respiratory mucosa which are mainly innervated by sensory neurons. Therefore, this thesis will focus on the sensory innervation of the respiratory tract and its role in the pathogenesis of influenza virus associated CNS disease.

In the nasal cavity, two types of ciliated mucosa exist, the respiratory mucosa and olfactory mucosa [198]. The main function of respiratory mucosa is moistening and protection of the airways by secreting mucus and mucocilliary clearance [197]. The respiratory mucosa is innervated by the trigeminal nerve (cranial nerve V) and responsible for sensation of the nasal cavity [198]. The olfactory mucosa is mainly innervated by the olfactory nerve (cranial nerve I) but also by the trigeminal nerve and are important for olfaction and sensation respectively[197, 199]. The free nerve endings of the trigeminal nerve reside very close to the epithelial surface, just underneath the tight junctions (figure 3)[199]. The soma of the sensory neurons that innervate both the respiratory and olfactory mucosa are located in the trigeminal ganglion which directly connects with the brainstem [199].

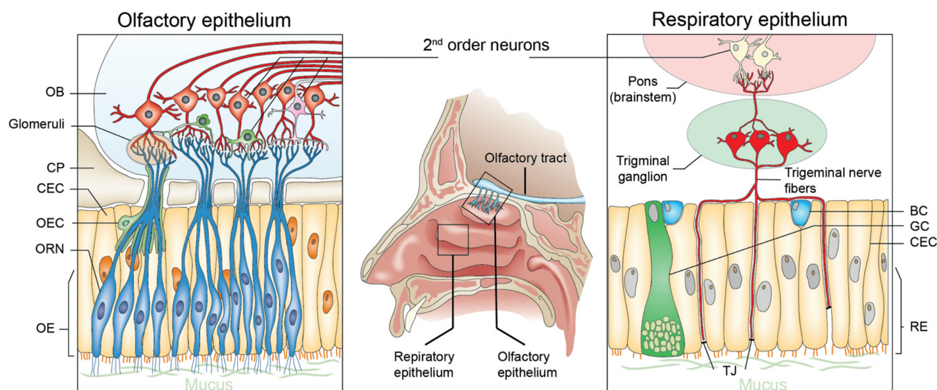


Figure 3. Sensory innervation of the nasal respiratory and olfactory mucosa. Simplified and schematic overview of the innervation of the nasal respiratory and olfactory mucosa by cranial nerves. OB; olfactory bulb, CP; cribriform plate, CEC; columnar epithelial cell, OEC; olfactory ensheathing cell, ORN; olfactory receptor neuron, OE; olfactory epithelium, BC; basal cell, GC; goblet cell, RE; respiratory epithelium, TJ; tight junction. Modified with permission from [228].

Olfactory mucosa is specialized epithelium involved in olfaction and contain specialized neuronal cells termed olfactory receptor neurons (ORN). These chemosensory ORNs are bipolar neurons, having apical dendrites facing the lumen of the nasal cavity where they sample odor molecules. On the basal side, the axons of ORNs passes through the cribriform plate and terminate in the glomeruli of the olfactory bulb (figure 3) [198].

In addition to the trigeminal and olfactory nerve, the respiratory tract is innervated by three types of vagal (cranial nerve X) afferents; stretch receptors, irritant receptors, and pulmonary C-fibers. Stretch receptors are found in the tracheal, bronchial, and bronchiole smooth muscles and are sensitive to changes in the lung volume (e.g. to prevent overstretching during heavy exercise) [197, 200, 201]. Irritant receptors are found between and below the epithelium of the larynx, trachea, bronchi, and bronchioles and are involved in the cough and sneeze-reflex [197, 200, and 201]. Lastly, pulmonary C-fiber are found in the pulmonary interstitium and respond to decreased oxygenation during events such as pulmonary edema and pneumonia [197, 200, and 201].

Influenza virus central nervous system invasion and spread

Viruses can invade the CNS via several ways including peripheral nerves and the hematogenous route. For influenza viruses, CNS invasion via peripheral nerves is most often described. Epithelial cells of the nasal respiratory mucosa are the primary target of many human influenza viruses [81]. Avian influenza viruses mainly target cells of the olfactory mucosa in the nasal cavity [168]. The free nerve endings of the trigeminal and olfactory nerves present in these tissues offer a potential site of entrance for influenza viruses into the CNS [202]. Influenza virus CNS invasion via cranial nerves has been observed for the olfactory

[203-205], trigeminal [154, 204, 206, 207], vagal [204, 206-208], and vestibulocochlear [203] nerves. Other nerves such as the sympathetic nerve have also been occasionally associated with CNS invasion [204, 208].

Of the peripheral nerves, the olfactory and trigeminal nerves are considered the main routes of entrance into the CNS since infection of their nuclei often precedes that of other nerves [168, 203]. In mice and ferrets infection of specifically ORNs, -based on morphology and location in the olfactory mucosa-, have been described for several viruses including zoonotic H5N1 [203, 209-213], seasonal H3N2 [209], pandemic 2009 H1N1 [209], and A/WSN/33[214] viruses. In ferrets, infection efficiency of the olfactory mucosa and ORNs seems strain dependent. Numerous infected cells are observed in H5N1 virus infections followed by intermediate and low numbers of infected cells with 2009 H1N1 and H3N2 virus respectively [168]. The opposite is observed for infection of nasal respiratory mucosa [168]. In humans, direct evidence for CNS invasion via the olfactory nerve was observed in an immunocompromised child. Here, a seasonal H3N2 virus was detected in the olfactory bulb and olfactory tract but not in other parts of CNS nor in plasma [161].

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Transport of the virus from the nasal cavity to the olfactory bulb and brain stem is not completely understood. The most efficient way for virus transport to the olfactory bulb seems via trans-axonal transport. Here the virus infects ORNs and is transported along the axons that synapse within the glomeruli of the olfactory bulb with second order neurons (figure 3) [215]. Second order neurons include mitral cells, periglomerular cells, and/or tufted cells in the olfactory bulb (figure 3) [216]. Subsequent infection of second, third, etc. order neurons allows the virus to travel along the olfactory tract (Figure 3). In addition to trans-axonal transport, the virus may reach the olfactory bulb via diffusion through channels formed by olfactory ensheathing cells (OEC) [217]. These OECs are glial, Schwann cell-like cells that support and line the axon from the olfactory epithelium to the olfactory bulb and create a fluid-filled perineural channel [217]. Virus infected cells in the olfactory submucosa surrounding the nerve twigs indicate that these cells are most likely OECs [212].

The mechanism of viral CNS invasion via the trigeminal nerve is poorly understood. One hypothesis is based on infection of respiratory mucosal cells in the nasal cavity. Subsequent cell death of infected mucosa cells exposes the free nerve endings that lie in between nasal epithelial cells. The virus then enters the axon either via binding to receptors on the axon termini or membrane fusion, similar to some herpes viruses [218, 219]. From here the virus is transported trans-axonally[215] via the trigeminal ganglion to the trigeminal nerve nuclei in the brain stem (figure 3). A second hypothesis, -similar to OECs in the olfactory epithelium-, is based on virus transport throughout channels formed by trigeminal nerve Schwann cells [220, 221]. In two human case reports, -although no direct evidence of peripheral nerve invasion was observed-, CNS invasion of a seasonal H3N2 and pandemic 2009 H1N1 virus was reported by PCR from a brain biopsy in immunocompetent adults[155, 222]. These reports suggest virus invasion via cranial nerves due to the absence of clinical respiratory

tract disease and extensive systemic infection.The mechanism of viral CNS invasion via the vagal nerve is likewise poorly understood. Similar to the trigeminal nerve, the virus may enter the CNS via exposed axon termini after epithelial cell death or via channels formed by vagal nerve Schwann cells.

The hematogenous route involves infection and/or disruption of the blood-brain barrier allowing virus dissemination or a 'Trojan horse' mechanism [223-225]. The 'Trojan horse' mechanism of CNS invasion involves infection of leukocytes that transmigrate the blood-brain barrier and/or the blood-CSF barrier [223-225]. However, for influenza viruses it seems that hematogenous spread plays a more important role in extra-respiratory spread to other organs than the CNS [203, 212]. Further investigations into the mechanism(s) and frequency of hematogenous spread and CNS invasion are warranted.

Once the virus reaches the CNS, virus dissemination appears centrifugal from cell to cell in the brain parenchyma and via the cerebrospinal fluid (CSF) [203, 205, 226, and 227]. Viral spread via the CSF is thought to occur due to the weak barrier between the CSF and olfactory bulb [203, 205, 226, and 227]. This weak barrier allows virus access to tissues around the subarachnoid space and ventricular system and would explain infection of the leptomeninges, choroid plexi, and ependyma observed in ferrets [205].

Taken together, it seems that different influenza virus subtypes are able to invade the CNS with variable efficiencies and via different routes. Gaining a better understanding on the exact route of CNS invasion by these viruses could result in the development of more efficient therapeutics to block and or reduce virus spread to the CNS.

Risk groups for severe influenza

The morbidity and mortality rates of influenza are not evenly distributed in the population during seasonal epidemics and pandemics. During seasonal influenza epidemics, young children and the elderly are at the highest risk of being hospitalized [229-232]. Next are individuals with chronic medical conditions such as cardiac, pulmonary, metabolic, renal, neurodevelopmental, liver or hematologic diseases and the immunocompromised [229-232].

During the 2009 H1N1 pandemic, a shift in age distribution towards older children and younger adults was observed whilst complications in adults older than 60 years were rare. In adults older than 60 years, this phenomenon was attributed to preexisting immunity to viruses of the same subtype circulating before 1950[233, 234]. During this pandemic, obesity was identified as a novel and independent risk factor for severe influenza [233, 235-240]. Amongst patients with severe obesity, a body mass index (BMI) of ≥35 or morbid obesity (BMI ≥40), severe or fatal influenza was reported 5-15 times higher compared to the general population [240]. In addition to obesity, excess weight (BMI ≥25) was also identified as a risk factor for hospitalization, admission to the intensive care unit, mechanical ventilation and death after influenza virus infection [241-243]. This increased susceptibility

has been associated with an impaired and altered functioning of the immune system in obese individuals [244]. However, during subsequent seasonal influenza virus epidemics, obesity as a risk factor for severe disease seemed less prominent [245, 246] or even absent [247, 248]. Given that more than 1.9 billion people are currently overweight or obese and excess weight will affect 90% of adults by the year 2050[249, 250], the role of excess weight on the pathogenesis of influenza is essential information for patient management as well as for future influenza (pandemic) preparedness.

Influenza virus CNS disease: prevention and treatment

Treatments for influenza are primarily designed to prevent or reduce respiratory tract disease. The most effective way of preventing influenza virus disease is avoiding infection through vaccine-induced neutralizing antibodies. Unfortunately, seasonal influenza vaccine effectiveness varies each season. In adults, the efficacy of trivalent inactivated vaccines was between 51-67% over eight influenza seasons [251]. In addition to vaccination, antivirals are developed that interfere with functioning of the virus. Currently, the FDA-approved antivirals comprise three neuraminidase inhibitors (oseltamivir, zanamivir, peramivir) and one polymerase inhibitor (baloxavir marboxil). In order to fulfill the antiviral effect, the drugs need to reach the CNS. Unfortunately, only little is known about the absorption in CNS of these FDA-approved antivirals. For oseltamivir, absorption of the bioactive oseltamivir carboxylate into the CSF in humans is low [252, 253]. And whether the concentration that penetrates the CSF also reaches therapeutic levels remains unknown. In addition, it is likely that the immune system too, plays an important role in the pathogenesis of IAE. It is proposed that modulation of the immune response might influence IAE disease severity, although this remains poorly evaluated [195, 254]. Collectively, to this day, it remains unclear whether vaccines, antiviral drugs or immunomodulatory therapies have the potential to prevent or reduce influenza virus CNS invasion and disease.

AIMS AND OUTLINE OF THIS THESIS

The aim of the research described within this thesis is to expand our knowledge and shed light on the mechanism of extra-respiratory complications of influenza virus infection. Large differences in the pathogenesis, including the ability to cause extra-respiratory disease exist between seasonal, pandemic, and zoonotic viruses, briefly introduced in **chapter 1**.

To better understand the systemic pathogenesis of the 1918 H1N1 virus, the most devastating pandemic influenza virus, we studied the pathogenesis in ferrets in **chapter 2**. In this chapter, we focused on CNS invasion and the involvement of extra-respiratory tissues.

Chronic medical conditions can influence the pathogenesis of respiratory virus infections. **Chapter 3** evaluated the effect of excess weight on the (systemic) pathogenesis of pandemic 2009 H1N1 virus infection in mice, with a focus on the cardiovascular system.

Until this doctoral thesis, it was unknown whether standard medical interventions such as vaccination and oseltamivir could prevent CNS invasion and disease by HPAI H5N1 virus. In **chapter 4**, we evaluated the effect of vaccination and oseltamivir on viral CNS invasion and disease in ferrets.

Seasonal, pandemic, and zoonotic influenza viruses differ in their ability to invade and replicate in the CNS. In order to get more insight into the viral factors important for the ability to invade and replicate in the CNS, the replication kinetics of these different influenza viruses was studied in vitro in **chapter 5**.

Zoonotic influenza viruses are able to adapt to new hosts and tissues. Potential within host adaptation of HPAI H5N1 virus to the ferret CNS was identified and identified mutations were comprehensively characterized using in vitro and in vivo models in **chapter 6**.

A summarizing discussion of the work presented in this thesis is given and potential implications of these studies are discussed in **chapter 7** to advance our understanding of influenza virus associated CNS invasion and disease.

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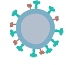

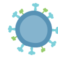

1918 H1N1 Influenza Virus Replicates and Induces Proinflammatory Cytokine Responses in Extrarespiratory Tissues of Ferrets

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ABSTRACT

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 extra-respiratory tract complications—which are often severe or even fatal—the potential contribution of extra-respiratory 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 extra-respiratory tissues. Respiratory and extra-respiratory 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 extra-respiratory 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

INTRODUCTION

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 extra-respiratory virus replication, systemic proinflammatory cytokines, which can be detected in the circulation of infected patients, or in extra-respiratory 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 extra-respiratory tissues.

2

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, extra-respiratory 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 extra-respiratory 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 extra-respiratory tissues. We show that 1918 H1N1 virus replicated efficiently in the upper and lower respiratory tract and spread to extra-respiratory 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 extra-respiratory 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 post-inoculation (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*).

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

Clinical Sign	No./Total (Days Post-inoculation)
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 post-inoculation these clinical signs were observed).

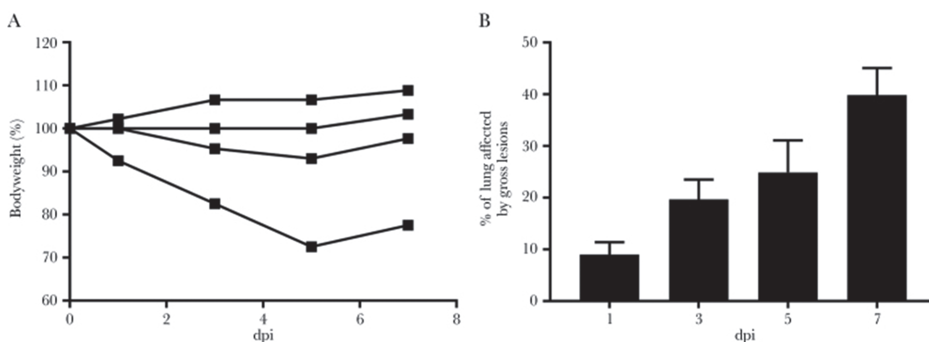

Figure 1. Bodyweight loss and gross lung lesions in 1918 H1N1 virus–inoculated ferrets. Ferrets were inoculated intranasally with 10^6 50% tissue culture infectious dose ($TCID_{50}$) of 1918 H1N1 influenza virus. A, Bodyweight of ferrets throughout the experiment was calculated as the percentage of start weight. Each line represents an individual ferret euthanized at 7 days post-inoculation (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.

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*).

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*).

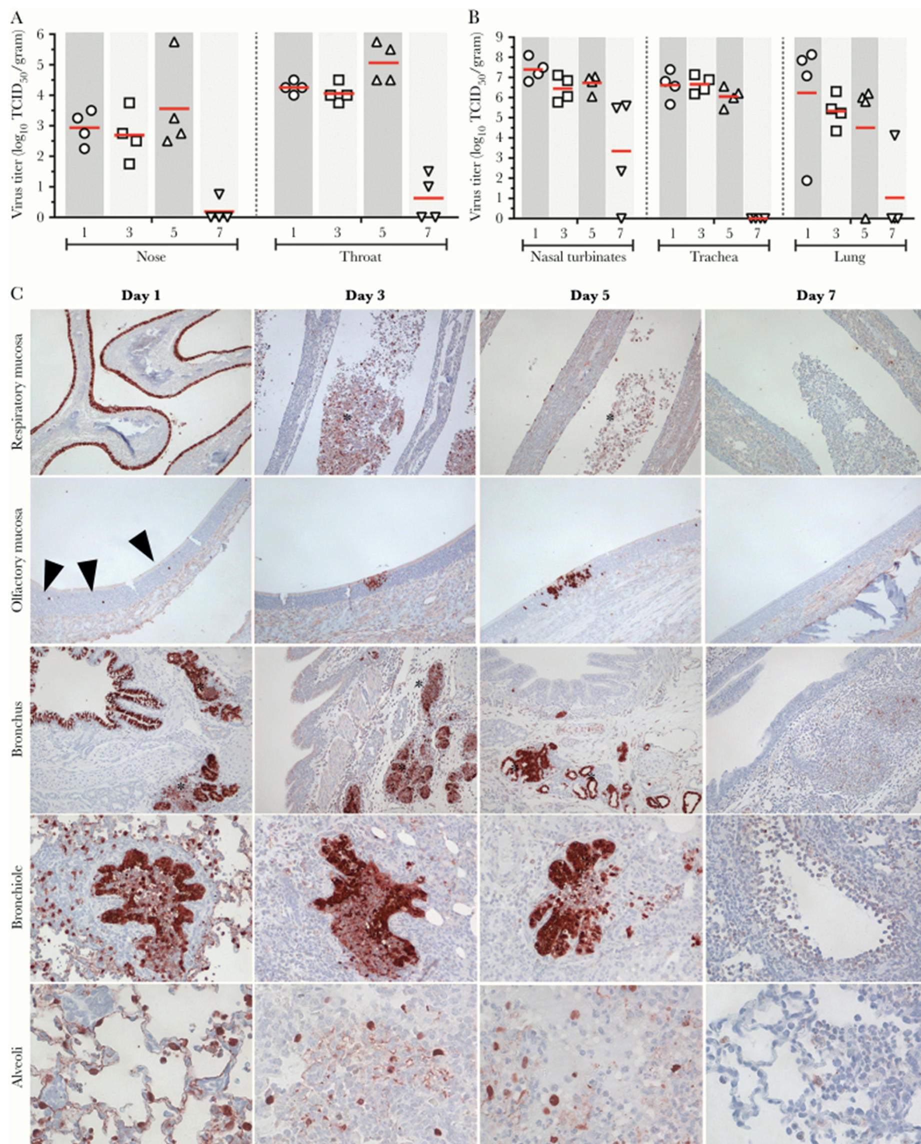

Figure 2. Virus shedding and replication in the respiratory tract of ferrets inoculated with 1918 influenza H1N1 virus. Ferrets were inoculated intranasally with 10^6 50% tissue culture infectious dose (TCID$_{50}$) of 1918 H1N1 influenza virus. A, Virus titers in nose and throat swabs collected at 1-, 3-, 5-, and 7-days post-inoculation (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 post-inoculation.
[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-days post-inoculation, respectively.

In the lower respiratory tract, there was a severe necrotizing suppurative broncho-interstitial pneumonia and broncho-adenitis 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*).

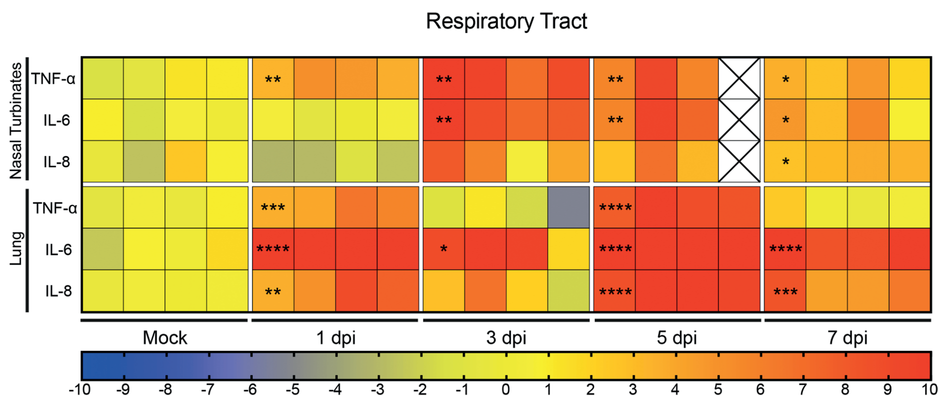

Figure 3. Induction of tumor necrosis factor alpha (TNF-α), interleukin 6 (IL-6), and interleukin 8 (IL-8) in 1918 H1N1 virus–inoculated ferrets. Ferrets were inoculated intranasally with 10^6 50% tissue culture infectious dose ($TCID_{50}$) of 1918 H1N1 influenza virus. Four ferrets were euthanized at each of the time points 1-, 3-, 5-, and 7-days post-inoculation (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.

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. 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.

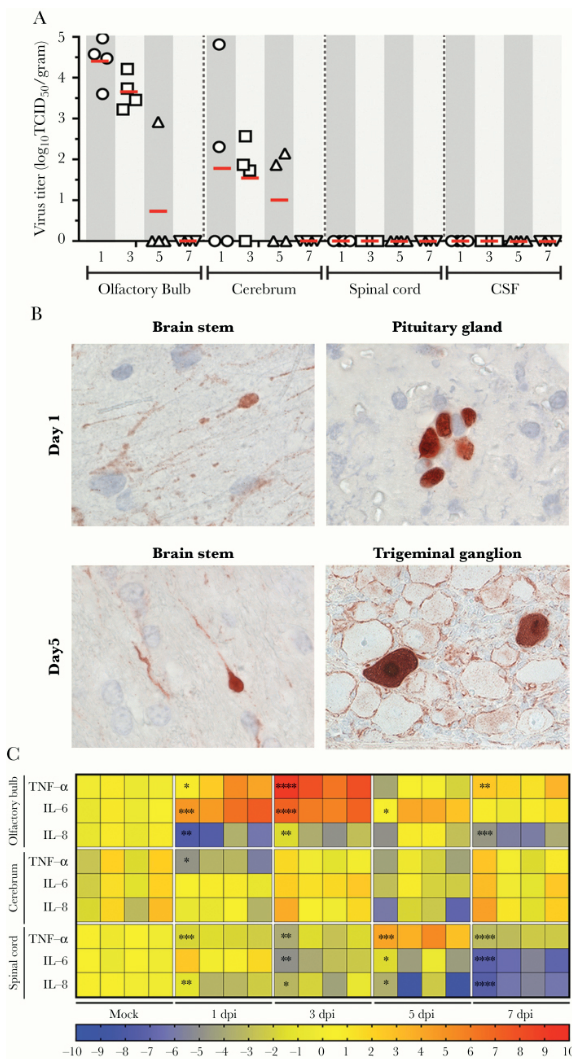

Figure 4. Virus titers, virus antigen, and cytokine expression in the nervous system of 1918 H1N1 virus–inoculated ferrets. Ferrets were inoculated intranasally with 10^6 50% tissue culture infectious dose ($TCID_{50}$) of 1918 H1N1 influenza virus. Four ferrets were euthanized at each of the time points 1-, 3-, 5-, and 7-days post-inoculation (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. $^*P \leq .05$; $^{**}P \leq .01$; $^{***}P \leq .001$; $^{****}P \leq .0001$.

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 Extra-respiratory 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 extra-respiratory 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).

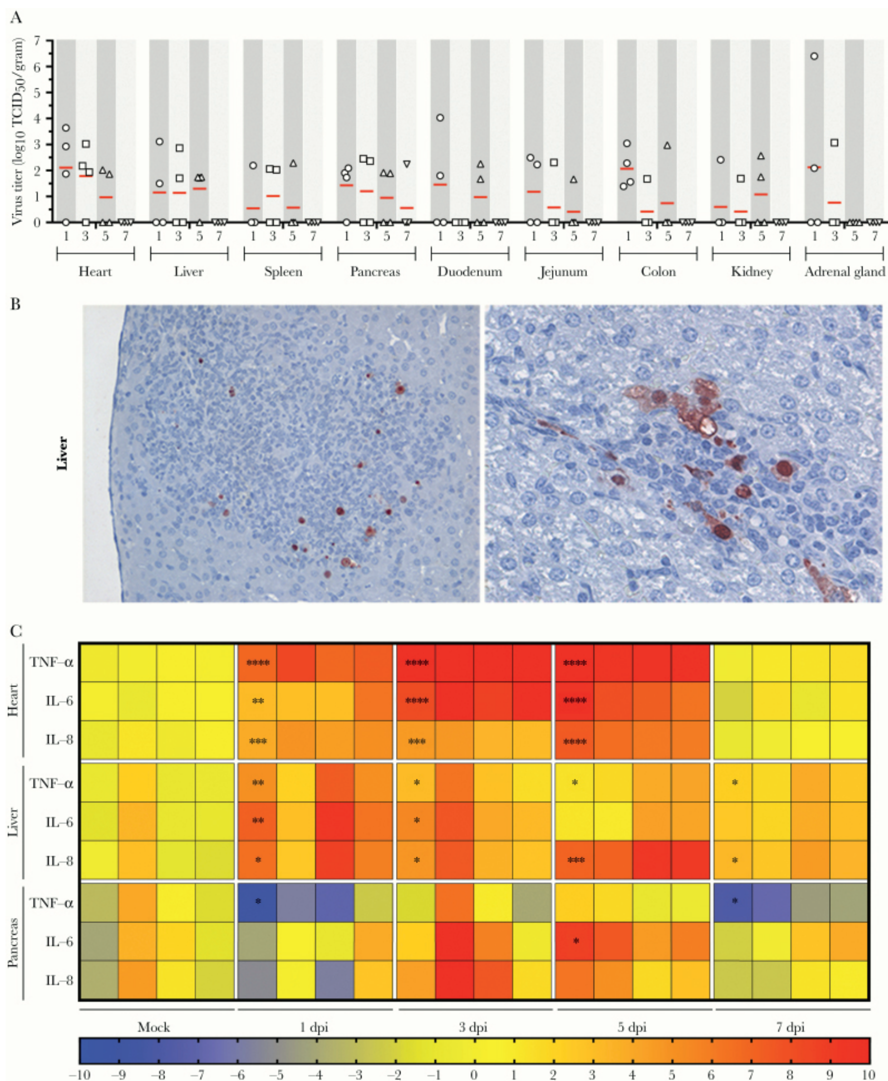

Figure 5. Virus titers, virus antigen, and cytokine expression in 1918 H1N1 influenza virus–inoculated ferrets. Ferrets were inoculated intranasally with 10^6 50% tissue culture infectious dose ($TCID_{50}$) of 1918 H1N1 influenza virus. Four ferrets were euthanized at each of the time points 1-, 3-, 5-, and 7-days post-inoculation (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. *$P \le .05$; **$P \le .01$; ***$P \le .001$; ****$P \le .0001$.

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 extra-respiratory tissues are involved in pathogenesis of 1918 H1N1 virus infection in ferrets. Virus was isolated from almost all extra-respiratory tissues sampled, and active virus replication—evidenced by the detection of nucleoprotein in the nucleus by immunohistochemistry—occurred in the nervous system, pituitary gland, and liver. In addition, proinflammatory cytokines were detected in respiratory as well as extra-respiratory 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 post-encephalitis 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 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 extra-respiratory tissues. Virus has also been isolated from extra-respiratory 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 extra-respiratory 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 extra-respiratory 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 extra-respiratory 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 extra-respiratory 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 extra-respiratory tissues has not previously been observed for pandemic 2009 H1N1 virus [*23*]. Extra-respiratory replication has been observed in ferrets inoculated with

HPAI H5N1 virus, which was dependent on the presence of a multi-basic cleavage site [*25*]. Although the 1918 H1N1 virus HA does not contain a multi-basic cleavage site, it is not dependent on cleavage by trypsin for replication, suggesting that the cleavage of HA may be an important determinant of extra-respiratory virus replication.

Extra-respiratory 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

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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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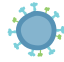

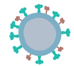
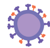
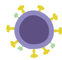

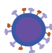

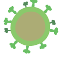

3

A High-Fat Diet Increases Influenza A Virus-Associated Cardiovascular Damage

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ABSTRACT

Background

Influenza A virus (IAV) causes a wide range of extra-respiratory complications. However, the role of host factors in these complications of influenza virus infection remains to be defined.

Methods

Here, we sought to use transcriptional profiling, virology, histology, and echocardiograms to investigate the role of a high-fat diet in IAV-associated cardiac damage.

Results

Transcriptional profiling showed that, compared to their low-fat counterparts (LF mice), mice fed a high-fat diet (HF mice) had impairments in inflammatory signaling in the lung and heart after IAV infection. This was associated with increased viral titers in the heart, increased left ventricular mass, and thickening of the left ventricular wall in IAV-infected HF mice compared to both IAV-infected LF mice and uninfected HF mice. Retrospective analysis of clinical data revealed that cardiac complications were more common in patients with excess weight, an association which was significant in 2 out of 4 studies.

Conclusions

Together, these data provide the first evidence that a high-fat diet may be a risk factor for the development of IAV-associated cardiovascular damage and emphasizes the need for further clinical research in this area.

Keywords: body mass index; cardiac disease; extra-respiratory complications; high-fat diet; influenza; obesity; overweight

INTRODUCTION

The 2009 influenza virus (IAV) pandemic infected 24% of the global population, resulting in > 500 000 deaths [1]. During this pandemic, overweight (body mass index [BMI] ≥ 25), obesity (BMI ≥ 30), and morbid obesity (BMI ≥ 35) emerged as novel risk factors for severe influenza [2–8]. Moreover, mice fed a high-fat diet (HF mice) are more susceptible to severe respiratory disease following IAV infection [9]. This susceptibility has been associated with altered interferon and cytokine production, impaired leukocyte function, and a reduced response to antigen stimulation in overweight individuals [9–11].

Whilst primarily a respiratory pathogen, IAV is often associated with extra-respiratory complications, the most frequent of which are cardiovascular and central nervous system disease [12–15]. Cardiac complications include endocarditis, myocarditis, and myocardial infarction [12]. For example, in the first 7 days after laboratory-confirmed IAV infection, hospitalization for acute myocardial infarction is 6 times higher than a control interval [16]. However, the role of host factors in IAV-associated heart disease remains to be elucidated.

At present, no study has investigated the role of a high-fat diet in IAV-associated cardiovascular damage. This is in spite of the fact that a high-fat diet and excess weight are associated with both increased influenza severity and increased cardiovascular disease in the absence of a viral infection. Given that > 1.9 billion people are currently overweight/obese [17], the role of excess weight in IAV-associated cardiovascular damage is essential information for patient management as well as pandemic preparedness.

Here, we provide the first evidence that a high-fat diet decreases the antiviral response to IAV whilst increasing IAV replication and structural changes to the heart in mice. These data further advance our understanding of the systemic pathogenesis of influenza.

METHODS

Cells

Madin Darby Canine Kidney (MDCK) cells were obtained from ATCC and used between passages 20 and 50. Cells were cultured in a humidified incubator at 37°C with 95% O2 and 5% CO2.

IAV Strain

Virus stocks of A/H1N1/Auckland/1/2009(H1N1) (Auckland/09) were prepared in embryonated chicken eggs and titers of infectious virus were determined by plaque assays on MDCK cells [18].

Mice

Four-week-old C57BL/6 male and female mice were obtained from the Animal Resources Centre (Australia). Mice were then randomly assigned to be fed either a high-fat diet (HF mice) consisting of 40% calories from fat or a low-fat diet (LF mice) consisting of 12% calories from fat (Specialty Feeds) for 10 weeks and supplied with water ad libitum. All animal work was approved by the University of Queensland Animal Ethics Committee (071/17).

IAV Inoculation of Mice

Mice were anesthetized with isoflurane then inoculated intranasally with 5500 or 100 plaque forming units (PFU) of influenza egg-grown A/Auckland/09(H1N1) or naive allantoic fluid (NAF) in 50 µL.

Blood Oxygen Saturation

Blood oxygen saturation was measured using a MouseOx Plus collar oximeter (Starr).

Body Composition

Whole body composition of live mice was determined using a nuclear magnetic resonance analyzer, Minispec LF50H (Bruker Optik).

Echocardiography

Cardiac function was assessed using a Vevo 2100 high-frequency ultrasound system with a 40 MHz center frequency transducer (Visualsonics). Depilated mice were anesthetized by 1.5% isoflurane, kept warm on a heated stage, with respiration and heart rate monitored on echocardiography (ECG) pads. ECG analysis is described in the Supplementary Material.

Viral Titers

Tissues were collected in DMEM (Gibco) and homogenized using a Qiagen Tissuelyser II (Qiagen). The homogenate was centrifuged and the supernatant collected and stored at −80°C. Viral titers were then measured by plaque assay on MDCK cells [18].

Histology

Hearts were fixed in 10% neutral-buffered formalin, processed to paraffin, and 5-µm sections were stained with H&E. Left ventricular thickness was measured by viewing the sections with a 4× objective with projection to a computer screen and then measuring the left ventricular width in mm. Measurements were performed by a veterinary pathologist (H.B.O.) blinded to treatment groups.

Immunohistochemistry

Immunolabeling for HIF-1α was performed as previously described [19, 20]. Assessment of labelling distribution and intensity are described in the Supplementary Material.

RNA Extraction and cDNA Synthesis

RNA was extracted using NucleoZOL (Macherey-Nagel) and an RNAeasy plus Minielute kit (Qiagen). cDNA for host expression analysis and viral mRNA quantification was synthesized using oligo(dT) (Roche) and a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative polymerase chain reaction (qPCR) was performed using SYBR green (Applied Biosystems). The relevant primers are listed in Supplementary Table 1. Gene expression was normalized relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression and fold change was calculated using the ΔΔCt method relative to NAF-infected animals. Outliers were removed using Grubbs outlier test. Viral copy number was determined as previously described [21].

RNA-seq Library Preparation and Sequencing

Library preparation and sequencing was performed at the Institute for Molecular Bioscience Sequencing Facility, University of Queensland. RNA-Seq libraries were generated using the Illumina TruSeq Stranded mRNA LT Sample Prep Kit (Illumina, RS-122–2101/RS-122–2102). Sequencing was performed using the Illumina NextSeq500 (NextSeq control software v2.1.0/Real Time Analysis v2.4.11). The library pool was diluted and denatured according to the standard NextSeq protocol and sequenced to generate single-end 76 bp reads using a 75 cycle NextSeq500/550 High Output reagent Kit v2 (Illumina). Analysis of RNA-seq data is described in the Supplementary Material.

Serum Cytokines

Serum samples were screened for proinflammatory cytokines using the mouse antiviral response LegendPlex according to the manufacturer's instructions (Biolegend). Outliers were removed using Grubbs outlier test.

Patient Data

Data on IAV patients were obtained from previously published sources [22–25]. Patients were excluded from the analysis if their BMI < 20, as low BMI may be associated with cardiovascular disease [26].

Statistics and Data Availability

Statistical analyses were performed using GraphPad Prism. Full details of statistical analysis and data availability can be found in the Supplementary Material.

RESULTS

A High-Fat Diet in Mice Results in Increased Body and Fat Weight

To study the effect of excess weight on IAV-associated cardiovascular damage, mice were fed either a high-fat or low-fat diet for 10 weeks. Following 10 weeks of a differential diet, HF mice had significantly greater body weight and fat percentage compared to LF mice

(Supplementary Figure 1A and 1B). A small but significant increase in lean weight was also observed in HF mice (Supplementary Figure 1C). This was accompanied by a decreased heart weight in HF mice, consistent with the fact that muscle weighs more than fat (Supplementary Figure 1D). Together, these data demonstrate that a high-fat diet for 10 weeks is sufficient to generate a murine model of excess weight.

HF Mice Are More Susceptible to Severe Respiratory Disease Following IAV Infection

We next sought to characterize the pulmonary response to IAV in HF and LF mice. To do so in a comprehensive manner, mice were infected with either a high dose (5500 PFU) or a low dose (100 PFU) of Auckland/09(H1N1) and euthanized at 4 days (high dose) or 6 days (low dose) post infection. Following IAV infection, there was a significant difference in percentage weight loss between HF and LF mice after a low-dose IAV infection (4 days post infection [dpi]; Figure 1A). Consistent with these data, HF mice had significantly lower blood oxygen saturation at 4 dpi (high dose) and 6 dpi (low dose) compared to LF mice (Figure 1B). At both viral inoculums and time points there was increased alveolar inflammation in HF mice (Figure 1C). HF mice infected with 100 PFU of IAV also had a small but significant increase in pulmonary viral load at 6 dpi relative to LF mice (Figure 1D). Together, these data are consistent with previous findings [27] that HF mice are more susceptible to respiratory disease after IAV infection than LF mice.

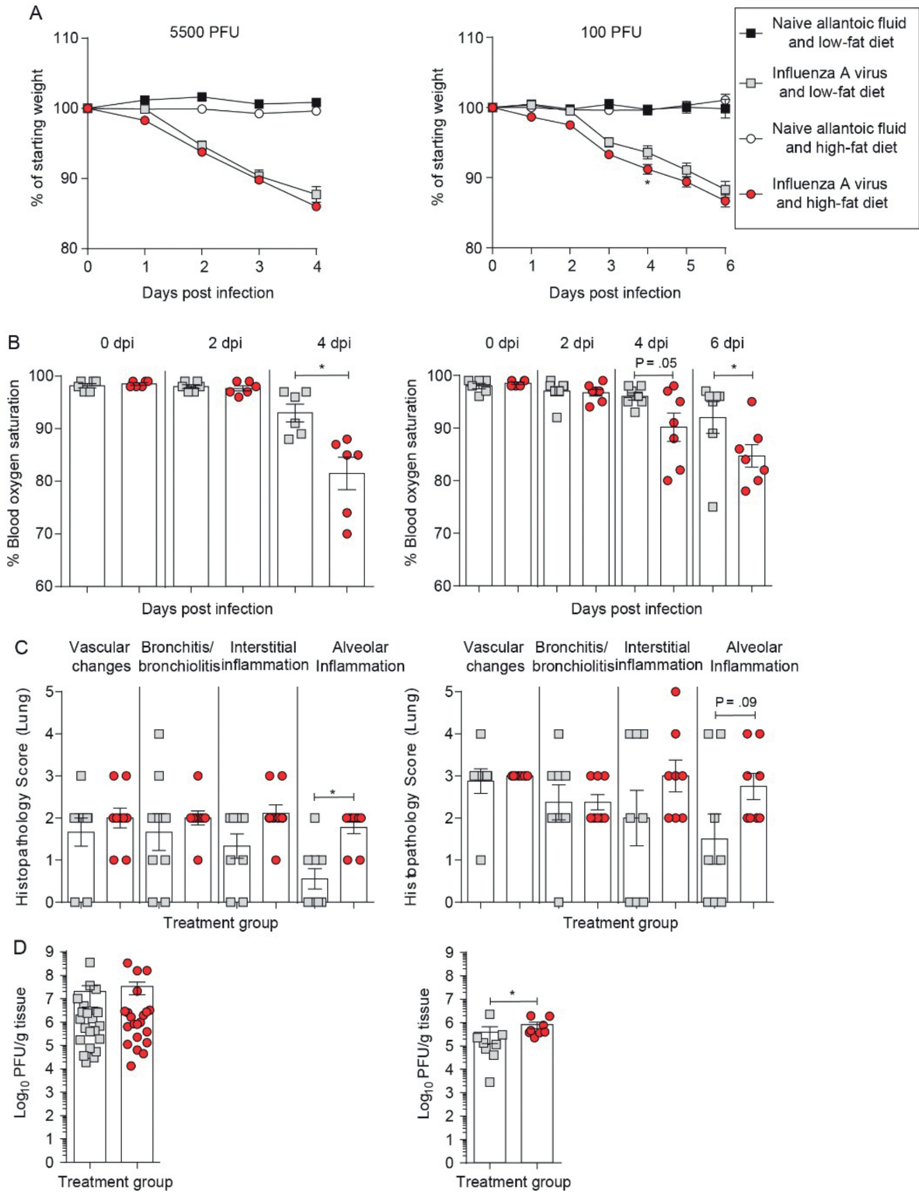

Figure 1. Mice fed a high-fat diet are more susceptible to severe respiratory disease following influenza A virus (Auckland/09) inoculation. (A) Weight loss, (B) percentage blood oxygen, (C) histopathological examination of pulmonary inflammation, and (D) pulmonary PFU of mice following inoculation with either 5500 PFU (left) or 100 PFU (right) of Auckland/09. Unless otherwise indicated, data were obtained at 4 dpi (5500 PFU) or 6 dpi (100 PFU). All graphs show the mean and standard error of mean of data pooled from at least 2 independent experiments. Statistical analysis was performed as described in "Materials and Methods" with *P < .05. Abbreviations: dpi, days post infection; PFU, plaque forming unit.

To gain a deeper insight into the localized response to IAV, RNAseq was performed on uninfected and infected lung tissue from HF and LF mice 2 dpi with 5500 PFU of IAV (Supplementary Figure 2). This timepoint and viral inoculum was selected to capture the early transcriptional events in the lungs of infected mice. The effects of a high-fat diet on gene expression in the lungs of mice, in the absence of IAV, have been established [28–30]. Consistent with these findings, several differentially expressed genes were noted when the lung transcriptome of NAF-infected HF and LF mice were compared (Supplementary Table 2). However, as the focus of the present study is the specific effects of IAV in different metabolic backgrounds, gene expression data in IAV-infected mice were analyzed relative to the relevant uninfected controls (Figure 2). In general, LF mice showed a stronger transcriptional response to IAV compared to HF mice (Figure 2A and 2B). Pathway analyses of differentially expressed genes indicated that the majority of genes were associated with immune system function and that these were poorly expressed in HF mice following IAV infection (Figure 2B). qPCR confirmed the validity of these analyses at 2 dpi (Figure 2C). Taken together, these data show that HF mice fail to induce the same number and mRNA levels of proinflammatory mediators in the lung early after IAV infection.

Serum Cytokine Levels Are Reduced in HF Mice After IAV Infection

The immunological response in the respiratory system to IAV in HF mice has been well described early (in this study) as well as later during infection [31]. However, there is currently limited information available on the inflammatory response to IAV outside of the respiratory tract. Therefore, serum cytokines in both LF and HF mice after IAV infection were assessed at 2 dpi (5500 PFU) and 6 dpi (100 PFU) (Figure 3). At 2 dpi, levels of interleukin-6 (IL-6), interferon-γ (IFN-γ), IFN-α, and IFN-γ inducible protein-10 (IP-10) were significantly higher in IAV-infected LF mice compared to the uninfected LF mice, whilst this was not observed in IAV-infected HF mice when compared to uninfected HF mice (Figure 3A). Moreover, at 2 dpi, LF mice infected with IAV had significantly higher serum levels of IP-10 compared to IAV-infected HF mice (Figure 3A). At 6 dpi, monocyte chemoattractant protein-1 (MCP-1), IL-6, IFN-γ, IP-10, and tumor necrosis factor-α (TNF-α) were significantly higher in IAV-infected LF mice compared to uninfected LF mice, whilst this was only observed with MCP-1 in IAV-infected HF mice when compared to uninfected HF mice (Figure 3B). Overall, these observations are consistent with a decreased proinflammatory response to IAV in the serum of HF mice.

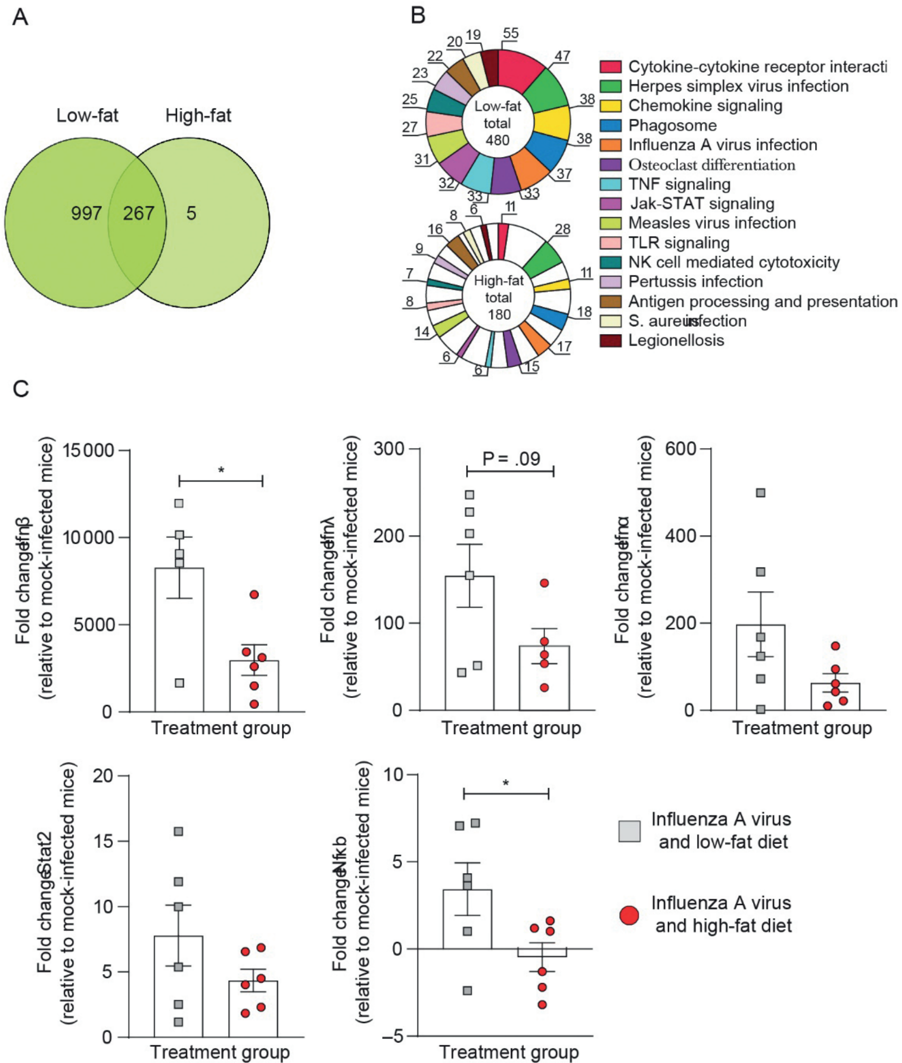

Figure 2. Reduced expression of inflammatory genes after IAV (Auckland/09) infection in the lungs of HF mice compared to the lungs of LF mice. A, Venn diagram of differentially expressed genes in IAV-infected LF and HF mice at 2 dpi (5500 PFU). B, The top 15 KEGG pathways identified in the analysis of the differentially expressed genes. C, qPCR analysis for different pro-inflammatory genes at 2 dpi (5500 PFU). All qPCR graphs show the mean and standard error of mean of data pooled from at least 2 independent experiments. Statistical analysis was performed as described in "Materials and Methods" with *P < .05. Abbreviations: dpi, days post infection; HF, high fat; IAV, influenza A virus; LF, low fat; NK cell, natural killer cell; PFU, plaque forming unit; qPCR, quantitative polymerase chain reaction; TLR, Toll-like receptor; TNF, tumor necrosis factor.

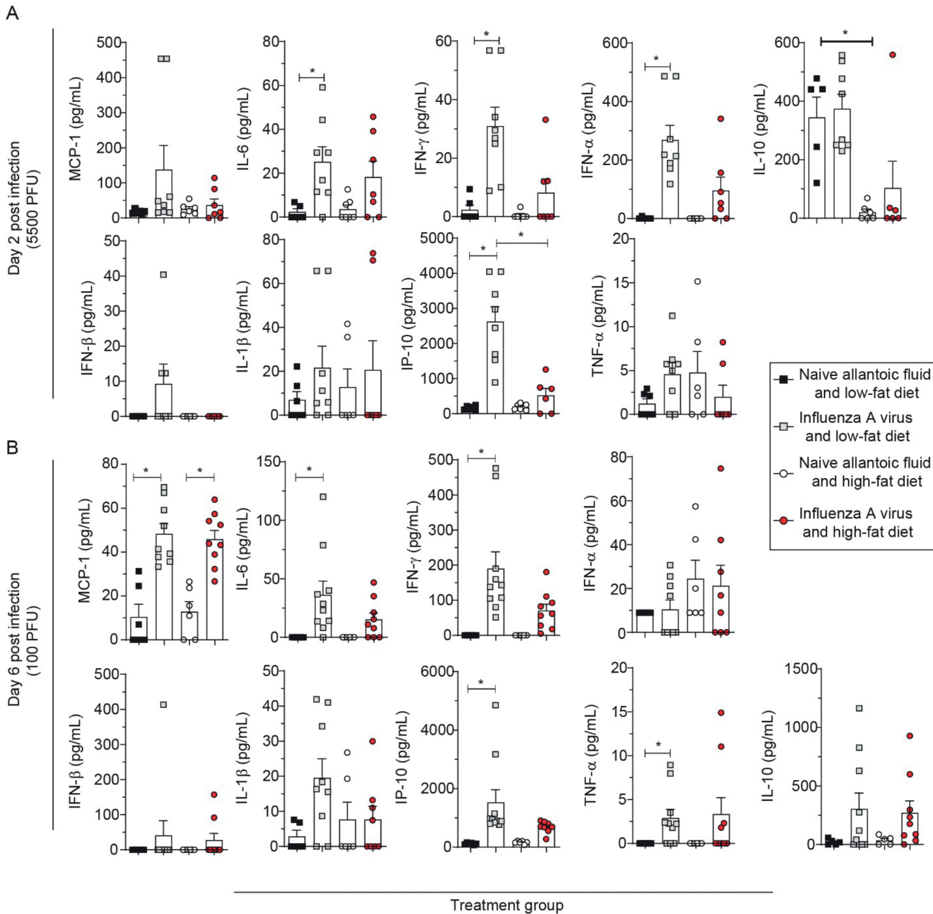

Figure 3. LF mice have a more pronounced pro-inflammatory response in the serum follow influenza A virus (Auckland/09) infection compared to HF mice. Data were obtained at (A) 2 dpi (following infection with 5500 PFU of virus) and (B) 6 dpi (following infection with 100 PFU of virus). All graphs show mean and standard error of mean of data pooled from at least 2 independent experiments. Statistical analysis was performed as described in the "Materials and Methods" with *$P < .05$. Abbreviations: dpi, days post infection; HF, high fat; IFN, interferon; IL, interleukin; IP-10, IFN-γ inducible protein-10; LF, low fat; MCP-1, monocyte chemoattractant protein-1; PFU, plaque forming unit; TNF, tumor necrosis factor.

The Antiviral Response in the Heart Is Reduced in HF Mice After IAV Infection

The above data suggest that, in addition to a differential respiratory response to IAV, HF and LF mice also display a differential systemic response to IAV. To further study extra-respiratory responses, we first investigated the replication of IAV outside of the respiratory tract of HF and LF mice at 2 dpi (5500 PFU). IAV was not detected in the brain, spleen, pancreas, tracheobronchial lymph nodes, duodenum, jejunum, ileum, and blood, whilst virus was sporadically detected in the kidney, liver, and adrenal gland at low titers (data not

shown). However, in both LF and HF mice, virus was consistently detected in the heart at 2 dpi (Figure 4A). Similarly, infectious virus was detected in the hearts of LF and HF mice at 6 dpi following infection with 100 PFU of IAV (Figure 4A). Strikingly, significantly higher viral loads were detected in the hearts of HF mice compared to LF mice at 6 dpi (Figure 4A).

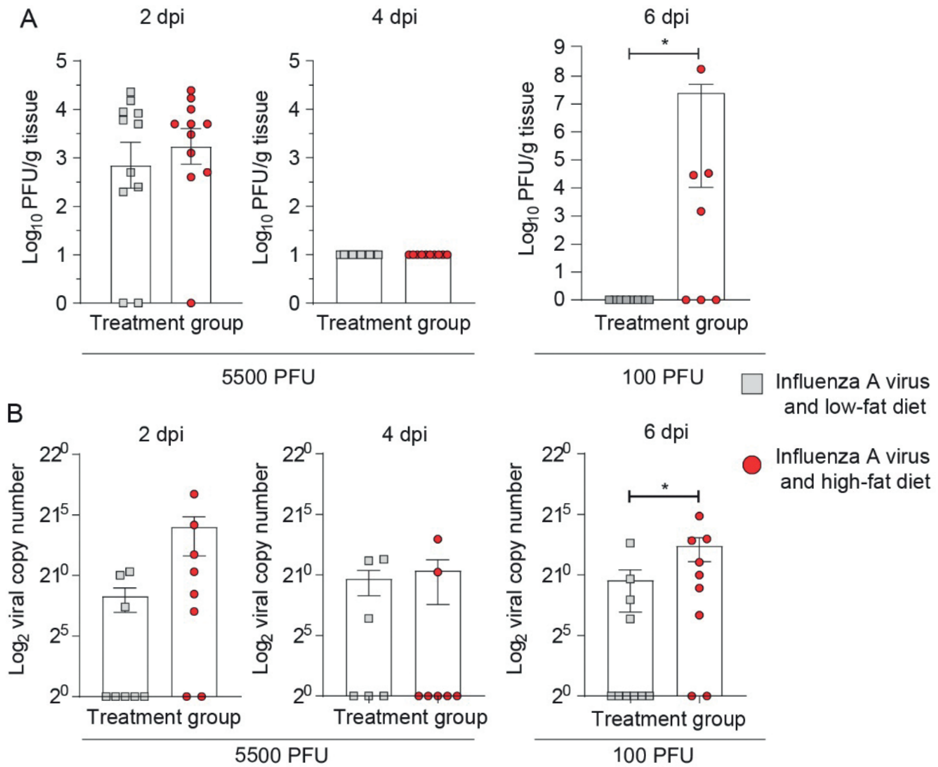

Figure 4. Influenza A virus (Auckland/09) can be detected in the hearts of LF and HF mice. A, Viral PFU in the heart of LF and HF mice at 2 dpi (following infection with 5500 PFU of virus) and 6 dpi (following infection with 100 PFU of virus). B, Viral mRNA in the heart of LF and HF mice at 2 dpi (following infection with 5500 PFU of virus) and 6 dpi (following infection with 100 PFU of virus). All graphs show mean and standard error of mean of data pooled from at least 2 independent experiments. Statistical analysis was performed as described in the "Materials and Methods" with *$P < .05$. Abbreviations: dpi, days post infection; HF, high fat; LF, low fat; PFU, plaque forming units.

To differentiate locally replicating virus from infectious virions simply present in the organ of interest, qPCR for viral mRNA was performed (Figure 4B). Viral mRNA could be detected in the hearts of infected mice at 2 dpi (5500 PFU), although the amount of mRNA detected was not significantly different between LF and HF mice (Figure 4B). In contrast, at 6 dpi (100 PFU) there was significantly more viral mRNA in the hearts of IAV-infected HF mice compared to IAV-infected LF mice (Figure 4B).

To investigate the early host response to IAV in the heart, the transcriptome of the heart of IAV-infected LF and HF mice was assessed by RNASeq at 2 dpi (5500 PFU) (Supplementary Figure 3). Consistent with our previous analysis of the lung, there was a difference in gene expression between the hearts of uninfected LF and HF mice (Supplementary Table 3). However, as the focus of this study is the specific effects of IAV in HF and LF mice, all subsequent analyses were performed relative to the relevant uninfected controls. In the heart, genes differentially expressed in infected LF mice were also expressed to a lower level, or not at all, in infected HF mice (Figure 5A). Moreover, with the exception of *Entpd4*, differentially expressed genes in infected HF mice were also differentially expressed in infected LF mice (Figure 5A). Consistent with our previous observations in the lung (Figure 2), the majority of differentially expressed genes identified in infected LF mice belonged to pathways associated with the inflammatory response (Figure 5B). To determine if this gene expression profile (reduced proinflammatory and antiviral genes) was specific to this particular viral infectious dose/time point, the expression of key genes identified by RNASeq was assessed by qPCR at both 4 dpi (5500 PFU) and 6 dpi (100 PFU) (Figure 5C). Consistent with the RNASeq data at 2 dpi, at 4 and 6 dpi IAV-infected LF mice had higher relative expression of *Irf7*, *Isg15*, and *Il-1*β in the heart compared to IAV-infected HF mice (Figure 5C).

Changes in the Structural Status of the Heart of IAV-Infected HF Mice

The presence of viral mRNA in the hearts of infected mice, and the strong transcriptional response observed in the same organ, suggested that IAV may affect cardiac function. To determine if this was the case, echocardiography was performed on LF and HF mice at 4 dpi (5500 PFU). There was no significant difference in various measures of cardiac function after IAV infection in both LF and HF mice when compared to their NAF-infected counterpart (Table 1). In contrast, IAV-infected HF mice had a significantly thicker left posterior ventricular wall compared to both IAV-infected LF mice and NAF-infected HF mice (Table 1). Similarly, IAV-infected HF mice had a significantly greater left ventricular mass compared to both IAV-infected LF mice and NAF-infected HF mice (Table 1). In contrast, in LF mice, IAV virus induced a significant decrease in left ventricular mass relative to NAF-infected LF mice (Table 1). To confirm these data, histology was used to assess left ventricle thickness at 4 dpi in IAV-infected LF and HF mice. Consistent with the echocardiographic data, IAV-infected HF mice had significantly thicker left ventricles than IAV-infected LF (Figure 6A), whilst a trend was seen towards increased left ventricle thickness in IAV-infected HF mice relative to NAF-infected HF mice. In contrast, there was no notable difference in the left ventricular mass of infected and uninfected LF mice (Figure 6A; $P > .99$).

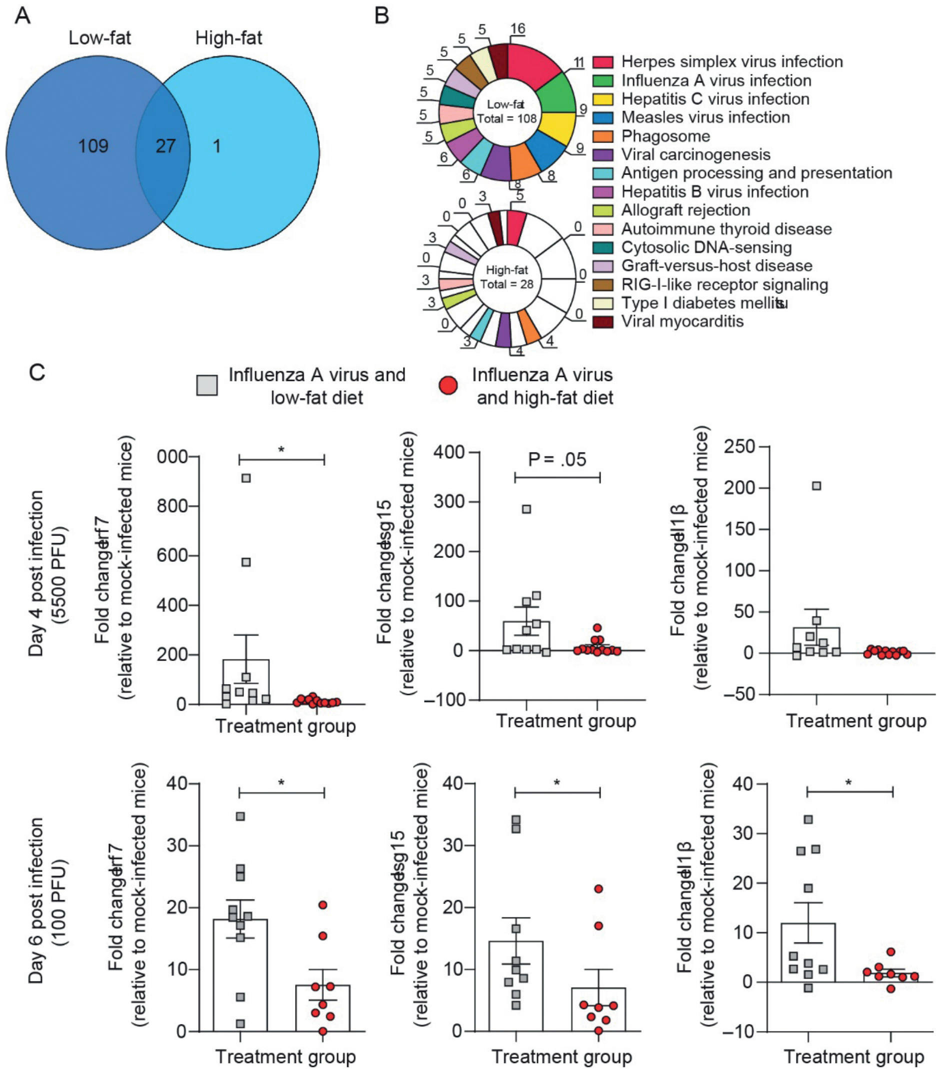

Figure 5. Reduced expression of inflammatory genes after influenza A virus (Auckland/09) infection in the hearts of HF mice compared to the hearts of LF mice. A, Venn diagram of differentially expressed genes in influenza A virus-infected LF and HF mice at 2 dpi (5500 PFU). B, KEGG pathway analysis of the differentially expressed genes. C, qPCR analysis for different pro-inflammatory genes at 4 dpi (5500 PFU) or 6 dpi (100 PFU). All qPCR graphs show the mean and standard error of mean of data pooled from at least 2 independent experiments. Statistical analysis was performed as described in the "Materials and Methods" with *P < .05. Abbreviations: dpi, days post infection; HF, high fat; LF, low fat; PFU, plaque forming units; qPCR, quantitative polymerase chain reaction.

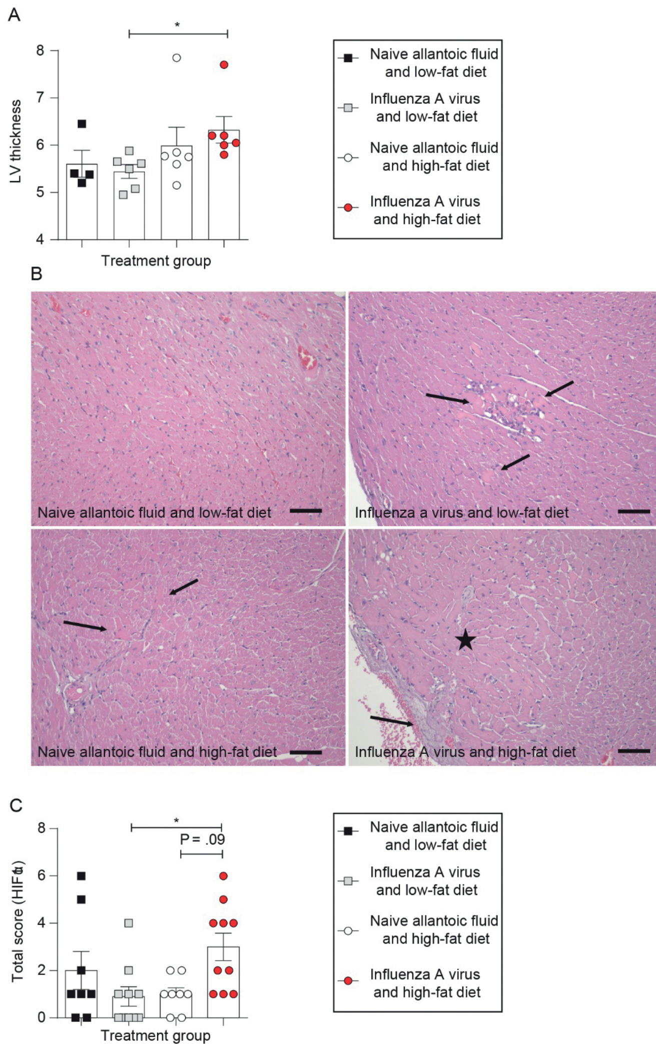

Figure 6. Structural changes in the hearts of IAV-infected HF mice. A, Left ventricle thickness (arbitrary units) at 4 dpi following infection with 5500 PFU of Auckland/09(H1N1). Graph shows the mean and standard error of mean and data are representative of 2 independent experiments. B, Representative images of cardiomyocyte degeneration in the hearts of IAV- and NAF-infected LF and HF mice. Top left: Histologically normal heart from NAF-infected LF mouse. Top right: heart from IAV-infected LF mouse. There are scattered and small clusters of degenerating cardiomyocytes (arrows). Bottom left: Heart from NAF-infected HF mouse showing randomly scattered but relatively rare degenerating cardiomyocytes. Bottom right: Heart from IAV-infected HF mice. There is focally severe cardiomyocyte degeneration (star), mild perivascular edema, and a small fibrin-thrombus attached to the endocardium of the left ventricle (arrow). Images are representative of n > 5 per group (100 μm). C, Increased HIF-1α in the hearts of IAV-infected HF mice. Graph shows the mean and standard error of mean of data pooled from a minimum of 2 independent experiments. Statistical analysis in (A) and (C) was performed as described in the "Materials and Methods" with *P < .05. Abbreviations: dpi, days post infection; HF, high fat; IAV, influenza A virus; LF, low fat; LV, left ventricle; NAF, naive allantoic fluid.

Table 1. Cardiovascular Parameters Measured by Echocardiogram at 4 dpi (Infectious Dose: 5500 PFU).

	Low Fat			High Fat		
	Mock (n = 8)	IAV (n = 10)	% difference	Mock (n = 8)	IAV (n = 9)	% difference
Global parameters						
Body weight, g	31.6±3.2	25.6±3.6	−19%	43.2±3[a]	37.8±3[c]	−13%
Heart rate, bpm	407.6±68.6	334.6±81.6	−18%	403.9±51.3	347.8±50.7	−16%
Functional status of the heart						
Cardiac output, mL/min	12.7±2.4	10.8±8.7	−15%	13.3±1.8	10.5±2.5	−21%
Ejection Fraction, %	47.4±8.6	55.8±11.7	+18%	57.5±4	60.9±9.4	+6%
Stroke Volume	31.3±4.9	26±8.6	−17%	33.1±2.9	30.2±5	−9%
Structural status of the heart						
LVAW−s, mm	1.2±0.2	1.2±0.2	0	1.2±0.1	1.5±0.3	+25%
LVPW−s, mm	1.2±0.2	1.2±0.2	0	1.1±0.1	1.4±0.2[b,c]	+27%
LV mass, g	135.9±16.6	104.6±19.1[b]	−23%	108.8±11	135.7±29[b,c]	+25%

[a]$P < .05$ vs mock treated, low fat mice (Kruskal-Wallis with Dunn's multiple comparison test).
[b]$P < .05$ vs mock treated mice (same treatment group) (One-Way ANOVA with Sidak's multiple comparison test).
[c]$P < .05$ vs IAV inoculated, low fat mice (One-Way ANOVA with Sidak's multiple comparison test).

To further investigate cardiomyopathy in these mice, heart sections were assessed for cardiomyocyte degeneration. Hearts from NAF-infected LF mice had no obvious histological abnormalities (Figure 6B). IAV-infected LF mice had signs of mild cardiomyopathy, with some samples showing mild, focal cardiomyocyte degeneration (Figure 6B). In contrast to NAF-infected LF mice, hearts from NAF-infected HF mice typically showed minimal cardiomyocyte degeneration with scattered and small clusters of hyalinized cardiomyocytes detected in the majority of samples (Figure 6B). However, the HF IAV-infected mice were the only treatment group where widespread and extensive cardiomyocyte degeneration was observed (Figure 6B). None of these lesions had a focal association with active virus replication, based on the lack of AIV nucleoprotein detected at the exact same site of the lesion in a serial tissue section (data not shown).

Increased HIF-1α in the Hearts of HF Mice After IAV Infection

HIF-1α expression in the heart has been associated with cardiomyopathy and increased left ventricular mass, and implicated in the pathogenesis of cardiovascular disease [32, 33]. Given the structural changes observed in the hearts of HF mice after IAV infection, we sought to determine if this was associated with increased HIF-1α expression in the heart. Accordingly, sections of murine hearts were immunolabeled for HIF-1α at 4 dpi (following infection with 5500 PFU Auckland/09) and semi-quantitatively assessed by a veterinary pathologist who was

blinded to the respective treatment groups. Consistent with the observed structural changes, the hearts of HF infected mice had increased HIF-1α immunolabelling compared to both their LF infected ($P < .05$) and naive HF counterparts ($P = .09$) (Figure 6C).

An Elevated BMI Is Associated With an Increased Risk of Influenza-Associated Cardiac Complications

Finally, we sought to validate the observations from our murine model of disease in the human population. To do so, we analyzed previously published data [22–25]. In all studies an increased percentage of overweight patients (compared to patients with a healthy BMI) experienced a cardiovascular complication of IAV. This association was statistically significant in 2 of the 4 surveyed studies (Supplementary Table 4). Taken together, these data suggest that the role of excess weight in the cardiac complications of influenza in patients warrants further investigation.

DISCUSSION

Here, we showed that the systemic pathogenesis of influenza differs between HF mice and LF mice. Specifically, our data demonstrate that mice fed a high-fat diet had impairments in systemic inflammatory signaling in the lungs, circulation, and heart after IAV infection. This was associated with increased viral titers, cardiomyocyte degeneration, and increased Hif-1α expression in the hearts of infected HF mice.

Impairments in the inflammatory response to IAV have previously been described in the lungs of HF mice [27]. Our data are the first to comprehensively characterize this pulmonary response early during infection (2 dpi) and demonstrate that these immune changes also occur outside of the respiratory tract. The exact trigger of the inflammatory transcriptome observed in the hearts of IAV-infected mice remains unclear. Given that we detected infectious virions in the hearts of both infected LF and HF mice, the associated inflammatory response could be a direct response to virus and/or viral antigens. This would be consistent with previous reports of viral antigen in the hearts of IAV-infected patients and mice [34–36]. Alternatively, the observed transcriptome could be induced indirectly, that is via exposure to proinflammatory mediators in blood that have been generated in response to the pulmonary infection. Regardless of the mechanism, the impaired cardiac inflammatory response in HF mice was associated with increased viral replication in the hearts of these mice at later time points post infection. This is consistent with recent findings that in the absence of IFITM3, there is increased IAV replication in the hearts of mice [37].

The altered transcriptome and increased viral replication in the hearts of HF mice was further associated with increased left ventricular mass and thickening of the left ventricle posterior wall. At present, the precise mechanism(s) by which influenza increases left ventricular mass in HF mice remains to be defined. Increased left ventricular mass can be the result

of numerous different physiological changes, including increased cardiomyocyte mass, endothelial cell proliferation, fibrosis, and/or inflammatory signaling/immune cell activation [38]. Several studies have shown that IFN-γ plays a protective role in the development of cardiac hypertrophy [39–42]. Because HF mice fail to induce similar levels of IFN-γ relative to LF mice, it is possible that this drove the observed increase in left ventricular mass. Alternatively, it is possible that as IAV causes hypoxemia in HF mice, this triggered cardiac hypoxia and increased left ventricular mass [43–45]. It is also important to note that whilst left ventricular function is a critical parameter in predicting cardiac morbidity and mortality [38], RV function is a more clinically relevant and sensitive measurement to examine the pathological changes associated with the cardiopulmonary diseases (ie, influenza). Unfortunately, consistent and reproducible echocardiographic evaluation of the RV is inherently difficult due to its retrosternal position and crescent-shaped structure [46]. It is therefore important for future studies to overcome these technical limitations and investigate RV function in both HF and LF mice.

Mice can never fully recapitulate the complexity of disease that occurs in the human population. We therefore sought to validate the observed association between excess weight and the cardiovascular complications of IAV across a variety of different published clinical studies. Unfortunately, of those studies that were available and recorded the cardiovascular complications of influenza, few also recorded patient BMI, therefore reducing the power of the analysis. Similarly, in the absence of a specifically designed clinical study, it is not possible to assess the rate of spontaneous cardiovascular disease in overweight patients (ie, in the absence of IAV). Nevertheless, in all 4 clinical studies that were obtained and had recorded the relevant data, a higher percentage of cardiovascular complications were recorded in influenza patients who had excess weight compared to those with a healthy BMI. This association was statistically significant in 2 of the 4 available studies. Whilst these data are certainly not definitive, they do suggest that there is sufficient preliminary evidence to warrant a targeted clinical assessment of the role of excess weight in the extra-respiratory complications of influenza and other infectious diseases. When one considers the fact that it is now predicted that in the year 2030 nearly 1 in 2 US adults will be living with obesity [47], a complete understanding of the role of excess weight in the cardiac complications of IAV becomes particularly pertinent.

NOTES

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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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SUPPLEMENTAL MATERIALS AND METHODS

Immunohistochemistry

Labelling distribution and intensity for HIF-1α was assessed for both cardiomyocytes and endocardium (total value shown) on a scale of 0-4 (0 = no signal, 1 = weak signal and/or <25% of cells positive, 2 = moderate signal and/or 25-50% of cells positive, 3 = strong signal in < 50% of cells, 4 = strong signal in >50% of cells) by a veterinary pathologist blinded to the groupings. Digital microphotographs were taken using a Nikon DS-Fi1 camera with a DS-U2 unit and NIS elements F4.60 software.

RNA-seq Analysis

After sequencing, fastq files were generated using bcl2fastq2 (v2.18.0). RNA-seq reads were aligned to the mm10 mouse transcriptome using Bowtie 1. Quantification of gene expression was performed using MMSEQ. Differential expression was determined using DEseq using RStudio (v1.1.436). Differentially expressed genes were determined using pairwise comparison between treatments, with a fold change > 1.5, adjusted p value of < 0.1, and RPKM > 5. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis was performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) web-tool [23].

Echocardiography (ECG) analysis

Ultrasound images of the left ventricle were obtained from a parasternal long axis view in B-mode and M-mode. Ultrasound parameters including cardiac output, ejection fraction, and left ventricular (LV) mass were calculated based on averaging three cardiac cycles using Vevolab software. LV mass was calculated based on formula by [19].

Statistics and data availability

Unless otherwise indicated, data were tested for normality using the Shapiro-Wilk test. Where data was normally distributed, data was analysed using a Two-Way ANOVA (with Tukey's multiple comparison test), a One-Way ANOVA (with Tukey's multiple comparison test) or an unpaired two-tailed student's t-test as appropriate. Where data was not normally distributed, data was analysed using a Kruskal-Wallis test (with Dunn's multiple comparisons test) or a Mann-Whitney U test as appropriate., the following treatment groups were compared: IAV HF vs IAV LF, NAF LF vs IAV LF, NAF HF vs IAV HF and NAF LF vs NAF HF. Categorical data was analysed using Fisher's exact test.

RNA sequencing data generated in this study are deposited in the Gene Expression Omnibus repository with the accession number GSE134685.

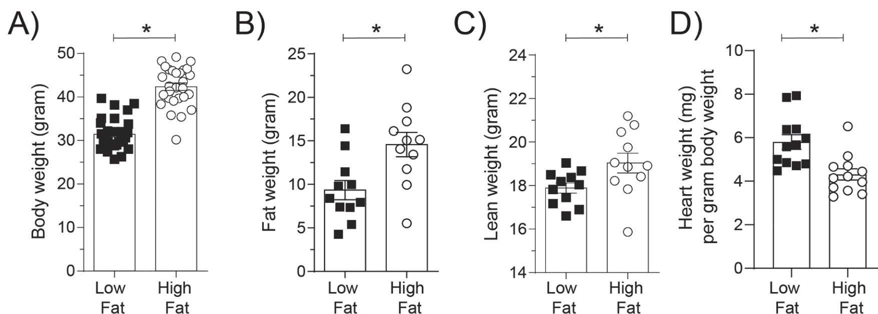
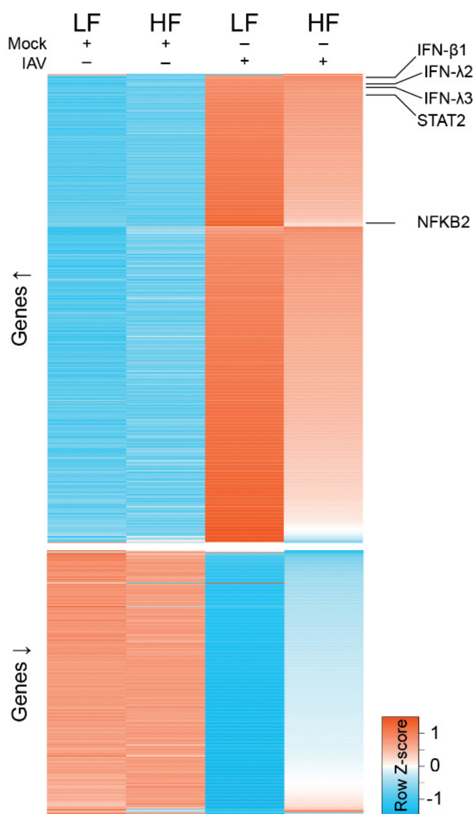
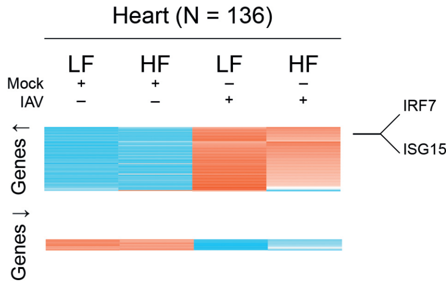


Figure S1. Ten weeks of a high fat diet results in increased body weight in mice. (A) Total body weight of mice fed a low fat or high fat diet for 10 weeks. (B) Total fat weight and (C) lean weight in grams. D) Heart weight relative to body weight in mice fed differential diets for 10 weeks. All graphs show the mean and standard error of mean of data pooled from at least three independent experiments. Statistical analysis was performed as described in the Materials and Methods with *P < 0.05.



Supplemental Figure 2. Heatmap of differentially expressed genes in the lungs of low fat (LF) and high fat (HF) mice 2 days after mock or influenza A virus (IAV) infection (5500 PFU). Specific genes subsequently validated by qPCR are highlighted.



Supplemental Figure 3. Heatmap of differentially expressed genes in the hearts of low fat (LF) and high fat (HF) mice 2 days after mock or influenza A virus (IAV) infection (5500 PFU). Specific genes subsequently validated by qPCR are highlighted.

3

Table S1. Primers used in the present study

	FORWARD	REVERSE
Gapdh	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGT
Ifnα	ACAACAGATCCAGAAGGCTCAAG	AGTCTTCCTGGGTCAGAGGAGGTT
Ifnβ	TCCAGAATGAGTGGTGGTTCC	GACCTTTCAAATGCAGTAGAT
Stat2	CAGGAACAGGCTGTCAAGGT	CGCTTGGAGAATTGGAAGTT
Ifnγ	ACTGGCAAAAGGATGGTGAC	TGAGCTCATTGAATGCTTGG
Ifnλ	AGCTGCAGGCCTTCAAAAAG	TGGGAGTGAATGTGGCTCAG
Il1β	CATGGAATCCGTGTCTTCCT	GAGCTGTCTGCTCATTCACG
NfκB	CCAGCTTCCGTGTTTGTTCA	AGGGTTTCGGTTCACTAGTTT
Irf7	GCCAGGAGCAAGACCGTGTT	TGCCCCACCACTGCCTGTA
Isg15	GAGCTAGAGCCTGCAGCAAT	TTCTGGGCAATCTGCTTCTT
Matrix	AAGACCAATCTTGTCACCTCTGA	TCCTCGCTCACTGGGCA

Table S2. Differentially expressed genes in the lungs between mock-infected LF and HF mice.

Gene	Mean log2 RPKM low fat (±SD)	Mean log2 RPKM high fat (±SD)
mt-Nd3	4.87 (± 0.76)	7.57 (± 1.07)
Cyp26b1	0.74 (± 0.72)	2.59 (± 0.65)
Fabp4	6.00 (± 1.59)	7.45 (± 2.13)
Pvalb	-4.95 (± 8.51)	-6.53 (± 5.33)
Klhl41	0.72 (± 2.33)	0.12 (± 0.97)
Ckm	5.38 (± 2.84)	4.52 (± 1.18)
Apobec2	2.69 (± 2.41)	1.70 (± 1.10)
Myl3	3.87 (± 2.68)	2.69 (± 1.50)
Prrt2	2.54 (± 0.86)	1.79 (± 1.26)
Myot	-1.84 (± 4.24)	-9.05 (± 6.45)
Gm4737	2.69 (± 0.24)	-1.17 (± 2.30)
Hspa1b	4.55 (± 0.21)	3.07 (± 0.37)

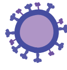
Table S3. Differentially expressed genes in the heart between mock-infected LF and HF mice.

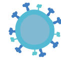
Gene	Mean log2 RPKM low fat (±SD)	Mean log2 RPKM high fat (±SD)
Hmgcs2	2.36 (± 0.31)	3.64 (± 0.38)
Cdo1	-0.03 (± 1.19)	2.08 (± 1.96)
Fasn	1.70 (± 0.74)	2.87 (± 1.99)
Scd1	1.72 (± 0.88)	3.02 (± 2.73)
Hp	-0.02 (± 1.86)	1.56 (± 3.19)

Table S4. Association between BMI and cardiovascular (CV) complications of influenza A virus in previously published clinical studies

Study location (reference)	Definition of CV complication of influenza virus	% of patients with a healthy BMI[a] with CV complications of influenza	% of overweight[b] patients with CV complications of influenza	P value[c]
Austria (Pawelka et al., 2019)	Any one of the following after influenza: acute heart failure, myocardial infarction, supraventricular tachycardia, hypotension, hypertension	4% (1/23)	25% (10/49)	0.04
the Netherlands (Beumer et al., 2019)	Circulatory collapse following influenza A virus infection	28% (14/50)	40% (22/55)	0.22
Austria (Pizzini et al., 2019).	Any one of the following after influenza: Troponin T levels >28ng/L (2x the upper limit of normal); Acute cardiac event; thoracic pain	22% (8/36)	46% (31/68)	0.02
UK (Dunning et al., 2018)	Cardiovascular SOFA (sequential organ failure assessment) score >2 on the of hospital admission for influenza	57% (4/7)	83% (10/12)	0.3

[a]Defined as a BMI 20-25
[b]Defined as a BMI >25
[c]Determined using Fisher's exact test





5

Viral Factors Important for Efficient Replication of Influenza A Viruses in Cells of the Central Nervous System

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ABSTRACT

Central nervous system (CNS) disease is one of the most common extra-respiratory tract complications of influenza A virus infections. Remarkably, zoonotic H5N1 virus infections are more frequently associated with CNS disease than seasonal or pandemic influenza viruses. Little is known about the interaction between influenza A viruses and cells of the CNS; therefore, it is currently unknown which viral factors are important for efficient replication. Here, we determined the replication kinetics of a seasonal, pandemic, zoonotic, and lab-adapted influenza A virus in human neuron-like (SK-N-SH) and astrocyte-like (U87-MG) cells and primary mouse cortex neurons. In general, highly pathogenic avian influenza (HPAI) H5N1 virus replicated most efficiently in all cells, which was associated with efficient attachment and infection. Seasonal H3N2 and to a lesser extent pandemic H1N1 virus replicated in a trypsin-dependent manner in SK-N-SH but not in U87-MG cells. In the absence of trypsin, only HPAI H5N1 and WSN viruses replicated. Removal of the multi-basic cleavage site (MBCS) from HPAI H5N1 virus attenuated, but did not abrogate, replication. Taken together, our results showed that the MBCS and, to a lesser extent, the ability to attach are important determinants for efficient replication of HPAI H5N1 virus in cells of the CNS. This suggests that both an alternative hemagglutinin (HA) cleavage mechanism and preference for α-2,3-linked sialic acids allowing efficient attachment contribute to the ability of influenza A viruses to replicate efficiently in cells of the CNS. This study further improves our knowledge on potential viral factors important for the neurotropic potential of influenza A viruses.

IMPORTANCE Central nervous system (CNS) disease is one of the most common extra-respiratory tract complications of influenza A virus infections, and the frequency and severity differ between seasonal, pandemic, and zoonotic influenza viruses. However, little is known about the interaction of these viruses with cells of the CNS. Differences among seasonal, pandemic, and zoonotic influenza viruses in replication efficacy in CNS cells, in vitro, suggest that the presence of an alternative HA cleavage mechanism and ability to attach are important viral factors. Identifying these viral factors and detailed knowledge of the interaction between influenza virus and CNS cells are important to prevent and treat this potentially lethal CNS disease.

Keywords: CNS disease, H1N1, H3N2, H5N1, influenza A virus, encephalitis, extrarespiratory, pathogenesis, viral replication, virus attachment

INTRODUCTION

One of the most common extra-respiratory complications of influenza virus infection is central nervous system (CNS) disease (1, 2). Clinically, CNS disease can range from mild febrile seizures to severe or even fatal meningoencephalitis (2, 3). Although most studies on influenza virus-associated CNS disease have focused on influenza A viruses, viruses of type B are also able to cause CNS disease. This is, however, less frequently observed (2, 4). Influenza A viruses, hereinafter referred to as influenza virus, have been linked to CNS disease since the 1918 H1N1 pandemic (5, 6), and CNS disease has been observed during all subsequent pandemics (7,–12) as well as during seasonal epidemics, with sporadic detection of influenza virus in the CNS or cerebral spinal fluid (CSF) of humans (13,–15). Zoonotic influenza viruses only occasionally infect humans, but when they do, they are frequently associated with severe and systemic disease (1). Highly pathogenic avian influenza (HPAI) H5N1 and H7N9 viruses, two recent zoonotic influenza viruses, are both associated with CNS disease (16,–19). The HPAI H5N1 virus is possibly the most neurotropic influenza virus known and has frequently been associated with CNS disease in humans and in other naturally (20,–23) and experimentally (24,–27) infected mammalian species.

In order to infect, replicate in, and spread throughout the CNS, influenza viruses first have to be able to enter the CNS. Entry of influenza viruses into the CNS can occur via, for example, the olfactory (24, 26,–28), trigeminal (6, 27, 29, 30), vagus (29,–31), and sympathetic (27, 31) nerves and possibly other cranial nerves. The primary targets of influenza viruses are, however, epithelial cells of the respiratory tract (32), which differ from cells of the CNS. Influenza virus infection starts with attachment of the virus to sialic acids (SA) present on host cells (33). Human and avian influenza viruses attach preferentially to α-2,6- and α-2,3-linked SA, respectively, present in the upper and lower respiratory tracts of humans, respectively (33). In cells of the CNS, little is known about SA distribution on the different cells at different anatomical locations. One comparative study using lectin immunohistochemistry suggested that in humans, both α-2,6 and α-2,3 SA are present on neurons and glial cells in many different regions, including cerebral cortex, hippocampus, brainstem, and cerebellum (34). In the mouse brain, however, SA distribution is less widespread, and regions with and without detectable SA are infected with influenza viruses (34). In another study, it was found that in human cortex tissue, some neurons only express α-2,3 SA, oligodendrocytes mainly express α-2,6 SA, while astrocytes appear to express both receptors (35). Moreover, both α-2,3 and α-2,6 SA receptors have been found to be present on human neuroblastoma SK-N-SH and SH-SY5Y and human glioblastoma T98G cell lines (36, 37). Given these differential results as well as the fact that SA usage depends on more than α-2,3 and α-2,6 SA linkage, e.g., α-2,8 SA linkage (38, 39) or even SA-independent entry of the virus (40), more studies should reveal which viruses are able to attach to cells in the CNS.

In order for progeny viruses to infect new cells, cleavage of the immature surface protein hemagglutinin (HA) into the biologically activated and infectious form is required (41).

Influenza viruses that contain a monobasic cleavage site can be cleaved by trypsin-like serine proteases such as human airway trypsin-like protease (HAT), transmembrane serine protease 2 (TMPRSS2), TMPRSS4, or matriptase present in the human respiratory tract (42,–44). In the human CNS, expression of HAT in the cerebellum (45) and matriptase mRNA in the frontal and temporal cortices, hippocampus, and cerebellum have been reported (46). Viruses that contain a multibasic cleavage site (MBCS), such as the HPAI H5N1 virus, can be cleaved by ubiquitously expressed subtilisin-like proteases such as furin and PC5/6 (41, 47). This MBCS is an important factor contributing to the ability to spread systemically, including in the CNS. Although extra-respiratory spread of HPAI H5N1 virus depends on the presence of the MBCS in ferrets, insertion of an MBCS into a seasonal H3N2 virus did not result in efficient systemic replication in ferrets, suggesting that more factors are necessary (24, 48). Other viruses that are associated with CNS invasion in mice or ferrets are 1918 H1N1 and A/WSN/33 viruses (6, 49, 50). These viruses do not possess an MBCS but use a different protease-mediated mechanism for HA cleavage, allowing trypsin-independent replication. Taken together, virus receptor specificity, receptor availability on host cells, protease distribution and availability, and HA cleavage mechanism all seem to play important roles in influenza virus infection and cell tropism as well as replication efficiency in the respiratory tract and beyond.

To date, not much is known about the replication efficiency of different influenza viruses, especially seasonal viruses, in cells of the CNS. Thus far, evidence from both in vivo and in vitro studies suggests that HPAI H5N1 viruses are able to infect and replicate in neurons and astrocytes (17, 20,–27, 36, 51,–54), but a direct comparison of replication efficiency in cells of the CNS between seasonal, zoonotic, and pandemic influenza viruses is currently lacking. Similarly, insights into the roles of attachment, protease availability, and presence of an MBCS on replication efficiency in cells of the CNS for these viruses are lacking. Therefore, we here determined the virus attachment, infectivity, and replication kinetics of a seasonal H3N2, 2009 pandemic H1N1 (pH1N1), HPAI H5N1, and WSN viruses in human neuroblastoma (SK-N-SH), human astrocytoma (U87-MG), primary mouse cortex neurons (pmCortex), and Madin-Darby canine kidney (MDCK) cells. Subsequently, we established the importance of the MBCS for the replication efficiency of HPAI H5N1 virus in cells of the CNS.

RESULTS

HPAI H5N1 virus replicates more efficiently in CNS cells than H3N2 and pH1N1 viruses

The replication kinetics of pH1N1, H3N2, HPAI H5N1, and WSN viruses were determined in SK-N-SH, U87-MG, pmCortex, and MDCK cells in the presence of trypsin. All viruses replicated efficiently in MDCK cells, where HPAI H5N1 virus and H3N2 virus replicated to higher titers than pH1N1 virus and WSN virus (Fig. 1D). In both SK-N-SH and U87-MG cells, HPAI H5N1 virus replicated to a significantly higher titer (~7 $\log_{10}$ 50% tissue culture infective dose [$TCID_{50}$]/ml) than all other viruses (Fig. 1A and 1B). In U87-MG and

pmCortex cells, only HPAI H5N1 and WSN viruses were able to replicate (Fig. 1B and 1C). In addition to HPAI H5N1 virus, SK-N-SH cells supported replication of pH1N1, H3N2, and WSN viruses, reaching virus titers of ~5.7, ~3.9, and ~3.3, respectively (Fig. 1A). Overall, our results show that HPAI H5N1 and WSN viruses replicated in all cells investigated and that H3N2 and pH1N1 viruses replicated less efficiently in SK-N-SH cells and not at all in U87-MG cells and pmCortex cells.

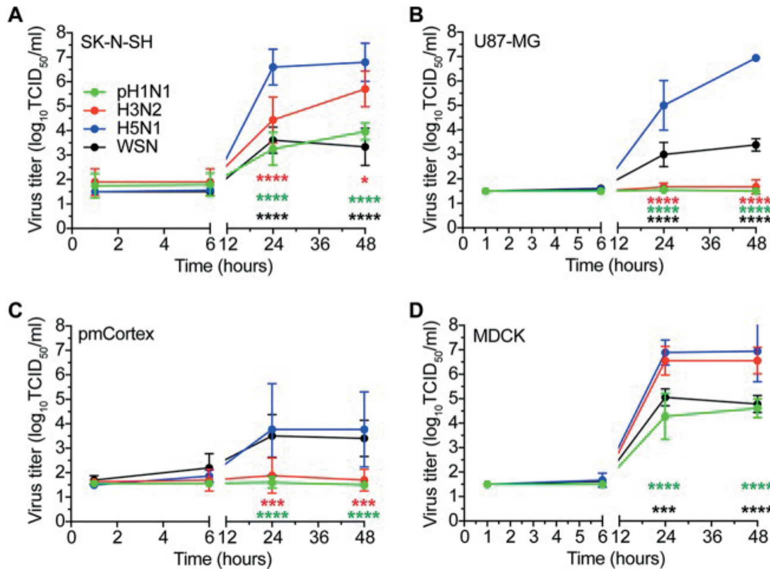

Figure 1. In the presence of trypsin, HPAI H5N1 virus replicates most efficiently in SK-N-SH, U87-MG, pmCortex, and MDCK cells. (A to D) Growth kinetics of pH1N1, H3N2, HPAI H5N1, and WSN viruses in SK-N-SH, U87-MG, pmCortex, and MDCK cells (MOI of 0.1) in the presence of trypsin. Data are presented as means ± SDs from at least three independent experiments. Two-way analysis of variance (ANOVA) with Dunnett's multiple-comparison tests for individual viruses against HPAI H5N1 virus. *, $P \leq 0.05$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$.

HPAI H5N1 and WSN viruses infected cells more efficiently than H3N2 and pH1N1 viruses

To determine whether efficient replication was associated with the ability of the virus to enter and infect host cells, we determined the percentage of infection 8 hours post-infection (hpi) (multiplicity of infection [MOI] of 3) in SK-N-SH, U87-MG, and MDCK cells, measured by flow cytometry (Fig. 2). In MDCK cells, pH1N1, HPAI H5N1, and WSN viruses infected significantly more cells than H3N2 virus. In SK-N-SH and U87-MG cells, HPAI H5N1 and WSN viruses infected significantly more cells than H3N2 or pH1N1 viruses.

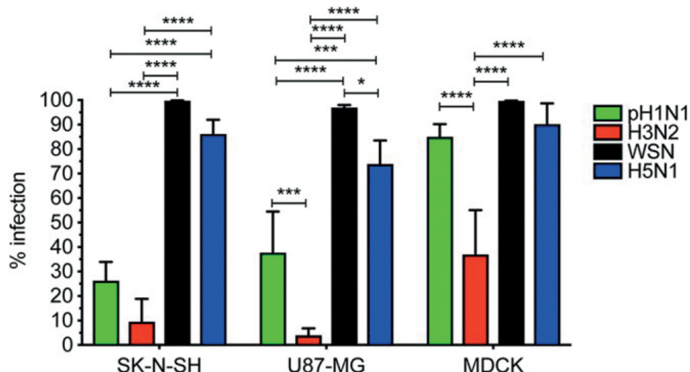

Figure 2. HPAI H5N1 and WSN viruses infect cells more efficiently than pH1N1 and H3N2 viruses. Percentages of infection in SK-N-SH, U87-MG, and MDCK cells were determined by FACS 8 hpi with either pH1N1, H3N2, WSN, or HPAI H5N1 virus (MOI of 3). Data are presented as means ± SDs from at least three independent experiments. Statistical analysis was performed using the two-way ANOVA with Tukey's multiple-comparison test. *, $P \leq 0.05$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$.

pH1N1 and H3N2 viruses attach less efficiently to SK-N-SH and U87-MG cells than HPAI H5N1 and WSN viruses

To determine whether there were differences in attachment between the viruses and whether this was associated with the infection percentages, we performed a virus attachment assay. The attachment efficiency was scored as follows: inefficient attachment (0% to 5%), low attachment (6% to 25%), intermediate attachment (26% to 75%), and efficient attachment (>76%). HPAI H5N1 virus attached efficiently (>90%) to all cell lines investigated (Fig. 3). WSN virus attached efficiently (>95%) to SK-N-SH and U87-MG cells and intermediately (70%) to MDCK cells (Fig. 3). Pandemic H1N1 virus attached with intermediate efficiency to SK-N-SH (37%) and MDCK (30%) cells (Fig. 3A, 3B, and 3D). Seasonal H3N2 virus attached with low efficiency to SK-N-SH (12%) and intermediate efficiency to MDCK (39%) cells (Fig. 3A, 3B, and 3D). Neither pH1N1 nor H3N2 virus attached to U87-MG cells (<2%) (Fig. 3A and 3C). Overall, these results show that pH1N1 and H3N2 viruses attach less efficiently to SK-N-SH and U87-MG cells than H5N1 and WSN viruses.

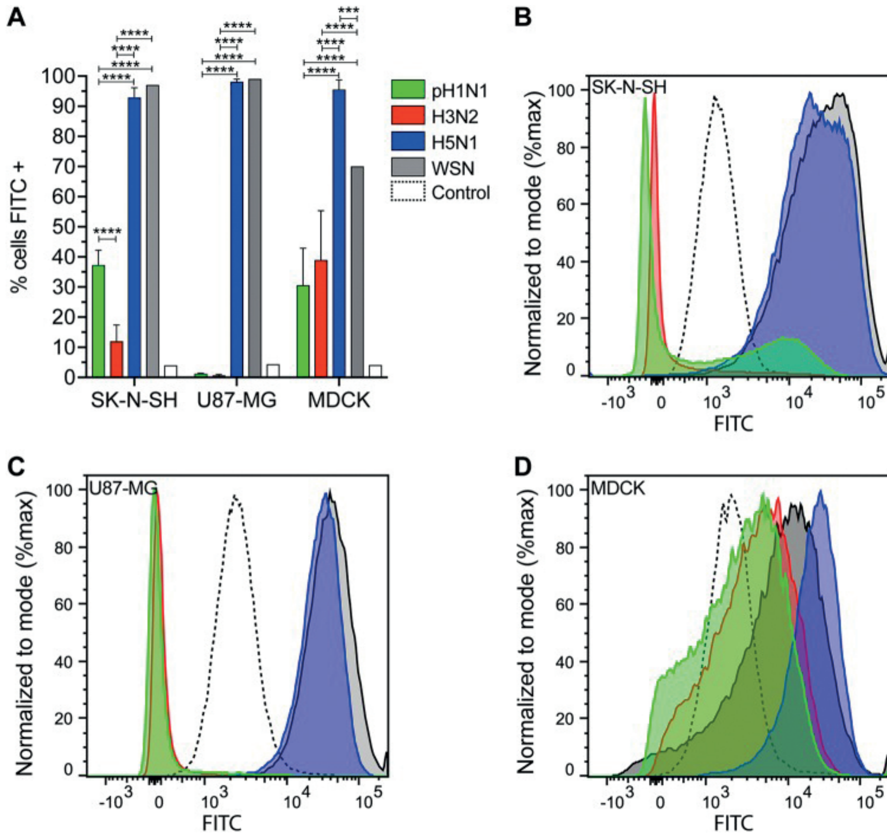

Figure 3. HPAI H5N1 and WSN viruses attach more efficiently to SK-N-SH and U87-MG cells than pH1N1 and H3N2 viruses. Virus attachment of pH1N1, H3N2, HPAI H5N1, and WSN viruses (using 100 hemagglutination units [HAU] units) to SK-N-SH, U87-MG, and MDCK cells. (A) Percentages of cells to which viruses attached. (B to D) Representative histograms of SK-N-SH, U87-MG, and MDCK cells. Dotted lines indicate cell control. Data in panel A are presented as means ± SDs from at least three independent experiments. Two-way ANOVA with Tukey's multiple-comparison test. ***, $P \leq 0.001$; ****, $P \leq 0.0001$.

HPAI H5N1 and WSN viruses replicate in the absence of trypsin, but H3N2 and pH1N1 viruses do not

To test whether efficient replication is dependent on the presence of trypsin, we determined the replication kinetics in the absence of trypsin. Replication of HPAI H5N1 and WSN viruses was not affected by the absence of trypsin (Fig. 4). In the presence of trypsin, H3N2 and pH1N1 viruses replicated efficiently in SK-N-SH cells but not in the absence of trypsin (Fig. 4A). To further understand this finding, we analyzed the presence of specific host cell proteases known to cleave the HA protein of H3N2 and pH1N1 viruses (43). We found that neither SK-N-SH nor U87-MG cells expressed HAT, TMPRSS2, nor TMPRSS4 mRNA, whereas these transcripts were present in human nasal cell (HN) cultures and human

bronchial/tracheal epithelial (HBTE) cultures (Fig. 5). These results show that HPAI H5N1 and WSN viruses replicate independently of trypsin and that pH1N1 and H3N2 viruses are dependent on trypsin for replication in SK-N-SH cells.

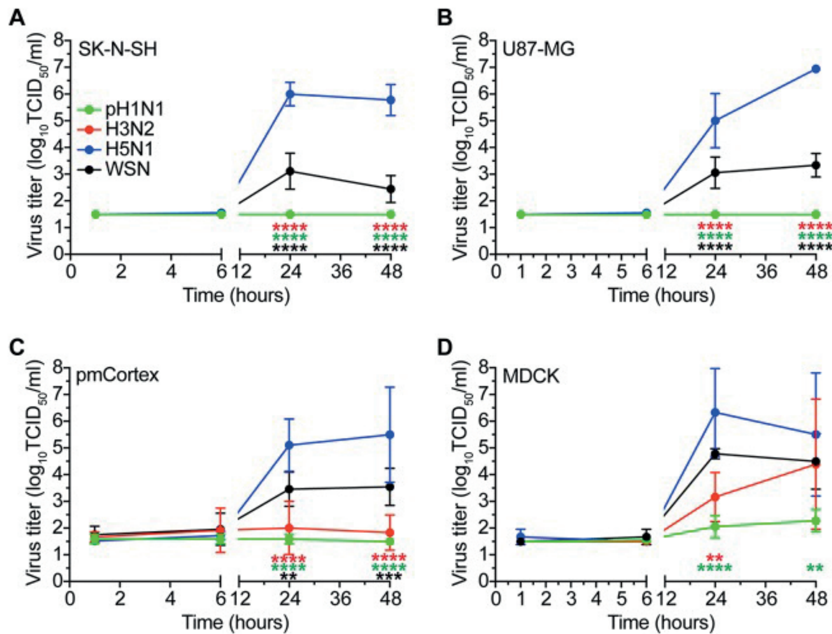

Figure 4. In the absence of trypsin, HPAI H5N1 virus grows most efficiently in SK-N-SH, U87-MG, MDCK, and pmCortex cells. (A to D) Growth kinetics of pH1N1, H3N2, HPAI H5N1, and WSN viruses in SK-N-SH, U87-MG, MDCK, and pmCortex cells (MOI of 0.1) in the absence of trypsin. Data are presented as means ± SDs from at least three independent experiments. Two-way ANOVA with Dunnett's multiple-comparison test against H5N1 virus. **, $P \leq 0.01$ ***, $P \leq 0.001$; ****, $P \leq 0.0001$.

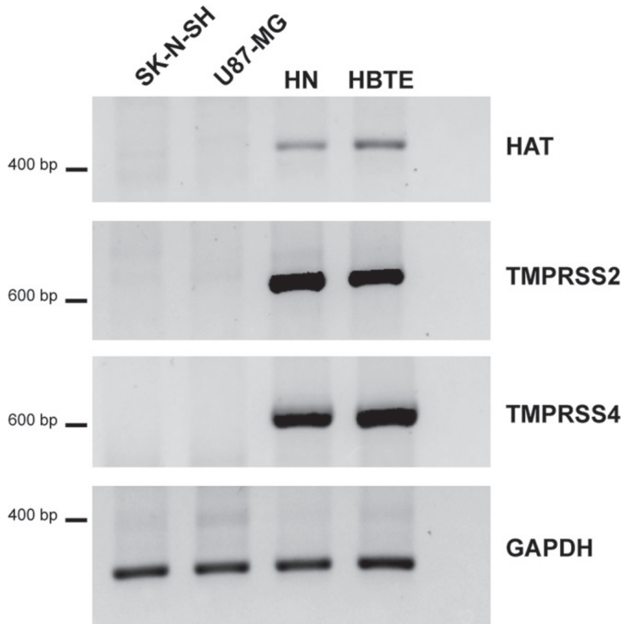

Figure 5. HAT, TMPRSS2, and TMPRSS4 mRNAs are not present in SK-N-SH and U87-MG cells. Presence of three known HA cleaving enzymes: human airway trypsin (HAT), transmembrane serine protease 2 (TMPRSS2), and TMPRSS4. Positive-control cell lines human nasal cells (HN) and human bronchial/tracheal epithelial (HBTE) cells did express HAT, TMPRSS2, and TMPRSS4 mRNA.

The MBCS of HPAI H5N1 virus is important but not solely responsible for replication in SK-N-SH cells

To determine whether efficient replication of HPAI H5N1 virus in cell culture solely depends on the presence of the MBCS, we generated an H5N1 virus without the MBCS. The replication kinetics of the HPAI H5N1^{WT} (wild-type) virus was not affected by the presence or absence of trypsin in all cell lines (Fig. 6A). However, the H5N1$^{\Delta MBCS}$ virus, without trypsin, replicated to a lower titer on each cell line investigated. This phenotype was restored by the addition of trypsin to the culture medium, allowing the virus to replicate to wild-type levels (Fig. 6A). The reduced replication efficiency of H5N1$^{\Delta MBCS}$ virus was not explained by the ability of the virus to infect cells, since this was not affected (Fig. 6B). In order to determine if there were multiple rounds of infection and that virus detected in the supernatant was not solely the result of primary infected cells, we investigated the percentage of infection at 8 and 24 hpi with an MOI of 0.1, without trypsin, measured by flow cytometry. We found that HPAI H5N1^{WT} virus efficiently replicated in MDCK, SK-N-SH, and U87-MG cells as indicated by the increase of infection percentages (Fig. 6C). In contrast, a significant increase for H5N1$^{\Delta MBCS}$ virus only was observed in MDCK and U87-MG cells. In SK-N-SH cells, no increased infection percentage was observed. These results reveal that the MBCS is

important but not solely responsible for efficient replication in MDCK and U87-MG cells in the absence of trypsin.

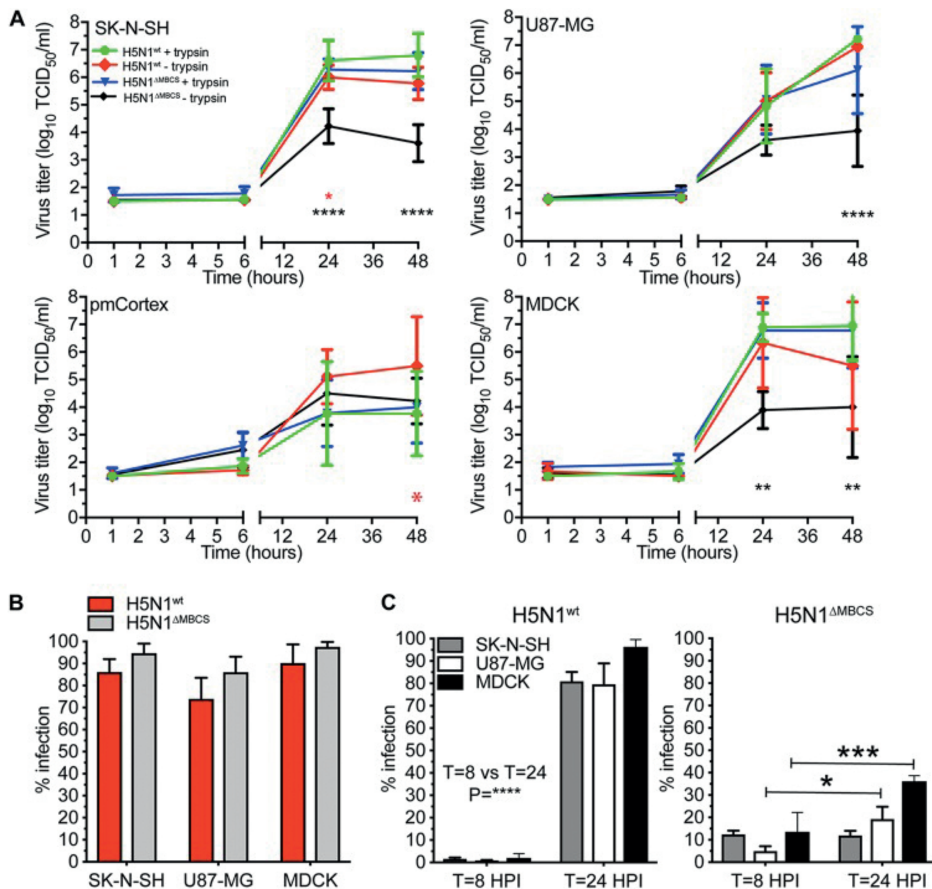

Figure 6. H5N1 virus without an MBCS replicates less efficiently in the absence of trypsin. (A) Replication kinetics of HPAI H5N1WT and H5N1ΔMBCS viruses in SK-N-SH, U87-MG, and MDCK cells (MOI of 0.1) in the presence or absence of trypsin. Statistical analysis was performed using a two-way ANOVA with Tukey's multiple-comparison test against "$H5N1^{WT}$ + trypsin." (B) Percentages of infection in SK-N-SH, U87-MG, and MDCK cells were determined by FACS at 8 hpi with HPAI $H5N1^{WT}$ and $H5N1^{\Delta MBCS}$ viruses (MOI of 3). Statistical analysis was performed using the two-way ANOVA with Bonferroni's multiple-comparison test. (C) Percentages of infection in SK-N-SH, U87-MG, and MDCK cells were determined by FACS at 8 and 24 hpi with HPAI $H5N1^{WT}$ and $H5N1^{\Delta MBCS}$ viruses at an MOI of 0.1 in the absence of trypsin. Statistical analysis was performed using the two-way ANOVA with Bonferroni's multiple-comparison test (8 versus 24 hpi). All data are presented as means ± SDs from at least three independent experiments. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$.

DISCUSSION

Here we show that HPAI H5N1 virus replicates more efficiently in human and mouse neuronal cells than seasonal H3N2 and 2009 pandemic H1N1 viruses. Both the ability to attach efficiently and the presence of an MBCS of HPAI H5N1 virus contributed to efficient replication in cells of the CNS, indicative that these are viral factors that contribute to the neurotropic potential of influenza viruses. This fits with the facts that HPAI H5N1 virus is more frequently associated with CNS disease in humans than seasonal and pandemic viruses (1) and that this virus is also more often detected in tissues of the CNS in experimentally inoculated laboratory animals than seasonal and pandemic viruses (17, 20,–27, 51, 55,–58).

The ability of HPAI H5N1 and WSN viruses to replicate efficiently in cells of the CNS seems to be associated with the ability to attach to and infect host cells efficiently. Especially, HPAI H5N1 virus which replicated efficiently in SK-N-SH and U87-MG cells, attached to high percentages of cells with high intensity, and infected these cells efficiently. WSN virus attached to and infected high percentages of neuronal cells, which resulted in multiple rounds of infection in all cells, although to lower titers on SK-N-SH and U87-MG cells than observed for HPAI H5N1 virus. The latter could be due to the fact that WSN virus is extensively passaged in suckling mouse brains, thereby adapting to mouse neuronal cells and not human neuronal cells. Seasonal H3N2 viruses replicated in SK-N-SH cells, even in the absence of efficient attachment or infection. Whether this is due to low-affinity binding of H3N2 virus, which cannot be detected by our assays, is unknown, but it does suggest that efficient attachment, as observed for H5N1 and WSN viruses on neuronal cells, is not the only viral factor involved in replication of influenza viruses in cells of the CNS. The lack of replication of H3N2 and pH1N1 viruses in U87-MG cells could be explained by both inefficient attachment and infection.

Efficient replication of influenza viruses in cells of the CNS seems to depend in part on the presence of an MBCS or alternative HA cleaving mechanisms. Studies in ferrets, mice, macaques, and chickens show that introduction or removal of an MBCS has different outcomes based on the virus backbone and host species (24, 48, 59,–61). In vitro, in the absence of trypsin, only HPAI H5N1 and WSN viruses were able to replicate, indicating that pH1N1 and H3N2 viruses are not able to circumvent the need for trypsin-like protease for HA cleavage. Removal of the MBCS from HPAI H5N1 virus resulted in attenuated replication in SK-N-SH and U87-MG cells in the absence of trypsin but not in pmCortex cells. Viruses without an MBCS that are associated with replication in cells of the CNS in vivo, such as the 1918 H1N1 and WSN viruses, have an alternative HA cleavage mechanism (6, 62). The WSN virus lacks a conserved glycosylation site in the neuraminidase making the virus trypsin independent (63) by using the serine-protease plasmin, which is present in many organ systems besides the respiratory tract (64). The 1918 H1N1 virus grows trypsin independent and neuraminidase dependent in MDCK cells and polarized Calu-3 cells but not in Huh-7 cells (65, 66). Our observation that neither HAT, TMPRSS2, nor TMPRSS4

is found in SK-N-SH and U87-MG cells supports the hypothesis that for efficient replication in cells of the CNS, influenza viruses require alternative HA cleavage, as shown for the HPAI H5N1, WSN, and 1918 H1N1 viruses. However, it must be noted that there is limited knowledge on the expression and accessibility of proteases in tissues other than the respiratory tract.

Previous studies on the replication kinetics of influenza viruses in cells of the CNS revealed some differences, which can in part be explained by the use of different cells, virus isolates, and experimental approaches (36, 54). Replication of HPAI H5N1 viruses in differentiated astrocytic cell lines resulted in efficient replication, similar to our observations. However, in SH-SY5Y cells, a subclone cell line derived from SK-N-SH cells, two HPAI H5N1 viruses did not replicate efficiently. This discrepancy could be due to the relatively high MOI used in this study compared to the low MOI we used and which resulted in efficient replication (36). Furthermore, two pandemic H1N1 viruses did not replicate in the neuronal or astrocytic cell lines, which fits with our observation, in the absence of trypsin (54).

Pandemic H1N1 and seasonal H3N2 viruses are occasionally detected in the CNS or CSF in humans and from experimentally inoculated ferrets and mice, even though pH1N1 and H3N2 viruses only replicated in SK-N-SH cells in the presence of trypsin (11,–15, 67,–70). However, these viruses are rarely isolated in high titers or detected by immunohistochemistry in the CNS of humans or experimentally inoculated ferrets and mice, indicating that these viruses might be able to enter the CNS but that replication is inefficient. This could be attributed to the limited attachment and infection and lack of an alternative HA cleavage mechanism allowing efficient replication. Previously, we showed that even in the absence of active virus replication, pro-inflammatory cytokines, such as interleukin 6 (IL-6), IL-8, and tumor necrosis factor alpha (TNF-α), are induced in the CNS of pH1N1 experimentally inoculated ferrets (71). Future studies should reveal how both efficient and inefficient replication in neuronal cells can trigger local pro-inflammatory responses, for which HPAI H5N1 and H5N1$^{\Delta MBCS}$ viruses might be a good model.

Taken together, results of our study have shown that the presence of an MBCS and, to a lesser extent, the ability to attach are important determinants for replication of HPAI H5N1 virus in cells of the CNS. This suggests that, at least for replication within the CNS, neurotropic influenza viruses contain an alternative HA cleavage mechanism and prefer α-2,3-linked sialic acids.

MATERIALS AND METHOD

Cells

Human neuroblastoma (neuron like, SK-N-SH) and human glioblastoma (astrocyte like, U87-MG) cells were purchased from Sigma-Aldrich and maintained in Eagle minimal

essential medium (EMEM; Lonza, Breda, the Netherlands) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO, USA), 100 IU/ml penicillin (Lonza, Basel, Switzerland), 100 µg/ml streptomycin (Lonza), 2 mM glutamine (Lonza), 1.5 mg/ml sodium bicarbonate (Cambrex, Wiesbaden, Germany), sodium pyruvate (Thermo Fisher Scientific, Waltham, MA, USA) and 1× (0.1 mM) nonessential amino acids(MP Biomedicals Europe, Illkirch, France). As a control cell line, we have included Madin-Darby canine kidney (MDCK) cells, since these cells are extensively used for influenza virus propagation. MDCK cells were maintained in EMEM supplemented with 10% FBS, 100 IU/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine, 1.5 mg/ml sodium bicarbonate, 1 mM, 10 mM HEPES (Cambrex), and 1× (0.1 mM) nonessential amino acids.

Viruses

Five viruses were included in this study, a seasonal H3N2 virus (A/Netherlands/213/2003), pH1N1 virus (A/Netherlands/602/2009), and zoonotic HPAI H5N1 virus (A/Indonesia/5/2005) all isolated from humans. Neurotropic WSN virus (A/WSN/33) and H5N1 virus lacking an MBCS (H5N1ΔMBCS) were generated using reverse genetics as described before (72) and passed once on 293T cells and once on MDCK cells. Experiments involving HPAI H5N1 and H5N1$^{\Delta MBCS}$ viruses were performed under biosafety level 3 conditions.

5

Isolation and culture of primary mouse cortex neurons

Animals were housed and experiments were conducted in strict compliance with European guidelines (EU directive on animal testing 86/609/EEC) and Dutch legislation (Experiments on Animals Act, 1997). Primary mouse cortex tissue was isolated from embryonic day 17 (E17) to E19 C57BL6 mouse embryos (Charles River Laboratories, Wilmington, MA, USA). The cultures were pooled cortexes of several mouse embryos originating from one mother. In brief, the cortex was dissected in ice-cold Hanks' balanced salt solution (HBSS; Life Technologies) supplemented with 20 µg/ml gentamicin (Life Technologies) under guidance with a stereomicroscope (Nikon). Next, tissues were cut to ~1 mm3 using a scalpel and digested using medium consisting of HBSS supplemented with 10 U/ml papain (Sigma), 2.5 U/ml DNase I (Roche), and 4 mM MgCl2 (Sigma-Aldrich) at 33°C for 15 min. After incubation, cells were washed once in 1 ml of 10% FBS (Life Technologies) in HBSS to stop the digestion. A second "mechanical digestion" was performed by carefully pipetting up and down in digestion buffer (without papain). After washing in HBSS twice, cells were counted using a Moxi Go cell counter (Orflo, Ketchum, ID, USA) and seeded on laminin (500 µg/ml; Sigma)-coated 1.5H 96-well glass-bottomed plates (Cellvis, Sunnyvale, CA, USA) at a density of 1.0 × 104 cells/well. For the first 2 h, the cells were cultured in culture medium containing 10% FBS. After 2 h, medium was replaced with fresh culture medium, without FBS. The culture medium contains primary neuron growth medium (PNBM; Lonza), GS-21 supplement (Tebu-Bio, Le-Perray-en-Yvelines, France), 5 µg/ml gentamicin (Thermo Fisher), and 2 mM GlutaMAX (Life Technologies). Half of the medium was changed once per week, and cells were cultured for 7 to 10 days before use.

Replication kinetics

Cells were infected at a multiplicity of infection (MOI) of 0.1. Virus dilutions were prepared in the cell-specific culture medium without serum (infection medium, see "Virus titrations"). After 1 h of virus absorption, cells were washed once with fresh infection medium and cultured in infection medium in the presence or absence of l-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin (see "Virus titration"). At time points 1, 6, 24, and 48 hours post-infection (hpi), 100 µl supernatant was collected and stored at −80°C for subsequent virus titration. All experiments were performed three times (biological replicates), and each experiment was performed with duplicates (technical replicates) from which the average was used for statistical analysis.

Virus titrations

The 50% tissue culture infectious dose ($TCID_{50}$) in cell supernatant was determined by endpoint titration on MDCK cells, as described before (73). Briefly, 10-fold serial dilutions of cell supernatants were prepared in infection medium. Infection medium consisted of EMEM, 100 IU/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine, 1.5 mg/ml sodium bicarbonate, 10 mM HEPES, 1× (0.1 mM) nonessential amino acids, and 1 µg/µl TPCK-treated trypsin (Sigma-Aldrich). Before inoculation, MDCK cells were washed twice with phosphate-buffered saline (PBS) to remove remaining FBS. One hundred microliters of the diluted supernatant was used to inoculate a confluent monolayer of MDCK cells in 96-well plates. After 1 h at 37°C, the cells were washed once with infection medium, and 200 µl new infection medium was added to each well. Three days after infection, supernatants of infected cell cultures were tested for agglutinating activity using turkey erythrocytes as an indicator of virus replication. The titers of infectivity were calculated from three replicates according to the method of Kärber (74). An initial 1:10 dilution of supernatant resulted in a detection limit of $10^{1.5}$ $TCID_{50}$/ml.

Percentage of infection

After 8 hpi (with an MOI of 3) or after 8 and 24 hpi (with an MOI of 0.1), in the absence of trypsin, cells were collected, fixed, and permeabilized using BD Cytofix/Cytoperm solution (BD Biosciences, Franklin Lakes, NJ, USA) according to the manufacturer's instructions. Cells were incubated with 2% normal goat serum (NGS; Dako, Denmark) in PBS for 10 min on ice. Next, influenza A virus was detected using a monoclonal antibody against influenza A virus nucleoprotein (clone HB-65, 1 µg/ml; ATCC) or mouse IgG2a isotype control (MAB003, 1 µg/ml; Dako) in BD Perm/Wash containing 2% NGS and incubated for 1 h on ice and in the dark. Cells were washed twice and incubated with goat anti-mouse IgG2a conjugated to Alexa Fluor 488 (8 µg/ml; Life Technologies, Inc., the Netherlands) for 1 h in the dark and on ice. After incubation, cells were washed twice and resuspended in fluorescence-activated cell sorting (FACS) buffer. Cells were measured and data collected using a BD FACSCanto II (BD Biosciences, USA). Data were analyzed using FlowJo 10 software (Ashland, OR, USA). All experiments were performed three times (biological

replicates), and each experiment included duplicate (technical replicate) measurements from which the average was calculated and used for further analysis.

Virus attachment

For influenza virus histochemistry, viruses were grown, inactivated, and labeled as described previously (32). As a control, uninfected MDCK cells and cell debris were harvested and processed similarly. Subsequently, in a 12-well plate, 2 × 105 cells were seeded, and 1 day later, the near confluent monolayers of MDCK, SK-N-SH, and U87-MG cells were harvested, washed in FACS buffer, and incubated with fluorescein isothiocyanate (FITC)-labeled virus for 1 h at 4°C. After incubation, the cells were washed twice in FACS buffer and measured using a BD FACSCanto II (BD Biosciences, USA). Data were analyzed using FlowJo 10 software (Ashland, OR, USA). All experiments were performed three times (biological replicates), and each experiment was performed with duplicates (technical replicates) from which the average was used for statistical analysis.

PCR proteases

Since MDCK cells are of canine origin, we have included primary human nasal (HN) cells (MucilAir, pool of 14 donors; Epithelix, Geneva, Switzerland), and primary human bronchial/tracheal epithelial cells (HBTE) (catalog number CC-2540, lot 97366, donor 97366: male, Caucasian, 57 years, healthy; Lonza) as control cell types for the expression of human HAT, human TMPRSS2, and human TMPRSS4. Total RNA was isolated from SK-N-SH, U87-MG, HN, and HBTE cells using the High Pure RNA isolation kit (Roche, Basel, Switzerland) according to the manufacturer's protocol. cDNA synthesis was performed using oligo(dT) primers and Superscript IV (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. For detection of HAT-, TMPRSS2-, and TMPRSS4-specific mRNAs, primers were used from Böttcher-Friebertshäuser et al. (75). The GAPDH (glyceraldehyde-3-phosphate dehydrogenase) mRNA was detected using primers GAPDH-FW (5′-TGA ACG GGA AGC TCA CTG G-3′) and GAPDH-RV (5′-TCC ACC ACC CTG TTG CTG TA-3′) as a control for sample quality. PCR products were resolved on a 1.5% agarose gel stained with SYBR Safe (Thermo Fisher) and imaged using a ChemiDoc MP imaging system and ImageLab 5.1 (Bio-Rad, Hercules, CA, USA). To confirm the specificity of the primers, PCR products were extracted from the gel and sequenced using a BigDye Terminator v3.1 Cycle sequencing kit (Applied Biosystems) and a 3130XL genetic analyzer (Applied Biosystems), according to the instructions of the manufacturer.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 6.0h software (La Jolla, CA, USA) for Mac. Each specific test is indicated in the figure legends. P values of ≤0.05 were considered significant. All data are presented as means ± standard deviations (SDs) from at least three independent experiments.

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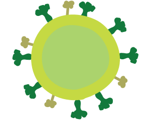

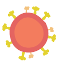

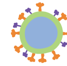

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Vaccination Is More Effective Than Prophylactic Oseltamivir in Preventing CNS Invasion by H5N1 Virus via the Olfactory Nerve

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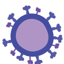

ABSTRACT

Background

Influenza A viruses can replicate in the olfactory mucosa and subsequently use the olfactory nerve to enter the central nervous system (CNS). It is currently unknown whether intervention strategies are able to reduce or prevent influenza virus replication within the olfactory mucosa and subsequent spread to the CNS. Therefore, we tested the efficacy of homologous vaccination and prophylactic oseltamivir to prevent H5N1 virus CNS invasion via the olfactory nerve in our ferret model.

Methods

Ferrets were vaccinated intramuscularly or received oseltamivir (5 mg/kg twice daily) prophylactically before intranasal inoculation of highly pathogenic H5N1 virus (A/Indonesia/05/2005) and were examined using virology and pathology.

Results

Homologous vaccination reduced H5N1 virus replication in the olfactory mucosa and prevented subsequent virus spread to the CNS. However, prophylactic oseltamivir did not prevent H5N1 virus replication in the olfactory mucosa sufficiently, resulting in CNS invasion via the olfactory nerve causing a severe meningoencephalitis.

Conclusions

Within our ferret model, vaccination is more effective than prophylactic oseltamivir in preventing CNS invasion by H5N1 virus via the olfactory nerve. This study highlights the importance of including the olfactory mucosa, olfactory nerve, and CNS tissues in future vaccine and antiviral studies, especially for viruses with a known neurotropic potential.

Keywords: olfactory nerve; encephalitis; vaccine; oseltamivir; H5N1; virus; influenza; ferret; olfactory mucosa

INTRODUCTION

The most common and potentially fatal extra-respiratory tract complication of influenza A virus infections is central nervous system (CNS) disease [1]. Clinical manifestations are diverse and can vary from febrile seizures to a severe or even fatal encephalitis (or meningoencephalitis). In addition, influenza virus infections have been linked to chronic CNS diseases, such as Guillain-Barré syndrome, post-encephalitis Parkinsonism, Parkinson-like disease, and Kleine-Levin syndrome, [2, 3]. Throughout history, CNS disease has been linked to pandemic, seasonal, and zoonotic influenza viruses. The 1918 H1N1 pandemic was associated with encephalitis lethargica and post-encephalitis Parkinsonism [4]. Furthermore, CNS disease was associated with the 1957 H2N2 “Asian flu” pandemic [5], the 2009 H1N1 “Swine flu” pandemic [6], seasonal influenza viruses [7, 8], zoonotic highly pathogenic avian influenza (HPAI) H5N1 virus [9, 10] and zoonotic avian H7N9 virus [11].

Human HPAI H5N1 virus infection was first identified in 1997 [12] and has resulted in >800 human infections thus far, with a mortality rate of >50% [13]. Evidence that HPAI H5N1 virus is able to enter the CNS and cause severe or even fatal encephalitis (or meningoencephalitis) in humans, is based on detection of whole virus, virus RNA, or virus antigen in the CNS or cerebrospinal fluid (CSF) of patients [9, 10, 14–16]. CNS invasion of HPAI H5N1 virus has also been observed in naturally infected tigers [17], leopards [18], cats [19], a stone marten [20], and experimentally infected ferrets [21–23], mice [24], and red foxes [25], indicating the neurotropic potential of this virus across a wide range of mammalian species.

The pathogenesis of influenza virus-associated CNS disease has not been studied extensively. Others and we have shown that influenza viruses can enter the CNS via the olfactory nerve in mice [24, 26], ferrets [21, 23, 27, 28], and humans [8]. The olfactory nerve starts in the nasal cavity at the olfactory mucosa, which contains olfactory receptor neurons (ORNs) [29]. These ORNs have an apical dendrite that samples the nasal cavity and an axon that extends through the cribriform plate and terminates in the olfactory bulb, synapsing with second-order neurons and interneurons [30]. In ferrets, intranasal inoculation with HPAI H5N1 virus results in infection of ORNs in the olfactory mucosa from as early as 1 day after inoculation [23]. Subsequently, HPAI H5N1 virus can be detected in the olfactory bulb 3 days after inoculation, after which the virus disseminates throughout the CNS, causing severe meningoencephalitis without severe lower respiratory tract involvement [23]. Although the ability or efficiency of different influenza viruses to travel along the olfactory nerve might vary, we have shown virus attachment to olfactory mucosa—the first step in the influenza virus replication cycle—not only for HPAI H5N1 virus, but also for pandemic H1N1 and seasonal H3N2 viruses [8].

Prophylactic strategies for influenza viruses are designed to reduce morbidity and mortality rates from respiratory tract disease in humans. Vaccination based on antigenic homologous virus remains the most effective prophylactic measure against influenza virus infection [31].

However, during an outbreak of a novel and potentially pandemic influenza virus, homologous vaccines are not readily available. In these cases, prophylactic antiviral therapy could be used to prevent or reduce infection and virus spread. The most commonly used antiviral drug against influenza virus infection is the orally administered neuraminidase inhibitor, oseltamivir [32, 33]. The efficacy of these prophylactic strategies on virus replication in the olfactory mucosa and subsequent CNS invasion is poorly studied, however. In the current study, we investigated the efficacy of homologous vaccination or prophylactic oseltamivir on HPAI H5N1 virus replication in the olfactory mucosa and subsequent CNS invasion in ferrets.

METHODS

Virus Preparation

HPAI H5N1 virus (A/Indonesia/5/05) was isolated from a human patient and passaged once in embryonated chicken eggs and twice in Madin-Darby canine kidney cells to a titer of $10^{8.3}$ 50% tissue culture infectious dose ($TCID_{50}$)/mL, as described elsewhere [34].

Ferret Experiment

All animal experiments were performed according to Dutch regulations, approved by an independent animal ethics committee, and performed under biosafety level 3 conditions. In total, 18 influenza virus seronegative and Aleutian disease-negative female ferrets (*Mustela putorius furo*) were divided among 3 groups of 6 ferrets each: a control group, a vaccination group, and an oseltamivir-treated (OST) group. Control group ferrets did not receive any pretreatment. Vaccine group ferrets were vaccinated intramuscularly on days −56 and −28 with AS03A-adjuvanted H5N1 A/Indonesia/5/05 split vaccine (3.75 μg of hemagglutinin per dose; Prepandrix, GlaxoSmithKline Biologicals). Ferrets in the OST group received prophylactic oral oseltamivir, 5 mg/kg twice daily, from day −1 until 7 days after inoculation (kindly provided by Hoffman-La Roche). This yields a systemic drug exposure equivalent to 75 mg, as recommended by the World Health Organization for prophylactic oseltamivir in humans [35]. Serum samples were collected at days −56 and −1 and at the end of the experiment.

On day 0, all ferrets were sedated with ketamine, inoculated intranasally with 10^6 $TCID_{50}$ of HPAI H5N1 virus (A/Indonesia/05/05), divided between both nostrils (250 μL to each nostril), and kept sedated for 10–15 minutes while on their backs. Nasal and pharyngeal swab samples were collected daily for virology. Nasal swab sample were collected from a single nostril to keep the respiratory mucosa of the other nostril intact for pathology. Ferrets were weighed daily and observed for clinical signs beginning 2 days after inoculation. At 3 and 7 days after inoculation, 3 randomly selected ferrets from each group were euthanized by exsanguination after anesthesia with ketamine, and tissues were collected for virological and/or pathological analysis, including nasal turbinates (NTs; respiratory and olfactory mucosa), trachea, lungs, tonsil, adrenal gland, tracheobronchial lymph node, liver, spleen, kidney,

heart, pancreas, duodenum, jejunum, olfactory bulb, cerebrum, cerebellum, cervical spinal cord, blood, and CSF.

Antibody Titers and Oseltamivir Levels

Serum samples from days −56 and −1 were tested for the presence of antibodies against A/Indonesia/05/2005 by hemagglutination inhibition assay, as described elsewhere [31], and results were expressed as reciprocals of the titers. Serum samples from OST ferrets (day −1 and day 3 or 7 after inoculation, 12–14 hours after the last dose) were tested with mass spectrometry for the presence of oseltamivir phosphate and oseltamivir carboxylate, the latter the biologically active component, as described elsewhere [36].

Virus Titrations

Virus titers ($TCID_{50}$) in nasal swab, pharyngeal swab, blood, CSF, and homogenized tissue samples from inoculated ferrets were determined by end point titration on Madin-Darby canine kidney cells, as described elsewhere [34].

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 antigen by immunohistochemistry, tissues were stained with a monoclonal antibody against influenza A virus nucleoprotein (clone HB-65; ATCC), as described elsewhere [37].

Detection of Neuraminidase-Resistant Mutations

To test whether HPAI H5N1 virus had acquired oseltamivir resistance mutation H275Y (N1 numbering) [38] in OST ferrets, viral RNA was extracted from tissues of the NTs and cerebrum using the High Pure RNA Isolation Kit (Roche) and reverse-transcribed to complementary DNA (SuperScript III Reverse Transcriptase,; ThermoFisher Scientific). A primer pair covering the region of mutation H275Y was used for amplification by polymerase chain reaction and sequenced using a BigDye Terminator v3.1 Cycle sequencing kit and a 3130XL genetic analyzer (both Applied Biosystems), according to the manufacturer's instructions.

Statistics

Statistical analysis (Mann–Whitney test) comparing control versus vaccine, control versus OST, and vaccine versus OST groups as performed using GraphPad Prism software 6.0h. A probability value of Differences were considered significant at $P \leq .05$.

6

RESULTS

Antibody Titers and Oseltamivir Levels

Before vaccination, none of the ferrets had any detectable antibodies against A/Indonesia/5/05 (Table 1). After the second vaccination, and 1 day before virus inoculation, A/Indonesia/5/05 virus–specific antibody titers were detected in all vaccinated ferrets. In all ferrets except ferret 8, hemagglutination inhibition titers were >40, which is considered protective [39]. In serum samples from OST ferrets collected during autopsy, oseltamivir phosphate and oseltamivir carboxylate concentrations were measured by mass spectrometry. Oseltamivir phosphate was undetectable, but oseltamivir carboxylate, the biologically active component, could be detected (mean [standard deviation {SD}], 102.1 [15.5] ng/mL) in serum samples from all OST ferrets (data not shown).

Table 1. Hemagglutination Inhibition Antibody Titers Against A/Indonesia/5/05 Before and After Vaccination[a]

	A/Indonesia/5/05 antibody titers	
Ferret number	Day -56	Day -1
Control		
3	<5	<5
6	<5	<5
9	<5	<5
12	<5	<5
14	<5	<5
15	<5	<5
Vaccine		
1	<5	320
4	<5	320
8	<5	25
10	<5	80
16	<5	240
18	<5	480
Oseltamivir		
2	<5	<5
5	<5	<5
7	<5	<5
11	<5	<5
13	<5	<5
17	<5	<5

[a] Ferrets in the vaccine group were vaccinated on days −56 and −28; control and oseltamivir-treated ferrets were not vaccinated.

Clinical Signs, Gross Pathology, and Weight Loss

Clinical signs were scored beginning 2 days after inoculation. Control ferrets were lethargic beginning 3 or 4 days after inoculation, although they remained alert when stimulated. Two

of 3 control ferrets (ferrets 6 and 12) had ataxia at 7 days after inoculation. In the vaccinated ferrets, no clinical signs of disease were observed. Clinical signs in OST ferrets were comparable to those in the control group; beginning 3 days after inoculation, the ferrets were lethargic, but remained alert on stimulation; beginning 6 days after inoculation, 2 of 3 (ferrets 5 and 7) had ataxia.

Gross pathology in control ferrets showed that at 3 days after inoculation up to 10% of the lungs were consolidated, and all ferrets had mild lipidosis of the liver. At 7 days after inoculation, up to 5% of the lungs were consolidated in all ferrets, and ferret 12 showed mild hepatic lipidosis. In vaccinated ferrets at 3 days after inoculation, up to 5% of the lungs were consolidated. At 7 days after inoculation, ferrets 10 and 16 had up to 5% consolidation of the lungs. Ferret 8 showed 20% consolidation of the lungs, an enlarged spleen and lymph nodes, and hemorrhage in the rostral meninges. In the OST group, up to 5% of the lungs were consolidated, and all ferrets showed mild hepatic lipidosis at 3 days after inoculation. At 7 days after inoculation, up to 10% of the lungs were consolidated.

Control ferrets lost significantly more weight than vaccinated ferrets at 2 and 3 days after inoculation, a trend that continued until the end of the experiment (Figure 1A). The weight loss in OST ferrets was comparable to that of the control ferrets. At 7 days after inoculation, the mean (SD) weight loss compared with day 0 was 11.9% (2.7%) in control ferrets, 3.6% (1.2%) in vaccinated ferrets, and 14.1% (4.0%) in OST ferrets.

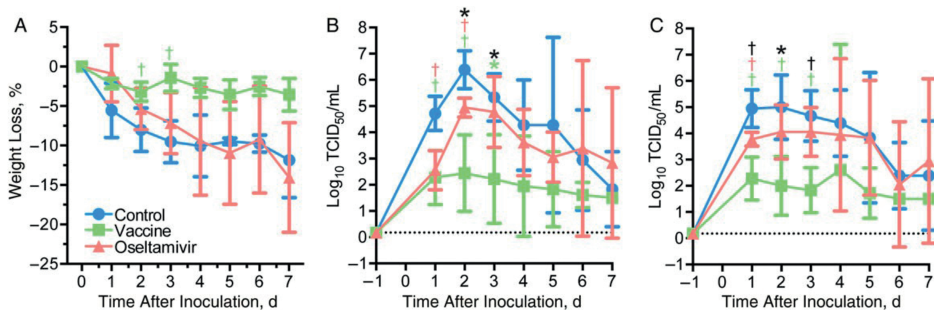

Figure 1. Vaccination reduced weight loss and virus shedding more effective than prophylactic oseltamivir treatment. Ferrets were inoculated with highly pathogenic avian influenza (HPAI) H5N1 virus and weighed daily to assess disease progression. (**A**) Mean weight loss during 7-day experiment. Weight loss or gain was calculated as percentage change from baseline (day 0). B, C, Nasal (**B**) and pharyngeal (**C**) swab samples were collected daily to assess virus titers. Data are presented as means (geometric, for B and C) with standard deviations for 6 ferrets (day 0–3) or 3 ferrets (day 4–7). Statistical significance was calculated using the Mann–Whitney test for comparison with control ferrets (green and red) or between vaccine- and oseltamivir-treated ferrets (black). $^{*}P < .05$; $^{\dagger}P < .01$. Abbreviation: $TCID_{50}$, 50% tissue culture infectious dose.

Virology

Virus Isolation From Nasal and Pharyngeal Swab Samples

Control ferrets shed virus up to 7 days after inoculation, peaking at 2 days after inoculation in the nasal (mean [SD], 6.4 [0.7] log_{10} $TCID_{50}$/mL) and pharyngeal (5.0 [1.2]) (Figure 1B and 1C) swab samples. In vaccinated ferrets, low virus titers could be isolated from the nasal and pharyngeal swab samples until 3 days after inoculation, except in ferret 8, which shed until 6–5 days after inoculation. Virus titers isolated from the nasal and pharyngeal swab samples were significantly lower than from control ferrets at 1–3 days after inoculation. Virus shedding in OST ferrets occurred from 1 to 7 days after inoculation in both nasal and pharyngeal swab samples, peaking at 2 (mean [SD], 4.9 [0.3]) and 3 (4.1 [0.9]) days after inoculation, respectively. In nasal swab samples, virus titers were significantly lower at 1 and 2 days after inoculation than in control ferrets. In pharyngeal swab samples, virus titers were significantly lower at 1 day after inoculation than in control ferrets. Virus shedding between vaccinated and OST ferrets was significantly lower in vaccinated ferrets on days 2 and 3 in nasal and on day 1–3 in pharyngeal swab samples.

Virus Isolation From the Respiratory Tract

In control ferrets at 3 days after inoculation, the highest virus titers were found in the NTs (Table 2). In other parts of the respiratory tract, virus titers were lower or undetectable. At 7 days after inoculation, virus could be isolated only from the NTs. In vaccinated ferrets at 3 days after inoculation, virus could be isolated from the NTs of ferret 10. Virus could not be isolated from any other part of the respiratory tract in other vaccinated ferrets at 3 or 7 days after inoculation. From all OST ferrets at 3 days after inoculation, virus was isolated from the NTs. In other parts of the respiratory tract, virus titers were lower or undetectable. At 7 days after inoculation, virus was isolated from the NTs of ferrets 2 and 5.

Table 2. Virus titers in respiratory tract tissues, CNS tissues, CSF, and other tissuesa.

Tissue	Virus Titer, Mean ±SD, Log_{10} $TCID_{50}$/g Tissue (Ferrets With Virus Isolated/ Total No.)					
	Day 3			Day 7		
	Control	Vaccine	Oseltamivir	Control	Vaccine	Oseltamivir
Respiratory system						
Nasal turbinates	8.2 ± 0.2 (3/3)	4.1 (1/3)	9.1 ± 0.1 (3/3)	4.9 ± 1.4 (3/3)	-	5.8 ± 0.9 (2/3)
Trachea	5.4 ± 0.2 (2/3)	-[b]	3.6 (1/3)	-	-	-
Bronchus	4.5 (1/3)	-	3.2 ± 0.1 (2/3)	-	-	-
Lung	2.8 ± 0.0 (2/3)	-	3.3 ± 0.5 (2/3)	-	-	-
CNS						
Olfactory bulb	6.0 ± 0.7 (3/3)	-	7.1 ± 0.2 (3/3)	5.0 (1/3)	-	4.4 (1/3)
Cerebrum	4.8 ± 0.5 (3/3)	-	5.9 ± 1.1 (3/3)	4.9 (1/3)	-	3.6 ± 0.3 (2/3)
Cerebellum	4.9 ± 0.3 (3/3)	-	4.5 ± 0.7 (3/3)	5.0 ± 2.4 (2/3)	-	3.5 ± 0.5 (2/3)
CSF[c]	-	-	-	7.2 (1/3)	-	-
Other						
Tracheo-bronchial LN	-	-	-	-	-	-
Tonsil	5.1 ± 06 (3/3)	-	6.1 ± 0.3 (3/3)	4.0 (1/3)	-	4.0 ± 0.0 (2/3)
Heart	-	-	-	-	-	-
Liver	-	-	-	6.9 (1/3)	-	-
Spleen	-	-	-	-	-	-
Kidney	-	-	3.3 (1/3)	-	-	-
Adrenal gland	3.3 (1/3)	-	-	-	-	-
Pancreas	-	-	-	-	-	-
Jejunum	4.6 ± 0.2 (2/3)	-	6.6 (1/3)	-	-	-
Blood[c]	-	-	-	-	-	-

Abbreviations: CNS, central nervous system; CSF, cerebrospinal fluid; SD, standard deviation; $TCID_{50}$, 50% tissue culture infectious dose.
[a] Control, oseltamivir-treated, and vaccinated ferrets inoculated with highly pathogenic avian influenza (HPAI) H5N1 virus were euthanized 3 or 7 days after inoculation, and virus titers were determined.
[b] Dash denote virus titer below detection limit.
[c] Virus titers for CSF and blood are measured in log_{10} $TCID_{50}$/mL.

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Virus Isolation From the CNS

In the CNS of control ferrets at 3 days after inoculation, virus was recovered from the olfactory bulb, cerebrum, and cerebellum (Table 2). At 7 days after inoculation, virus was recovered from the olfactory bulb, cerebrum, and CSF of ferret 12 and the cerebellum of ferrets 6 and 12. From ferret 9, no virus could be isolated from the CNS at 7 days after inoculation. From vaccinated ferrets, no virus could be isolated from any part of the CNS, 3 or 7 days after inoculation. From OST ferrets, virus was recovered from the olfactory bulb, cerebrum, and cerebellum at 3 days after inoculation. At 7 days after inoculation, virus was isolated from the olfactory bulb of ferret 7, the cerebrum of ferrets 2 and 7, and the cerebellum of ferrets 5 and 7 (Table 2).

Virus Isolation From the Other Tissues

From the tonsils, virus was isolated from all control and OST ferrets at 3 days after inoculation (Table 2). At 7 days after inoculation, virus was isolated from the tonsils of control ferret 6 and OST ferrets 5 and 7, the jejunum of control ferrets 3 and 15 and OST ferret 17 at 3 days after inoculation, and from the liver of control ferret 12 at 7 days after inoculation. From the adrenal gland and kidney, virus was isolated at 3 days after inoculation in control ferret 15 and OST ferret 17, respectively.

Detection of Oseltamivir Resistance Mutation

By sequencing, we found no evidence for the oseltamivir resis- tance mutation H275Y (N1 numbering) in any of the viruses isolated from the NTs or cerebrum of OST ferrets.

Immunohistochemistry

Respiratory Tract

In the respiratory tract of control ferrets at 3 days after inoculation, virus antigen was detected in few respiratory epithelial cells of the NTs in control ferrets 3 and 15 (Table 3, Figure 2). In the olfactory mucosa, focal areas with many virus antigen-positive cells, most likely ORNs based on location and shape and occasional in Bowman glands, were observed in all ferrets. In addition, in all ferrets, virus antigen-positive cells were found in lymphoid structures within the larynx at 3 days after inoculation. At 7 days after inoculation, virus antigen was detected in few individual cells in the olfactory mucosa and lungs of ferret 12. In vaccinated ferrets at 3 days after inoculation, virus antigen was detected in a few cells of the olfactory mucosa of ferret 16; at 7 days after inoculation, no virus antigen was detected in the respiratory tract. In OST ferrets, virus antigen was focally detected in the olfactory mucosa, most likely ORNs based on location and shape and occasional in the Bowman glands of all ferrets at 3 days after inoculation. At 7 days after inoculation, virus antigen was observed in a few cells of the olfactory mucosa.

Central Nervous System

In control ferrets at 3 days after inoculation, virus antigen was detected in cells surrounding the glomeruli of the olfactory bulb (Table 3, Figure 3). At 7 days after inoculation, ferrets 9

and 12 had virus antigen-positive cells in the olfactory bulb (Table 3, Figure 4). All ferrets had foci with virus antigen-positive neurons and glial cells within the cerebrum. In addition, meningeal cells surrounding the cerebrum and cerebellum, ependymal cells of the cerebellum, and ependymal cells and neurons of the spinal cord were found positive in ferret 12. In vaccinated ferrets, virus antigen was not detected in tissue of the CNS. In the OST ferrets at 3 days after inoculation, virus antigen was detected in cells surrounding the glomeruli of the olfactory bulb of all ferrets. At 7 days after inoculation, virus antigen was detected in the olfactory bulb of ferret 7. The cerebrum contained foci with virus antigen-positive neurons and glial cells in all ferrets.

Other Tissues

Virus antigen was detected in the tonsil of control ferret 3 and OST ferret 13 at 3 days after inoculation. In addition, virus antigen was detected in Peyer patches lining the jejunum and duodenum of control ferret 3. At 7 days after inoculation, the liver of control ferret 12 contained virus antigen-positive cells in the bile duct (Table 3).

Histology

At histopathology, the lesions in the respiratory and extra-respiratory tissues were moderate to severe in the control ferrets, mild in the vaccinated ferrets, and mild to moderate in the OST ferrets, whereas the lesions in the CNS were comparable in the control and OST ferrets and less severe in the vaccinated ferrets (Supplementary Figure 1). In general, histological lesions were associated with the presence of virus antigen. A detailed histopathological report can be found in the supplementary material (Supplementary Data).

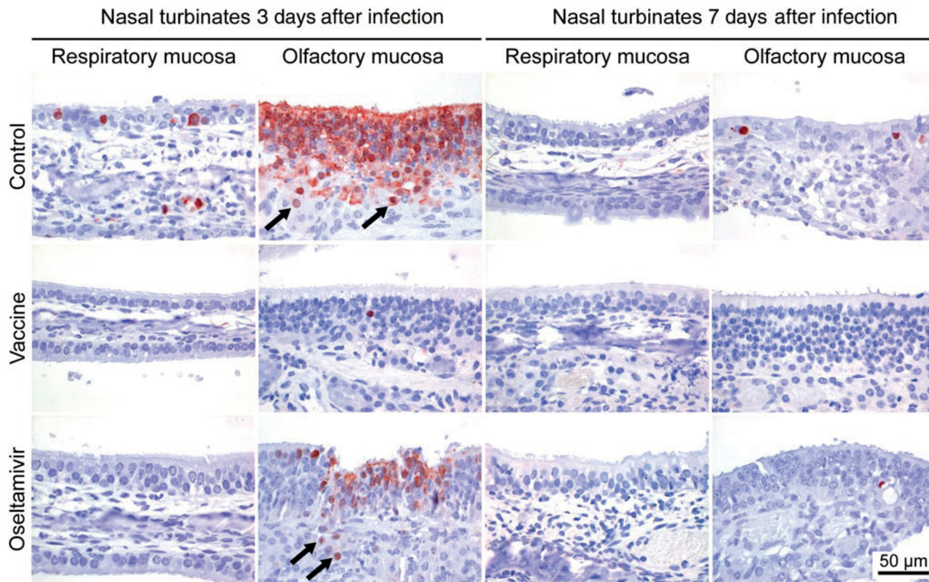

Figure 2. Vaccination, but not oseltamivir treatment, reduced virus antigen expression in the nasal turbinates of ferrets inoculated with highly pathogenic avian influenza (HPAI) H5N1 virus. Influenza virus antigen was detected by immunohistochemistry in the olfactory and respiratory mucosa of the nasal turbinates in control, vaccinated, and oseltamivir-treated ferrets 3 or 7 days after inoculation with HPAI H5N1 virus. Arrows indicate Bowman glands.

Table 3. Influenza virus antigen expression in respiratory tract tissues, CNS tissues, and other tissuesa.

	Influenza A Virus Antigen Presence (Ferrets With Virus Antigen Detected/Total No.)					
	Day 3			Day 7		
Tissue	Control	Vaccine	Oseltamivir	Control	Vaccine	Oseltamivir
Respiratory system						
Nasal turbinates						
Respiratory mucosa	+ (2/3)	-	-	-	-	-
Olfactory mucosa	+ (3/3) [b]	+ (1/3)	+ (3/3) [b]	+ (1/3)	-	+ (3/3)
Larynx	+ (3/3) [c]	-	-	-	-	-
Trachea	-	-	-	-	-	-
Bronchus	-	-	-	-	-	-
Lung	-	-	-	+ (1/3)	-	-
CNS						
Olfactory bulb	+ (3/3)	-	+ (3/3)	+ (2/3)	-	+ (1/3)
Cerebrum	-	-	-	+ (3/3)	-	+ (3/3)
Cerebellum	-	-	-	-	-	-
Ependymal cells	-	-	-	+ (1/3)	-	-
Choroid plexus	-	-	-	-	-	-
Meninges	-	-	-	+ (1/3)	-	-
Trigeminal nerve	-	-	-	-	-	-
Spinal cord	-	-	-	+ (1/3)	-	-
Other						
Tracheo-bronchial LN	- [d]	- [e]	- [d]	- [e]	-	- [d]
Tonsil	+ (1/1) [d]	- [e]	+ (1/2) [e]	-	-	- [e]
Heart	-	-	-	-	- [e]	- [e]
Liver	-	-	-	+ (1/3)	-	-
Spleen	-	-	-	-	-	-
Kidney	-	-	-	-	-	-
Adrenal gland	-	-	-	-	-	-
Pancreas	-	-	-	-	-	-
Jejunum	+ (1/3) [c]	-	-	-	-	-
Duodenum	+ (1/3) [c]	-	-	-	-	-

Abbreviation: CNS, central nervous system.
[a] Control, vaccinated, and oseltamivir-treated ferrets inoculated with HPAI H5N1 virus were euthanized 3 or 7 days after inoculation, and virus antigen was detected with immunohistochemistry. Plus signs represent the detection of virus antigen.
[b] Virus antigen detected in Bowman glands.
[c] Virus antigen detected in lymphoid structures.
[d] Tissue from 1 ferret available.
[e] Tissue from 2 ferrets available.

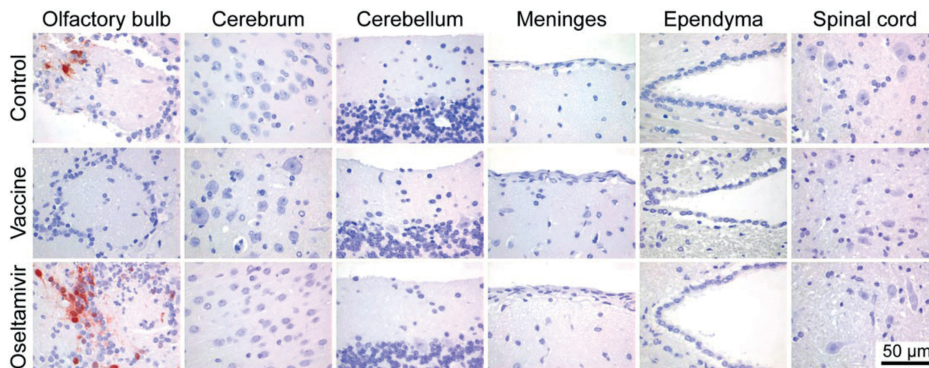

Figure 3. Virus antigen detection in central nervous system (CNS) tissues of control and oseltamivir-treated but not in the CNS of vaccinated ferrets 3 days after inoculation with highly pathogenic avian influenza (HPAI) H5N1 virus. Influenza virus antigen was detected by immunohistochemistry of the olfactory bulb, cerebrum, cerebellum, meniges, ependyma and spinal cord of control, vaccinated, and oseltamivir-treated ferrets 3 days after inoculation with HPAI H5N1 virus.

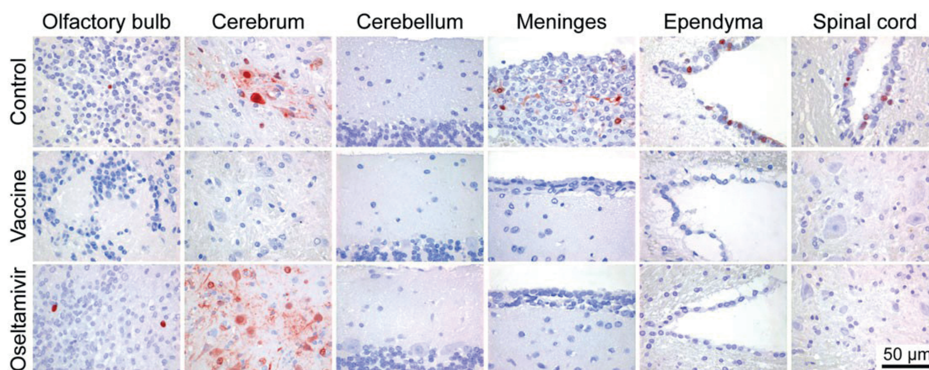

Figure 4. Virus antigen detection in central nervous system (CNS) tissues of control and oseltamivir-treated but not in the CNS of vaccinated ferrets 7 days after inoculation with highly pathogenic avian influenza (HPAI) H5N1 virus. Influenza virus antigen was detected by immunohistochemistry of the olfactory bulb, cerebrum, cerebellum, meniges, ependyma and spinal cord of control, vaccinated, and oseltamivir-treated ferrets 7 days after inoculation with HPAI H5N1 virus.

DISCUSSION

In this study, we show that homologous vaccination reduced replication of HPAI H5N1 virus in the olfactory mucosa and prevented subsequent spread to the olfactory bulb and the rest of the CNS. In contrast, in OST ferrets HPAI H5N1 virus was able to spread to the CNS via the olfactory nerve, resulting in a severe meningoencephalitis comparable to that

observed in control ferrets. Homologous vaccination reduced virus replication within the NTs significantly early after infection. Moreover, virus antigen and associated lesions were rarely detected in the olfactory mucosa of vaccinated ferrets. Together, these factors probably prevented CNS invasion via the olfactory nerve. In ferret 8, antibody titers were <40 (>40 is considered protective [39]), which might explain the prolonged virus shedding in this ferret. However, this low level of virus replication was not sufficient for virus spread along the olfactory nerve to the CNS.

In our study, ferrets were vaccinated with an adjuvanted inactivated split HPAI H5N1 virus (A/Indonesia/5/05) vaccine and challenged with the homologues virus, a clade 2.1 virus. Using the same vaccine, studies by others have shown that cross-clade antibody responses were detected against clade 1 (A/Vietnam/1203/04), clade 2.2 (A/turkey/Turkey/1/2005) and clade 2.3 (A/Anhui/1/2005 viruses) [40, 41]. However, whether these cross-clade antibody responses would be sufficient to prevent CNS invasion via the olfactory nerve remains to be elucidated.

Prophylactic oseltamivir treatment did not prevent virus replication within the olfactory mucosa and subsequent CNS invasion via the olfactory nerve. In general, the effect of oseltamivir was mild, although there were some differences between the OST and control groups, which include (1) lower virus titers in pharyngeal and nasal swabs early after infection; (2) reduction of lung lesions at 7 days after inoculation; and (3) lack of virus antigen in the respiratory mucosa, larynx, liver, and intestinal tract. The inability of oseltamivir to prevent CNS invasion via the olfactory nerve was most likely not the result of a low susceptibility of this isolate to oseltamivir, because A/Indonesia/5/05 is equally susceptible to oseltamivir as pandemic H1N1 virus (A/Netherlands/602/2009) [42]. Furthermore, Sanger sequencing did not reveal the H275Y mutation associated with oseltamivir resistance in viruses isolated from the NTs and cerebrum of OST ferrets. Once the virus has entered the CNS, it is unlikely that oseltamivir has an effect on virus replication, because oseltamivir cannot pass the blood-brain barrier efficiently. The active metabolite of oseltamivir, oseltamivir carboxylate, is found only at very low and subtherapeutic concentration in the CNS of humans [43] and rats [44] after oral administration.

This study focuses on the efficacy of prophylactic strategies on the ability of influenza viruses to replicate in the olfactory mucosa and spread via the olfactory nerve to the olfactory bulb, and subsequently throughout the rest of the CNS. Some studies have included CNS tissues after intranasal challenge, to evaluate the efficacy of vaccines and antiviral therapies. This includes a 2014 intranasal vaccine study, which showed that A/Vietnam/1194/04 could be isolated from the olfactory bulb in 2 of 6 ferrets after intranasal challenge [45]. The difference in findings between this study and ours cannot be explained by the lack of virus-specific antibodies but could be related to differences in vaccination approach, virus isolate, or inoculum dose. Studies on oseltamivir prophylaxis revealed that the dose and administration (once or twice a day) as well as the start of treatment affected the neuroinvasiveness of A/Vietnam/1203/04 [46, 47]. Interestingly, a similar approach using 5 mg/kg twice daily, starting

1 day before virus inoculation, did prevent CNS invasion to the CNS in a study by Boltz et al [47]. Unfortunately, it is difficult to compare these studies directly with ours because they did not include the olfactory mucosa and used a different virus clade, a different virus inoculation dose, and a larger inoculum volume.

CNS invasion by influenza viruses via the olfactory nerve seems to depend on several factors, one being efficient replication within the olfactory mucosa. However, virus replication within the olfactory mucosa does not necessarily result in virus spread along the olfactory nerve. For example, both 2009 H1N1 virus and low pathogenic H5N1 virus lacking a multibasic cleavage site replicated within the olfactory mucosa of ferrets but did not spread via the olfactory nerve to the CNS [23, 48]. Other factors important for CNS invasion along the olfactory nerve might include the role of local innate immunity and the presence of hemagglutinin-cleaving enzymes.

Taken together, we show here that within our ferret model vaccination is more effective than prophylactic oseltamivir in preventing CNS invasion by HPAI H5N1 virus via the olfactory nerve. Furthermore, there is substantial evidence that many other viruses besides influenza viruses, including paramyxoviruses, herpes viruses, and picornaviruses, can enter the CNS via the olfactory nerve in animal models [29]. It is therefore important to include the olfactory mucosa, nerve, and bulb in future pathogenesis, antiviral, and vaccine studies involving influenza viruses and other viruses that replicate within the nasal cavity, especially viruses with a known neurotropic potential.

NOTES

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Potential conflicts of interest

A. D. M. E. O. participated in the IRIS project to study antiviral resistance within the Erasmus MC, which was funded by Roche, is part-time CSO at Viroclinics, and is an ad hoc consultant for public and private entities. T. K. owns share certificates in Viroclinics. All other authors report no potential conflicts. 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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SUPPLEMENTARY DATA

Histology

By histopathology, the lesions in the (extra)-respiratory tissues were moderate to severe in the control ferrets, mild in the vaccinated ferrets and mild to moderate in the OST ferrets, while the lesions in the CNS were comparable in the control and OST ferrets and less severe in the vaccinated ferrets.

Respiratory tract

In the control ferrets at 3 dpi, in the nose there was moderate to severe epithelial necrosis with moderate numbers of neutrophilis in the olfactory epithelium and lamina propria and infiltration of few neutrophils in the respiratory epithelium and lamina propria. At 7 dpi, there were more neutrophils, more epithelial necrosis, proteinaceous exudate, and respiratory epithelial hypertrophy and hyperplasia, all associated with antigen presence. In the larynx, there was a mild to moderate neutrophilic laryngitis in all control ferrets at 3 dpi and in one ferret (#12) at 7 dpi and mild neutrophilic tonsillitis in one ferret (#03) at 3 dpi, all associated with virus antigen. In the lungs, no lesions were seen at 3 dpi and at 7 dpi there was a multifocal mild thickening of the alveolar septa with infiltration of few neutrophils and macrophages in the septa, bronchiolar epithelium and perivascular and peribronchiolar areas of two ferrets (#09 and #12).

In the vaccinated ferrets at 3 and 7 dpi, in the nose there were fewer neutrophils and no necrosis in the epithelium when compared to control ferrets. No lesions were seen in the larynx and tonsil. In the lungs, there were few neutrophils in the alveolar septa with mild epithelial necrosis and mild perivascular and peribronchiolar lymphoplasmacytic infiltrates at 3 dpi. At 7 dpi in one ferret (#18) there were few perivascular lymphoplasmacytic infiltrates and in one ferret (#08) there was a mild to severe neutrophilic bronchopneumonia with infiltrates of many neutrophils, epithelial necrosis and perivascular lymphoplasmacytic infiltrates, not associated with presence of antigen.

In OST ferrets, lesions in the nasal turbinates were comparable to those in control ferrets at 3 and 7 dpi. There were no lesions in the larynx and tonsils. In the lungs of one OST ferret (#17) at 3 dpi, there was focal thickening of the septa with infiltration of few neutrophils, the rest of the ferrets showed no lung lesions.

Central nervous system

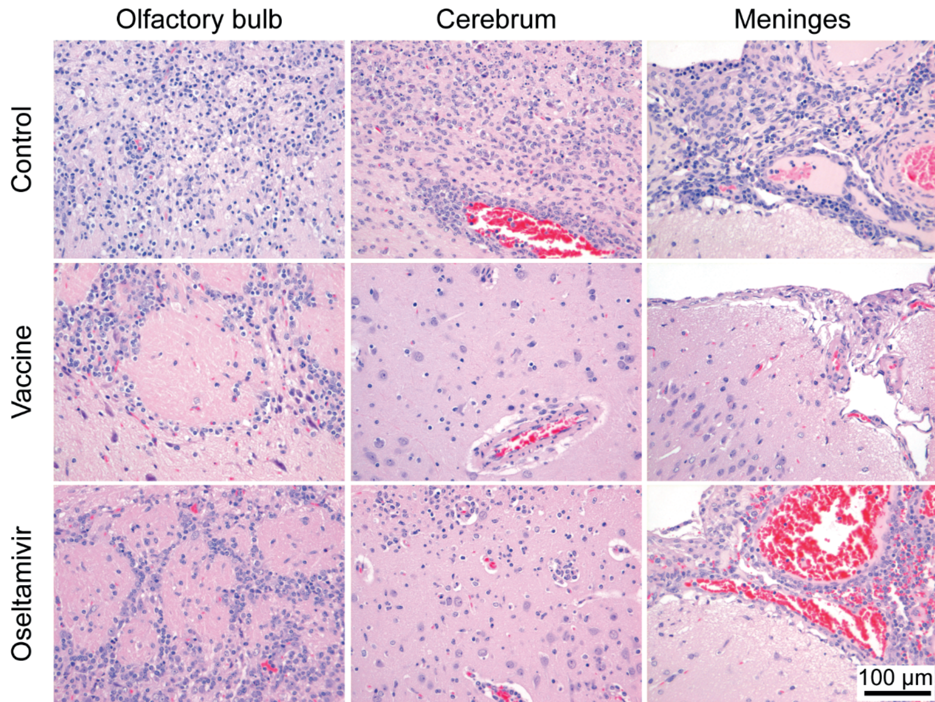
In the CNS of control ferrets at 3 dpi, there were multifocal infiltrates of few neutrophils (some of which were degenerate), macrophages and lymphocytes within the glomerular layer of the olfactory bulb associated with virus antigen in the olfactory bulb, and less infiltrates in the cerebral gray and white matter, meninges and choroid plexus of two ferrets (#03 and #14). In all control ferrets at 7 dpi, the lesions were more severe with more inflammatory cells, multifocal gliosis, mild astrocytosis, necrosis and satellitosis of neurons, perivascular cuffing

and thickening of the meninges with neutrophils, macrophages, lymphocytes and plasma cells. In the trigeminal nerve of one control ferret (#12) there were hemorrhage and perineuronal infiltrates of few neutrophils, macrophages, lymphocytes, and necrosis of neurons. In this ferret, the meninges of the spinal cord had similar lesions as those in the brain with associated virus antigen.

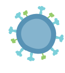
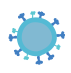
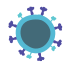
In the vaccinated ferrets, the lesions were very mild, with infiltrations of few macrophages and lymphocytes in the olfactory bulb and in the cerebrum. In ferret #8 there were multifocal perivascular inflammatory infiltrates, gliosis and mild neuronal necrosis in in all parts of the brain including the cerebellum. No lesions were seen in the trigeminal nerve or spinal cord except for ferret #8 with perivascular inflammatory infiltrates surrounding the trigeminal nerve. In the OST ferrets, the lesions were comparable to those in the control ferrets but showed more necrosis in the olfactory bulb at 3 dpi in all ferrets and more inflammatory cells in the meninges and in the white matter of the cerebellum and brainstem often associated with the presence of virus antigen. No lesions were seen in the trigeminal nerve or spinal cord.

Other tissues

In extra-respiratory tissues of control ferrets, the liver showed multifocal mild to moderate hepatocellular necrosis with infiltration of few neutrophils and macrophages in one ferret (#03), and periportal infiltrates of macrophages, lymphocytes and plasma cells in all ferrets at 3 dpi. At 7 dpi, there were fewer neutrophils and more macrophages, lymphocytes and plasma cells and more severe necrosis associated with virus antigen in the liver of one ferret (#14). In the lymphoid tissue of the Peyer's patches in the duodenum and jejunum of one ferret (#03) at 3 dpi, there was multifocal necrosis with few to moderate numbers of neutrophils associated with virus antigen. In the vaccinated ferrets, there was mild to moderate periportal lymphoplasmacytic infiltrates in the liver liver of all ferrets and in ferret #8 enlarged lymphatic nodules in the spleen and severe histiocytosis in the mesenterial lymph node. In OST ferrets, there is mild to moderate periportal cuffing and necrosis was only seen in one ferret (#13) at 3 dpi in the liver. In OST ferrets, focal mild hepatocytic necrosis with infiltration of few neutrophils was seen in one ferret (#13) at 3 dpi in the liver. In all groups, there was mild to moderate hepatic lipidosis characterized by vacuoles in the cytoplasm of the hepatocytes consistent with hepatic lipidosis. No lesions were seen in other tissues.

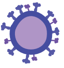


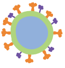
Supplementary Figure 1. Histological lesions in the CNS of control, vaccinated and oseltamivir-treated ferrets, 7 days post inoculation with HPAI H5N1 virus. Hematoxylin and Eosin staining of different anatomical sites in the CNS of control, vaccinated, and oseltamivir-treated ferrets.

7

Summarising Discussion





Influenza virus infections are a major cause of respiratory tract disease in humans. Each year, seasonal influenza epidemics infect about 5-10% of adults and 20-30% of children globally with ~1 billion cases, 3-5 million hospitalizations, and 290-650 thousand deaths [1, 2]. The most common and under-recognized extra-respiratory complications of influenza are central nervous system (CNS) and cardiac diseases [3]. The frequency and severity of influenza virus associated CNS disease differs between subtypes and strains [3, 4]. Highly pathogenic avian influenza (HPAI) H5N1 virus infections [5-8] are more often associated with extra-respiratory disease than seasonal H3N2 or 2009 pandemic H1N1 influenza viruses [9]. So far, it is poorly understood which viral factors are important for the ability to spread to, replicate in, and spread throughout extra-respiratory tissues, including the CNS. In this thesis, I studied the ability of different pandemic and zoonotic influenza viruses to spread & cause disease outside the respiratory tract, with a focus on the CNS and cardiovascular system (**chapter 2, chapter 3, and chapter 6**). In addition, I studied the effect of diet induced obesity on extra-respiratory disease of 2009 H1N1 influenza virus infections (**chapter 3**). To get more insight into viral factors important for efficient replication inside the CNS, I studied the ability of different influenza viruses to replicate in cells of the CNS, **chapter 4, chapter 5**. Finally, in **chapter 6**, I evaluated whether vaccination or antivirals could prevent or reduce influenza virus to spread outside the respiratory tract.

INVOLVEMENT OF EXTRA-RESPIRATORY TISSUES BY DIFFERENT INFLUENZA A VIRUSES

Many different influenza viruses affect organs other than the respiratory tract, some viruses more often and with more serious consequences than others [3]. Of these viruses, some spread efficiently to and invade the CNS via cranial nerves while other influenza viruses can (in) directly affect the cardiac system or other organs. In this thesis, I explored the neuroinvasive potential of different influenza viruses and spread to other extra-respiratory organs. I investigated the possible and preferential routes of CNS invasion for different influenza viruses into more detail and proposed a hypothesis why certain viruses act differently from others.

Involvement of the Central Nervous System

The neuroinvasive potential of HPAI H5N1 viruses has been recognized for many years both in avian and mammalian species including humans [10-12]. The H5N1 subtype currently serves as the most neurotropic influenza virus without the need of prior adaptation to brain tissues. Disease of the CNS caused by the H5N1 subtype is a combination of direct infection of cells (predominantly neurons) and local and systemic immune responses inflammatory cytokines produced locally but also systemically (this thesis, [10, 13]). Although pandemic 2009 H1N1, pandemic 1918 H1N1 and seasonal H3N2 viruses are associated with CNS disease, experimental and human evidence indicates that the frequency and severity are lower compared to that of CNS disease associated with HPAI H5N1 viruses (this thesis, [4, 10]).

The first part of this thesis focuses on one of the most devastating pandemics in human history, the 1918 H1N1 influenza pandemic. Infection with this virus was associated with the development of neurological diseases such as encephalitis lethargica[14-16]. However, whether these neurological diseases were caused by, enhanced by, or just coincided with the virus infection remained a topic of debate for many years with the influenza virus etiology being more often refuted than supported[17, 18].

Chapter 2 provided the first evidence that the 1918 H1N1 virus is able to directly invade and replicate within the ferret CNS. In addition, infection with the 1918 H1N1 virus induces pro-inflammatory cytokines in respiratory and extra-respiratory tissues that likely contribute to the disease severity. In this study, infected cells were observed in the brainstem and pituitary gland, a protrusion of the hypothalamus. Both the brainstem and hypothalamus are, amongst others, involved in regulation of sleep cycles and consciousness [19]. This may be relevant for encephalitic lethargica, which was associated with hypersomnia and therefor also called 'sleeping sickness'. The infected cells found in the brainstem and pituitary gland provide an attractive hypothesis for local influenza virus replication as the causative agent of this 'sleeping sickness'. However, further research should reveal the full extent of disease caused by infection of the brainstem, pituitary gland and possibly hypothalamus with the 1918 H1N1 influenza virus. Taken together, **chapter 2** broadens our knowledge on the (extra-) respiratory pathogenesis and supports the suggested link between neurological complications and 1918 H1N1 influenza virus infection. This study highlights the need for awareness of potential neurological complications in future influenza virus outbreaks and pandemics.

CNS invasion

Seasonal, pandemic, and zoonotic viruses differ in their ability to invade, replicate efficiently in the CNS and cause disease (**chapter 2, chapter 3, chapter 4, chapter 5, and chapter 6**) [4]. Viral CNS invasion and efficient replication in the CNS are two different things. It remains unclear whether a virus that is not able to replicate efficiently in cells of CNS is still able to invade the CNS. Distinguishing which viral factors contribute to efficient neuroinvasion and/or replication in the CNS is pivotal for identifying and classifying the neurotropic potential of circulating and future influenza viruses.

Influenza viruses replicate within the respiratory tract of humans and the anatomical location of replication differs between strains. In general, human influenza viruses replicate efficiently in the nasal cavity and in the trachea, whereas avian influenza viruses that infect humans predominantly replicate within the bronchioles and alveoli [4]. However, avian influenza viruses do infect cells in the human nasal cavity, albeit to a lesser extent and in different cells than human influenza viruses. More specifically, within the nasal cavity, human influenza viruses mainly infect and replicate in the respiratory epithelium whereas avian influenza viruses that infect humans replicate predominantly in the olfactory epithelium, as described in **chapter 2**, **chapter 4, and chapter 6** and by others [9]. Respiratory mucosa is mainly innervated by the trigeminal nerve where olfactory mucosa is mainly innervated by

the olfactory nerve but also the trigeminal nerve [20-22]. These differences could result in strain-, or subtype-specific, routes of CNS invasion, depending on the preferred replication site of the virus. Indeed, it seems that HPAI H5N1 virus invades the CNS primarily via the olfactory nerve as indicated by early detection in the olfactory bulb after extensive replication within the olfactory mucosa (**chapter 4, chapter 6** and [6]), but also via the trigeminal nerve (**chapter 4**). In support of this hypothesis, a HPAI H5N1 virus that lacked a MBCS failed to replicate extensively in the olfactory epithelium and consequently failed to invade the CNS [6]. In contrast, I showed in **chapter 2** that the 1918 H1N1 virus, which predominantly replicates in the respiratory mucosa of the nasal cavity, appears to invade the CNS mainly via the trigeminal nerve. This conclusion is based on detection of infectious virus and viral antigens mainly in the trigeminal ganglion and brain stem, although limited virus replication was also detected in the olfactory mucosa and olfactory bulb. The lower respiratory tract (LRT) is furthermore innervated by the vagal nerve [23]. Since influenza viruses are also able to replicate within the LRT, extensive replication in the lung fits with CNS invasion along the vagal nerve to the brainstem [24-26].

The 2009 H1N1 pandemic virus does replicate in the olfactory mucosa and is more often than seasonal H3N2 virus, detected, and to higher viral loads, in the olfactory bulb of experimentally inoculated animals [9, 27]. Seasonal H3N2 virus does not replicate efficiently within the olfactory mucosa and is rarely found in the olfactory bulb, and if so, only at very low levels [9, 28, 29]. On the other hand, both seasonal H3N2 virus and pandemic 2009 H1N1 replicates efficiently in the respiratory mucosa of the URT[9] but whether virus can invade the CNS via the trigeminal nerve and be detected in the brainstem remains to be investigated.

Presence of HPAI H5N1 or 1918 H1N1 viruses in richly vasculated organs such as the heart, liver, pancreas, kidney and spleen suggests hematogenous spread of these viruses, **chapter 2, chapter 4, and chapter 6**. However, in ferrets, based on histopathological examination and distribution of virus antigens, the hematogenous route does not seem to be the route of CNS invasion. In contrast, in cats invasion of the CNS via the hematogenous route is observed after intraintestinal inoculation with HPAI H5N1 virus, but not after intratracheal inoculation or by by feeding on virus-infected chicks of HPAI H5N1 virus[30, 31]. Overall, it seems that for influenza viruses, hematogenous spread plays a more important role in spread to extra-respiratory spread organs other than the CNS which contain a blood-brain and blood-CSF barrier (this thesis, [5, 6]).

Overall, these observations suggest that a subtype and strain specific 'preferred route' of CNS invasion exists for HPAI H5N1, 1918 H1N1 virus and potentially other viruses based on their primary site of replication in the respiratory tract. Collectively, the studies performed in this thesis have contributed to knowledge about how influenza viruses spread to the CNS and indicate a prominent role for cranial nerves.

Replication in the central nervrous system

In this thesis, **chapter 5** showed that, *in vitro*, HPAI H5N1 and WSN viruses replicate efficiently within cells of the CNS. This efficient replication was dependent on the preference for α-2,3 SA linkage for high percentage of virus attachment resulting in high infectivity, and the presence of an alternative HA cleavage mechanism. In particular, the mechanism of HA cleavage seemed to be important for efficient replication of viruses in the CNS. One of the last stages of the influenza virus replication cycle is cleavage of the immature and non-infectious HA into a mature and infectious HA through proteolytic modifications [32]. Seasonal and pandemic viruses such as the H3N2 and 2009 H1N1 virus that contain a mono-basic cleavage site are cleaved by trypsin-like serine proteases such as human airway trypsin-like protease (HAT), transmembrane serine protease 2 (TMPRSS2), TMPRSS4, tryptase Clara, (mini)plasmin, ectopic anionic trypsin I or matriptase[33-36], that are present intracellular or in the lumen of the human respiratory tract. Although serine proteases HAT and matriptase mRNAs are produced in the human CNS, and even enriched in neurons in vivo[37, 38], in vitro SK-N-SH and U87-MG cells did not express HAT, TMPRSS2 nor TMPRSS4 mRNA, **chapter 5**. *In vitro*, H3N2 and 2009 H1N1 viruses were only able to replicate in SK-N-SH cells but not in U87-MG cells in the presence of trypsin proteases added to the culture medium, **chapter 5**. However, it seems that *in vivo*, these proteases are not available for or accessible to H3N2 and 2009 H1N1 viruses, these proteases are expressed in a cell type specific manner or that presence of these proteases alone is not sufficient for efficient replication, **chapter 2** [9, 13]. Another possibility could be that production of HA-cleaving proteases is cell-type specific. Further research should determine the biological availability of proteases present in the mammalian CNS that can cleave mono-basic cleavage sites of influenza viruses.

The first step of the influenza virus replication cycle is attachment to a host cell [32]. In the human respiratory tract, human and avian influenza viruses attach to epithelial cells that express α-2,6- and α-2,3-linked SA, respectively[39, 40]. However, once inside the CNS, cells are encountered that differ from respiratory epithelial cells. Little is known about SA distribution on cells in the CNS at different anatomical locations. Several studies looked into the α-2,6- and α-2,3-linked SA distribution on cells of the human and mouse CNS and reported similar but also different findings. In general, both α-2,6 and α-2,3 SA are present on neurons and glial cells in many different brain regions, including the cerebral cortex, hippocampus, brainstem, and cerebellum[41, 42]. In cell lines, both α-2,3 and α-2,6 SA receptor expression are reported in human neuroblastoma SK-N-SH and SH-SY5Y and human glioblastoma T98G cells[43, 44]. In **chapter 5,** I have shown that viruses that attach and infect cells of the CNS more efficiently prefer α-2,3 linked SA for attachment. However, the linkage preference alone is not sufficient to explain the attachment differences. The affinity and avidity of HA binding is influenced by factors such as sialic acid fucosylation and sulphation but also by the length, branching and sialic acid modifications of the glycan itself [45]. In addition, it remains unknown whether receptor preferences such as α-2,8 SA or even SA-independent attachment of the virus[46-48] broadens the virus cell tropism in the CNS.

Influenza viruses that have been associated with CNS disease, such as HPAI H5N1, A/WSN/33, and 1918 H1N1, all have different mechanisms of HA cleavage compared to human adapted non-neurotropic viruses. The A/WSN/33 virus replicates independently of trypsin due to the loss of a conserved glycosylation site in the neuraminidase, and uses the serine-protease plasmin present in serum and many other organ systems besides the respiratory tract [49, 50]. Trypsin-independent replication of 1918 H1N1 virus is cell-line-dependent and not fully understood. The 1918 H1N1 virus replicates neuraminidase-dependently and trypsin-independently in MDCK and Calu-3 cells but not in Huh-7 cells [51, 52]. In lung tissues, it has only been reported that 1918 H1N1 virus can be cleaved by the serine proteases TMPRSS 2 and 4[52]. Therefore, the mechanism of trypsin-independent replication and the full range of proteases able to cleave the 1918 H1N1 HA remains to be elucidated. Viruses of the H5N1 subtype that contain a multi-basic cleavage site (MBCS) are cleaved by ubiquitously expressed subtilisin-like proteases such as furin and PC5/6[36, 53]. For H5N1 viruses, the MBCS is critical for extra-respiratory spread and CNS invasion[6], but insertion of a MBCS into viruses other than H5N1, did not consistently result in extra-respiratory spread of the virus[54-58]. In **chapter 5**, we also demonstrated that removal of the MBCS from the H5N1 virus resulted in attenuated but not abrogated replication in the absence of added trypsin. In general, this indicated that other viral factors, such as SA preference, are necessary for efficient replication besides a MBCS, and that having a MBCS does not automatically confers increase pathogenicity, extra-respiratory spread and replication. These data fit with what is observed experimentally, where seasonal H3N2 and pandemic 2009 H1N1 viruses are rarely detected in the CNS of ferrets and mice, 1918 H1N1 virus being occasionally detected and frequent detection of A/WSN/33 and HPAI H5N1 viruses **chapter 2**, **chapter 3**, **chapter 4, chapter 6,** [9, 13]. It seems that not all influenza virus subtypes benefit in the same way from insertion of a MBCS and it is likely that other HA cleaving mechanisms that have not yet been described exist. Observations made in this thesis and from other studies led to the development of the following hypothesis. Once inside the CNS, influenza viruses that replicate efficiently, preferentially attach to α-2,3 linked SAs and must contain an alternative HA cleavage mechanism that allows for trypsin-independent replication of the virus.

The fact that HPAI H5N1 virus infection in ferrets could result in the acquisition of mutations in the polymerase complex and NP genes suggest that these viruses can adapt to extra-respiratory tissues, **chapter 4** [59]. Comprehensive characterizations of the wildtype and CNS-mutant virus revealed different phenotypic characteristics *in vitro* but not *in vivo*. Even though neither adaptation nor attenuation of H5N1 virus to CNS tissues could be confirmed *in vivo*, our data suggest that selection of viruses in the CNS could occur, and that this involves mutations in the viral replication machinery. The polymerase complex has many functions and is important for host and cell type switching [59-61]. These host and cell type switches can include processes such as replication temperature adaptation and interaction with host factors (e.g. nuclear import) [60-62]. Collectively, these studies indicate that sialic acid preference, viral polymerase compatibility and/or adaptations, and the presence of a

MBCS or other ways of HA cleavage are all viral factors that contribute to the neurotropic potential of influenza viruses.

Involvement of the heart and other organs

In mammals, some influenza viruses affect only the respiratory tract whereas others spread more systemically, e.g. to the the heart, liver, kidney and pancreas, presumably via the hematogenous route (this thesis, [30, 31]). Influenza associated cardiovascular disease (CVD) is an underappreciated complication of influenza and could potentially have a bigger impact on the disease burden of seasonal influenza than currently thought [3]. Notably, in humans, in the first week after a laboratory-confirmed influenza virus infection, hospitalization for myocardial infarctions is up to six times higher[63, 64]. Understanding the pathogenesis of influenza associated CVD is important, especially for those at risk of CVD which include individuals with pre-existing heart conditions, hypertension, diabetes, high cholesterol, smoking, or obesity [65].

Influenza viruses have been previously found in the liver, kidney, and pancreas of fatal human cases and of experimentally infected ferrets with HPAI H5N1 or 2009 H1N1 virus infections, **chapter 2, chapter 4, chapter 6** [9, 66-68]. In mice infected with a HPAI H5N1 virus, cardiac myocytes contained viral antigens indicating direct infection of these cells [69]. Influenza virus detection in the heart, liver, and pancreas is compatible with hematogenous spread of the virus to extra-respiratory organs. Replication in any of these extra-respiratory tissues, as determined by virus antigen presence in the parenchyma, likely contributes to the disease severity of both HPAI H5N1 and 1918 H1N1 virus [3]. A plausible route for influenza to reach the blood is through passing the alveolar-capillary barrier, which is damaged directly by influenza virus infection of type I or type II pneumocytes and/or indirectly as a consequence of the viral pneumonia [3, 4]. However, the frequency of influenza viremia during pandemic or seasonal infections is heavily debated since both the presence and absence of viremia is reported [4]. This could indicate that influenza virus viremia occurs infrequently and/or is not sustained for long periods explaining the inconsistency between studies. Taken together, understanding the mechanism of extra-respiratory spread to and replication in organs other than the CNS is crucial to understand the complete pathogenesis of influenza virus infection.

SYSTEMIC INFLAMMATORY RESPONSES AND EXTRA-RESPIRATORY DISEASE

Extra-respiratory complications of influenza virus infection are often caused by a combination of viral and host factors. One such factor is the ability of a virus to spread systemically, resulting in direct virus infection of the CNS parenchyma and other organs leading to cellular damage **chapter 2, chapter 4, and chapter 6**. Another factor is the immune response of the host that could be damaging. The host immune response could have a local or systemic effect,

or both. A local effect, at the site of infection, is where infected cells produce proinflammatory cytokines leading to the influx of immune cells which cause pathological changes in the tissue. A systemic effect is when virus replication in for example the lungs causes a hyperreaction of the immune response. As a consequence, circulating proinflammatory cytokines can activate non-infected parenchymal cells and immune cells that wreak havoc in organs other than the lungs [70]. This hypercytokinemia is termed a 'cytokine storm' [70]. The exact mechanism and initial trigger of this hyperreaction is not known [70]. One could think of proinflammatory cytokines or viral proteins or RNA produced in the lungs that go systemic as the initial trigger for the involvement of endothelial cells and/or parenchymal cells in other tissues. Other factors, such as tissue hypoxia caused by respiratory dysfunction, also could play a role in the development of a cytokine storm.

Cells infected with influenza virus typically start producing antiviral cytokines called interferons and other inflammatory mediators to restrict viral replication and spread [71]. However, influenza viruses are equipped with an interferon antagonist, the NS1 protein, to partially counteract these interferon responses [72]. Whilst interferon responses can be partially counteracted, the induction of other proinflammatory cytokines cannot. The immune response is further amplified by unrestricted viral replication and spread and could contribute to cytokine-mediated organ dysfunction and tissue damage. Several cytokines including IL-6 and TNF-α, have been implicated in the pathogenesis of extra-respiratory disease and associated with neurotoxic effects [73]. In the CNS, IL-6 and TNF-α play a role in the development of seizures [74], one of the most common clinical findings in influenza associated encephalopathies [3]. In the heart, TNF-α plays an important role in the pathogenesis of viral myocarditis and may affect the severity of cardiac dysfunction [75].

Different influenza viruses trigger different immune responses. Viruses that cause severe disease are often associated with high levels of proinflammatory cytokines in the respiratory tract and circulation. Avian influenza viruses such as HPAI H5N1 are well known for extensive replication in the lungs and extra-respiratory organs in ferrets, mice, and humans, resulting in the production of large amounts of proinflammatory cytokines [13, 76-78]. Although less wel characterized, human infections with H7N9 virus often lead to severe disease and high mortality rates. Both clinical and experimental evidence also suggest that proinflammatory cytokines underlie the immunopathology and subsequent severity of disease [79-81]. Even for viruses that in general cause mild disease, hypercytokinemia is observed in a subset of patients. During the 2009 H1N1 pandemic, hypercytokinemia was more often observed in severe/critical patients compared to hospitalized but non-critical and non-hospitalized patients [82-84]. Experimentally it has been shown that not only are proinflammatory cytokines detected in the blood but also in extra-respiratory organs such as the CNS, heart, spleen, liver, pancreas, and jejunum, and are produced by both infected and non-infected parenchymal cells **chapter 2, chapter 3** [13, 85, 86]. The induction of proinflammatory cytokines by the 1918 H1N1 pandemic virus in the respiratory system and extra-respiratory tissues likely contributed to the disease severity and lethality, **chapter 2** [87].

Recently it has been reported that in contrast to completely human adapted influenza viruses, the polymerases of pathogenic influenza viruses such as HPAI H5N1 virus and 1918 H1N1 virus generate aberrant RNA species called mini viral RNAs (mvRNA). These mvRNAs are potent inducers of the interferon signaling cascade and associated with the enhanced pathogenicity of these viruses [88]. Taken together, these data suggest that both local virus presence and/or (dysregulated) systemic immune responses are able to induce pro-inflammatory immune response in extra-respiratory tissues. In general, higher levels of proinflammatory cytokines are associated with cytokine-mediated organ dysfunction and tissue damage and therefor disease severity [13, 77, 89-91]. Altogether, there is a complex interplay between influenza virus and the host immune response that influences the development and severity of respiratory and extra-respiratory disease.

OBESITY AS A RISK FACTOR FOR SEVERE INFLUENZA

During seasonal epidemics and pandemics, the morbidity and mortality rates in the population are not evenly distributed. Young children and elderly are at the highest risk of being hospitalized followed by individuals with chronic medical conditions such as cardiac, pulmonary, metabolic, renal, neurodevelopmental, liver or hematologic diseases, immunocompromised people and pregnant women[92-97]. Interestingly, during the 2009 H1N1 pandemic, obesity emerged as a novel risk factor for severe influenza [98-107]. Both excess weight and obesity were associated with severe disease, hospitalization, admission to the intensive care unit, mechanical ventilation and death [98-100, 107].

Obesity: risk factors on the individual level

In this thesis, I have shown that in mice fed a high-fat diet, the immune response to 2009 H1N1 influenza virus infection is hampered in multiple organs. Furthermore, I observed structural changes in the heart of these mice after influenza virus infection, **chapter 3**. This high-fat diet resulted in a significantly higher total body weight and fat weight and was termed diet induced obesity, or obesity in short. For humans, classification for obesity is based on body mass index (BMI). However, for mice (and rats) this is not as simple and classification is based on the presence of hyperphagia, decreased energy expenditure, hyperglycemia, and insulin resistance [108]. Having an international standard for obesity in laboratory animals is therefore warranted.

In the lungs, obesity has a broad dampening effect on the antiviral immune response, which is unlikely a consequence of decreased viral replication, consistent with other reports [109-111]. This decreased antiviral response is likely caused by a reduction in the number and functioning of alveolar macrophages and plasmacytoid dendritic cells in the lung [112-115]. However, the few studies that evaluated the pro-inflammatory response in airway epithelial cells from obese individuals also found a dampened cytokine response [116], indicating a potential role for epithelial cells as well. Not only are antiviral responses affected in obesity,

wound repair and regeneration of damaged epithelial surfaces are also impaired resulting in a prolonged recovery phase [111]. Further studies into the innate immune responses of airway epithelial cells in obese individuals and/or animal models are warranted.

Obese mice infected with 2009 H1N1 virus had an increased left ventricular mass, **chapter 3**. In humans, an increased left ventricle mass increases the risk of myocardial infarction [117, 118]. Strikingly, in humans, in the first week after laboratory confirmed influenza virus infection, hospitalization for acute myocardial infarction was up to six times higher[63, 64]. In two out of four clinical trials analyzed retrospectively, I found evidence that patients experiencing influenza associated respiratory distress also had higher rates of obesity and influenza associated cardiac complications_**chapter 2**. Results from **chapter 2** and others should provide enough evidence to warrant a targeted clinical assessment into the role of obesity on new or worsened influenza virus associated CVD [119]. This is particularly important since ~15% of the global population is infected with influenza virus every year and ~39% of adults are overweight and ~13% of the world adult population is obese [120, 121]. Therefore, understanding the role of obesity on influenza virus associated cardiac disease is important for patient management in future epidemics and pandemics.

Obesity impairs the antiviral response to influenza virus in the lungs and this thesis provided the first evidence that this immune impairment also occurs in extra-respiratory organs such as the heart, **chapter 3** [109]. The immune response to influenza virus infection is hampered in the hearts of mice fed a high-fat diet alongside with structural changes of the heart, **chapter 3**. Recently it has been shown that mice lacking a critical antiviral factor (IFITM3) and infected with influenza virus suffer from cardiac complications which include the development of cardiac fibrosis and abnormal cardiac electrophysiology [122]. This raises the possibility that impairment of the antiviral response, not only in IFITM3 but more generally, as observed in our obese mice, contributes to the development of cardiac complications during influenza virus infection. In addition, interferon gamma (IFN-γ) seems to play a protective role in the development of cardiac hypertrophy [123-126]. Since obese mice failed to induce similar IFN-γ levels after influenza virus infection as lean mice did, one could speculate whether this lack is related to the observed cardiac changes. Collectively, the effect of obesity on influenza virus associated cardiac disease is likely a complex interplay between the virus and host where direct infection of the heart parenchyma, systemic proinflammatory cytokines and tissue hypoxia all play a role.

Obesity: risk factors on the population level

Not only does obesity pose a risk on the individual level but also on a population level. For example, the duration of influenza virus replication in and shedding from the respiratory tract in obese humans and mice is prolonged [110-112, 127, 128]. In addition, recently it has been shown that obesity promotes the rapid emergence of more virulent variants in mice and human cells [109]. Several other studies have shown that obesity can dampen T cell and B cell responses and affect influenza vaccination efficacy[129-134]. Consequently, it seems that

obesity negatively impacts vaccine efficacy and contributes to increased waning immunity over time [130, 135-138]. The prolonged recovery phase, prolonged mechanical ventilation time, longer intensive care unit stay and longer hospitalization time of obese individuals puts a higher burden on hospital capacity [105, 111]. Collectively, these data indicate that obesity likely plays an important role in influenza virus transmission dynamics and contributes to shape influenza virus evolution on a global scale [139-142]. Future investigations should determine the impact of obesity, and potentially other co-morbidities on influenza virus evolution within the host and on a population level.

THERAPEUTICS FOR INFLUENZA VIRUS EXTRA-RESPIRATORY COMPLICATIONS

In humans, influenza virus vaccines and antivirals are designed to prevent and reduce morbidity and mortality rates from influenza-associated respiratory tract disease [143-145]. Clinical symptoms and sequelae of influenza associated CNS disease are diverse and can range from febrile seizures to a potentially fatal encephalitis and chronic neuropathies such as Guillain-Barré syndrome and post-encephalitis Parkinsonism [3]. However, specific therapies for influenza virus associated CNS disease and other extra-respiratory complications do not exist. Until this thesis, the efficacy of intervention strategies on influenza virus associated CNS invasion and disease specifically had not been studied. In **chapter 6**, I investigated whether homologous vaccination or prophylactic oseltamivir, -a neuraminidase inhibitor-, could prevent or reduce CNS invasion or disease.

Vaccines

Homologous vaccination against HPAI H5N1 virus prevented extra-respiratory spread and CNS invasion in ferrets by significantly reducing virus replication in the olfactory mucosa, **chapter 6**. This indicates that serum antibodies, induced by the vaccine, ‘leak’ through the olfactory mucosa thereby neutralizing the virus. Not only is vaccination able to prevent CNS disease in ferrets, but vaccination can also reduce the incidence of cardiovascular events in humans [3]. However, during future outbreaks with a novel influenza virus, homologous vaccines will not be readily available and patients and physicians will rely mainly on antiviral therapies and possibly heterosubtypic immunity from natural infections and/or vaccine induced cross-clade protection. While current vaccines can induce some heterosubtypic immunity in humans, it remains unknown whether this antibody mediated immunity, is sufficient to prevent CNS invasion and disease [146, 147]. In animal experiments, vaccination against human influenza viruses (H3N2, H1N1 or influenza B Brisbane) reduced cell-mediated heterosubtypic immunity compared to infection with H3N2 or H1N1 and increased the pathogenicity of HPAI H5N1 virus infection in ferrets and mice[148-152]. Similar observations were found in humans during the 2009 H1N1 pandemic, where patients vaccinated previously with a seasonal vaccine were more likely to develop severe disease [153, 154]. This is likely due to a complete mismatch in the vaccine and challenge virus HA and

NA subtypes and highlights the need for vaccines that can induce a broader protection and/or vaccines for subtypes currently not circulating in humans.

Vaccination with an inactivated influenza virus vaccine induces antibody mediated immunity but no cell mediated immunity. In contract, infection with an influenza virus generates systemic antibody responses as well as cell-mediated immunity and local mucosal immunity. Ferrets previously infected with seasonal H3N2 or pandemic 2009 H1N1 virus developed cross-reactive T cells to HPAI H5N1 viruses and were protected from severe respiratory disease and extra-respiratory disease [66, 148-152]. This indicates that heterosubtypic immunity relies mainly on cross-reactive T cells and not on serum antibodies induced by vaccination. However, in these studies the role of mucosal immunity on the heterosubtypic immunity should be further investigated. Not only were animals protected from CNS disease, viral replication in other extra-respiratory organs such as the intestinal tract, liver, and spleen was also reduced or absent [66, 148, 152]. It is likely that a reduction of the viral load in the upper and lower respiratory tracts, including olfactory mucosa, is enough to prevent spread to extra-respiratory organs.

Intranasal vaccination with a live attenuated influenza vaccine (LAIV) could be promising since this vaccination strategy provides both cellular and mucosal immunity to the vaccine strain and a broader heterosubtypic immunity [155]. Compared to a seasonal trivalent inactivated influenza virus vaccine, ferrets vaccinated with a (chimeric HA) LAIV had significantly reduced virus replication in both the respiratory tract and olfactory bulb after challenge with a pandemic H1N1 2009 virus [156, 157]. However, all vaccine constructs contained at least one match with the challenge strain such as the HA stalk domain, neuraminidase (N1) or other (internal) proteins of PR8 which is the same subtype. Future studies should evaluate the efficacy of different LAIVs on extra-respiratory spread by influenza virus infections including subtype mismatches. Establishing a repository of (chimeric) LAIVs from different influenza virus subtypes with a high zoonotic and/or pandemic potential will aid the global pandemic preparedness.

Antivirals

As mentioned before, during future outbreaks with a novel influenza virus, patients and physicians will rely at first mainly on antiviral therapies. Currently advised and FDA-approved antivirals comprise three neuraminidase inhibitors (oseltamivir, zanamivir, peramivir) and one polymerase inhibitor (baloxavir marboxil). However, their effect on extra-respiratory spread and CNS invasion of a virus is not known. Prophylactic treatment with oseltamivir reduced virus replication of HPAI H5N1 virus in the nasal and pharyngeal swabs, but extra-respiratory spread, CNS invasion and development of a meningo-encephalitis were not prevented, **chapter 6**. This is likely due to the low absorption of the bioactive oseltamivir carboxylate into the CSF [158, 159]. It is hypothesized that influenza virus-associated acute cardiac events are triggered by proinflammatory and prothrombotic cytokines, which cause endothelial cell dysfunction, tachycardia and increased blood viscosity [160]. One study

found that patients with a history of cardiovascular disease and an influenza diagnosis that were prescribed oseltamivir experienced significantly fewer recurrent cardiac events such as myocardial infarction, stroke, angina, heart failure and sudden cardiac death[161]. The exact mechanism by which oseltamivir reduces the recurrence or occurance of cardiac events remains to be elucidated. It is likely that, since olseltamivir is distributed via the blood, virus replication within the cardiovascular system, and other extra-respiratory tissues (excluding the CNS), would be reduced by the presence of oseltamivir. It would be important to evaluate peramivir, zanamivir, and baloxavir marboxil for their effect on virus replication in the olfactory mucosa, extra-respiratory tissues, and the absorption into the CNS. In addition, specific antiviral drugs against influenza virus-associated CNS disease should be designed that allow efficient entry into the CNS [162].

Immunomodulation

Apart from damage cause by direct infection of cells, it is likely the immune system too plays an important role in the pathogenesis of influenza virus extra-respiratory disease. For influenza associated encephalopathy, it is proposed that modulation of the immune response may reduce disease severity [163, 164], although this remains poorly evaluated. Several studies encourage the use of oseltamivir, often in combination with corticosteroids, against pandemic 2009 H1N1 virus infection with neurological complications [165-168]. However, further research and controlled human clinical studies are warranted that should determine the effect of immunomodulatory therapeutics on influenza-associated extra-respiratory disease.

GENERAL CONCLUSIONS

The research described in this thesis was set out to gain a better understanding of extra-respiratory disease of influenza A virus infections. In this thesis, I show that we should not think of influenza virus as purely a respiratory pathogen, but need to consider its ability to spread to other organs, where it can cause severe damage. This thesis provides a hypothesis for the ability and efficiency of different influenza viruses to spread to and replicate in cells of the CNS. Future studies should investigate the mechanism by which influenza virus spreads throughout the brain and whether this happens via an extracellular phase or via direct cell-to-cell contact. In addition, a hypothesis is posed for different routes of CNS invasion used by different influenza viruses. These new insights provide a fundament for future research into CNS invasion by influenza virus but also by other respiratory viruses with a neurotropic potential. Identifying and categorizing important viral factors for different subtypes and strains of influenza viruses and other respiratory viruses for their neurotropism would be useful in risk assessment of future outbreaks.

I have shown that this extra-respiratory inflammation is not only a direct result of local virus infection in extra-respiratory organs, but also by the host immune system. Studies from this thesis revealed that extra-respiratory organs and systemic cytokines are involved in the

pathogenesis of pandemic 1918 H1N1 virus and pandemic 2009 H1N1 virus infections and potentially contribute to disease severity. Not only did I study the host immune system under "normal" conditions, I also evaluated the effect of obesity on extra-respiratory disease of influenza virus infection. Future studies should investigate the impact of other chronic medical conditions such as diabetes, asthma, cardiovascular disease, and others on influenza virus extra-respiratory disease.

The ability of influenza virus to cause systemic disease needs to be kept in mind during clinical evaluation and management of patients and considered in vaccine and antiviral development. Vaccines should be evaluated for their inhibitory effect on replication in the respiratory and olfactory mucosa and therefor viral CNS invasion via cranial nerves, as well as their ability to spread systemically to other extra-respiratory tissues. Preferentially, antiviral drugs should be further investigated and potentially redesigned for their potential to penetrate into the CNS in cases of (suspected) neurotropic virus infection. Developing a platform for specific and generic neurotropic antiviral drugs would greatly improve patient's management where vaccines are not readily available.

Together, the interdisciplinary approach of combining virology, pathology, genomics and immunology revealed that both viral and host factors contribute to influenza virus associated extra-respiratory disease. Furthermore, it showed that the severity of this extra-respiratory disease can differ significantly according toi viral subtypes and host immune status.

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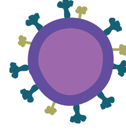

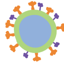

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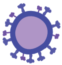

PHD PORTFOLIO

Name:	Ynze Jurre Siegers
Research department:	Viroscience, Erasmus MC
Research school:	Postgraduate school Molecular Medicine
PhD Period:	January 2015- January 2020
Promotor:	Prof. Dr. Thijs Kuiken
Co-promotor:	Assoc. Prof. Dr. Debby van Riel

EDUCATION

2015 – 2020 **DOCTOR OF PHILOSOPHY (PhD)**
Dept. of Viroscience, Erasmus Medical Center Rotterdam, the Netherlands

2011 – 2013 **MASTER OF SCIENCE (MSc)**
Infection & Immunity, Erasmus University Rotterdam, the Netherlands

2007 – 2011 **BACHELOR OF APPLIED SCIENCE (BASc)**
Biotechnology, University of Applied Sciences van Hall Larenstein, Leeuwarden, the Netherlands

PHD TRAINING

General Courses

2019	CADDE Genomic Epidemiology Workshop. Sao Paulo, Brazil
2019	Course Scientific Integrity. Erasmus Univeristy, Rotterdam, Netherlands
2018	Species-specific Carnivores course. Utrecht University
2018	Gene expression data analysis using "R". MolMed
2017	Basic Course on "R". MolMed
2017	Ingenuity Pathway Analysis (IPA) Workshop. MolMed
2017	Workshop Presenting Skills for Scientists. MolMed
2017	Biomedical English Writing. MolMed
2017	Introduction in GraphPad Prism Version 7. MolMed
2016	Course in Virology. MolMed

Attended Symposia / Conferences

2021	European Scientific Working Group on Influenza. Virtual
2020	European Scientific Working Group on Influenza. Virtual
2019	American Society for Virology. Minneapolis, MN, USA
2019	European Congress of Virology. Rotterdam, Netherlands
2019	Dutch Annual Virology Symposium. Amsterdam, Netherlands

2019	The 1918 influenza pandemic: Historical and biomedical reflections. Ypres, Belgium
2019	23rd Molecular Medicine Day. Rotterdam, Netherlands
2018	8th Orthomyxovirus Research Conference. Hanoi, Vietnam
2017	5th European Seminar in Virology. Bertinoro, Italy
2017	European Scientific Working Group on Influenza. Riga, Latvia
2016	Options X for the Control of Influenza. Chicago, IL, USA
2016	Young Antigone Meeting. Cambridge, UK
2016	20th Molecular Medicine Day. Rotterdam, Netherlands
2015	Young Predigone (Predemics/Antigone). Rotterdam, Netherlands
2015	7th Orthomyxovirus Research Conference. Toulouse, France
2015	19th Molecular Medicine Day. Rotterdam, Netherlands

Attended meetings / seminars

2015-2020	Viroscience department seminars
2015-2020	Viroscience workgroup meetings
2015-2020	Viroscience journal club

Oral presentations

2021	European Scientific Working Group on Influenza. Virtual
2020	European Scientific Working Group on Influenza. Virtual
2019	American Society for Virology. Minneapolis, MN, USA
2019	Dutch Annual Virology Symposium. Amsterdam, Netherlands
2019	The 1918 influenza pandemic: Historical and biomedical reflections. Ypres, Belgium
2018	8th Orthomyxovirus Research Conference (chair). Hanoi, Vietnam
2017	5th European Seminar in Virology. Bertinoro, Italy
2016	Options X for the Control of Influenza. Chicago, IL, USA
2016	Young Antigone Meeting. Cambridge, UK
2015	7th Orthomyxovirus Research Conference (co-chair). Toulouse, France

Poster presentations

2019	CADDE Genomic Epidemiology Workshop. Sao Paulo, Brazil
2019	7th European Congress for Virology. Rotterdam, Netherlands
2017	European Scientific Working Group on Influenza. Riga, Latvia
2015	Young Predigone (Predemics/Antigone). Rotterdam, Netherlands
2015	18th Molecular Medicine Day. Rotterdam, Netherlands

Attended meetings / seminars

2015-2020 Viroscience department seminars

2015-2020 Viroscience workgroup meetings

2015-2020 Viroscience journal club

SCHOLARSHIPS, AWARDS AND ACHIEVEMENTS

2019 Travel grant award. CADDE Genomic Epidemiology Workshop

2018 Travel grant award. Visiting Scientist, University of Queensland, Australia

2017 Travel grant award, The sixth ESWI conference

2017 Travel grant award, European Seminar in Virology

2016 Travel grant award, Options XI for the control of Influenza

2015 Best poster award, Young Predigone (Predemix/Antigone)

2015 Best abstract award, 7th Orthomyxovirus Research Conference

2015 Travel grant award, 7th Orthomyxovirus Research Conference

TEACHING AND SUPERVISION ACTIVITIES

2018-2019 Teaching: MSc Infection & Immunity. Erasmus University

2017 Co-supervision: MSc Student, Mr Lukas Wilken. Erasmus University

2017 Co-supervision: MSc Student, Ms Syriam Sooksawasdi. Erasmus University

2016 Co-supervision: BSc student, Mr Rémon Lavrijssen. Erasmus University

2015-2019 Lab rotations MSc students; Comparative Pathology. Erasmus University

COMMUNITY OUTREACH

2015-2016 Viruskenner (virus knowledge transfer for high school students). Rotterdam, Netherlands

2015 Clinical Laboratory Technician (Ebola Virus Diagnostics). Koidu, Sierra Leone

PUBLICATIONS

2020 Sooksawasdi Na Ayudhya S, Meijer A, Bauer L, Oude Munnink B, Embregts C, Leijten L, **Siegers JY**, Laksono BM, van Kuppeveld F, Kuiken T, Geurts-van Kessel C, van Riel D. Enhanced Enterovirus D68 Replication in Neuroblastoma Cells Is Associated with a Cell Culture-Adaptive Amino Acid Substitution in VP1. mSphere. 2020 Nov 4;5(6):e00941-20. doi: 10.1128/mSphere.00941-20.

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2018 Te Velthuis AJW, Long JC, Bauer DLV, Fan RLY, Yen HL, Sharps J, **Siegers JY**, Killip MJ, French H, Oliva-Martín MJ, Randall RE, de Wit E, van Riel D, Poon LLM, Fodor E. Mini viral RNAs act as innate immune agonists during influenza virus infection. Nat Microbiol. 2018 Nov;3(11):1234-1242. doi: 10.1038/s41564-018-0240-5. Epub 2018 Sep 17.

2018 de Wit E, **Siegers JY**, Cronin JM, Weatherman S, van den Brand JM, Leijten LM, van Run P, Begeman L, van den Ham HJ, Andeweg AC, Bushmaker T, Scott DP, Saturday G, Munster VJ, Feldmann H, van Riel D. 1918 H1N1 Influenza Virus Replicates and Induces Proinflammatory Cytokine Responses in Extrarespiratory Tissues of Ferrets. J Infect Dis. 2018 Mar 28;217(8):1237-1246. doi: 10.1093/infdis/jiy003.

2017 Anfasa F*, **Siegers JY***, van der Kroeg M, Mumtaz N, Stalin Raj V, de Vrij FMS, Widagdo W, Gabriel G, Salinas S, Simonin Y, Reusken C, Kushner SA, Koopmans MPG, Haagmans B, Martina BEE, van Riel D. Phenotypic Differences between Asian and African Lineage Zika Viruses in Human Neural Progenitor Cells. mSphere. 2017 Jul 26;2(4):e00292-17. doi: 10.1128/mSphere.00292-17. eCollection 2017 Jul-Aug.

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2016 Corneth OB, Reijmers RM, Mus AM, Asmawidjaja PS, van Hamburg JP, Papazian N, **Siegers JY**, Mourcin F, Amin R, Tarte K, Hendriks RW, Cupedo T, Lubberts E. Loss of IL-22 inhibits autoantibody formation in collagen-induced arthritis in mice. Eur J Immunol. 2016 Jun;46(6):1404-14. doi: 10.1002/eji.201546241. Epub 2016 May 12.

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2014 **Siegers JY**, Short KR, Leijten LM, de Graaf M, Spronken MI, Schrauwen EJ, Marshall N, Lowen AC, Gabriel G, Osterhaus AD, Kuiken T, van Riel D. Novel avian-origin influenza A (H7N9) virus attachment to the respiratory tract of five animal models. J Virol. 2014 Apr;88(8):4595-9. doi: 10.1128/JVI.03190-13. Epub 2014 Jan 29.

2013 van Riel D, Leijten LME, de Graaf M, **Siegers JY**, Short KR, Spronken MIJ, Schrauwen EJA, Fouchier RAM, Osterhaus ADME, Kuiken T. Novel avian-origin influenza A (H7N9) virus attaches to epithelium in both upper and lower respiratory tract of humans. Am J Pathol. 2013 Oct;183(4):1137-1143. doi: 10.1016/j.ajpath.2013.06.011. Epub 2013 Sep 10.

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DUTCH SUMMARY / NEDERLANDSE SAMENVATTING

Influenza A virussen, -griepvirussen-, zijn een belangrijke oorzaak van luchtweginfecties bij de mens, met meestal een relatief mild ziektebeloop. In enkele gevallen leidt infectie tot ernstigere complicaties zoals een longontsteking. Jaarlijks worden 5-10% van de volwassenen en 20-30% van de kinderen geïnfecteerd met een influenzavirus wat wereldwijd zorgt voort ~1 miljard infecties, ~3-5 miljoen ziekenhuisopnames, en ~290-650 duizend sterfgevallen. Tijdens een influenzapandemie kunnen deze cijfers vele malen hoger liggen. Een influenza pandemie is een wereldwijde uitbraak van een voor mensen nieuw influenzavirus dat zich efficiënt verspreidt. Sinds begin vorige eeuw hebben er vier influenzapandemieën plaatsgevonden; de Spaanse griep in 1918 (H1N1, ~50 miljoen doden), de Aziatische griep in 1957 (H2N2, ~2-3 miljoen doden), de Hongkong griep in 1968 (H3N2, ~1 miljoen doden) en de Mexicaanse griep in 2009 (H1N1, 150–578 duizend doden).

Influenza A virussen worden gekarakteriseerd op basis van twee oppervlakte-eiwitten, de hemagglutinine (HA) en neuraminidase (NA). Tot op heden zijn er zijn 18 verschillende HA subtypes (H1-H18) en 11 NA subtypes (N1-N11) bekend. De naam van een influenzavirus wordt gegeven op basis van de combinatie van deze subtypes, bijvoorbeeld H1N1, H3N2 of H5N1. Verschillende diersoorten, zoals vogels, varkens, paarden, en de mens, hebben hun "eigen" influenza A virussen, die circuleren in de populatie. Soms kan een dergelijk virus overspringen van dieren naar mensen, een zoönose (of zoönotische infectie). Een belangrijk zoönotische virus is het hoog pathogene aviaire influenza (HPAI) H5N1 virus. Sinds 1997 veroorzaakt dit virus uitbraken bij pluimvee dat gepaard gaat met ernstige ziekte, een hoge mortaliteit, en grote economische schade. Alhoewel het HPAI H5N1 virus niet efficiënt wordt overgedragen op mensen, kan dit af en toe gebeuren na intensief contact met pluimvee. Omdat het HPAIV H5N1 zich niet eenvoudig verspreidt van mens op mens heeft dit virus tot nu toe geen influenzapandemie veroorzaakt.

Naast de luchtwegen kunnen influenzavirussen ook elders in het lichaam schade veroorzaken. De meest voorkomende complicatie van influenzavirus infectie buiten de luchtwegen zijn ziekte van de hersenen (encefalopathie) of aandoeningen van het hart. Deze influenzavirus-geassocieerde encefalopathieën kunnen uiteenlopen van relatief milde verschijnselen zoals koortsstuipen en verwarring tot aan levensbedreigende complicaties zoals hersen(vlies) ontsteking. Cardiovasculaire aandoeningen includeren complicaties zoals hartfalen, ischemie, hartritmestoornis, en myocarditis (ontsteking van de hartspier). Omdat er een groot verschil zit in de potentie van verschillende influenzavirussen om complicaties buiten de luchtwegen te veroorzaken heb ik onderzocht: (1) hoe verschillende virussen zich buiten de luchtwegen kunnen verspreiden en schade kunnen veroorzaken; (2) welke virale factoren belangrijk zijn voor replicatie in cellen van het centrale zenuwstelsel (CZS); (3) of risicofactoren zoals obesitas, bedragen aan verspreiding en schade buiten de luchtwegen; (4) of virussen als ze eenmaal het CZS binnendringen zich ook aanpassen aan de nieuwe omgeving (adaptatie); en (5) welke interventie strategieën mogelijk herseninvasie kunnen voorkomen.

Verspreiding van influenzavirussen en ontstekingsreacties buiten de luchtwegen
Sommige influenzavirussen tasten organen buiten de luchtwegen vaker aan en met ernstigere gevolgen dan andere. Van deze virussen verspreiden sommige zich efficiënt naar het CZS, terwijl andere influenzavirussen het hart, de lever en/of andere organen kunnen aantasten. In dit proefschrift heb ik de verschillen van verspreiding buiten de luchtwegen onderzocht en beschreven waarom bepaalde influenzavirussen zich anders gedragen dan andere. Om hier meer inzicht in te krijgen heb ik dit experimenteel onderzocht in voornamelijk fretten en deels in muizen. Ik heb gekeken naar het pandemische H1N1 influenzavirus uit 1918, HPAI H5N1 virus en een pandemisch H1N1 virus uit 2009.

Het H1N1 influenzavirus uit 1918, ook wel het Spaanse griepvirus genoemd veroorzaakte de meest ernstige pandemie die we tot nu toe kennen met 20-50 miljoen doden wereldwijd. Alhoewel er indicaties waren dat dit virus mogelijk de hersenen zou kunnen aantasten, was dit nooit uitgebreid onderzocht en aangetoond. Experimenteel onderzoek beschreven in hoofdstuk 2 toont onomstotelijk aan dat, in fretten, het virus zich buiten de luchtwegen kon verspreiden. Buiten de luchtwegen bevond het 1918 H1N1 virus zich in het perifere en centrale zenuwstelsel en in de lever. Ook het HPAI H5N1 virus is geassocieerd met verspreiding buiten de luchtwegen en zelfs efficiënter dan 1918 H1N1 virus, naar zowel het CZS als andere organen waaronder de lever. Eerdere studies hebben laten zien dat het pandemische 2009 H1N1 virus en seizoens H3N2 virus zich niet efficiënt buiten de luchtwegen verspreiden. Kortom, er zijn grote verschillen tussen verschillende influenzavirussen in hun capaciteit om te verspreiden buiten de luchtwegen.

Niet alleen kan het virus zelf schade veroorzaken in en buiten de luchtwegen door cellen te infecteren. Ook kan een te heftige reactie van het immuunsysteem schade veroorzaken. Tijdens een infectie van de luchtwegen kunnen er immuunreacties in andere organen plaatsvinden zonder dat er aanwijzingen zijn voor actieve virus replicatie in deze organen. Nadat dit fenomeen was beschreven voor het HPAI H5N1 virus en het 2009 pandemische griepvirus, hebben ik dit voor het eerst beschreven voor het 1918 Spaanse griepvirus (hoofdstuk 2). Samengevat, influenzavirussen die ernstige ziekte veroorzaken zijn vaak geassocieerd met immuunreacties in zowel de luchtwegen als daarbuiten.

Verschillende influenzavirussen, verschillend neuroinvasief vermogen
Het tropisme van een influenzavirus is de voorkeur voor het infecteren van specifieke cel soorten of weefseltypen. Influenzavirussen repliceren in epitheelcellen in de luchtwegen van mensen en de locatie van replicatie verschilt tussen humane, pandemische en vogelgriepvirussen. In het algemeen repliceren humane influenzavirussen efficiënt in epitheelcellen in de neusholte en in de luchtpijp, terwijl vogelgriepvirussen voornamelijk repliceren in epitheelcellen van de bronchioli en alveoli (longblaasjes). Daarnaast kunnen influenzavirussen ook cellen infecteren van het reukslijmvlies, waarin vooral neuronen zitten die geurstoffen binden. Vooral het HPAI H5N1 virus kan deze cellen efficiënt infecteren.

Het respiratoir slijmvlies van de neusholte wordt voornamelijk geïnnerveerd door de trigeminuszenuw, terwijl het reukslijmvlies voornamelijk wordt geïnnerveerd door de reukzenuw. Deze verschillen in innervatie kunnen resulteren in virus-specifieke routes van CZS-invasie op basis van hun primaire replicatieplaats. In hoofdstukken 4 & 6 heb ik aangetoond dat het HPAI H5N1-virus het CZS voornamelijk binnendringt via de reukzenuw, terwijl het Spaanse griepvirus (hoofdstuk 2), dat voornamelijk repliceert in het respiratoire epitheel, het CZS voornamelijk via de nervus trigeminus lijkt binnen te dringen. Gezamenlijk hebben de onderzoeken die in dit proefschrift zijn uitgevoerd bijgedragen aan de kennis over hoe influenzavirussen het CZS binnendringen met aanwijzingen voor een prominente rol voor hersenzenuwen.

Virale factoren belangrijk voor replicatie in cellen van het CZS

Als influenzavirussen het CZS binnendringen, is de volgende stap het infecteren van en repliceren in cellen van het CZS. In mijn onderzoek heb ik gekeken welke virale factoren bijdragen aan efficiënte replicatie van influenzavirussen in cellen van het CZS. De eerste stap van de replicatiecyclus van het influenzavirus is hechten aan een gastheercel. Hoofdstuk 5 laat zien dat het HPAI H5N1 virus en het laboratorium geadapteerde “H1N1 WSN” virus efficiënt hechten aan cellen van het CZS, zowel aan neuronen en gliacellen. Dit in tegenstelling tot de seizoensgriepvirus H3N2 en pandemisch 2009 H1N1 virus, die inefficiënt aanhechten.

Een tweede belangrijke stap in de replicatiecyclus is het “activeren” van het oppervlakeiwit hemagglutinine. Zonder activatie van dit eiwit kan het nieuwgevormde virus niet fuseren met een nieuwe gastheercel. Deze activatie wordt uitgevoerd door specifieke proteasen, enzymen van de gastheer. Verschillende influenzavirussen kunnen geactiveerd worden door verschillende enzymen. In hoofdstuk 5 heb ik aangetoond dat virussen die efficiënt repliceren in cellen van het CZS, het H5N1 hoog-pathogene vogelgriepvirus en het H1N1 WSN virus, gebruik maken van andere enzymen dan seizoens en pandemische influenzavirussen. HPAI H5N1 en H1N1 WSN virussen gebruiken enzymen die beschikbaar zijn in vele orgaansystemen. Kortom, het identificeren van virale factoren die bijdragen aan efficiënte herseninvasie en/of replicatie in het CZS is cruciaal voor het classificeren van circulerende en toekomstige influenzavirussen op hun neurotropisme.

Het polymerase complex van influenzavirussen is belangrijk voor de vermenigvuldiging van het virus. Genetische veranderingen in het polymerase complex dragen bij aan adaptatie aan bijvoorbeeld een nieuwe gastheer in het geval van een zoönose. Maar of dit ook gebeurt wanneer een virus eenmaal in het CZS terecht komt, waar zich cellen bevinden die enorm verschillen van cellen in de luchtwegen, was nooit onderzocht. In hoofdstuk 4 heb ik laten zien dat een HPAI H5N1 virus dat het CZS is binnengedrongen, genetische veranderingen heeft ondergaan in genen die behoren tot dit polymerase complex. Alhoewel deze genetische veranderingen alleen werden aangetroffen in virussen die in de hersenen werden gevonden kon ik niet onomstotelijk aantonen dat CZS-virussen een “fitness” voordeel hadden t.o.v. het wildtype virus.

Obesitas als risicofactor voor ernstige complicaties van influenzavirus infectie

Tijdens de 2009 H1N1 pandemie bleek dat mensen met obesitas disproportioneel vaak ernstige ziekte ontwikkelden met meer ziekenhuisopnames, IC-opnames en overlijdens. In hoofdstuk 3 heb ik de rol onderzocht van een vetrijk dieet op complicaties buiten de luchtwegen na een infectie met het 2009 H1N1 virus. In muizen op een vetrijk dieet was de afweerreactie tegen het virus verminderd in de longen en andere organen. Dit ging gepaard met verhoogde virus replicatie in en functionele verandering aan het hart van muizen op een vetrijk dieet t.o.v. muizen met een "normaal" dieet. Verder liet een retrospectieve analyse van klinische studies zien dat hartcomplicaties relatief vaker voorkwamen bij mensen met overgewicht en influenzavirus infectie dan bij mensen zonder overgewicht en een influenzavirus infectie. Gerichte klinische studies zijn noodzakelijk om de bijdrage van overgewicht tijdens infectieziekten en hartcomplicaties verder in kaart te brengen. Gezien het feit dat >1,9 miljard mensen momenteel overgewicht/obesitas hebben, is de rol van overgewicht op influenza-geassocieerde hartschade belangrijke informatie voor het behandelen van de patiënt en ter voorbereiding op toekomstige influenza uitbraken en pandemieën.

Interventie strategieën om influenzavirus herseninvasie te voorkomen

Influenzavirusvaccins en antivirale medicijnen zijn ontwikkeld om de frequentie en ernst van ziekte te verminderen. Interessant genoeg verlagen seizoensgriep vaccinatie en het antivirale medicijn oseltamivir het risico op een hartaanval, beroerte en overlijden bij mensen met hartaandoeningen. Voor influenza-geassocieerde CZS-ziekte en andere extra-respiratoire complicaties bestaan echter geen specifieke therapieën. In dit proefschrift (hoofdstuk 6) heb ik onderzocht of vaccinatie of profylactische toedienen van oseltamivir, -een neuraminidase-remmer-, invasie of ziekte van het CZS kan voorkomen of verminderen. Hoofdstuk 6 liet zien dat vaccinatie tegen HPAI H5N1-virus extra-respiratoire verspreiding en CZS-invasie voorkwam bij fretten d.m.v. verminderde virusreplicatie in het reukslijmvlies. Oseltamivir daarentegen zorgde alleen voor een vermindering van virusreplicatie in de luchtwegen maar niet in het reukslijmvlies, waardoor het virus kon verspreiden naar de hersenen. Dit komt hoogstwaarschijnlijk door de lage penetratie van oseltamivir in het reukslijmvlies en in de bloed-hersenbarrière en daardoor een lage concentratie in het CZS. Tijdens uitbraken met nieuwe influenzavirussen zijn vaccins echter niet direct beschikbaar en zullen patiënten en artsen voornamelijk aangewezen zijn op antivirale medicijnen. Momenteel door de FDA goedgekeurde antivirale middelen zijn drie neuraminidaseremmers (oseltamivir, zanamivir, peramivir) en één polymeraseremmer (baloxavir marboxil). Buiten oseltamivir om is er echter weinig tot niets bekend wat het effect is van deze medicijnen op virusverspreiding buiten de luchtwegen. Meer onderzoek is daarom nodig op het gebied van antivirale middelen en hun effect op virusverspreiding en replicatie buiten de luchtwegen.

Dankzij de studies beschreven in dit proefschrift hebben we meer inzicht gekregen in de systemische pathogenese van seizoensgriep-, pandemische griep- en hoogpathogene vogelgriepvirussen en de verspreiding van deze virussen naar extra-respiratoire organen. Dit proefschrift laat zien dat zowel virale alsmede gastheerfactoren bijdragen aan de frequentie

en ernst van extra-respiratoire verspreiding van influenzavirussen en dat er meer onderzoek nodig is voor de evaluatie en ontwikkeling van interventietherapieën naast vaccineren.

CURRICULUM VITAE / ABOUT THE AUTHOR

Jurre Siegers was born on the 20th of February 1989 in Doetinchem, the Netherlands. After completing his secondary education at the Rietveld Lyceum in the same city in 2006, Jurre started a bachelor degree in biotechnology at the van Hall Larenstein, University of Applied Sciences in Leeuwarden, the Netherlands which he completed in 2011. During his final year internship of his bachelor, -performed at the Murdoch Children's Research Institute in Melbourne, Australia-, Jurre developed an interest in the field of infectious diseases while working on a project that investigated how the inherited blood disorder beta-thalassemia contributes to aberrant innate immune responses. His interest resulted in pursuing a Research Master of Science degree in "Infection and Immunity" at the Postgraduate School Molecular Medicine from the Erasmus University in Rotterdam, the Netherlands which he completed in 2013. During his final MSc internship, Jurre studied the attachment pattern of a novel avian influenza A virus H7N9 to the respiratory tract of humans and animals which sparked his interest in the field of virology and in particular influenza A viruses. After travelling the world for one year, Jurre started a PhD in Virology at the department of Viroscience of the Erasmus Medical Center in Rotterdam in 2014 under supervision of Prof. Dr. Thijs Kuiken and Dr. Debby van Riel investigating the pathogenesis of extra-respiratory tract complication of influenza resulting in this thesis. In October 2021, Jurre continued his scientific career at Institute Pasteur in Cambodia focussing on emerging respiratory viruses at the human-animal interface.

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