University of Natural Resources and Life Sciences
Department of Biotechnology

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DISSERTATION

Title of the Dissertation

“Countermeasures against emerging avian influenza subtype H7N9 viruses and insights for vaccine design”

submitted by

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in partial fulfillment of the requirements for the degree of

Dr.nat.techn.

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ABSTRACT

Influenza viruses remain a major public health risk. Besides globally circulating seasonal influenza virus strains of the H1N1 subtype, H3N2 subtype, and influenza B lineages, avian influenza viruses can result in zoonotic infections. In 2013, an epizootic influenza A H7N9 virus emerged in China that resulted in significant morbidity and an associated high case fatality rate in humans (about 40%). The H7N9 virus was established in the avian reservoir in China and led to annual periodic epidemics in humans. From February 2013 until August 2018 there were approximately 1500 laboratory-confirmed cases of human infection resulting in over 600 deaths. To date, there have been no cases of confirmed sustained human-to-human transmission. Nevertheless, the virus is constantly changing, and may adapt to the human host or it could undergo reassortment with seasonal viruses, allowing the virus to further spread within the human population. Typically, humans are immunologically naïve to H7 hemagglutinin subtype viruses. Therefore, developing and testing H7N9 vaccines and H7 hemagglutinin specific monoclonal antibodies constitutes a priority for pandemic preparedness. In this study we show that human vaccination with a recombinant H7 hemagglutinin induced H7 specific antibodies in addition to broadly reactive antibodies towards the hemagglutinin stalk domain, the conserved part of the influenza virus hemagglutinin. In addition, we found that the passive transfer of human sera into mice conferred protection against H7N9 virus challenge. Finally, we characterized murine H7-specific monoclonal antibodies and demonstrated that antibodies raised against the H7 hemagglutinin of the 2013 H7N9 isolate can bind and neutralize recent H7NX isolates, despite changes in the target site. Our studies help inform the development of H7 prepandemic vaccines and could guide the selection of prepandemic H7 vaccine strains.

Keywords: Influenza A virus – H7N9 – humoral immunity - vaccines – monoclonal antibodies

ZUSAMMENFASSUNG

Influenzaviren stellen ein großes öffentliches Gesundheitsrisiko dar. Neben den global zirkulierenden saisonalen Influenzaviren vom H1N1 Subtyp, H3N2 Subtyp und den Influenza B Stamm-Linien können Vogelgrippe-Viren zu zoonotischen Infektionen führen. Im Jahr 2013 trat in China ein epizootischer Influenza A H7N9 Virus auf, der zu einer hohen Morbiditätsrate und damit verbundenen hohen Letalität (circa 40%) im Menschen führte. Der H7N9 Virus etablierte sich im Vogel-Reservoir in China und führte zu jährlichen periodischen Epidemien im Menschen. Von Februar 2013 bis August 2018 wurden im Labor 1500 Fälle von Infektionen im Menschen nachgewiesen, welche zu über 600 Todesfällen führten. Bis jetzt sind keine Fälle von sich fortsetzenden Mensch-zu-Mensch Übertragungen nachgewiesen worden. Dennoch ändert sich der Virus ständig, was dazu führen könnte, dass der Virus sich dem menschlichen Wirt anpasst oder es könnte zu einem Reassortment mit saisonalen Viren kommen, wodurch der Virus sich besser in der menschlichen Population ausbreiten könnte. Üblicherweise sind Menschen immunologisch naiv gegenüber Viren des H7 Hämagglutinin Subtyps. Deswegen stellt die Entwicklung und Untersuchung von H7N9 Impfungen sowie H7 spezifischen monoklonalen Antikörpern eine Priorität dar, um für eine Pandemie gerüstet zu sein. In dieser Studie zeigen wir, dass eine Impfung mit einem rekombinanten H7 Hämagglutinin H7 spezifische monoklonale Antikörper induzieren kann. Außerdem werden Antikörper gebildet, die mit der Hämagglutininstammdomäne, dem wenig variablen Teil des Influenzavirus-Hämagglutinins, reagieren. Zusätzlich konnte festgestellt werden, dass der passive Transfer von menschlichem Serum in Mäusen Schutz vor einer tödlichen H7N9 Infektion bietet. Schlussendlich wurden H7 spezifische murine Antikörper

charakterisiert und damit wurde bewiesen, dass Antikörper, die durch Immunisierung mit einem H7 Hämagglutinin eines Isolates aus 2013 gebildet wurden, imstande sind, neue H7NX Isolate zu neutralisieren, obwohl die Zielstruktur verändert ist. Diese vorliegenden Studien können die Entwicklung von präpandemischen H7 Impfstoffen unterstützen und Hilfestellung für die Auswahl von präpandemischen H7 Impfstoff-Stämmen geben.

Schlagworte: Influenza A Virus – H7N9 - Humorale Immunantwort – Impfstoffe – Monoklonale Antikörper

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INTRODUCTION

Influenza viruses

The influenza viruses are part of the *Orthomyxoviridae* family and consists of a negative-sense, single-stranded, segmented RNA genome. The viruses are divided into four different genera, influenza A, B, C and D. Influenza A and B viruses can cause severe disease in humans, whereas influenza C viruses only cause mild disease and are not associated with epidemic outbreaks. The predominant natural reservoir of influenza A viruses is wild waterfowl, but also many mammals can be infected. Influenza B viruses are known to infect humans only.[1-3] A recently identified influenza virus, classified as influenza D virus, was found in swine, cattle, sheep, and goats. Among these hosts, cattle have been proposed as the natural reservoir.[4] Therefore, vaccine development and research are directed mostly to influenza A and B viruses, as these virus types cause disease and pose a major global public health burden.[2,3]

The influenza A genome consists of eight segments that encode at least 11 proteins. The most predominant proteins include the polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2), polymerase acidic protein (PA), nucleoprotein (NP), hemagglutinin (HA), neuraminidase (NA), matrix protein 1 (M1), matrix protein 2 (M2), the nonstructural protein (NS1) and the nuclear export protein (NEP).[2] Based on the surface glycoproteins HA and NA, influenza A viruses are further classified into different subtypes. Currently, there are 16 different HA (H1-H16) subtypes and 9 NA (N1-N9) subtypes described for influenza A. RNA encoding for two HA-like and NA-like proteins has also been discovered in bats (HA-like H17-H18, NA-like N10-

N11 respectively).[5,6] The subtypes of influenza A viruses can be further divided into two distinct phylogenetic groups based on the differences in their HA sequence. Influenza A group 1 consists of H1, H2, H5, H6, H8, H9, H11, H12, H13, H16, HA-like H17 and HA-like H18 and group 2 encompasses H3, H4, H7, H10, H14, H15 (Fig. 1A).[3]

Seasonal epidemics of influenza virus infections in humans are caused by influenza A strains of the H1N1 subtype and H3N2 subtype. Additionally, influenza B viruses of two distinct lineages (B/Victoria/2/87-like and B/Yamagata/16/88-like lineage) circulate in the human population, causing regular epidemics. In temperate climates, seasonal epidemics occur mostly during winter months (October-May in the Northern Hemisphere, May-October in the Southern Hemisphere), while in tropical climates, influenza virus infections may occur throughout the year, causing more irregular outbreaks.[7] Infection with either seasonal influenza A or B viruses can cause severe disease. This is particularly problematic in the elderly and other at-risk groups such as pregnant women, the pediatric cohort and people with underlying conditions like immunocompromised individuals. They might develop life-threatening complications requiring hospitalization, in the worst case resulting in death. It is estimated that seasonal influenza viruses are responsible for the death of more than 500,000 people worldwide per year.[7,8]

In theory, influenza is a vaccine-preventable disease and the most effective strategy for preventing influenza virus infections is annual vaccination.[9-11] However, low vaccination coverage (overall about 40% or less among adults in the USA in the 2017-18 influenza season, and as low as 35% in older people in Europe in the 2014-15 season) and the ability of the virus to rapidly evolve leads to millions of infections every year.[7,12,13] Similar to many other RNA viruses, the influenza virus has a high

mutation rate caused by its error-prone RNA-dependent RNA polymerases (RdRp) lacking proofreading activity.[2,3] Random mutations that occur during viral replication in combination with immunological pressure lead to a constant change of the viral proteins. For instance, the major viral surface glycoprotein HA is the main target for the host adaptive immune system and antibody responses upon influenza virus infection or vaccination.[14] The HA of the influenza A virus consists of two distinct parts: the very plastic, immunodominant, membrane-distal globular head domain and the highly conserved, immunosubdominant stalk domain (Fig. 1B).[15,16] The HA head domain harbors the receptor binding site of the virus and antibodies that block this site prevent the virus from attaching to host cells and therefore neutralize it. For that reason it is advantageous for the virus that the head domain is constantly changing. It allows the virus to escape the antibody response by introducing point mutations that lead to changes in the major antigenic sites on the head domain. Hence, viruses that evade immune recognition are fitter and can spread more easily, resulting in novel, dominant virus populations. This process is called antigenic drift and is responsible for the need of optimized, new vaccines virtually every year.[1,2,17] Indeed, from 1998 to 2018 at least one of the influenza vaccine strain components for the Northern Hemisphere influenza vaccine had to be changed 17 out of 21 times.[18,19]

In addition to antigenic drift, the segmented genome of influenza viruses allows for the exchange of gene segments when two different viruses co-infect the same cell, a term called virus reassortment. Virus reassortment can further lead to viruses with new HA and/or NA proteins which is commonly referred to as antigenic shift.[2,3] Antigenic shift can cause a more drastic genetic change than antigenic drift, resulting in completely different antigenic phenotypes of viruses.[20] Moreover, antigenic shift of viruses by reassortment can facilitate the adaptation of influenza viruses to new host

species and can be a pathway that viruses follow to enter a new host. In theory, novel reassortant viruses, to which humans are naïve, have the potential to cause pandemics in humans.[21]

The year 2018 marks the 100-year anniversary of the 1918 influenza pandemic, which has caused about 50-100 million deaths worldwide. The 1918 pandemic virus was the first dated influenza pandemic for which a causative agent (influenza A H1N1) could be isolated and it was the most severe influenza pandemic to date.[22,23] In 1957 a new influenza virus strain of the H2N2 subtype emerged in East Asia, triggering a pandemic termed “Asian Flu”.[24] Eleven years later, the next pandemic was caused by an emerging influenza A H3N2 virus (“Hong Kong Flu”) in 1968. Drifted virus strains of this H3N2 virus have been continuously circulating in humans since then.[25] The most recent pandemic occurred in 2009 termed the 2009 H1N1 pandemic (“Swine Flu”). As far as we know, influenza B viruses have never caused a pandemic thus far and have been circulating in humans since at least 1940 (Fig. 2). The common theme for the four documented pandemics was that the human population had little to no pre-existing immunity to the emerging virus strains and that the pandemic strains with changes in the HA subtype arose from genetic reassortment of circulating human viruses with animal influenza A viruses.[24,26]

Zoonotic influenza

Zoonotic influenza viruses are defined as influenza viruses of animal origin that cross the animal-human species barrier to infect people. Humans have been infected with a variety of avian, swine or other zoonotic influenza viruses, whereas infections with

avian influenza A viruses occur most frequently. Typically, human infections are only acquired through close exposure to infected animals or contaminated environments.

In the past, avian influenza A viruses of the H5N1, H5N6, H6N1, H7N2, H7N3, H7N4 H7N7, H7N9, H9N2 and H10N8 subtype resulted in infections in humans.[27-36] Other zoonotic viruses that have been reported to infect humans are swine influenza viruses of the H1N1, H1N2 and H3N2 subtype (denoted as variant viruses H1N1v, H1N2v and H3N2v respectively), which have been largely associated with exposure to infected swine at country fairs.[37-39] Additionally, an outbreak of an H7N2 subtype virus in cats in an animal shelter in New York City was reported in 2016. The feline virus caused one confirmed human zoonotic event by infecting a veterinarian, who subsequently experienced influenza-like illness.[40] To date, no confirmed cases of equine or canine influenza virus infections in humans have been reported, however, these viruses pose a zoonotic risk and are under close surveillance. In particular, canine influenza viruses require enhanced surveillance due to increasing influenza A virus diversity detected in canines that are in close contact with humans.[41-43]

Zoonotic influenza virus subtypes of considerable concern include highly pathogenic avian influenza (HPAI) H5N1 viruses and low pathogenic influenza (LPAI) H7N9 subtype viruses. HPAI viruses cause systemic infections in avian species and can induce multi-organ failure and high mortality of poultry and birds. HPAI viruses have caused huge economic loss for the poultry industry and resulted in international trade restrictions.[44] The HA of these viruses features a polybasic cleavage site (a string of multiple basic amino acid residues) that can be cleaved by ubiquitous furin-like proteases, that are present in all host tissues. In contrast, the HA of LPAI viruses can only be cleaved by host proteases localized in respiratory and intestinal organs of avian species. Proteolytic cleavage and ensuing activation of the HA mediates

fusion between the viral envelope and the host cell endosomal membrane, consequently influencing viral infectivity and dissemination.[45,46] Since 2003, highly pathogenic H5N1 avian influenza viruses have caused over 850 laboratory confirmed human cases with a high case fatality rate among these cases.[27,47] The numbers of new infections decreased rapidly after 2015 and there have been no reported cases in humans in 2018.[48] Recently, in 2013, an avian influenza A H7N9 virus (LPAI) emerged and frequently caused severe lower respiratory tract infections in humans in China.[32] Since 2013, the H7N9 virus displayed seasonality, comparable to seasonal human influenza viruses, and led to annual epidemics in China (Fig. 3). During these five waves of H7N9 epidemics over 1500 laboratory confirmed cases, with a case fatality rate of about 40% have been reported. These viruses reappeared each year, gained some virulence determinants that enhance their risk for humans and increased their spread in the human population, attributing this virus subtype a major pandemic potential.[48,49] Moreover, during the fifth wave of H7N9 epidemics the virus evolved further and split into two distinct phylogenetic lineages, the Pearl River Delta clade and the Yangtze River Delta clade. Stockpiled prepandemic H7 vaccines were generated against the original H7N9 isolate of 2013. However, when tested against ferret antisera, it was shown that the antigenically distinct lineages did not match stockpiled H7 vaccines well, rendering these vaccines less effective.[50] Additionally, highly pathogenic influenza A H7N9 viruses emerged and caused human infections.[51] These HPAI viruses are usually not causing more severe disease than LPAI viruses in humans, but it is believed that HPAI viruses grow to higher titers in animals and therefore increase the risk of animal-to-human transmission. LPAI viruses may not be detected in infected poultry, whereas HPAI viruses cause death among poultry and are more easily detected. [50-54]

Even though there have been large numbers of zoonotic infections in humans, zoonotic influenza viruses have not yet gained the capacity for effective and sustained human-to-human transmission. Human-to-human transmission has been occasionally reported in family clusters, but many of these cases likely describe situations where more than one individual was in contact with same non-human virus source.[55] Zoonotic influenza viruses capable of replicating in the human host have already surmounted host species barriers, however, gaining the ability for sustained transmission in a new host species poses a major adaptive challenge because a number of mutations are required.[56] In controversial laboratory experiments, zoonotic avian viruses (HPAI H5N1) were serially passaged in ferrets and subsequently acquired the ability to be transmitted by aerosol or respiratory droplet between mammals.[57,58] Another concern, as already mentioned, is genetic reassortment of zoonotic viruses with human seasonal influenza viruses, thereby gaining the capability of airborne transmission as well as enhanced virus replication properties in the human host.[59] Cases of people infected with both H7N9 and seasonal influenza strains have already been reported during the period of overlapping H7N9 and seasonal influenza epidemics in China.[60]

If, when and which zoonotic viruses adapt to humans or reassort with seasonal human viruses is impossible to predict. Thus, it is of the utmost importance to be armed with effective vaccines, therapeutic antibodies, reagents and antivirals in the event of a pandemic.[61,62]

Influenza virus vaccines and therapeutics

Seasonal influenza virus vaccines include the surface proteins HA and NA of all circulating strains, resulting in trivalent (TIV) or quadrivalent influenza virus vaccines

(QIV) containing an H1N1, H3N2 and either one or two lineages of influenza B viruses.[63] These vaccines induce subtype-specific antibody responses towards the glycoproteins HA and NA. Therefore, regular influenza virus vaccines offer very little to no protection against emerging zoonotic influenza viruses with drifted seasonal subtypes or entirely different subtypes.[64] Indeed, during the 2009 H1N1 pandemic the H1N1 component of the seasonal vaccine did not protect against the novel pandemic virus strain. As such, a new vaccine had to be made, but the pandemic virus strain was only isolated when the first wave of infections had already hit the human population. Subsequently, it took about half a year until the pandemic H1N1 vaccine was available and could be administered at which point the second wave of pH1N1 virus infections had already begun. The human population was vulnerable to infection and disease during this time. For this reason, vaccines need to be manufactured in a less time consuming manner, the manufacturing process altered or better vaccine generated. Additionally, prepandemic vaccines, for viruses that are predicted to have pandemic potential, need to be developed and tested prior to a potential pandemic outbreak.[61,65]

A promising technology to produce influenza vaccines in a more timely fashion is the cell-based production of recombinant influenza vaccines utilizing the baculovirus expression vector system. Insect cells can be harnessed to express different recombinant HAs, which are purified and formulated into vaccines. The production process can easily be modified to express HAs of choice, including HAs of zoonotic influenza viruses with pandemic potential.[66-68] Furthermore, DNA and mRNA vaccines represent promising and versatile alternatives to conventional vaccine approaches. These vaccines have the potential for rapid, inexpensive and scalable manufacturing, which can be quickly upscaled in response to emerging pandemic

influenza viruses. A standalone mRNA influenza vaccine is currently in human clinical trials and encouraging results from preclinical trials in large animal models in the fields of DNA influenza vaccines have been achieved.[69-72]

A prospective strategy to induce protection against both future seasonal influenza strains and emerging zoonotic influenza strains is the development of universal influenza virus vaccines. Current proposed targets for universal influenza virus vaccines are conserved regions of the influenza virus such as the HA stalk domain, the ectodomain of the M2 ion channel or the internal matrix and nucleoproteins.[64,73] Furthermore, addition of NA to current vaccines could broaden the provided protective effect. The first human trials testing universal vaccines targeting the HA stalk domain are currently ongoing. As mentioned earlier, the hemagglutinin head is constantly changing and thereby evading the immune system, whereas the stalk domain does not tolerate mutations easily and evolves slower.[15,74] Importantly, the HA stalk domain is highly conserved within the groups of influenza A and within influenza B viruses (Fig. 1A). Therefore, antibodies raised against the HA head domain are specific and only protect against well-matched strains, however, antibodies that target the highly conserved stalk domain are broadly reactive towards multiple HAs of different subtypes. One approach to re-focus the immune response towards the conserved stalk domain is to vaccinate with chimeric HAs (Fig. 4). The idea is to introduce the immune system to chimeric HAs that possess an exotic head domain, to which humans are naïve, and a stalk domain of a seasonal influenza virus that the human immune system has already encountered. Upon vaccination with a chimeric HA the immune system will recognize conserved epitopes in the HA stalk domain and mount a re-call response towards these epitopes. The human immune system is naïve to the exotic head domain and during the first encounter

with this HA subtype a rather weak primary antibody response occurs. Upon follow-up vaccination with another chimeric HA, with a different exotic HA head domain and the same HA stalk domain, the antibody response towards the stalk domain gets boosted further, ideally resulting in protective titers against seasonal influenza as well as possible pandemic viruses.[73,75-80]

Other countermeasures against influenza virus infection are scarce and include three types of licensed anti-influenza drugs to prevent or treat influenza virus infections. These drugs act by inhibiting the action of the M2 protein (adamantanes), by inhibiting the NA enzyme (oseltamivir, zanamivir) or by inhibiting the influenza cap-dependent endonuclease (baloxavir marboxil). Adamantanes are only active against influenza A viruses and due to a marked increase in resistant influenza A viruses the use of this drug is currently not recommended. Oseltamivir or zanamivir show activity against both influenza A and B, but again influenza viruses can develop resistance by the introduction of mutations in the NA sequence.[81] Baloxavir marboxil is a new antiviral drug that received approval from the US Food and Drug Administration (FDA) in October 2018. Baloxavir marboxil is effective against viruses resistant to NA inhibitors or adamantanes and mutations hampering the activity of the new drug have not yet been described.[82,83]

An alternative or an addition to these treatment options is the use of passive immunization with broadly protective monoclonal antibodies (mAbs). In recent years several cross-reactive anti-influenza antibodies have been identified that neutralize a wide range of influenza virus strains. Furthermore, a number of human clinical studies showed promising results upon testing the therapeutic efficacy of anti-influenza antibodies in human challenge studies.[84,85] A large scale prophylactic administration of antibodies to the population might not be feasible, but the

antibodies could help curb the immediate health threat of an emerging pandemic virus strain (e.g. H7N9) while matched vaccines are being developed and deployed. Additionally, novel antibody-based universal influenza virus therapeutics can be used for pre- or post-exposure prophylaxis, to reduce symptoms and to treat severe seasonal infection in at-risk individuals or to treat zoonotic influenza infection.[73,86,87]

Pandemic preparedness

The manuscripts in this thesis demonstrate the results of a detailed analysis of human serum samples from a clinical trial testing a prepandemic H7 vaccine and the characterization of broadly reactive H7 HA specific monoclonal antibodies. Additionally, the immune response on a monoclonal and polyclonal level after H7 exposure is being reviewed.

FIGURES

Figure 1

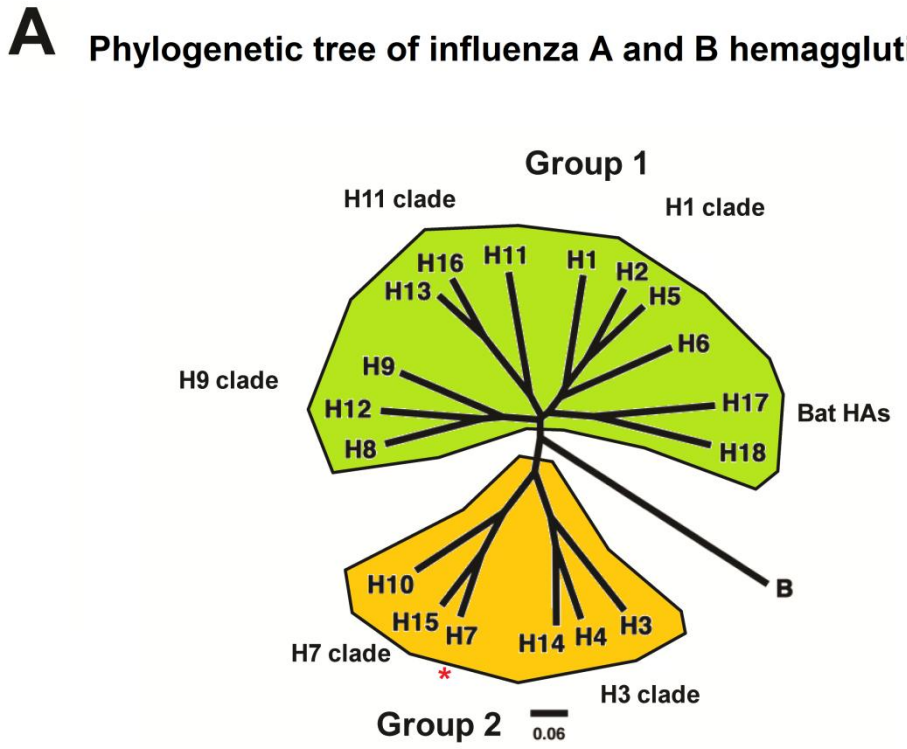

Figure 1 Phylogenetic tree of influenza A and B hemagglutinins and structure of an influenza A virus hemagglutinin trimer **(A)** Phylogenetic tree of influenza A and B HA based on amino acid sequence. Influenza A HAs are separated into group 1 (highlighted in green) and group 2 (highlighted in orange) based on their sequence. HA clades and subtypes are annotated. H7 HA (group 2) is marked by a red star. The scale bar represents % amino acid difference. **(B)** Visualization of an influenza A H7 HA trimer with the membrane distal globular head domain visualized in dark red and the membrane proximal stalk domain shown in blue. The amino acid residues (cysteins) that separate the head domain and the stalk domain are shown in yellow. The figure is based on Protein Data Bank (PDB) structure code 1TI8.[88] The figure was adapted from Stadlbauer et al., Frontiers of Medicine, 2017.[89]

Figure 2

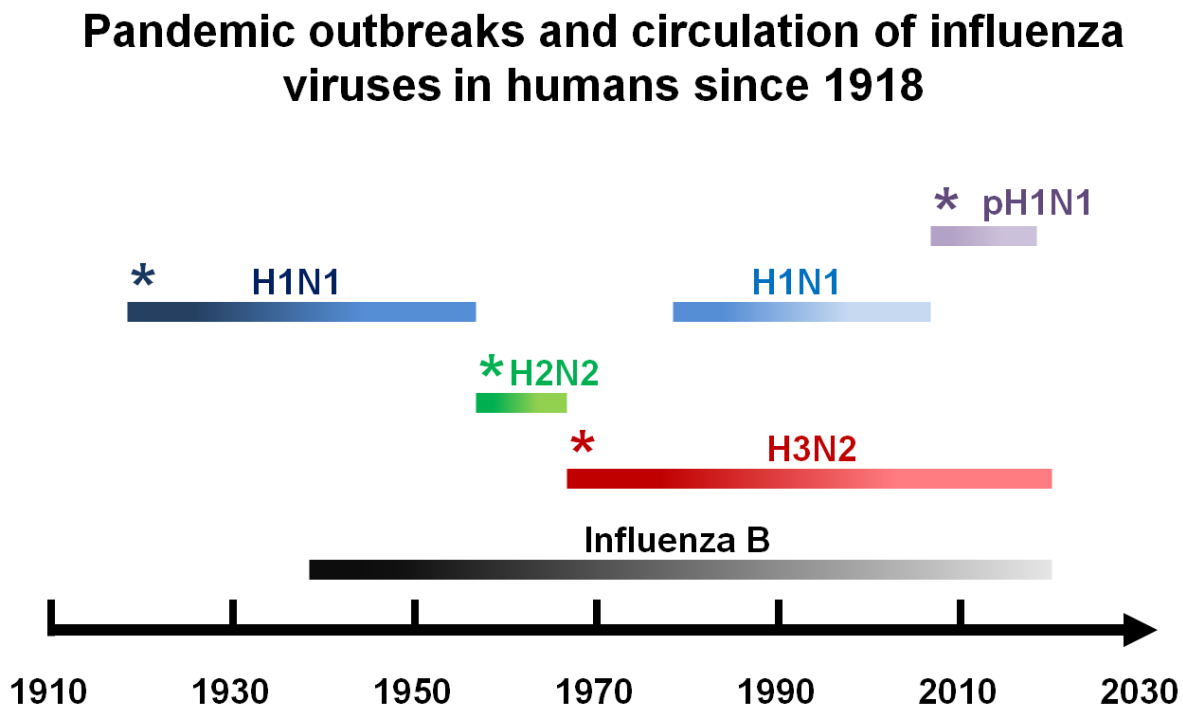

Figure 2 Pandemic outbreaks and circulation of influenza viruses in humans since 1918. Two subtypes of influenza A, pH1N1 (purple) and H3N2 (red), as well as influenza B (black) are currently circulating in the population globally. The first recorded influenza pandemic occurred in 1918 (blue star) caused by an influenza A H1N1 virus. Influenza B was first isolated in 1940, did not cause a pandemic and has been constantly circulating since. In 1957 influenza A H2N2 (green) replaced H1N1, causing a pandemic (green star). In 1968 (red star) influenza A H3N2 replaced H2N2 resulting in another pandemic. Usually, the introduction and establishment of a new pandemic virus leads to the replacement of the previously circulating strain. However, in 1977 influenza A H1N1 re-emerged. This H1N1 strain was genetically nearly identical to the H1N1 virus that was replaced in 1950s, which hints at a possible lab accident. In 2009 H1N1 got replaced with a new H1N1 strain termed pH1N1 that caused the most recent pandemic to date (purple star).

Figure 3

Epidemiological curve of avian influenza A H7N9 cases in humans

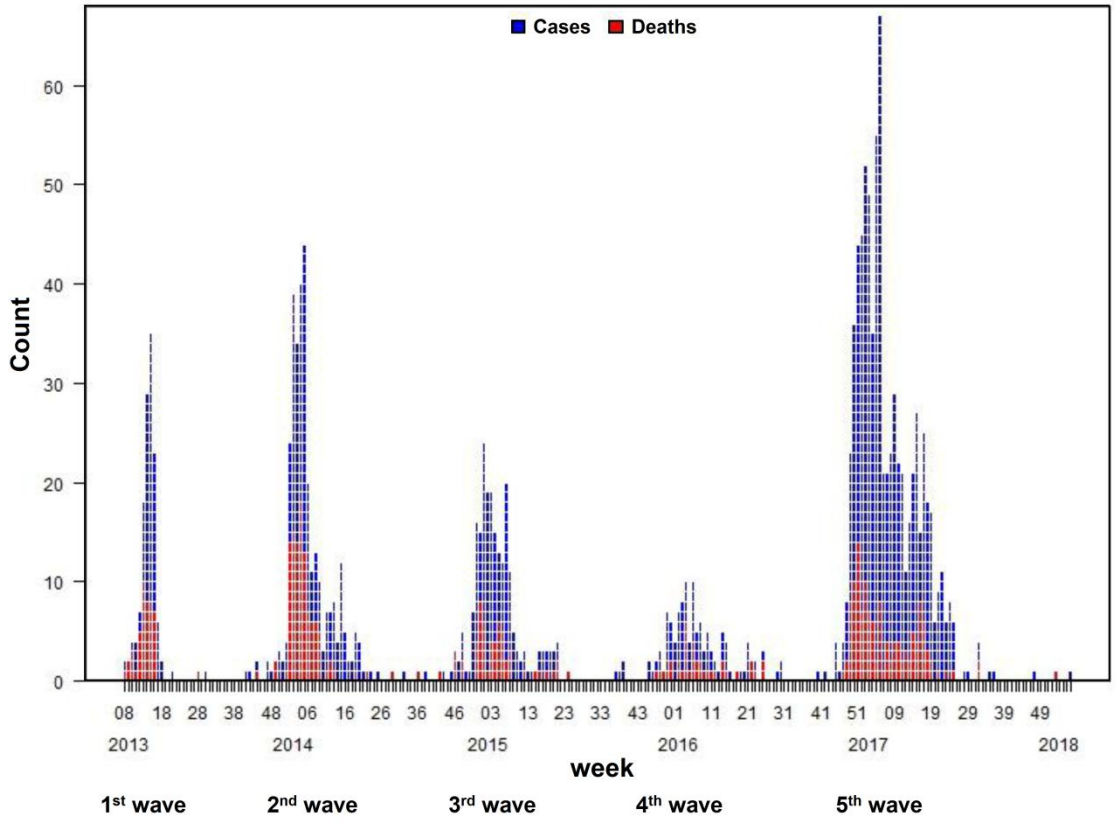

Figure 3 Epidemiological curve of avian influenza A H7N9 cases in humans. The X-axis indicates the years and weeks by onset of infection from 2013 to 2018. The Y-axis shows the count of laboratory confirmed cases. The number of cases of infection is highlighted in blue and the cases with fatal outcome are highlighted in red. The five waves of epidemics, typically starting at the end/beginning of each year, are annotated. From 2013 until 2018, the total count of laboratory confirmed infections with avian influenza A H7N9 virus was 1567, including 615 deaths. The figure was adapted from WHO.com [48].

Figure 4

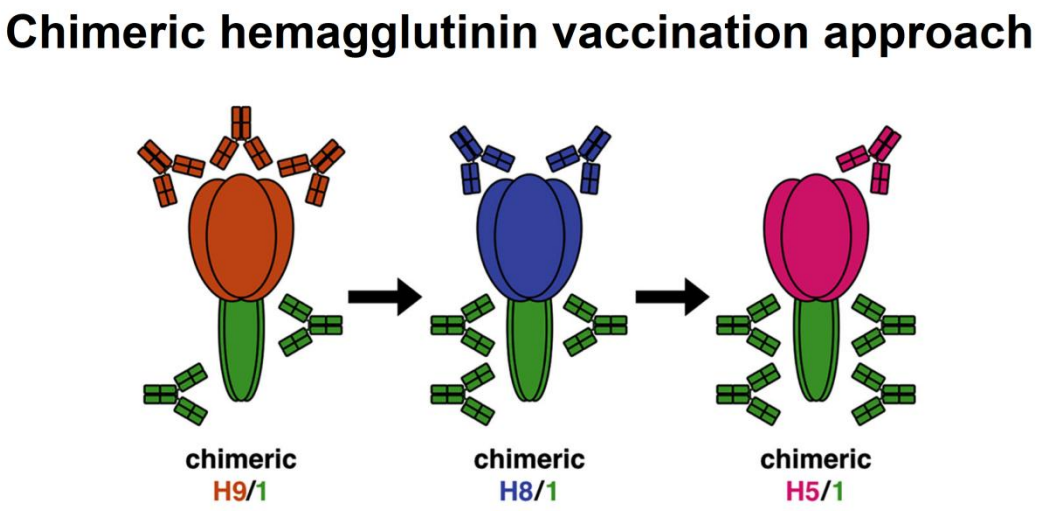

Figure 4 Illustration of a chimeric hemagglutinin vaccination approach. The chimeric HAs consist of different exotic head domains of avian influenza viruses shown in brown (H9 HA), blue (H8 HA) and pink (H5 HA). The stalk domain (green) is derived from the seasonal, human influenza A H1N1 virus. Upon serial exposure to chimeric HAs with different head domains but the same stalk domain, the antibody response gets redirected from the variable, immunodominant head domain to the conserved but immunosubdominant stalk domain. The figure was adapted from Nachbagauer *et al*., NPJ vaccines, 2017. [90]

1) **“Vaccination with a Recombinant H7 Hemagglutinin-Based Influenza Virus Vaccine Induces Broadly Reactive Antibodies in Humans.” (mSphere, 2017)** [91]

Daniel Stadlbauer, Arvind Rajabhathor, Fatima Amanat, Daniel Kaplan, Abu Masud, John J. Treanor, Ruvim Izikson, Manon M. Cox, Raffael Nachbagauer, Florian Krammer

In this study we demonstrated that individuals vaccinated with a recombinant H7 HA-based influenza virus vaccine generate broadly reactive antibodies that target all group 2 HAs (H3, H4, H7 H10, H13, H14) and to a small extent group 1 H1 HA. Importantly, we showed protection of mice in a viral challenge model after passive human serum transfer.

2) **“Cross-reactive mouse monoclonal antibodies raised against the hemagglutinin of A/Shanghai/1/2013 (H7N9) protect against novel H7 virus isolates in the mouse model.” (*Emerging Microbes & Infections, 2018)*** [92]

Daniel Stadlbauer, Fatima Amanat, Shirin Strohmeier, Raffael Nachbagauer, Florian Krammer

This study showed that H7 HA specific mAbs raised against the HA of the H7N9 virus that was first detected in humans in China in 2013 are still reactive and protective against novel, drifted H7N9 viruses that emerged in 2017. Moreover, these antibodies also show cross-reactivity towards other H7 viruses, like a feline H7N2 virus and a HPAI H7N8 virus. The applicability of these mAbs as therapeutics and prophylactics was tested in an animal challenge model.

3) **“Universal influenza virus vaccines: what can we learn from the human immune response following exposure to H7 subtype viruses?” (Frontiers of Medicine, 2017)** [89]

Daniel Stadlbauer, Raffael Nachbagauer, Philip Meade, Florian Krammer

In this review article we discussed what was known about the human humoral immune response to H7 vaccines and to infection with H7 subtype influenza viruses on a monoclonal and polyclonal level. Additionally, differences and similarities of the immune response to H7 subtype exposure or H5 subtype vaccination were reviewed.

MANUSCRIPT 1

Vaccination with a Recombinant H7 Hemagglutinin-Based Influenza Virus Vaccine Induces Broadly Reactive Antibodies in Humans.

Published in mSphere on December 13, 2017

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Daniel Stadlbauer was involved in designing and executing experiments and writing the manuscript for this study.

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RESEARCH ARTICLE
Therapeutics and Prevention

Vaccination with a Recombinant H7 Hemagglutinin-Based Influenza Virus Vaccine Induces Broadly Reactive Antibodies in Humans

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ABSTRACT Human influenza virus infections with avian subtype H7N9 viruses are a major public health concern and have encouraged the development of effective H7 prepandemic vaccines. In this study, baseline and postvaccination serum samples of individuals aged 18 years and older who received a recombinant H7 hemagglutinin vaccine with and without an oil-in-water emulsion (SE) adjuvant were analyzed using a panel of serological assays. While only a small proportion of individuals seroconverted to H7N9 as measured by the conventional hemagglutination inhibition assay, our data show strong induction of anti-H7 hemagglutinin antibodies as measured by an enzyme-linked immunosorbent assay (ELISA). In addition, cross-reactive antibodies against phylogenetically distant group 2 hemagglutinins were induced, presumably targeting the conserved stalk domain of the hemagglutinin. Further analysis confirmed an induction of stalk-specific antibodies, suggesting that epitopes outside the classical antigenic sites are targeted by this vaccine in the context of preexisting immunity to related H3 hemagglutinin. Antibodies induced by H7 vaccination also showed functional activity in antibody-dependent cell-mediated cytotoxicity reporter assays and microneutralization assays. Additionally, our data show that sera from hemagglutination inhibition seroconverters conferred protection in a passive serum transfer experiment against lethal H7N9 virus challenge in mice. Interestingly, sera from hemagglutination inhibition nonseroconverters also conferred partial protection in the lethal animal challenge model. In conclusion, while recombinant H7 vaccination fails to induce measurable levels of hemagglutination-inhibiting antibodies in most subjects, this vaccination regime induces homosubtypic and heterosubtypic cross-reactive binding antibodies that are functional and partly protective in a murine passive transfer challenge model.

IMPORTANCE Zoonotic infections with high case fatality rates caused by avian H7N9 influenza viruses have been reported since early 2013 in China. Since then, the fifth wave of the H7N9 epidemic emerged in China, resulting in higher numbers of laboratory-confirmed cases than in previous years. Recently, H7N9 has started to antigenically drift and split into two new lineages, the Pearl River Delta and Yangtze River Delta clades, which do not match stockpiled H7 vaccines well. Humans are immunologically naive to these subtypes, and an H7N9 strain that acquires the capability of efficient human-to-human transmission poses a credible pandemic threat. Other characteristics of H7N9 are raising concerns as well, like its ability to bind to receptors in the human upper respiratory tract, the recent emergence of highly

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pathogenic variants, and the ability to quickly gain resistance to neuraminidase inhibitors. Therefore, developing and testing H7N9 vaccines constitutes a priority for pandemic preparedness.

KEYWORDS H7N9, HA stalk, influenza, influenza virus vaccine

In addition to circulating human seasonal influenza virus strains, avian influenza A (H7N9) viruses emerged as a public health concern in 2013 (1). H7N9 viruses frequently cause severe lower respiratory tract infections in humans in China but have not yet gained the capability of sustained human-to-human transmission (2). During the recent 2016–2017 Northern Hemisphere winter season, the fifth wave of the H7N9 epidemic hit China, causing more cases than in previous years. Currently, over 1,500 laboratory-confirmed cases of H7N9 with a case fatality rate of almost 40% have been reported (3). During the fifth wave, a highly pathogenic variant (for poultry) of the H7N9 virus which features a polybasic cleavage site in hemagglutinin (HA) emerged (4, 5). In addition, H7N9 has split into two antigenic lineages, the Pearl River Delta (PRD) and the Yangtze River Delta (YRD) lineages, which have been shown to not match H7N9 stockpiled vaccines well when tested with ferret antisera (4). If the avian virus either adapts to humans through mutations or undergoes reassortment with seasonal influenza virus strains circulating in the human population (6, 7), H7N9 could gain pandemic potential (8). Therefore, it is important to have a good understanding of the human immune response to the H7 HA and H7 vaccines that are being developed for pandemic preparedness.

Humoral responses to influenza virus vaccine candidates are traditionally evaluated in a hemagglutination inhibition (HI) assay. Humans are immunologically naive to the H7N9 subtype and have very low baseline immunity and HI titers (9). The HI assay measures titers of strain-specific antibodies binding to the HA head domain which inhibit binding of the HA to sialylated host receptors by steric hindrance (10, 11). In human trials, a serum HI antibody titer of $\geq$1:40 was established as a correlate of protection from seasonal influenza viruses and is now used as a criterion for vaccine licensure (11, 12). However, it is unclear if this surrogate of protection is adequate for avian influenza virus strains. Additionally, antibodies that bind the highly conserved HA stalk are not detected in this assay, because they do not interfere with receptor binding. These HA stalk antibodies were previously shown to be broadly cross-reactive against multiple influenza virus strains. While cross-group HA stalk binding antibodies exist, most stalk-reactive antibodies are restricted in binding to either group 1 HAs (H1, H2, H5, H6, H8, H9, H11, H12, H13, H16, HA-like H17, and HA-like H18) or group 2 HAs (H3, H4, H7, H10, H14, and H15) (13–15). Cross-reactive stalk-based antibodies neutralize the virus by binding to the membrane-proximal stalk domain and prevent infection by inhibiting the fusion of viral and endosomal membranes or reducing viral titers by other mechanisms like Fc-mediated effector functions (antibody-dependent cell-mediated cytotoxicity [ADCC], antibody-dependent cellular phagocytosis [ADCP], or complement-dependent lysis [CDL]). These antibodies also show neutralizing activity *in vitro* (although at a lower potency than HI-active antibodies) and confer protection *in vivo* (11, 16, 17). In the present study, we analyzed the titers, breadth, functionality, and protective efficacy of antibodies induced by two doses of a prepandemic recombinant H7 HA vaccine in humans. Information about the potential to elicit broad antibody responses could aid the development of novel universal or broadly protective influenza virus vaccine candidates and guide pandemic preparedness efforts directed against emerging influenza viruses (18–20).

RESULTS

Recombinant H7 vaccination induces robust anti-H7 binding antibody titers. Healthy subjects received two doses of a recombinant monovalent full-length H7 HA vaccine intramuscularly 21 days apart. In this study, 407 subjects were enrolled. Out of those, 382 met the evaluable criteria, which were defined as two immunizations and

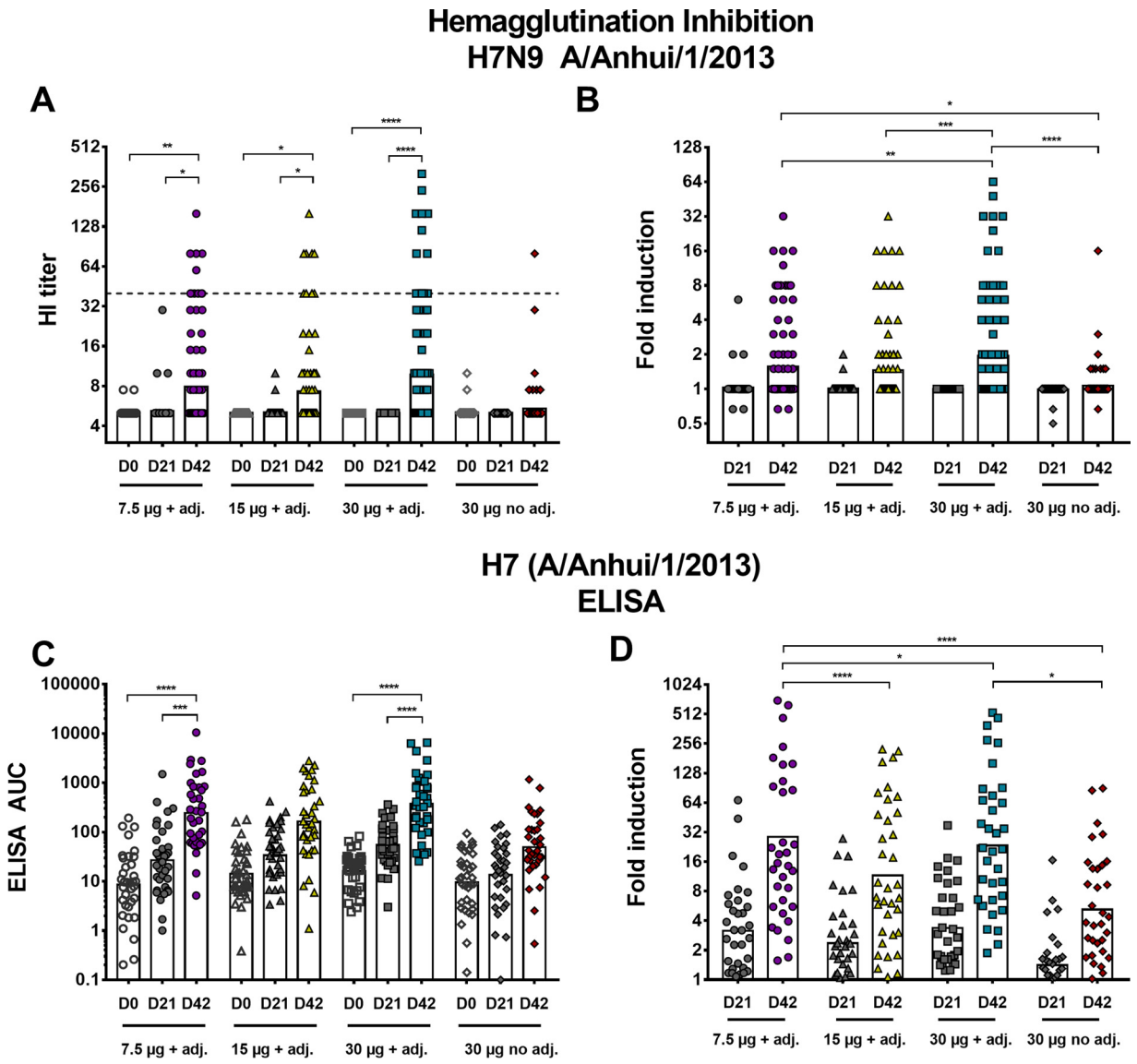

FIG 1 Human antibody response to vaccination with recombinant H7 HA as measured by HI assay (A and B) and ELISA (C and D). (A) HI titers of enrolled subjects (n = 382) at time points day 0 (D0), day 21, and day 42 postprime for the four different treatment groups. The dashed line represents an HI titer of 1:40, which was defined as seroconversion (4-fold increase in HI titer or HI titer of ≥1:40). The bars indicate the geometric mean (GM) of all data points. (B) Induction of HI titers over baseline after one vaccination (D21) and two vaccinations (D42). (C) Absolute ELISA AUC values of antibodies binding to matched HA of A/Anhui/1/2013 after vaccination with recombinant H7 HA. (D) Induction for the time points day 21 and day 42 postvaccination for the four different treatment groups. The results are presented as GM values relative to baseline. In panels A and C, time points day 0, day 21, and day 42 were compared to each other within a treatment group in a one-way ANOVA. In panels B and D, each day 21 time point was compared to every day 21 time point of all other treatment groups. The same comparison was performed for the day 42 time point. Significance is indicated as follows: no symbol, $P > 0.05$; *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$. adj., adjuvant.

serology draws at two predefined time points (days 0 and 42 postprime). Additionally, blood was drawn at day 21 postvaccination. The 407 participants were split up into four different treatment groups. One group received 30 μg of nonadjuvanted recombinant HA, and the other three groups received various amounts of recombinant HA (7.5, 15, or 30 μg) adjuvanted with a 2% stable oil-in-water emulsion (SE) (21). Only 36 (9.4%) of the 382 evaluable individuals seroconverted (≥1:40) to H7N9 as measured by the conventional hemagglutination inhibition (HI) assay (Fig. 1A and B). An even distribution of seroconverters, subjects with a rise from baseline not meeting the seroconversion definition, and subjects with no change from baseline were randomly selected for further analysis (n = 35 per treatment group) (see Fig. S1A in the supplemental material). Our data show strong induction of anti-H7 HA antibodies by enzyme-linked

immunosorbent assay (ELISA) (Fig. 1C and D). Only low induction of antibodies was observed after one vaccination for all groups (3.2-fold [95% confidence interval {CI}, 2.2 to 4.6] for 7.5 μg plus adjuvant, 2.4-fold [95% CI, 1.7 to 3.3] for 15 μg plus adjuvant, 3.7-fold [95% CI, 2.4 to 4.7] for 30 μg plus adjuvant, and 1.4-fold [95% CI, 1.1 to 1.8] for 30 μg, nonadjuvanted). For the 7.5-μg recombinant HA adjuvanted group, an induction of 28.6-fold (95% CI, 14.7 to 55.5) over baseline was measured after 2 vaccinations at day 42. For the 15-μg recombinant HA adjuvanted group, an induction of 11.5-fold was detected (95% CI, 6.5 to 20.4), and for the 30-μg recombinant HA adjuvanted group, an induction of 23.3-fold was detected (95% CI, 13.1 to 41.4). The nonadjuvanted group (30-μg recombinant HA) showed much lower induction of 5.2-fold (95% CI, 3.3 to 8.1) at day 42 postprime. This highlights the need for the administration of at least two doses of the vaccine and shows that the addition of adjuvant increases the immunogenicity, leading to higher titers of measurable binding antibodies. No clear dose dependence was observed. In fact, the induction was highest (28.6-fold) for the lowest-dose (7.5 μg plus adjuvant) recombinant HA group within the subselection of samples ($n = 35$).

Antibodies induced by vaccination with recombinant H7 HA from the A/Anhui/1/2013 H7N9 strain bind to HAs of emerging H7 viruses. Cross-reactivity of antibodies induced by recombinant HA vaccination within subtype H7 HAs was determined by performing ELISAs with HAs from viruses that emerged in 2016 and 2017 from both the Eurasian and North American H7 lineages. Testing was restricted to sera from a subselection of subjects ($n = 35$) of the high-dose (30-μg) adjuvanted treatment group. It is of interest to know if the antibodies induced by the vaccine strain of 2013 are reactive to drifted, evolving strains from both the Pearl River Delta (PRD) and Yangtze River Delta (YRD) lineages that are currently found in infected humans in China. Additionally, it was investigated if there is cross-reactivity to an H7 HA from the North American lineage highly pathogenic avian H7N8 virus as well as to the H7 of an H7N2 feline virus strain that led to an outbreak in cats (with one human zoonotic event) in an animal shelter in New York City (22–24). Our data showed that there was a 16.2-fold induction of binding to A/Hong Kong/2014/2017 (Hong Kong, PRD) HA, a 17.9-fold induction of binding to A/Hunan/02285/2017 (Hunan, YRD) HA, and a 15.2-fold induction of binding to A/Guangdong/17SF003/2016 (Guangdong, YRD, highly pathogenic isolate) HA after vaccination (Fig. 2A and B). The increase of antibodies that bound to the North American lineage H7N2 feline virus HA (6.0-fold increase at day 42 for A/feline/New York/16-040082-1/2016) and to H7 from the avian H7N8 A/turkey/Indiana/16-001403-1/2016 virus isolate (13.0-fold induction at day 42) was lower, likely due to their larger phylogenetic distance to the A/Anhui/1/2013 strain (Eurasian lineage). These data indicate persistent reactivity of induced antibodies to emerging H7 viruses of both lineages.

Recombinant H7 HA vaccination induces antibodies that cross-react to all other group 2 HAs. The antibody response to HAs from all other group 2 subtypes (H3, H4, H10, H14, and H15) and to H1 HA (A/California/4/2009 [Cal09]) was measured at three time points (days 0, 21, and 42), and geometric mean (GM) titers of all treatment groups combined are shown as a heat map (Fig. 3A). The area under the curve (AUC) values, measured by ELISA, are high for the H3 clade HAs (H3, H4, and H14) at day 0, which is likely caused by preexisting antibodies to the globally circulating seasonal H3N2 influenza A virus strains (9, 25). The participants in the vaccine trial were 18 years and older and had most likely previously been exposed to multiple H3N2 viruses (26). The AUC values for the H7 clade HAs (H7, H10, and H15) were low on day 0, and they were boosted only to levels that reach the H3 clade AUC values at baseline. The low antibody levels that were detected on day 0 for the H7 clade HAs were presumably antibodies that bind to the highly conserved HA stalk domain. Low baseline titers of H7 clade HA (H7, H10, and H15) antibodies in humans, including the absence of head-specific immunity to H7 virus strains, have previously been reported (9, 27, 28). For the group 1 hemagglutinin H1 (Cal09), the AUC values were higher on day 0 than H3 HA baseline AUC values, which suggests a strong preexposure to H1N1 and/or other group

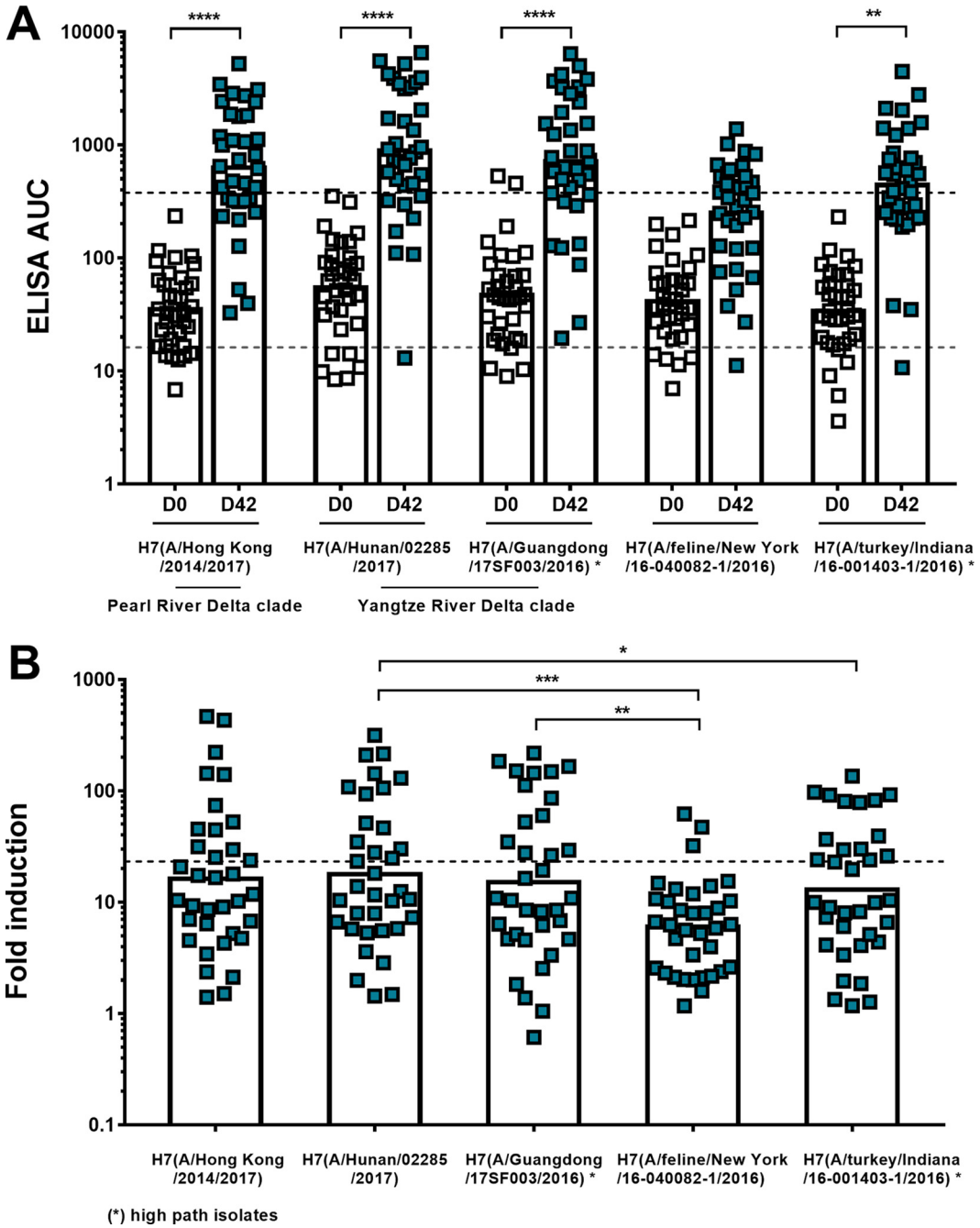

FIG 2 Cross-reactive antibody response to HAs from emerging Eurasian and American lineage H7 viruses after H7 A/Anhui/1/2013 vaccination as measured by ELISA. (A) Serum samples from a subselection of samples (high-dose 30-μg recombinant HA adjuvanted group) were tested for binding to H7 HAs of different H7NX virus isolates (H7N9 A/Hunan/02285/2017, H7N9 A/Hong Kong/2014/2017, H7N9 A/Guangdong/17SF003/2016, H7N2 A/feline/New York/16-040082-1/2016, and H7N8 A/turkey/Indiana/16-001403-1/2016). Absolute ELISA area under the curve (AUC) values were determined. Data for baseline (D0, white) and postvaccination (D42, red) serum samples are shown. The dashed lines represent the GM titer of serum antibodies binding to A/Anhui/1/2013 at day 0 (gray line) and day 42 (black line) as shown in Fig. 1C. (B) Fold induction of cross-reactive H7 antibodies based on ELISA AUC values postvaccination (D42). The dashed line represents the induction based on the ELISA AUC values of serum antibodies binding to A/Anhui/1/2013 as shown in Fig. 1D. Time points day 0 and day 42 were compared within each treatment group in a one-way ANOVA. Significance is indicated as follows: no symbol, $P > 0.05$; *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$.

1 HA viruses (9). The fold induction over baseline on day 42, based on the ELISA AUC values, was highest (14.2-fold) for H7 HA compared to the other HAs (Fig. 3B). The antibodies reactive to the two other H7 clade HAs—H10 and H15—increased by 3.5-fold and 5.0-fold, respectively. The induction of antibodies binding to the H3 clade

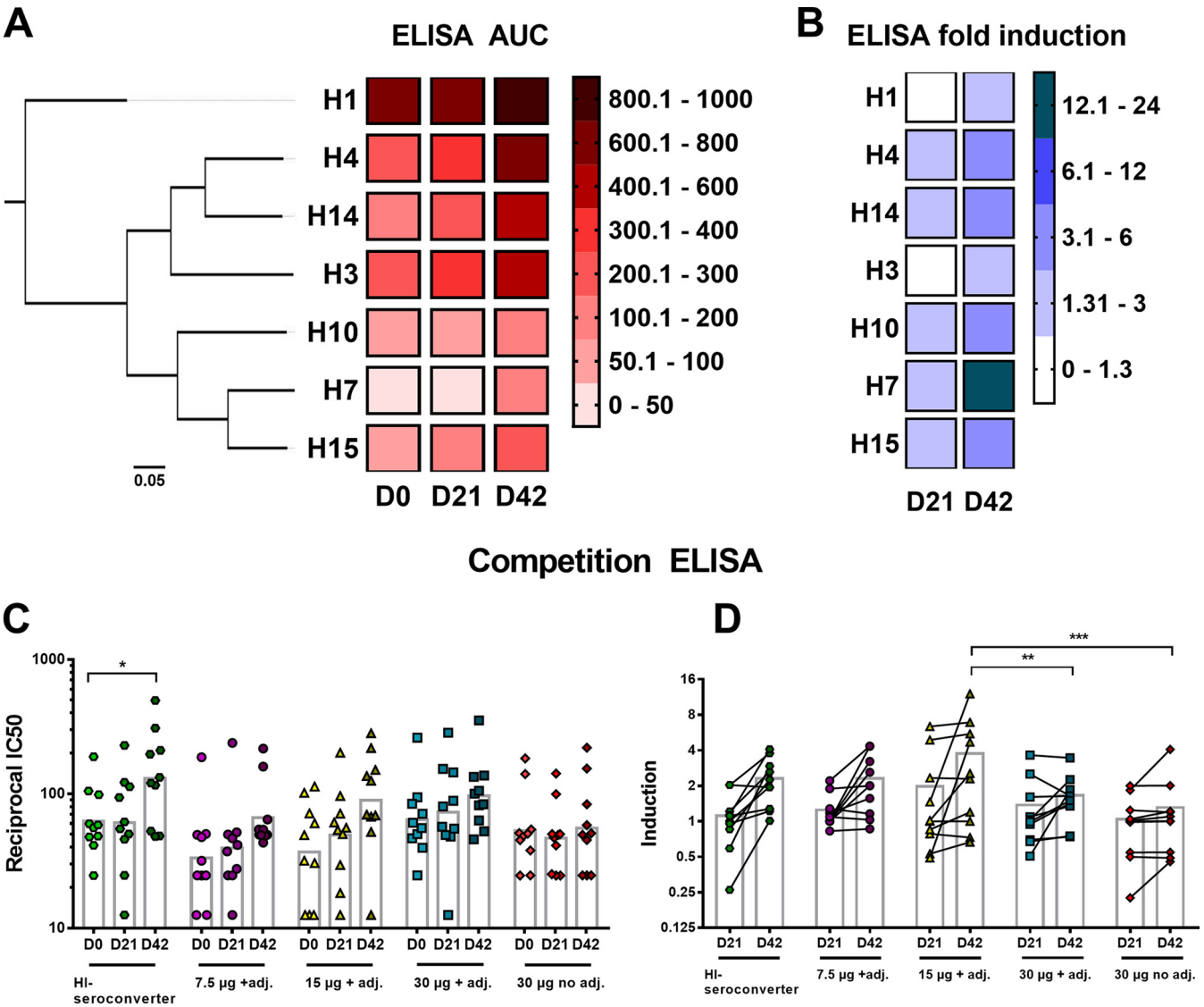

FIG 3 Cross-group reactivity. (A) A phylogenetic tree of group 2 HAs is depicted on the left. The scale bar at the bottom shows 5% difference in amino acid identity. Heat map showing the ELISA area under the curve values for H1, H4, H14, H3, H10, H7, and H15 for time points day 0 (D0), day 21 (D21), and day 42 (D42) postvaccination. The color key (AUC values of 0 to 1,000) is shown on the right. For analysis, all treatment groups ($n = 35$ per group) were combined. (B) Heat map representing the fold induction based on absolute ELISA values over baseline at time points day 21 (D21) and day 42 (D42) postvaccination for all group 2 HAs and group 1 HA H1 (Cal09). The color key is shown (fold induction of 0 to 24) on the right. The four different treatment groups were combined for analysis ($n = 35$ per group). (C) Reciprocal IC_{50} values for the five different groups as measured in an H3/stalk mAb competition ELISA are shown for time points day 0, day 21, and day 42. The bars represent the geometric mean values. Time points day 0, day 21, and day 42 were compared to each other within a treatment group in a one-way ANOVA. (D) Induction of reciprocal IC_{50} values for day 21 and day 42 serum samples over baseline (day 0). The bars show the GM values. Statistical significance was analyzed for each day 21 time point compared to every other day 21 time point. The same procedure was applied for time point day 42. Significance is indicated as follows: no symbol, $P > 0.05$; *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$. adj., adjuvant.

HAs was lower (3.1-fold for H4, 2.9-fold for H14, and 1.9-fold for H3), most likely because they are phylogenetically more distant from H7 and because baseline antibody levels were higher. For H1, a fold induction of 1.3 over baseline on day 42 was detected, possibly mediated by the induction of cross-group-reactive stalk-specific antibodies (25, 29, 30).

To confirm that the observed cross-reactive responses were actually mediated by an increase of group 2 stalk-specific antibodies, competition ELISAs for H3 with the well-characterized stalk binding mouse monoclonal antibody (MAb) 9H10 (31) were performed (Fig. 3C and D). Sera from 10 individuals per treatment group and sera from 10 subjects who seroconverted by HI (see below) were randomly selected. Preincubation of H3 with human serum resulted in a decrease of binding of MAb 9H10 (see Fig. S2

in the supplemental material) to H3. 9H10-competing serum antibody levels were indirectly measured and are depicted as reciprocal half-maximal inhibitory concentration (IC_{50}). The increase of geometric mean (GM) IC_{50}s over baseline (Fig. 3D) after one vaccination at day 21 was 0.98-fold for the HI seroconverter group, 1.19-fold for 7.5 μg plus adjuvant, 1.34-fold for 15 μg plus adjuvant, 1.14-fold for 30 μg plus adjuvant, and 0.88-fold for the 30-μg unadjuvanted group. After two vaccinations (day 42), a higher increase of GM IC_{50}s could be observed (2.09-fold for the HI seroconverters, 2.00-fold for the 7.5-μg-plus-adjuvant group, 2.43-fold for the 15-μg-plus-adjuvant group, 1.51-fold for the 30-μg-plus-adjuvant group, and 1.05-fold for the 30-μg unadjuvanted group). The competing serum antibodies measured in this assay are not representative of the whole repertoire of stalk-specific serum antibodies. Only antibodies with the same epitope as MAb 9H10, or epitopes close to the footprint of 9H10, are being detected.

Antibodies induced by H7 vaccination are functional *in vitro* and protective *in vivo*. To test the functional activity of antibodies induced by H7 vaccination, ADCC reporter and microneutralization assays were performed. Protectivity *in vivo* was assessed in a murine passive transfer challenge model. For the ADCC reporter assay, microneutralization, and passive serum transfer challenge experiments, HI seroconverters (34 subjects) were removed from all four initial groups and defined as an individual fifth group (Fig. S1). The seroconverters were excluded to be able to tease out differences in antibody responses to H7 HA between seroconverters and nonconverters in different assays. Sera from 10 individuals per group, as mentioned above, were tested in an ADCC reporter assay. We observed a slight increase in ADCC reporter activity after recombinant H7 vaccination for all groups (Fig. 4A). Again, the 7.5-μg-plus-adjuvant group had the highest induction of activity (2.8-fold [95% CI, 1.0 to 8.0]), as measured in the ADCC bioreporter assay (Fig. 4B), followed by the newly defined HI seroconverter group (1.9-fold [95% CI, 1.0 to 3.8]). The increase in activity for the other groups, 15 μg plus adjuvant, 30 μg plus adjuvant, and 30 μg unadjuvanted, was lower with fold inductions of 1.4 (95% CI, 1.0 to 2.0), 1.8 (95% CI, 1.0 to 3.3), and 1.2 (95% CI, 0.8 to 1.8), respectively. It must be noted that the spread of values was high in all groups. Therefore, it is not possible to draw definite conclusions.

Subsequently, microneutralization assays were performed with pre- (day 0) and postvaccination (day 42) sera of the HI seroconverter, 30-μg-plus-adjuvant, and 30-μg unadjuvanted groups using the A/Shanghai/1/2013 strain, which is closely related to the vaccine strain and was used for technical reasons (Fig. 4C). The sera from the HI seroconverter group had the highest increase (16.7-fold) in microneutralization titers, whereas the titers for the high-dose adjuvanted group increased 2.1-fold and those for the unadjuvanted group without seroconverters increased 1.6-fold (Fig. 4D). These results indicate that neutralization is mostly, but not exclusively, mediated by HI-active antibodies that bind to the receptor binding site and thereby prevent viral attachment to host cell receptors (10, 11).

Next, the protective efficacy of the vaccine-induced antibodies was determined *in vivo* in a murine passive transfer challenge model. Day 0 and day 42 serum pools were generated for each of the selected subsets of samples and transferred into mice via intraperitoneal injection. After 2 h, the mice were infected intranasally with an H7N9 (A/Shanghai/1/2013) challenge virus (Fig. 5A). This virus is an A/Puerto Rico/8/34 (PR8)-based reassortant virus that consists of the six internal segments of PR8 and the HA and NA of the A/Shanghai/1/2013 isolate. The HA of this virus shares 98% amino acid sequence identity with the HA of A/Anhui/1/2013 and was selected because it induces morbidity and mortality in the mouse model (32). Sera from the HI seroconverter postvaccination group (day 42) conferred full protection from lethal H7N9 challenge (Fig. 5B), whereas mice that received the postvaccination sera from the high-dose adjuvanted group (without seroconverters) were partially protected. The mice showed morbidity, but 6 out of 10 mice recovered (Fig. 5C). All mice that received prevaccination sera (day 0) and mice that received the high-dose nonadjuvanted postvaccination (day 42) sera succumbed to infection at day 7 or 8 postinfection, except

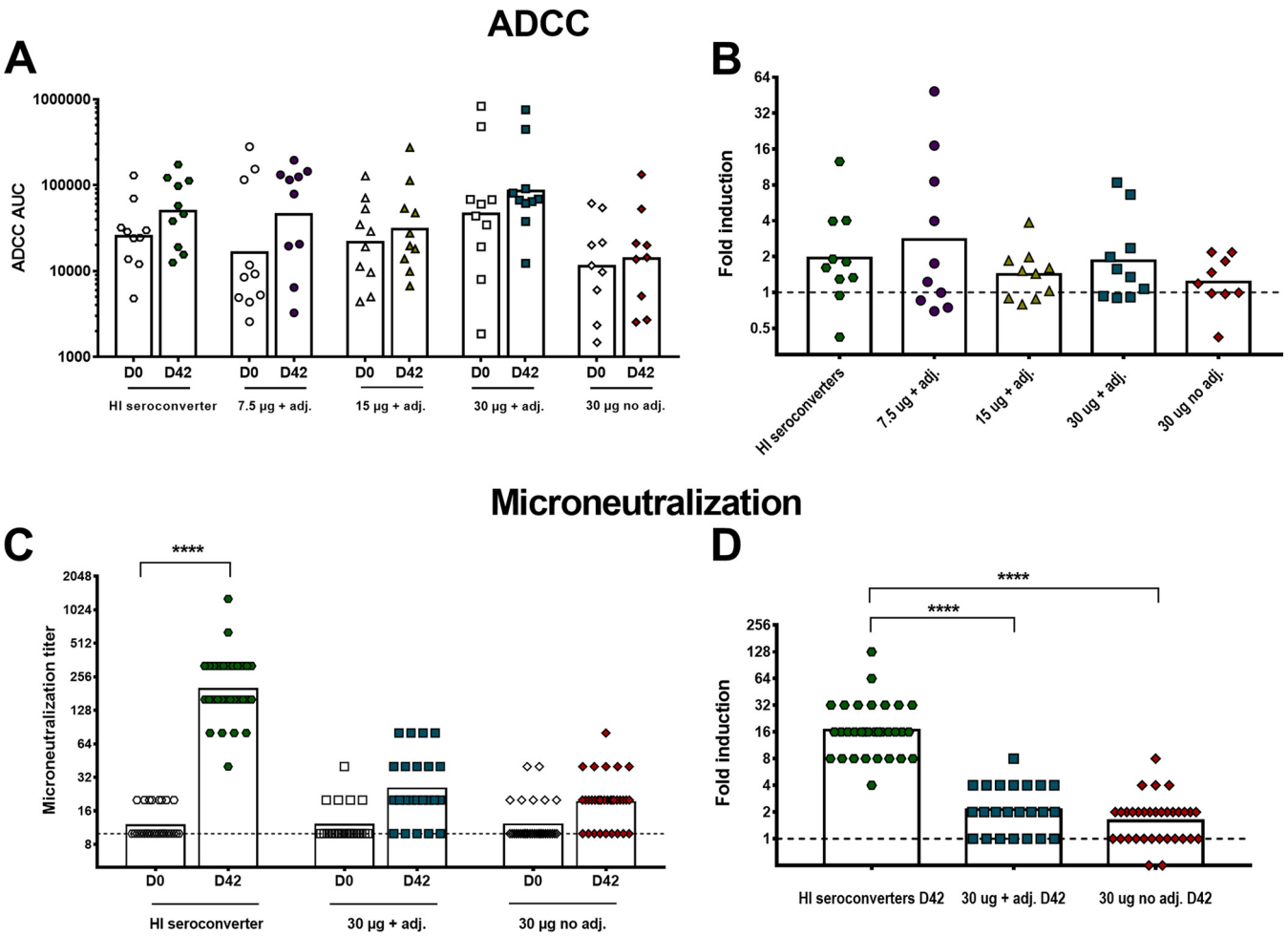

FIG 4 *In vitro* functionality of human serum antibodies induced by H7 vaccination. (A) ADCC AUC values measured for the four different treatment groups and the HI seroconverter group. The bars show the geometric means of the AUC values. (B) GMs of induction over baseline at day 42 after two vaccinations based on AUC values are represented for the HI seroconverter group (1.9-fold, green hexagons), 7.5-μg-plus-adjuvant group (2.8-fold, purple circles), 15-μg-plus-adjuvant (adj.) group (1.4-fold, yellow triangles), 30-μg-plus-adjuvant group (1.8-fold, blue squares), and 30-μg no-adjuvant group (1.2-fold, red diamonds). The dashed line represents a 1-fold increase in ADCC activity. (C) Individual titers of neutralizing serum antibodies in a microneutralization assay using the H7N9 A/Shanghai/1/2013 strain. The HI seroconverter group (green hexagons), high-dose (30-μg) adjuvanted group (blue squares), and 30-μg nonadjuvanted group (red diamonds) were selected for analysis. The dashed line represents the limit of detection (titer of 1:10), and the bars show the geometric means. (D) Increase in microneutralization (MN) titers over baseline at day 42. The dashed line represents a 1-fold increase in microneutralization titers. In panels A and C, time points day 0 and day 42 within one group were analyzed for significance values in a one-way ANOVA. In panels B and D, each column was compared to every other column. Significance is indicated as follows: no symbol, $P > 0.05$; *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$.

for one survivor in the 30-μg nonadjuvant postvaccination group and one in the 30-μg adjuvanted prevaccination group (Fig. 5C). These data indicate that the sera from the HI seroconverters, which also had the highest overall anti-H7 antibody levels (Fig. 5D) measured by ELISA, contain functionally active antibodies that are protective in an animal challenge model. However, sera from HI nonseroconverters also conferred partial protection, likely mediated by mechanisms based on antibody effector functions like ADCC, ADCP, and/or complement-dependent cytotoxicity in addition to HI-active antibodies below the limit of detection in the HI assay. Additionally, neutralization might be mediated by antibodies binding to the membrane-proximal HA stalk domain (10, 11, 17). The ELISA data corresponding to the groups in the challenge experiment showed that postvaccination geometric mean ELISA AUC values (against the matched H7 HA antigen) of approximately 1,000 (HI seroconverters) conferred full protection, whereas values of approximately 100 (high-dose adjuvant group) conferred partial protection and AUC values below 100 (high-dose nonadjuvanted) conferred no protection *in vivo* (Fig. 5D).

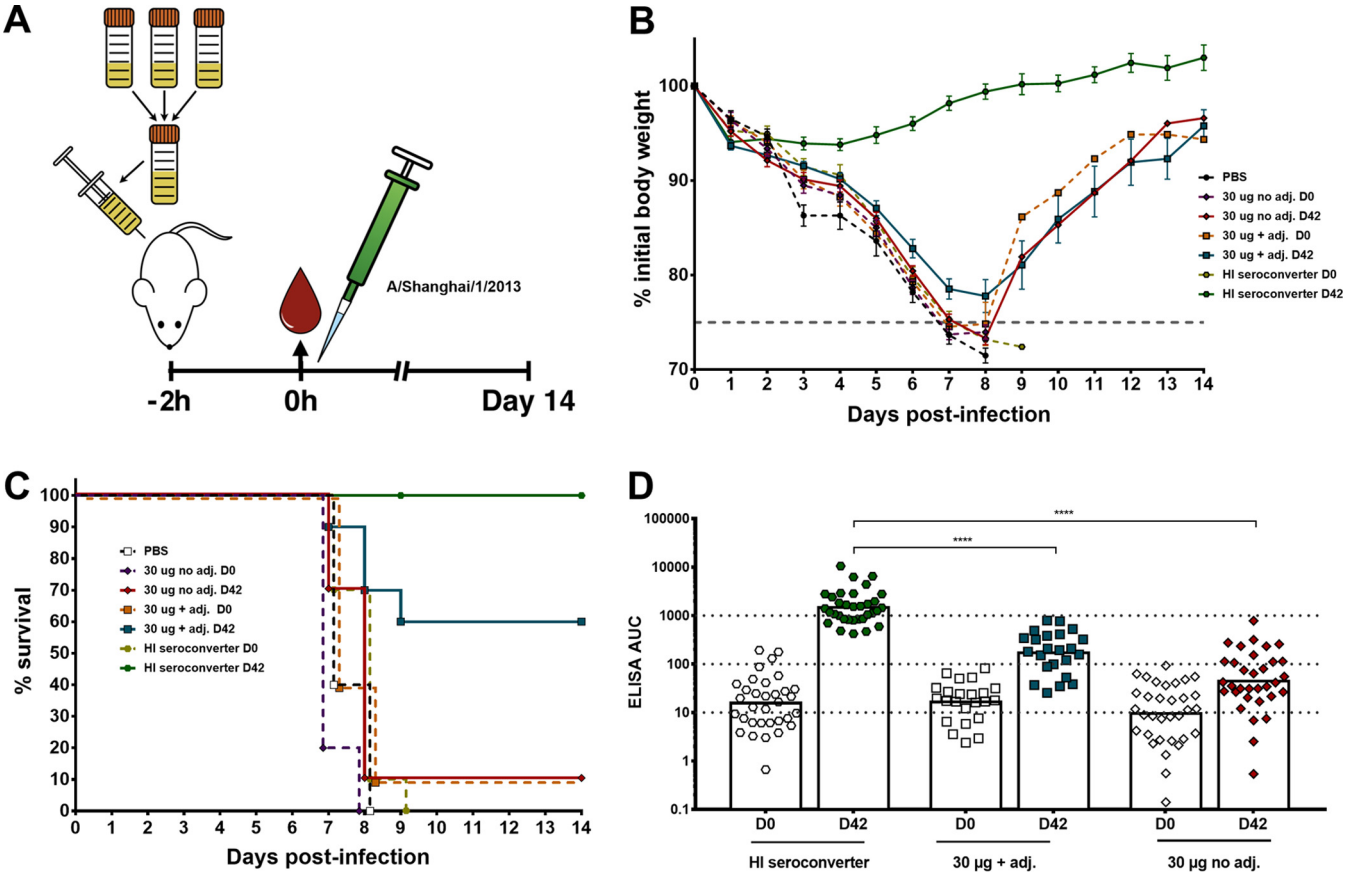

FIG 5 *In vivo* protectivity of human serum antibodies in a lethal mouse challenge model. (A) Graphic representation of the serum transfer and lethal challenge animal experiment. Sera from all individuals of a selected group were pooled and given to female BALB/c mice ($n = 10$ per group). After 2 h, the mice were infected intranasally with 5 50% lethal doses (2×10^4 PFU) of H7N9 virus (A/Shanghai/1/13, PR8-based 6:2 reassortant). Weight loss and survival were monitored for 14 days. (B) The weight loss curve of the mouse challenge experiment is shown. The dashed colored lines represent the prevaccination sera. The dashed gray line represents 75% of initial body weight, which was used as the humane endpoint. (C) Survival graph showing percent survival in the different groups used in the animal experiment. The dashed lines represent prevaccination sera. (D) Anti-H7 antibody levels of the groups used in the challenge experiment are shown as ELISA AUC values. The dotted lines at titers of 10, 100, and 1,000 indicate differences of 1 log. Time point day 0 of each group was tested against every other time point day 0 in a one-way ANOVA with a Sidak posttest for multiple comparisons. The same analysis was applied for time point day 42. Significance is indicated as follows: no symbol, $P > 0.05$; *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$. adj., adjuvant.

DISCUSSION

Avian H7N9 viruses are a risk to public health and pose a potential pandemic threat. Besides the large number of human cases, several attributes of H7N9 influenza viruses are raising concerns, including their ability to bind to human receptor orthologs (33), to quickly gain resistance to neuraminidase inhibitors under treatment without substantial loss of fitness (34), and to often asymptomatically spread among poultry (22). They also have acquired mutations that are facilitating growth in human tissues (35), and there is evidence of limited human-to-human transmission within family clusters through direct contact (36) and for transmission via aerosol exposure in a ferret model (37). Therefore, it is important to develop and test H7 vaccines that can be used as a preventive measure to rapidly respond to a potential pandemic. It has been previously shown that H7 vaccines often fail to induce seroconversion (HI titer of ≥1:40 or 4-fold increase in HI titers postvaccination) (28, 38, 39). However, it remains unclear what level of HI titer is associated with protection against emerging influenza virus strains, including the H7N9 subtype. A serum HI antibody titer of ≥1:40 was defined as a correlate of protection from seasonal influenza viruses, but whether this also applies to avian zoonotic virus strains is unclear (10). Additionally, the H7 HA seems to have a less immunodominant head domain, leading to the preferential induction of antibodies directed against nonclassical antigenic sites of the HA and consequently a lower HI titer

(10, 28, 29, 38). Humans have negligible preexisting head-specific HI-based immunity to nonseasonal influenza virus strains. Upon exposure to such virus strains, responses to the conserved HA stalk domain, which hosts epitopes shared with seasonal influenza virus strains, are preferably elicited (18, 19, 40, 41).

Recently, it was shown that H7N9 vaccination induces both group-specific and cross-group-reactive HA stalk binding B cells (29). In the present study, we detected a high induction of anti-H7 antibodies in an ELISA in individuals without HI seroconversion. A portion of these antibodies were broadly cross-reactive, and we detected binding to all other group 2 HAs (H3, H4, H14, H10, and H15). These cross-reactive responses were likely mediated by stalk-specific cross-reactive group 2 antibodies (26, 42, 43) as confirmed in a stalk-based competition ELISA with titers that increased noticeably after vaccination. The induction of antistalk antibodies was more apparent in the adjuvanted groups. It is unlikely that the use of adjuvant redirected the antibody response to the stalk domain. However, the stronger immune response induced by the adjuvanted vaccine might have made the presence of these antibodies more apparent and easier to detect. Interestingly, insect cell-produced recombinant HA vaccines have in the past been shown to induce broader protection than classical, egg-derived vaccines (44). It has been hypothesized that this might be caused by the smaller glycan size of insect cell-derived HA (45), which might allow a higher accessibility of stalk epitopes that are shielded by conserved glycans (46, 47). However, due to the lack of an egg-derived comparator vaccine, this hypothesis could not be tested in the current study. The vaccinees also exhibited heterologous cross-reactivity within the H7 HA subtype. Tested individuals showed high antibody levels measured by ELISA to HAs of three novel H7N9 virus isolates from China (Eurasian lineage), of one North American H7N2 cat virus isolate, and of one North American avian high-pathogenicity H7N8 isolate. This demonstrates the potential of a vaccine against the A/Anhui/1/2013 strain to elicit cross-reactive antibodies against novel, evolving H7N9 viruses and zoonotic H7NX viruses.

Furthermore, the *in vitro* functionality and *in vivo* protective efficacy of these antibodies were demonstrated. Sera from the HI seroconverter group showed the highest levels of antibodies measured by ELISA and microneutralization and conferred full protection in the animal H7N9 challenge model. However, we also found that sera from HI nonseroconverters conferred partial protection from mortality in a serum transfer mouse model. In this subset, protection might have been mediated by stalk-reactive antibodies and Fc-mediated effector functions like ADCC, ADCP, and CDL even though only low levels of ADCC activity were detected in a reporter assay (17, 48). The protective efficacy mediated by human nonneutralizing antibodies might be lower in mice than in humans even though the binding affinities of human IgG to murine Fc receptors and human IgG to human Fc receptors are somewhat similar (49). As demonstrated, a big part of the immune response to vaccination can be overlooked when only HI titers are taken into account. This further emphasizes the need to develop additional correlates of protection for influenza virus infections. Natural infection with H7N9 viruses has been shown to elicit strong humoral responses, including HI titers in humans (25, 30), but so far it has not been possible to mimic these responses by vaccination schemes with either live attenuated influenza vaccines (LAIV), inactivated influenza vaccines (IIV), or recombinant H7 HA formulations (27, 28, 38, 39, 50–53). Other vaccine candidates, e.g., those based on virus-like particles (VLPs) (54), could not elicit robust immune responses to H7 either, as shown in past clinical trials in humans.

Additionally, it has become clear that, in the absence of an H7-primed immune system, at least two vaccinations with any H7N9 vaccine are necessary (53) and that vaccine formulations need to be supplemented with strong adjuvants to boost immune responses to high levels (55). This is reminiscent of H5 vaccines, which also show lower immunogenicity in humans than seasonal influenza virus vaccines (56, 57). However, the immunogenicity of H5-based vaccines seems to be higher than that of H7-based vaccines. Further development of new vaccination strategies, like DNA or LAIV prime followed by boosting with monovalent inactivated virus vaccines (52, 53, 58, 59) or

strategies based on mRNA administration (60), and enhanced understanding of the immune response to emerging viruses are needed to tackle pandemic threats. Moreover, the addition of recombinant influenza virus neuraminidase to recombinant HA-based but also conventional inactivated vaccines could add another independent path for protection against influenza virus infections (61).

MATERIALS AND METHODS

Vaccine. The vaccine consists of monovalent pandemic H7N9 recombinant HA influenza virus vaccine derived from A/Anhui/1/2013 manufactured in the baculovirus expression vector system (62). The unadjuvanted formulation consists of recombinant HA alone, while the adjuvanted formulations were mixed at a 1:1 ratio with a stable oil-in-water emulsion (SE). Recombinant HA content for formulation was determined by the single radial immunodiffusion assay. The antigen was stored in sodium phosphate buffer with 0.005% Tween 20, pH 7.0, and 0.01% thimerosal. A final 0.36-ml dose of adjuvanted recombinant HA supplemented with SE or unadjuvanted recombinant HA was administered intramuscularly.

SE (Infectious Disease Research Institute, Seattle, WA) is an oil-in-water formulation that appears as a milky-white emulsion. The emulsion contains squalene (oil), glycerol, phosphatidylcholine, surfactant (poloxamer), and buffer (ammonium phosphate). Squalene is sourced from sharks; the other components are synthesized chemically.

Cells, viruses, and proteins. BTI-TN5B1-4 (*Trichoplusia ni*) cells were maintained in serum-free SFX medium (HyClone) supplemented with antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin; Gibco). Madin-Darby canine kidney (MDCK) cells were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco). DMEM was supplemented with a penicillin-streptomycin antibiotic mix (100 U/ml penicillin, 100 μg/ml streptomycin; Gibco) and fetal bovine serum (FBS, 10%; HyClone). Single-use aliquots of ADCC bioeffector FcγRIIIa cells (Promega) were thawed before usage. The H7N9 viruses were grown in 8- to 10-day-old embryonated chicken eggs (Charles River Laboratories) at 37°C for 48 h. The viral titers were determined by plaque assay using MDCK cells as previously described (41). The viruses consist of the HA and NA segments of the original virus isolates, A/Shanghai/1/2013 and A/Anhui/1/2013, respectively, combined with the backbone of the A/Puerto Rico/8/1934 (PR8) virus isolate. The recombinant proteins, including H1 from A/California/04/2009 virus; H3 from A/Hong Kong/4801/2014 virus; H4 from A/duck/Czech/1956 virus; H7 from A/Anhui/1/2013, A/Hunan/02285/2017, A/Guangdong/17SF003/2016, A/Hong Kong/2014/2017, A/feline/New York/16-040082-1/2016, and A/turkey/Indiana/16-001403-1/2016 virus; H10 from A/Jiangxi-Donghu/346/2013 virus; H14 from A/mallard/Gurjev/263/1982 virus; and H15 from A/shearwater/West Australia/2576/1979 virus, were produced in the baculovirus expression system as described before (63, 64). For the highly pathogenic avian A/Guangdong/17SF003/2016 and A/turkey/Indiana/16-001403-1/2016 virus isolates, the polybasic cleavage site was removed to increase recombinant protein stability, resulting in sequences with regular low-pathogenic avian influenza (LPAI) H7N9 cleavage sites.

Human serum samples. The tested human serum samples were obtained during a phase I/II double-blind, adaptive-design clinical trial to evaluate the immunogenicity and safety of Panblok, conducted with Protein Sciences Corporation's recombinant pandemic H7 HA vaccine (ClinicalTrials.gov identifier NCT02464163). Informed consent was obtained from all 407 enrolled subjects, who were then randomized equally into four different treatment groups, receiving 7.5, 15, or 30 μg recombinant HA adjuvanted with 2.0% SE or 30 μg unadjuvanted recombinant HA twice intramuscularly. The participants were healthy adults aged 18 years or older. Serum samples ($n = 35$ per group) before vaccination (day 0) and after one (day 21) and two (day 42) vaccinations were provided as deidentified samples for analysis. The subselected samples contained an even distribution of seroconverters, subjects with a rise from baseline not meeting the seroconversion definition, and subjects with no change from baseline. Because the study was limited in the number of seroconverters and subjects with a rise from baseline, the samples chosen included almost all seroconverters and subjects with a rise from baseline. Subjects with no rise from baseline were chosen randomly. For the microneutralization assay, ADCC assay, and passive serum transfer challenge experiments, HI seroconverters were excluded from all four groups and defined as an individual fifth group (baseline and day 42). Seroconverters are defined as subjects with either a prevaccination HI titer of $<$1:10 and a postvaccination HI titer of $>$1:40 or a prevaccination titer of $>$1:10 and a minimum 4-fold rise in postvaccination HI antibody titer (as defined by the FDA). Subjects with HI titers below the limit of detection at baseline need HI titers of at least 1:40 postvaccination to be considered seroconverters.

Hemagglutination inhibition assay. HI antibody testing was carried out by a central laboratory (Southern Research Institute [SRI]) using a qualified assay that employed a whole-virus antigen. The influenza virus A/Anhui/1/2013 isolate was obtained from the Centers for Disease Control and Prevention (CDC) and amplified in eggs at SRI under appropriate biocontainment conditions. Serum samples were treated initially with receptor-destroying enzyme (RDE; Denka Seiken, Tokyo, Japan) to remove nonspecific inhibitors of hemagglutination. Sera were tested at an initial dilution of 1:10 (lower limit of detection [LOD] of the assay), with subsequent 2-fold serial dilutions (1:20, 1:40, 1:80, 1:160, etc.). The assays were performed using 1.0% equine red blood cells (RBCs; Lampire Biologicals) diluted in phosphate-buffered saline (PBS). Titers of 1:5 were assigned to HI-negative subjects to facilitate data analysis and data representation.

ELISA. Microtiter plates (96-well plates; Thermo Fisher) were coated with 50 μl of recombinant HA diluted to a concentration of 2 μg/ml in coating buffer (SeraCare) overnight at 4°C. The next day, the

plates were blocked with 220 μl of blocking solution consisting of phosphate-buffered saline (PBS; pH 7.4; Gibco) supplemented with 0.1% Tween 20 (PBS-T), 3% goat serum (Life Technologies), and 0.5% milk powder (American Bio) for at least 1 h at room temperature. Human serum samples were diluted to a starting concentration of 1:100, serially diluted 1:2 in blocking solution, and incubated at room temperature for 2 h. The plates were washed three times with PBS-T, and 50 μl of secondary antibody, anti-human IgG (Fab specific) that was conjugated with horseradish peroxidase (HRP), produced in goat (Sigma catalog no. A0293), and diluted 1:3,000 in blocking solution, was added to each well. After 1 h, plates were washed four times with PBS-T. The plates were developed with SigmaFast *o*-phenylenediamine dihydrochloride (OPD; Sigma) for 10 min, and the reaction was stopped with 3 M HCl (Thermo Fisher). The plates were read at 490 nm with a microplate reader (BioTek). The data were analyzed in Microsoft Excel and GraphPad Prism 7, and the area under the curve (AUC) values were determined. The cutoff value was defined as the average of the values of blank wells plus 3 times the standard deviation of the blank wells.

Competition ELISA. Microtiter plates (96-well plates; Thermo Fisher) were coated with 50 μl of recombinant H3 A/Hong Kong/4801/2014 protein at a concentration of 2 μg/ml in coating buffer (KPL) overnight at 4°C. The following day, the plates were washed three times and blocked with 220 μl PBS-T per well for 1 h at room temperature. Human serum samples were diluted to a starting concentration of 1:25, serially diluted 1:2 in blocking solution, and incubated for 2 h at room temperature. The plates were washed three times with PBS-T, and 100 μl of competing anti-group 2 stalk biotinylated mouse MAb 9H10 (31) diluted to a concentration of 0.20 μg/ml in blocking solution was added to all wells. After 1 h, the plates were washed with PBS-T, and 50 μl of streptavidin labeled with HRP (Thermo Fisher catalog no. 21130) diluted 1:3,000 in blocking solution was added to all wells and incubated for 1 h at room temperature. The plates were washed four times with PBS-T and developed with OPD for 10 min, and the reaction was stopped with 3 M HCl (Thermo Fisher). The plates were read at a wavelength of 490 nm with a microplate reader (BioTek), and the data were analyzed in GraphPad Prism 7 and Microsoft Excel. The cutoff value was defined as the average of the values of blank wells plus 3 times the standard deviation of the blank wells. Percent competition was calculated based on the average signal of the mouse MAb 9H10-only wells on each plate.

Microneutralization assay. Serum samples were treated with receptor-destroying enzyme (RDE; Denka Seiken) for 18 h at 37°C. To stop RDE treatment, sodium citrate (2.5%) was added and serum was incubated at 56°C for 1 h. The inactivated serum samples (dilution of 1:10) were serially diluted 2-fold in UltraMDCK medium (Lonza), supplemented with tosyl phenylalanyl chloromethyl ketone (TPCK)-treated trypsin (infection medium; Sigma) at a concentration of 1 μg/ml, in 96-well cell culture plates (Sigma). The A/Shanghai/1/2013 H7N9 virus was diluted to a concentration of 100 50% cell culture infectious doses ($TCID_{50}$) in infection medium. Sixty microliters of serially diluted serum was incubated with 60 μl of virus dilution (1,250 PFU/60 μl) for 1 h at room temperature on a shaker. MDCK cells were washed once with 220 μl of PBS, and 100 μl of the virus-serum mixture was added to MDCK cells. The cells were incubated for 48 h at 33°C. The readout was performed by the means of a hemagglutination assay. In brief, chicken red blood cells (RBCs; Lampire) were washed once with PBS and diluted to a concentration of 0.5% RBCs in PBS, and 50 μl of RBCs was added to 50 μl of cell supernatant in V-bottom plates (Corning). The plates were kept at 4°C for 30 to 45 min and scanned, and the results were analyzed in Microsoft Excel and GraphPad Prism 7.

ADCC reporter assay. MDCK cells (100 μl) at a concentration of 2×10^5 cells/ml were seeded in white polystyrene 96-well plates (Costar Corning). The next day, the cells were washed once with PBS, and 100 μl of H7N9 A/Anhui/1/2013 virus diluted to a concentration of 2.8×10^5 PFU/100 μl (multiplicity of infection [MOI] of about 1) in UltraMDCK medium (Lonza) was added to each well and incubated for 24 h at 37°C. In 96-well cell culture plates, sera (baseline and day 42 sera of 10 randomly selected individuals) were serially diluted 2-fold (1:10 starting concentration) in Roswell Park Memorial Institute (RPMI) 1640 medium (Life Technologies), and ADCC bioeffector FcγRIIIa cells (Promega) were thawed. The MDCK cells were washed once with 220 μl PBS, and 25 μl of RPMI 1640 medium, 25 μl of bioeffector FcγRIIIa cells (6.25×10^4 cells/25 μl), and 25 μl of serially diluted sera were added. After incubation for 6 h at 37°C, 75 μl of Bio-Glo luciferase (Promega) was added to each well. The cells were incubated for 10 min in the dark before measuring the luciferase-induced luminescence with a microplate reader (BioTek). The results were analyzed in GraphPad Prism 7, and the AUC values were determined. The cutoff was defined as the average of the values of the blank wells plus 5 times the standard deviation of the blank wells.

Passive transfer challenge experiments in mice. Pre- (baseline) and postvaccination (day 42) serum samples of the different treatment groups were pooled separately, and 150 μl of the serum per pool was administered intraperitoneally to 6- to 8-week-old female BALB/c mice (10 mice per group). After 2 h, the mice were anesthetized with a ketamine-xylazine-water mixture (0.15 mg ketamine/kg of body weight and 0.03 mg/kg xylazine; 100 μl intraperitoneally) and challenged with 2×10^4 PFU of H7N9 A/Shanghai/1/2013 virus (PR8 reassortant; corresponds to 5 50% murine lethal doses) in 50 μl PBS intranasally. All mice were bled to verify successful serum transfer by ELISA as previously described (65). Weight was monitored daily for 14 days, and a weight loss of 25% of initial weight was used as the humane endpoint. All procedures were performed in accordance with the Icahn School of Medicine at Mount Sinai Institutional Animal Care and Use Committee (IACUC) guidelines.

Ethics statement. The original clinical study was approved by the Western Institutional Review Board (Seattle, WA), and the protocol was registered at ClinicalTrials.gov (NCT02464163), carried out in accordance with the standards of the International Conference on Harmonization-Good Clinical Practices

(ICH-GCP), and followed the ethical principles established in the Declaration of Helsinki. All subjects provided written informed consent prior to enrollment.

Statistical analysis. Statistical analysis was performed in GraphPad Prism 7. Data are shown as geometric means. Confidence intervals were calculated as 95% of the GM. Different time points and treatment groups were compared in a one-way analysis of variance (ANOVA) with a Sidak posttest for multiple comparisons. Detailed descriptions of groups compared are provided in the corresponding figure legends. The sequences for the phylogenetic tree were assembled in Clustal Omega and visualized in FigTree.

Data availability. The data that support the findings of this study are available from the corresponding author upon request.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/mSphere.00502-17.

FIG S1, TIF file, 0.5 MB.

FIG S2, TIF file, 0.5 MB.

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REFERENCES

1. Gao R, Cao B, Hu Y, Feng Z, Wang D, Hu W, Chen J, Jie Z, Qiu H, Xu K, Xu X, Lu H, Zhu W, Gao Z, Xiang N, Shen Y, He Z, Gu Y, Zhang Z, Yang Y, Zhao X, Zhou L, Li X, Zou S, Zhang Y, Li X, Yang L, Guo J, Dong J, Li Q, Dong L, Zhu Y, Bai T, Wang S, Hao P, Yang W, Zhang Y, Han J, Yu H, Li D. 2013. Human infection with a novel avian-origin influenza A (H7N9) virus. N Engl J Med 368:1888–1897. https://doi.org/10.1056/NEJMoa1304459.
2. Danqi B, Li Z, Liu Q, Richt JA. 2017. H7N9 avian influenza A virus in China: a short report on its circulation, drug resistant mutants and novel antiviral drugs. Expert Rev Anti Infect Ther 15:723–727. https://doi.org/10.1080/14787210.2017.1353419.
3. World Health Organization. 2017. Influenza at the human-animal interface. Summary and assessment. World Health Organization, Geneva, Switzerland.
4. Iuliano AD, Jang Y, Jones J, Davis CT, Wentworth DE, Uyeki TM, Roguski K, Thompson MG, Gubareva L, Fry AM, Burns E, Trock S, Zhou S, Katz JM, Jernigan DB. 2017. Increase in human infections with avian influenza A(H7N9) virus during the fifth epidemic—China, October 2016–February 2017. MMWR Morb Mortal Wkly Rep 66:254–255. https://doi.org/10.15585/mmwr.mm6609e2.
5. Zhang F, Bi Y, Wang J, Wong G, Shi W, Hu F, Yang Y, Yang L, Deng X, Jiang S, He X, Liu Y, Yin C, Zhong N, Gao GF. 2017. Human infections with recently-emerging highly pathogenic H7N9 avian influenza in China. J Infect 75:71–75. https://doi.org/10.1016/j.jinf.2017.04.001.
6. Zhang W, Zhu D, Tian D, Xu L, Zhu Z, Teng Z, He J, Shan S, Liu Y, Wang W, Yuan Z, Ren T, Hu Y. 2015. Co-infection with avian (H7N9) and pandemic (H1N1) 2009 influenza viruses, China. Emerg Infect Dis 21:715–718. https://doi.org/10.3201/eid2104.141560.
7. Li J, Kou Y, Yu X, Sun Y, Zhou Y, Pu X, Jin T, Pan J, Gao GF. 2014. Human co-infection with avian and seasonal influenza viruses, China. Emerg Infect Dis 20:1953–1955. https://doi.org/10.3201/eid2011.140897.
8. Osterholm MT, Ballering KS, Kelley NS. 2013. Major challenges in providing an effective and timely pandemic vaccine for influenza A(H7N9). JAMA 309:2557–2558. https://doi.org/10.1001/jama.2013.6589.
9. Nachbagauer R, Choi A, Hirsh A, Margine I, Iida S, Barrera A, Ferres M, Albrecht RA, García-Sastre A, Bouvier NM, Ito K, Medina RA, Palese P, Krammer F. 2017. Defining the antibody cross-reactome directed against the influenza virus surface glycoproteins. Nat Immunol 18:464–473. https://doi.org/10.1038/ni.3684.
10. Krammer F, Cox RJ. 2013. The emergence of H7N9 viruses: a chance to redefine correlates of protection for influenza virus vaccines. Expert Rev Vaccines 12:1369–1372. https://doi.org/10.1586/14760584.2013.850036.
11. Cox RJ. 2013. Correlates of protection to influenza virus, where do we go from here? Hum Vaccin Immunother 9:405–408. https://doi.org/10.4161/hv.22908.
12. Hobson D, Curry RL, Beare AS, Ward-Gardner A. 1972. The role of serum haemagglutination-inhibiting antibody in protection against challenge infection with influenza A2 and B viruses. J Hyg 70:767–777. https://doi.org/10.1017/S0022172400022610.
13. Dreyfus C, Laursen NS, Kwaks T, Zuijdgeest D, Khayat R, Ekiert DC, Lee JH, Metlagel Z, Bujny MV, Jongeneelen M, van der Vlugt R, Lamrani M, Korse HJ, Geelen E, Sahin Ö, Sieuwerts M, Brakenhoff JP, Vogels R, Li OT, Poon LL, Peiris M, Koudstaal W, Ward AB, Wilson IA, Goudsmit J, Friesen RH. 2012. Highly conserved protective epitopes on influenza B viruses. Science 337:1343–1348. https://doi.org/10.1126/science.1222908.
14. Ekiert DC, Friesen RH, Bhabha G, Kwaks T, Jongeneelen M, Yu W, Ophorst C, Cox F, Korse HJ, Brandenburg B, Vogels R, Brakenhoff JP, Kompier R, Koldijk MH, Cornelissen LA, Poon LL, Peiris M, Koudstaal W, Wilson IA, Goudsmit J. 2011. A highly conserved neutralizing epitope on group 2 influenza A viruses. Science 333:843–850. https://doi.org/10.1126/science.1204839.
15. Sui J, Hwang WC, Perez S, Wei G, Aird D, Chen LM, Santelli E, Stec B, Cadwell G, Ali M, Wan H, Murakami A, Yammanuru A, Han T, Cox NJ, Bankston LA, Donis RO, Liddington RC, Marasco WA. 2009. Structural and functional bases for broad-spectrum neutralization of avian and human influenza A viruses. Nat Struct Mol Biol 16:265–273. https://doi.org/10.1038/nsmb.1566.
16. Henry Dunand CJ, Leon PE, Huang M, Choi A, Chromikova V, Ho IY, Tan GS, Cruz J, Hirsh A, Zheng NY, Mullarkey CE, Ennis FA, Terajima M, Treanor JJ, Topham DJ, Subbarao K, Palese P, Krammer F, Wilson PC. 2016. Both neutralizing and non-neutralizing human H7N9 influenza vaccine-induced monoclonal antibodies confer protection. Cell Host Microbe 19:800–813. https://doi.org/10.1016/j.chom.2016.05.014.
17. Dilillo DJ, Tan GS, Palese P, Ravetch JV. 2014. Broadly neutralizing hemagglutinin stalk-specific antibodies require FcγR interactions for protection against influenza virus in vivo. Nat Med 20:143–151. https://doi.org/10.1038/nm.3443.
18. Krammer F, García-Sastre A, Palese P. 2017. Is it possible to develop a "universal" influenza virus vaccine? Toward a universal influenza virus vaccine: potential target antigens and critical aspects for vaccine development. Cold Spring Harb Perspect Biol https://doi.org/10.1101/cshperspect.a028845.
19. Krammer F. 2017. Strategies to induce broadly protective antibody

responses to viral glycoproteins. Expert Rev Vaccines 16:503–513. https://doi.org/10.1080/14760584.2017.1299576.
20. Nachbagauer R, Krammer F. 2017. Universal influenza virus vaccines and therapeutic antibodies. Clin Microbiol Infect 23:222–228. https://doi.org/10.1016/j.cmi.2017.02.009.
21. Fox CB, Barnes VL, Evers T, Chesko JD, Vedvick TS, Coler RN, Reed SG, Baldwin SL. 2013. Adjuvanted pandemic influenza vaccine: variation of emulsion components affects stability, antigen structure, and vaccine efficacy. Influenza Other Respir Viruses 7:815–826. https://doi.org/10.1111/irv.12031.
22. Wang D, Yang L, Zhu W, Zhang Y, Zou S, Bo H, Gao R, Dong J, Huang W, Guo J, Li Z, Zhao X, Li X, Xin L, Zhou J, Chen T, Dong L, Wei H, Li X, Liu L, Tang J, Lan Y, Yang J, Shu Y. 2016. Two outbreak sources of influenza A (H7N9) viruses have been established in China. J Virol 90:5561–5573. https://doi.org/10.1128/JVI.03173-15.
23. Lee DH, Torchetti MK, Killian ML, Swayne DE. 2017. Deep sequencing of H7N8 avian influenza viruses from surveillance zone supports H7N8 high pathogenicity avian influenza was limited to a single outbreak farm in Indiana during 2016. Virology 507:216–219. https://doi.org/10.1016/j.virol.2017.04.025.
24. Newbury SP, Cigel F, Killian ML, Leutenegger CM, Seguin MA, Crossley B, Brennen R, Suarez DL, Torchetti M, Toohey-Kurth K. 2017. First detection of avian lineage H7N2 in Felis catus. Genome Announc 5:e00457-17. https://doi.org/10.1128/genomeA.00457-17.
25. Liu L, Nachbagauer R, Zhu L, Huang Y, Xie X, Jin S, Zhang A, Wan Y, Hirsh A, Tian D, Shi X, Dong Z, Yuan S, Hu Y, Krammer F, Zhang X, Xu J. 2017. Induction of broadly cross-reactive stalk-specific antibody responses to influenza group 1 and group 2 hemagglutinins by natural H7N9 virus infection in humans. J Infect Dis 215:518–528. https://doi.org/10.1093/infdis/jiw608.
26. Margine I, Hai R, Albrecht RA, Obermoser G, Harrod AC, Banchereau J, Palucka K, García-Sastre A, Palese P, Treanor JJ, Krammer F. 2013. H3N2 influenza virus infection induces broadly reactive hemagglutinin stalk antibodies in humans and mice. J Virol 87:4728–4737. https://doi.org/10.1128/JVI.03509-12.
27. Mulligan MJ, Bernstein DI, Winokur P, Rupp R, Anderson E, Rouphael N, Dickey M, Stapleton JT, Edupuganti S, Spearman P, Ince D, Noah DL, Hill H, Bellamy AR, DMID 13-0032 H7N9 Vaccine Study Group. 2014. Serological responses to an avian influenza A/H7N9 vaccine mixed at the point-of-use with MF59 adjuvant: a randomized clinical trial. JAMA 312:1409–1419. https://doi.org/10.1001/jama.2014.12854.
28. Krammer F, Jul-Larsen A, Margine I, Hirsh A, Sjursen H, Zambon M, Cox RJ. 2014. An H7N1 influenza virus vaccine induces broadly reactive antibody responses against H7N9 in humans. Clin Vaccine Immunol 21:1153–1163. https://doi.org/10.1128/CVI.00272-14.
29. Andrews SF, Joyce MG, Chambers MJ, Gillespie RA, Kanekiyo M, Leung K, Yang ES, Tsybovsky Y, Wheatley AK, Crank MC, Boyington JC, Prabhakaran MS, Narpala SR, Chen X, Bailer RT, Chen G, Coates E, Kwong PD, Koup RA, Mascola JR, Graham BS, Ledgerwood JE, McDermott AB. 2017. Preferential induction of cross-group influenza A hemagglutinin stem–specific memory B cells after H7N9 immunization in humans. Sci Immunol 2:eaan2676. https://doi.org/10.1126/sciimmunol.aan2676.
30. Guo L, Zhang X, Ren L, Yu X, Chen L, Zhou H, Gao X, Teng Z, Li J, Hu J, Wu C, Xiao X, Zhu Y, Wang Q, Pang X, Jin Q, Wu F, Wang J. 2014. Human antibody responses to avian influenza A(H7N9) virus, 2013. Emerg Infect Dis 20:192–200. https://doi.org/10.3201/eid2002.131094.
31. Tan GS, Lee PS, Hoffman RM, Mazel-Sanchez B, Krammer F, Leon PE, Ward AB, Wilson IA, Palese P. 2014. Characterization of a broadly neutralizing monoclonal antibody that targets the fusion domain of group 2 influenza A virus hemagglutinin. J Virol 88:13580–13592. https://doi.org/10.1128/JVI.02289-14.
32. Krammer F, Albrecht RA, Tan GS, Margine I, Hai R, Schmolke M, Runstadler J, Andrews SF, Wilson PC, Cox RJ, Treanor JJ, García-Sastre A, Palese P. 2014. Divergent H7. immunogens offer protection from H7N9 virus challenge. J Virol 88:3976–3985. https://doi.org/10.1128/JVI.03095-13.
33. Shi Y, Zhang W, Wang F, Qi J, Wu Y, Song H, Gao F, Bi Y, Zhang Y, Fan Z, Qin C, Sun H, Liu J, Haywood J, Liu W, Gong W, Wang D, Shu Y, Wang Y, Yan J, Gao GF. 2013. Structures and receptor binding of hemagglutinins from human-infecting H7N9 influenza viruses. Science 342:243–247. https://doi.org/10.1126/science.1242917.
34. Hai R, Schmolke M, Leyva-Grado VH, Thangavel RR, Margine I, Jaffe EL, Krammer F, Solórzano A, García-Sastre A, Palese P, Bouvier NM. 2013. Influenza A(H7N9) virus gains neuraminidase inhibitor resistance without loss of in vivo virulence or transmissibility. Nat Commun 4:2854. https://doi.org/10.1038/ncomms3854.
35. Knepper J, Schierhorn KL, Becher A, Budt M, Tönnies M, Bauer TT, Schneider P, Neudecker J, Rückert JC, Gruber AD, Suttorp N, Schweiger B, Hippenstiel S, Hocke AC, Wolff T. 2013. The novel human influenza A(H7N9) virus is naturally adapted to efficient growth in human lung tissue. mBio 4:e00601-13. https://doi.org/10.1128/mBio.00601-13.
36. Yi L, Guan D, Kang M, Wu J, Zeng X, Lu J, Rutherford S, Zou L, Liang L, Ni H, Zhang X, Zhong H, He J, Lin J, Ke C. 2015. Family clusters of avian influenza A H7N9 virus infection in Guangdong Province, China. J Clin Microbiol 53:22–28. https://doi.org/10.1128/JCM.02322-14.
37. Zhu H, Wang D, Kelvin DJ, Li L, Zheng Z, Yoon SW, Wong SS, Farooqui A, Wang J, Banner D, Chen R, Zheng R, Zhou J, Zhang Y, Hong W, Dong W, Cai Q, Roehrl MH, Huang SS, Kelvin AA, Yao T, Zhou B, Chen X, Leung GM, Poon LL, Webster RG, Webby RJ, Peiris JS, Guan Y, Shu Y. 2013. Infectivity, transmission, and pathology of human-isolated H7N9 influenza virus in ferrets and pigs. Science 341:183–186. https://doi.org/10.1126/science.1239844.
38. Cox RJ, Madhun AS, Hauge S, Sjursen H, Major D, Kuhne M, Höschler K, Saville M, Vogel FR, Barclay W, Donatelli I, Zambon M, Wood J, Haaheim LR. 2009. A phase I clinical trial of a PER.C6 cell grown influenza H7 virus vaccine. Vaccine 27:1889–1897. https://doi.org/10.1016/j.vaccine.2009.01.116.
39. Couch RB, Patel SM, Wade-Bowers CL, Niño D. 2012. A randomized clinical trial of an inactivated avian influenza A (H7N7) vaccine. PLoS One 7:e49704. https://doi.org/10.1371/journal.pone.0049704.
40. Ellebedy AH, Krammer F, Li GM, Miller MS, Chiu C, Wrammert J, Chang CY, Davis CW, McCausland M, Elbein R, Edupuganti S, Spearman P, Andrews SF, Wilson PC, García-Sastre A, Mulligan MJ, Mehta AK, Palese P, Ahmed R. 2014. Induction of broadly cross-reactive antibody responses to the influenza HA stem region following H5N1 vaccination in humans. Proc Natl Acad Sci U S A 111:13133–13138. https://doi.org/10.1073/pnas.1414070111.
41. Nachbagauer R, Wohlbold TJ, Hirsh A, Hai R, Sjursen H, Palese P, Cox RJ, Krammer F. 2014. Induction of broadly reactive anti-hemagglutinin stalk antibodies by an H5N1 vaccine in humans. J Virol 88:13260–13268. https://doi.org/10.1128/JVI.02133-14.
42. Lee PS, Wilson IA. 2015. Structural characterization of viral epitopes recognized by broadly cross-reactive antibodies. Curr Top Microbiol Immunol 386:323–341. https://doi.org/10.1007/82_2014_413.
43. Henry Dunand CJ, Leon PE, Kaur K, Tan GS, Zheng NY, Andrews S, Huang M, Qu X, Huang Y, Salgado-Ferrer M, Ho IY, Taylor W, Hai R, Wrammert J, Ahmed R, García-Sastre A, Palese P, Krammer F, Wilson PC. 2015. Preexisting human antibodies neutralize recently emerged H7N9 influenza strains. J Clin Invest 125:1255–1268. https://doi.org/10.1172/JCI74374.
44. Dunkle LM, Izikson R, Patriarca P, Goldenthal KL, Muse D, Callahan J, Cox MMJ, PSC12 Study Team. 2017. Efficacy of recombinant influenza vaccine in adults 50 years of age or older. N Engl J Med 376:2427–2436. https://doi.org/10.1056/NEJMoa1608862.
45. An Y, Rininger JA, Jarvis DL, Jing X, Ye Z, Aumiller JJ, Eichelberger M, Cipollo JF. 2013. Comparative glycomics analysis of influenza hemagglutinin (H5N1) produced in vaccine relevant cell platforms. J Proteome Res 12:3707–3720. https://doi.org/10.1021/pr400329k.
46. Krammer F, Palese P. 2015. Advances in the development of influenza virus vaccines. Nat Rev Drug Discov 14:167–182. https://doi.org/10.1038/nrd4529.
47. Magadán JG, Altman MO, Ince WL, Hickman HD, Stevens J, Chevalier A, Baker D, Wilson PC, Ahmed R, Bennink JR, Yewdell JW. 2014. Biogenesis of influenza A virus hemagglutinin cross-protective stem epitopes. PLoS Pathog 10:e1004204. https://doi.org/10.1371/journal.ppat.1004204.
48. Terajima M, Cruz J, Co MD, Lee JH, Kaur K, Wrammert J, Wilson PC, Ennis FA. 2011. Complement-dependent lysis of influenza A virus-infected cells by broadly cross-reactive human monoclonal antibodies. J Virol 85:13463–13467. https://doi.org/10.1128/JVI.05193-11.
49. Dekkers G, Bentlage AEH, Stegmann TC, Howie HL, Lissenberg-Thunnissen S, Zimring J, Rispens T, Vidarsson G. 2017. Affinity of human IgG subclasses to mouse Fc gamma receptors. MAbs 9:767–773. https://doi.org/10.1080/19420862.2017.1323159.
50. Rudenko L, Kiseleva I, Naykhin AN, Erofeeva M, Stukova M, Donina S, Petukhova G, Pisareva M, Krivitskaya V, Grudinin M, Buzitskaya Z, Isakova-Sivak I, Kuznetsova S, Larionova N, Desheva J, Dubrovina I, Nikiforova A, Victor JC, Neuzil K, Flores J, Tsvetnitsky V, Kiselev O. 2014. Assessment of human immune responses to H7 avian influenza virus of pandemic potential: results from a placebo-controlled, randomized

double-blind phase I study of live attenuated H7N3 influenza vaccine. PLoS One 9:e87962. https://doi.org/10.1371/journal.pone.0087962.
51. Rudenko L, Isakova-Sivak I, Naykhin A, Kiseleva I, Stukova M, Erofeeva M, Korenkov D, Matyushenko V, Sparrow E, Kieny MP. 2016. H7N9 live attenuated influenza vaccine in healthy adults: a randomised, double-blind, placebo-controlled, phase 1 trial. Lancet Infect Dis 16:303–310. https://doi.org/10.1016/S1473-3099(15)00378-3.
52. Sobhanie M, Matsuoka Y, Jegaskanda S, Fitzgerald T, Mallory R, Chen Z, Luke C, Treanor J, Subbarao K. 2016. Evaluation of the safety and immunogenicity of a candidate pandemic live attenuated influenza vaccine (pLAIV) against influenza A(H7N9). J Infect Dis 213:922–929. https://doi.org/10.1093/infdis/jiv526.
53. DeZure AD, Coates EE, Hu Z, Yamshchikov GV, Zephir KL, Enama ME, Plummer SH, Gordon IJ, Kaltovich F, Andrews S, McDermott A, Crank MC, Koup RA, Schwartz RM, Bailer RT, Sun X, Mascola JR, Tumpey TM, Graham BS, Ledgerwood JE. 2017. An avian influenza H7 DNA priming vaccine is safe and immunogenic in a randomized phase I clinical trial. NPJ Vaccines 2:15. https://doi.org/10.1038/s41541-017-0016-6.
54. Fries LF, Smith GE, Glenn GM. 2013. A recombinant viruslike particle influenza A (H7N9) vaccine. N Engl J Med 369:2564–2566. https://doi.org/10.1056/NEJMc1313186.
55. O'Hagan DT, Friedland LR, Hanon E, Didierlaurent AM. 2017. Towards an evidence based approach for the development of adjuvanted vaccines. Curr Opin Immunol 47:93–102. https://doi.org/10.1016/j.coi.2017.07.010.
56. Treanor JJ, Chu L, Essink B, Muse D, El Sahly HM, Izikson R, Goldenthal KL, Patriarca P, Dunkle LM. 2017. Stable emulsion (SE) alone is an effective adjuvant for a recombinant, baculovirus-expressed H5 influenza vaccine in healthy adults: a phase 2 trial. Vaccine 35:923–928. https://doi.org/10.1016/j.vaccine.2016.12.053.
57. Baz M, Luke CJ, Cheng X, Jin H, Subbarao K. 2013. H5N1 vaccines in humans. Virus Res 178:78–98. https://doi.org/10.1016/j.virusres.2013.05.006.
58. Babu TM, Levine M, Fitzgerald T, Luke C, Sangster MY, Jin H, Topham D, Katz J, Treanor J, Subbarao K. 2014. Live attenuated H7N7 influenza vaccine primes for a vigorous antibody response to inactivated H7N7 influenza vaccine. Vaccine 32:6798–6804. https://doi.org/10.1016/j.vaccine.2014.09.070.
59. Halliley JL, Khurana S, Krammer F, Fitzgerald T, Coyle EM, Chung KY, Baker SF, Yang H, Martínez-Sobrido L, Treanor JJ, Subbarao K, Golding H, Topham DJ, Sangster MY. 2015. High-affinity H7 head and stalk domain-specific antibody responses to an inactivated influenza H7N7 vaccine after priming with live attenuated influenza vaccine. J Infect Dis 212:1270–1278. https://doi.org/10.1093/infdis/jiv210.
60. Bahl K, Senn JJ, Yuzhakov O, Bulychev A, Brito LA, Hassett KJ, Laska ME, Smith M, Almarsson Ö, Thompson J, Ribeiro AM, Watson M, Zaks T, Ciaramella G. 2017. Preclinical and clinical demonstration of immunogenicity by mRNA vaccines against H10N8 and H7N9 influenza viruses. Mol Ther 25:1316–1327. https://doi.org/10.1016/j.ymthe.2017.03.035.
61. Wohlbold TJ, Nachbagauer R, Xu H, Tan GS, Hirsh A, Brokstad KA, Cox RJ, Palese P, Krammer F. 2015. Vaccination with adjuvanted recombinant neuraminidase induces broad heterologous, but not heterosubtypic, cross-protection against influenza virus infection in mice. mBio 6:e02556-14. https://doi.org/10.1128/mBio.02556-14.
62. Cox MM, Hashimoto Y. 2011. A fast track influenza virus vaccine produced in insect cells. J Invertebr Pathol 107(Suppl):S31–S41. https://doi.org/10.1016/j.jip.2011.05.003.
63. Krammer F, Margine I, Tan GS, Pica N, Krause JC, Palese P. 2012. A carboxy-terminal trimerization domain stabilizes conformational epitopes on the stalk domain of soluble recombinant hemagglutinin substrates. PLoS One 7:e43603. https://doi.org/10.1371/journal.pone.0043603.
64. Margine I, Palese P, Krammer F. 2013. Expression of functional recombinant hemagglutinin and neuraminidase proteins from the novel H7N9 influenza virus using the baculovirus expression system. J Vis Exp (81):e51112. https://doi.org/10.3791/51112.
65. Jacobsen H, Rajendran M, Choi A, Sjursen H, Brokstad KA, Cox RJ, Palese P, Krammer F, Nachbagauer R. 2017. Influenza virus hemagglutinin stalk-specific antibodies in human serum are a surrogate marker for in vivo protection in a serum transfer mouse challenge model. mBio 8:e01463-17. https://doi.org/10.1128/mBio.01463-17.

MANUSCRIPT 2

Cross-reactive mouse monoclonal antibodies raised against the hemagglutinin of A/Shanghai/1/2013 (H7N9) protect against novel H7 virus isolates in the mouse model.

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Cross-reactive mouse monoclonal antibodies raised against the hemagglutinin of A/Shanghai/1/2013 (H7N9) protect against novel H7 virus isolates in the mouse model

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Abstract

Influenza viruses remain a major global public health risk. In addition to seasonal influenza viruses, epizootic influenza A H7 subtype viruses of both the Asian and North American lineage are of concern due to their pandemic potential. In China, the simultaneous occurrence of H7N9 zoonotic episodes and seasonal influenza virus epidemics could potentially lead to novel reassortant viruses with the ability to efficiently spread among humans. Recently, the H7N9 virus has evolved into two new lineages, the Pearl River Delta and the Yangtze River Delta clade. This development has also resulted in viruses with a polybasic cleavage site in the hemagglutinin that are highly pathogenic in avian species and have caused human infections. In addition, an outbreak of a highly pathogenic H7N8 strain was reported in the US state of Indiana in 2016. Furthermore, an H7N2 feline virus strain caused an outbreak in cats in an animal shelter in New York City in 2016, resulting in one human zoonotic event. In this study, mouse monoclonal antibodies previously raised against the hemagglutinin of the A/Shanghai/1/2013 (H7N9) virus were tested for their (cross-) reactivity to these novel H7 viruses. Moreover, the functionality of these antibodies was assessed in vitro in hemagglutination inhibition and microneutralization assays. The therapeutic and prophylactic efficacy of the broadly reactive antibodies against novel H7 viruses was determined in vivo in mouse passive transfer-viral challenge experiments. Our results provide data about the conservation of critical H7 epitopes and could inform the selection of pre-pandemic H7 vaccine strains.

Introduction

Influenza viruses are a public health concern on a global scale[1]. Annually, influenza viruses infect millions of people worldwide resulting in 290,000 to 650,000 influenza-related deaths[2]. Besides globally circulating seasonal influenza strains of the H1N1 subtype, H3N2 subtype, or influenza B strains, avian influenza viruses of the H7 subtype can result in zoonotic infections[3]. In 2017, the fifth wave of a zoonotic H7N9 epidemic emerged in China, resulting in higher numbers of laboratory-confirmed human infections (over 1500) than in previous years, coupled with a high case fatality rate (almost 40%)[4]. While these viruses have not yet gained the capability of sustained human-to-human transmission, they do pose a pandemic risk if the avian virus were to adapt to humans or undergo reassortment with seasonal viruses[5,6]. Human infections with highly pathogenic avian influenza (HPAI) H7N9 viruses with polybasic cleavage sites in the hemagglutinin (HA) have been reported during the most

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recent epidemic[6]. These HPAI H7N9 virus isolates contained dual receptor binding properties, allowing them to bind to α2,6-linked sialic acid receptors (prevalent in the human upper airways) as well as α2,3-linked sialic acid receptors (prevalent in many avian species)[7]. Additionally, during the 2016–2017 Northern Hemisphere winter season, the A/H7N9 virus evolved and clustered into antigenically distinct lineages[7,8] the Yangtze River Delta (YRD) lineage and Pearl River Delta (PRD) lineage. When tested against ferret antisera, it was shown that these two lineages did not match H7 stockpiled vaccines well[9]. Outside Mainland China, a highly pathogenic avian H7N8 virus was isolated from commercial turkeys in the US state of Indiana in 2016, causing severe systemic disease and high mortality in these animals[10,11]. Additionally, in New York City, an outbreak of an H7N2 virus in cats in an animal shelter led to public health concerns at the end of 2016. The feline virus caused one known human zoonotic event by infecting a human healthcare worker, who subsequently experienced influenza-like illness[12].

Humans are immunologically naive to subtype H7 viruses[13]. If zoonotic H7 viruses from animal reservoirs were to adapt to humans through mutations, H7 viruses could gain pandemic potential[14,15]. Vaccination regimens to protect against H7 viruses often only elicit low levels of hemagglutination inhibiting antibody titers and require further development[16–21]. However, the hemagglutination inhibition (HI) assay may not be sufficient to measure the full extent of the antibody response against H7 viruses[19,22,23]. Antibodies that target other regions of the HA, such as the membrane proximal stalk domain, can contribute to protection by mechanisms other than HI, but can only be detected in other types of assays[24–26].

We have previously generated a set of four murine monoclonal antibodies (mAbs) against the HA of the A/Shanghai/1/2013 H7N9 virus[27]. The panel includes two HI-active and neutralizing mouse mAbs, as well as two non-HI-active and non-neutralizing mouse mAbs which have all been shown to be protective against H7N9 challenge in vivo. Here we tested their (cross-) reactivity and in vitro and in vivo functionality against the newly emerged Eurasian and American lineage H7 viruses described above.

Results

Mouse mAbs bind to the HA of novel H7 virus isolates of the Eurasian and North American lineages

The minimal binding concentrations of four broadly reactive mAbs raised against the H7 HA of the A/Shanghai/1/2013 (Shanghai) virus strain were assessed using enzyme-linked immunosorbent assays (ELISAs). The mAbs 1A8, 1B2, 1H5, and 1H10 have been previously generated in our laboratory using hybridoma technology and have been described in detail[27]. It was shown that mAbs 1A8 and 1B2 bind to a wide range of H7 HAs of both the Eurasian and North American lineage. Antibodies 1H5 and 1H10 showed strong binding to Eurasian lineage H7 HAs but displayed weak binding to North American lineage HAs. In this study, we tested binding of these mAbs to H7 HAs of emerging viruses from both lineages (Fig. 1). Here, we detected the minimal binding concentrations performing ELISAs using the recombinantly expressed HA of novel H7 virus strains of the Asian PRD and YRD clade (Fig. 1a–c). The minimal binding concentration for antibodies 1A8, 1B2, 1H5, and 1H10 ranged between 0.51 and 1.52 ng/mL for all tested novel H7 HAs of the Eurasian lineage (A/Hunan/02285/2017 (Hunan), A/Guangdong/17SF003/2016 (Guangdong), A/Hong Kong/2014/2017 (Hong Kong)). Binding to novel H7 HAs of the North American lineage (A/feline/New York/16-040082-1/2016 (New York), (A/turkey/Indiana/16-001403-1/2016 (Indiana)) was weaker (Fig. 1d, e). As expected, mAbs 1A8 and 1B2 had low minimal binding concentrations for the North American lineage HAs (4.57–13.72 ng/mL for the feline H7 HA (Fig. 1d) and 1.52 ng/mL for Indiana H7 (Fig. 1e)) indicating a strong binding phenotype. The binding of 1H5 and 1H10 to these isolates was lower, but still detectable (1H5 to feline H7 0.10 μg/mL, 1H10 to feline H7 0.37 μg/mL (Fig. 1d), 1H5 to Indiana H7 0.37 μg/mL, 1H10 to Indiana H7 3.33 μg/mL) (Fig. 1e). These data confirmed that the broadly reactive antibodies raised against the A/Shanghai/1/2013 virus isolate can bind to the H7 HA of novel virus isolates of 2016 and 2017. In fact, the mAbs had similar minimal binding concentrations for novel Eurasian lineage HAs as compared to the Shanghai H7 HA (depicted by the vertical dashed line; Fig. 1a–c). As expected, the binding to the phylogenetically more distant North American lineage HAs was weaker (Fig. 1d, e).

Characterization of in vitro functionality of mAbs in HI and microneutralization assays

In order to characterize the in vitro functionality of the mAbs, we generated (low pathogenic) H7 virus reassortants that express the surface glycoprotein segment (HA) of novel H7 virus isolates by plasmid-based reverse genetic techniques[28]. The HAs of two H7N9 (Hong Kong and Hunan, both low pathogenic variants) virus isolates were each recombined with the six internal segments and the neuraminidase (NA) of laboratory strain PR8, resulting in 7:1 reassortant viruses. For the generation of the New York (A/feline/New York/16-040082-1/2016) reassortant virus, the HA (H7) and NA (N2) were rescued in a PR8 backbone, leading to a PR8-6:2 reassortant. Successful virus rescue and a lack of mutations were confirmed by deep-sequencing. Following the successful virus rescue, they were used to assess antibody functionality. The mAbs 1A8 and 1B2 showed HI activity (Fig. 2a–c)

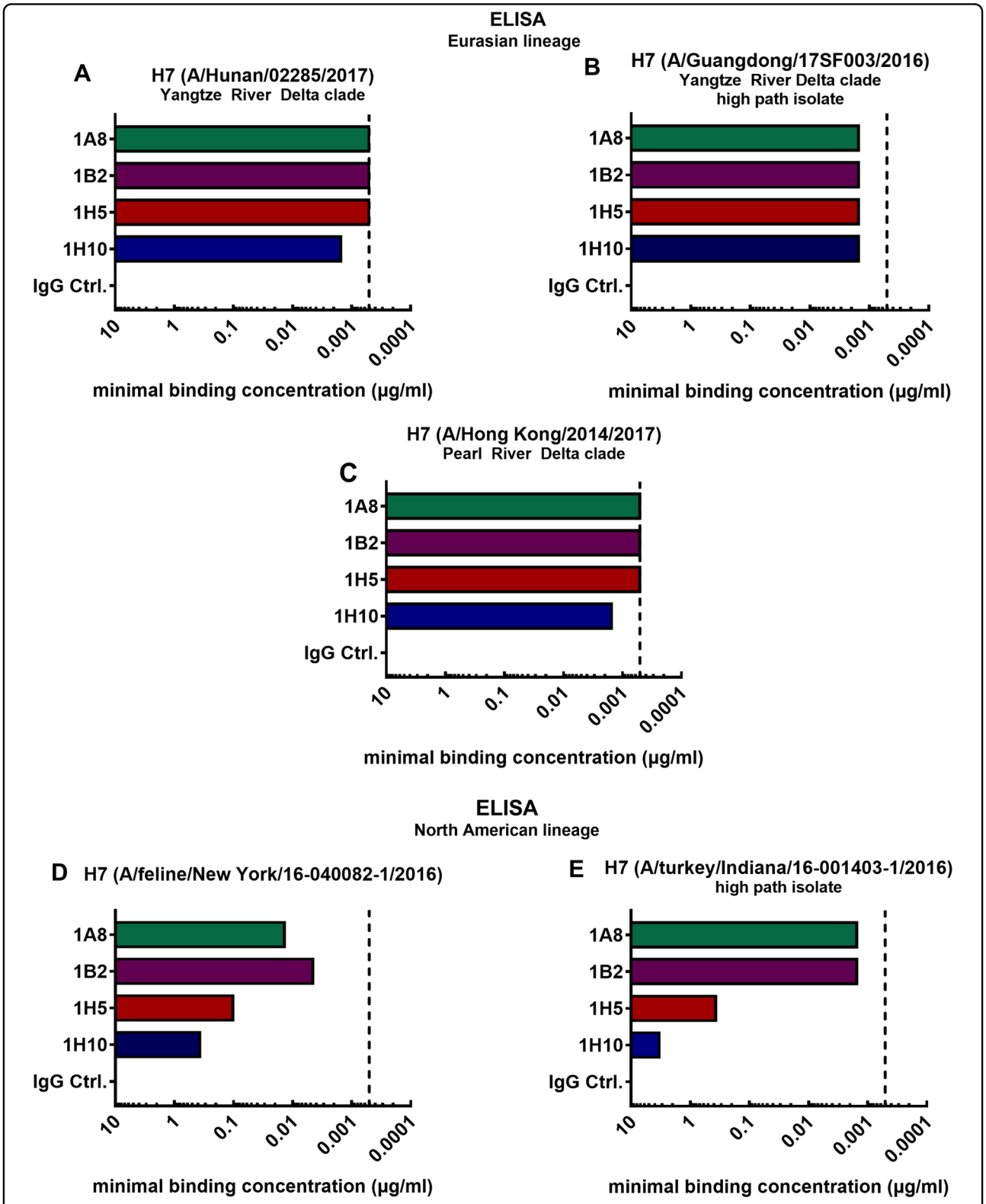

Fig. 1 Reactivity of broadly reactive mouse mAbs to HAs of novel H7 virus isolates as measured by ELISA. Minimal binding concentrations of mAbs 1A8, 1B2, 1H5, 1H10 and negative IgG control binding to recombinant H7 HAs of Eurasian lineage virus isolates A/Hunan/02285/2017 (**a**), A/Hong Kong/2014/2017 (**b**), and A/Guangdong/17SF003/2016 (**c**). Minimal binding concentrations of mAbs binding to the H7 HAs of North American lineage HAs of virus isolates A/feline/New York/16-040082/2016 (**d**) and A/turkey/Indiana/16-001403-1/2016 (**e**). The vertical dashed line represents the minimal binding concentration of all four mAbs to H7 HA of A/Anhui/1/2013, which is antigenically similar to the strain they were raised to

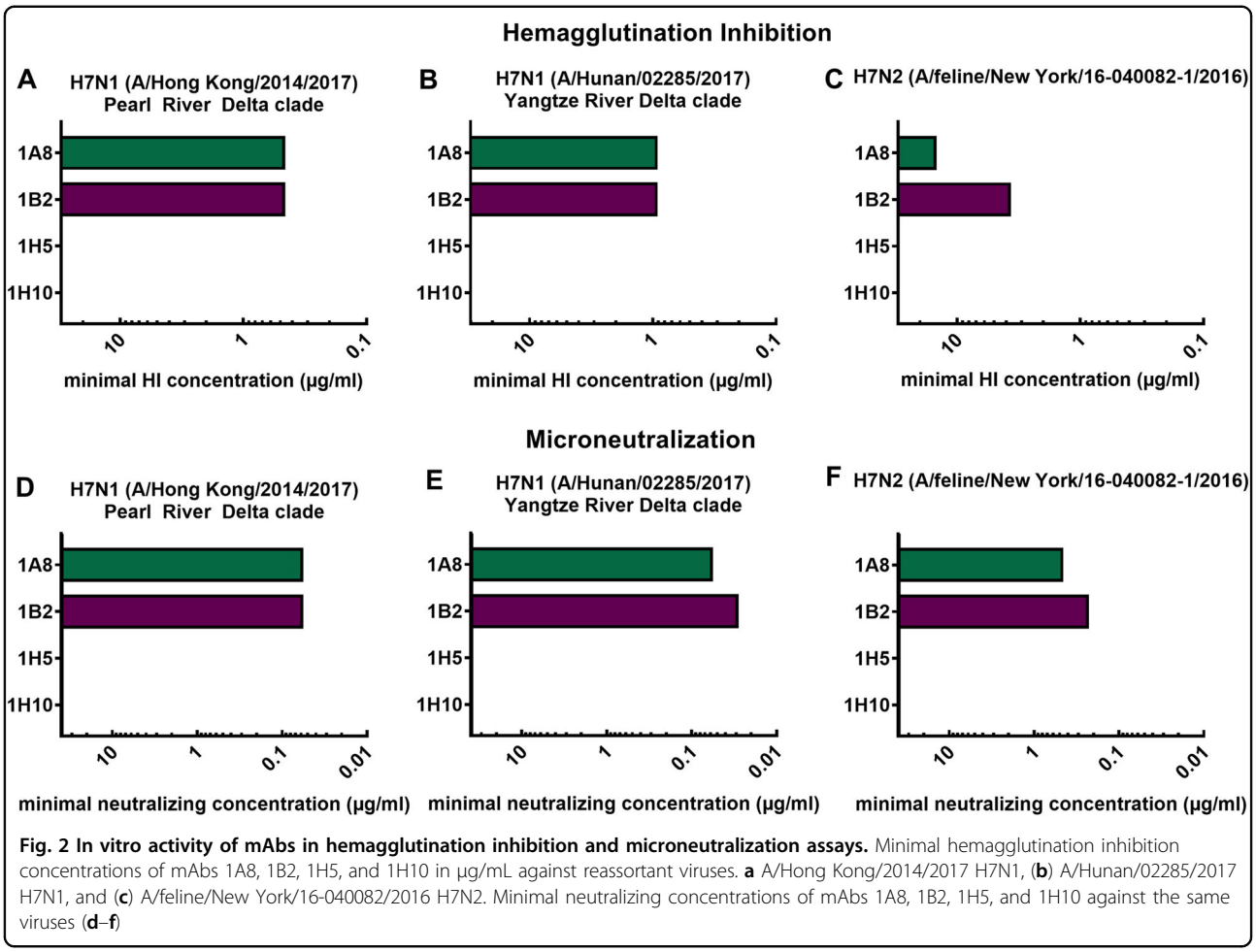

Fig. 2 In vitro activity of mAbs in hemagglutination inhibition and microneutralization assays. Minimal hemagglutination inhibition concentrations of mAbs 1A8, 1B2, 1H5, and 1H10 in µg/mL against reassortant viruses. **a** A/Hong Kong/2014/2017 H7N1, (**b**) A/Hunan/02285/2017 H7N1, and (**c**) A/feline/New York/16-040082/2016 H7N2. Minimal neutralizing concentrations of mAbs 1A8, 1B2, 1H5, and 1H10 against the same viruses (**d**–**f**)

and inhibited novel H7 reassortant viruses at low minimal HI concentrations (1A8 0.47 µg/mL, 1B2 0.47 µg/mL for Hong Kong; 1A8 0.94 µg/mL, 1B2 0.94 µg/mL for Hunan). These concentrations are in the same range as previously reported[27] for a Shanghai H7N9 (xPR8) virus. The minimal HI concentrations of the mAbs against the feline virus (New York) were higher (1A8 15 µg/mL, 1B2 3.75 µg/mL) (Fig. 2c), and are comparable to an avian H7 virus (A/rhea/North Carolina/39482/1993) of the North American lineage previously tested. As expected from previous results, mAbs 1H5 and 1H10 had no detectable HI activity for the viruses tested (Fig. 2a–c). This is consistent with previously conducted epitope mapping that showed potential binding of these mAbs to the lateral part of the globular head domain in close proximity to the stalk domain[27]. Furthermore, neutralizing activity was assessed using microneutralization assays (Fig. 2d–f). The HI-active antibodies (1A8, 1B2) could also neutralize all three tested viruses (Fig. 2d–f). The minimal neutralizing concentration of 1A8 and 1B2 was 0.06 µg/mL against the Hong Kong virus. Against the Hunan virus isolate, 1A8 neutralized at a concentration of 0.06 µg/mL and 1B2 at 0.03 µg/mL (Fig. 2e). The minimal neutralizing concentration of 1A8 and 1B2 against the New York isolate was 0.47 and 0.23 µg/mL, respectively (Fig. 2f). The non-HI-active mAbs 1H5 and 1H10—as expected—did not show neutralizing potential for the viruses at the tested concentrations (Fig. 2d–f).

Novel H7 viruses in a PR8 backbone cause morbidity and mortality in the BALB/c mouse model

Subsequently, to prepare for in vivo mAb protection studies, we tested whether the rescued H7 viruses were able to infect and replicate in mice. Female BALB/c mice were intranasally infected with the H7 PR8 reassortants (PR8-7:1 or PR8-6:2) and weight loss was monitored daily for 14 days (Fig. 3a–c). All three H7 viruses: the YRD clade (Hunan), the PRD clade (Hong Kong), and the New York (PR8-6:2) virus conferred morbidity and mortality (Fig. 3). The murine lethal dose 50 (LD_{50}) for the H7 Hong Kong isolate was reached at a viral input of 250 times the tissue culture infection dose 50 ($TCID_{50}$) per 50 µL. For the Hunan isolate the LD_{50} was at 253 × $TCID_{50}$s/50 µL. The LD_{50} value of the New York feline

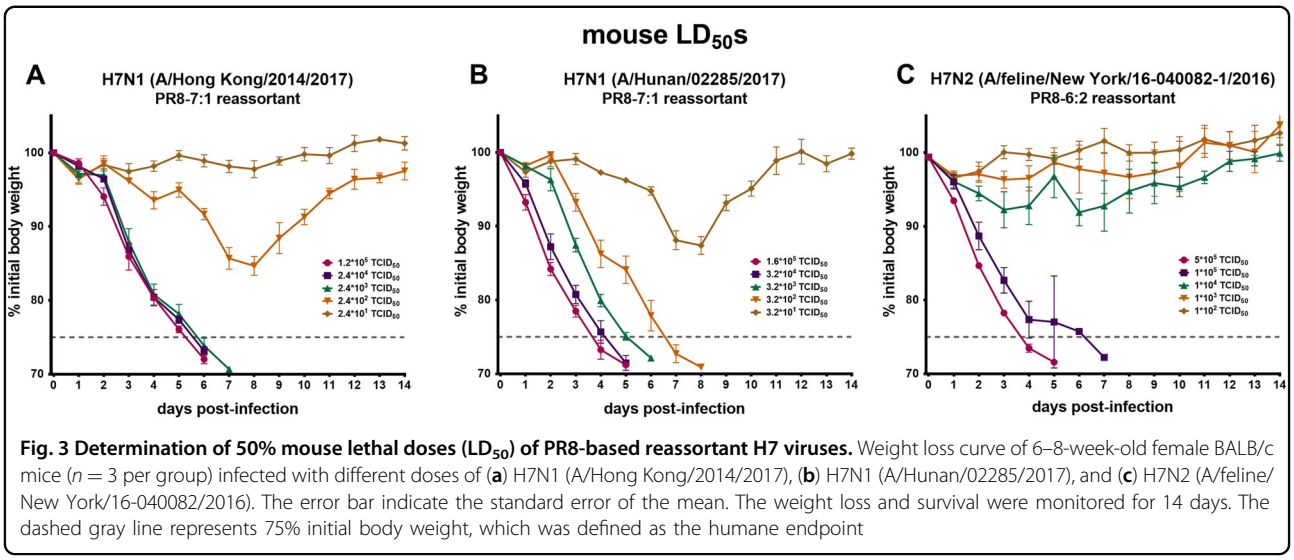

Fig. 3 Determination of 50% mouse lethal doses (LD_{50}) of PR8-based reassortant H7 viruses. Weight loss curve of 6–8-week-old female BALB/c mice ($n = 3$ per group) infected with different doses of (**a**) H7N1 (A/Hong Kong/2014/2017), (**b**) H7N1 (A/Hunan/02285/2017), and (**c**) H7N2 (A/feline/New York/16-040082/2016). The error bar indicate the standard error of the mean. The weight loss and survival were monitored for 14 days. The dashed gray line represents 75% initial body weight, which was defined as the humane endpoint

virus was $3.16 \times 10^4 \times TCID_{50}/50\,\mu L$. The mice dropped below 75% initial body weight starting from days 4 to 7 (Hong Kong, New York) or day 8 (Hunan) and had to be euthanized. These data indicate that all three reassortant H7 viruses infected mice, resulting in morbidity and mortality and could be used for subsequent experiments.

Cross-reactive H7 mAbs confer protection from lethal virus challenge in prophylactic and therapeutic settings in the mouse model

After assessing inhibition and neutralization activity of the antibodies in vitro the novel H7 virus reassortants were used to investigate whether the four broadly reactive H7 mAbs confer protection in vivo. The protective effect of the mAbs was tested both in a prophylactic setting and by therapeutic administration of mAbs post infection. For the prophylactic treatment female 6–8-week-old BALB/c mice received 1 mg/kg of a mAb and were challenged with $5 \times LD_{50}$ (Hunan and Hong Kong). Both the neutralizing mAbs 1A8 and 1B2 and the non-neutralizing, non-HI-active antibodies 1H5 and 1H10 fully protected against lethal challenge with H7N1 Hong Kong and Hunan reassortant viruses (Fig. 4a–d). The negative control mice lost weight and succumbed to infection on days 5 to 7 (Hong Kong) and on days 7 to 8 (Hunan) post infection (Fig. 4a–d). To test the protective efficacy of the mAbs against a North American lineage virus, mice were challenged with $2 \times LD_{50}$ of feline PR8 reassortant virus. The viral input dose needed to induce mortality in mice for the New York isolate was substantial (LD_{50} of $3.16 \times 10^4 \times TCID_{50}/50\,\mu L$; Fig. 3c). To avoid losing sensitivity and challenging the mice with too much virus (which could lead to unwanted morbidity early after infection due to innate immune responses triggered by massive virus input), a lower viral input dose ($2 \times LD_{50}$ instead of $5 \times LD_{50}$) was selected. All mice that received broadly reactive H7 antibodies were fully protected from lethal challenge but showed morbidity (10–15% weight loss) before recovering at day 8 (Fig. 4e). The negative control mice succumbed to infection on day 6. (Fig. 4f). As described above, mAb-treated mice challenged with Eurasian lineage H7 viruses showed no morbidity, whereas the mAb-treated mice challenged with the North American lineage feline H7 virus showed weight loss. Therefore, the reduction of lung virus titers was assessed for the New York virus to determine the mAbs' ability to clear infection and to investigate if there are differences between neutralizing and non-neutralizing mAbs. Mice were given 5 mg/kg mAbs (1A8, 1B2, 1H5, 1H10, or immunoglobulin G (IgG) control). After 2 h, the mice were infected with $0.1 \times LD_{50}$. This low dose was selected to avoid that the (control) mice succumb to infection before day 3 or more importantly day 6 and to allow for a more sensitive readout. To assess lung titers, the lungs were harvested at 3 or 6 days post infection and the lung titers determined in the form of egg infectious dose 50s (EID_{50}s). All H7-specific mAbs reduced viral lung titers on days 3 and 6 (Fig. 4g) as compared to a control IgG. It has to be noted that 1A8 and 1B2 had lower minimal binding concentrations than 1H5 and 1H10 as demonstrated before (indicating stronger binding), which might explain the subtle differences observed in lung virus reduction. However, non-neutralizing, non-HI-active antibodies 1H5 and 1H10 significantly reduced viral lung titers on day 3 and reduced the titers on day 6 as well.

To determine if the mAbs were also protective in a therapeutic setting, mice were challenged with $5 \times LD_{50}$ of Hunan virus (YRD). Viruses of the YRD clade were predominantly detected in individuals with H7N9 infections within the fifth wave of the H7N9 epidemics and the

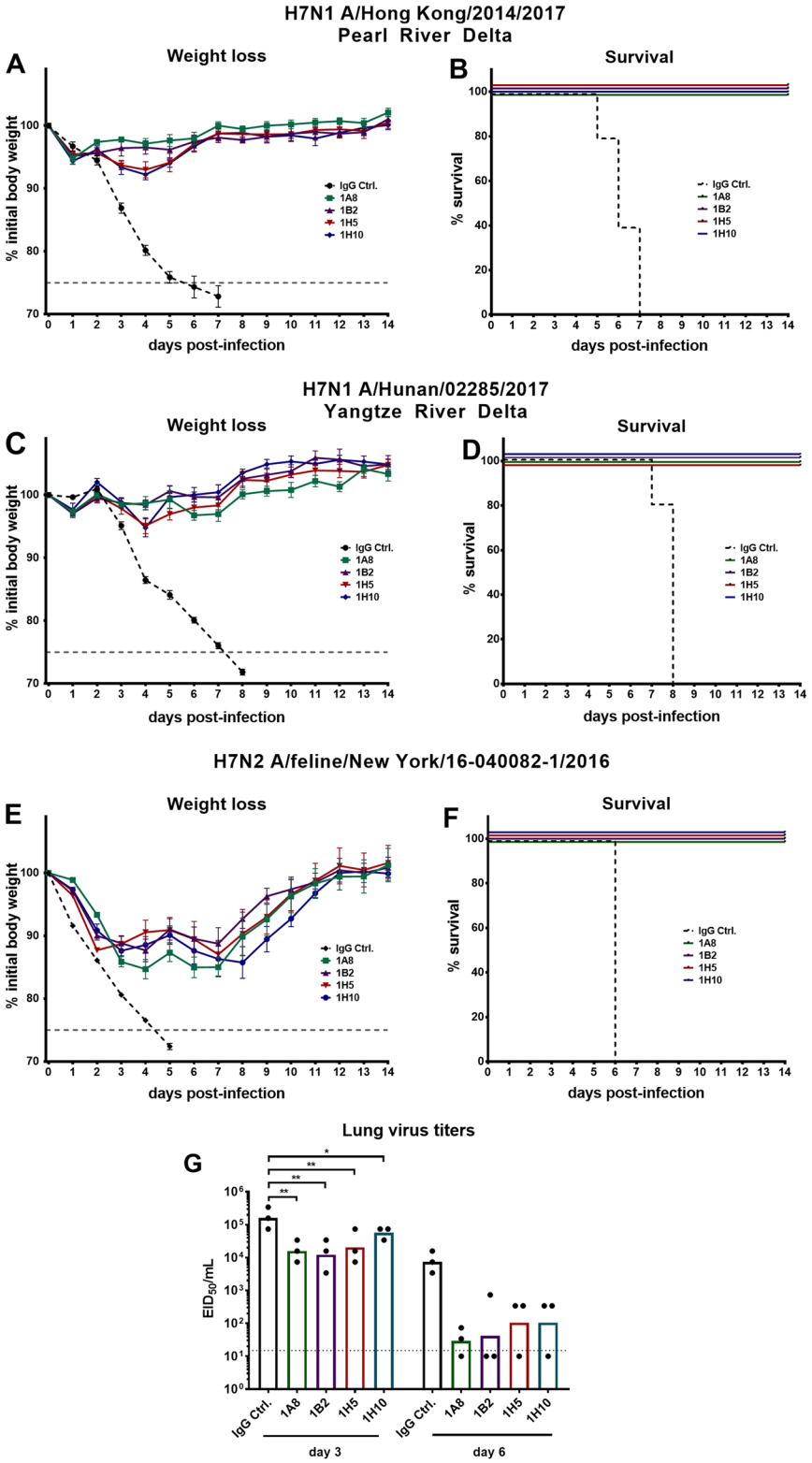

Fig. 4 (See legend on next page.)

(see figure on previous page)
Fig. 4 Protective efficacy of mAbs in a prophylactic setting against lethal virus challenge in the mouse model. a, **c**, **e** show weight loss curves of animals pretreated ($n = 5$ per group) with monoclonal antibodies at a concentration of 1 mg/kg and challenged with H7N1 A/Hong Kong/2014/2017, H7N1 A/Hunan/02285/2017, or H7N2 A/feline/New York/16-040082/2016 reassortant viruses 2 h post mAb transfer. The error bars represent the standard error of the mean. The dashed black line represents treatment with a negative control IgG and the dashed gray line represents 75% weight loss. **b**, **d**, **f** Survival graphs showing percent survival in the different groups used in the prophylactic mouse challenge model. **g** The lung viral titers on days 3 and 6 post infection are shown as EID_{50}/mL for IgG control and mAbs 1A8, 1B2, 1H5, and 1H10 ($n = 3$ per group). The dotted line represents the limit of detection ($10 \times EID_{50}$/mL). Lung virus titers of the IgG control were compared for the same day (3 or 6) in a one-way ANOVA with a Sidak post test for multiple comparison. Significance is indicated as follows: $P > 0.05$; $^{*}P \leq 0.05$; $^{**}P \leq 0.01$

Hunan virus was selected for therapeutic testing for this reason[9]. After 48 or 72 h 5 mg/kg 1B2 or 1H5 mAb were administered intraperitoneally. Here, one neutralizing (1B2) and one non-neutralizing (1H5) antibody was selected. The selection was based on ELISA data that showed slightly stronger binding of neutralizing mAb 1B2 over 1A8 and non-neutralizing 1H5 over 1H10 to novel H7 HAs, and to reduce the number of mice used. The mice that received mAbs, either 48 or 72 h post infection, recovered shortly after administration, gained weight, and were fully protected from lethal challenge (Fig. 5a–d). The IgG control mice succumbed to infection on days 7 to 9, except for one mouse in the 72 h post infection IgG control group that survived the challenge. These data show that all four mAbs were protective in vivo, reduced lung virus titers, and could be applied as prophylactics and/or therapeutics.

mAbs 1A8 and 1B2 crossreact to the HA of emerging H7N9 viruses despite changes in their target antigenic site A

In our previous report[27], epitope analysis showed that neutralizing mAbs 1A8 and 1B2 target an epitope that overlaps antigenic site A of H7 HA (Fig. 6c). To further investigate the conservation of this critical H7 epitope, we generated a phylogenetic tree based on the amino acid sequences of H7 HAs, which showed high divergence between different isolates (Fig. 6a). A stark contrast could be observed between North American and Eurasian lineage H7 HA sequences. Nevertheless, a common, conserved motive found in all H7 HA sequences was antigenic site A. As previously reported[27], the amino acid sequence of antigenic site A found in Eurasian lineage H7 HAs was RRSGSS in about 83% of isolates, and 49% in North American isolates. The second major sequence found in North American isolates was TRSGSS (38% of isolates). Previously generated escape mutants[27] indicated that the mAbs bound to the sequence RRSGSS (antigenic site A), and mutations in amino acids within that site (Fig. 6d) led to a loss or reduction of binding. The HA of the Hunan and Hong Kong virus isolates used in this study had a mutation in antigenic site A in position 148 (according to H3 HA numbering) changing arginine (R) to lysine (K) (Fig. 6c). As shown by ELISA, in vitro, and in vivo experiments, a mutation in this position had no detectable influence on mAb binding and function. It has to be noted that R and K are similar amino acids and that a change to another amino acid might have a different impact. The HA of the New York virus isolate had a different amino acid in position 148 (threonine (T) instead of arginine (R)). This variant of antigenic site A is the second most commonly found sequence in North American isolates as mentioned above. Again, the antibodies did bind to the HA and did not lose function, but showed increased minimal binding concentrations as compared to Eurasian lineage HAs. This indicates that the usually highly conserved antigenic site A was changing. So far, mAbs raised against the Shanghai HA isolate were still reactive and functional. It is however unclear what might happen if other amino acids mutate, like those that led to an escape in previous experiments[27] or if other positions within site A change.

Discussion

Influenza viruses of the H7 subtype pose a pandemic threat. Zoonotic H7 viruses have shown to possess two out of three major factors that drive the pandemic potential of an influenza virus including their ability to cause human disease and the fact that immunity of the population to these virus strains is very low to non-existent[5]. So far, no substantial transmission of H7 influenza A viruses (IAVs) between humans has been observed, which would be the third major requirement for a virus to cause widespread human disease and potentially become pandemic. Reassortment of zoonotic H7 viruses with seasonal human IAV strains could, in theory, facilitate the generation of viruses with high transmission potential[15,29]. To date, no reassortment events have been reported and incompatibilities at the RNA or protein level, called segment mismatch, might potentially prevent certain human IAV strains from easily recombining with zoonotic influenza virus strains[30]. Nevertheless, it is important to better understand the antigenicity of H7 viruses[31] and to establish potential therapeutics for pandemic preparedness[32].

In the present study, we used mouse mAbs as a tool to characterize the conservation of epitopes between novel H7 viruses. H7N9 viruses in China are evolving, but at

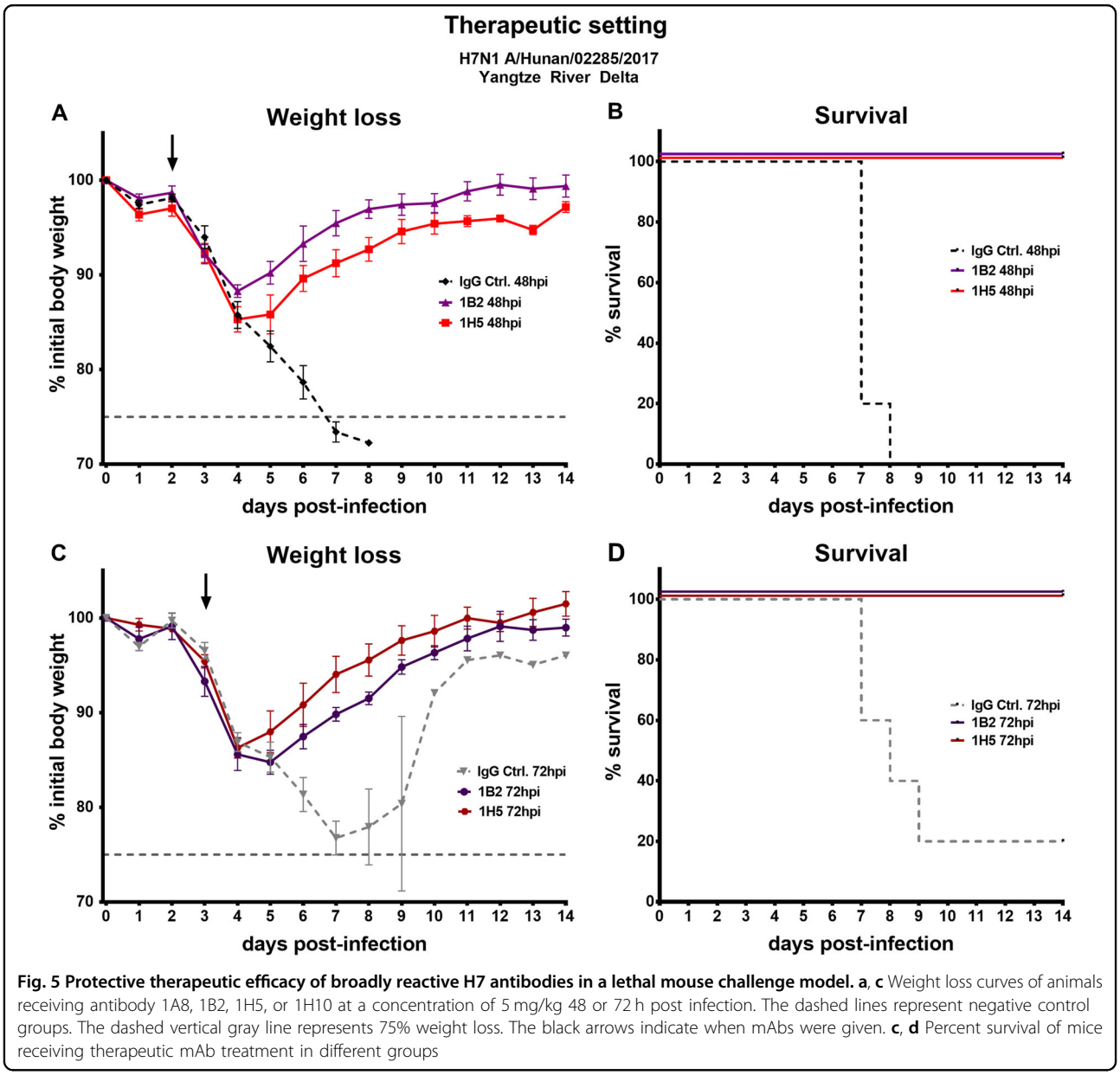

Fig. 5 Protective therapeutic efficacy of broadly reactive H7 antibodies in a lethal mouse challenge model. a, **c** Weight loss curves of animals receiving antibody 1A8, 1B2, 1H5, or 1H10 at a concentration of 5 mg/kg 48 or 72 h post infection. The dashed lines represent negative control groups. The dashed vertical gray line represents 75% weight loss. The black arrows indicate when mAbs were given. **c**, **d** Percent survival of mice receiving therapeutic mAb treatment in different groups

least two epitopes of the H7 HA are unchanged and can still be targeted by broadly reactive antibodies. These findings are consistent with the high sequence identity (95–98%) of A/Shanghai/1/2013 and the novel Asian lineage H7 HAs. Interestingly, the mAbs also bound to the more diverged HA of a feline H7N2 virus and the H7 HA of a highly pathogenic avian H7N8 isolate. The isolates of the North American lineage tested were phylogenetically very distinct from the H7 A/Shanghai/1/2013 HA used for the generation of the mAbs and antibody binding in ELISA to North American isolate HAs was weaker. Importantly, we showed that cross-reactive mouse mAbs can still be used as effective prophylactic or therapeutic agents in animal challenge experiments. This suggests that humanized or human mAbs targeting the same or similar epitopes could be developed for potential human application. It typically takes about at least 6 months from the preparation of a seed virus strain until a vaccine can be shipped and administered[14]. In case of a pandemic outbreak, the population is vulnerable to infection and disease during this time frame[33]. A readily available cocktail of broadly reactive anti-H7 mAbs could help to bridge this gap and serve as an anti-viral agent while matched vaccines are being developed[34].

Previously generated neutralizing as well as non-neutralizing, non-HI-active H7 HA-reactive mAbs conferred protection in mouse passive transfer experiments.

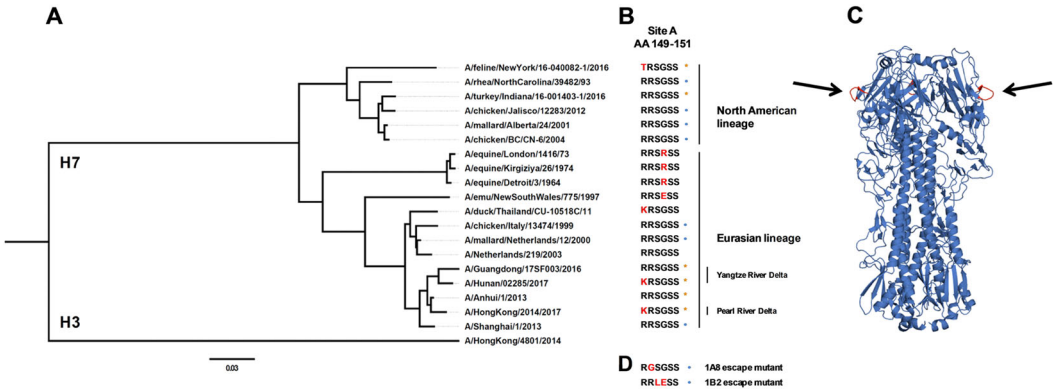

Fig. 6 Phylogenetic tree of H7 HAs, antigenic site A, and structure of influenza A H7 HA. a Phylogenetic tree based on the amino acid sequence of H7 HAs. The amino acid sequence of H3 (A/Hong Kong/4801/2014) was used as an outgroup. The scale bar at the bottom shows a 3% difference in amino acid identity. **b** Amino acids of H7 HA at position 149 to 151 (antigenic site A; H3 numbering) are shown. Red letters represent differences to A/Anhui/1/2013 (and A/Shanghai/1/2013). Orange stars represent H7 HAs used in this study and blue circles H7 HAs tested in the previous report[27]. The different lineages (North American vs. Eurasian) and clades (Pearl River Delta vs. Yangtze River Delta) are indicated. **c** Graphic representation of the crystal structure of the A/Anhui/1/2013 H7 HA trimer. Black arrows are pointing at the antigenic site A shown in red. The structure is based on PDB # 4R8W[48]. **d** Mutations of antigenic site A that resulted in loss of binding of mAbs 1A8 and 1B2 as determined by escape mutagenesis (as previously reported)

Specifically, non-HI-active antibodies that confer protection by Fc-mediated effector functions, like antibody-dependent cell-mediated cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP), and complement-dependent lysis (CDL), are of recent interest[23,24,26,35]. These mAbs cannot readily be detected by classical HI assays. For instance, it has been demonstrated that the non-HI-active, non-neutralizing mAbs 1H5-mediated and 1H10-mediated ADCC activity as measured in a bioreporter assay[27].

The North American and Eurasian lineage H7 HAs are genetically highly divergent[36]. However, antigenic site A is conserved, present on HAs of both lineages and a potentially important protective epitope[37]. As previously shown, antigenic site A is also recognized and targeted by the human immune system after vaccination with an H7N9 LAIV and IIV boost regimens[24,38]. The mAbs 1A8 and 1B2 that target antigenic site A were neutralizing, which allowed for the generation of escape mutants. Sequence analysis of the escape mutants showed that a change of amino acid at position 2 (H3 numbering position 149) or 3 and 4 (position 150, 151) of antigenic site A led to an escape. Another group showed the same amino acid change (R149G), leading to a loss of neutralization activity[37]. As demonstrated, naturally occurring mutations of amino acids at position 148 did not influence binding, function, or protective efficacy of mAbs raised against the wild-type sequence of the 2013 H7 HA (Shanghai).

In conclusion, we showed that antibodies raised against the H7 HA from a 2013 strain bound to H7 HAs of novel zoonotic viruses isolated in 2016 and 2017. These findings suggest that while the H7 HAs evolve; highly conserved epitopes on the H7 HA are still maintained, possibly because of a lack of antigenic pressure on the site in the avian host species. Ideally, the human immune response could be directed against these epitopes by vaccination to elicit cross-protection against several different H7 virus strains. Based on our data, it is likely that stockpiled vaccines based on the Shanghai virus strain could confer at least some protection against divergent, novel H7 viruses. Indeed, we recently showed that antibodies induced by vaccination with recombinant H7 HA from the A/Anhui/1/2013 strain react to the HAs of emerging H7 viruses[19]. Nevertheless, assessment of the antigenicity of emerging strains is of utmost importance to detect mismatches of vaccine strain and novel virus strains. Our findings help inform the development of pre-pandemic influenza vaccines and offer tools to characterize and evaluate new zoonotic or human IAV H7 virus strains and candidate vaccine viruses.

Material and methods

Cells, viruses, and proteins

Madin Darby canine kidney (MDCK) cells were grown and maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco) containing penicillin–streptomycin antibiotics mix (100 U/mL of penicillin, 100 µg/mL streptomycin; Gibco) and fetal bovine serum (10%; HyClone), resulting in complete DMEM (cDMEM). BTI-TN5B1-4 (*Trichoplusia ni*) cells were grown in serum-free SFX medium (HyClone) supplemented with antibiotics (100 U/mL of penicillin, 100 µg/mL streptomycin; Gibco).

Human embryonic kidney cells (293T) were grown in cDMEM. The H7 low pathogenic virus reassortants were generated by plasmid-based reverse genetic techniques as described previously[28]. Briefly, the H7 HA of A/Hunan/02285/2017 and A/Hong Kong/2014/2017 were combined, respectively, with seven genomic segments of A/Puerto Rico/8/1934 (PR8) resulting in 7:1 reassortants. The H7 and N2 of the A/feline/New York/16-040082/2016 were combined with the six internal segments of PR8 producing a 6:2 reassortant. The PR8 backbone was chosen because it is attenuated in humans (but not in mice), does not confer a transmittable phenotype and is generally regarded as safe in humans, poultry, and ferrets[39–43]. The HA cDNAs were synthetically generated (Thermo Fisher), and all recombinant viruses produced by reverse genetics were sequenced to confirm genotype. None of the rescued viruses contained a polybasic cleavage site in their HA sequence. The viruses were grown in 8–10-day-old embryonated chicken eggs (Charles River Laboratories) for 48 h at 37 °C and the allantoic fluid harvested. The recombinant proteins, including H7 from A/Hunan/02285/2017, A/Hong Kong/2014/2017, A/feline/New York/16-040082/2016, A/turkey/Indiana/16-001403-1/2016, and A/Guangdong/17SF003/2016 were expressed in the baculovirus expression system as described previously[44,45]. To increase recombinant protein stability, the polybasic cleavage sites of the HA of the highly pathogenic isolates A/turkey/Indiana/16-001403-1/2016 and A/Guangdong/17SF003/2016 were removed. The resulting sequences have regular monobasic low pathogenic avian influenza H7 cleavage sites.

mAb generation and purification

The H7-specific mAbs 1A8, 1B2, 1H5, and 1H10 were generated by hybridoma technology as previously described[27]. The antibodies were purified from 800 mL culture supernatant via sepharose G columns using a standard protocol[46].

Enzyme-linked immunosorbent assay

Ninety six-well microtiter plates (Thermo Fisher) were coated with 50 µL recombinant protein at a concentration of 2 µg/mL in coating buffer (KPL) overnight at 4 °C. The next day, 220 µL blocking solution (phosphate-buffered saline (PBS; Gibco) supplemented with 0.1% Tween-20 (T-PBS; Fisher Scientific), 0.5% milk powder (American-Bio), and 3% goat serum (Life Technologies)) were added to all wells of the microtiter plates and incubated for 1 h at room temperature. mAbs were diluted to a starting concentration of 10 µg/mL, serially diluted 1:3, and incubated for 2 h at room temperature. The microtiter plates were washed three times with T-PBS and 50 µL anti-mouse IgG (whole molecule) peroxidase antibody (produced in rabbit; Sigma, #A9044) diluted 1:3000 in blocking solution was added to all wells and incubated for 1 h at room temperature. The microtiter 96-well plates were washed four times with T-PBS and were developed with 100 µL/well SigmaFast *o*-phenylenediamine dihydrochloride (Sigma). After 10 min the reaction was stopped with 50 µL 3 M hydrochloric acid (Thermo Fisher) and the plates were read at 490 nm with a microtiter plate reader (Bio-Tek). The data were analyzed in Microsoft Excel and GraphPad Prism. The cutoff value was defined as the average of all blank wells plus three times the standard deviation of the blank wells and the area under curve values were calculated.

HI assay

The mAbs were diluted to an initial concentration of 30 µg/mL in PBS and serially diluted 1:3 in V-bottom plates 96-well plates (Thermo Fisher). The viruses were diluted to 8 hemagglutination units/50 µL in PBS and 25 µL of virus was added to the serially diluted 25 µL/well of mAb dilutions. The plates were incubated at room temperature for 30 min on a shaker. Chicken red blood cells (RBCs; Lampire Biologicals) were diluted to a concentration of 0.5% in PBS and 50 µL was added to each well of the V-bottom plates. The plates were incubated at 4 °C until the formation of red pellets on the bottom of the wells of the negative control wells were visible (45–60 min). The minimal HI concentration was defined as the last dilution (concentration of antibody) in which hemagglutination does not occur. The results were analyzed in Microsoft Excel and GraphPad Prism 7.

Microneutralization assay

MDCK cells (100 µL/well) were seeded at a concentration of 2×10^5 cells/mL in 96-well cell culture plates (Sigma) and incubated at 37 °C for 12 h. The mAbs were diluted to a starting concentration of 30 µg/mL in PBS and serially diluted 1:2 in UltraMDCK media (Lonza) supplemented with tosyl phenylalanyl chloromethyl ketone-treated trypsin (infection media; Sigma) at a concentration of 1 µg/mL, in 96-well cell culture plates (Sigma). The viruses were diluted to a concentration of $100 \times TCID_{50}$/50 µL (A/Hunan/02285/2017 (Hunan), A/feline/New York/16-040082/2016 (New York), A/Hong Kong/2014/2017 (Hong Kong)) in infection medium. Next, 60 µL of virus dilution was incubated with 60 µL of mAb serial dilution and incubated on the shaker at room temperature for 1 h. The plates were incubated at 33 °C for 48h (New York) or 72 h (Hunan, Hong Kong). The readout was performed by the means of classical hemagglutination assay. This readout was chosen because it is more objective than assessment of cytopathic effects but easier to perform than staining for virus antigen (e.g., for nucleoprotein). In brief, chicken RBCs (Lampire Biological Laboratories) was diluted to a concentration of

0.5% in PBS and added to 50 µL of cell supernatant in v-bottom plates (Corning). After 45–60 min the plates were scanned and the results analyzed in Microsoft Excel and GraphPad Prism 7.

Passive transfer experiments in mice

Passive transfer experiments were performed to test prophylactic and therapeutic efficacy of the mAbs. For the prophylactic setting, 6–8-week-old female BALB/c mice ($n = 5$ mice/group) were given 150 µL mAb 1A8, 1B2, 1H5, or 1H10, at a concentration of 1 mg/kg intraperitoneally. The negative control mice received 150 µL of irrelevant IgG (anti-Ebola virus glycoprotein mAb 2E5[47]) control at a concentration of 1 mg/kg. Two hours post transfer, the mice were anesthetized with a ketamine–xylazine–water mixture (0.15 mg ketamine/kg of body weight and 0.03 mg/kg xylazine; 100 µl intraperitoneally) and challenged intranasally with $5 \times LD_{50}$ (1265 $TCID_{50}$/50 µL H7N1; A/Hunan/02285/2017), $5 \times LD_{50}$ (1250 $TCID_{50}$/50 µL H7N1; A/Hong Kong/2014/2017), or $2 \times LD_{50}$ (6.32×10^4 $TCID_{50}$/50 µL H7N2; A/feline/New York/16-040082/2016). Blood was drawn and analyzed by ELISA as previously described to confirm successful antibody transfer[26]. The weight loss was monitored daily for 14 days. The humane endpoint was defined as a loss of 25% of the day 0 weight. To test the therapeutic effect of the antibodies mice were infected with $5 \times LD_{50}$. MAbs 1H5, 1B2 and negative control mAb 2E5 (anti-Ebola virus glycoprotein mAb) were administered 48 or 72 h post infection at a concentration of 5 mg/kg. Weight loss was monitored for 14 days and mice that lost 25% or more of their initial body weight were euthanized according to institutional guidelines. For the determination of reduction of lung viral titers, mice were infected with $0.1 \times LD_{50}$ (3.16×10^3 $TCID_{50}$/50 µL) H7N2 A/feline/New York/16-040082/2016 (xPR8) virus. Two hours prior infection, mAbs 1A8, 1B2, 1H5, and 1H10, at a concentration of 5 mg/kg and a negative IgG control (anti-Ebola virus glycoprotein), were transferred via intraperitoneal injection. At day 3 ($n = 3$ mice/mAb) and day 6 ($n = 3$ mice/mAb) post infection, lungs were harvested and homogenized using a BeadBlaster24 (Benchmark). Lung virus titers were assessed by injecting dilutions (1:5, 1:50, 1:500, 1:5000, 1:50,000, 1:500,000) of lung homogenate into 8-day-old embryonated chicken eggs (Charles River Laboratories) and incubation for 48 h at 37 °C. The eggs were harvested and presence or absence of virus determined by classical HA readout as described before[19]. The EID_{50} was calculated in Microsoft Excel and GraphPad Prism 7.

HA sequences

The sequences for the analysis of antigenic site A of the H7 HA were downloaded from the Influenza Resource Database (www.fludb.org) and the Global Initiative on Sharing Avian Influenza Data (www.gisaid.org).

Statistical analysis

Statistical analysis was performed in GraphPad Prism 7. Data are shown as geometric means. Differences in lung virus titers were compared in a one-way analysis of variance (ANOVA) with a Sidak post test for multiple comparisons. The sequences for the phylogenetic tree were assembled in Clustal Omega and visualized in FigTree.

Data availability

The data that support the findings of this study are available from the corresponding author upon request.

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Conflict of interest

The authors declare that they have no conflict of interest.

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References

1. Molinari, N.-A. M. et al. The annual impact of seasonal influenza in the US: measuring disease burden and costs. *Vaccine* **25**, 5086–5096 (2007).
2. http://www.who.int/mediacentre/factsheets/fs211/en/ (2017).
3. Danqi, B., Li, Z., Liu, Q. & Richt, J. A. H7N9 avian influenza A virus in China: a short report on its circulation, drug resistant mutants and novel antiviral drugs. *Expert. Rev. Anti. Infect. Ther.* **15**, 723–727 (2017).
4. Organisation, W. H. (2017). http://www.who.int/influenza/human_animal_interface/Influenza_Summary_IRA_HA_interface_09_27_2017.pdf?ua=1
5. Tanner, W. D., Toth, D. J. & Gundlapalli, A. V. The pandemic potential of avian influenza A(H7N9) virus: a review. *Epidemiol. Infect.* **143**, 3359–3374 (2015).
6. Subbarao, K. Avian influenza H7N9 viruses: a rare second warning. *Cell Res.* **28**, 1–2 (2017).
7. Yang, L. et al. Genesis and spread of newly emerged highly pathogenic H7N9 avian viruses in Mainland China. *J Virol* **91**, e01277–17 (2017).
8. Wang, D. et al. Two outbreak sources of influenza A (H7N9) viruses have been established in China. *J. Virol.* **90**, 5561–5573 (2016).
9. Kile, J. C. et al. Update: increase in human infections with novel Asian lineage avian influenza A(H7N9) viruses during the fifth epidemic—China, October 1, 2016–August 7, 2017. *Morb. Mortal. Wkly. Rep.* **66**, 928–932 (2017).
10. Lee, D. H., Torchetti, M. K., Killian, M. L. & Swayne, D. E. Deep sequencing of H7N8 avian influenza viruses from surveillance zone supports H7N8 high pathogenicity avian influenza was limited to a single outbreak farm in Indiana during 2016. *Virology* **507**, 216–219 (2017).
11. Pantin-Jackwood, M. J., Stephens, C. B., Bertran, K., Swayne, D. E. & Spackman, E. The pathogenesis of H7N8 low and highly pathogenic avian influenza viruses from the United States 2016 outbreak in chickens, turkeys and mallards. *PLoS ONE* **12**, e0177265, (2017).
12. Marinova-Petkova, A. et al. Avian influenza A(H7N2) virus in human exposed to sick cats, New York, USA, 2016. *Emerg. Infect. Dis.* **23**, 2046–2049 (2017).
13. Nachbagauer, R. et al. Defining the antibody cross-reactome directed against the influenza virus surface glycoproteins. *Nat. Immunol.* **18**, 464–473 (2017).
14. Osterholm, M. T., Ballering, K. S. & Kelley, N. S. Major challenges in providing an effective and timely pandemic vaccine for influenza A(H7N9). *JAMA* **309**, 2557–2558 (2017).
15. Zhang, W. et al. Co-infection with avian (H7N9) and pandemic (H1N1) 2009 influenza viruses, China. *Emerg. Infect. Dis.* **21**, 715–718 (2015).

16. Couch, R. B., Patel, S. M., Wade-Bowers, C. L. & Nino, D. A randomized clinical trial of an inactivated avian influenza A (H7N7) vaccine. *PLoS ONE* **7**, e49704 (2012).
17. Cox, R. J. et al. A phase I clinical trial of a PER.C6 cell grown influenza H7 virus vaccine. *Vaccine* **27**, 1889–1897 (2009).
18. Krammer, F. et al. An H7N1 influenza virus vaccine induces broadly reactive antibody responses against H7N9 in humans. *Clin. Vaccin. Immunol.* **21**, 1153–1163 (2014).
19. Stadlbauer, D. et al. Vaccination with a recombinant H7 hemagglutinin-based influenza virus vaccine induces broadly reactive antibodies in humans. *mSphere* **2**, e00502-17, (2017).
20. Rudenko, L. et al. H7N9 live attenuated influenza vaccine in healthy adults: a randomised, double-blind, placebo-controlled, phase 1 trial. *Lancet Infect. Dis.* **16**, 303–310 (2016).
21. Rudenko, L. et al. Assessment of human immune responses to H7 avian influenza virus of pandemic potential: results from a placebo-controlled, randomized double-blind phase I study of live attenuated H7N3 influenza vaccine. *PLoS ONE* **9**, e87962 (2014).
22. Krammer, F. & Cox, R. J. The emergence of H7N9 viruses: a chance to redefine correlates of protection for influenza virus vaccines. *Expert. Rev. Vaccin.* **12**, 1369–1372 (2013).
23. Cox, R. J. Correlates of protection to influenza virus, where do we go from here? *Hum. Vaccin. Immunother.* **9**, 405–408 (2013).
24. Henry Dunand, C. J. et al. Both neutralizing and non-neutralizing human H7N9 influenza vaccine-induced monoclonal antibodies confer protection. *Cell Host Microbe* **19**, 800–813 (2016).
25. Leon, P. E. et al. Optimal activation of Fc-mediated effector functions by influenza virus hemagglutinin antibodies requires two points of contact. *Proc. Natl Acad. Sci. USA* **113**, E5944–e5951 (2016).
26. Jacobsen, H. et al. Influenza virus hemagglutinin stalk-specific antibodies in human serum are a surrogate marker for in vivo protection in a serum transfer mouse challenge model. *MBio* **8**, e01463-17, (2017).
27. Tan, G. S. et al. Broadly-reactive neutralizing and non-neutralizing antibodies directed against the H7 influenza virus hemagglutinin reveal divergent mechanisms of protection. *PLoS Pathog.* **12**, e1005578 (2016).
28. Martínez-Sobrido, L. & García-Sastre, A. Generation of recombinant influenza virus from plasmid DNA. *J. Vis. Exp.* (2010).
29. Li, J. et al. Human co-infection with avian and seasonal influenza viruses, China. *Emerg. Infect. Dis.* **20**, 1953–1955 (2014).
30. White, M. C. & Lowen, A. C. Implications of segment mismatch for influenza A virus evolution. *J. Gen. Virol.* **99**, 3–16 (2017).
31. Stadlbauer, D., Nachbagauer, R., Meade, P. & Krammer, F. Universal influenza virus vaccines: what can we learn from the human immune response following exposure to H7 subtype viruses? *Front. Med.* **11**, 471–479 (2017).
32. Nachbagauer, R. & Krammer, F. Universal influenza virus vaccines and therapeutic antibodies. *Clin. Microbiol. Infect.* **23**, 222–228 (2017).
33. Krammer, F. & Palese, P. Advances in the development of influenza virus vaccines. *Nat. Rev. Drug. Discov.* **14**, 167–182 (2015).
34. Salazar, G., Zhang, N., Fu, T.-M. & An, Z. Antibody therapies for the prevention and treatment of viral infections. *NPJ Vaccin.* **2**, 19 (2017).
35. DiLillo, D. J., Palese, P., Wilson, P. C. & Ravetch, J. V. Broadly neutralizing anti-influenza antibodies require Fc receptor engagement for in vivo protection. *J. Clin. Invest.* **126**, 605–610 (2016).
36. Krammer, F. et al. Divergent H7 immunogens offer protection from H7N9 virus challenge. *J. Virol.* **88**, 3976–3985 (2014).
37. Schmeisser, F. et al. Antibodies to Antigenic Site A of Influenza H7 Hemagglutinin Provide Protection against H7N9 Challenge. *PLoS ONE* **10**, e0117108 (2015)
38. Chen, Z. et al. Development of a high-yield live attenuated H7N9 influenza virus vaccine that provides protection against homologous and heterologous H7 wild-type viruses in ferrets. *J. Virol.* **88**, 7016–7023 (2014).
39. Matsuoka, Y. et al. Safety evaluation in chickens of candidate human vaccines against potential pandemic strains of influenza. *Avian Dis.* **47**, 926–930 (2003).
40. Beare, A. S., Schild, G. C. & Craig, J. W. Trials in man with live recombinants made from A/PR/8/34 (H0 N1) and wild H3 N2 influenza viruses. *Lancet* **2**, 729–732 (1975).
41. Rodriguez, A. et al. Attenuated strains of influenza A viruses do not induce degradation of RNA polymerase II. *J. Virol.* **83**, 11166–11174 (2009).
42. Campbell, P. J. et al. The M segment of the 2009 pandemic influenza virus confers increased neuraminidase activity, filamentous morphology, and efficient contact transmissibility to A/Puerto Rico/8/1934-based reassortant viruses. *J. Virol.* **88**, 3802–3814 (2014).
43. Belser, J. A. et al. Pathogenicity testing of influenza candidate vaccine viruses in the ferret model. *Virology* **511**, 135–141 (2017).
44. Krammer, F. et al. A carboxy-terminal trimerization domain stabilizes conformational epitopes on the stalk domain of soluble recombinant hemagglutinin substrates. *PLoS ONE* **7**, e43603 (2012).
45. Margine, I., Palese, P. & Krammer, F. Expression of functional recombinant hemagglutinin and neuraminidase proteins from the novel H7N9 influenza virus using the baculovirus expression system. *J. Vis. Exp.* **81**, e51112, (2013).
46. Tan, G. S. et al. A pan-H1 anti-hemagglutinin monoclonal antibody with potent broad-spectrum efficacy in vivo. *J. Virol.* **86**, 6179–6188 (2012).
47. Duehr, J. et al. Novel cross-reactive monoclonal antibodies against Ebola virus glycoproteins show protection in a murine challenge model. *J. Virol.* **91**, e00652-17, (2017).
48. Wu, Y. et al. A potent broad-spectrum protective human monoclonal antibody crosslinking two haemagglutinin monomers of influenza A virus. *Nat. Commun.* **6**, 7708, https://doi.org/10.1038/ncomms8708 (2015).

MANUSCRIPT 3

Universal influenza virus vaccines: what can we learn from the human immune response following exposure to H7 subtype viruses?

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REVIEW

Universal influenza virus vaccines: what can we learn from the human immune response following exposure to H7 subtype viruses?

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Abstract **Several universal influenza virus vaccine candidates based on eliciting antibodies against the hemagglutinin stalk domain are in development. Typically, these vaccines induce responses that target group 1 or group 2 hemagglutinins with little to no cross-group reactivity and protection. Similarly, the majority of human anti-stalk monoclonal antibodies that have been isolated are directed against group 1 or group 2 hemagglutinins with very few that bind to hemagglutinins of both groups. Here we review what is known about the human humoral immune response to vaccination and infection with H7 subtype influenza viruses on a polyclonal and monoclonal level. It seems that unlike vaccination with H5 hemagglutinin, which induces antibody responses mostly restricted to the group 1 stalk domain, H7 exposure induces both group 2 and cross-group antibody responses. A better understanding of this phenomenon and the underlying mechanisms might help to develop future universal influenza virus vaccine candidates.**

Keywords universal influenza virus vaccine; hemagglutinin stalk; H7N9

Introduction

Influenza viruses cause annual epidemics and, in irregular intervals, pandemics in the human population. Viral infection in humans leads to respiratory disease that can be associated with severe morbidity and mortality with up to half a million deaths caused by seasonal influenza viruses every year globally [1]. Pandemics are usually associated with higher numbers of deaths. An example is the 1918/19 H1N1 pandemic which caused an estimated 40 million deaths worldwide [2]. Influenza viruses are a significant challenge for vaccine design due to their ability to mutate and escape antibody-based immunity through introduction of antigenic changes in their surface glycoproteins hemagglutinin (HA) and neuraminidase (NA). The HA, specifically the immunodominant, membrane distal globular head domain, is the major target of influenza virus vaccines (Fig. 1A) [2]. The globular head domain harbors the receptor binding site of the virus and antibodies that block this site prevent the virus from attaching to host cells and therefore neutralize it. Unfortunately, this domain has a very high plasticity [3,4] and the virus escapes the antibody response by introducing point mutations that lead to changes in the major antigenic sites on the head domain, a phenomenon called antigenic drift. Therefore, seasonal influenza virus vaccines need to be updated frequently [5]. The situation is even worse for emerging pandemic viruses for which matched vaccines need to be manufactured, a process that usually takes up to six months during which the population is vulnerable to infection [2]. These problems have led to major initiatives to develop a universal influenza virus vaccine that would protect from all antigenically drifted seasonal influenza viruses, zoonotic influenza virus infections, and pandemic influenza viruses [6,7]. Several conserved targets for such a vaccine have been proposed including the membrane proximal stalk domain (Fig. 1A) of the HA which evolves much slower than the head domain [8]. In fact, antibodies against this domain have been isolated from mice and humans and can neutralize a

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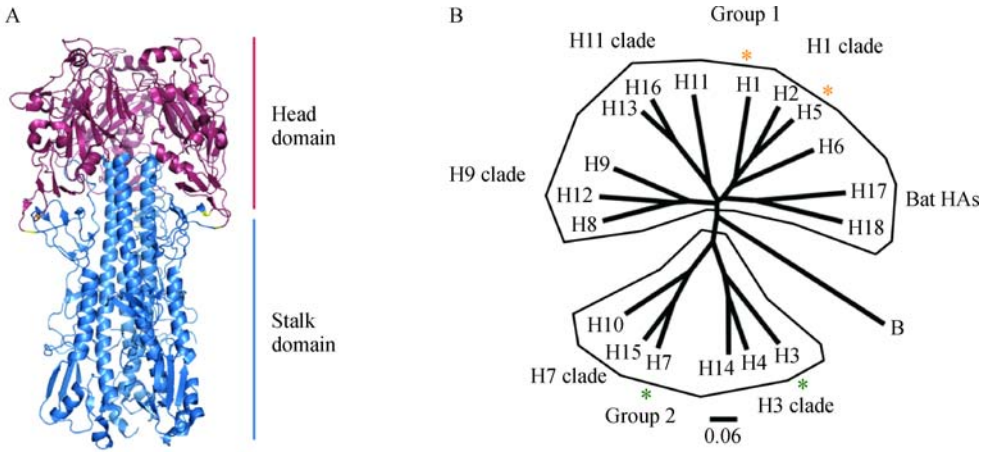

Fig. 1 Structure of influenza A virus hemagglutinin and phylogenetic tree of influenza A and B hemagglutinins. (A) An HA timer with the membrane distal globular head domain visualized in dark red and the membrane proximal stalk domain shown in blue. Cysteines 52 and 277 (H3 numbering), which are the demarcation line between head and stalk, are shown in yellow. The figure is based on PDB # 1TI8 [27]. (B) Phylogenetic tree of influenza A and B hemagglutinins based on amino acid sequence. Influenza A HAs are separated into groups 1 and 2 based on their sequence. HA clades and subtypes are annotated. H1 and H5 (group 1) and H3 and H7 (group 2) are marked by stars. The scale bar represents % amino acid difference. The sequences were assembled in Clustal Omega and visualized in FigTree.

broad panel of influenza virus subtypes and strains. The majority of these broadly neutralizing antibodies bind to either the stalk of influenza A group 1 HAs (H1, H2, H5, H6, H8, H9, H11, H12, H13, H16, H17, H18) [9–11] or to the stalk of influenza A group 2 HAs (H3, H4, H7, H10, H14, H15) [12,13] with some notable exceptions that bind to both groups [14–16] or even all influenza A and B HAs [17]. It has been noted that exposure of humans with pre-existing immunity to pre-pandemic seasonal H1N1 to novel influenza virus strains/subtypes from group 1 that feature drastically different head domains, but conserved epitopes in the stalk domain leads to preferential induction of these broadly neutralizing stalk-reactive antibodies. This has been shown for infection and vaccination with 2009 pandemic H1N1 (which has a very different head domain compared to pre-pandemic seasonal H1N1) and for vaccination with H5N1 vaccines [18–25]. These observations have led to vaccine strategies based on chimeric HAs which have "exotic" head domains, but stalk domains from e.g. H1 or H3 HAs. Sequential vaccination with these constructs in animal models also leads to the preferential induction of broadly neutralizing antibodies [26]. While several studies have elucidated the immune response after sequential exposure to divergent group 1 HAs in humans, studies about sequential exposure to group 2 HAs are sparse. Here we will review what is known about the immune response after H7 (group 2) exposure in humans that have been primed by infection and/or vaccination with seasonal H3N2 (group 2) viruses. Lessons learned from these studies might inform further development of group 2 targeted universal influenza virus vaccine candidates.

Pre-existing immunity to H7 HA on a polyclonal and monoclonal level

The human population is constantly exposed to seasonal H3N2 viruses through both vaccination and infection. H3N2 has been circulating in humans ever since it caused a pandemic in 1968 ("Hong Kong Flu") and can cause severe morbidity and also mortality. Therefore, the human population is primed for group 2 HAs. However, sera from human individuals have little or no baseline neutralizing activity to H7 subtype viruses [28–30]. This is not very surprising since most of the immune response induced by H3N2 exposure is directed toward the globular head domain [31] and H3 and H7 globular head domains only share approximately 30% amino acid identity [32]. In addition, H3 and H7 HAs are relatively far apart phylogenetically within group 2 HAs, with H3 being a member of the H3 clade (with H4 and H14) and H7 being a member of the H7 clade (with H10 and H15) (Fig. 1B). However, H3N2 infections (and potentially to a lower degree, vaccination) induces low levels of group 2 stalk-reactive antibodies in humans [28,33]. Of note, these levels are substantially lower than group 1 stalk reactive antibodies, likely because circulation of very different group 1 HA expressing viruses (H1N1, H2N2, pandemic H1N1) has boosted stalk titers against group 1 HAs to higher levels [34,35]. In fact, the reactivity to H7 is lower than most other group 2 or group 1 HA independently of the age group (Fig. 2). Nevertheless, some cross-reactivity to H7 HA has been detected in serum of human individuals [28]. This also translates to the monoclonal level. In a

recent study Henry Dunand and colleagues found that of 83 H3 reactive antibodies isolated from plasmablasts after vaccination only six reacted to H7 [36]. Three of these six antibodies showed neutralizing activity and protected mice from a lethal challenge with H7N9 virus. Interestingly, two of the mAbs also reacted with group 1 HAs while one had a pan-group 2 profile. It is interesting to note that two of the mAbs were isolated after seasonal influenza virus vaccination (containing H1N1, H3N2 and influenza B components) while the third, cross-group mAb was isolated after vaccination with monovalent pH1N1 vaccine. The cross-group mAbs both featured variable heavy (VH) segments from the VH1-18 heavy chain germline [37]. Similarly, low numbers of H7 reactive memory-B cells were found in the same study. While human serum has low reactivity to H7, this reactivity is boosted to some degree after H3N2 infection [28]. However, even then antibody levels remain low. In summary, low levels of antibodies and B cells with specificities to H7 HA exist in humans, and the majority of this reactivity is likely induced by H3N2 exposure and directed to the stalk domain of HA. Of note, recent work by Gostic and colleagues hypothesized that group 2 imprinting by H3N2 infection during childhood could explain protection of certain segments of the population from severe infections and death caused by H7N9 viruses [38]. The authors speculate that this could be driven by antibodies to the group 2 stalk. This is an intriguing thought and while reactivity to H7 HA is very

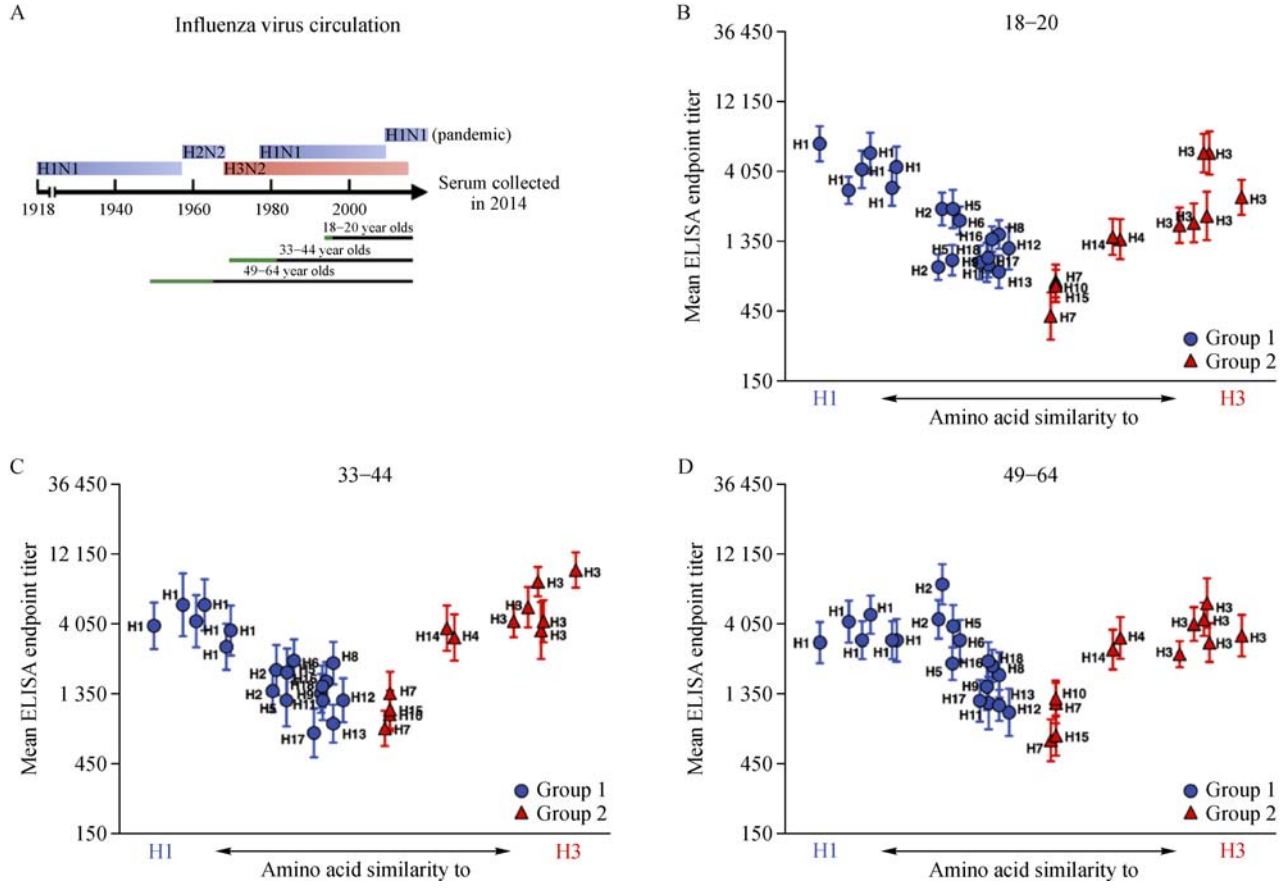

Fig. 2 Reactivity of human sera of different age groups to diverse influenza virus HAs. (A) Human serum samples from three different age cohorts were tested by ELISA against recombinant HA proteins including all influenza A subtypes. The birth ranges for each cohort are indicated in green for 18 – 20 year olds (n = 30), 33 – 44 year olds (n = 30) and 49 – 64 year olds (n = 30). The blue bars indicate circulating group 1 viruses and the red bars indicate circulating group 2 viruses. Serum samples were collected in 2014. (B–D) The ELISA endpoint titers are shown on the y-axis. Each point indicates the geometric mean titer of 30 individuals. The error bars show the 95% confidence intervals. Blue circles show group 1 HAs and red triangles show group 2 HAs. The x-axis indicates the difference of the analyzed HAs to both H1 (A/New Caledonia/20/99; NC99) and H3 (A/Philippines/2/1982; Phil82). The percent similarities for each strain were calculated and the percent difference to Phil82 was subtracted from the percent difference to NC99 for each HA. This resulted in an alignment that shows HAs more closely related to H1, but more distantly related to H3 on the left side and vice versa. HAs that are distantly related to both H1 and H3 are shown toward in the middle of the graph. (B) Sera from 18 to 20 year olds. (C) Sera from 33 to 44 year olds. (D) Sera from 49 to 64 year olds. Figures are adapted from Ref. [28].

low in all age groups (including those likely first exposed in life to H3N2; Fig. 2) this phenomenon might be driven by memory B cells for which antibody products are not readily detected in serum.

Breadth of the polyclonal immune response after H7 vaccination

Before H7N9 emerged in 2013 [39,40], very few vaccine trials with H7 HA had been conducted. The general consensus from these trials was that H7 HAs are of very low immunogenicity, even lower than H5 HAs [29,41,42]. Similar observations have been made with H7N9 vaccines. However, the use of adjuvants [30], virus-like particles [43], or heterologous prime-boost regimens [44,45] improved the immunogenicity but results still lagged behind what is typically observed for seasonal influenza virus vaccines. Importantly, the typical readout used in these trials is the hemagglutination inhibition (HI) assay. In addition, microneutralization (MN) assays are now used to assess the induction of H7 specific immunity, but results from these assays — unlike results from the HI assay— are not accepted by regulatory agencies as "surrogates" or "correlates" of protection. The HI assay only detects antibodies against the globular head domain, while the MN assay detects mostly antibodies against the head domain but might detect stalk-reactive antibodies as well, when they are present at high levels. Both assays usually miss the induction of binding but non-neutralizing antibodies as well as levels of neutralizing antibodies below the limit of detection. Importantly, these types of antibodies might still provide protection, e.g. through effector functions, as demonstrated by several studies [46–49]. Enzyme-linked immunosorbent assays (ELISAs) were used to assess the immune response in only very few studies. These assays detect all antibodies that bind a certain HA, including antibodies that are not detected in HI and MN assays. A study based on an H7N1 inactivated vaccine prime-boost regimen found strong cross-reactivity induced by ELISA, while HI or MN activity was negligible [50]. The antibody response also extended to H15 and an induction of group 2 stalk-reactive antibodies was detected (albeit at low levels). An induction of antibodies to seasonal H1 HA was not detected. Another study examined the antibody response in humans vaccinated twice with an H7N7 live attenuated vaccines (LAIV) followed by an H7N7 inactivated vaccine (IIV) [51]. An antibody response against both the H7 head domain as well as the stalk domain was detected by several methods including analysis of memory B cell frequency and by mapping via a phage library. The memory B cell frequency against pandemic H1 HA was also measured but no significant increase was detected. From these limited data it can be concluded that H7 vaccines induce detectible levels of group 2 stalk-reactive antibodies.

Breadth of the polyclonal immune response after H7N9 infection

So far, two studies have analyzed the breadth of the antibody response after H7N9 infection in humans. In an early paper in 2014 Guo and colleagues analyzed sera from samples from 21 patients and found an increase of H7 binding antibodies, HI and MN titers post H7N9 infection [52]. Interestingly, they also detected an increase in H3 and pandemic H1 antibody binding by ELISA. The increase was higher and longer lasting for H3, which would be expected since it can be hypothesized that H7 exposure in H3 experienced individuals would boost stalk-reactive antibodies. The induction of antibodies to pandemic H1 HA however is highly unusual. A second, more detailed study that examined sero-reactivity of 18 individuals infected with H7N9 was recently published [53]. Liu and colleagues tested binding to all HA subtypes and found that H7N9 infection boosted binding antibody titers (measured by ELISA) against group 1 and group 2 HAs (but not influenza B virus HA). As expected, overall stronger induction (and higher absolute titers) was observed for group 2 HAs but reactivity against several group 1 HAs, specifically pandemic H1, was strongly induced as well. Of note, this was also reflected in neutralization titers which increased against many subtypes including pandemic H1N1.

These are important findings since they suggest that infection with H7N9 induces a much broader immune response than infection with H3N2 or even vaccination with H7 vaccines, which induce some group 2 specific stalk antibodies on a polyclonal basis. The response was comparable to the primary response to infection with pandemic H1N1 in humans previously exposed to seasonal HIN1, which can also trigger cross-group HA responses [28]. A direct comparison with the immune response after H5N1 vaccination cannot be made since only very limited data from H5N1 survivors exists [54].

It is important to keep in mind that the analyzed cohorts typically consisted of elderly individuals who were likely exposed to influenza viruses many times. In addition, many of the H7N9 infected individuals frequented wet markets, which increases the chance that they were exposed to avian influenza viruses of different subtypes in the past and had therefore a skewed immune response already. Finally, most of these individuals suffered from co-morbidities which might have influenced their immune responses in unknown ways.

Analysis of the monoclonal immune response after H7N9 vaccination

Three studies have so far analyzed the monoclonal antibody response to H7N9 vaccination. An initial study

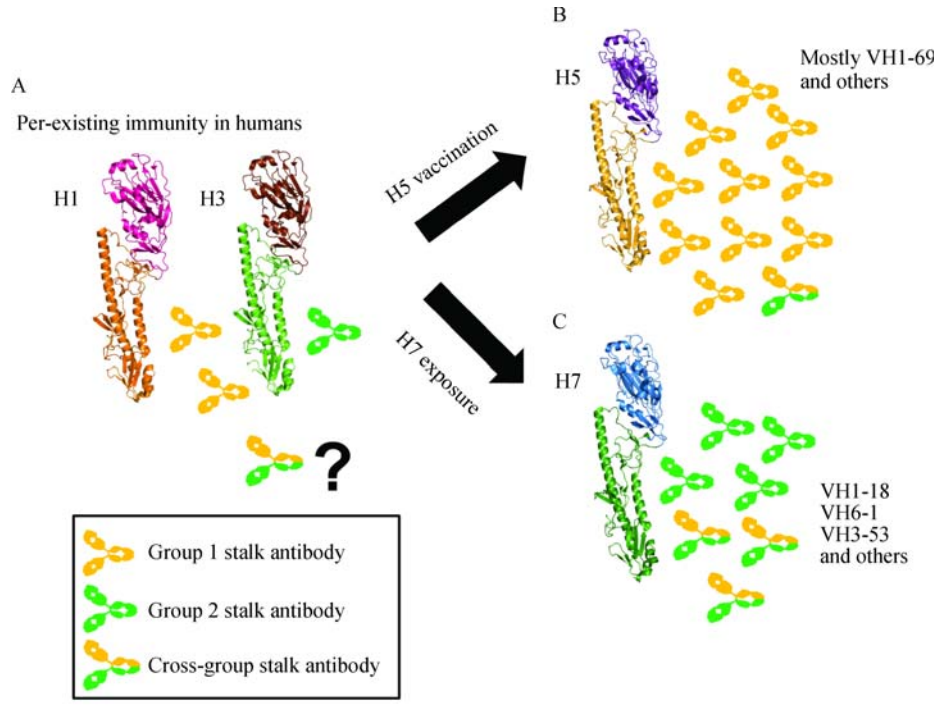

Fig. 3 The immune response of human individuals with pre-existing H1 and H3 immunity to H5 vaccination or H7 exposure. Hemagglutinins (H1, H3, H5, H7) are shown as monomers (based on PDB # 1TI8) for simplicity. (A) Low levels of pre-existing anti-stalk immunity to H1 (pink head domain, orange stalk domain, group 1 HA) and to H3 (brown head, green stalk, group 2) exists in the human population. Typically anti-group 1 HA stalk antibody (yellow) levels are higher than anti-group 2 HA stalk antibody (green) levels. The baseline level of cross-group stalk antibodies (yellow and green) is unclear and likely very low. Antibodies binding to the globular head domain are not shown. (B) Vaccination with H5 HA (purple head, orange stalk) leads to a strong increase of mostly group 1 reactive stalk antibodies (biased toward the VH1-69 germline) and very few cross-group stalk antibodies. (C) Exposure to H7 HA (blue head, green stalk) induces fewer anti-stalk antibodies overall but a relatively larger proportion of cross-group reactive stalk antibodies (focused toward VH1-18, VH6-1 and other germlines).

analyzed mAbs derived from memory B cells of 12 donors who had received an inactivated split H7N9 vaccine (adjuvanted with MF59 or AS03) twice. Twenty hybridomas were obtained and 11 mAbs were further characterized [55]. Eight mAbs showed binding that was restricted to H7 HAs, with 5 of them having classical HI activity. Three mAbs showed broader binding activity with two binding broadly to group 2 HAs and one binding to both group 2 and group 1 HAs. One of the group 2 cross-reactive mAbs showed strong neutralization against H3N2 while the other two seemed to be non-neutralizing antibodies, with the caveat that neutralizing activity was only tested up to a concentration of 2 μg/mL. This concentration might not be in the range of many anti-stalk antibodies which are typically less potent neutralizers as compared to HI-active antibodies [56,57]. Another study analyzed mAbs isolated from plasmablasts after a vaccination regimen that included two H7N9 LAIV inoculations followed by an inactivated H7N9 vaccine [46]. Of 12 analyzed mAbs, eight showed specificity (albeit mostly broad) for H7 and had HI and/or MN activity. One of these mAbs, an HI active antibody, cross-reacted to H15. Three mAbs had no neutralizing activity but showed broad binding to group 2 HAs, and in two cases to group 1 HAs. While these mAbs did not neutralize, they were still capable of protecting mice from a lethal challenge with H7N9 virus. The last antibody showed no HI activity but was neutralizing, and was identified as bona fide anti-stalk mAb. It bound strongly to group 2 HAs as well as pandemic H1 HA (and with lower affinity to some other group 1 HAs). Of note, this mAb featured a VH from the VH3-53 germline (see below). A third, very recent study [58] reported mAbs derived from memory B cells from a vaccine trial that tested H7 DNA prime-H7N9 inactivated vaccine boost vaccination regimens (with H7N9 inactivated vaccine prime-boost regimens or DNA plus inactivated vaccine twice as controls) [45]. Cells were obtained from all three vaccine groups and the authors stated that no difference was found between the groups. Memory B cells were sorted for reactivity to H7 and other HAs. The authors reported that the overall amount of cross-reactive cells was lower for H7 as compared to an H5 trial with a similar setup. However, while H5N1 vaccination induced approximately 50% group 1 stalk-reactive antibodies and very

little cross-group reactivity, the percentage of cross-group reactive antibodies was close to 25% in the H7 vaccinees. The response after H5N1 vaccination was skewed toward the VH1-69 germline, which is heavily over-represented in group 1 stalk-reactive antibodies. After H7N9 vaccination the response was skewed toward a VH1-18 response. In this study, several mAbs were cloned out and analyzed for signatures. mAbs with a VH1-18 germline, or a VH6-1 germline typically showed cross-group reactivity. However, while VH6-1 mAbs neutralized both H1N1 and H3N2, VH1-18 mAbs either neutralized H1N1 or H3N2 (more often). Another signature found were VH3-53 mAbs, which showed pan group 2 activity and neutralized H3N2, but also had an affinity toward pandemic H1 HA (with no binding to seasonal H1 and very little binding to other group 1 HAs). Interestingly these VH1-18 and VH3-53 mAbs with similar signatures had also been isolated in earlier studies from plasmablast responses to seasonal trivalent, pandemic H1N1 and H7N9 vaccines [36,46].

These data are highly interesting and suggest that the human immune system is capable of reacting to H7 exposure with both group 2 specific as well as cross-group anti-stalk responses. Of note, some bona fide group 2 stalk antibodies (VH3-53) also seem to have an unusual, selective affinity for pandemic H1— a signature that was also detected in the polyclonal response after H7N9 infection.

Conclusions

Based on the limited data available it becomes clear that the human immune response to H7 HA is — like the response to H5 HA — strongly influenced by H1 and H3 primed pre-existing memory-B cells that have specificities for conserved epitopes which are shared between human and avian influenza viruses. While H5N1 vaccination seems to produce strong responses of stalk antibodies that cross-react between group 1 HAs (with a skewed, VH1-69 signature), exposure to H7 HA seems to trigger a slightly different response. H7 vaccination, as analyzed so far, seems to induce a polyclonal serological response that mostly targets H7 and other group 2 HAs with a substantial proportion of group 2 stalk antibodies present. H7N9 infection triggers a polyclonal serum response that is much broader than the one induced by vaccination and includes induction of antibodies against group 2 and group 1 HAs with a notable reactivity to H1. On a monoclonal level, broadly reactive mAbs with VH1-18 signatures can be isolated from plasmablasts even after vaccination with seasonal trivalent inactivated or monovalent pH1N1 vaccine. Plasmablast responses after H7N9 vaccination also reveal that neutralizing (VH3-53) and non-neutralizing broadly protective antibodies are induced. Similarly, analysis of the memory B cell compartment after H7N9 vaccination revealed broadly binding and neutralizing mAbs, in many cases spanning group 1 and group 2 (VH6-1, VH1-18) or with pan-group 2 plus pH1 reactivity (VH3-53). After infection and on a cellular, monoclonal level these responses seem to be much broader than the VH1-69 dominated group 1 stalk response after H5N1 vaccination. Currently, it is unclear why there is a difference in the response to H5 as compared to the response to H7. The stalk structure of group 1 and group 2 HAs is slightly different and the positions of conserved glycans differ as well. It might be that germlines like VH1-18, VH3-53 and VH6-1 have structure/sequence constellations that facilitate interactions with the H7 (group 2) stalk while binding is not well supported by the VH1-69 germline.

It is important to realize that both plasmablast and memory B responses — on which the current data are mostly based — are not necessarily reflective of long-term serum antibody responses that are driven by long lived plasma cells in the bone marrow. Just because a memory B cell or plasmablast response produces antibodies with broad specificities does not necessarily mean that the same cells (or clonally related cells) will migrate to the bone marrow to become long lived plasma cells that provide constant levels of serum antibody. Additional serological analysis of H7N9 vaccine trials (including heterologous prime-boost regimens and adjuvanted vaccination) are needed to reach a better understanding of the long-term serum response. Nevertheless, these data — although based on only a handful of studies — suggest that a group 2 stalk antigen might be a viable option to induce broad protection against group 2 HA expressing viruses including H3N2, H7N9 and H10N8. These limited data also suggest that a very strong stalk-based immunity induced by a group 2 construct might provide some protection against group 1 HA expressing viruses, specifically pH1N1.

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Compliance with ethics guidelines

Daniel Stadlbauer declares no conflict of interest. Raffael Nachbagauer is named as inventor on patent applications filed by the Icahn School of Medicine at Mount Sinai regarding influenza virus vaccines. Philip Meade declares no conflict of interest. Florian Krammer is named as inventor patent on applications filed by the Icahn School of Medicine at Mount Sinai regarding influenza virus vaccines. This manuscript is a review article and does not involve a research protocol requiring approval by the relevant institutional review board or ethics committee.

References

1. WHO. WHO Fact sheet N°211. 2014
2. Krammer F, Palese P. Advances in the development of influenza virus vaccines. Nat Rev Drug Discov 2015; 14(3): 167–182
3. Heaton NS, Sachs D, Chen CJ, Hai R, Palese P. Genome-wide mutagenesis of influenza virus reveals unique plasticity of the hemagglutinin and NS1 proteins. Proc Natl Acad Sci USA 2013; 110(50): 20248–20253
4. Doud MB, Bloom JD. Accurate measurement of the effects of all amino-acid mutations on influenza hemagglutinin. Viruses 2016; 8 (6): E155
5. Gerdil C. The annual production cycle for influenza vaccine. Vaccine 2003; 21(16): 1776–1779
6. Berlanda Scorza F, Tsvetnitsky V, Donnelly JJ. Universal influenza vaccines: shifting to better vaccines. Vaccine 2016; 34(26): 2926–2933
7. Krammer F, García-Sastre A, Palese P. Is it possible to develop a "Universal" influenza virus vaccine? Toward a universal influenza virus vaccine: potential target antigens and critical aspects for vaccine development. Cold Spring Harb Perspect Biol 2017; a028845
8. Nachbagauer R, Krammer F. Universal influenza virus vaccines and therapeutic antibodies. Clin Microbiol Infect 2017; 23(4): 222–228
9. Ekiert DC, Bhabha G, Elsliger MA, Friesen RH, Jongeneelen M, Throsby M, Goudsmit J, Wilson IA. Antibody recognition of a highly conserved influenza virus epitope. Science 2009; 324(5924): 246–251
10. Sui J, Hwang WC, Perez S, Wei G, Aird D, Chen LM, Santelli E, Stec B, Cadwell G, Ali M, Wan H, Murakami A, Yammanuru A, Han T, Cox NJ, Bankston LA, Donis RO, Liddington RC, Marasco WA. Structural and functional bases for broad-spectrum neutralization of avian and human influenza A viruses. Nat Struct Mol Biol 2009; 16(3): 265–273
11. Throsby M, van den Brink E, Jongeneelen M, Poon LL, Alard P, Cornelissen L, Bakker A, Cox F, van Deventer E, Guan Y, Cinatl J, ter Meulen J, Lasters I, Carsetti R, Peiris M, de Kruif J, Goudsmit J. Heterosubtypic neutralizing monoclonal antibodies cross-protective against H5N1 and H1N1 recovered from human IgM + memory B cells. PLoS One 2008; 3(12): e3942
12. Friesen RH, Lee PS, Stoop EJ, Hoffman RM, Ekiert DC, Bhabha G, Yu W, Juraszek J, Koudstaal W, Jongeneelen M, Korse HJ, Ophorst C, Brinkman-van der Linden EC, Throsby M, Kwakkenbos MJ, Bakker AQ, Beaumont T, Spits H, Kwaks T, Vogels R, Ward AB, Goudsmit J, Wilson IA. A common solution to group 2 influenza virus neutralization. Proc Natl Acad Sci USA 2014; 111(1): 445–450
13. Ekiert DC, Friesen RH, Bhabha G, Kwaks T, Jongeneelen M, Yu W, Ophorst C, Cox F, Korse HJ, Brandenburg B, Vogels R, Brakenhoff JP, Kompier R, Koldijk MH, Cornelissen LA, Poon LL, Peiris M, Koudstaal W, Wilson IA, Goudsmit J. A highly conserved neutralizing epitope on group 2 influenza A viruses. Science 2011; 333(6044): 843–850
14. Corti D, Voss J, Gamblin SJ, Codoni G, Macagno A, Jarrossay D, Vachieri SG, Pinna D, Minola A, Vanzetta F, Silacci C, Fernandez-Rodriguez BM, Agatic G, Bianchi S, Giacchetto-Sasselli I, Calder L, Sallusto F, Collins P, Haire LF, Temperton N, Langedijk JP, Skehel JJ, Lanzavecchia A. A neutralizing antibody selected from plasma cells that binds to group 1 and group 2 influenza A hemagglutinins. Science 2011; 333(6044): 850–856
15. Wu Y, Cho M, Shore D, Song M, Choi J, Jiang T, Deng YQ, Bourgeois M, Almli L, Yang H, Chen LM, Shi Y, Qi J, Li A, Yi KS, Chang M, Bae JS, Lee H, Shin J, Stevens J, Hong S, Qin CF, Gao GF, Chang SJ, Donis RO. A potent broad-spectrum protective human monoclonal antibody crosslinking two haemagglutinin monomers of influenza A virus. Nat Commun 2015; 6: 7708
16. Gupta P, Kamath AV, Park S, Chiu H, Lutman J, Maia M, Tan MW, Xu M, Swem L, Deng R. Preclinical pharmacokinetics of MHAA4549A, a human monoclonal antibody to influenza A virus, and the prediction of its efficacious clinical dose for the treatment of patients hospitalized with influenza A. MAbs 2016; 8 (5): 991–997
17. Dreyfus C, Laursen NS, Kwaks T, Zuijdgeest D, Khayat R, Ekiert DC, Lee JH, Metlagel Z, Bujny MV, Jongeneelen M, van der Vlugt R, Lamrani M, Korse HJ, Geelen E, Sahin Ö, Sieuwerts M, Brakenhoff JP, Vogels R, Li OT, Poon LL, Peiris M, Koudstaal W, Ward AB, Wilson IA, Goudsmit J, Friesen RH. Highly conserved protective epitopes on influenza B viruses. Science 2012; 337 (6100): 1343–1348
18. Nachbagauer R, Wohlbold TJ, Hirsh A, Hai R, Sjursen H, Palese P, Cox RJ, Krammer F. Induction of broadly reactive anti-hemagglutinin stalk antibodies by an H5N1 vaccine in humans. J Virol 2014; 88(22): 13260–13268
19. Ellebedy AH, Krammer F, Li GM, Miller MS, Chiu C, Wrammert J, Chang CY, Davis CW, McCausland M, Elbein R, Edupuganti S, Spearman P, Andrews SF, Wilson PC, García-Sastre A, Mulligan MJ, Mehta AK, Palese P, Ahmed R. Induction of broadly cross-reactive antibody responses to the influenza HA stem region following H5N1 vaccination in humans. Proc Natl Acad Sci USA 2014; 111(36): 13133–13138
20. Wrammert J, Koutsonanos D, Li GM, Edupuganti S, Sui J, Morrissey M, McCausland M, Skountzou I, Hornig M, Lipkin WI, Mehta A, Razavi B, Del Rio C, Zheng NY, Lee JH, Huang M, Ali Z, Kaur K, Andrews S, Amara RR, Wang Y, Das SR, O'Donnell CD, Yewdell JW, Subbarao K, Marasco WA, Mulligan MJ, Compans R, Ahmed R, Wilson PC. Broadly cross-reactive antibodies dominate the human B cell response against 2009 pandemic H1N1 influenza virus infection. J Exp Med 2011; 208(1): 181–193
21. Li GM, Chiu C, Wrammert J, McCausland M, Andrews SF, Zheng NY, Lee JH, Huang M, Qu X, Edupuganti S, Mulligan M, Das SR, Yewdell JW, Mehta AK, Wilson PC, Ahmed R. Pandemic H1N1 influenza vaccine induces a recall response in humans that favors broadly cross-reactive memory B cells. Proc Natl Acad Sci USA 2012; 109(23): 9047–9052
22. Thomson CA, Wang Y, Jackson LM, Olson M, Wang W, Liavonchanka A, Keleta L, Silva V, Diederich S, Jones RB, Gubbay J, Pasick J, Petric M, Jean F, Allen VG, Brown EG, Rini JM, Schrader JW. Pandemic H1N1 influenza infection and vaccination in humans induces cross-protective antibodies that target the hemagglutinin stem. Front Immunol 2012; 3: 87
23. Pica N, Hai R, Krammer F, Wang TT, Maamary J, Eggink D, Tan GS, Krause JC, Moran T, Stein CR, Banach D, Wrammert J, Belshe RB, García-Sastre A, Palese P. Hemagglutinin stalk antibodies elicited by the 2009 pandemic influenza virus as a mechanism for

the extinction of seasonal H1N1 viruses. Proc Natl Acad Sci USA 2012; 109(7): 2573–2578

24. Qiu C, Huang Y, Wang Q, Tian D, Zhang W, Hu Y, Yuan Z, Zhang X, Xu J. Boosting heterosubtypic neutralization antibodies in recipients of 2009 pandemic H1N1 influenza vaccine. Clin Infect Dis 2012; 54(1): 17–24
25. Andrews SF, Huang Y, Kaur K, Popova LI, Ho IY, Pauli NT, Henry Dunand CJ, Taylor WM, Lim S, Huang M, Qu X, Lee JH, Salgado-Ferrer M, Krammer F, Palese P, Wrammert J, Ahmed R, Wilson PC. Immune history profoundly affects broadly protective B cell responses to influenza. Sci Transl Med 2015; 7(316): 316ra192
26. Krammer F, Palese P. Influenza virus hemagglutinin stalk-based antibodies and vaccines. Curr Opin Virol 2013; 3(5): 521–530
27. Russell RJ, Kerry PS, Stevens DJ, Steinhauer DA, Martin SR, Gamblin SJ, Skehel JJ. Structure of influenza hemagglutinin in complex with an inhibitor of membrane fusion. Proc Natl Acad Sci USA 2008; 105(46): 17736–17741
28. Nachbagauer R, Choi A, Hirsh A, Margine I, Iida S, Barrera A, Ferres M, Albrecht RA, García-Sastre A, Bouvier NM, Ito K, Medina RA, Palese P, Krammer F. Defining the antibody cross-reactome directed against the influenza virus surface glycoproteins. Nat Immunol 2017; 18(4): 464–473
29. Cox RJ, Madhun AS, Hauge S, Sjursen H, Major D, Kuhne M, Höschler K, Saville M, Vogel FR, Barclay W, Donatelli I, Zambon M, Wood J, Haaheim LR. A phase I clinical trial of a PER.C6 cell grown influenza H7 virus vaccine. Vaccine 2009; 27(13): 1889–1897
30. Mulligan MJ, Bernstein DI, Winokur P, Rupp R, Anderson E, Rouphael N, Dickey M, Stapleton JT, Edupuganti S, Spearman P, Ince D, Noah DL, Hill H, Bellamy AR; DMID 13-0032 H7N9 Vaccine Study Group. Serological responses to an avian influenza A/H7N9 vaccine mixed at the point-of-use with MF59 adjuvant: a randomized clinical trial. JAMA 2014; 312(14): 1409–1419
31. Moody MA, Zhang R, Walter EB, Woods CW, Ginsburg GS, McClain MT, Denny TN, Chen X, Munshaw S, Marshall DJ, Whitesides JF, Drinker MS, Amos JD, Gurley TC, Eudailey JA, Foulger A, DeRosa KR, Parks R, Meyerhoff RR, Yu JS, Kozink DM, Barefoot BE, Ramsburg EA, Khurana S, Golding H, Vandergrift NA, Alam SM, Tomaras GD, Kepler TB, Kelsoe G, Liao HX, Haynes BF. H3N2 influenza infection elicits more cross-reactive and less clonally expanded anti-hemagglutinin antibodies than influenza vaccination. PLoS One 2011; 6(10): e25797
32. Hai R, Krammer F, Tan GS, Pica N, Eggink D, Maamary J, Margine I, Albrecht RA, Palese P. Influenza viruses expressing chimeric hemagglutinins: globular head and stalk domains derived from different subtypes. J Virol 2012; 86(10): 5774–5781
33. Margine I, Hai R, Albrecht RA, Obermoser G, Harrod AC, Banchereau J, Palucka K, García-Sastre A, Palese P, Treanor JJ, Krammer F. H3N2 influenza virus infection induces broadly reactive hemagglutinin stalk antibodies in humans and mice. J Virol 2013; 87(8): 4728–4737
34. Miller MS, Gardner TJ, Krammer F, Aguado LC, Tortorella D, Basler CF, Palese P. Neutralizing antibodies against previously encountered influenza virus strains increase over time: a longitudinal analysis. Sci Transl Med 2013; 5(198): 198ra107
35. Nachbagauer R, Choi A, Izikson R, Cox MM, Palese P, Krammer F. Age dependence and isotype specificity of influenza virus hemagglutinin stalk-reactive antibodies in humans. MBio 2016; 7 (1): e01996–e15
36. Henry Dunand CJ, Leon PE, Kaur K, Tan GS, Zheng NY, Andrews S, Huang M, Qu X, Huang Y, Salgado-Ferrer M, Ho IY, Taylor W, Hai R, Wrammert J, Ahmed R, García-Sastre A, Palese P, Krammer F, Wilson PC. Preexisting human antibodies neutralize recently emerged H7N9 influenza strains. J Clin Invest 2015; 125(3): 1255–1268
37. Watson CT, Breden F. The immunoglobulin heavy chain locus: genetic variation, missing data, and implications for human disease. Genes Immun 2012; 13(5): 363–373
38. Gostic KM, Ambrose M, Worobey M, Lloyd-Smith JO. Potent protection against H5N1 and H7N9 influenza via childhood hemagglutinin imprinting. Science 2016; 354(6313): 722–726
39. Liu D, Shi W, Shi Y, Wang D, Xiao H, Li W, Bi Y, Wu Y, Li X, Yan J, Liu W, Zhao G, Yang W, Wang Y, Ma J, Shu Y, Lei F, Gao GF. Origin and diversity of novel avian influenza A H7N9 viruses causing human infection: phylogenetic, structural, and coalescent analyses. Lancet 2013; 381(9881): 1926–1932
40. Gao R, Cao B, Hu Y, Feng Z, Wang D, Hu W, Chen J, Jie Z, Qiu H, Xu K, Xu X, Lu H, Zhu W, Gao Z, Xiang N, Shen Y, He Z, Gu Y, Zhang Z, Yang Y, Zhao X, Zhou L, Li X, Zou S, Zhang Y, Li X, Yang L, Guo J, Dong J, Li Q, Dong L, Zhu Y, Bai T, Wang S, Hao P, Yang W, Zhang Y, Han J, Yu H, Li D, Gao GF, Wu G, Wang Y, Yuan Z, Shu Y. Human infection with a novel avian-origin influenza A (H7N9) virus. N Engl J Med 2013; 368(20): 1888–1897
41. Talaat KR, Karron RA, Callahan KA, Luke CJ, DiLorenzo SC, Chen GL, Lamirande EW, Jin H, Coelingh KL, Murphy BR, Kemble G, Subbarao K. A live attenuated H7N3 influenza virus vaccine is well tolerated and immunogenic in a Phase I trial in healthy adults. Vaccine 2009; 27(28): 3744–3753
42. Couch RB, Patel SM, Wade-Bowers CL, Niño D. A randomized clinical trial of an inactivated avian influenza A (H7N7) vaccine. PLoS One 2012; 7(12): e49704
43. Fries LF, Smith GE, Glenn GM. A recombinant viruslike particle influenza A (H7N9) vaccine. N Engl J Med 2013; 369(26): 2564–2566
44. Babu TM, Levine M, Fitzgerald T, Luke C, Sangster MY, Jin H, Topham D, Katz J, Treanor J, Subbarao K. Live attenuated H7N7 influenza vaccine primes for a vigorous antibody response to inactivated H7N7 influenza vaccine. Vaccine 2014; 32(50): 6798–6804
45. DeZure AD, Coates EE, Hu Z, Yamshchikov GV, Zephir KL, Enama ME, Plummer SH, Gordon IJ, Kaltovich F, Andrews S, McDermott A, Crank MC, Koup RA, Schwartz RM, Bailer RT, Sun X, Mascola JR, Tumpey TM, Graham BS, Ledgerwood JE. An avian influenza H7 DNA priming vaccine is safe and immunogenic in a randomized phase I clinical trial. Vaccines (Basel) 2017; 2(1): 15
46. Henry Dunand CJ, Leon PE, Huang M, Choi A, Chromikova V, Ho IY, Tan GS, Cruz J, Hirsh A, Zheng NY, Mullarkey CE, Ennis FA, Terajima M, Treanor JJ, Topham DJ, Subbarao K, Palese P, Krammer F, Wilson PC. Both neutralizing and non-neutralizing human H7N9 influenza vaccine-induced monoclonal antibodies confer protection. Cell Host Microbe 2016; 19(6): 800–813
47. Tan GS, Leon PE, Albrecht RA, Margine I, Hirsh A, Bahl J, Krammer F. Broadly-reactive neutralizing and non-neutralizing antibodies directed against the H7 influenza virus hemagglutinin reveal divergent mechanisms of protection. PLoS Pathog 2016; 12

(4): e1005578

48. Jegaskanda S, Job ER, Kramski M, Laurie K, Isitman G, de Rose R, Winnall WR, Stratov I, Brooks AG, Reading PC, Kent SJ. Cross-reactive influenza-specific antibody-dependent cellular cytotoxicity antibodies in the absence of neutralizing antibodies. J Immunol 2013; 190(4): 1837–1848
49. Jegaskanda S, Weinfurter JT, Friedrich TC, Kent SJ. Antibody-dependent cellular cytotoxicity is associated with control of pandemic H1N1 influenza virus infection of macaques. J Virol 2013; 87(10): 5512–5522
50. Krammer F, Jul-Larsen A, Margine I, Hirsh A, Sjursen H, Zambon M, Cox RJ. An H7N1 influenza virus vaccine induces broadly reactive antibody responses against H7N9 in humans. Clin Vaccine Immunol 2014; 21(8): 1153–1163
51. Halliley JL, Khurana S, Krammer F, Fitzgerald T, Coyle EM, Chung KY, Baker SF, Yang H, Martínez-Sobrido L, Treanor JJ, Subbarao K, Golding H, Topham DJ, Sangster MY. High-affinity H7 head and stalk domain-specific antibody responses to an inactivated influenza H7N7 vaccine after priming with live attenuated influenza vaccine. J Infect Dis 2015; 212(8): 1270–1278
52. Guo L, Zhang X, Ren L, Yu X, Chen L, Zhou H, Gao X, Teng Z, Li J, Hu J, Wu C, Xiao X, Zhu Y, Wang Q, Pang X, Jin Q, Wu F, Wang J. Human antibody responses to avian influenza A(H7N9) virus, 2013. Emerg Infect Dis 2014; 20(2): 192–200
53. Liu L, Nachbagauer R, Zhu L, Huang Y, Xie X, Jin S, Zhang A, Wan Y, Hirsh A, Tian D, Shi X, Dong Z, Yuan S, Hu Y, Krammer F, Zhang X, Xu J. Induction of broadly cross-reactive stalk-specific antibody responses to influenza group 1 and group 2 hemagglutinins by natural H7N9 virus infection in humans. J Infect Dis 2017; 215 (4): 518–528
54. Kashyap AK, Steel J, Oner AF, Dillon MA, Swale RE, Wall KM, Perry KJ, Faynboym A, Ilhan M, Horowitz M, Horowitz L, Palese P, Bhatt RR, Lerner RA. Combinatorial antibody libraries from survivors of the Turkish H5N1 avian influenza outbreak reveal virus neutralization strategies. Proc Natl Acad Sci USA 2008; 105(16): 5986–5991
55. Thornburg NJ, Zhang H, Bangaru S, Sapparapu G, Kose N, Lampley RM, Bombardi RG, Yu Y, Graham S, Branchizio A, Yoder SM, Rock MT, Creech CB, Edwards KM, Lee D, Li S, Wilson IA, García-Sastre A, Albrecht RA, Crowe JE Jr. H7N9 influenza virus neutralizing antibodies that possess few somatic mutations. J Clin Invest 2016; 126(4): 1482–1494
56. He W, Mullarkey CE, Duty JA, Moran TM, Palese P, Miller MS. Broadly neutralizing anti-influenza virus antibodies: enhancement of neutralizing potency in polyclonal mixtures and IgA backbones. J Virol 2015; 89(7): 3610–3618
57. DiLillo DJ, Tan GS, Palese P, Ravetch JV. Broadly neutralizing hemagglutinin stalk-specific antibodies require FcγR interactions for protection against influenza virus *in vivo*. Nat Med 2014; 20(2): 143–151
58. Andrews SF, Joyce MG, Chambers MJ, Gillespie RA, Kanekiyo M, Leung K, Yang ES, Tsybovsky Y, Wheatley AK, Crank MC, Boyington JC, Prabhakaran MS, Narpala SR, Chen X, Bailer RT, Chen G, Coates E, Kwong PD, Koup RA, Mascola JR, Graham BS, Ledgerwood JE, McDermott AB. Preferential induction of cross-group influenza A hemagglutinin stem-specific memory B cells after H7N9 immunization in humans. Sci Immunol 2017; 2(13): eaan2676

CONCLUSIONS

The emergence of zoonotic H7N9 viruses, that have repeatedly crossed the species barrier and infected humans, pose a substantial pandemic threat. These avian influenza viruses cause disease in humans with high case fatality rates. The immunity of the human population to these emerging virus strains is very low to non-existent. However, sustained human-to-human transmission has not been reported, yet the versatile and adaptive nature of the influenza virus might result in readily transmissible strains. Therefore, a proactive approach, testing and developing effective H7 prepandemic vaccines and antivirals, is highly desirable.[32,48,49,93,94]

We analyzed human serum samples from a clinical trial with recombinant H7 HA and showed that H7 vaccination induced a broad response against other group 2 HAs, such as H3, H4, H10, H14 and H15 (Figure 3A-B from manuscript 1[91]). H7 is an influenza A group 2 subtype HA which has shared epitopes in its stalk domain with commonly circulating H3 subtype viruses (Fig. 1). Therefore, the broad reactivity is likely based on a recall response of memory B-cells that differentiate into plasmablasts, secreting antibodies that target the conserved HA stalk domain, which might have been primed by exposure to human, seasonal H3N2 viruses.[95,96] Additionally, vaccination with recombinant H7 HA derived from one of the first isolated H7N9 viruses in humans (A/Anhui/1/2013) induced strong reactivity to the H7 HA of more recent isolates from 2016/2017. More precisely, we confirmed binding to the H7 HA of isolates including the antigenically distinct Pearl River Delta and Yangtze River Delta lineage and a highly pathogenic H7N9 isolate. Interestingly, we found strong induction of antibodies binding to even more antigenically distinct H7 HAs of North American lineage H7 viruses like a highly pathogenic avian H7N8

virus and a feline H7N2 virus. These findings suggest the existence of shared epitopes among divergent H7 HAs and demonstrate the potential of a recombinant H7 HA only vaccine to elicit cross-reactive antibodies against both novel H7N9 viruses and zoonotic H7NX viruses. Notably, we demonstrated that passive transfer of human sera into mice which were subsequently challenged with a lethal dose of H7N9 virus conferred partial protection, even in the absence of serum antibodies with hemagglutination inhibition (HI) activity. This highlights the importance of non HI-active antibodies which likely mediate protection based on antibody effector functions or bind to the HA stalk domain, thus preventing fusion and neutralizing the virus.[97,98] These findings have implications for the development of HA-stalk (group 2) based universal influenza virus vaccines and help inform the generation of prepandemic H7N9 influenza vaccines.

Next, we evaluated mouse monoclonal antibodies that had been raised against the H7 HA of another, closely related H7N9 strain (A/Shanghai/1/2013) and showed binding to novel H7N9 variants as well as emerging, zoonotic North American lineage H7NX viruses. Applying reverse genetics techniques we proceeded to generate novel H7N9 viruses (both a Pearl River Delta and Yangtze River Delta lineage virus) and a feline H7N2 virus in order to characterize the broadly-reactive H7 specific antibodies *in vitro* and *in vivo*. A proportion of the tested antibodies bound to a major antigenic site (antigenic site A) of influenza H7 HA. Interestingly, despite sequence analysis indicating that novel H7N9 isolates had changes in site A (Figure 6 from manuscript 2[92]), the antibodies bound and neutralized these viruses. The tested antibodies protected from lethal challenge with the new virus variants in a murine passive transfer challenge model. We hypothesize that, due to their breadth and protective efficacy, the characterized monoclonal antibodies are potential

candidates for the development of mAb-based therapeutics for H7N9 infections. Furthermore, we provided data for antibody-guided vaccine design. We showed that antigenic site A is a highly conserved, protective epitope among divergent H7 subtype viruses and therefore, a prime target for vaccination approaches.

Finally, we reviewed available data on the humoral immune response to vaccination and infection with H7 subtype viruses and compared these findings to H5 vaccination. We found that natural H7N9 infection seems to elicit broadly-reactive antibodies to group 2 as well as group 1 HAs with a decent reactivity to H1. H7 HA vaccination triggers a polyclonal serum antibody response that mostly targets H7 HA and other group 2 HAs as well as moderate levels of stalk antibodies targeting group 1 HAs. However, vaccination with H5 HA leads to an increase of H5 specific antibodies and group 1 anti-stalk antibodies, but elicits very low levels of cross-group reactive stalk antibodies (Fig. 3 from manuscript 3[86]).[89,91,99,100] This data suggests that for a stalk-based universal influenza vaccine more than one component will be needed. Nevertheless, a strong group 2 stalk immunogen could induce broad protection against group 2 viruses, including H3N2, H7N9 or H10N8, and to some extent group 1 viruses like pH1N1.

In conclusion, our findings help to develop future universal influenza virus vaccine candidates, specifically a group 2 stalk component. Besides, we provide data for H7 vaccine strategies since our results suggest that stockpiled H7N9 vaccines might be efficacious against drifted H7N9 variants as well as divergent H7 strains. Finally, we provide insights for antibody-guided vaccine design, facilitate the selection of prepandemic H7 vaccine strains and provide a potential therapeutic resource.

REFERENCES

1. Webster RG, Bean WJ, Gorman OT, Chambers TM, Kawaoka Y. Evolution and ecology of influenza A viruses. *Microbiol Rev.* 1992;56(1):152-179.
2. Bouvier NM, Palese P. The biology of influenza viruses. *Vaccine.* 2008;26 Suppl 4:D49-53.
3. Shaw M, Palese P. Fields Virology (6th edition). In: *Fields Virology (6th edition).*2013:p. 1151-1185.
4. Ferguson L, Olivier AK, Genova S, et al. Pathogenesis of Influenza D Virus in Cattle. *J Virol.* 2016;90(12):5636-5642.
5. Tong S, Li Y, Rivailler P, et al. A distinct lineage of influenza A virus from bats. *Proc Natl Acad Sci U S A.* 2012;109(11):4269-4274.
6. Tong S, Zhu X, Li Y, et al. New world bats harbor diverse influenza A viruses. *PLoS Pathog.* 2013;9(10):e1003657.
7. World Health Organization. 2017. Influenza Seasonal Fact sheet. World Health Organization, Geneva, Switzerland.
8. Lozano R, Naghavi M, Foreman K, et al. Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: a systematic analysis for the Global Burden of Disease Study 2010. *Lancet.* 2012;380(9859):2095-2128.
9. Nichol KL, Treanor JJ. Vaccines for seasonal and pandemic influenza. *J Infect Dis.* 2006;194 Suppl 2:S111-118.
10. Cox NJ, Subbarao K. Influenza. *Lancet.* 1999;354(9186):1277-1282.
11. Grohskopf LA, Sokolow LZ, Broder KR, Walter EB, Fry AM, Jernigan DB. Prevention and Control of Seasonal Influenza with Vaccines: Recommendations of the Advisory Committee on Immunization Practices-United States, 2018-19 Influenza Season. *MMWR Recomm Rep.* 2018;67(3):1-20.
12. Jorgensen P, Mereckiene J, Cotter S, Johansen K, Tsolova S, Brown C. How close are countries of the WHO European Region to achieving the goal of vaccinating 75% of key risk groups against influenza? Results from national surveys on seasonal influenza vaccination programmes, 2008/2009 to 2014/2015. In: *Vaccine.* Vol 36.2018:442-452.
13. Center for Disease Control and Prevention. 2018. FluVaxView - Estimates of flu vaccination coverage among children–United States, 2017-18 flu season. Center for Disease Control and Prevention, Atlanta, United States of America.
14. Subbarao K, Joseph T. Scientific barriers to developing vaccines against avian influenza viruses. *Nature Reviews Immunology.* 2007;7(4):267-278.
15. Heaton NS, Sachs D, Chen CJ, Hai R, Palese P. Genome-wide mutagenesis of influenza virus reveals unique plasticity of the hemagglutinin and NS1 proteins. *Proc Natl Acad Sci U S A.* 2013;110(50):20248-20253.
16. Doud MB, Bloom JD. Accurate Measurement of the Effects of All Amino-Acid Mutations on Influenza Hemagglutinin. *Viruses.* 2016;8(6).
17. Shaw M, Palese P. Fields Virology. In: 6th edition ed.2013:p. 1151-1185.
18. Weir JP, Gruber MF. An overview of the regulation of influenza vaccines in the United States. In: *Influenza Other Respir Viruses.* Vol 10.2016:354-360.

19. World Health Organization. 2018. WHO recommendations on the composition of influenza virus vaccines. World Health Organization, Geneva, Switzerland.

20. Morens DM, Taubenberger JK. Pandemic influenza: certain uncertainties. *Rev Med Virol.* 2011;21(5):262-284.

21. Kasowski EJ, Garten RJ, Bridges CB. Influenza pandemic epidemiologic and virologic diversity: reminding ourselves of the possibilities. *Clin Infect Dis.* 2011;52 Suppl 1:S44-49.

22. Short KR, Kedzierska K, van de Sandt CE. Back to the Future: Lessons Learned From the 1918 Influenza Pandemic. *Front Cell Infect Microbiol.* 2018;8.

23. Johnson NP, Mueller J. Updating the accounts: global mortality of the 1918-1920 "Spanish" influenza pandemic. *Bull Hist Med.* 2002;76(1):105-115.

24. Kilbourne ED. Influenza Pandemics of the 20th Century. In: *Emerg Infect Dis.* Vol 12.2006:9-14.

25. Glezen WP. Emerging infections: pandemic influenza. *Epidemiol Rev.* 1996;18(1):64-76.

26. Reperant LA, Moesker FM, Osterhaus AD. Influenza: from zoonosis to pandemic. In: *ERJ Open Res.* Vol 2.2016.

27. Beigel JH, Farrar J, Han AM, et al. Avian influenza A (H5N1) infection in humans. *N Engl J Med.* 2005;353(13):1374-1385.

28. He J, Liu BY, Gong L, et al. Genetic characterization of the first detected human case of avian influenza A (H5N6) in Anhui Province, East China. *Sci Rep.* 2018;8(1):15282.

29. Shi W, Shi Y, Wu Y, Liu D, Gao GF. Origin and molecular characterization of the human-infecting H6N1 influenza virus in Taiwan. *Protein Cell.* 2013;4(11):846-853.

30. Lopez-Martinez I, Balish A, Barrera-Badillo G, et al. Highly pathogenic avian influenza A(H7N3) virus in poultry workers, Mexico, 2012. *Emerg Infect Dis.* 2013;19(9):1531-1534.

31. Puzelli S, Rizzo C, Fabiani C, et al. Influenza A(H7N7) Virus among Poultry Workers, Italy, 2013. *Emerg Infect Dis.* 2016;22(8):1512-1513.

32. Gao R, Cao B, Hu Y, et al. Human infection with a novel avian-origin influenza A (H7N9) virus. *N Engl J Med.* 2013;368(20):1888-1897.

33. Butt KM, Smith GJ, Chen H, et al. Human infection with an avian H9N2 influenza A virus in Hong Kong in 2003. *J Clin Microbiol.* 2005;43(11):5760-5767.

34. Chen H, Yuan H, Gao R, et al. Clinical and epidemiological characteristics of a fatal case of avian influenza A H10N8 virus infection: a descriptive study. *Lancet.* 2014;383(9918):714-721.

35. Ostrowsky B, Huang A, Terry W, et al. Low pathogenic avian influenza A (H7N2) virus infection in immunocompromised adult, New York, USA, 2003. *Emerg Infect Dis.* 2012;18(7):1128-1131.

36. Tong XC, Weng SS, Xue F, Wu X, Xu TM, Zhang WH. First human infection by a novel avian influenza A(H7N4) virus. *J Infect.* 2018;77(3):249-257.

37. Jhung MA, Epperson S, Biggerstaff M, et al. Outbreak of Variant Influenza A(H3N2) Virus in the United States. *Clin Infect Dis.* 2013;57(12):1703-1712.

38. Bowman AS, Nolting JM, Nelson SW, Slemons RD. Subclinical Influenza Virus A Infections in Pigs Exhibited at Agricultural Fairs, Ohio, USA, 2009–2011. In: *Emerg Infect Dis.* Vol 18.2012:1945-1950.

39. Karasin AI, Landgraf J, Swenson S, et al. Genetic characterization of H1N2 influenza A viruses isolated from pigs throughout the United States. *J Clin Microbiol.* 2002;40(3):1073-1079.

40. Marinova-Petkova A, Laplante J, Jang Y, et al. Avian Influenza A(H7N2) Virus in Human Exposed to Sick Cats, New York, USA, 2016. In: *Emerg Infect Dis.* Vol 23.2017:2046-2049.

41. Chambers TM. A brief introduction to equine influenza and equine influenza viruses. *Methods Mol Biol.* 2014;1161:365-370.

42. Burnell FJ, Holmes MA, Roiko AH, et al. Little evidence of human infection with equine influenza during the 2007 epizootic, Queensland, Australia. *J Clin Virol.* 2014;59(2):100-103.

43. Chen Y, Trovao NS, Wang G, et al. Emergence and Evolution of Novel Reassortant Influenza A Viruses in Canines in Southern China. *MBio.* 2018;9(3).

44. Capua I, Marangon S. Control of Avian Influenza in Poultry. In: *Emerg Infect Dis.* Vol 12.2006:1319-1324.

45. Horimoto T, Kawaoka Y. Influenza: lessons from past pandemics, warnings from current incidents. *Nat Rev Microbiol.* 2005;3(8):591-600.

46. Webster RG, Rott R. Influenza virus A pathogenicity: the pivotal role of hemagglutinin. *Cell.* 1987;50(5):665-666.

47. Peiris JSM, de Jong MD, Guan Y. Avian Influenza Virus (H5N1): a Threat to Human Health. In: *Clin Microbiol Rev.* Vol 20.2007:243-267.

48. World Health Organization. 2017. Influenza at the human-animal interface. Summary and assessment. World Health Organization, Geneva, Switzerland.

49. Subbarao K. Avian influenza H7N9 viruses: a rare second warning. *Cell Research.* 2017.

50. Kile JC, Ren R, Liu L, et al. Update: Increase in Human Infections with Novel Asian Lineage Avian Influenza A(H7N9) Viruses During the Fifth Epidemic - China, October 1, 2016-August 7, 2017. *MMWR Morb Mortal Wkly Rep.* 2017;66(35):928-932.

51. Imai M, Watanabe T, Kiso M, et al. A Highly Pathogenic Avian H7N9 Influenza Virus Isolated from A Human Is Lethal in Some Ferrets Infected via Respiratory Droplets. *Cell Host Microbe.* 2017.

52. Wang D, Yang L, Zhu W, et al. Two Outbreak Sources of Influenza A (H7N9) Viruses Have Been Established in China. *J Virol.* 2016;90(12):5561-5573.

53. Quan C, Shi W, Yang Y, et al. New Threats from H7N9 Influenza Virus: Spread and Evolution of High- and Low-Pathogenicity Variants with High Genomic Diversity in Wave Five. 2018.

54. Yang L, Zhu W, Li X, et al. Genesis and Spread of Newly Emerged Highly Pathogenic H7N9 Avian Viruses in Mainland China. *J Virol.* 2017.

55. Yi L, Guan D, Kang M, et al. Family Clusters of Avian Influenza A H7N9 Virus Infection in Guangdong Province, China. 2015.

56. Kuiken T, Holmes EC, McCauley J, Rimmelzwaan GF, Williams CS, Grenfell BT. Host species barriers to influenza virus infections. *Science.* 2006;312(5772):394-397.

57. Herfst S, Schrauwen EJ, Linster M, et al. Airborne transmission of influenza A/H5N1 virus between ferrets. *Science.* 2012;336(6088):1534-1541.

58. Imai M, Watanabe T, Hatta M, et al. Experimental adaptation of an influenza H5 HA confers respiratory droplet transmission to a reassortant H5 HA/H1N1 virus in ferrets. *Nature.* 2012;486(7403):420-428.

59. Herfst S, Imai M, Kawaoka Y, Fouchier RA. Avian influenza virus transmission to mammals. *Curr Top Microbiol Immunol.* 2014;385:137-155.

60. Li J, Kou Y, Yu X, et al. Human co-infection with avian and seasonal influenza viruses, China. *Emerg Infect Dis.* 2014;20(11):1953-1955.

61. Fineberg HV. Pandemic preparedness and response--lessons from the H1N1 influenza of 2009. *N Engl J Med.* 2014;370(14):1335-1342.

62. Gensheimer KF, Meltzer MI, Postema AS, Strikas RA. Influenza Pandemic Preparedness. In: *Emerg Infect Dis.* Vol 9.2003:1645-1648.

63. Graaf H, Faust SN. Fluarix quadrivalent vaccine for influenza. *Expert Rev Vaccines.* 2015;14(8):1055-1063.

64. Krammer F, Palese P. Advances in the development of influenza virus vaccines. *Nat Rev Drug Discov.* 2015;14(3):167-182.

65. Broadbent AJ, Subbarao K. Influenza Virus Vaccines: Lessons from the 2009 H1N1 pandemic. *Curr Opin Virol.* 2011;1(4):254-262.

66. Cox MM. Cell-based protein vaccines for influenza. *Curr Opin Mol Ther.* 2005;7(1):24-29.

67. Cox MM, Hollister JR. FluBlok, a next generation influenza vaccine manufactured in insect cells. *Biologicals.* 2009;37(3):182-189.

68. Cox MM, Hashimoto Y. A fast track influenza virus vaccine produced in insect cells. *J Invertebr Pathol.* 2011;107 Suppl:S31-41.

69. Pardi N, Hogan MJ, Porter FW, Weissman D. mRNA vaccines — a new era in vaccinology. *Nat Rev Drug Discov.* 2018;17(4):261-279.

70. Lee LYY, Izzard L, Hurt AC. A Review of DNA Vaccines Against Influenza. *Front Immunol.* 2018;9.

71. Bahl K, Senn JJ, Yuzhakov O, et al. Preclinical and Clinical Demonstration of Immunogenicity by mRNA Vaccines against H10N8 and H7N9 Influenza Viruses. *Mol Ther.* 2017;25(6):1316-1327.

72. Yan J, Morrow MP, Chu JS, et al. Broad cross-protective anti-hemagglutination responses elicited by influenza microconsensus DNA vaccine. *Vaccine.* 2018;36(22):3079-3089.

73. Nachbagauer R, Krammer F. Universal influenza virus vaccines and therapeutic antibodies. *Clin Microbiol Infect.* 2017;23(4):222-228.

74. Kirkpatrick E, Qiu X, Wilson PC, Bahl J, Krammer F. The influenza virus hemagglutinin head evolves faster than the stalk domain. *Sci Rep.* 2018;8(1):10432.

75. Krammer F, Garcia-Sastre A, Palese P. Is It Possible to Develop a Universal Influenza Virus Vaccine? Toward a Universal Influenza Virus Vaccine: Potential Target Antigens and Critical Aspects for Vaccine Development. *Cold Spring Harb Perspect Biol.* 2017.

76. Krammer F. Strategies to induce broadly protective antibody responses to viral glycoproteins. *Expert Rev Vaccines.* 2017;16(5):503-513.

77. Krammer F, Palese P. Universal influenza virus vaccines: need for clinical trials. *Nature Immunology.* 2013;15:3-5.

78. Margine I, Krammer F, Hai R, et al. Hemagglutinin stalk-based universal vaccine constructs protect against group 2 influenza A viruses. *J Virol.* 2013;87(19):10435-10446.

79. Goff PH, Eggink D, Seibert CW, et al. Adjuvants and immunization strategies to induce influenza virus hemagglutinin stalk antibodies. *PLoS One.* 2013;8(11):e79194.

80. Nachbagauer R, Krammer F, Albrecht RA. A Live-Attenuated Prime, Inactivated Boost Vaccination Strategy with Chimeric Hemagglutinin-Based Universal Influenza Virus Vaccines Provides Protection in Ferrets: A Confirmatory Study. *Vaccines (Basel).* 2018;6(3).

81. Ison MG. Finding the right combination antiviral therapy for influenza. *Lancet Infect Dis.* 2017;17(12):1221-1222.

82. Hayden FG, Sugaya N, Hirotsu N, et al. Baloxavir Marboxil for Uncomplicated Influenza in Adults and Adolescents. *N Engl J Med.* 2018;379(10):913-923.

83. Koshimichi H, Ishibashi T, Kawaguchi N, Sato C, Kawasaki A, Wajima T. Safety, Tolerability, and Pharmacokinetics of the Novel Anti-influenza Agent Baloxavir Marboxil in Healthy Adults: Phase I Study Findings. *Clin Drug Investig.* 2018.

84. Deng R, Lee AP, Maia M, et al. Pharmacokinetics of MHAA4549A, an Anti-Influenza A Monoclonal Antibody, in Healthy Subjects Challenged with Influenza A Virus in a Phase IIa Randomized Trial. *Clin Pharmacokinet.* 2018;57(3):367-377.

85. Hershberger E, Sloan S, Narayan K, et al. Safety and efficacy of monoclonal antibody VIS410 in adults with uncomplicated influenza A infection: Results from a randomized, double-blind, phase-2, placebo-controlled study. *EBioMedicine.* 2019.

86. Sparrow E, Friede M, Sheikh M, Torvaldsen S, Newall AT. Passive immunization for influenza through antibody therapies, a review of the pipeline, challenges and potential applications. In: *Vaccine.* Vol 34.2016:5442-5448.

87. Shriver Z, Trevejo JM, Sasisekharan R. Antibody-Based Strategies to Prevent and Treat Influenza. *Front Immunol.* 2015;6.

88. Russell RJ, Gamblin SJ, Haire LF, et al. H1 and H7 influenza haemagglutinin structures extend a structural classification of haemagglutinin subtypes. *Virology.* 2004;325(2):287-296.

89. Stadlbauer D, Nachbagauer R, Meade P, Krammer F. Universal influenza virus vaccines: what can we learn from the human immune response following exposure to H7 subtype viruses? *Frontiers of Medicine.* 2017.

90. Nachbagauer R, Liu W-C, Choi A, et al. A universal influenza virus vaccine candidate confers protection against pandemic H1N1 infection in preclinical ferret studies. *npj Vaccines.* 2017;2(1):26.

91. Stadlbauer D, Rajabhathor A, Amanat F, et al. Vaccination with a Recombinant H7 Hemagglutinin-Based Influenza Virus Vaccine Induces Broadly Reactive Antibodies in Humans. *mSphere.* 2017;2(6).

92. Stadlbauer D, Amanat F, Strohmeier S, Nachbagauer R, Krammer F. Cross-reactive mouse monoclonal antibodies raised against the hemagglutinin of A/Shanghai/1/2013 (H7N9) protect against novel H7 virus isolates in the mouse model. *Emerging Microbes & Infections.* 2018;7(1):110.

93. Krammer F, Department of Microbiology ISoMaMS, New York, NY, USA. Emerging influenza viruses and the prospect of a universal influenza virus vaccine. *Biotechnology Journal.* 2017;10(5):690-701.

94. Danqi B, Li Z, Liu Q, Richt JA. H7N9 avian influenza A virus in China: a short report on its circulation, drug resistant mutants and novel antiviral drugs. *Expert Rev Anti Infect Ther.* 2017;15(8):723-727.

95. Henry Dunand CJ, Leon PE, Kaur K, et al. Preexisting human antibodies neutralize recently emerged H7N9 influenza strains. *J Clin Invest.* 2015;125(3):1255-1268.

96. Kaur K, Sullivan M, Wilson PC. Targeting B cell responses in universal influenza vaccine design. *Trends Immunol.* 2011;32(11):524-531.

97. DiLillo DJ, Tan GS, Palese P, Ravetch JV. Broadly neutralizing hemagglutinin stalk-specific antibodies require FcgammaR interactions for protection against influenza virus in vivo. *Nat Med.* 2014;20(2):143-151.

98. Henry Dunand CJ, Leon PE, Huang M, et al. Both Neutralizing and Non-Neutralizing Human H7N9 Influenza Vaccine-Induced Monoclonal Antibodies Confer Protection. *Cell Host Microbe.* 2016;19(6):800-813.

99. Liu L, Nachbagauer R, Zhu L, et al. Induction of Broadly Cross-Reactive Stalk-Specific Antibody Responses to Influenza Group 1 and Group 2 Hemagglutinins by Natural H7N9 Virus Infection in Humans. *J Infect Dis.* 2017;215(4):518-528.

100. Nachbagauer R, Wohlbold TJ, Hirsh A, et al. Induction of broadly reactive anti-hemagglutinin stalk antibodies by an H5N1 vaccine in humans. *J Virol.* 2014;88(22):13260-13268.

LIST OF PUBLICATIONS

Research articles listed in chronological order:

1. **Universal influenza virus vaccines: what can we learn from the human immune response following exposure to H7 subtype viruses?**
   Stadlbauer D, Nachbagauer R, Meade P, Krammer F.
   Frontiers of Medicine. 2017.

2. **Vaccination with a Recombinant H7 Hemagglutinin-Based Influenza Virus Vaccine Induces Broadly Reactive Antibodies in Humans.**
   Stadlbauer D, Rajabhathor A, Amanat F, Kaplan D, Masud A, Treanor JJ, Izikson R, Cox MM, Nachbagauer R, Krammer F.
   mSphere. 2017.

3. **Cross-reactive mouse monoclonal antibodies raised against the hemagglutinin of A/Shanghai/1/2013 (H7N9).**
   Stadlbauer D, Amanat F, Strohmeier S, Nachbagauer R, Krammer F.
   Emerging Microbes & Infections. 2018.

4. **Sequential immunization with live-attenuated chimeric hemagglutinin-based vaccines confers heterosubtypic immunity against influenza A viruses in a preclinical ferret model.**
   Liu W-C, Nachbagauer R, Stadlbauer D, Solórzano A, Berlanda-Scorza F, García-Sastre A, Palese P, Krammer F, Albrecht RA.
   Frontiers in Immunology. 2019.

5. **Chimeric hemagglutinin-based influenza virus vaccines induce protective stalk-specific humoral immunity and cellular responses in mice.**
   Choi A, Bouzya B, Cortes Franco K-D, Stadlbauer D, Rajabhathor A, Rouxel RN, Mainil R, Van der Wielen M, Palese P, García-Sastre A, Innis BL, Krammer F, Schotsaert M, Mallett CP, Nachbagauer R.
   ImmunoHorizons. (accepted in principle) 2019.

6. **Novel correlates of protection against influenza A(H1N1)pdm virus infection.**
   Ng S, Nachbagauer R, Balmaseda A, Stadlbauer D, Ojeda S, Patel M, Rajabhathor A, Lopez R, Guglia AF, Sanchez N, Amanat F, Gresh L, Kuan G, Krammer F, Gordon A.
   Nature Medicine. (accepted in principle) 2019.