

Characterization of pre-existing humoral immunity to H5N1 clade 2.3.4.4b influenza viruses in unexposed individuals



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List of Abbreviations

aa	Amino acid
AbRAT	Antibody repertoire analysis toolkit
ACE2	Angiotensin-converting enzyme 2
ADCC	Antibody-dependent cellular cytotoxicity
ADCP	Antibody-dependent cellular phagocytosis
AI	Artificial intelligence
AID	Activation induced deaminase
AIV	Avian influenza virus
ATP	Adenosine triphosphate
BCR	B cell receptor
Bis-Tris	Bis(2-hydroxyethyl)amino-tris(hydroxymethyl)methane
bnAb	Broadly neutralizing antibody
CDC	Centers for Disease Control and Prevention
cDNA	Complementary DNA
CDR	Complementarity-determining region
CEN	Cap-dependent endonuclease
C	Constant region
C _H	Constant domain of the heavy chain
C _L	Constant domain of the light chain
COVID-19	Coronavirus disease of 2019
CPE	Cytopathic effect
cRNA	Complementary RNA
CSR	Class switch recombination
D	Diversity gene segment
DAPI	4',6-diamindin-2-phenylindol
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphates
DPBS	Dulbecco's phosphate buffered saline
Dpi	Days post-infection
DTT	Dithiothreitol
<i>E. coli</i>	<i>Escherichia coli</i>
EC ₅₀	Half-maximal effective concentration
ED ₅₀	Half-maximal effective dilution
EDTA	Ethylenediamine tetraacetic acid

ELISA	Enzyme-linked immunosorbent assay
euDG	DE23-11-N1.3 euDG A/wild goose/Germany-NW/2024AI02730/2024
Fab	Antigen-binding fragment
FACS	Fluorescence-activated cell sorting
Fc	Fragment crystallizable
FCS	Fetal calf serum
FSC	Forward scatter
FSC-A	Forward scatter area
FSC-H	Forward scatter height
fwd	Forward
FWR	Framework region
<i>g</i>	gravitational acceleration
GFP	Green fluorescent protein
Gs/Gd	A/Goose/Guangdong/1/1996 lineage
H1-H19	Hemagglutinin subtypes 1-19
H1N1pdm09	New H1N1 lineage of the 2009 H1N1 pandemic
HA	Hemagglutinin
HA0	Uncleaved precursor of HA
HA1	Hemagglutinin subunit 1
HA2	Hemagglutinin subunit 2
HAI	Hemagglutination inhibition
HC	Heavy chain
HEK	Human embryonic kidney
HIV	Human immunodeficiency virus
HPAIV	Highly pathogenic avian influenza virus
HRP	Horseradish peroxidase
i.n.	intranasal
i.p.	intraperitoneally
IAV	Influenza A virus
IC ₁₀₀	Complete inhibitory concentration
IC ₅₀	Half-maximal inhibitory concentration
ID ₁₀₀	Complete inhibitory dilution
ID ₅₀	Half-maximal inhibitory dilution
Ig	Immunoglobulin
IgA	Immunoglobulin A
IgD	Immunoglobulin D
IgE	Immunoglobulin E

IgG	Immunoglobulin G
IgM	Immunoglobulin M
IRAT	Influenza Risk Assessment Tool
J	Joining gene segment
kDa	Kilodalton
LB	Lysogeny broth
LC	Light chain
LLOD	Lower limit of detection
LPAIV	Low pathogenic avian influenza virus
M1	Matrix protein 1
M2	Matrix protein 2
mAb	Monoclonal antibody
MACS	Magnetic-activated cell sorting
MDCK	Madin-Darby Canine Kidney cell line
MDCK-SIAT1	MDCK cells that overexpress the α 2,6-sialyltransferase ST6Gal1
MEM	Minimum Essential Medium Eagle
MN	Microneutralization
MOPS	3-(N-morpholino)propanesulfonic acid
mRNA	Messenger ribonucleic acid
N1-N11	Neuraminidase subtypes 1-11
N/A	Not applicable
NA	Neuraminidase
nAbs	Neutralizing antibodies
NEP	Nuclear export protein
NP	Nucleoprotein
NS	Non-structural gene segment
OAS	Original antigenic sin
OD	Optical density
PA	Polymerase acidic protein
PB1	Polymerase basic protein 1
PB2	Polymerase basic protein 2
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PBST	PBS with Tween 20
PCR	Polymerase chain reaction
PEI	Polyethylenimine
PES	Polyethersulfone

plgH	Heavy chain expression plasmid
plgK	Kappa chain expression plasmid
plgL	Lambda chain expression plasmid
r_p	Pearson's r
PS	Penicillin-Streptomycin
RBS	Receptor binding site
rev	Reverse
RNA	Ribonucleic acid
RLU	Relative light units
RSV	Respiratory syncytial virus
RT	Room temperature
RT-PCR	Reverse transcription PCR
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
sfGFP	SuperfolderGFP
SHM	Somatic hypermutation
SIAT1	α 2,6-sialyltransferase
SLIC	Sequence- and ligation-independent cloning
SOC	Super optimal broth with catabolite repression medium
SSC	Side scatter
SSC-A	Side scatter area
TAE	Tris-Acetate-EDTA buffer
TCID ₅₀	50% tissue culture infectious dose
TRIS	Tris(hydroxymethyl)aminomethane
ULOD	Upper limit of detection
US	United States
V	Variable gene segment
VE	Vestigial esterase
V _H domain	Variable domain of the heavy chain
V _L domain	Variable domain of the light chain
vRNA	Viral RNA
vRNP	Viral ribonucleoprotein
WHO	World Health Organization

Preamble

Parts of this doctoral thesis are based on the following publication:

Daniel, K., Ullrich, L., Ruchnewitz, D., Meijers, M., Halwe, N.J., Wild, U., Eberhardt, J., Schön, J., Stumpf, R., Schlotz, M., Wunsch, M., Girao Lessa, L., Abdelwhab, E.M., Kuryshko, M., Dietrich, C., Pinger, A., Schumacher, A.-L., Germer, M., Rohde, M., Kukat, C., Gieselmann, L., Gruell, H., Hoffmann, D., Beer, M., Erren, T., Lässig, M., Kreer, C., Klein, F., 2026. Pre-existing neutralizing antibodies against cattle-transmitted influenza A virus H5N1 are detectable in unexposed individuals. *Immunity* 59, 494-508.e8. <https://doi.org/10.1016/j.immuni.2025.12.013>

Together with the establishment of an HA-pseudovirus neutralization assay, this publication represents a central component of this thesis. All data from the publication are included in this thesis in a revised and expanded form. The publication was conducted under shared first authorship between me and Leon Ullrich. Prof. Dr. Florian Klein and PD Dr. Christoph Kreer supervised the study and provided scientific guidance throughout the project.

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Abstract

Influenza A viruses remain a major challenge for global public health. Beyond the annual burden of seasonal influenza, influenza A viruses circulating in animal populations represent an unpredictable risk, as they can give rise to novel variants with pandemic potential. Among these zoonotic variants, highly pathogenic avian H5N1 clade 2.3.4.4b influenza viruses are of major concern due to their rapid global spread in birds and their frequent detection in mammalian species. In particular, the 2024 outbreak of these viruses in dairy cattle in the United States, accompanied by repeated transmission to humans, represents a notable shift in host range, thereby increasing opportunities for zoonotic transmission and viral adaptation. Pre-existing immunity plays a key role in susceptibility to infection with emerging zoonotic variants and is an important component of pandemic risk assessment. Therefore, this thesis assessed pre-existing antibodies against the H5N1 clade 2.3.4.4b A/Texas/37/2024 isolate associated with the cattle outbreak in H5N1-naïve individuals.

For this purpose, a highly sensitive hemagglutinin(HA)-pseudovirus neutralization assay was initially established, which showed strong positive correlation with authentic virus neutralization and enabled quantification of functional antibody reactivity against this and other influenza strains. By applying this assay and complementary binding and neutralization assays, A/Texas/37/2024-reactive antibodies were detected in all 66 H5N1-naïve individuals analyzed at the serum and plasma level. However, neutralizing titers against A/Texas/37/2024 were consistently lower compared to seasonal H1 and H3 viruses. To further dissect the underlying B cell response, H5-reactive memory B cells were isolated from six H5N1-naïve individuals. Functional characterization of the encoded antibodies revealed highly potent A/Texas/37/2024-neutralizing antibodies in five out of the six individuals. Subsequent analyses showed that these antibodies cross-react with H1, compete with antibodies directed against the conserved stem region of HA and protect mice from lethal H5N1 challenge. Together, these findings indicate that the observed serum-level H5-reactivity is largely mediated by group 1 cross-reactive HA stem antibodies. In conclusion, this work demonstrates the presence of pre-existing cross-reactive antibodies to the H5N1 A/Texas/37/2024 isolate in individuals without prior exposure to this subtype. Further studies are required to better define the functional relevance of such antibody responses for protection against infection with emerging zoonotic influenza variants.

Zusammenfassung

Influenza A Viren stellen eine erhebliche Herausforderung für die globale öffentliche Gesundheit dar. Neben der jährlichen Belastung durch saisonale Grippewellen stellen in Tierpopulationen zirkulierende Influenza A Viren ein unvorhersehbares Risiko dar, da aus ihnen neue Varianten mit pandemischem Potenzial entstehen können. Unter diesen zoonotischen Varianten sind hochpathogene aviäre Influenza A Viren des Subtyps H5N1 der Klade 2.3.4.4b aufgrund ihrer raschen globalen Ausbreitung in Vögeln und ihres häufigen Nachweises in Säugetieren von besonderer Bedeutung. Insbesondere der Ausbruch dieser Viren im Jahr 2024 in Milchkühen in den Vereinigten Staaten, begleitet von wiederholten Übertragungen auf den Menschen, stellt eine deutliche Veränderung des Wirtsspektrums dar und erhöht somit die Möglichkeiten für zoonotische Übertragungen und Anpassungen der Viren. Eine bereits bestehende Immunität spielt eine zentrale Rolle für die Empfindlichkeit gegenüber Infektionen mit neu auftretenden zoonotischen Varianten und ist eine wichtige Komponente bei der Einschätzung des Pandemierisikos. Diese Arbeit hat daher bereits existierende Antikörper gegen das H5N1-Isolat A/Texas/37/2024, das mit dem Ausbruch in Rindern in Verbindung steht, in H5N1-naiven Individuen untersucht.

Zunächst wurde ein hochsensitiver Hämagglutinin(HA)-Pseudovirus-Neutralisationstest etabliert, der eine starke positive Korrelation mit der Neutralisation authentischer Influenzaviren zeigte und die Quantifizierung funktioneller Antikörperreaktivität gegen dieses Isolat sowie weitere Influenzastämme ermöglichte. Mithilfe dieses Tests und ergänzender Bindungs- und Neutralisationstests konnten in allen 66 untersuchten H5N1-naiven Individuen A/Texas/37/2024-reaktive Antikörper im Serum und Plasma nachgewiesen werden. Die Neutralisationstiter gegen A/Texas/37/2024 waren jedoch durchweg niedriger als die Titer gegen saisonale H1- und H3-Viren. Zur weiteren Untersuchung der zugrunde liegenden B-Zell-Antwort wurden H5-reaktive B-Gedächtniszellen aus sechs H5N1-naiven Individuen isoliert. Die funktionelle Charakterisierung der von diesen Zellen kodierten Antikörper zeigte hochpotente, A/Texas/37/2024-neutralisierende Antikörper in fünf der sechs Individuen. Weitere Analysen zeigten, dass diese Antikörper mit H1 kreuzreagieren, mit Antikörpern gegen die konservierte Stammregion des HA konkurrieren und Mäuse vor einer letalen H5N1-Infektion schützen. Zusammen deuten diese Ergebnisse darauf hin, dass die auf Serumebene beobachtete H5-Reaktivität überwiegend durch Gruppe 1-kreuzreaktive HA-Stamm-Antikörper vermittelt wird. Diese Arbeit konnte somit die Existenz kreuzreaktiver Antikörper gegen das H5N1 A/Texas/37/2024-Isolat in Personen ohne vorherigen Kontakt mit diesem Influenzasubtyp nachweisen. Weitere Untersuchungen sind erforderlich, um die funktionelle Relevanz dieser Antikörper für den Schutz vor Infektionen mit neu auftretenden zoonotischen Influenzaviren besser beurteilen zu können.

1 Introduction

The interaction between pathogens – such as viruses, bacteria, fungi, or parasites – and their hosts represents a continuous evolutionary challenge for both sides. Host immune systems have evolved to detect and eliminate invading microorganisms. In turn, pathogens have developed strategies to evade immune recognition, thereby ensuring their survival and continued transmission within host populations.

Over the last two centuries, scientific advances in microbiology, immunology and vaccinology have greatly improved our understanding of infectious pathogens, their associated diseases and host immune responses. This knowledge has facilitated the development of preventive and therapeutic measures, such as vaccines, antibiotics or antiviral drugs, which have substantially reduced the burden of many infectious diseases. In the case of smallpox, global eradication has even been achieved through worldwide vaccination campaigns (World Health Organization, 1980).

Nevertheless, infectious diseases continue to pose a major risk for global health, as clearly underlined by the recent COVID-19 pandemic. Viruses, in particular, remain difficult to control as they can quickly adapt to new hosts and environments. Moreover, viruses often circulate in animal populations and zoonotic transmission to humans poses a risk for the emergence of new infectious diseases. Influenza viruses represent a prominent example, as they continuously circulate in both human and animal populations and remain a major public health concern.

1.1 Influenza A virus

Influenza viruses are enveloped viruses with a segmented, single-stranded, negative-sense RNA genome and belong to the family of *Orthomyxoviridae*. They are classified into four genera – influenza A, B, C, and D – which differ in genome structure and the encoded viral proteins (Treanor, 2023). Among them, only influenza A, B, and C viruses are known to infect humans. Influenza C viruses cause only mild respiratory infections with cold-like symptoms, particularly in young children (Sederdahl and Williams, 2020). In contrast, influenza A and B viruses constantly circulate in the human population and cause annual epidemic outbreaks of the respiratory disease influenza, also known as the flu. Seasonal infections are associated with respiratory symptoms like cough, sore throat, and nasal congestion as well as fever, myalgia, or headache (Monto et al., 2000). In healthy individuals, symptoms typically resolve within one to two weeks. However, influenza can lead to severe disease or may be fatal in the presence of complications, such as bacterial co-infection, or in individuals belonging to risk groups. These include pregnant women, young children, the elderly, individuals with chronic illnesses, or immunocompromised patients (Uyeki et al., 2022). According to current estimations, seasonal influenza epidemics are responsible for about 5.7 million hospitalizations and 290,000 to 650,000 deaths worldwide every year (Iuliano et al., 2018; Lafond et al., 2021; World Health Organization, 2026a).

Influenza A viruses (IAV) are further divided into subtypes based on antigenic differences in their surface glycoproteins hemagglutinin (HA) and neuraminidase (NA) with 19 HA (H1-H19) and 11 NA (N1-N11) subtypes identified so far (Fereidouni et al., 2023; Karakus et al., 2024). Among these, only a limited number of subtypes have established sustained circulation in humans. Currently, H1N1 and H3N2 co-circulate worldwide, while H2N2 circulated in humans from 1957 until 1968 (Treanor, 2023). As a zoonotic pathogen, IAV can infect not only humans but also diverse animal species including birds, cats, pigs, horses, or marine mammals. This broad host range enables the emergence of novel virus variants with pandemic potential for humans. Such zoonotic viruses have repeatedly caused pandemics in the human population, like the Spanish influenza pandemic in 1918 or the 2009 H1N1 pandemic (Fong, 2017).

In contrast to IAVs, influenza B viruses show less antigenic and genetic variation and are therefore not divided into subtypes but into two lineages termed Victoria and Yamagata. Both lineages have emerged in the 1970s and have co-circulated in humans since then. However, the Yamagata lineage has not been detected since 2020. Although influenza B viruses can infect several animal species, they primarily circulate in humans and are therefore not considered to have the same pandemic potential as IAV (Ashraf et al., 2024; Treanor, 2023).

Influenza virus isolates are named using a standardized nomenclature including the antigenic type (A-D), the host species (omitted for human isolates), the geographic origin, the isolate serial number, the year of isolation, and, for IAV, the subtype information (World Health Organization, 2026b). For example, the isolate A/Hawaii/70/2019 (H1N1) refers to a human influenza A H1N1 virus isolated in Hawaii in 2019.

1.1.1 Structure of the viral particle

IAV virions are pleiomorphic and can appear as spherical particles of approximately 100 nm or as elongated filamentous forms (Harris et al., 2006). The viral envelope is composed of a lipid bilayer that is derived from the plasma membrane of the host cell (Figure 1). Embedded within this membrane are the three transmembrane proteins HA, NA, and the matrix protein 2 (M2) (Noda, 2012). HA is the most abundant surface protein on the virion, whereas NA is present at lower density, with an approximate HA:NA ratio of 7:1. Only a small number of M2 molecules are incorporated (Harris et al., 2006; Rossman and Lamb, 2011). Both glycoproteins, HA and NA, form spikes on the viral envelope, representing the major antigenic determinants of the virus. The integral M2 functions as an ion channel that mediates proton transport, essential for viral particle acidification during entry of the host cell (Pinto et al., 1992; Shimbo et al., 1996; Wang et al., 1994). Beneath the viral envelope there is a layer of matrix protein 1 (M1) which provides structural support and interacts with the viral ribonucleoprotein (vRNP) complexes (Noda, 2012).

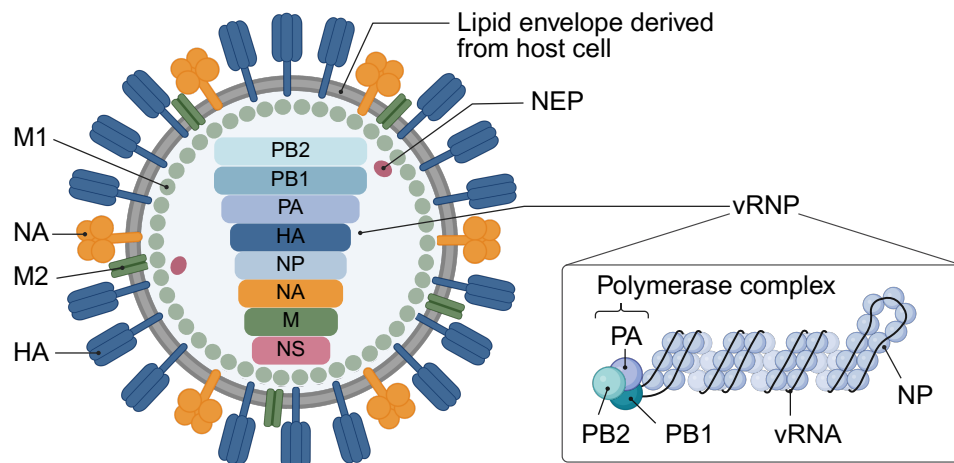


Figure 1. Structure of the influenza A virus particle. The viral particle contains the three transmembrane proteins hemagglutinin (HA), neuraminidase (NA), and the M2 ion channel, embedded in a host-derived lipid membrane that forms the viral envelope. The inner surface of the envelope is lined by matrix protein 1 (M1). The viral core comprises eight single-stranded, negative-sense viral RNA (vRNA) segments. Each vRNA is encapsidated by nucleoprotein (NP) and associated with the viral polymerase complex consisting of polymerase basic proteins 1 and 2 (PB1, PB2) and the polymerase acidic protein (PA), to form viral ribonucleoprotein (vRNP) complexes. The nuclear export protein (NEP) is encoded by the NS segment, which also encodes for the non-structural protein 1 (NS1), one of several non-structural influenza A proteins (adapted from Medina and García-Sastre, 2011, created with BioRender.com).

The viral genome lies in the core of the virion and is composed of eight negative-sense single-stranded viral RNA (vRNA) segments that encode for up to 18 viral proteins (Jiang et al., 2023; Yamayoshi et al., 2015). Each segment is coated with nucleoprotein (NP) and is associated with the heterotrimeric viral RNA-dependent RNA polymerase, consisting of the polymerase acidic protein (PA) and the polymerase basic proteins 1 and 2 (PB1, PB2). These helical structures of vRNA, NP, and the polymerase form the vRNP complexes. In addition to the proteins incorporated into the viral particle, IAV expresses several non-structural proteins during replication that regulate viral replication and contribute to pathogenicity (Hao et al., 2020).

1.1.2 Hemagglutinin structure and antigenic organization

Among the viral proteins, HA plays a central role as it mediates receptor binding and membrane fusion during host cell entry. It is thus also the major antigenic protein. Its structure and major antigenic sites were first reported in 1981 and have since been further characterized (Wiley et al., 1981; Wilson et al., 1981). The mature protein is a homotrimer consisting of three identical subunits (Figure 2A, Wilson et al., 1981). Each monomer is synthesized as a single polypeptide chain (HA0) and then cleaved by host cell proteases into the HA1 and HA2 subunits, which stay connected through a single disulfide bond (Figure 2B). The cleavage site can be monobasic or polybasic. This determines which host proteases can process the protein thereby influencing tissue tropism and pathogenicity (Skehel and Waterfield, 1975; Wu and Wilson, 2020). Based on genetic, antigenic, and structural differences, HA subtypes are divided into two groups, with group 1 comprising subtypes H1, H2, H5, H6, H8, H9, H11, H12, H13, and H16, and group 2 comprising H3, H4, H7, H10, H14, and H15 (see phylogenetic tree in Figure 23, Gamblin et al., 2021).

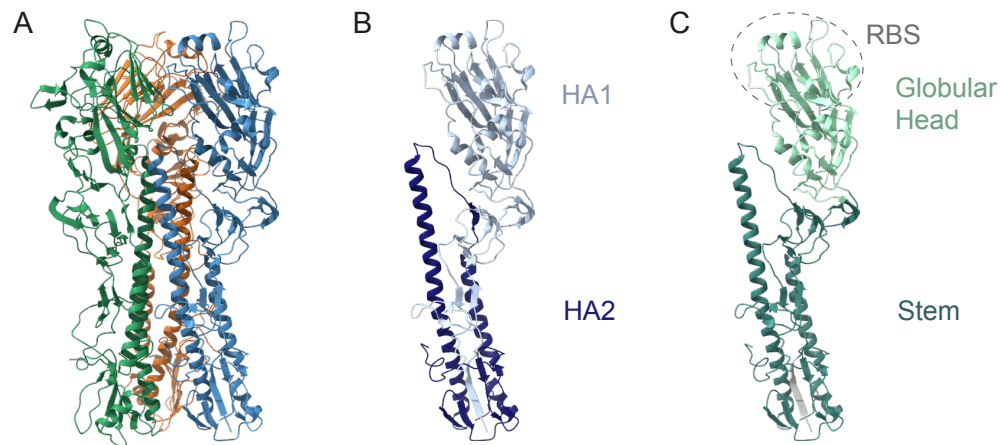


Figure 2. Hemagglutinin (HA) structure. (A) Structure of an HA homotrimer from the H1N1 strain A/Puerto Rico/8/1934 (Gamblin et al., 2004; PDB identifier: 1RU7). (B) Monomeric structure of the HA from (A) showing the two polypeptide chains HA1 (light blue) and HA2 (dark blue). (C) Monomeric structure of the HA from (A) showing the globular head domain (light green) containing the receptor binding site (RBS) and the stem domain (dark green). Structures were displayed and annotated using ChimeraX.

The HA monomers each are divided into a membrane-distal globular head domain and a membrane-proximal stem domain (Figure 2C). The head domain is formed by HA1 and comprises the receptor binding subdomain and the vestigial esterase (VE) subdomain. The RB subdomain also includes the receptor binding site (RBS) which mediates attachment to the host cell receptors (Jiao et al., 2023). The stem domain mainly consists of HA2 and small parts of HA1 and is structurally divided into the fusion subdomain and the membrane anchor subdomain (Gamblin et al., 2021; Tan et al., 2019). During host cell entry, it mediates fusion with the host cells' plasma membrane. In contrast to the HA head, the stem domain shows increased conservation and contains sites that are highly conserved across most HA subtypes (Wu and Wilson, 2020).

1.1.3 Influenza A virus replication cycle

The structural characteristics of HA are directly linked to its role in the viral replication cycle. Entry is initiated by binding of HA to sialic acid-containing receptors on the host cell surface via its RBS in the head domain (Figure 3, Carter and Iqbal, 2024). Binding triggers clathrin-mediated endocytosis of the virion, however, especially larger filamentous particles can also be taken up by macropinocytosis (de Vries et al., 2011; Rossman et al., 2012; Rust et al., 2004). Following transport through early and late endosomes, the gradual acidification of these compartments triggers pH-dependent conformational changes of the HA protein, resulting in exposure of the HA2 fusion peptide. The fusion peptide is inserted into the endosomal membrane and leads to fusion of the viral and endosomal membranes (Hamilton et al., 2012; Wilson et al., 1981). The low pH inside the endosome also triggers activation of the M2 ion channel causing protons to enter the virus particle (Pinto et al., 1992; Shimbo et al., 1996). As a result, the viral core becomes acidified which allows dissociation of M1 and the vRNP complexes and release of the genome into the cytoplasm (Martin and Helenius, 1991).

For genome replication and transcription, vRNPs are imported into the host cell nucleus (Hutchinson and Fodor, 2012). Within the nucleus, the viral RNA-dependent RNA polymerase uses the negative-sense vRNA as a template to synthesize both positive-sense messenger RNA (mRNA) for protein production and complementary RNA (cRNA). The cRNA then serves as a template to generate new genomic vRNA for new viral particles. Synthesis of viral mRNA requires a primer, which the viral polymerase obtains from host pre-mRNAs in a process known as cap snatching. The PB2 subunit binds to the 5' cap of nascent host mRNAs and the PA subunit then cleaves the RNA downstream of the cap structure, which then serves as a primer for PB1 for transcription of viral mRNA. The 5' cap is also required for recognition of viral mRNA by the cellular translation machinery (Carter and Iqbal, 2024; Dou et al., 2018).

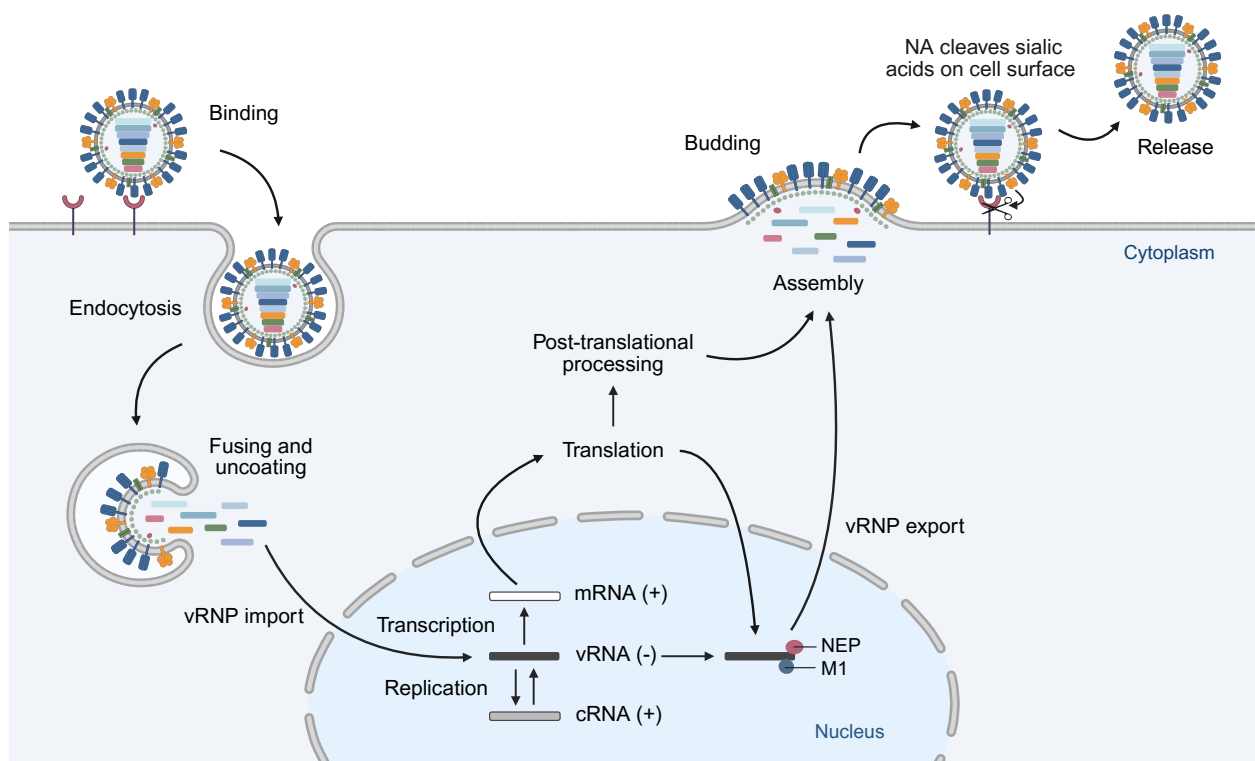


Figure 3. Influenza A virus life cycle. The influenza life cycle starts with hemagglutinin (HA) binding to $\alpha(2,3)$ - or $\alpha(2,6)$ -linked sialic acid-containing receptors on the host cell surface, initiating receptor-mediated endocytosis. Following endosomal uptake, fusion of the viral and endosomal membranes enables release of viral ribonucleoproteins (vRNPs) into the cytoplasm and subsequent import into the nucleus. There, negative-sense viral RNA (vRNA) is transcribed into positive-sense complementary RNA (cRNA) and viral mRNA, with cRNA serving as a template for genome replication. Viral mRNAs are exported for protein synthesis and the proteins of the viral polymerase complex re-enter the nucleus to assemble with vRNA to build new vRNPs. Matrix protein 1 (M1) and the nuclear export protein (NEP) also re-enter the nucleus and mediate nuclear export of vRNPs. Assembly of viral components occurs at lipid raft domains of the apical plasma membrane, followed by budding and release of progeny virions, a process facilitated by neuraminidase (adapted from Medina and García-Sastre, 2011, created with BioRender.com).

Following export from the nucleus, viral mRNA is then translated at cytosolic ribosomes or, in case of the membrane proteins HA, NA, and M2, at ribosomes associated with the endoplasmic reticulum. Proteins PB1, PB2, PA, and NP are then re-imported into the nucleus and support vRNA transcription and replication and assemble with vRNA to form new vRNPs (Dou et

al., 2018). NEP and M1 also enter the nucleus and mediate export of the vRNPs from the nucleus (Akarsu et al., 2003; Brunotte et al., 2014; Huang et al., 2013; Shimizu et al., 2011).

Assembly and budding of new viral particles occur at the apical plasma membrane in a multistep process involving both viral and cellular factors. First, the glycoproteins HA and NA concentrate in lipid rafts in the membrane which leads to membrane curvature and the formation of budding sites. M1, M2, and the vRNPs are then recruited to these sites to form complete virus particles. Following membrane scission, efficient release of newly formed virions depends on NA, as particles may otherwise remain attached to the cell surface through HA binding to sialic acids. Using its sialidase activity, NA then cleaves these sialic acid residues from the cell surface thereby releasing the new virions (Rossman and Lamb, 2011).

1.1.4 Epidemiology and the zoonotic potential of influenza A viruses

Although the replication cycle of IAV follows a defined series of intracellular steps and is tightly regulated, the virus exhibits a high degree of genetic variability with important consequences for its evolution and epidemiology. This genetic variability is largely driven by the error-prone RNA-dependent RNA polymerase of IAV, which lacks proofreading function (Steinhauer et al., 1992). Thus, frequent point mutations are introduced during viral genome replication, including in the antigenic regions of HA and NA. Population immunity then drives the selection of virus variants with antigenic changes in HA and NA that confer escape from pre-existing antibodies (Carrat and Flahault, 2007). This gradual accumulation of minor antigenic changes is called *antigenic drift* (Both et al., 1983). It is responsible for seasonal epidemic outbreaks of influenza, as it allows the emergence of new virus variants that can evade pre-existing immunity acquired during previous seasons. Consequently, individuals can be repeatedly infected with viruses of the same subtype, enabling influenza viruses to circulate in the human population year after year (Webby and Webster, 2001).

Another factor contributing to the genetic variability of IAV is its segmented genome. If one cell is infected with two different influenza viruses, entire genome segments can be exchanged. This process, known as *reassortment*, can generate progeny viruses with new combinations of gene segments, including complete replacement of the HA and/or NA segments. Such major antigenic changes are referred to as *antigenic shift* and occur with a much lower frequency. The resulting viruses can spread rapidly due to limited population immunity, and have been the cause of pandemics with substantial morbidity and mortality (Treanor, 2023).

1.1.4.1 Influenza pandemics and subtype circulation in the human population

Three influenza pandemics occurred during the 20th century and one in the 21st century (Figure 4). All of these resulted from major antigenic shifts involving the introduction of novel HA and/or NA subtypes from animal hosts into the human population (Fong, 2017). The most severe

influenza pandemic was the Spanish influenza pandemic in 1918/1919, also known as the “Spanish flu”. It was caused by IAV of the subtype H1N1 and affected approximately one third of the world population (approx. 500 million people) leading to approximately 50–100 million deaths (Johnson and Mueller, 2002; Taubenberger and Morens, 2020). The pandemic was associated with unusually high mortality in young adults. Frequent bacterial co-infections contributed to a large proportion of the fatalities (Morens et al., 2008; Taubenberger and Morens, 2006). Similar to later pandemics, population immunity gradually developed over the following years. The typical pattern of seasonal epidemics re-emerged, caused by antigenically drifted strains and associated with lower mortality (Ferreira et al., 2023).

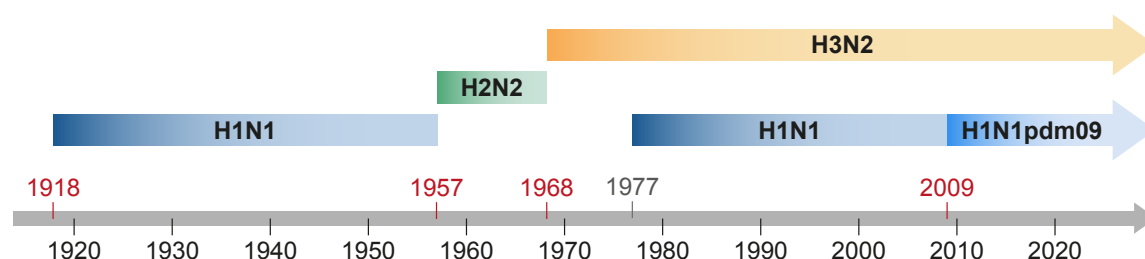


Figure 4. Timeline of influenza A virus subtype circulation in the human population. Depicted is the circulation of influenza A virus subtypes in the human population over time. Years highlighted in red indicate influenza pandemics associated with the emergence of novel subtypes (adapted from Treanor, 2023).

In 1957, H1N1 was replaced by an H2N2 virus causing the “Asian flu” pandemic, which was less severe due to improved medical care, availability of antibiotics, and emerging vaccine development (Ferreira et al., 2023). H2N2 circulated in the population afterwards until it was replaced in 1968 by an H3N2 virus, causing the “Hong Kong flu” pandemic. This pandemic again appeared to be less severe, possibly because only the HA subtype had changed, while the retained N2 allowed for partial immunity against NA from previous H2N2 infections (Monto and Kendal, 1973; Murphy et al., 1972). In 1977, H1N1 re-emerged from an uncertain origin and spread globally, however, infections were largely limited to younger individuals without previous H1N1 exposure. Hence, the outbreak was not classified as a pandemic (Kilbourne, 2006; Monto and Fukuda, 2020). Since then, H1N1 and H3N2 co-circulate in the human population.

In 2009, a novel H1N1 virus from swine origin caused another relatively mild influenza pandemic with an estimated 100,000–400,000 respiratory fatalities worldwide, predominantly in individuals younger than 65 years (Dawood et al., 2012; Simonsen et al., 2013). This virus was the result of multiple reassortment events between avian, human, and swine influenza viruses and was antigenically distinct from the previously circulating seasonal H1N1 strains (Garten et al., 2009). To distinguish it from earlier H1N1 viruses, this lineage and its descendants are referred to as H1N1pdm09. The H1N1pdm09 virus replaced the previously circulating seasonal H1N1 strain and continues to co-circulate in humans together with H3N2 viruses (Fong, 2017).

1.1.4.2 Avian influenza viruses and zoonotic transmission to humans

As demonstrated by past influenza pandemics, IAVs circulating in animal reservoirs pose a continuous threat to both human and animal health. Wild aquatic birds, such as ducks, geese, swans, gulls, and waders, serve as the primary natural reservoir for IAV with 17 HA (H1-H16 and H19) and 9 NA (N1-N9) subtypes identified in birds so far (Bellido-Martín et al., 2026; Karakus et al., 2024; Webster et al., 1992). In aquatic birds, these avian influenza viruses (AIVs) mainly infect the intestinal tract, causing only mild or asymptomatic infection (Causey and Edwards, 2008). Viruses are shed in the feces and are primarily transmitted via the fecal-oral route. This allows them to spread through contaminated water. Due to the long-distance migration of wild birds, AIVs can rapidly be disseminated across continents along migratory flyways, facilitating the global spread of newly emerging strains (Olsen et al., 2006).

AIVs are further classified based on their pathogenicity in chickens into low pathogenic AIVs (LPAIVs) and highly pathogenic AIVs (HPAIVs). Most AIVs are of low pathogenicity and cause only mild disease after transmission from wild birds to poultry. After introduction into poultry, however, LPAIVs of the subtypes H5 and H7 can evolve into HPAIVs. This transition is linked to the cleavability of the HA precursor HA0 into HA1 and HA2 (see chapter 1.1.2). LPAIVs have a monobasic cleavage site that is processed by trypsin-like proteases, which are mainly restricted to the intestinal and respiratory tract of chickens. In contrast, HPAIVs contain a polybasic cleavage site that can be cleaved by furin-like proteases expressed in a wide range of tissues, thereby enabling systemic infection in chicken associated with severe disease and high mortality (Bellido-Martín et al., 2026; Munster and Fouchier, 2009). The pathogenicity of these HPAIVs for other (wild) birds and mammals varies between different species and virus strains (Alexander et al., 1986, 1978; Kuiken et al., 2010).

Several HPAIV lineages, especially those of the subtype H5N1, have spread globally. They repeatedly cause outbreaks in wild birds and poultry that come along with high mortality and substantial economic losses. Moreover, infections have been observed in a broad range of wild and domestic animals and occasionally also in humans (Peacock et al., 2025). In general, however, AIVs primarily infect avian hosts because of their receptor specificity. AIVs preferentially bind to α 2,3-linked sialic acids, which are highly expressed on epithelial cells of the intestinal and respiratory tract in birds. In contrast, human influenza viruses prefer α 2,6-linked sialic acids as receptors, which are found predominantly in epithelial cells of the upper respiratory tract in humans. α 2,3-linked sialic acids, in turn, are mostly found in the human lower respiratory tract (Rogers and Paulson, 1983; Shinya et al., 2006; van Riel et al., 2006).

These differences in receptor specificity and distribution represent an important species-barrier, which constrains the pandemic potential of IAV. Swine however, express both α 2,3- and α 2,6-linked sialic acids in their respiratory tract and can thus be infected with both avian and

human IAV, making them an important intermediate host for the emergence of novel reassortant viruses with pandemic potential (Krammer et al., 2025).

Despite the species barrier, sporadic infections of humans with AIVs have been reported. These include both LPAIVs of the subtypes H7N2, H7N3, H7N9, H9N2, H6N1, H10N7, and H10N8, as well as HPAIVs of the subtypes H5N1, H5N6, H7N3, H7N7, and H7N9. However, most of these infections represent dead-end events, as the viruses replicate mainly in the human lower respiratory tract. This likely limits the amount of virus released during breathing or coughing and thereby prevents efficient human-to-human transmission (Krammer et al., 2025; Wang et al., 2021). Most human cases occur after close contact with infected animals, suggesting that infection requires inhalation of a high infectious dose into the lower respiratory tract, where α 2,3-linked sialic acids are present. Clinical symptoms range from mild respiratory illness and conjunctivitis to severe pneumonia or death (Wang et al., 2021).

Among the AIVs, H5 subtype viruses have attracted particular attention, as they are frequently associated with high pathogenicity and zoonotic infections. The first confirmed human infections with highly pathogenic H5N1 viruses were detected in 1997 in Hong Kong, where 18 cases were reported, six of which were fatal (Claas et al., 1998; Subbarao et al., 1998; Xu et al., 1999). Subsequent human cases were reported in 2003. Between 2003 and January 2025, a total of 964 human H5N1 infections with 466 deaths (case-fatality rate: 48.3%) were reported to the World Health Organization (WHO) from 24 countries (World Health Organization, 2025). Most HPAIV H5N1 viruses belong to the A/Goose/Guangdong/1/1996 (Gs/Gd) lineage, which emerged in the 1990s. The global spread of this lineage was accompanied by genetic diversification, resulting in a complex classification system that groups H5 subtype viruses into clades and subclades based on their HA sequence (Krammer et al., 2025). Several of these clades have been associated with infections in poultry, mammals and occasionally humans.

In recent years, H5 viruses have been increasingly detected in a broad range of mammalian species, raising concerns about adaptation to mammalian hosts (Bellido-Martín et al., 2026). Studies on H5 transmission have shown that, in principle, AIVs can overcome the avian-human species barrier through specific mutations in different viral proteins. These include for example changes in HA (e.g. Q226L) that alter receptor binding specificity towards α 2,6-linked sialic acids, thereby facilitating airborne transmission in mammalian models. Moreover, mutations in the polymerase (e.g. PB2 E627K) can enhance replication in mammalian cells (Chutinimitkul et al., 2010; Herfst et al., 2012; Imai et al., 2012; Linster et al., 2014; Subbarao et al., 1993; Suttie et al., 2019). Some of these mutations have been observed in virus isolates from natural outbreaks, highlighting again the pandemic potential of AIVs (Russell et al., 2012). Continuous surveillance of circulating strains is therefore crucial to detect the emergence of viruses with increased zoonotic or pandemic potential.

1.1.4.3 H5N1 clade 2.3.4.4b viruses and the dairy cattle outbreak

Among the currently circulating H5 viruses of the Gs/Gd lineage, clade 2.3.4.4b has emerged as a group of major epidemiological relevance. Over the past years, viruses of this clade have undergone extensive diversification through frequent reassortments with other AIVs and dominate outbreaks in wild birds and poultry (Xie et al., 2023). In 2021, a new H5N1 reassortant of clade 2.3.4.4b emerged in Europe and rapidly spread across Europe, Asia, and Africa, replacing previously circulating H5 viruses. These novel H5N1 viruses were also introduced into North America by migratory birds in late 2021 and subsequently spread to South America and Antarctica (Banyard et al., 2024; Bevins et al., 2022; Caliendo et al., 2022; Jimenez-Bluhm et al., 2023). In the United States (US), this was followed by repeated outbreaks in commercial and backyard poultry flocks, affecting more than 200 million birds to date, including both natural deaths and culling (US Department of Agriculture Animal and Plant Health Inspection Service, 2026a). Beyond poultry, clade 2.3.4.4b H5N1 viruses have also been the source of an unprecedented number of spillover events to a broad range of wild and domestic mammalian species, especially carnivores and marine mammals (Peacock et al., 2025; Webby and Uyeki, 2024). In several cases during 2022-2023, including outbreaks in European fur farms and mass mortality events in South American sea lions and seals, the scale and clustering of infections suggest that mammal-to-mammal transmission has occurred (Bellido-Martín et al., 2026; Peacock et al., 2025).

In March 2024, clade 2.3.4.4b H5N1 infections in dairy cattle were first detected in Texas and subsequently developed into a multistate outbreak across the US (Caserta et al., 2024). This represented an unexpected expansion of the host range because cattle have previously not been considered susceptible to influenza infections (Krammer et al., 2025). The infections were caused by a new H5N1 reassortant, referred to as genotype B3.13. Sequence analysis suggests that this virus was likely introduced by a single spillover event from infected birds to cattle in late 2023 (Nguyen et al., 2025).

In cattle, the infection primarily targets the mammary glands rather than the respiratory tract, causing mastitis and reduced milk production (Halwe et al., 2025). Consequently, high virus titers can be found in the raw milk, however, pasteurization effectively inactivates infectious virus and ensures safety of dairy products (Alkie et al., 2025; Halwe et al., 2025; Schafers et al., 2025). The transmission between animals likely occurred via contaminated milking machines (Le Sage et al., 2024). In combination with the transport of infected animals between farms, this facilitated rapid spread of the virus across multiple US states. As of May 2026, 1,106 cases in dairy cattle across 19 US states have been confirmed, with the most recent case reported on 12 May 2026 (US Department of Agriculture Animal and Plant Health Inspection Service, 2026b). Viral RNA continues to be detected in milk samples in Idaho and California, indicating ongoing circulation in these states (US Department of Agriculture Animal and Plant Health Inspection Service, 2026c).

The bovine H5N1 virus has also been transmitted to other animals on and near the affected dairy farms, including cats, raccoons, wild birds, alpacas, and poultry, highlighting the continued cross-species transmission (Caserta et al., 2024; Lehman et al., 2026; Nguyen et al., 2025). Shortly after the initial detection of infections in cattle, the first human case associated with this outbreak was reported in March 2024 (Uyeki et al., 2024). Since February 2024, a total of 71 human H5N1 cases have been reported in the US, of which 41 were linked to contact with dairy cattle, 24 to outbreaks on poultry farms, and 6 cases to other animal or unknown exposures (CDC, 2026a). However, serological data suggests that additional undetected infections have occurred among exposed individuals, as 7% seropositivity was detected among dairy farm workers (Mellis et al., 2024). Clinical symptoms in identified human cases in the US have generally been mild, most commonly presenting as conjunctivitis. So far, no human-to-human transmission has been observed (CDC, 2026b; Garg et al., 2025).

In response to the outbreak, control measures such as culling and movement restrictions have been implemented. Nevertheless, as of May 2026, the outbreak is still ongoing. The continued circulation in cattle, accompanied by spillback into wild birds and transmission to other mammals including humans, increases the risk of viral adaptation and raises concerns about the emergence of viruses with enhanced transmissibility in humans.

1.1.5 Countermeasures against influenza A infections

Despite these concerns, a key advantage in the context of human infections with H5N1 and other AIVs is the extensive existing knowledge of influenza viruses and the experience gained from seasonal and pandemic outbreaks. In contrast to the emergence of SARS-CoV-2, a range of countermeasures, including antiviral drugs and vaccines, is already available and licensed. Although primarily developed for seasonal influenza, some of these approaches have demonstrated activity against AIVs or show potential effectiveness, providing an important foundation for pandemic preparedness.

1.1.5.1 Antiviral therapy

Multiple classes of antiviral drugs are available for the treatment of seasonal influenza infections. The three main classes, currently approved in Europe and the US, comprise NA inhibitors, cap-dependent endonuclease (CEN) inhibitors, and M2 inhibitors (Kumari et al., 2023). NA inhibitors, like oseltamivir or zanamivir, inhibit the function of the viral NA and thereby prevent the release of newly produced virions (Gubareva and Mohan, 2022; Moscona, 2005). CEN inhibitors, such as baloxavir marboxil, target the viral replication machinery and inhibit the cap-snatching function of the PA protein (Takashita, 2021). In contrast, the M2 ion channel inhibitors amantadine and rimantadine interfere with viral uncoating and fusion of viral and host cell membranes. However,

they are no longer recommended for treatment as most circulating seasonal influenza strains are resistant (Kumari et al., 2023).

In addition to these three classes, antiviral approaches targeting other steps of the influenza replication cycle have been developed or are under investigation. These include HA inhibitors like umifenovir (licensed in China and Russia) or inhibitors of the viral polymerase complex such as favipiravir (limited approval in Japan). Furthermore, passive immunization strategies, including monoclonal antibody (mAb)-based therapies, are under evaluation. Broadly neutralizing antibodies (bnAbs) targeting conserved epitopes on the HA protein represent a promising complementary approach due to their cross-subtype reactivity and potential applicability to infections with AIVs (Jones et al., 2023). Several bnAbs have been evaluated in clinical studies, however, their clinical outcomes have been variable, and no candidate has yet been approved for treatment (Ali et al., 2018; Han et al., 2021; Hershberger et al., 2019; Jones et al., 2023; Lim et al., 2022, 2020; Tan et al., 2024).

Antiviral treatment is also highly relevant in the context of infections with AIVs. NA inhibitors have been used in the treatment of AIV infections, however clinical data are limited. Observational studies in patients with H5N1 infections suggest a potential survival benefit of oseltamivir treatment, whereas no association with improved survival was found in patients infected with H7N9 viruses (Adisasmito et al., 2010; Chan et al., 2012; Cheng et al., 2021; Kandun et al., 2008). Clinical data on baloxavir marboxil treatment is not available but *in vitro* and *in vivo* data indicates efficacy against H5, H7, and H9 subtypes (Andreev et al., 2024b; Takashita et al., 2020). *In vitro* susceptibility data also suggests that both NA and CEN inhibitors retain activity against clade 2.3.4.4b H5N1 viruses (Andreev et al., 2024a, 2024b).

For pandemic preparedness, anti-influenza drugs are thus stockpiled by countries worldwide, however, their efficacy depends strongly on early administration. Moreover, the emergence of virus strains with antiviral resistance may further limit their effectiveness, highlighting the need for complementary strategies such as vaccination (Jones et al., 2023).

1.1.5.2 Vaccination

Despite the availability of treatment options, vaccination remains the most important measure to control influenza infections, as it can prevent disease and reduce transmission. Currently available seasonal influenza vaccines primarily aim to induce protective immunity against the major antigenic protein HA, while also eliciting more limited responses against NA. These vaccines, which are available as live attenuated, inactivated, or recombinant formulations, are typically composed of one A(H3N2) strain, one A(H1N1)pdm09 strain, and one or two influenza B viruses of the Victoria and/or Yamagata lineage (Wong and Webby, 2013). To account for the constantly ongoing antigenic drift, seasonal influenza vaccines need to be updated annually based on WHO surveillance data (Mokalla et al., 2025). This represents a major limitation of

seasonal influenza vaccines, as their effectiveness strongly depends on the antigenic match between the predicted vaccine strains and the circulating viruses (McLean and Belongia, 2021). Consequently, vaccine effectiveness varies from year to year, emphasizing the need for improved vaccination approaches that provide broader protection.

In the context of pandemic preparedness, several vaccines against avian influenza subtypes have been developed and licensed, especially for H5N1 viruses (Focosi and Maggi, 2024; Taaffe et al., 2025). These vaccines are primarily licensed for use in pandemic settings and not used in routine immunization. Several available candidates are based on H5 strains from previously circulating clades (e.g. clade 1 or clade 2.2.1) and rely on strain updates to improve antigenic matching to emerging viruses. Notably, one vaccine is available which incorporates an HA from a clade 2.3.4.4b virus (Zoonotic Influenza Vaccine, Seqirus). In general, however, H5 vaccines show limited immunogenicity and often require adjuvants and booster immunization (Krammer et al., 2025).

To overcome the need for constant updating of seasonal and zoonotic influenza vaccines, considerable efforts have been directed toward the development of a universal influenza vaccine. Current strategies focus on targeting conserved viral epitopes such as the HA stem, NA, M2 or internal proteins. The goal is to induce cross-protective immune responses across different influenza subtypes, providing long-term protection against seasonal influenza and viruses with pandemic potential (Nguyen and Choi, 2021).

1.2 Humoral immunity against influenza viruses

Protection against influenza viruses is mediated by a coordinated interplay of innate and adaptive immune responses. While the innate immune system provides rapid, non-specific defense mechanisms, adaptive immune responses mediate long-term and antigen-specific protection. A central component of this adaptive response is the production of antibodies, which play a key role in antiviral immunity by binding to viral particles and contributing to their neutralization and clearance.

1.2.1 Antibody structure

Antibodies, also referred to as immunoglobulins (Ig), are glycoproteins that specifically recognize and bind to antigens, a diverse range of molecular structures targeted by the adaptive immune system. They are composed of two identical heavy chains (HC) and two identical light chains (LC). These polypeptide chains are linked by disulfide bonds and form a characteristic Y-shaped molecule (Figure 5). Each HC and LC consists of both a variable (V) and a constant (C) region and can be subdivided into distinct domains. The LC contains one variable (V_L) and one constant (C_L) domain, whereas the HC comprises one variable domain (V_H) followed by three or four constant domains (C_{H1-4}), depending on the antibody isotype (Murphy et al., 2022).

The two identical arms of the antibody, known as the antigen-binding fragments (Fab), comprise the variable regions of the HC and LC, as well as the first constant domain of the heavy chain (C_{H1}), and the constant domain of the light chain. Together, the variable domains of both chains form the antigen-binding site, which determines the specificity of an antibody. Each variable domain can be further divided into three complementarity-determining regions (CDR1-3) and four intervening framework regions (FWR1-4). The CDRs form flexible loops that originate from hypervariable sequences and primarily determine antigen specificity, whereas the FWRs are more conserved and provide structural support (Murphy et al., 2022; Schroeder and Cavacini, 2010).

The two Fabs are linked to the stem of the antibody, known as the fragment crystallizable (Fc) region, via a flexible hinge region, which allows movement of the Fab arms. The Fc region consists only of constant domains of the HC. It mediates interactions with various components of the immune system, thereby enabling distinct effector functions of the antibody (Chiu et al., 2019; Murphy et al., 2022).

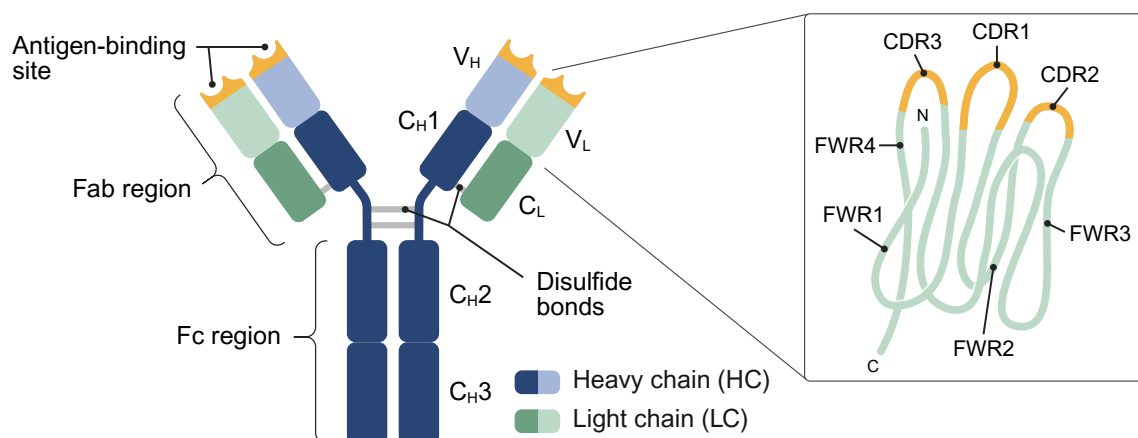


Figure 5. Antibody structure. Schematic illustration of an immunoglobulin G (IgG) antibody. The IgG is a Y-shaped molecule composed of two identical heavy chains (HC, blue) and two identical light chains (LC, green) linked by disulfide bonds. Each of these chains contains a constant region (C_H and C_L , respectively) and a variable region (V_H and V_L , respectively). The C_H region consists of three domains (C_{H1} -3). The two arms of the IgG form the antigen binding fragment (Fab), where V_H and V_L together form the antigen binding site. These variable regions contain three complementarity-determining regions (CDR1-3), hypervariable sequences that mainly determine antigen specificity, and four intervening framework regions (FWR1-4) that provide structural support. The stem of the IgG, known as the fragment crystallizable (Fc) regions, is responsible for effector functions (adapted from Murphy et al., 2022, created with BioRender.com).

Based on differences in their heavy chain constant region, antibodies can be classified into the five isotypes IgA, IgD, IgE, IgG, and IgM. While all isotypes follow the same overall antibody structure, they differ in their functional properties. Moreover, light chains occur in two types, kappa (κ) and lambda (λ), which differ in their amino acid sequence but have largely similar functional properties (Murphy et al., 2022).

1.2.2 B cell development and the generation of antibody diversity

Antibodies are produced by antibody-secreting cells, known as plasma cells and plasmablasts, which differentiate from B lymphocytes. These B lymphocytes, or B cells, are a central component of the adaptive immune system and originate from hematopoietic stem cells in the bone marrow, where they follow a highly regulated developmental program (Figure 6). During this process, they pass through defined developmental stages, including pro-B and pre-B cell stages, before they develop into immature B cells that express a functional B cell receptor (BCR) on their surface (Eibel et al., 2014).

This BCR represents a membrane-bound form of an antibody and allows the B cells to recognize its specific antigen. Importantly, each B cell expresses a unique BCR and can thus only recognize one specific antigen. A key feature of B cell development is therefore the generation of a highly diverse repertoire of B cells expressing different BCRs. This diversity facilitates the recognition of a broad range of potential antigens that arise from the extensive variability of pathogens and other foreign molecules. In humans, the repertoire is estimated to comprise at least 10^{11} distinct specificities and likely exceeds this by several orders of magnitude (Murphy et al., 2022).

Given the limited number of Ig gene segments in the genome, the generation of this highly diverse B cell and antibody repertoire relies on multiple diversification mechanisms that act during B cell development. A substantial proportion of this diversity is already established during early B cell development in the bone marrow through three mechanisms: V(D)J recombination, combinatorial diversification and junctional diversity (Murphy et al., 2022).

The variable region of the BCR, which determines its antigen binding specificity, is encoded by separate gene segments. A HC V region is assembled from one variable (V), one diversity (D), and one joining (J) gene segment, whereas in the LC V region, only one V and one J gene segment are combined. In the human genome, the Ig loci contain multiple different copies of these V, D, and J gene segments. The exact number of functional gene segments varies between loci (HC, κ or λ) and between individuals. These V, (D), and J gene segments are recombined and enzymatically joined to form an exon encoding the variable region. This process generates a highly diverse repertoire of V regions, although not all rearrangements result in productive gene products. In addition, BCR specificity depends on both the HC and LC V region, and random pairing of different HCs and LCs further increases combinatorial diversity. In addition, junctional diversity is generated by the insertion or deletion of nucleotides at the junctions between recombined V, (D), and J gene segments. This further enhances the diversity of antigen binding sites (Murphy et al., 2022; Rees, 2020; Roth, 2014; Tonegawa, 1983). As the extensive receptor diversification may generate autoreactive B cells, tolerance mechanisms ensure their elimination at multiple stages during B cell development (Meffre and Wardemann, 2008).

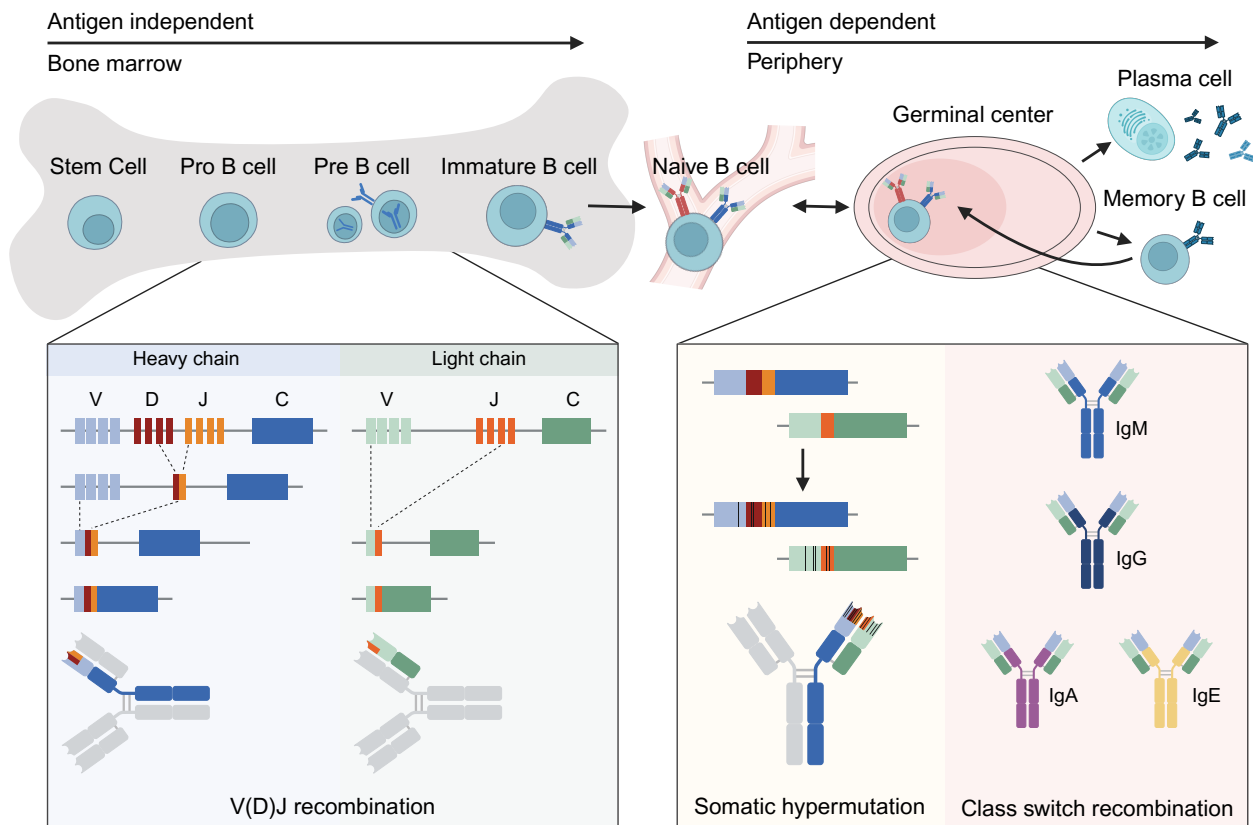


Figure 6. B cell development and generation of antibody diversity. B cells originate from hematopoietic stem cells in the bone marrow and progress through defined developmental stages, including pro-B and pre-B cells. During these stages, immunoglobulin (Ig) gene rearrangements occur. The variable region of the B cell receptor is generated through V(D)J recombination, involving the random assembly of variable (V), diversity (D), and joining (J) gene segments in the heavy chain locus, and V and J segments in the light chain loci. The constant region is encoded separately by downstream constant gene segments. This process, together with combinatorial pairing of heavy and light chains and junctional diversification at the recombination sites, establishes a highly diverse primary BCR repertoire. Immature B cells expressing a functional BCR undergo selection processes to eliminate autoreactive clones before exiting the bone marrow as naive B cells. These cells co-express IgM and IgD and circulate through secondary lymphoid organs. Upon antigen encounter, naive B cells become activated and undergo clonal expansion. A subset of activated B cells enters germinal centers, where further diversification occurs. Somatic hypermutation introduces point mutations into the variable regions of Ig genes, generating B cell clones with varying antigen affinities. In parallel, class switch recombination can replace the constant region of the heavy chain, enabling the transition from IgM to downstream isotypes such as IgG, IgA, or IgE. This process alters antibody effector function without affecting antigen specificity. The germinal center reaction ultimately gives rise to long-lived plasma cells, which secrete large amounts of antibodies, and memory B cells, which persist and mediate rapid and enhanced responses upon antigen re-exposure (adapted from Bai et al., 2018; Murphy et al., 2022; Schultheiß et al., 2022, created with BioRender.com).

After completing their development, the naive B cells leave the bone marrow and circulate through the bloodstream and the secondary lymphoid organs (i.e. the spleen and lymph nodes). Upon binding their specific antigen via the BCR, B cells become activated. This process commonly occurs in the B cell follicles of the secondary lymphoid organs, although antigen-specific B cell activation can also take place in extrafollicular sites. It often requires additional signals, particularly through interaction with antigen-specific helper T cells that provide co-stimulatory signals and cytokines. However, B cell activation can also occur in a T cell-independent manner (Cyster and Allen, 2019). Activated B cells then proliferate, generating a population of identical B cell clones with the same antigen specificity (clonal expansion). A subset of activated B cells subsequently undergoes further diversification and selection in the germinal centers of the secondary lymphoid organs in a process called affinity maturation. Within the

germinal centers, B cells undergo rapid proliferation and somatic hypermutation (SHM), whereby the enzyme activation-induced cytidine deaminase (AID) introduces point mutations in the Ig variable regions. This results in a heterogeneous population of B cells with varying affinities for the antigen. This heterogeneity creates a competitive environment in which B cells compete for limited antigen and T follicular helper cell-derived signals. B cells with higher antigen affinities more efficiently capture antigen, thereby gaining a selective advantage, whereas lower-affinity or non-functional variants fail to compete and undergo apoptosis. Through repeated cycles of proliferation, SHM, and selection, B cells undergo stepwise maturation resulting in the preferential expansion of high-affinity clones (De Silva and Klein, 2015; Victora and Nussenzweig, 2022).

In parallel, class switch recombination, also mediated by AID, enables the replacement of the constant region of the heavy chain. This leads to the expression of different antibody isotypes with distinct effector functions while maintaining antigen specificity (Murphy et al., 2022; Stavnezer and Schrader, 2014).

The final outcome of B cell activation is the differentiation into plasma cells and memory B cells. While plasma cells mediate immediate protection through the secretion of large quantities of antibodies, memory B cells persist long-term and form the basis for immunological memory. Upon re-exposure to the same antigen, memory B cells can rapidly reactivate and differentiate into antibody-secreting cells, thereby enabling a faster and more robust response (De Silva and Klein, 2015; Victora and Nussenzweig, 2022).

1.2.3 Antibody function

Antibodies mediate a range of distinct functions critical for immune defense, which are enabled by their Fab- and Fc-domains. One of the main functions of antibodies is neutralization. By binding to pathogens or toxins, antibodies can block their interaction with the host cell, thereby preventing infection or toxicity. This Fab-mediated process is particularly important during viral infections, like influenza, as neutralizing antibodies can directly prevent viral entry into the host cell, often through binding to the surface proteins of the virus (Burton, 2023; Lu et al., 2018).

In addition, after binding to their target antigen, antibodies can mediate Fc-dependent effector functions. The Fc region can interact with Fc receptors expressed on the surface of various immune cells, such as macrophages, neutrophils, and natural killer cells. This interaction activates macrophages and neutrophils to perform antibody-dependent cellular phagocytosis (ADCP). Natural killer cells, in turn, mediate antibody-dependent cellular cytotoxicity (ADCC) by releasing cytotoxic molecules that induce apoptosis in infected or target cells. Moreover, antibody binding can also activate the complement system, thereby linking adaptive immune responses to the innate immune system (Lu et al., 2018).

The functional properties of antibodies are further determined by their isotype, which influences their distribution across different body compartments, their half-life, and their effector

functions. For example, IgM is typically the first antibody produced during an immune response and is particularly efficient in activating the complement system. IgG represents the most abundant and long-lived antibody in the blood and extracellular fluids and mediates a wide range of effector functions. In contrast, IgA is the predominant antibody class at mucosal surfaces, such as the respiratory and gastrointestinal epithelia, where it mainly exerts its function through the neutralization of pathogens (Murphy et al., 2022).

1.2.4 Antibody responses targeting influenza viruses

Antibodies represent a central correlate of protection in influenza virus infections (Hobson et al., 1972; Krammer et al., 2020). Although all viral proteins are expressed during infection and can, in principle, elicit antibody responses, their accessibility to BCRs differs substantially. As a result, antibody responses are primarily directed against the viral surface proteins HA and NA (Krammer, 2019).

Antibody responses to HA dominate following natural infection and constitute the main source of neutralizing activity. Most of these antibodies target antigenic sites on the globular head domain, primarily those located in and around the RBS (Gamblin et al., 2021). Therefore, the HA head domain is immunodominant, which is consistent with the fact that it evolves faster than the stem region (Kirkpatrick et al., 2018). HA head-directed antibodies primarily prevent infection by blocking the interaction between HA and sialic acid receptors on the host cells. While HA responses can overall be very long-lived, continuous antigenic drift in the HA head domain results in substantial variability between circulating strains. Accordingly, head-directed antibodies are often strain-specific and provide only limited protection against newly emerging variants (Krammer, 2019; Wu and Wilson, 2020).

In addition, antibodies targeting the stem domain of HA can be detected in a substantial proportion of individuals, although typically at much lower levels (Nachbagauer et al., 2016; Sui et al., 2011). As this domain is relatively conserved, stem-specific antibodies often display broad reactivity across strains of multiple subtypes, often within the same HA group and in some cases across both group 1 and group 2 HA or even influenza B viruses (Krammer and Palese, 2013). Unlike head-directed antibodies, they generally do not interfere with receptor binding but mediate their antiviral activity through distinct mechanisms. These include the stabilization of HA in its prefusion conformation, thereby preventing fusion with the endosomal membrane, as well as blocking the proteolytic cleavage of HA required for viral infectivity (Brandenburg et al., 2013; Ekiert et al., 2009). In addition, stem-directed antibodies can recruit Fc-mediated effector functions, including ADCC, ADCP, and complement activation, contributing to viral clearance (DiLillo et al., 2014; Terajima et al., 2011).

Alongside HA-directed responses, antibodies targeting NA are also induced during infection, although typically at lower levels. These responses can display a certain degree of

breadth within a given NA subtype and may persist over extended periods. Functionally, they primarily act by inhibiting the enzymatic activity of NA, thus impairing the release of newly formed virions and limiting viral spread (Rajendran et al., 2021). Responses directed against other viral proteins, especially M2, M1, and NP, have likewise been detected, however, their contribution to protective immunity remains less well characterized (Krammer, 2019).

To assess influenza-specific antibody responses, a variety of experimental approaches has been established. Antibody binding to different influenza proteins is most commonly quantified using enzyme-linked immunosorbent assays (ELISA) (Reber and Katz, 2013). Most functional assays focus on HA-specific antibody activity, with the hemagglutination inhibition (HAI) assay representing the gold standard. It measures the ability of antibodies to prevent HA binding to sialic acid receptors on red blood cells, which normally leads to visible aggregation of these cells (Hirst, 1942; Spackman and Sitaras, 2020). Although HAI titers are widely used as a correlate of protection for seasonal influenza viruses, this assay predominantly measures head-directed responses. It therefore does not capture antibodies that neutralize the virus through alternative mechanisms, such as those targeting the HA stem (Reber and Katz, 2013). Alternative (micro)neutralization (MN) assays have also been developed, typically using live influenza virus to measure the ability of antibodies to prevent infection in cell culture by assessing cytopathic effects (Trombetta et al., 2014). Complementing these approaches, pseudovirus-based neutralization assays have been established as a flexible and widely used system to analyze antibody-mediated inhibition of viral entry. These assays employ replication-incompetent viral particles expressing influenza HA (and/or NA) on their surface and a reporter gene to quantify infection. Compared to assays using authentic viruses, pseudovirus systems allow more flexible and standardized neutralization analyses across diverse viral strains. At the same time, they reduce biosafety requirements and avoid the need for extensive collections of infectious virus isolates, which is particularly relevant for HPAIVs (Carnell et al., 2015; Trombetta et al., 2014).

As seasonal influenza viruses constantly circulate in the human population, most individuals acquire pre-existing immunity against influenza viruses through repeated infections and/or vaccinations, often beginning in early childhood (Bodewes et al., 2011; Sauerbrei et al., 2014). Accordingly, antibody responses to influenza viruses are strongly influenced by immune imprinting, which describes how previous exposures to a viral antigen shape immune responses to subsequent infections with antigenically related virus variants (King et al., 2023). Depending on the degree of antigenic similarity, subsequent exposures can preferentially recall immune responses to previously encountered epitopes, rather than induce *de novo* antibody responses (King et al., 2023; Maltseva et al., 2024). This phenomenon was first described in 1960 under the term original antigenic sin (OAS) in the context of influenza infections (Francis, 1960). Consequently, immune responses may become biased toward previously encountered epitopes, which may be detrimental when non-protective antibodies dominate and limit development of

responses to the antigenically distant virus variant. However, immune imprinting can also support protective immunity when recalled responses to shared epitopes are functionally neutralizing (King et al., 2023; Maltseva et al., 2024). Immune imprinting likely contributed to age-specific differences in disease burden during past influenza pandemics and has important implications for future vaccination strategies (Ma et al., 2025; Simonsen et al., 2004; Worobey et al., 2014).

Moreover, imprinting studies have demonstrated that pre-existing humoral immunity from seasonal influenza infections can substantially influence susceptibility and disease outcome following infection with zoonotic influenza viruses, as shown for H5N1 and H7N9 (Gostic et al., 2016). Pre-existing population immunity is therefore an important parameter in the Influenza Risk Assessment Tool (IRAT) developed by the Centers for Disease Control and Prevention (CDC) for pandemic risk assessment (CDC, 2024; Trock et al., 2015). In line with the observed impact of pre-existing immunity on zoonotic influenza infections, cross-reactive binding and neutralizing activity against H5 isolates have been detected in sera of individuals without prior H5 exposure (Bresson et al., 2006; Corti et al., 2010; Garcia et al., 2009; Nefkens et al., 2007; Pichyangkul et al., 2014; Sanz-Muñoz et al., 2024; Sui et al., 2011; Treanor et al., 2006; Yassine et al., 2018). Such cross-reactivity has been linked, at least in part, to antibodies targeting conserved epitopes, particularly within the HA stem domain (Corti et al., 2010; Sui et al., 2011). However, the level and prevalence of H5 cross-reactivity vary considerably between the above-mentioned studies, which may in part reflect differences in study cohorts, assay system, and the antigenic diversity of H5 strains. Importantly, the functional relevance of these cross-reactive responses remains incompletely understood, particularly in the context of continuous antigenic evolution of H5 viruses.

1.3 Aim of the thesis

IAVs remain a major challenge for global public health due to their broad host range and rapid evolution. Highly pathogenic avian H5N1 influenza viruses are of particular concern because of their zoonotic potential and their ability to cause severe disease in humans. When the outbreak of H5N1 clade 2.3.4.4b viruses in dairy cattle was reported in spring 2024, this marked an unexpected expansion of the host range of these viruses. The rapid spread and continuous circulation of H5N1 in mammals, accompanied by repeated spillovers to humans, raised concerns about ongoing viral adaptation and an increased pandemic risk. Pre-existing immunity is a key determinant of susceptibility to infection and disease severity in the context of emerging influenza viruses and an important parameter in pandemic risk assessment. However, at the onset of the outbreak, little was known about the presence of pre-existing immunity against the recently emerged bovine H5N1 clade 2.3.4.4b variant in individuals without prior exposure to H5N1. In particular, the prevalence and potency of pre-existing influenza-specific antibodies in H5N1-naïve individuals cross-reacting with this variant remained unclear.

The overall aim of this thesis was therefore to investigate pre-existing humoral immunity to the human H5N1 clade 2.3.4.4b isolate A/Texas/37/2024 originating from the cattle outbreak in H5N1-naïve individuals. To address this aim, three main objectives were pursued:

1) Establishment of an HA-pseudovirus neutralization assay for functional antibody analysis

To enable quantification of functional antibody reactivity against the emerging H5N1 strain as well as other IAV strains, a highly sensitive pseudovirus-based neutralization assay was established. The assay was optimized and validated to assess neutralizing activity of serum and monoclonal antibody samples. In addition, recombinant HA proteins were produced to allow complementary binding analysis.

2) Characterization of pre-existing A/Texas/37/2024-reactive antibody responses at the serum and plasma level

Pre-existing humoral immunity against the A/Texas/37/2024 strain was analyzed at the serum and plasma level using a combination of the established neutralization and binding assays. Serum reactivity was further compared across a panel of avian and seasonal influenza strains.

3) Analysis of the B cell origin and functional properties of pre-existing H5-reactive antibodies

The B cell origin of pre-existing H5-reactive antibodies was investigated by isolating A/Texas/37/2024-reactive memory B cells from five H5N1-naïve and one H5-vaccinated individuals. The antibodies encoded by these B cells were subsequently analyzed regarding their neutralization breadth, epitope specificity *in vitro*, as well as their protective capacity *in vivo*.

2 Material and Methods

2.1 Material

2.1.1 Chemicals, reagents, and media

Table 1. Chemicals, reagents, and media. Table adapted from Daniel et al., 2026.

Reagent	Manufacturer and order number
0.05% Trypsin-EDTA	Gibco, Cat.-No.: 25300096
0.25% Trypsin-EDTA	Sigma-Aldrich, Cat.-No.: T4049
4',6-diamindin-2-phenylindol (DAPI)	Miltenyi Biotec, Cat.-No.: 130-111-570
ABTS solution	Thermo Fisher Scientific, Cat.-No.: 002024
Agar Bacteriology grade	PanReac AppliChem, Cat.-No.: A0949,0500
Agarose, bacteriology grade	PanReac AppliChem, Cat.-No.: A8963,0500
Agel-HF	New England Biolabs, Cat.-No: R3552L
ATP	Sigma-Aldrich, Cat.-No.: A2383
Branched Polyethylenimine, 25 kDa	Sigma-Aldrich, Cat.-No.: 408727
BSA (Albumin Fraction V)	Sigma-Aldrich, Cat.-No.: 8076.1
BsiWI-HF	New England Biolabs, Cat.-No: R3553L
CD19 MicroBeads, human	Miltenyi Biotec, Cat.-No.: 130-050-301
Coenzyme A sodium salt hydrate	Sigma-Aldrich, Cat.-No.: C3144;
Cytofix™ fixation buffer	BD Biosciences, Cat.-No.: 554655
D-Luciferin, Sodium Salt	GoldBio, Cat.-No.: LUCNA-1G
DAPI Staining Solution	Miltenyi Biotec, Cat.-No.: 130-111-570
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich, Cat.-No.: D2650-100ML
Dithiothreitol (DTT)	Promega, Cat.-No.: P1171
dNTP Mix	Thermo Fisher Scientific, Cat.-No.: R1122
Dulbecco's Modified Eagle Medium (DMEM), high glucose, no glutamine	Thermo Fisher Scientific, Cat.-No.: 11960044
Dulbecco's PBS (DPBS)	Gibco, Cat.-No.: 14190169
EcoRI	New England Biolabs, Cat.-No: R3101S
Ethylenediamine tetraacetic acid (EDTA)	Carl Roth, Cat.-No.: CN06.3
Fetal calf serum (FCS)	Sigma-Aldrich, Cat.-No.: F9665
FreeStyle™ 293 Expression Medium	Thermo Fisher Scientific, Cat.-No.: 12338026
FuGENE® 6 Transfection Reagent	Promega, Cat.-No.: E2691
GeneRuler 1 kb Plus DNA Ladder	Thermo Fisher Scientific, Cat.-No.: SM1331
Glycine	Carl Roth, Cat.-No.: 3790.2
HindIII-HF	New England Biolabs, Cat.-No: R3104S
Histopaque-1077	Sigma-Aldrich, Cat.-No.: H8889
IGEPAL® CA-630	Sigma-Aldrich, Cat.-No.: I8896
KpnI-HF	New England Biolabs, Cat.-No: R3142S
L-Glutamine 200mM	Thermo Fisher Scientific, Cat.-No.: 25030024
LB-Medium	Carl Roth, Cat.-No.: X968.4
Magnesium chloride	Sigma-Aldrich, Cat.-No.: M8266
MEM non-essential amino acids	Gibco, Cat.-No.: 11140035
Minimum Essential Medium Eagle (MEM)	Sigma-Aldrich, Cat.-No.: M2279
Neuraminidase from <i>Clostridium perfringens</i>	Sigma-Aldrich, Cat.-No.: N2876
Nonfat dried milk powder	PanReac AppliChem, Cat.-No.: A0830,0500

Nuclease-free water	Thermo Fisher Scientific, Cat.-No.: AM9937
NuPAGE™ Bis-Tris Mini Protein Gels, 4-12%, 1.0-1.5 mm	Thermo Fisher Scientific, Cat.-No.: NP0322BOX
NuPAGE™ LDS Sample Buffer	Thermo Fisher Scientific, Cat.-No.: NP0007
NuPAGE™ Sample Reducing Agent	Thermo Fisher Scientific, Cat.-No.: NP0009
Nutridoma™-SP	Roche, Cat.-No.: 11011375001
PageRuler™ Plus Prestained Protein Ladder, 10 to 250 kDa	Thermo Fisher Scientific, Cat.-No.: 26619
Penicillin-Streptomycin	Gibco, Cat.-No.: 15070063
Phosphate buffered saline (PBS)	Gibco, Cat.-No.: 10010056
Pierce™ High Sensitivity Streptavidin-Horseradish peroxidase (HRP)	Thermo Fisher Scientific, Cat.-No.: 21130
Platinum™ Taq Green Hot Start DNA Polymerase	Thermo Fisher Scientific, Cat.-No.: 11966034
Polyethylenimine, branched	Sigma-Aldrich, Cat.-No.: 408727-100ML
Protein G Sepharose 4 Fast Flow	Cytiva, Cat.-No.: 17061805
Q5® High-Fidelity DNA Polymerase	New England Biolabs, Cat.-No.: M0491
Random hexamer primers	Thermo Fisher Scientific Cat.-No.: SO142
RNaseOUT™ Recombinant Ribonuclease Inhibitor	Thermo Fisher Scientific, Cat.-No.: 10777019
RNasin® Ribonuclease Inhibitor	Promega, Cat.-No.: N2515
Sall-HF	New England Biolabs, Cat.-No.: R3138S
SARS-CoV-2 S HexaPro	Hsieh et al., 2020
SOC Medium	Thermo Fisher Scientific, Cat.-No.: 15544034
Sodium chloride	Carl Roth, Cat.-No.: P029.3
Sodium pyruvate	Thermo Fisher Scientific, Cat.-No.: 11360070
Strep-TactinXT 4Flow resin	IBA Lifescience, Cat.-No.: 2-4010-025
SuperScript™ IV Reverse Transcriptase	Thermo Fisher Scientific, Cat.-No.: 18090200
SYBR™ Safe DNA Gel Stain	Thermo Fisher Scientific, Cat.-No.: S33102
T4 DNA Ligase	New England Biolabs, Cat.-No.: M0202S
T4 DNA Polymerase	New England Biolabs, Cat.-No.: M0203L
TPCK-treated Trypsin	Sigma-Aldrich, Cat.-No.: T1426
TRIS	Carl Roth, Cat.-No.: 4855.3
Trypsin Inhibitor from soybean	Roche, Cat.-No.: 10109886001
Tween® 20	Carl Roth, Cat.-No.: 9127.2
XhoI	New England Biolabs, Cat.-No.: R0146L
Zombie NIR™	BioLegend, Cat.-No.: 423106

2.1.2 Commercial kits

Table 2. Commercial kits. Table adapted from Daniel et al., 2026.

Kit	Manufacturer and order number
DyLight 488 Antibody Labeling Kit	Thermo Fisher Scientific, Cat.-No.: 53024
DyLight 650 Antibody Labeling Kit	Thermo Fisher Scientific, Cat.-No.: 84535
EZ-Link™ Sulfo-NHS-LC-Biotinylation Kit	Thermo Fisher Scientific, Cat.-No.: 21435
NucleoBond Xtra Midi Kit	Macherey-Nagel, Cat.-No.: 740410.100
Nucleospin Gel and PCR clean-up Kit	Macherey-Nagel, Cat.-No.: 740609
Q5® Site-Directed Mutagenesis Kit	New England Biolabs, Cat.-No.: E0554S

2.1.3 Buffers

Table 3. Buffers.

Buffer	Composition
1× Buffer W	100 mM TRIS-HCl, 150 mM NaCl, 1 mM EDTA, pH=8
1× MOPS	50 mM 3-(N-morpholino)propanesulfonic acid (MOPS), 50 mM TRIS base, 0.1% SDS (w/v), 1 mM EDTA
1× PBST	1× PBS, 0.05% Tween-20 (v/v)
1× Tris-Acetate-EDTA (TAE) buffer	40 mM TRIS base, 1mM EDTA, 20 mM acetic acid
10× PBS	1.37 M NaCl, 27 mM KCl, 100 mM Na ₂ HPO ₄ -12H ₂ O, 18 mM KH ₂ PO ₄
2× HEPES buffered Saline (HBS)	280 mM NaCl, 10 mM KCl, 1.5 mM Na ₂ HPO ₄ , 12 mM D-Glucose, 50 mM HEPES, pH=7.05
Blocking buffer	1× PBST, 2.5% BSA (w/v), 2.5% nonfat dried milk powder (w/v)
FACS buffer	1× PBS, 2% FCS (v/v), 2 mM EDTA
Luciferin lysis buffer	220 mM TRIS-HCl, 10 mM MgCl ₂ , 500 µM Coenzyme A, 300 µM ATP, 300 µg/mL D-Luciferin, 0.42 % IGEPAL (v/v), 12.6 mM NaCl
MACS buffer	1× PBS, 1% BSA (w/v), 2 mM EDTA

2.1.4 Antibodies

Table 4. Antibodies. Table adapted from Daniel et al., 2026.

Antibody	Manufacturer and order number
Affini Pure Goat Anti-Human IgG, Fcγ fragment specific	Jackson ImmunoResearch, Cat.-No.: 109-005-098
CD20 Alexa Fluor 700, clone 2H7	BD Biosciences, Cat.-No.: 560631
Goat Anti-Human IgG-HRP	Southern Biotech, Cat.-No.: 2040-05
IgG1, Kappa from human myeloma plasma	Sigma-Aldrich, Cat.-No.: I5154
IgG BV711, clone G18-145	BD Biosciences, Cat.-No.: 740796
IgG PE, clone G18-145	BD Biosciences, Cat.-No.: 555787

2.1.5 Cell lines

Table 5. Cell lines. Table adapted from Daniel et al., 2026.

Cell line	Source and order number
HEK293-6E cells	National Research Council of Canada, NRC file 11565
HEK293T cells	ATCC, Cat.-No.: CRL-11268
HEK293T-ACE2 cells	BEI Resources, Cat.-No.: NR-52511
MDCK	Merck, Cat.-No.: 84121903-1VL
MDCK-SIAT1	Sigma-Aldrich, Cat.-No.: 5071502
MDCK II	Collection of Cell Lines in Veterinary Medicine, CCLV-RIE 1061

2.1.6 Plasmids and oligonucleotides

Table 6. Plasmids and oligonucleotides. Primers are listed in the Appendix. Table adapted from Daniel et al., 2026.

DNA	Source
dTomato	Shaner et al., 2004
Human antibody expression vectors (IgG1, IgK, IgL)	Tiller et al., 2008
mTagBFP2	Subach et al., 2011
pCAGGS	Niwa et al., 1991
pcDNA TM 3.1/V5-His TOPO TM TA Expression Kit	Thermo Fisher Scientific; Cat.-No.: K480001
pHAGE-CMV-Luc2-IRES-ZsGreen-W	Jesse Bloom Lab; Crawford et al., 2020; RRID: Addgene_164432
pHDM-Hgpm2	Jesse Bloom Lab; Crawford et al., 2020; RRID: Addgene_164441
pHDM-tat1b	Jesse Bloom Lab; Crawford et al., 2020; RRID: Addgene_164442
pRC-CMV-Rev1b	Jesse Bloom Lab; Crawford et al., 2020; RRID: Addgene_164443
SuperfolderGFP (sfGFP)	Pédelacq et al., 2006

2.1.7 Laboratory equipment

Table 7. Laboratory equipment.

Equipment	Source
Cell analyzer LSRFortessa TM	BD Biosciences
CO ₂ incubator CB 260	Binder
CO ₂ shaking incubator New Brunswick S41i	Eppendorf
Flow cytometer FACSAria TM Fusion	BD Biosciences
Gel electrophoresis chamber Owl TM A3-1	Thermo Fisher Scientific
Gel electrophoresis chamber Owl TM D3-14	Thermo Fisher Scientific
Gel imager ChemStudio PLUS	Analytik Jena
High throughput sampler (HTS)	BD Biosciences

Microscope Primovert	Carl Zeiss
Microplate reader Sunrise	Tecan
Microplate reader Tristar 5	Berthold Technologies
Microplate stacker BioTek BioStack	Agilent Technologies
Microplate washer BioTek 405 TS	Agilent Technologies
MINI96 96 channel electronic pipette	Integra
pH meter inoLab® pH 7110	WTW
Protein gel electrophoresis system XCell SureLock Mini-Cell	Thermo Fisher Scientific
Thermo cycler Mastercycler® X50	Eppendorf
Thermo cycler Veriti™	Thermo Fisher Scientific
UVP ChemStudio PLUS imaging system	Analytik Jena

2.1.8 Software

Table 8. Software. Table adapted from Daniel et al., 2026.

Software	Source and identifier
AbRAT	https://github.com/ckreer/AbRAT ; 10.5281/zenodo.15313641
Adobe Illustrator	Adobe, https://www.adobe.com
BLAST	Camacho et al., 2009
FACSDiva 9.0.1	BD Biosciences, RRID: SCR_001456
FigTree	https://tree.bio.ed.ac.uk/software/figtree/ ; RRID: SCR_008515
FlowJo 10.10.0	https://www.flowjo.com ; RRID: SCR_008520
IgBlast	National Library of Medicine; Ye et al., 2013
IDT Codon Optimization Tool	https://eu.idtdna.com/CodonOpt ;
IQ-Tree	https://iqtree.github.io ; Minh et al., 2020; RRID: SCR_017254
MAFFT	Katoh and Standley, 2013; RRID: SCR_011811
Microsoft Excel for Mac (V16.78.3)	Microsoft Corporation
Prism 10	https://www.graphpad.com ; RRID: SCR_002798
Python	http://python.org ; RRID: SCR_008394
SciPy	https://scipy.org ; RRID: SCR_008058
SnapGene	https://www.snapgene.com ; RRID: SCR_015052
Vectorbuilder Codon Optimization Tool	https://en.vectorbuilder.com/tool/codon optimization.html

2.2 Methods

2.2.1 Human samples

2.2.1.1 Enrollment of study participants

Blood samples from healthy study participants from the local area of Cologne were collected longitudinally under the “EIKIM”-study protocol (DRKS00026266) approved by the Ethics Committee of the University of Cologne (#21-1468). All participants gave their written informed

consent and were asked to self-report previous vaccinations against SARS-CoV-2 or influenza virus, as well as previous respiratory infections with SARS-CoV-2, influenza or respiratory syncytial virus (RSV) at each sampling timepoint. One individual reported a previous vaccination with an experimental H5N1 vaccine (clade 1, A/Vietnam/1203/2004) in 2008/2009. Longitudinal sampling was performed at three timepoints per year (April-May, August-September and December-January) starting in August 2023. Serum and EDTA-blood were collected from all participants at each timepoint. For the experiments of this doctoral thesis, only samples of the first collection timepoint per individual, collected between August 2023 and June 2024, were analyzed. Detailed information about age, sex, vaccination status, and sampling of the study participants is presented in Table 16 in the Appendix.

2.2.1.2 Isolation of serum, plasma, and PBMCs

For serum isolation, blood collected in serum gel tubes (Sarstedt) was centrifuged at $1600 \times g$ for 15 min. The serum fraction was harvested and stored in aliquots at -80°C . For processing of plasma and peripheral blood mononuclear cells (PBMCs), blood samples collected in EDTA tubes (Sarstedt) were centrifuged at $400 \times g$ and 20°C for 10 min. The plasma fraction was collected and stored at -80°C . From the remaining fraction of the blood, PBMCs were obtained by density gradient centrifugation in Leucosep tubes (Greiner Bio-one) containing Histopaque-1077 (Sigma), following the manufacturer's instructions. Cells were washed three times and stored at 10^7 cells/mL in FCS with 10% DMSO at -150°C .

2.2.2 Cell culture

2.2.2.1 Cell lines

For cell culture experiments, the cell lines MDCK, MDCK II, MDCK-SIAT1, HEK293T, HEK293T-ACE2, and HEK293-6E were used (Table 5). All cell culture experiments were conducted under sterile conditions in a biosafety cabinet.

The Madin-Darby Canine Kidney (MDCK) cell line was isolated in 1958 of epithelial cells from the distal tubule of a kidney from a female Cocker Spaniel dog. The cells grow as a confluent monolayer and have become a common cell culture model for epithelial tissues in general and to study viral infection. From the parental MDCK cells line, various other cell strains have been derived, one of them being the MDCK II cell line, which was obtained from a higher passage of MDCK cells (Dukes et al., 2011). By stable transfection of MDCK cells with the human $\alpha 2,6$ -sialyltransferase (SIAT1), the MDCK-SIAT1 cell line was derived. It overexpresses $\alpha 2,6$ -linked sialic acids, the primary receptor of human influenza viruses (Matrosovich et al., 2003). In this doctoral thesis, MDCK and MDCK-SIAT1 cells were used for pseudovirus neutralization assays, while MDCK II cells were used for authentic virus neutralization assays. Both MDCK and MDCK-SIAT1 cells were maintained in Minimum Essential Medium Eagle (MEM, Sigma-Aldrich)

containing 10% FCS (Sigma Aldrich), 1 mM L-Glutamine (Gibco), 1% MEM non-essential amino acids (Gibco), and 1% Penicillin-Streptomycin (PS, Gibco).

Human embryonic kidney 293 (HEK293) cells are a widely used human cell line and commonly employed for transient transfection and recombinant protein expression. The derivative cell line HEK293T was generated by stable transfection with a plasmid encoding a temperature sensitive SV40 large T-antigen mutant (DuBridge et al., 1987) and was used for production of pseudotyped lentiviral particles and mAbs. HEK-293T-ACE2 cells, which stably express angiotensin-converting enzyme 2 (ACE2) on the cell surface, were used for SARS-CoV-2 pseudovirus assays. The suspension-adapted derivative HEK293-6E was employed for recombinant expression of HA proteins and mAbs. HEK293T and HEK293T-ACE2 cells were cultured in Dulbecco's Modified Eagle Medium (Gibco) with 10% FCS, 1% PS, 1 mM L-Glutamine and 1 mM Sodium pyruvate (Gibco). HEK293-6E cells were maintained in FreeStyle™ 293 Expression Medium supplemented with 0.2% PS.

2.2.2.2 Cultivation and passaging of adherent cells

The adherent cell lines MDCK, MDCK-SIAT1, HEK293T, and HEK293T-ACE2 were cultured in 15 cm tissue culture treated plates (TPP) using the appropriate growth medium in incubators at 37°C and 5% CO₂. Cells were passaged every 2-3 days upon reaching confluence. This involved PBS washing followed by incubation with trypsin-EDTA to induce detachment (3 mL of 0.05% trypsin-EDTA (Gibco) for HEK293T and HEK293T-ACE2 cells; 5 mL of 0.25% trypsin-EDTA (Sigma-Aldrich) for MDCK and MDCK-SIAT1). Trypsinization was stopped by resuspending the cells in the corresponding culture medium. Cells were then centrifuged for 5 min at $336 \times g$, resuspended in fresh medium, and seeded at the required density into new tissue culture dishes containing the corresponding growth medium.

2.2.2.3 Cultivation and passaging of HEK293-6E cells

HEK293-6E suspension cells were maintained in Erlenmeyer flasks (Corning) in incubators with constant shaking at 110 rpm and 6% CO₂. Cells were passaged every 2-3 days to keep the density between 0.2×10^6 and 1.2×10^6 cells/mL.

2.2.3 Phylogenetic analysis and selection of influenza A strains

Next to the H5N1 strain A/Texas/37/2024, that was isolated from an infected farm worker after spillover from cattle during the recent outbreak in the US, a panel of recent and historical influenza strains was created. The panel included strains of the subtypes H1, H2, and H3, which have been associated with seasonal human infections, as well as strains of the subtypes H5, H7, and H9, which are the avian subtypes most frequently linked to zoonotic spillover events. Phylogenetic analysis of influenza A HA sequences and the subsequent selection of representative strains

were performed by the research group of Prof. Dr. Michael Lässig from the Institute of Biological Physics at the University of Cologne.

2.2.3.1 Phylogenetic analysis of HA sequences

HA sequences of influenza A subtypes H1-H18 were downloaded from the GISAID EpiFlu data base. Sequences containing more than 1% ambiguous nucleotides were removed during an initial quality control step. The remaining sequences were aligned using MAFFT (v7.525), followed by an additional round of quality control. A global, unrooted maximum-likelihood phylogeny was inferred using IQ-TREE and the resulting tree visualized with FigTree (Daniel et al., 2026).

2.2.3.2 Selection of representative influenza A strains

To guide the selection of representative influenza A strains, individual phylogenetic sub-trees were generated for the HA subtypes H1, H2, H3, H5, H7, and H9. Each tree was separated into clades that were genetically distinct, reached a minimum frequency of 20% in the global population, and showed broad geographic distribution. For subtypes H3 and H1(pdm09), clades were additionally required to be antigenically distinct, as determined using an antigenic inference method based on ferret neutralization data (Worldwide Influenza Centre and Crick Institute, 2026). Then, a representative isolate from each clade was selected. For seasonal influenza subtypes H1 and H3, preference was given to vaccine strains or isolates serving as reference viruses for ferret neutralization assays. For H1(seasonal), H5, H7, and H9, strains had to differ from one another by a minimum of five amino acids or be recently collected from exposed individuals. Whenever possible, human-derived isolates or candidate vaccine viruses were selected for the avian subtypes. To further capture evolutionary diversity within H9, one animal-derived isolate from the Y280A lineage was incorporated (Daniel et al., 2026).

2.2.4 Molecular biology

2.2.4.1 HA cloning

Distinct HA expression constructs needed to be generated for pseudovirus production, the cell-based binding assay and recombinant HA protein production. Full-length HA sequences of the selected influenza strains (see Appendix, Table 17), including their native signal peptides, were codon optimized using the VectorBuilder Codon Optimization Tool and synthesized as gene fragments from Twist Bioscience. After reconstitution in nuclease-free water (Ambion) to a concentration of 50 ng/μL, the fragments were PCR-amplified using primers listed in Table 9 and the Q5® High-Fidelity DNA Polymerase (New England Biolabs) following the manufacturer's protocol. PCR products were separated on 0.7% agarose gels and purified prior to cloning. For strains differing only by minor amino acid substitutions, constructs were generated by site-

directed mutagenesis using the Q5[®] Site-Directed Mutagenesis Kit (New England Biolabs) as suggested by the manufacturer.

For pseudovirus production, HA segments were then digested with AgeI and HindIII and ligated into the mammalian expression vector pCDNA3.1 cut with the same enzymes. Ligation products were transformed, followed by colony PCR screening and plasmid preparation. To assess potential co-expression of NA during the establishment of particle production, the full-length NA sequence of H1N1 A/Hawaii/70/2019 was also ordered as gene fragment and cloned using the same strategy.

For the cell-based binding assay, HA sequences were cloned into a pCDNA3.1 expression vector via HindIII and XhoI restriction, resulting in a C-terminal fusion of HA to a 6×GS linker and one of the fluorescent proteins mTagBFP2, dTomato, or superfolderGFP (sfGFP) encoded in the vector backbone.

For recombinant protein production, HA sequences lacking the transmembrane and cytoplasmic domains (Δ TM) were amplified with primer sets listed in Table 1 using the Q5[®] High-Fidelity DNA Polymerase according to the manufacturer's instructions. After purification, HA segments were inserted into the pCAGGS expression vector via KpnI and XhoI restriction sites, resulting in C-terminal fusion to a Foldon trimerization domain, sfGFP, an HRV 3C cleavage site, an 8×His-tag, and a Twin-Strep-tag. To produce the A/Texas/37/2024 bait protein for the single B cell sort, the Y98F mutation was inserted by site-directed mutagenesis.

Table 9. Primer usage for HA and NA amplification. Primer sequences are listed in the Appendix.

HA amplification step	5' primer	3' primer
HA amplification for pCDNA3.1 cloning	LU P27	LUP28
NA amplification for pCDNA3.1 cloning	P1_NA_Mut	P0_NA_Mut_rev
A/Hawaii/70/2019 HA Δ TM amplification	CMV for	HA Rev L14
A/Hong Kong/4801/2014 HA Δ TM amplification	CMV for	HA Rev L12
A/Texas/37/2024 HA Δ TM amplification	Texas_for	Texas_rev

2.2.4.2 Restriction digest

Restriction enzymes were obtained from New England Biolabs and restriction digests of plasmid DNA or PCR products were performed according to the manufacturer's instructions. Briefly, DNA samples were mixed with 10× NEBuffer, the appropriate enzyme(s), and nuclease-free H₂O to achieve a final volume of 50 μ L, and incubated overnight at 37°C. To ensure complete digestion, restriction enzymes were used at a tenfold excess relative to the manufacturer's recommendations. Following digestion, DNA samples were either directly purified or separated by agarose gel electrophoresis prior to purification.

2.2.4.3 DNA gel electrophoresis

Following restriction digestion or PCR amplification, DNA samples were analyzed and separated by agarose gel electrophoresis. Agarose gels were prepared by heating agarose (Panreac AppliChem) in 1× TAE buffer using a microwave until completely dissolved. Depending on the expected DNA fragment size, gels containing either 0.7% or 2% (w/v) agarose were used. After the solution had cooled to approximately 60°C, SYBR Safe DNA stain (1:20,000) was added and the gel was poured into a casting chamber of appropriate size. PCR samples already contained loading dye in the PCR buffer. All other DNA samples were mixed with 6× gel loading dye (New England Biolabs) prior to loading onto the gel. For size determination, 6 µL of GeneRuler 1 kb Plus DNA ladder (Thermo Fisher Scientific) was applied to the gel. Electrophoresis was performed at 250 V and 200 mA in 1× TAE running buffer, followed by gel visualization and documentation using a UVP ChemStudio PLUS bioimaging system (Analytik Jena). When required, the band of the expected size was excised from the gel for subsequent purification.

2.2.4.4 DNA purification

To purify nucleic acid samples from gel after PCR or restriction digestion, the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel) was used as recommended by the manufacturer. Deviating from the protocol, samples were eluted in 15–30 µL nuclease-free H₂O in the final step. DNA concentrations were measured with a microvolume spectrophotometer (NanoDrop One, Thermo Fisher Scientific).

2.2.4.5 DNA ligation

To ligate digested vectors and inserts, DNA ligation was performed using a T4 DNA Ligase (New England Biolabs). 250 fmol of insert DNA was mixed with 50 fmol of vector DNA, 2 µL of 10× T4 ligase buffer, 1 µL of T4 DNA ligase and nuclease-free H₂O to a final volume of 20 µL. It was then incubated overnight at 16°C. The enzyme was subsequently inactivated by incubation at 65°C for 10 min prior to transformation into competent bacteria.

2.2.4.6 Transformation

For plasmid amplification, DNA samples were transformed into chemically competent *Escherichia coli* (*E. coli*, DH5α-strain) cells. 50 µL of bacteria was thawed on ice and mixed with 2.5 µL of ligation mix, 4 µL of SLIC reaction mix or 1 µL of plasmid DNA from midi preparations. After incubation for 30 min on ice, heat shock was performed for 45 sec at 42°C, following incubation on ice again for 2 min. 500 µL (50 µL in case of transformation in a 96-well format) of super optimal broth with catabolite repression (SOC) medium was added and samples incubated for 1 h

at 37°C and 250 rpm. 50–100 µL of the transformation mix was spread on LB agar plates containing 50 µg/mL ampicillin for selection. Plates were incubated overnight at 37°C.

2.2.4.7 Colony PCR

Colony PCR was performed to identify positive clones after transformation. The PCR reaction mix was prepared as described in Table 10 using the respective primers (Table 11) and dispensed into a 96-well PCR plate. Individual colonies were picked from the transformation agar plates using sterile pipette tips. They were first transferred into the corresponding wells of a liquid culture plate (96-well plate containing 50 µL/well of LB medium supplemented with 50 µg/mL ampicillin) and subsequently into the PCR plate containing the reaction mix. PCR was performed with the temperature profile listed in Table 12, followed by analysis of the product size by DNA gel electrophoresis. The liquid culture plate was incubated overnight at 37°C with shaking at 200 rpm.

Table 10. Colony PCR mastermix.

Reagent	Volume per well (µl)
Nuclease-free H ₂ O	17.44
10× Green PCR Buffer -Mg	2.2
KB extender	1.28
50 mM MgCl ₂	0.65
25 mM dNTPs	0.2
5' Primer (fwd)	0.08
3' Primer (rev)	0.08
Platinum <i>Taq</i> DNA Polymerase	0.08

Table 11. Primer usage during colony PCR. Primer sequences are listed in the Appendix.

Plasmid type	5' primer	3' primer
pCDNA-HA	LU P7	P13 rev
pCDNA-HA-6×GS-dTomato	LU P7	LU P13
pCDNA-HA-6×GS-mTagBFP2	LU P7	LU P12
pCDNA-HA-6×GS-sfGFP	LU P7	LU P11
pCAGGS-HAΔTM-FOLDON-sfGFP-8×His-2×Strep	oCKc51	pCAGGS_fwd_new
plgH	5'AB-sense	IgG internal
plgK	5'AB-sense	3' C _K 543
plgL	5'AB-sense	3' C _λ

Table 12. Colony PCR temperature profile.

Step of reaction	Temperature	Time	
Initial denaturation	94°C	5 min	
Denaturation	94°C	30 s	} 28 x
Annealing	52-57°C	30-60 s/kb	
Extension	72°C	1-2 min	
Final extension	72°C	5 min	
Cooling	10°C	hold	

2.2.4.8 Plasmid DNA preparation

Midi preparations of plasmid DNA were performed using the NucleoBond Xtra Midi Kit (Macherey-Nagel) according to the manufacturer's instructions for high copy plasmids. Bacterial cultures were inoculated in LB Medium supplemented with 50 µg/mL ampicillin and incubated at 140 rpm and 37°C overnight. Deviating from the manufacturer's protocol, DNA pellets were resuspended in H₂O in the final step. DNA concentrations were measured with a microvolume spectrophotometer (NanoDrop One or NanoDrop Eight, Thermo Fisher Scientific).

2.2.5 Protein biochemistry

2.2.5.1 Recombinant expression of HA proteins

HA proteins of the influenza strains A/Texas/37/2024, A/Hawaii/70/2019, and A/Hong Kong/4801/2014 were recombinantly expressed in HEK293-6E cells. On the day of transfection, the cell density was adjusted to 0.8×10^6 cells/mL. For each milliliter of cell culture volume, 1 µg HA expression plasmid was diluted in 45 µL DPBS (Gibco), followed by the addition of 3.4 µL of 0.45 mg/mL polyethylenimine (PEI, Sigma-Aldrich). The mixture was vortexed for 10 s and incubated for 10 min to allow DNA–PEI complex formation. The transfection mix was then added dropwise to the cells, which were subsequently incubated for 7 days at 37°C and 6% CO₂ under constant shaking at 110 rpm.

2.2.5.2 Purification of HA proteins

To purify the recombinantly expressed HA proteins from the cell culture supernatant, the supernatant was harvested by centrifugation at $500 \times g$ for 5 min and filtered through a 0.45 µm polyethersulfone (PES) membrane (Labsolute). Using Strep-Tactin®XT 4Flow® resin (IBA Lifesciences), HA proteins were then purified at 4°C on chromatography columns (Bio-Rad) following the manufacturer's instructions. After elution, buffer exchange to PBS was performed using 30K Amicon Ultra centrifugal filters (Merck Millipore) according to the manufacturer's protocol. Briefly, the filter units were equilibrated once with PBS before loading the sample. Columns were centrifuged at $4000 \times g$ until the sample volume was less than 500–1000 µL, then refilled with PBS and centrifuged again. The process was repeated for a total of three cycles.

Protein concentrations were measured with a microvolume spectrophotometer (NanoDrop One, Thermo Fisher Scientific).

2.2.5.3 Fluorescent and biotin labeling of HA proteins

Recombinantly produced HA proteins were labeled with biotin or fluorescent dyes for downstream applications. Biotin labeling was performed using the EZ-Link™ Sulfo-NHS-LC-Biotinylation Kit (Thermo Fisher Scientific) as specified in the supplied protocol. Fluorescent labeling with DyLight 488 or DyLight 650 was carried out using the DyLight™ Antibody Labeling Kit (Thermo Fisher Scientific) according to the manufacturer's instructions.

2.2.5.4 SDS-PAGE

Proteins were analyzed by separation according to their molecular mass using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Samples were supplemented with 4× NuPAGE™ LDS Sample Buffer (Thermo Fisher Scientific) and, as indicated, either with or without 10× NuPAGE™ Sample Reducing Agent (Thermo Fisher Scientific), followed by heating for 10 min at 70°C to induce denaturation. Subsequently, equal amounts of protein (typically 1 µg) were loaded onto NuPAGE™ Bis-Tris Mini Protein Gels, 4–12% (Thermo Fisher Scientific). PageRuler™ Prestained Protein Ladder (Thermo Fisher Scientific) served as a molecular weight marker. Electrophoresis was performed at 200 V in 1× MOPS running buffer. Following electrophoresis, gels were stained with InstantBlue® Coomassie Protein Stain (Abcam) for approximately 1 h and detained overnight in H₂O on a rocker.

2.2.5.5 Purification and depletion of poly-IgGs from plasma

Poly-IgGs were purified and depleted from plasma using protein G beads. Briefly, 1 mL of plasma was heat-inactivated by incubation at 56°C for 1 h. Samples were then supplemented with 0.8 mL of washed Protein G Sepharose™ 4 Fast Flow beads (Cytiva) and diluted to a final volume of 14 mL with PBS. After overnight incubation at 4°C under constant rotation, samples were transferred to PBS-equilibrated chromatography columns (Bio-Rad). The flow-through was collected and defined as IgG-depleted plasma. Columns were washed three times with DPBS, followed by elution of poly-IgGs with 4.5 mL of 0.1 M glycine buffer (pH 3) into tubes containing 500 µL of 1 M Tris buffer (pH 8). IgG-depleted plasma as well as purified poly-IgG samples were subsequently concentrated in PBS using 30K Amicon Ultra centrifugal filters (Merck Millipore) according to the manufacturer's protocol (see chapter 2.2.5.2).

2.2.5.6 Depletion of HA-reactive antibodies from poly-IgGs

Poly-IgG samples were depleted of HA-reactive antibodies using affinity chromatography. Chromatography columns were prepared with 1 mL Strep-Tactin®XT 4Flow® resin (IBA Lifesciences) and washed once using 1× Buffer W. Recombinant HA proteins from A/Hawaii/70/2019 (2mg), A/Hong Kong/4801/2014 (2 mg), and A/Texas/37/2024 (1 mg) were combined, added to the resin and incubated for 30 min. After discarding the initial flow-through, 1 mL of poly-IgG (2 mg/mL, in 1× Buffer W) was loaded to the column. The collected flow-through was subsequently buffer-exchanged into PBS using 30K Amicon Ultra centrifugal filters (Merck Millipore) according to the manufacturer's protocol (see chapter 2.2.5.2). IgG concentrations were measured with a microvolume spectrophotometer (NanoDrop One, Thermo Fisher Scientific).

2.2.6 Antibody isolation and production

2.2.6.1 Isolation of HA-specific IgG⁺ B cells

For isolation of HA-specific IgG⁺ B cells, PBMCs were rapidly thawed in a 37°C water bath and immediately transferred to a 50 mL reaction tube containing 40 mL cold PBS. Tubes were filled to a final volume of 50 mL with PBS, and a 10 µL aliquot was taken for cell counting by trypan blue exclusion. Cells were then centrifuged for 15 min. During B cell isolation and staining, all centrifugation steps were performed at 400 × *g* and 4°C and cells were always kept on ice during all intermediate steps.

Magnetic B cell enrichment was performed using CD19 MicroBeads and LS columns (both Miltenyi Biotec) according to the manufacturer's recommendations. Briefly, cells were resuspended in magnetic-activated cell separation (MACS) buffer (160 µL per 10⁷ cells) and CD19 MicroBeads (20 µL per 10⁷ cells) were added. Following incubation for 15 min, cells were washed with 20 mL MACS buffer and centrifuged for 15 min. The cell pellet was resuspended in 500 µL MACS buffer and applied to LS columns mounted in a magnetic separator and pre-equilibrated with 3 mL MACS buffer. Columns were washed three times with 3 mL MACS buffer, removed from the separator, and B cells were eluted with 5 mL MACS buffer into a fresh 15 mL reaction tube. Cells were counted again by trypan blue exclusion and centrifuged for 15 min.

For antigen staining, cells were resuspended in PBS (100 µL per 5 × 10⁶ cells) containing DyLight 488- and DyLight 650-labeled H5N1 A/Texas/37/2024(Y98F) HAΔTM protein (10 µg/mL each) and incubated for 30 min. Cells were washed with 1 mL PBS and centrifuged for 10 min. Subsequently, cells were resuspended in FACS buffer (100 µL per 5 × 10⁶ cells) supplemented with 4',6-diamindin-2-phenylindol (DAPI, Miltenyi Biotec; 1:100), Alexa Fluor® 700 Mouse Anti-Human CD20 (BD Biosciences, clone 2H7; 1:80), and PE Mouse Anti-Human IgG (BD Biosciences, clone G18-145; 1:5). After incubation for 30 min, cells were washed with 1 mL FACS buffer and centrifuged for 10 min.

Cells were resuspended in < 500 μL of FACS buffer and filtered through a 35 μm nylon mesh. Single DAPI⁻ CD20⁺ IgG⁺ cells that were double positive for H5N1 A/Texas/37/2024(Y98F) HA Δ TM protein were sorted into 96-well plates using a FACS Aria™ Fusion flow cytometer (BD Biosciences). Sorting plates contained 4 μL of 0.5 $\times$ PBS supplemented with 3 U/ μL RNasin (Promega) and 10 mM dithiothreitol (DTT, Thermo Fisher Scientific). Plates were stored at -80°C after sorting.

2.2.6.2 Single B cell receptor amplification and sequencing

After isolation of HA-specific IgG⁺ B cells by single cell sorting, BCR sequences of sorted cells were amplified to enable characterization of the encoded mAbs. RNA was first reverse transcribed into cDNA, followed by PCR amplification of IgG heavy and light chain genes.

2.2.6.2.1 Reverse transcription

Prior to amplification by PCR, RNA of single cell sorted B cells was reverse transcribed into cDNA as described in detail by Gieselmann et al., 2021. Briefly, 96-well plates containing sorted B cells were thawed on ice and 0.75 μL Random Hexamer Primers (200 ng/ μL ; Thermo Fisher Scientific), 0.5 μL NP-40 (10% v/v; Thermo Fisher Scientific), 0.15 μL RNasin Ribonuclease Inhibitor (Promega), and 5.6 μL nuclease-free H₂O was added per well. After mixing thoroughly by pipetting up and down several times, plates were incubated at 65°C for 2 min and placed on ice afterwards for a minimum of 2 min. Then, 3 μL 5 $\times$ SuperScript™ IV RT buffer, 0.5 μL dNTPs, 1 μL 100 mM DTT, 0.25 μL SuperScript™ IV Reverse Transcriptase (200 U/ μL ; all Thermo Fisher Scientific), 0.2 μL RNasin (Promega), and 2.05 μL nuclease-free H₂O were added per well. Plates were incubated for 15 min at 25°C, followed by 10 min at 42°C, 10 min at 25°C, 10 min at 50°C, and 5 min at 94°C. Afterwards, cDNA was diluted with 16 μL nuclease-free H₂O and stored at -20°C until further usage.

2.2.6.2.2 Single cell PCR for amplification of heavy and light chain genes

Semi-nested PCR was performed to amplify IgG heavy and light chain genes as previously described using human V gene segment specific forward primers together with reverse primers targeting the IgG constant region (Gieselmann et al., 2021; Kreer et al., 2020a). A combined first PCR for heavy, kappa, and lambda chains was performed using Platinum™ Taq DNA Polymerase (Thermo Fisher Scientific) with a reaction mixture described in Table 13 and 3.8 μL cDNA as template. Second PCRs were then carried out separately for each chain type using Platinum™ Taq Green Hot Start DNA Polymerase (Thermo Fisher Scientific) with the master mix described in Table 13 and 3 μL of first PCR product as template. PCR reactions were run under the conditions listed in Table 14 using the primers listed in Table 15. Four microliters of the second

PCR product were analyzed by DNA gel electrophoresis on a 2% agarose gel and the remaining product was sent for Sanger sequencing using the 3' primers from the second PCR.

Table 13. First and second single cell PCR mastermix.

Reagent	Volume/well (μl)
Nuclease-free H ₂ O	15.39
10× (Green) PCR Buffer -Mg	2.15
KB extender	1.29
50 mM MgCl ₂	0.64
25 mM dNTPs	0.17
5' Primer (fwd)	0.09
3' Primer (rev)	0.09
Platinum <i>Taq</i> DNA Polymerase	0.09

Table 14. First and second single cell PCR temperature profile.

Step of reaction	Temperature	Time
Initial denaturation	94°C	2 min
Denaturation	94°C	30 s
Annealing	57°C	30 s
Extension	72°C	1 st : 55 s, 2 nd : 45 s
Final extension	72°C	5 min
Cooling	10°C	hold

50 x

Table 15. Primer usage for single cell PCR. For composition of primer mixes and sequences see Appendix.

PCR type	5' primer mix	3' primer
1 st PCR combined	oPR-IGHV + oPR-IGKV + oPR-IGLV	Cg RT + 3' Cκ 543 + 3' Cλ
2 nd PCR heavy chain	oPR-IGHV	3' IgG internal
2 nd PCR kappa chain	oPR-IGKV	3' Cκ 494
2 nd PCR lambda chain	oPR-IGLV	3' XhoI Cλ

2.2.6.2.3 Single B cell receptor sequence analysis

Sequence analysis of single cell BCR sequences was performed using the Antibody Repertoire Analysis Toolkit (AbRAT; Kreer, 2025). Via this workflow, raw Sanger sequencing results from heavy and light chain PCR products were quality-controlled and annotated using IgBLAST-based germline gene assignment. Heavy and light chain sequences were paired to reconstruct individual BCRs and non-productive sequences were excluded. Clonal relationships between B cells were inferred based on sequence similarity using the clonal clustering module implemented in AbRAT. The resulting datasets were used to analyze repertoire features such as V gene usage, clonal expansion, and somatic hypermutation.

2.2.6.3 Cloning of monoclonal antibodies

For antibody production, antibody variable regions were cloned into heavy and light chain expression plasmids (pIgH, pIgK, and pIgL, Tiller et al., 2008) using sequence- and ligation-independent cloning (SLIC), as described by Giesemann et al., 2021. Variable regions were obtained either as synthetic gene fragments lacking the endogenous leader sequence but containing overhangs for SLIC (eBlocks, IDT), or by PCR amplification from first PCR products. Depending on the insert source, constructs either retained their native leader sequence in the case of PCR-amplified products, or utilized a murine leader sequence encoded by the vector backbone for synthetic gene fragments, as described previously by Giesemann et al., 2021.

PCR amplification was performed with Q5[®] High-Fidelity DNA Polymerase (New England Biolabs) according to the manufacturer's instructions, using 1 µL of first PCR product as template and previously described primer sets (see Appendix, Table 19). PCR products were analyzed on a 2% agarose gel and purified prior to SLIC.

Before SLIC, heavy and light chain expression plasmids were linearized by restriction digestion and purified from agarose gels. For cloning of synthetic gene fragments, plasmids were digested with AgeI and SalI (pIgH), AgeI and BsiWI (pIgK), or AgeI and XhoI (pIgL). For cloning of PCR-amplified fragments, plasmids were digested with EcoRI and SalI (pIgH), EcoRI and BsiWI (pIgK), or EcoRI and XhoI (pIgL).

For SLIC, 80 ng of linearized vector DNA was mixed with 20 ng of variable region insert (PCR-amplified or synthetic), 1 µL NEB Buffer 2.1, 0.2 µL T4 DNA Polymerase (both New England Biolabs), and nuclease-free H₂O to achieve a final volume of 10 µL. The mixture was first incubated for 2.5 min at 24°C, followed by incubation on ice for 10 min. Subsequently, 4 µL of the SLIC reaction was used for transformation into *E. coli* DH5α as described in chapter 2.2.4.6.

2.2.6.4 Expression and purification of monoclonal antibodies

Monoclonal antibodies were recombinantly expressed by transient transfection of HEK293-6E or HEK293T cells with plasmids encoding the antibody heavy and light chains. HEK293-6E were transfected at a density of 0.8×10^6 cells/mL. For each milliliter of cell suspension, 0.5 µg of heavy chain and 0.5 µg of light chain expression plasmid were mixed with 45 µL DPBS and 3.4 µL of 0.45 mg/mL PEI was added. After vortexing for 10 s, the mixture was incubated for 10 min to allow formation of DNA–PEI complexes and subsequently added dropwise to the cells. Cells were maintained for 7 days at 37°C and 6% CO₂ under constant shaking at 110 rpm. HEK293-T cells were transfected at approximately 80% confluency. On the day of transfection, cells were incubated in serum-free DMEM for 1 h before switching to DMEM containing 1 mM L-Glutamine, 1 mM sodium pyruvate, 1% Penicillin-Streptomycin (all Gibco), and 1% Nutridoma-SP (Roche). For transfection, plasmid DNA encoding heavy and light chains (15 µg each per 15 cm dish) was diluted in 1.2 mL DPBS, followed by addition of 200 µL of 0.45 mg/mL PEI. After brief vortexing

(20 s) and 10 min incubation at RT, DNA–PEI complexes were added dropwise to the cells. Cells were then cultured for 3 days at 37°C and 5% CO₂.

Antibodies were purified from cell culture supernatants using Protein G Sepharose™ 4 Fast Flow beads (Cytiva). Supernatants were cleared by centrifugation at 3740 × *g* and 4°C for 25 min and incubated with washed Protein G beads (200 µL/50 mL supernatant) overnight at 4°C on a rotating platform. The antibody-bound beads were pelleted by centrifugation at 336 × *g* and 4°C for 5 min without brakes and loaded onto PBS-equilibrated chromatography columns (Bio-Rad). Columns were washed three times with DPBS, then 0.1 M glycine buffer (pH 3) was applied for antibody elution, with the eluate being collected in tubes pre-filled with 1 M Tris (pH 8) for neutralization (9:1 ratio). The eluate was buffer-exchanged to PBS using 30K Amicon Ultra centrifugal filters (Merck Millipore) according to the manufacturer's protocol and as described in chapter 2.2.5.2. Purified antibodies were kept at 4°C and concentrations were determined using a microvolume spectrophotometer (NanoDrop One, Thermo Fisher Scientific).

2.2.7 Functional and immunological assays

2.2.7.1 Enzyme-linked immunosorbent assay

ELISAs were performed to assess the binding activity of plasma, purified poly-IgG or mAb samples. For coating, 45 µL per well of recombinant influenza HA or SARS-CoV-2 S HexaPro (2.5 µg/mL in PBS) was added to high binding 96-well plates (Corning) and incubated overnight at 4°C. On the following day, plates were washed three times with 150 µL PBST per well, as performed for all subsequent wash steps, and incubated with 180 µL per well of blocking buffer for 1 h at RT. After washing, serial dilutions of the respective primary antibody sample were prepared in blocking buffer and applied to the plate (45 µL per well), followed by a 2 h incubation. Plasma samples were analyzed starting at a dilution of 1:20 with 4-fold serial dilution, whereas purified or HA-depleted poly-IgGs and mAbs were tested starting at 5 µg/mL with 5-fold serial dilutions. Plates were then washed and incubated for 1 h with 45 µL per well of the goat anti-human IgG-HRP secondary antibody (Southern Biotech) diluted 1:2000 in PBS. Following a final wash, plates were developed with 150 µL ABTS substrate per well and absorbance was measured at 415 nm with reference at 695 nm using a microplate reader (Tecan). Each plate included a sandwich ELISA with serial dilutions (5–0.000064 µg/mL) of standardized human IgG (human myeloma IgG1, kappa; Sigma-Aldrich) as an internal reference. Plates were developed to reach a maximal OD_{415–695} of approximately 1.5 for the highest standard concentration.

For competition ELISAs, plates were incubated with the primary antibody samples for 1 h, followed by washing and incubation with defined concentrations of biotinylated competitive mAbs (100F4: 0.1 µg/mL, MEDI8852: 0.5 µg/mL, CR6261: 1 µg/mL) for an additional 1 h. Bound biotinylated antibodies were detected using Pierce™ High Sensitivity Streptavidin-HRP (Thermo

Fisher Scientific) diluted 1:5000 in blocking buffer and incubated for 30 min, prior to ABTS development as described above.

2.2.7.2 Cell-based binding assay

In addition to IgG ELISA, HA binding was also measured using a cell-based assay, thereby allowing assessment of antibody recognition of HA in its native-like membrane-associated conformation. Cell-based binding assays were performed by Leon Ullrich at the Institute of Virology in Cologne to measure the binding of plasma or mAb samples to HA proteins expressed on the surface of HEK293-6E cells.

For the assay, HEK293-6E cells were transfected at a density of 0.8×10^6 cells/mL with plasmids encoding for the respective HA protein fused to one of the fluorophores mTagBFP2, sfGFP, or dTomato. For each milliliter of cell suspension, 0.5 µg of plasmid, 45 µL DPBS, and 3.4 µL of 0.45 mg/mL PEI were mixed, incubated for 10 min at RT, and then added dropwise to the cell culture. After 48 h, cells were harvested by centrifugation for 15 min at $400 \times g$ and 4°C and 60,000 cells per HA-fluorophore construct were combined in one well in FACS buffer to enable simultaneous analysis of up to three different HA proteins. Cells were again centrifuged for 5 min at $400 \times g$ and 4°C. Following removal of the supernatant, they were incubated for 30 min on ice with 100 µL of serial dilutions of plasma or mAb samples in PBS. Plasma samples were analyzed starting at a dilution of 1:20 with 16-fold serial dilution, whereas purified poly-IgGs and mAbs were tested starting at 100 µg/mL with 20-fold serial dilutions. Cells were washed twice by addition of 200 µL FACS buffer and centrifugation for 5 min at $400 \times g$ and 4°C. All subsequent wash steps were performed in the same manner. Afterwards, the detecting antibody BD OptiBuild™ BV711 Mouse Anti-Human IgG (BD Biosciences) diluted 1:300 in FACS buffer was added to the cells following incubation for 30 min on ice. After another wash step, cells were stained with 100 µL of the live-dead marker Zombie NIR™ (BioLegend; 1:600 in PBS) for 30 min on ice, washed again, and then fixated by incubation with 100 µL BD Cytofix™ fixation buffer (BD Biosciences) for 30 min on ice. Following two additional wash steps, cells were resuspended in 150 µL FACS buffer and filtered using a 40 µm MultiScreen-MESH filter plate (Merck Millipore). Subsequently, cells were analyzed on a LSRFortessa™ cell analyzer (BD Biosciences) with a high-throughput sampler (HTS, BD Biosciences), collecting 10,000 viable cells per sample.

2.2.7.3 Production of HA-pseudotyped lentiviral particles

Pseudotyped viral particles allow the analysis of viral entry and neutralization in the absence of replication-competent virus. For the generation of HA-pseudotyped lentiviral particles, a previously established protocol for the production of SARS-CoV-2 pseudoviruses (Crawford et al., 2020; Vanshylla et al., 2021) was adapted to enable expression of influenza HA on the particle surface. In addition to plasmids encoding lentiviral components of human immunodeficiency virus

1 (HIV-1), namely Tat, Gag/Pol, and Rev, as well as a luciferase-IRES-ZsGreen reporter construct, an HA-encoding plasmid was co-transfected. To facilitate efficient particle release and HA processing, neuraminidase treatment and a post-production trypsinization step were included (Carnell et al., 2015). The influenza strains for which HA-pseudovirus particles were produced are listed in Table 17 in the Appendix.

Pseudovirus particles were produced in HEK293T cells. One day prior transfection, 8×10^6 cells were seeded in 15 cm cell culture dishes to reach approximately 80% confluency. Medium was replaced with 23 mL FreeStyle™ 293 Expression Medium 2-4 h prior to transfection. For the transfection mix, plasmids pHAGE-Luc-IRES-ZsGreen (20,000 ng), pHDM-Hgpm, pHDM-Tat, pHDM-Rev (4,400 ng each), and pCDNA-HA (6800 ng), encoding the HA of the respective influenza strain, were diluted in FreeStyle™ medium to a final volume of 250 μ L. For selected pseudoviruses, an NA-encoding plasmid was included, with the total amount of HA and NA plasmids kept constant at 6,800 ng. In parallel, 1,650 μ L FreeStyle™ medium was mixed with 100 μ L FuGENE® 6 Transfection Reagent (Promega) and incubated for 10 min at RT. The plasmid solution was then combined with the FuGENE® mixture, incubated for 30 min to allow DNA-reagent complex formation and added dropwise to the cells. Sixteen hours post-transfection, neuraminidase from *Clostridium perfringens* (Sigma-Aldrich) was added at a concentration of 25 mU per mL of culture medium. Supernatants containing pseudovirus particles were harvested 48 h post-transfection by centrifugation at $400 \times g$ for 5 min and subsequently filtered through a 0.45 μ M PVDF syringe filter. To cleave and activate HA, filtered supernatants were incubated with 250 μ g/mL TPCK-treated Trypsin (Sigma-Aldrich) for 1 h at 37°C. Trypsin activity was stopped by addition of 250 μ g/mL of Soybean Trypsin Inhibitor (Roche) and incubation for 30 min at 37°C. This procedure was roughly based on previously published protocols (Chaipan et al., 2009; Sawoo et al., 2014). Pseudovirus stocks were aliquoted and stored at -80°C. When pseudoviruses were produced in culture vessels smaller than 15 cm dishes, cell numbers and volumes of all reagents were scaled proportionally to the surface area of the respective culture vessel.

During establishment of the pseudovirus production protocol, several parameters were evaluated and optimized, including NA treatment, harvest timepoint, or transfection reagent. Any deviations from the protocol described here are specified in the respective figure legends.

2.2.7.4 Titration of HA-pseudotyped lentiviral particles

Each pseudovirus preparation was titrated to determine the optimal virus dilution for use in neutralization assays. For titration, a 2-fold serial dilution of the pseudovirus stock was prepared in 100 μ L DMEM (Gibco) supplemented with 1 mM L-Glutamine in a 96-well tissue culture plate (Corning), starting at a 1:2 dilution. MDCK-SIAT1 cells (1.25×10^4 cells in 50 μ L per well) were then added and plates were incubated for 48 h at 37°C and 5% CO₂. Each plate included two

negative controls consisting of cells only and virus only. For luciferase readout, 50 µL of culture medium was removed from each well and cells were lysed by incubation with 100 µL luciferin-lysis-buffer per well for 2 min and repeated pipetting. Subsequently, 150 µL of the lysate was transferred to a white 96-well microtiter plate (Thermo Fisher Scientific) and luciferase activity was measured using a Tristar 5 plate reader (Berthold Technologies) with a counting time of 1 s.

2.2.7.5 Pseudovirus neutralization assay

Pseudovirus neutralization assays were performed to assess the neutralizing activity of serum, purified (HA-depleted) poly-IgG or mAbs. First, samples were serially diluted in DMEM (Gibco) supplemented with 1 mM L-Glutamine in a 96-well tissue culture plate to achieve a final volume of 50 µL per well. After the addition of 50 µL pseudovirus solution per well (stock diluted in DMEM with 1 mM L-Glutamine as determined by titration), plates were incubated for 1 h at 37°C and 5% CO₂. MDCK-SIAT1 cells (1.25×10^4 cells in 50 µL per well) were then added and plates were incubated for 48 h at 37°C and 5% CO₂. During establishment, assays were also performed with MDCK or HEK293T cells, the latter additionally used for SARS-CoV-2 assays. Luciferase activity was determined as described in chapter 2.2.7.4. To establish background signal, two negative controls consisting of either cells only or virus only were included on each plate. In addition, wells containing virus and cells in the absence of serum or mAb sample served as virus positive control to determine the maximal signal. Background-corrected luminescence values were obtained by subtracting the mean signal of the two negative controls from all measurements. The corrected values were then used to calculate 50% inhibitory dilution (ID₅₀) and 50% inhibitory concentration (IC₅₀) values, defined as the serum dilution or mAb concentration achieving a 50% reduction in luminescence relative to the virus positive control.

2.2.7.6 Authentic virus neutralization test

Virus neutralization tests using authentic H5N1 and H1N1 influenza viruses were performed by collaboration partners of the Institute of Diagnostic Virology at the Friedrich-Loeffler-Institute in Greifswald, Germany. Serial dilutions of human sera (heat-inactivated for 1 h at 56°C, starting concentration 1:16, 2-fold dilution) or mAbs (starting concentration 100 µg/mL, 2-fold dilution) were prepared in DMEM. Samples were mixed with 100 TCID₅₀ of the respective influenza virus strain (either A/dairy cow/Texas/063224-24-1/2024 genotype B3.13 (H5N1, GISAID-No: EPI_ISL_19155861), the European HPAIV isolate A/wild goose/Germany-NW/2024AI02730/2024 genotype DE23-11-N1.3 euDG (H5N1, GISAID-No: EPI_ISL_19494411), or A/Sydney/5/2021 (H1N1, GISAID-No: EPI_ISL_6424984)) and incubated for 1 h at 37°C. Afterwards, 100 µL per well of MDCK II cells were added and plates were incubated for 72 h at 37°C. Neutralization was assessed by light microscopy and samples were classified as neutralizing when no cytopathic effect (CPE) was detectable. The complete inhibitory dilution

(ID₁₀₀) and the complete inhibitory concentration (IC₁₀₀) were determined as the highest dilution or concentration showing complete absence of CPE (100% neutralization). Samples were analyzed in triplicates, with inclusion of both negative and positive control sera, and the virus input was validated by titration.

2.2.8 *In vivo* challenge study

The *in vivo* challenge experiment was performed by collaboration partners of the Institute of Diagnostic Virology and the Institute of Molecular Virology and Cell Biology at the Friedrich-Loeffler-Institute in Greifswald, Germany. Governmental approval was obtained from the responsible ethics committee of the State Office of Agriculture, Food Safety, and Fishery in Mecklenburg-Western Pomerania (LALLF M-V) under the registration number LVL MV TSD/7221.3-1-042/24. The experiment was conducted using healthy female BALB/c mice (6–8 weeks old; 16.9–22.4 g) obtained from Charles River (CRL:028, genotype: Coat Color White (albino) MHC Haplotype H2d). Mice were maintained in individually ventilated cages (Tecniplast) at 20–24°C in groups of two to four animals. Group assignment was performed randomly prior to experimental intervention (Daniel et al., 2026).

On day -1, mice were inoculated intraperitoneally (i.p.) with 10 mg/kg of one mAb in 100 µL physiological saline (0.9% sodium chloride). Four animals each received one of the H5-neutralizing antibodies 48H5-1-1G9K or 47H5-1-2B12K, while five animals in the control group received the SARS-CoV-2-specific antibody DZIF-10c (Kreer et al., 2020b). On day 0, all mice were then infected intranasally with 10³ TCID₅₀ H5N1 (bovine isolate A/dairy cow/Texas/063224-24-1/2024, genotype B3.13). Body weight and clinical symptoms were scored in all animals on a daily basis. Mice meeting predefined humane endpoints, defined as a loss of ≥20% of initial body weight or the presence of severe clinical signs, were euthanized. Surviving animals were euthanized on day 10 post-challenge to allow collection of terminal blood samples and lung tissue. Euthanasia was performed by cervical dislocation under isoflurane anesthesia (Daniel et al., 2026).

2.2.9 Quantification and statistical analysis

Pseudovirus neutralization as well as ELISA and cell-based binding data were analyzed with GraphPad Prism 10. Using a four-parameter logistic (4PL) nonlinear regression model, serum ED₅₀ values and EC₅₀ values for poly-IgG and mAbs were calculated for ELISA binding data. For the calculation of serum ED₅₀/ID₅₀ values and EC₅₀/IC₅₀ values for poly-IgG and mAbs from HA-pseudovirus neutralization and cell-based binding data, a normalized nonlinear fit model (agonist vs. normalized response curve with variable slope) was used. Data points falling below the assay's detection limit were derived through extrapolation from partial dose-response curves.

Correlation analyses were performed between neutralization titers as well as between titers and other variables using Pearson's correlation coefficient in GraphPad Prism 10. Pearson's r (r_p) and the corresponding p-values are reported in the figures.

Statistical comparisons were performed using either an unpaired two-tailed Student's t-test or a two-sided Fisher permutation test (1,000 permutations), depending on the dataset. The specific test used is indicated in the respective figure legend.

For the data presented in Figure 17, statistical analysis was performed by collaboration partners of the research group of Prof. Dr. Michael Lässig from the Institute of Biological Physics at the University of Cologne, as described below.

To quantify statistical errors for titers of each individual, the following approach was used: based on the data shown in Figure 17C, growth curves were resampled under the assumption of normally distributed measurement errors with standard deviations of 0.5 ($\log_2$ dilution titers) and 5% (growth rates). These resampled datasets were used to infer posterior distributions of individual EC_{50} values. The standard deviation of the estimates was found to depend on the mean value. In particular, titers derived through extrapolating from partial dose-response curves exhibited a greater error margin than those obtained from full dose-response curves. An exponential increase in this error margin is observed as the estimated EC_{50} moves progressively outside the experimentally tested concentration range. The standard error of the $\log_2$ -titer was approximated as $\Delta\log(EC_{50}) \sim 0.18 \times (EC_{50}) - 0.34$, based on calculations performed using the SciPy optimize package. This error model derived from data of Figure 17C was then applied to resample ED_{50}/ID_{50} and EC_{50}/IC_{50} values of all remaining panels in Figure 17. For each individual, a normal probability distribution of $\log(EC_{50})$ values was defined, where the inferred $\log(EC_{50})$ defines the expectation value and $\Delta\log(EC_{50})$ from the error model determines the standard deviation. To obtain cohort population distributions of characteristic titers, the posterior $\log(EC_{50})$ distributions of all individuals within a given group were combined ($n=66$ for the upper panel and $n=20$ for the lower panel). From these combined distributions, characteristic titer ranges are reported as 50% percentile [25% percentile, 75% percentile] (adapted from Daniel et al., 2026).

2.3 List of contributions

Unless stated otherwise below, all experiments, data analyses, and figure preparation described in this thesis were planned, performed, and analyzed by me. The project was conducted under the scientific supervision of Prof. Dr. Florian Klein. PD Dr. Christoph Kreer provided methodological guidance and training in antibody-related techniques as well as conceptual guidance during the establishment of the pseudovirus neutralization assay.

In addition, this doctoral thesis was greatly supported by collaborations with several research groups and by the contributions of many colleagues who worked closely with me on the project. Their roles in the experimental work of the study are outlined below.

Study visit coordination, medical consultations and blood draws of EIKIM study participants were carried out by the team of Prof. Dr. Thomas Erren at the Institute and Policlinic for Occupational Medicine, Environmental Medicine and Prevention Research of the University Hospital Cologne, as well as Dr. Malena Rohde and Dr. Lutz Gieselmann. Blood samples were processed (isolation of serum, plasma and PBMCs) by Ricarda Stumpf and Maike Schlotz.

The phylogenetic analysis of HA sequences and selection of influenza strains was performed by Denis Ruchnewitz and Dr. Matthijs Meijers from the research group of Prof. Dr. Michael Lässig at the Institute of Biological Physics, University of Cologne. Jan Eberhardt from the group of Prof. Dr. Michael Lässig performed statistical error modelling for neutralization and binding curves of Figure 17.

Cloning of HA plasmids, production of recombinant HA proteins, ELISAs, the isolation of HA-specific IgG⁺ B cells as well as cloning and production of mAbs were performed together with Leon Ullrich and PD Dr. Christoph Kreer. Single BCR amplification and sequencing was conducted together with PD Dr. Christoph Kreer. BCR sequences were analyzed by PD Dr. Christoph Kreer. Cell-based binding assays were performed by Leon Ullrich. Luana Girao Lessa assisted during selected pseudovirus neutralization assays and performed one assay independently to assess inter-operator reproducibility (see Figure 12).

Authentic virus neutralization assays were performed by PD Dr. Donata Hoffman, Dr. Jacob Schön and Dr. Nico Joel Halwe from the research group of Prof. Dr. Martin Beer at the Institute of Diagnostic Virology at the Friedrich-Loeffler-Institute in Greifswald. The *in vivo* challenge study was also performed by the group of Prof. Dr. Martin Beer at the Institute of Diagnostic Virology in collaboration with the Institute of Molecular Virology and Cell Biology at the Friedrich-Loeffler-Institute in Greifswald.

During the project, Hanna Janicki, Lisa Kottege, Neslihan Mühlhans, Silvia Gulisano-Laudani and Banu Meiners supported DNA preparations, cell culture as well as antibody production and purification.

3 Results

The aim of this doctoral thesis was to analyze potential pre-existing humoral immunity of H5N1-naïve individuals against the recently transmitted human H5N1 clade 2.3.4.4b isolate A/Texas/37/2024, obtained during the HPAIV outbreak in cattle in 2024. The results of this study are presented in the following chapters. The first part describes the establishment of the required experimental systems, comprising a detailed description of the establishment and validation of a pseudovirus neutralization assay as well as the production of recombinant HA proteins. Afterwards, the A/Texas/37/2024-specific immune response is investigated, first on the serum and plasma level and later on the level of single B cells and their encoded mAbs. The results presented in this second part have largely been published in Daniel et al., 2026. However, further analyses and supplementary data are also included.

3.1 Establishment and validation of an HA-pseudovirus neutralization assay

To enable the analysis of the neutralizing activity of serum and monoclonal antibody samples against the H5N1 A/Texas/37/2024 strain as well as other influenza viruses, a lentivirus-based HA-pseudovirus neutralization assay was first established. For this purpose, a previously established protocol for a SARS-CoV-2 pseudovirus neutralization assay was adapted (Crawford et al., 2020; Vanshylla et al., 2021). However, the establishment of a robust HA-pseudovirus neutralization assay required optimization of multiple parameters influencing pseudovirus particle production and assay performance, like NA-treatment, target cell susceptibility, or virus dilution. In the following chapters, the stepwise establishment and validation of the lentivirus-based HA-pseudovirus neutralization assay are described. The final protocols for pseudovirus production, titration, and neutralization assay is provided in chapters 2.2.7.3 to 2.2.7.5 of the methods section.

3.1.1 Optimization of pseudovirus particle production

To adapt and optimize the production of HA-pseudotyped viral particles, the established pseudovirus system required adjustments to account for influenza-specific features influencing particle release and activation. During optimization of particle production, pseudovirus preparations were evaluated by infection of target cells followed by measurement of luciferase activity, which reflected the amount of functional, entry-competent pseudovirus particles present in the supernatant. Optimization experiments were performed using MDCK-SIAT1 cells, as preliminary tests indicated efficient infection of this cell line. A systematic comparison of target cell lines is presented in chapter 3.1.2.

3.1.1.1 Neuraminidase treatment

Like wild-type influenza viruses, influenza HA-pseudotyped particles require NA for efficient particle release since NA cleaves sialic acid residues on the surface of the producing cells, preventing particle retention (Carnell et al., 2015). NA can either be co-expressed via an NA-encoding plasmid or provided as exogenous bacterial NA during production. Both approaches were evaluated during optimization of pseudovirus production, but exogenous NA treatment was preferred to avoid additional neutralization signals from NA-directed antibodies.

To compare both methods, pseudoviruses for the H1N1 strain A/Hawaii/70/2019 were generated by co-transfecting HEK293T cells with different ratios of HA- and NA-encoding plasmids (1:0, 5:1, 6:1, 7:1, and 8:1) together with the lentiviral packaging and reporter constructs. For exogenous NA treatment, the cell culture supernatant was supplemented with 10 or 25 mU/mL neuraminidase from *Clostridium perfringens* 16 h post-transfection. Virus containing supernatants were harvested 48 h post-transfection and HA was subsequently activated by trypsin treatment. To determine functional pseudovirus yields, MDCK-SIAT1 cells were infected with the undiluted virus stocks and luciferase activity was measured 48 h post-infection as described in chapter 2.2.7.4.

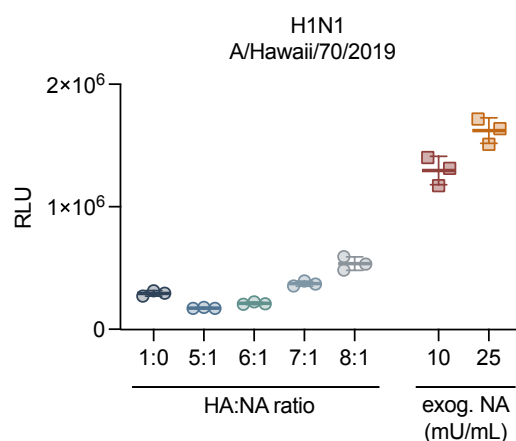


Figure 7. Comparison of NA expression and exogenous NA treatment during pseudovirus production. Pseudovirus particles of the H1N1 strain A/Hawaii/70/2019 were generated by co-transfecting HEK293T cells with HA- and NA-encoding plasmids at ratios 1:0, 5:1, 6:1, 7:1, and 8:1 together with lentiviral packaging and reporter constructs. For exogenous NA-treatment, NA from *Clostridium perfringens* (10 or 25 mU/mL) was added to the cell culture supernatant 16 h post-transfection. Viruses were harvested 48 h post-transfection and used to infect MDCK-SIAT1 cells in technical triplicates. Luciferase activity was measured 48 h post-infection. Depicted is the mean $\pm$ SD.

Pseudovirus particles produced without NA (1:0) yielded low luciferase signals of 2.9×10^5 relative light units (RLU), indicating limited presence of functional viral particles (Figure 7). Co-expression of NA at HA:NA plasmid ratios of 5:1 and 6:1 resulted in even lower signals. Ratios of 7:1 and 8:1, however, increased luciferase signal to 3.7×10^5 RLU and 5.4×10^5 RLU, respectively. In contrast, exogenous NA treatment resulted in substantially higher luciferase activity, reaching 1.3×10^6 RLU with 10 mU/mL NA and 1.6×10^6 RLU with 25 mU/mL NA,

suggesting enhanced release of functional pseudovirus particles. Based on these results, all subsequent pseudoviruses were produced with 25 mU/mL exogenous NA.

3.1.1.2 Harvest timepoint

A second parameter which needed optimization during assay establishment was the harvest timepoint for pseudovirus-containing supernatants. According to previously described protocols, supernatants are typically collected 24 h, 48 h, or 72 h after transfection (Carnell et al., 2015). To determine the optimal timepoint, pseudoviruses of the H1N1 strain A/Hawaii/70/2019 were harvested at 24 h, 48 h, and 72 h post-transfection and functional pseudovirus yield was determined by infection of MDCK-SIAT1 cells and measurement of luciferase activity.

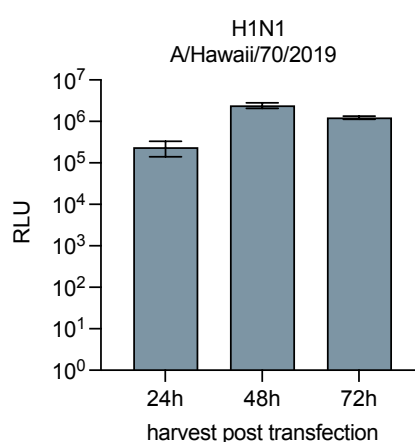


Figure 8. Comparison of pseudovirus production with virus harvest 24 h, 48 h, and 72 h post-transfection. Pseudovirus particles of the H1N1 strain A/Hawaii/70/2019 were produced and virus containing supernatants harvested either 24 h, 48 h, or 72 h post-transfection. MDCK-SIAT1 cells were infected in technical triplicates and luciferase activity determined 48 h post-infection. Depicted is the mean $\pm$ SD.

The yield of functional pseudovirus particles was lowest in supernatants harvested 24 h after transfection, with a luciferase activity of 2.4×10^5 RLU (Figure 8). It increased substantially after 48 h, reaching a maximum of 2.4×10^6 RLU and decreased again after 72 h to 1.2×10^6 RLU. Consequently, 48 h post-transfection was chosen as the standard harvest timepoint for all subsequent productions.

3.1.1.3 Transfection reagent

For all previous pseudovirus productions, FuGENE 6 was used as the transfection reagent. However, calcium phosphate (CaPO_4) precipitation was evaluated as a cost-effective alternative. Pseudovirus particles for a small panel of influenza strains from the HA subtypes H1, H2, H3, H5 and H7 were produced in parallel using either FuGENE 6 or CaPO_4 precipitation. Functional pseudovirus yield was assessed by infection of MDCK-SIAT1 cells and measurement of luciferase activity.

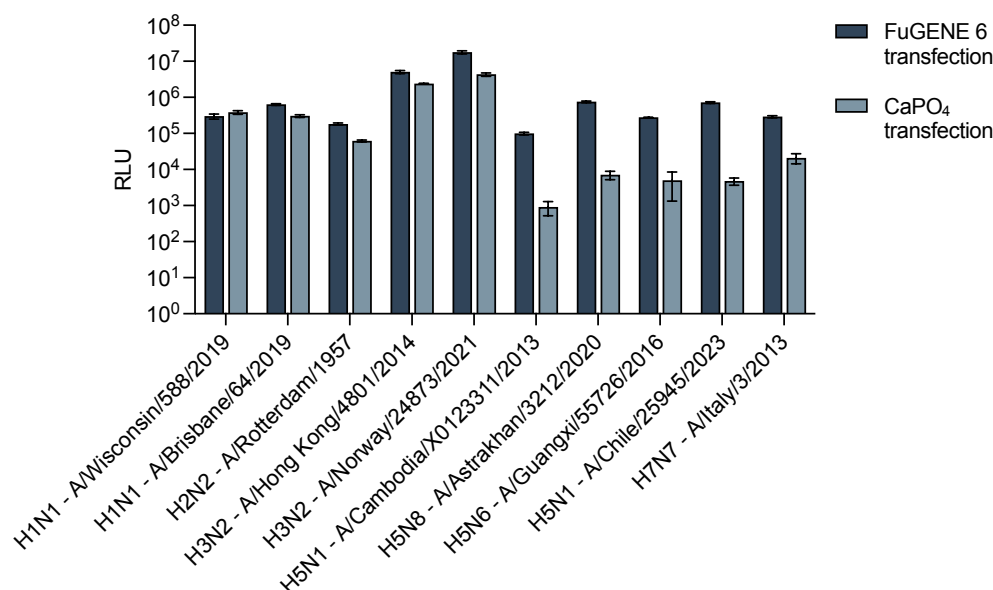


Figure 9. Comparison of pseudovirus production using transfection via FuGENE 6 or CaPO₄ precipitation. HA-pseudovirus particles for the indicated influenza strains were produced in HEK293T cells either using the standard pseudovirus production protocol with FuGENE 6 transfection or, alternatively, CaPO₄ precipitation. For CaPO₄ transfection, the same plasmid amounts as in the standard protocol were mixed with 1 mL of 250 mM CaCl₂ followed by dropwise addition of 1 mL 2×HBS buffer. After mixing and incubation for 2 min at room temperature, the solution was added dropwise to the cells. Supernatants were harvested 48 h post-transfection and used to infect MDCK-SIAT1 cells in technical triplicates. Luciferase activity was determined 48 h post-infection. Data are shown as mean ± SD.

Comparison of the two transfection methods revealed strain-specific differences in luciferase signals (Figure 9). Except for the H1N1 strain A/Wisconsin/588/2019, which showed comparable signals with both transfection methods, all other strains yielded higher luciferase signals when pseudoviruses were produced using FuGENE 6. The highest signal was observed for the H3N2 strain A/Hong Kong/4801/2014, reaching 1.8×10^7 RLU. Across the panel of non-H1 strains, the fold increase with FuGENE 6 compared with CaPO₄ ranged from 2- to 151-fold, with the largest differences observed for the avian H5 and H7 strains. For some of these strains, CaPO₄-mediated transfection generated luciferase signals that were insufficiently distinct from background signal, making them unsuitable for downstream assays. Based on these results, pseudovirus production was continued using FuGENE 6 as the transfection reagent.

3.1.2 Evaluation of different target cell lines for infection

Following optimization of pseudovirus production, the susceptibility of different cell lines to pseudovirus infection was tested to identify the most suitable cell line for use in neutralization assays. Susceptibility was assessed for HEK293T, MDCK, and MDCK-SIAT1 cells. All three cell lines have previously been described for use in HA-pseudovirus neutralization assays (Carnell et al., 2015; Garcia et al., 2010; Wang et al., 2008; Yu et al., 2025).

For comparison, each cell line was infected with undiluted pseudoviruses of different HA-subtypes. In addition to strains from the human subtypes H1, H2, and H3, pseudoviruses of the avian subtypes H5, H7, and H9 were included to account for differences in viral receptor

preference for $\alpha 2,3$ - or $\alpha 2,6$ -linked sialic acid residues. Luciferase activity was measured 48 h post-infection and used as a measure of relative transduction efficiency, reflecting the susceptibility of the tested cell lines to pseudovirus entry.

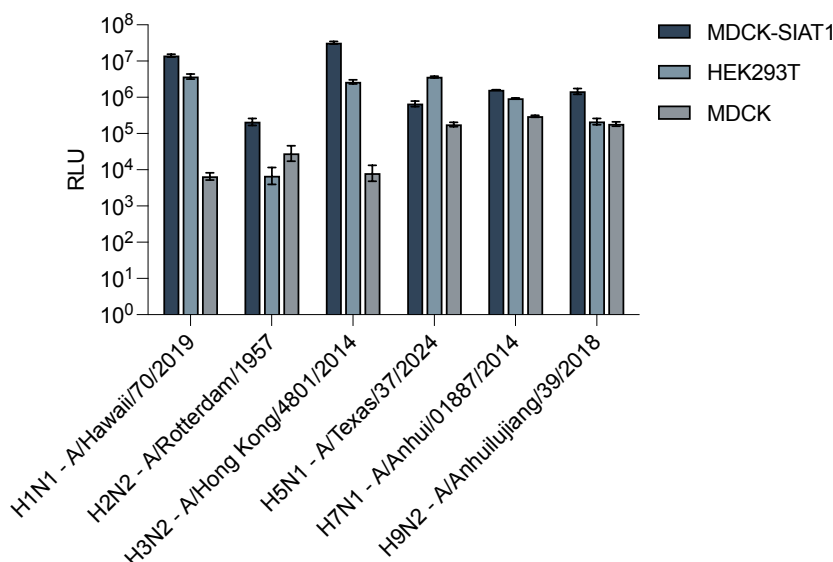


Figure 10. Susceptibility of MDCK-SIAT1, MDCK, and HEK293T cells to HA-pseudovirus infection. HA-pseudovirus particles of the indicated influenza strains were generated and used to infect MDCK-SIAT1, HEK293T, and MDCK cells using equal virus input. Cells were infected in technical triplicates, and luciferase activity was determined 48 h post-infection. Data represent the mean $\pm$ SD.

For pseudoviruses of the subtypes H1, H2, H3, H7, and H9, MDCK-SIAT1 cells consistently produced the highest luciferase signals, ranging from 2.1×10^5 RLU for the H2N2 strain A/Rotterdam/1957 to 3.2×10^7 RLU for the H3N2 strain A/Hong Kong/4801/2014, indicating superior transduction efficiency in this cell line (Figure 10). The H5N1 strain A/Texas/37/2024 represented the only exception, yielding higher luciferase activity in HEK293T cells (3.6×10^6 RLU) compared with MDCK-SIAT1 (6.6×10^5 RLU) and MDCK cells (1.8×10^5 RLU). To avoid introducing additional variability between assays, a single cell line was selected for all subsequent experiments. Based on the overall performance across the tested strains, MDCK-SIAT1 cells were used for all further neutralization assays.

3.1.3 Validation of the HA-pseudovirus neutralization assay with HA-specific antibodies and serum samples

After the establishment of pseudovirus production and the selection of a target cell line, the performance of the neutralization assay was evaluated. Neutralization of H1 and H3 pseudoviruses was tested using a panel of well-characterized influenza HA antibodies with known neutralization specificities. In addition, serum samples from healthy human blood donors were included as a validation control to confirm that the assay can detect neutralizing activity in polyclonal human sera, in which immunity against circulating seasonal H1N1 and H3N2 strains

can be expected. The antibody panel comprised the five antibodies MEDI8852, 39.29, 31.a.83, 5J8, and CR8020 (Figure 11). The antibodies MEDI8852, 39.29, and 31.a.83 are broadly reactive stem-directed antibodies that recognize the conserved hydrophobic groove region across both group 1 and group 2 influenza viruses (see Table 18 in the Appendix for antibody information; Jiao et al., 2023; Joyce et al., 2016; Kallewaard et al., 2016; Nakamura et al., 2013). In contrast, CR8020 and 5J8 exhibit more restricted subtype specificity and were included to validate assay specificity (Ekiert et al., 2011; Krause et al., 2011). The antibodies were recombinantly produced. Neutralization capacity of antibodies and sera was determined by pseudovirus neutralization assay as described in chapter 2.2.7.5.

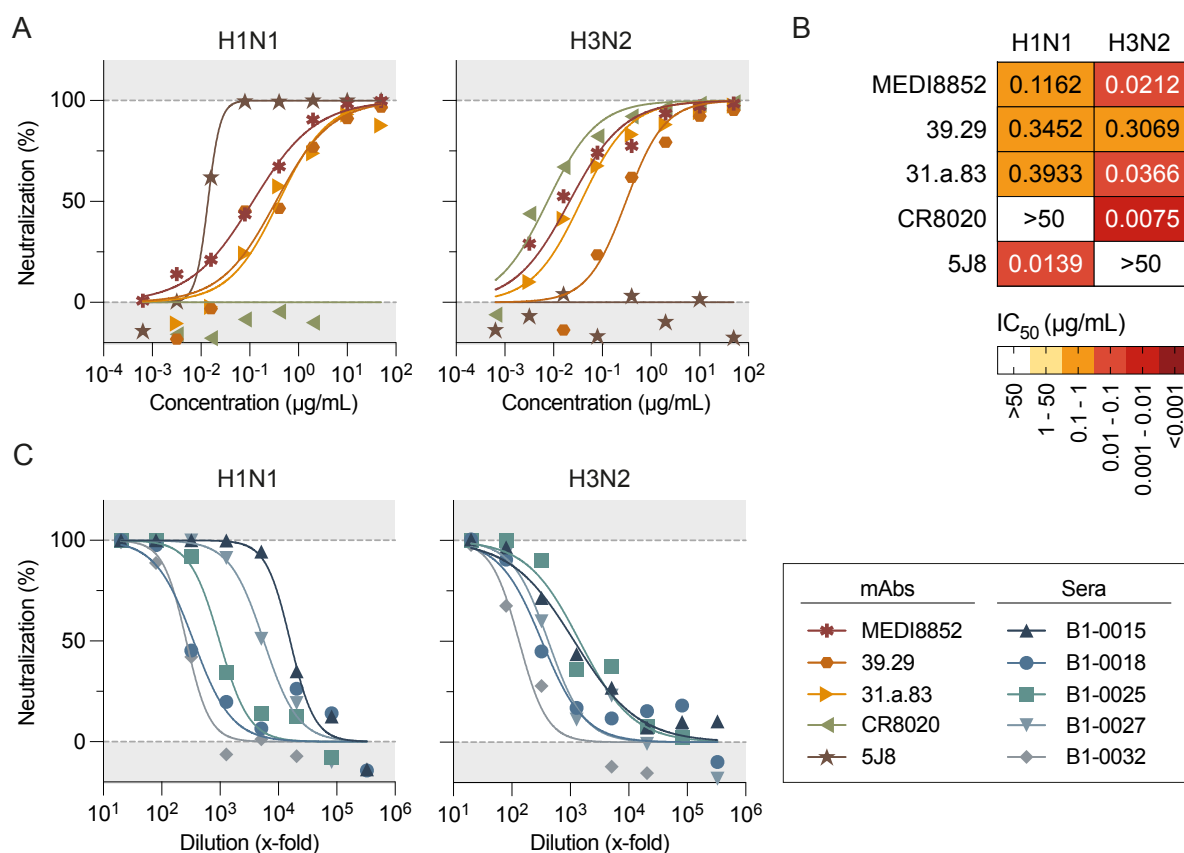


Figure 11. Neutralization of HA-pseudoviruses by monoclonal antibodies and serum samples. (A) Monoclonal antibodies MEDI8852, 39.29, 31.a.83, CR8020, and 5J8 were tested for their neutralizing activity against pseudoviruses expressing the HA of H1N1 A/Hawaii/70/2019 or H3N2 A/Hong Kong/45/2019. Symbols represent normalized neutralization from $n=2$ technical replicates per antibody with lines showing non-linear curve fits. The upper limit of detection was a concentration of 50 $\mu\text{g/mL}$. (B) Half-maximal inhibitory concentration (IC_{50}) values determined in (A). (C) Serum samples from $n=5$ healthy individuals were tested for their neutralizing activity as described in (A). The lower limit of detection was a 20-fold serum dilution.

Neutralization of H1N1 and H3N2 pseudoviruses by the panel of mAbs demonstrated the expected reactivity patterns. The broadly reactive stem antibodies MEDI8852, 39.29, and 31.a.83 efficiently neutralized both influenza strains with half-maximal inhibitory concentration (IC_{50}) values ranging from 0.393 to 0.021 $\mu\text{g/mL}$ (Figure 11A and B). The subtype-restricted antibodies exhibited distinct specificity with CR8020 neutralizing only the H3N2 pseudovirus and 5J8 neutralizing only H1N1. No off-target effects were detected, confirming assay specificity.

Neutralization was also detected for all human sera against both strains with half-maximal inhibitory dilution (ID₅₀) values between 133 and 15,937 (Figure 11C).

3.1.4 Reproducibility of the HA-pseudovirus neutralization assay

In the next step, reproducibility of the HA-pseudovirus neutralization assay was evaluated. Neutralizing activity of ten serum samples against ten different pseudoviruses was determined in independent replicates performed by two different operators. Different pseudoviruses of the subtype H3N2 were selected to cover a broad range of neutralizing activity, including low, medium and high ID₅₀ values.

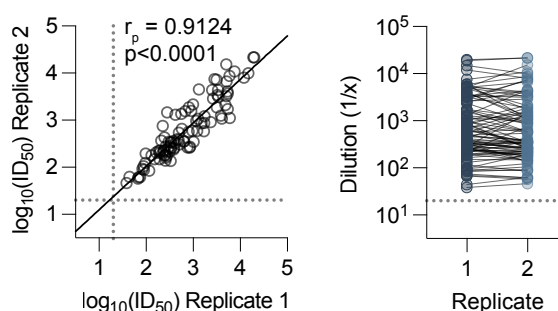


Figure 12. Reproducibility of the HA-pseudovirus neutralization assay. Neutralizing activity was measured for $n=10$ sera from healthy individuals against ten different pseudoviruses of the subtypes H3N2 (A/Victoria/2030/2017, A/Tunisia/1597/2017, A/Victoria/723/2017, A/Victoria/601/2017, A/South Australia/34/2019_S1AT1, A/South Australia/34/2019_E4, A/Hong Kong/45/2019, A/Sydney/549/2017, A/Sydney/473/2019, A/Darwin/9/2021_S1AT1) in independent replicates performed by two different operators. Depicted is the correlation of log₁₀-transformed half-maximal inhibitory dilution (ID₅₀) values for both replicates, along with a direct comparison of the untransformed values. ID₅₀ values below the lower limit of detection of a 20-fold serum dilution (dotted lines) were excluded from the analysis. Pearson correlation coefficient (r_p) and the associated p -value are indicated in the figure.

Log-transformed ID₅₀ values were analyzed and showed statistically significant correlation ($r_p=0.9124$, $p<0.0001$) between the replicates performed by different operators (Figure 12). The mean fold-change between replicates was 1.763-fold and no systematic shift toward higher or lower titers was observed. These findings demonstrate high inter-operator reproducibility of the assay and provide insights into the assay's resolution across a broad range of neutralizing titers.

3.1.5 Selection of virus dilution for neutralization assays

In a previous study, a threshold of approximately 10^5 RLU was proposed for pseudovirus neutralization assays (Garcia et al., 2010). However, this recommendation was based on assays with pseudoviruses of only the H5 subtype using a production protocol that differs from the one established in the present study. Therefore, the dependency of IC₅₀ values on pseudovirus input was systematically evaluated in the present assay setup.

Pseudovirus neutralization assays were performed to determine IC₅₀ values for the antibodies MEDI8852 and 39.29 against one strain each of the subtypes H1N1, H3N2, and H5N1 using different pseudovirus dilutions. These dilutions resulted in positive control signals ranging

from 6,000 to 950,000 RLU for H1N1, 25,000 to 800,000 RLU for H3N2, and 9,000 to 300,000 RLU for H5N1 in only infected cells (Figure 13A).

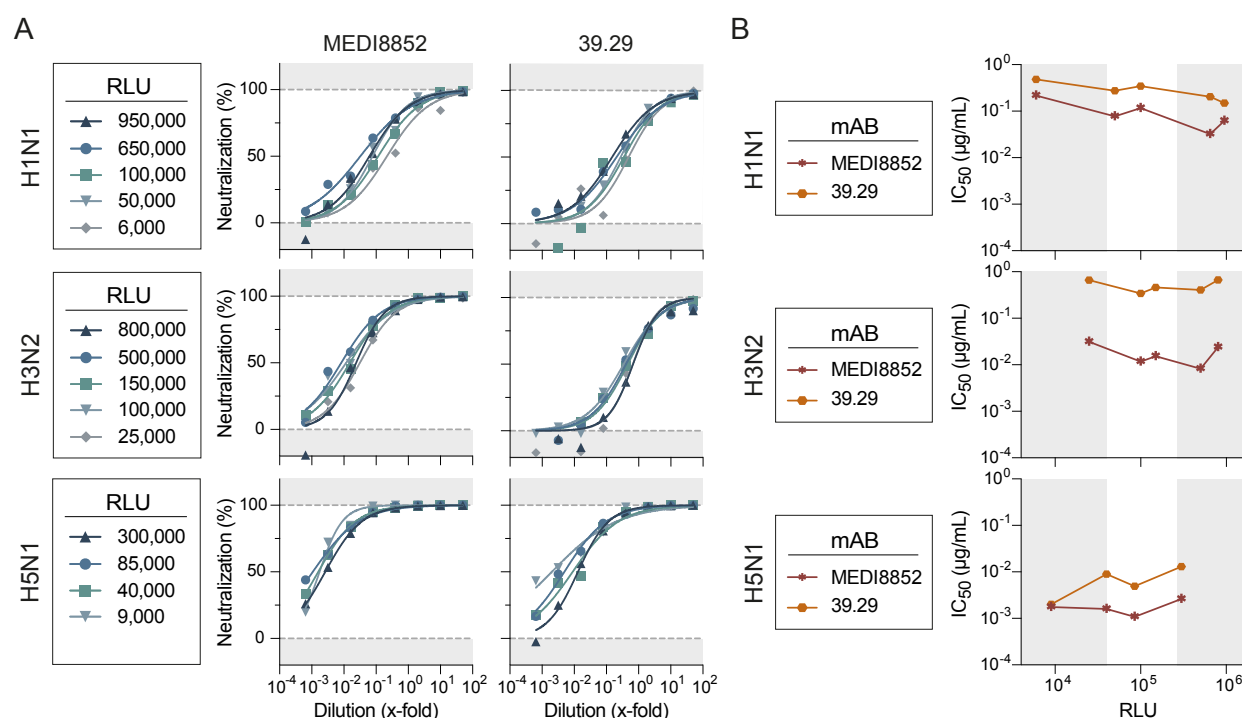


Figure 13. Neutralization of HA-pseudoviruses across varying virus inputs. (A) Monoclonal antibodies MEDI8852 and 39.29 were tested for their neutralizing activity against pseudoviruses expressing the HA of H1N1 A/Hawaii/70/2019, H3N2 A/Hong Kong/4801/2014, or H5N1 A/Texas/37/2024 using different pseudovirus dilutions, resulting in different positive control signals (6,000–950,000 RLU). Symbols represent normalized neutralization from n=2 technical replicates per antibody and virus dilution, and lines show non-linear curve fits. (B) Half-maximal inhibitory concentration (IC₅₀) values determined in (A) for the different virus dilutions.

Across all tested pseudovirus inputs, sigmoidal neutralization curves were obtained, allowing reliable determination of IC₅₀ values for both antibodies (Figure 13A). Full neutralization was observed at all virus dilutions. Although IC₅₀ values showed minor variation between the different pseudovirus inputs, no systematic trend toward higher or lower IC₅₀ values with increasing signal intensity was observed (Figure 13B). Similar patterns were detected for both MEDI8852 and 39.29 across all tested subtypes. The maximal difference in IC₅₀ values between conditions was 6.73-fold for H1N1, 3.8-fold for H3N2, and 6.49-fold for H5N1.

Increased variability was observed for very low and very high pseudovirus inputs. Therefore, a working range of pseudovirus inputs generating positive-control signals between 40,000 and 270,000 RLU was selected for subsequent experiments (indicated by the white area in Figure 13B). Within this range, the maximal IC₅₀ variation was 1.51-fold for the H1N1 strain, 1.34-fold for the H3N2 strain, and 2.65-fold for the H5N1 strain, which more closely reflects the variability observed between independent replicates (see chapter 3.1.4). This range was therefore considered appropriate to maintain assay robustness while ensuring a reliable signal-to-background ratio and minimizing virus consumption.

3.1.6 Pseudovirus and authentic virus neutralization assays show strong correlation

As a final step of assay validation, the pseudovirus neutralization assay was compared with an authentic virus neutralization test. Beyond validation, this comparison also allowed assessment of the relative sensitivity of the pseudovirus assay.

Authentic virus neutralization assays were performed at the Institute of Diagnostic Virology of the Friedrich-Loeffler-Institute in Greifswald using a CPE-based microneutralization assay. For direct comparison, the H1N1 strain A/Sydney/5/2021, for which both HA-pseudovirus and authentic virus were available, was selected and neutralization analyzed using a panel of twenty human serum samples and six previously described HA antibodies. ID₅₀ values determined by pseudovirus assay were compared to authentic virus ID₁₀₀ titers. Log-transformed titers were analyzed using Pearson correlation.

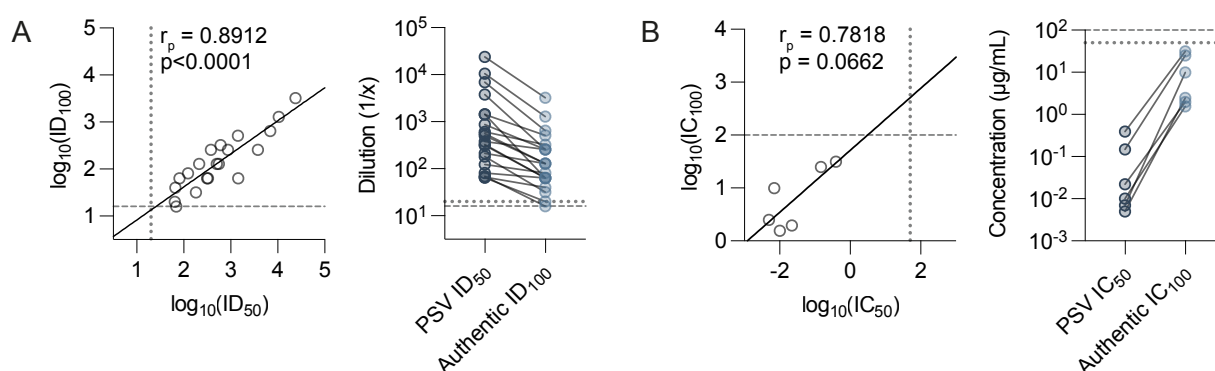


Figure 14. Comparison of neutralization levels determined by HA-pseudovirus vs. authentic neutralization assay. (A) Comparison of the neutralizing activity of $n=20$ sera from healthy individuals against HA-pseudoviruses (PSV; ID₅₀) or authentic viruses (ID₁₀₀) of the H1N1 strain A/Sydney/5/2021. Figure adapted from Daniel et al., 2026 (B) Comparison of the neutralizing activity of antibodies MEDI8852, CR6261, 39.29, CR9114, F045-092, and 5J8 against HA-pseudoviruses (PSV, ID₅₀) or authentic viruses (ID₁₀₀) of the H1N1 strain A/Sydney/5/2021. In (A)–(B) correlations of log₁₀-transformed neutralization values (ID₅₀/ID₁₀₀ or IC₅₀/IC₁₀₀) are depicted. Pearson correlation coefficient (r_p) and the associated p-value are indicated in the figure. Detection limits are marked by dotted (HA-pseudovirus) and dashed (authentic virus) lines, representing a 20-fold serum dilution or an antibody concentration of 50 µg/mL for the pseudovirus assay, and a 16-fold serum dilution or 100 µg/mL for the authentic virus assay.

A strong positive correlation was observed between pseudovirus and authentic virus neutralization assays for serum ($r_p=0.8912$) and antibody samples ($r_p=0.7818$), demonstrating comparable results between the assays (Figure 14). However, a systematic shift in titers was observed. ID₁₀₀ values were on average 5.5-fold lower than ID₅₀ values, whereas IC₁₀₀ values were correspondingly 401-fold higher than IC₅₀ values, reflecting differences in assay setup and readout. Notably, the pseudovirus assay detected neutralizing activity in serum samples below the authentic virus assay's detection limit.

3.2 Production and validation of HA proteins

To be able to analyze human samples not only for their neutralization capacity but also for their binding reactivity to influenza HA and to isolate HA-reactive single B cells from these samples, HA proteins were recombinantly produced using HEK293-6E cells. After production, HA proteins were validated by measuring binding to previously described HA antibodies with known epitopes and subtype specificities.

3.2.1 Design and production of HA proteins

Recombinant HA proteins were produced for the human H5N1 clade 2.3.4.4b isolate A/Texas/37/2024 associated with the 2024 cattle outbreak, as well as for the seasonal strains A/Hawaii/70/2019 (H1N1) and A/Hong Kong/4801/2014 (H3N2). HA ectodomain constructs for recombinant production were generated as schematically shown in Figure 15A and described in chapter 2.2.4.1. After purification, the proteins were analyzed by SDS-PAGE.

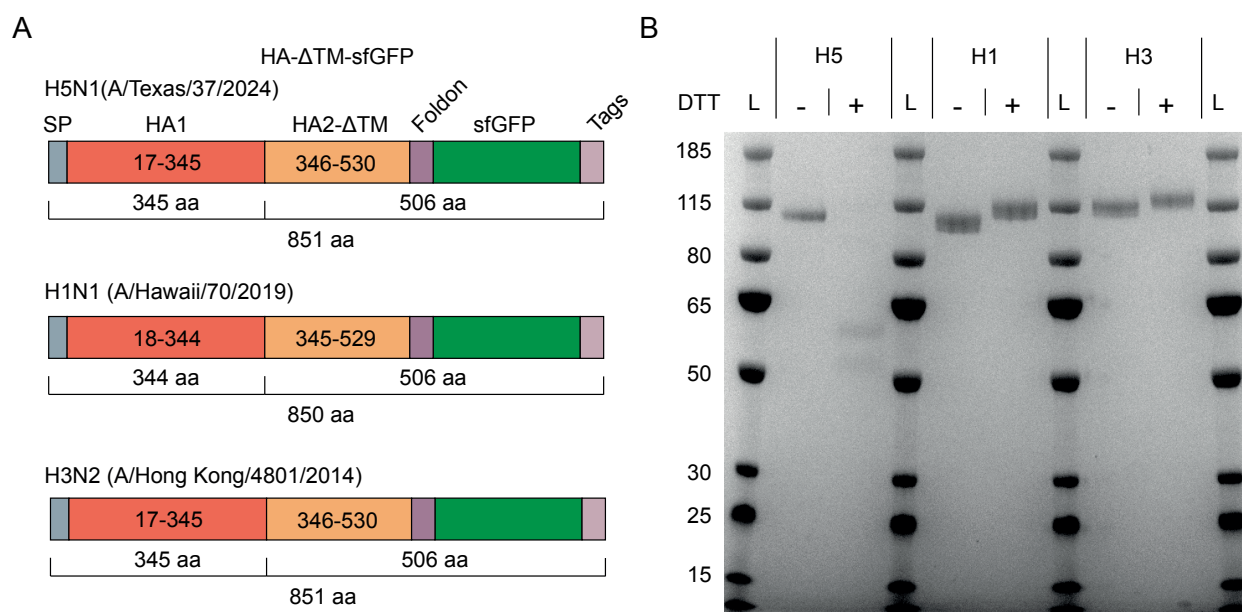


Figure 15. Cloning and purification of recombinant H5N1, H1N1, and H3N2 HA proteins. (A) Recombinant HA constructs contained the native HA coding sequence including the endogenous signal peptide (SP) but lacking the transmembrane and intracellular domain (ΔTM). The resulting ectodomains comprised amino acids (aa) 1-530 for H5, aa 1-529 for H1, and aa 1-530 for H3. For stabilization and trimer formation, the proteins were fused to a Foldon trimerization motif, a super-folder (sf)GFP, and C-terminal tags including an HRV 3C protease cleavage site, an 8×His-tag, and a Twin-Strep tag. (B) Proteins were produced in HEK293-6E suspension cells and purified by Strep-Tactin affinity chromatography. Depicted is the SDS-PAGE analysis of purified proteins performed under reducing (+) and non-reducing (-) conditions using dithiothreitol (DTT). L=protein ladder. Figure previously shown in Daniel et al., 2025.

SDS-PAGE confirmed successful expression and purification of all HA proteins (Figure 15B). Under non-reducing conditions (DTT -), all proteins migrated as a single band at approximately 105 kDa, consistent with the HA-ectodomain fused to sfGFP and C-terminal tags. Under reducing conditions (DTT +), the H1 and H3 protein appeared as a single band at ~115 kDa, corresponding to the uncleaved HA0 form, given their monobasic HA1/HA2 cleavage

site. In contrast, the H5 protein from A/Texas/37/2024 displayed two bands at ~55 kDa (HA1) and ~60 kDa (HA2), reflecting cleavage at the polybasic HA1/HA2 site and proper processing of this protein.

3.2.2 Recombinant HA proteins bind known HA-specific antibodies in ELISA

In addition to size-based analysis, the recombinantly produced HA proteins were assessed for correct epitope presentation by measuring their binding to a panel of well-characterized antibodies targeting distinct regions and epitopes of the influenza HA. The antibody panel was selected to confirm proper folding and epitope presentation of both conserved and subtype-specific HA domains. It comprised the antibodies MEDI8852, 5J8, and CR8020, already used for validation of the pseudovirus neutralization assays, as well as the antibodies FLU-A20 and 100F4.

Like MEDI8852, FLU-A20 is a broadly reactive antibody recognizing HA proteins across both group 1 and group 2 subtypes. However, it targets the monomer interface within the HA head domain while MEDI8852 is stem-directed (Bangaru et al., 2019; Kallewaard et al., 2016). 100F4 recognizes the VE subdomain of only H5 HAs and was chosen for its restricted specificity (Zuo et al., 2015). Together with 5J8 and CR8020 it served to validate subtype- or group-specific epitope integrity. Antibody binding to the HA proteins of A/Texas/37/2024 (H5), A/Hawaii/70/2019 (H1), and A/Hong Kong/4801/2014 (H3) was assessed by ELISA.

	MEDI8852	FLU-A20	5J8	CR8020	100F4	EC ₅₀ (µg/mL)
H1N1 - A/Hawaii/70/2019	0.0372	0.0126	0.0406	>5	>5	>5
H3N2 - A/Hong Kong/4801/2014	0.0808	0.0320	>5	0.0772	>5	1 - 5
H5N1 - A/Texas/37/2024	0.1612	0.1447	>5	>5	0.0002	0.1 - 1

Figure 16. Binding of reference monoclonal antibodies to recombinant influenza HA proteins. Half-maximal effective concentration (EC₅₀) values for antibodies MEDI8852, FLU-A20, 5J8, CR8020, and 100F4 determined by IgG enzyme-linked immunosorbent assay (ELISA) against recombinantly produced HA proteins of the influenza strains A/Hawaii/70/2019, A/Hong Kong/4801/2014, and A/Texas/37/2024. The upper limit of detection was an antibody concentration of 5 µg/mL.

Binding analysis demonstrated the expected reactivity pattern for all combinations of antibodies and proteins (Figure 16). All HA proteins were efficiently bound by the broadly reactive stem antibody MEDI8852 and the head-directed antibody FLU-A20, with EC₅₀ values ranging from 0.015 µg/mL to 0.162 µg/mL. In addition, subtype- and group-specific antibodies displayed the anticipated binding specificity, with 5J8 binding only to H1 HA, 100F4 selectively recognizing H5 HA, and CR8020 binding only to the group 2 H3 HA, while showing no detectable binding to the other HA subtypes. Together this indicates that epitopes targeted by the tested antibodies on both the head and stem domains were preserved and accessible across all HA proteins, supporting their use in subsequent binding and functional assays.

3.3 Assessment of H5-specific binding and neutralization in H5-naive individuals

Having established the required experimental tools, the question of whether H5N1-naive individuals possess pre-existing humoral immunity against the recently emerged H5N1 strain from the US dairy cattle outbreak could be addressed. All following chapters of the results section present experiments that were conducted in the framework of the study published in Daniel et al., 2026. The structure of the following chapters largely follows the original publication, therefore, some descriptions might be similar to those used in the publication. In addition to the published data, further results are included, some of which were previously shown as supplementary material.

3.3.1 H5-naive individuals exhibit measurable IgG binding and serum neutralization against A/Texas/37/2024 HA

To address pre-existing H5-specific humoral immunity in H5N1-naive individuals, blood samples from a cohort of $n=66$ healthy study participants from the local area of Cologne were analyzed (Figure 17A). Given that no human H5N1 infections have been reported in Germany and the very low number of human cases worldwide, prior exposure to H5 viruses is highly unlikely. Sample collection took place between August 2023 and June 2024. Among the participants, 32% were male and 68% were female. Based on self-reported vaccination status, 70% of participants had received an influenza vaccination at some point in their life, however, timepoint and frequency of vaccinations were not documented. Participants were born between 1952 and 2004, with a median age of 38 years at the timepoint of blood collection.

Serum and plasma samples from all individuals were assessed for binding and neutralizing reactivity against HA using three complementary assays. IgG binding of plasma from the 66 H5N1-naive individuals to recombinant HA protein of the spilled-over A/Texas/37/2024 strain was first measured by ELISA. H5-reactive IgGs were detectable in the majority of samples, with 62 of 66 individuals showing binding at the lowest plasma dilution tested (1:20) and a median ED_{50} of 93 (Figure 17B). To compare this with responses to seasonal influenza viruses, plasma reactivity was also assessed against the HA protein of H1N1 A/Hawaii/2019 and H3N2 Hong/Kong/4801/2014. Median ED_{50} values of 656 and 338 were observed for these seasonal controls, respectively, indicating substantially higher plasma reactivity compared to H5.

Next, binding reactivity was measured against the same three HA proteins using a cell-based binding assay. This assay measures IgG binding to HA expressed on the surface of HEK293-6E cells by flow cytometry, allowing assessment of antibody recognition of HA in a native-like membrane-associated conformation. With a median ED_{50} of 19, plasma IgG binding to H5 was more than 100-fold lower than to the seasonal H1N1 and H3N2 controls (Figure 17C). However, in 37 samples, plasma ED_{50} values remained below the assay's lower limit of detection (LLOD), corresponding to a 1:20 plasma dilution.

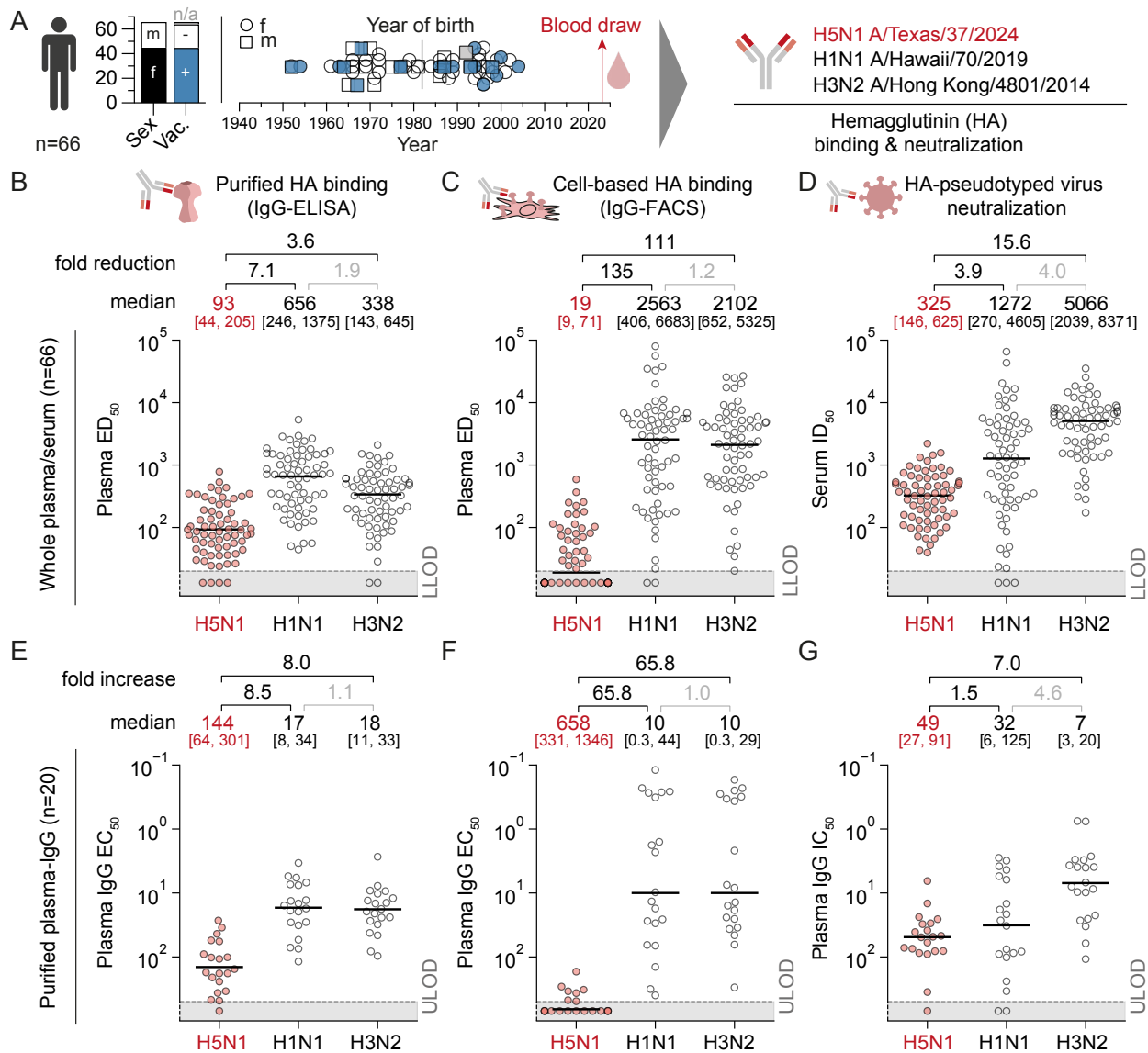


Figure 17. Plasma binding and serum neutralization against H5N1 A/Texas/37/2024 and seasonal influenza strains in H5-naïve individuals. (A) Overview of the study cohort of n=66 H5N1-naïve individuals, including sex, vaccination (Vac.) status, and birth year. The panel of influenza strains used to evaluate HA binding and neutralization is indicated on the right. (B) Plasma IgG binding titers measured by ELISA against recombinant HA proteins of the strains listed in (A). Half-maximal effective dilution (ED_{50}) values are shown for all 66 participants. (C) Plasma IgG binding titers measured by cell-based binding assay against the strains listed in (A). ED_{50} values are shown for all 66 participants. (D) Neutralizing activity of serum samples against strains listed in (A), determined by HA-pseudovirus neutralization assay. Half-maximal inhibitory dilution (ID_{50}) values are shown for all 66 participants. In panels (B)-(D), the lower limit of detection (LLOD, dashed line) was a 20-fold plasma/serum dilution, while the lowest dilution tested was 1:327,680. Data points under the LLOD were assigned a fixed value to allow visualization. Fold reductions between sample pairs are shown relative to the higher geometric mean. (E)-(G) Poly-IgG purified from plasma for a subset of n=20 study participants was analyzed in the same three assays as in (B)-(D). Half-maximal effective concentration (EC_{50}) values were measured by ELISA (E) or cell-based binding assay (F) while half-maximal inhibitory concentration (IC_{50}) values were determined by pseudovirus assay (G). The upper limit of detection (ULOD, dashed line) was 500 μ g/mL, while the lowest concentration tested was 0.0064 μ g/mL. Data points above the ULOD were assigned a fixed value to allow visualization. Fold increases between sample pairs are shown relative to the lower median. In panels (B)-(G) the error-corrected median (horizontal bar) is reported on top of each graph with the 25th and 75th percentile indicated in brackets. Figure taken from Daniel et al., 2026.

In addition to binding, neutralizing activity against the H5N1 A/Texas/37/2024 variant and the seasonal controls was assessed in the H5-naïve cohort using the established pseudovirus neutralization assay (Figure 17D). H5 serum neutralization was detected in all individuals, with a median ID_{50} of 325. Consistent with the binding results, neutralizing activity against the seasonal controls was substantially higher, with median ID_{50} values of 1,272 for H1N1 and 5,066 for H3N2.

In summary, the results demonstrated that most H5N1-naive individuals displayed measurable binding and neutralizing activity against A/Texas/37/2024 in plasma and serum samples.

To reduce the impact of non-IgG components in the plasma and serum and to standardize antibody input for better comparison between samples, poly-IgGs were isolated from plasma of $n=10$ male and $n=10$ female individuals representing the age diversity of the cohort. Purified IgGs were subsequently analyzed using the same binding (Figure 17E and 17F) and neutralization assays (Figure 17G) and showed comparable results to those observed with plasma or serum. H5-specific binding and neutralization were detected in the majority of samples by ELISA and pseudovirus neutralization assay. In the cell-based binding assay, 13 out of 20 samples reached EC_{50} values above the assay's ULOD. Across all three assays, H5 reactivity was lower than for the seasonal controls, with fold reductions ranging between 1.5-fold to 65.8-fold depending on the assay and subtype.

To further evaluate the contribution of IgG antibodies to plasma and serum reactivity against H5, neutralizing activity measured in serum was compared with that of the corresponding purified poly-IgG fractions. The comparison was performed for the previously analyzed H5N1, H1N1 and H3N2 strains, as well as for the H2N2 strain A/Japan/305/1957 (Figure 18A).

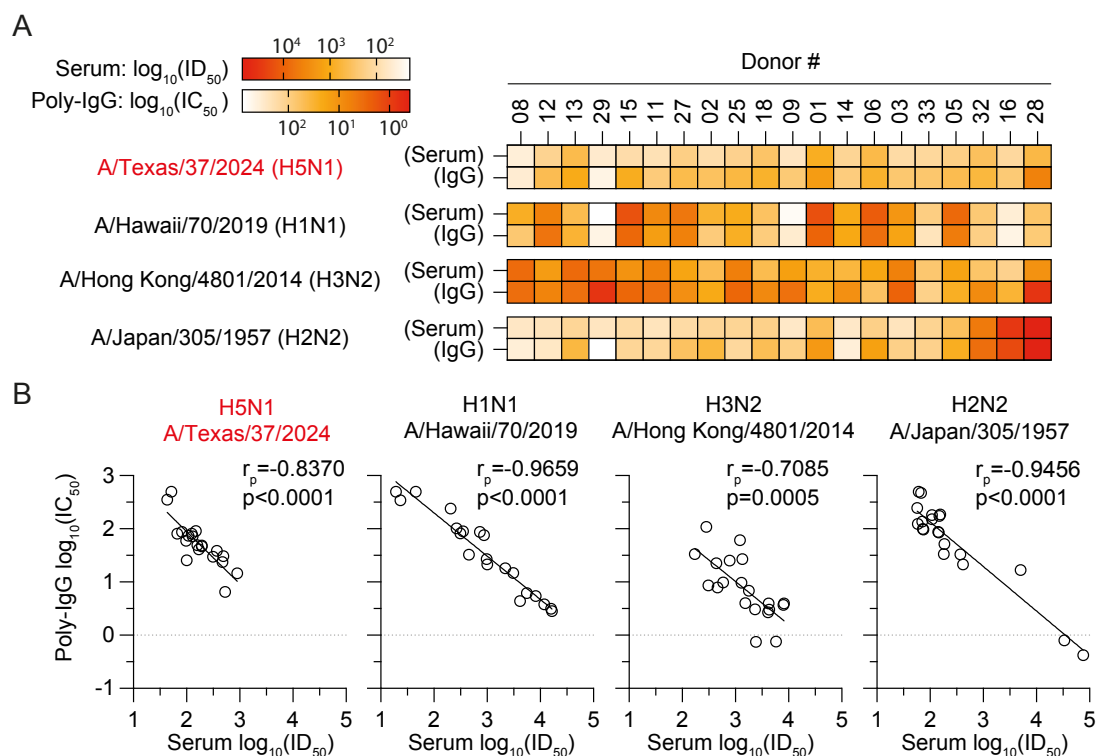


Figure 18. Comparison of pseudovirus-neutralizing activity measured for serum and poly-IgG samples. (A) Neutralizing activity against the indicated strains was measured in parallel for serum and poly-IgG samples for a subset of $n=20$ H5N1-naive individuals. Half-maximal inhibitory dilution (ID₅₀) or concentration (IC₅₀) values are depicted. **(B)** Correlations of log₁₀-transformed neutralization values (ID₅₀ and IC₅₀) determined in (A). Pearson correlation coefficients (r_p) and the associated p-values are indicated in the figure. The line corresponds to a linear regression calculated for the dataset. Figure taken from Daniel et al., 2026.

A significant correlation between serum ID_{50} and poly-IgG IC_{50} values was observed across all four subtypes, with Pearson correlation coefficients ranging from -0.709 and -0.966 (Figure 18B). In addition, plasma samples from a subset of four individuals were depleted of total IgG using protein G-based affinity chromatography. Reactivity of untreated and IgG-depleted plasma was assessed by pseudovirus neutralization assays and ELISA against the previously analyzed H5N1, H1N1, and H3N2 strains, as well as SARS-CoV-2.

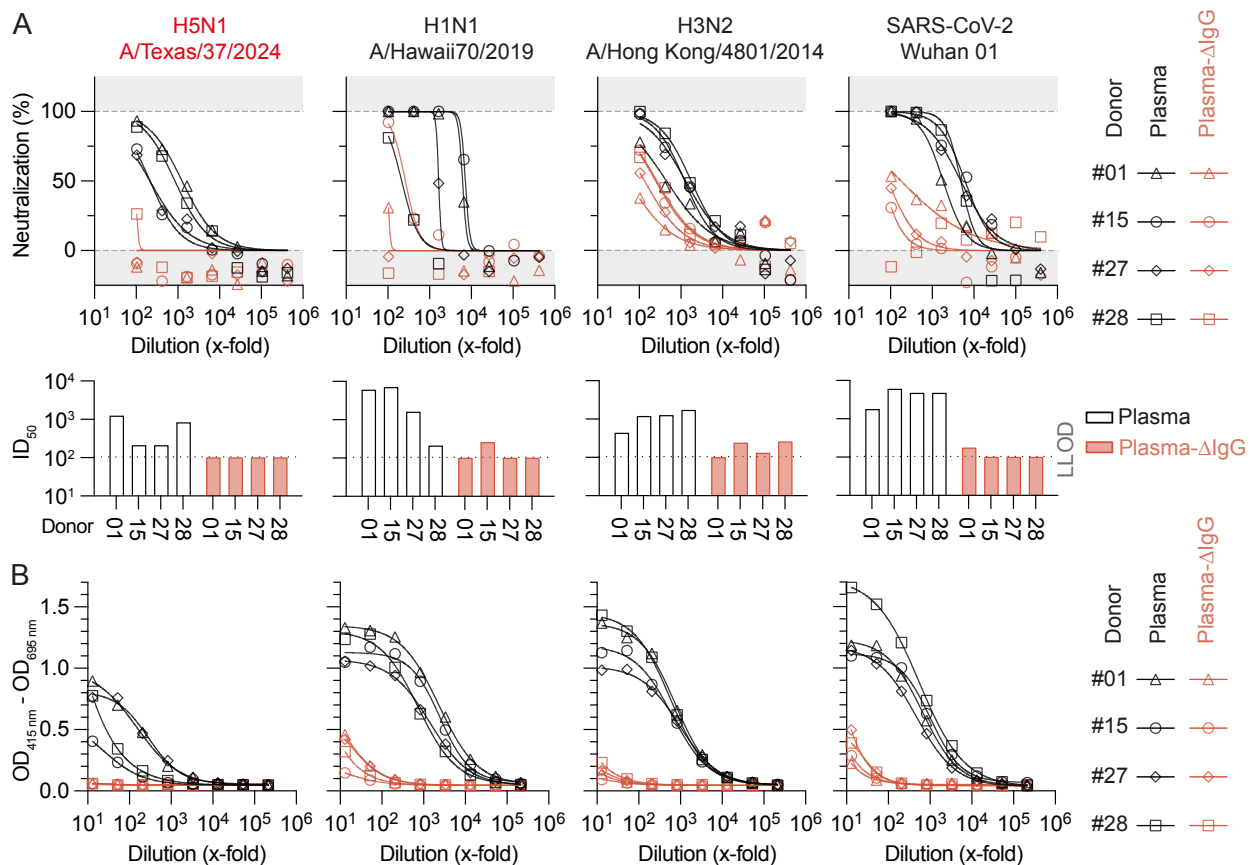


Figure 19. Neutralization and binding before and after IgG depletion from plasma. (A) Plasma samples from $n=4$ individuals (Donors #01, 15, 27, and 28) were depleted from IgG via protein G-based affinity chromatography. Pseudovirus neutralization of untreated and poly-IgG depleted plasma samples was measured against the indicated influenza strains or a SARS-CoV-2 (Wuhan 01) pseudovirus. In the upper panel, symbols represent normalized neutralization from $n=2$ technical replicates per samples and lines show non-linear curve fits. In the lower panel, bars show half-maximal inhibitory dilutions (ID_{50}). The lower limit of detection (LLOD) was a 100-fold dilution. (B) Binding activity of untreated and poly-IgG depleted plasma samples was assessed by ELISA against the HA proteins of the indicated influenza strains or SARS-CoV-2 spike protein. Symbols represent $OD_{415 nm} - OD_{695 nm}$ values from $n=2$ technical replicates and lines show non-linear curve fits. Figure adapted from Daniel et al., 2026.

Following IgG depletion, H5-neutralizing activity was almost completely abolished with IC_{50} values below the LLOD for all four samples (Figure 19A). Consistent with this observation, H5 binding was no longer detectable after depletion (Figure 19B). For H1, H3 and SARS-CoV-2, IgG depletion also resulted in a pronounced reduction in both neutralization and binding, although residual activity remained detectable in some samples. In conclusion, these results demonstrated that IgG antibodies are the primary mediators of the observed binding and neutralizing activity in serum and plasma samples.

3.3.2 HA binding and neutralization is mediated by HA-reactive antibodies

To further confirm that the observed plasma and serum activity is mediated by HA-specific antibodies, purified poly-IgG samples from the same four individuals were depleted of HA-reactive antibodies by affinity chromatography. Binding and neutralization of untreated poly-IgG and poly-IgG depleted of HA-reactive antibodies were compared by ELISA and pseudovirus neutralization assays against the previously analyzed H5N1, H1N1, and H3N2 strains, as well as SARS-CoV-2.

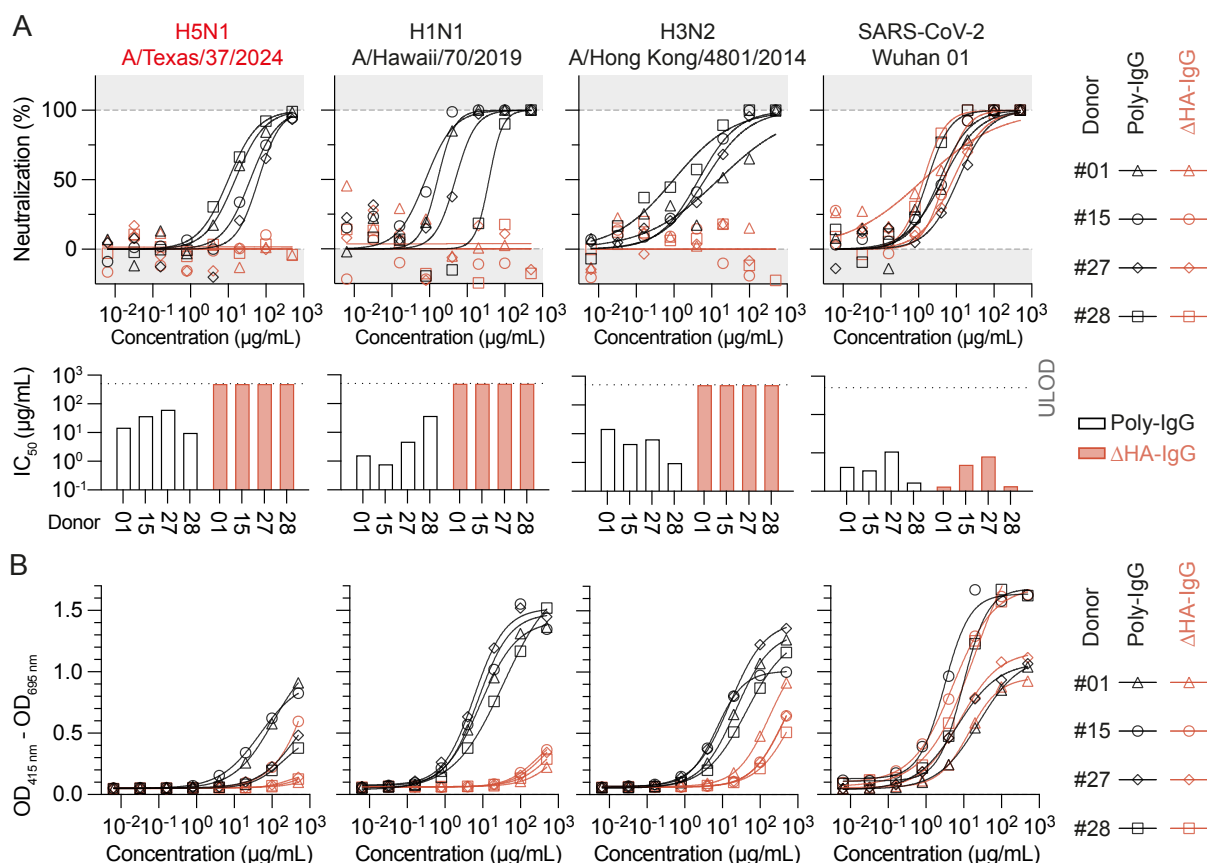


Figure 20. Neutralization and binding of poly-IgG before and after depletion of HA-reactive antibodies. (A) Poly-IgG samples from $n=4$ individuals (Donors #01, 15, 27, and 28) were depleted of HA-reactive antibodies via Step-Tactin-based affinity chromatography. Pseudovirus neutralization of untreated poly-IgG samples and poly-IgG depleted from HA-reactive antibodies ($\Delta\text{HA-IgG}$) was measured against the indicated influenza strains or a SARS-CoV-2 (Wuhan 01) pseudovirus. In the upper panel, symbols represent normalized neutralization from $n=2$ technical replicates per samples and lines show non-linear curve fits. In the lower panel, bars show half-maximal inhibitory concentrations (IC_{50}). The upper limit of detection (ULOD) was a $500 \mu\text{g/mL}$. (B) Binding activity of untreated poly-IgG samples and poly-IgG depleted from HA-reactive antibodies ($\Delta\text{HA-IgG}$) was assessed by ELISA against the HA proteins of the indicated influenza strains or SARS-CoV-2 spike protein. Symbols represent $\text{OD}_{415\text{ nm}} - \text{OD}_{695\text{ nm}}$ values from $n=2$ technical replicates and lines show non-linear curve fits. Figure adapted from Daniel et al., 2026.

Depletion of HA-reactive antibodies resulted in a complete loss of neutralizing activity against H5N1, H1N1, and H3N2, with IC_{50} values exceeding the ULOD of $500 \mu\text{g/mL}$ in all samples (Figure 20A). Binding activity was also strongly reduced for all three subtypes, particularly H5N1 and H1N1, however, low levels of binding activity remained detectable in most samples (Figure 20B). In contrast to depletion of total IgG from plasma, depletion of only HA-reactive antibodies did not affect SARS-CoV-2 neutralization and binding. Together, these results

demonstrate that HA-specific IgG antibodies are responsible for the observed H5-specific binding and neutralization in plasma and serum from H5N1-naïve individuals.

3.3.3 Vaccination and age but not sex affect serum neutralization

Next, the influence of sex, birth year, and influenza vaccination history on the observed serum neutralization was analyzed. Neutralization titers against the H5N1 A/Texas/37/2024 strain, as well as the seasonal H1N1 and H3N2 control strains (see Figure 17D), were compared between male and female participants, vaccinated and unvaccinated individuals, and evaluated in relation to the participant's birth year.

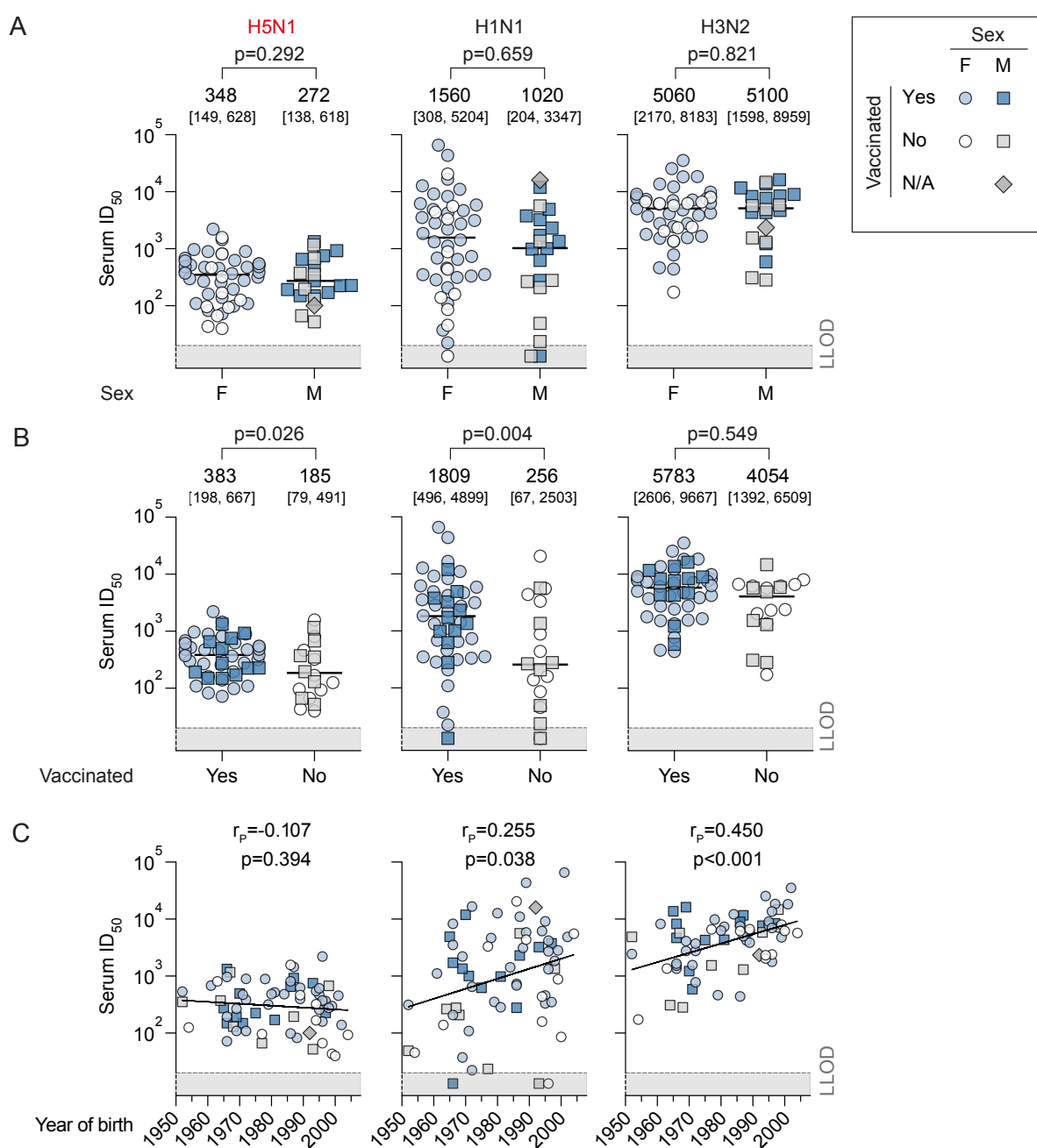


Figure 21. Impact of sex, vaccination status and year of birth on neutralization of A/Texas/37/2024 and seasonal influenza strains. (A) Comparison of serum neutralization, expressed as half-maximal inhibitory dilutions (ID_{50}), of the 45

female and 21 male individuals of the H5N1-naive cohort against pseudoviruses of the influenza strains A/Texas/37/2024 (H5N1), A/Hawaii/70/2019 (H1N1), and A/Hong Kong/4801/2014 (H3N2). (B) Comparison of serum neutralization of vaccinated (yes, n=46) and unvaccinated (no, n=19) participants of the cohort determined against the strains from (A). Vaccination status of previous influenza vaccination was self-reported, however, timepoint and frequency of vaccinations were not documented. In (A)-(B), the error-corrected median (horizontal bar) is reported on top of each graph with the 25th and 75th percentile indicated in brackets. Statistical significance was evaluated using a two-sided Fisher permutation test with 1,000 permutations. P values were calculated assuming that both groups follow the same underlying distribution and describe the probability of observing differences in the median. (C) Correlation of log₁₀-transformed ID₅₀ values of all 66 H5N1-naive individuals with their year of birth. Pearson correlation coefficients (r_p) and the associated p-values are indicated in the figure. The line corresponds to a linear regression calculated for the dataset. In (A)-(C) the lower limit of detection (LLOD, dashed line) was a 20-fold serum dilution. Data points under the LLOD were assigned a fixed value to allow visualization. Figure adapted from Daniel et al., 2026.

No significant differences in serum neutralization were observed between male and female participants for any of the strains (Figure 21A). Neutralization titers tended to be higher in vaccinated individuals across all three strains (Figure 21B). This difference was significant for H1N1, with a 7.1-fold higher titer compared with unvaccinated participants (two-sided Fisher permutation test, $p=0.004$). For H5N1, a modest but significant 2.1-fold increase was observed in the vaccinated group ($p=0.026$). For H3N2, mean ID₅₀ values were only slightly higher in vaccinated participants (5,783) compared with unvaccinated participants (4,054). Serum neutralization correlated with participant's birth year for H1N1 ($r_p=0.255$, $p=0.038$) and H3N2 ($r_p=0.450$, $p<0.001$), indicating that younger individuals tended to have higher neutralizing activity against these seasonal influenza strains (Figure 21C). In contrast, no significant age-dependent effect was observed for the H5N1 variant. Overall, vaccination status and age influenced serum neutralization in a strain-dependent manner, whereas no effect of sex was observed.

3.4 Ranking of H5 serum neutralization

To place the widespread detection of H5-reactive antibodies in H5N1-naive individuals into a broader context and provide a framework for evaluating its magnitude, neutralization activity was compared across different human and avian influenza strains and serum sources.

3.4.1 H5 serum neutralization is lower in H5-naive individuals than in infected cows

First, H5 serum neutralization in H5N1-naive individuals was compared with serum neutralization in cows experimentally infected with H5N1 clade 2.3.4.4b viruses. The animals had been infected in the framework of a study by our collaboration partners from the Friedrich-Loeffler-Institute with either an H5N1 isolate obtained from a dairy cow during the US outbreak (A/dairy cow/Texas/063224-24-1/ 2024, genotype B3.13) or with DE23-11-N1.3 euDG (A/wild goose/Germany-NW/2024AI02730/2024), a German isolate belonging to the same clade (Halwe et al., 2025). The B3.13 virus had an HA sequence identical to that of the human A/Texas/37/2024 isolate, whereas the DE23-11-N1.3 euDG virus differed by seven amino acid substitutions in the HA, including one located in the signal peptide, four in the head domain, and two in the stalk domain.

Sera from infected cows, as well as commercial FCS as a negative control, were first analyzed via the pseudovirus neutralization assay against A/Texas/37/2024 HA (Figure 22A). No H5 neutralization was detected for FCS and for sera collected from cows three days post-infection, consistent with the seronegative status at this early timepoint. In contrast, cow sera obtained at 9, 13, and 21 days post H5N1 infection (dpi) showed strong neutralizing activity with ID₅₀ values ranging from 9,843 to 116,984. In comparison, sera from H5N1-naïve humans showed markedly lower neutralization titers, with ID₅₀ values between 40 and 2,199.

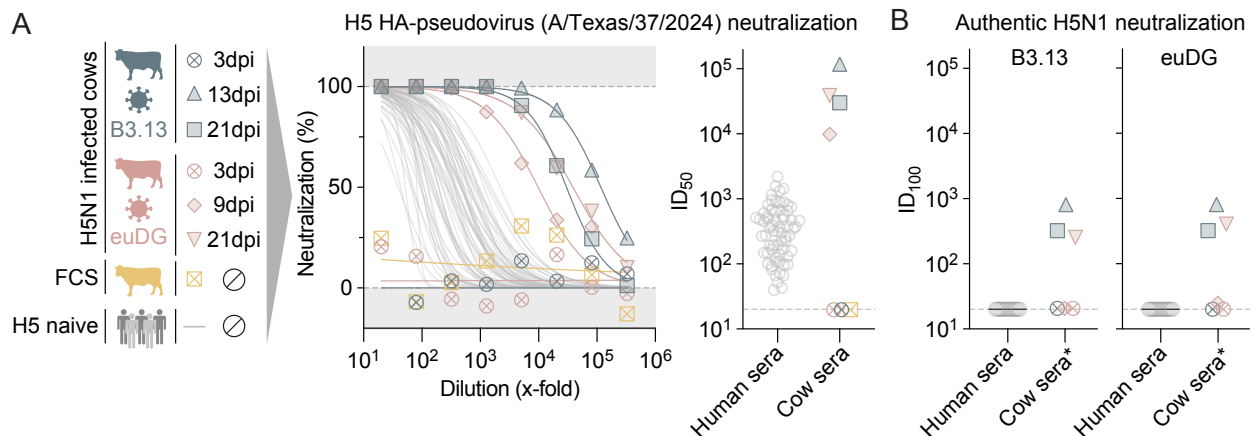


Figure 22. Comparison of H5 neutralization by sera from H5N1-naïve individuals and cows experimentally infected with H5N1 virus. (A) Neutralization of A/Texas/37/2024 HA-pseudovirus by sera from cows experimentally infected with H5N1 (clade 2.3.4.4b, genotypes B3.13 or DE23-11-N1.3 euDG [euDG]), commercial fetal calf serum (FCS), and sera of n=66 H5N1-naïve individuals. Symbols represent normalized neutralization from n=2 technical replicates per sample and lines show non-linear curve fits. In the right panel, half-maximal inhibitory dilutions (ID₅₀) values are shown for all human and cow sera. **(B)** Authentic virus neutralization, expressed as complete inhibitory dilution (ID₁₀₀), against H5N1 B3.13 and euDG for sera from infected cows from (A) and a subset of n=20 sera from H5N1-naïve individuals. Figure taken from Daniel et al., 2026.

Next, cow sera and sera from a subset of 20 H5N1-naïve individuals were compared in an authentic virus neutralization assay against the two H5N1 isolates with which the cows had been infected (genotype B3.13 (A/dairy cow/Texas/063224-24-1/2024) and DE23-11-N1.3 euDG (A/wild goose/Germany-NW/2024A102730/2024)). As observed in the pseudovirus assay, sera from seronegative cows collected 3 days post-infection showed no neutralizing activity (Figure 22B). Serum obtained from a euDG-infected cow at 9 days post-infection showed no neutralization against authentic B3.13 and only weak neutralization against euDG, with an ID₁₀₀ of 25. In contrast, the three remaining cow sera displayed potent neutralization of both authentic viruses, with ID₁₀₀ values ranging from 323 to 813. Overall, ID₁₀₀ values measured in the authentic virus assay were approximately 100-fold lower than the corresponding ID₅₀ values obtained in the pseudovirus neutralization assay. Unlike for the cow sera, neutralization of authentic B3.13 or euDG virus could not be detected for any of the human serum samples. These results indicate that neutralizing activity in sera from H5N1-naïve individuals is markedly lower than in sera from experimentally infected cows.

3.4.2 A/Texas/37/2024 serum neutralization is comparable to other H5 strains but lower than against H1 and H3

Next, the observed A/Texas/37/2024 serum neutralization was placed into a broader context by comparing it with the serum reactivity against a large panel of IAV strains representing both seasonal and avian subtypes. To guide strain selection, a phylogenetic tree was generated from 83,118 HA sequences of human and animal origin, covering influenza A subtypes H1 to H18 (Figure 23A). Based on this analysis, a panel of 77 influenza strains was selected for the comparative analysis, including the human subtypes H1, H2, and H3 as well as the avian subtypes H5, H7 and H9, most frequently linked to zoonotic spillover events. Within each subtype, strains were selected based on genetic diversity, global prevalence, and geographic distribution. Preference was given to antigenically distinct clades and to reference, vaccine, or human-derived isolates (see chapter 2.2.3.2).

Pseudoviruses were generated for all 77 selected strains and serum from the previously defined subset of 20 study participants was tested for neutralization against the complete panel (Figure 23B). Neutralizing activity was detected in the serum from most individuals against the majority of tested strains, with ID₅₀ values ranging from below the LLOD at a 1:20 serum dilution up to an ID₅₀ of 111,102. For each strain, the geometric mean ID₅₀ across all 20 individuals was calculated and is plotted in Figure 23C. Mean neutralization titers were then grouped according to HA subtype (Figure 23D). H1N1 strains were analyzed separately for viruses circulating before and after the 2009 pandemic, as the H1pdm09 lineage that emerged in 2009 is antigenically distinct from previously circulating H1N1 viruses.

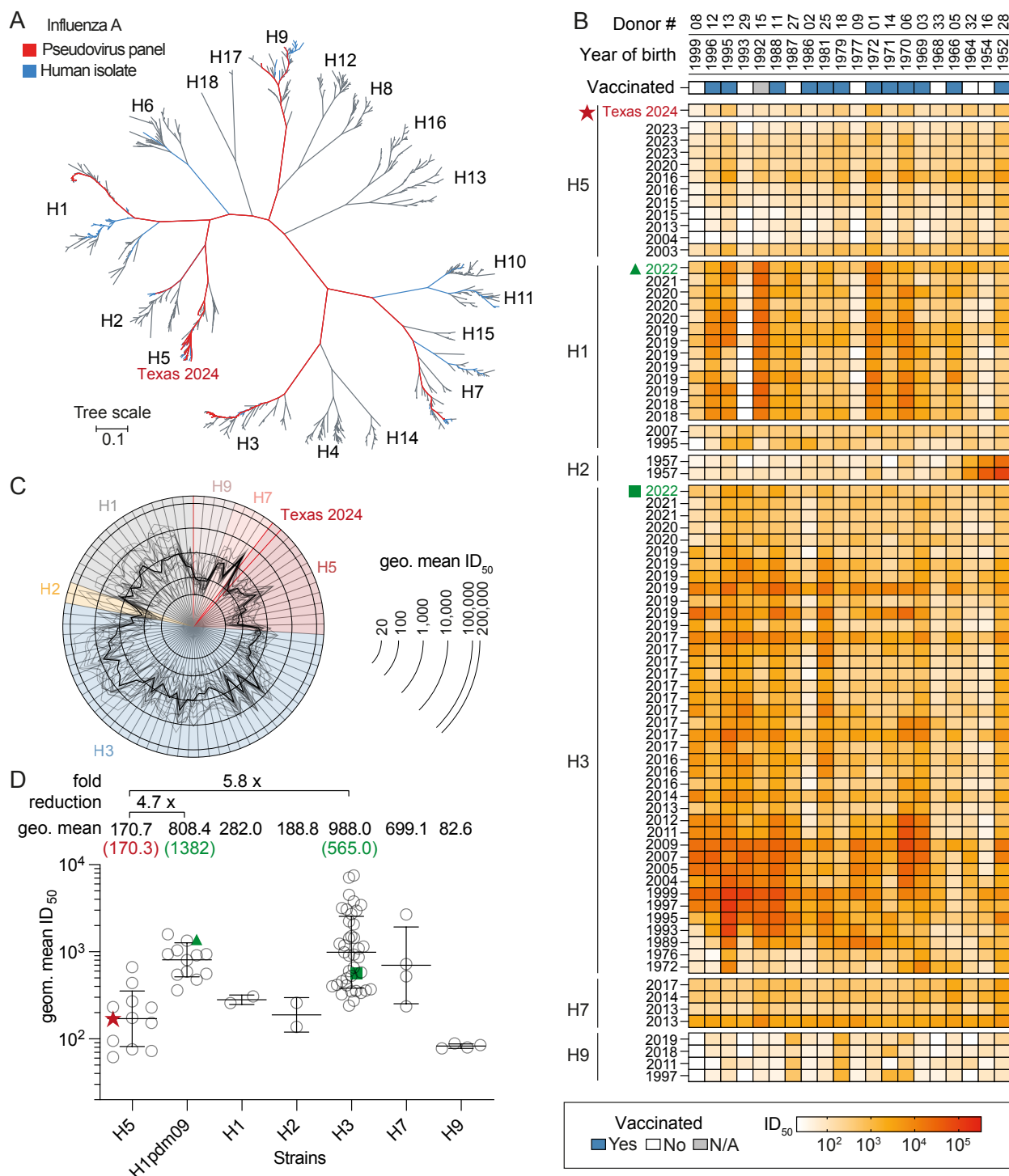


Figure 23. Ranking of pseudovirus neutralization for A/Texas/37/2024 against a panel of 76 seasonal and avian influenza strains. (A) Phylogenetic tree created from 83,118 HA sequences of the subtypes H1 to H18. The 77 strains of the subtypes H1, H2, H3, H5, H7, and H9 that were selected for the representative pseudovirus panel are highlighted in red. Human isolates are highlighted in blue. (B) Half-maximal inhibitory dilutions (ID₅₀) determined by pseudovirus neutralization assay for a subpanel of 20 sera from H5N1-naïve individuals against the representative panel of 77 influenza strains highlighted in (A). A/Texas/37/2024 is marked by a red star, whereas the most recent H1 and H3 strains are marked by a green triangle and square, respectively. (C) Geometric mean ID₅₀ values were calculated for each strain in (B) across all 20 individuals (for each row in (B)) and are indicated by the black line, with each segment of the pie chart corresponding to one influenza strain. Light grey lines indicate ID₅₀ values for the individual donors. (D) Comparison of geometric mean ID₅₀ values grouped by HA subtype. Each symbol corresponds to the geometric mean as calculated in (C) for one influenza strain from (B). A/Texas/37/2024 is marked by a red star, while the most recent H1 and H3 strains are marked by a green triangle and square, respectively. The corresponding geometric mean ID₅₀ values are given above the graph in matching colors. Horizontal lines show the average geometric mean for each subtype (values given above the graph) and error bars indicate geometric standard deviation. Figure taken from Daniel et al., 2026.

The highest geometric mean neutralization at the subtype level was observed for the H3N2 strains (988.0), whereas the lowest level was detected for the group of H9 strains (82.6; Figure 23D). For the 12 tested H5N1 strains, geometric mean ID₅₀ values ranged from 61.7 to 665.7, resulting in a group mean of 170.7. Neutralization of A/Texas/37/2024 was therefore similar to the overall mean of the H5 group and comparable to titers observed for H2N2 viruses. Compared with H5, significantly higher neutralization was measured against the seasonal subtypes H1pdm09 (4.7-fold increase, $p=0.003$) and H3N2 (5.8-fold increase, $p<0.001$), as determined by Kruskal-Wallis test with Dunn's correction for multiple comparisons.

Building on the analysis in chapter 3.3.3, the large serum neutralization dataset further allowed to extend the analysis of the effects of birth year and prior influenza vaccination from individual strains to influenza subtypes. To assess the effect of both parameters, geometric mean ID₅₀ values were additionally calculated for each individual for the tested strains of each subtype separately. Comparison between vaccinated and unvaccinated individuals revealed significantly higher neutralization levels against H1 viruses in participants who reported previous influenza vaccination (Figure 24A). For the other tested subtypes, no significant differences were observed between vaccinated and unvaccinated individuals, although titers tended to be higher in vaccinated individuals across H3, H5, and H9 strains. To better visualize the effect of participants' year of birth, the calculated geometric mean ID₅₀ values of each individual for strains of each subtype were sorted by birth year (Figure 24B). Neutralization against subtypes H2 and H3 showed a clear association with participants' year of birth. H2N2 mean neutralization was remarkably high for the three individuals born between 1952 and 1964, corresponding to the period shortly before or during circulation of H2N2 viruses in the human population (1957–1968). Interestingly, two of those individuals displayed very low H3 neutralization in comparison to the other study participants. In contrast, higher H3N2 neutralization was observed predominantly in younger individuals, with the strongest responses detected for individuals born in the 1990s against H3 strains circulating during that decade (see Figure 23B).

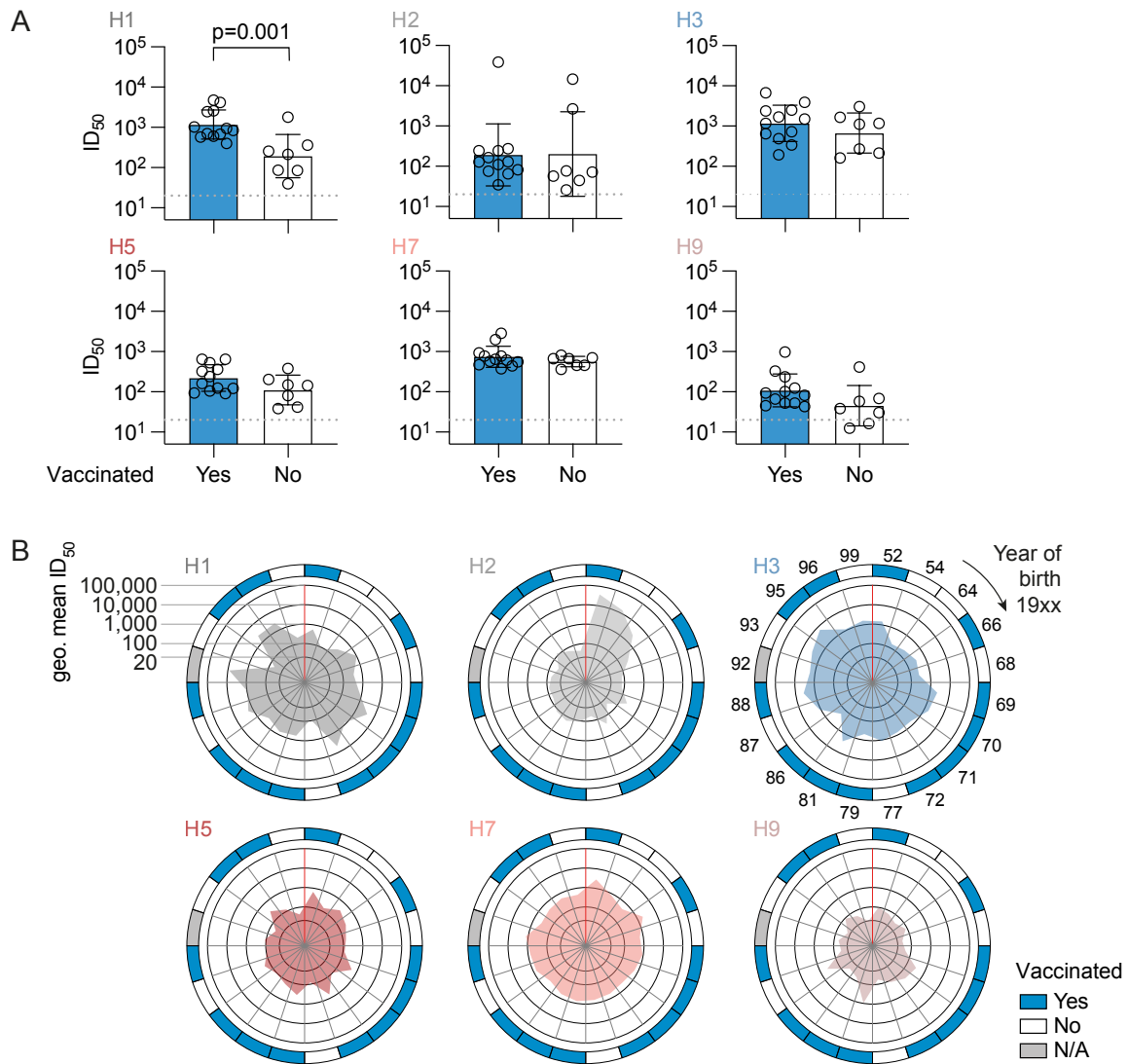


Figure 24. Impact of influenza vaccination and year of birth on neutralization of pseudoviruses from seasonal and avian subtypes. (A) Geometric mean half-maximal inhibitory dilution (ID_{50}) values were calculated from data in Figure 23B for each of the 20 individuals for all strains of a certain subtype separately. Symbols represent geometric mean ID_{50} values of vaccinated (yes, $n=12$) and unvaccinated (no, $n=7$) individuals. Vaccination status of previous influenza vaccination was self-reported, however, timepoint and frequency of vaccinations were not documented. Bars show geometric mean values and geometric standard deviation. One individual with unknown vaccination status (N/A) was not included in the analysis. ID_{50} values were set to 10 if calculated values were <10 or curve fitting failed. P values, determined by unpaired two-tailed t-test are indicated if ≤ 0.05 . (B) Geometric mean ID_{50} values calculated in (A) arranged clockwise according to the individuals' year of birth. Each segment of the pie chart corresponds to one individual. Figure adapted from Daniel et al., 2026.

In summary, the comparative analysis of serum neutralization against the panel of 77 different strains showed that A/Texas/37/2024 neutralization fell within the range observed for other H5 variants. However, it was clearly lower than neutralization activity against seasonal subtypes. Moreover, neutralization against H2 and H3 was influenced by the participants' year of birth, suggesting an association with prior influenza exposure history.

3.5 Isolation of A/Texas/37/2024-specific single B cells from H5-naïve individuals

Following the detailed analysis of serum and plasma antibody responses to A/Texas/37/2024 in H5N1-naïve individuals, the humoral immune response was further investigated at the level of single B cells.

3.5.1 H5-naïve individuals carry H5-reactive memory B cells

For antigen-specific B cell sorting, a recombinant HA protein of A/Texas/37/2024 modified by introduction of the Y98F mutation was used as bait. This mutation has previously been described to reduce sialic acid binding (Martín et al., 1998) and has been used in HA-specific B cell sorting to minimize non-specific binding of the HA bait to sialic acids present on the surface of B cells (Whittle et al., 2014). To further improve specificity, a dual-color staining strategy using differently labeled HA proteins (DyLight 488 and DyLight 650) was applied (see chapter 2.2.6.1).

Single B cell analysis was performed for six H5N1-naïve individuals (Donors #08, 09, 10, 20, 47, and 48) with A/Texas/37/2024 ID₅₀ values between 43 and 2199, covering the broad range of serum neutralization titers observed in the cohort. In addition, one individual previously vaccinated with an experimental H5N1 vaccine in 2008/2009 (clade 1, A/Vietnam/1203/2004; ID₅₀=2343) was included for comparison. H5-specific IgG⁺ B cells were identified as double-positive for A/Texas/37/2024(Y98F) HA Δ TM based on the gating strategy shown in Figure 25.

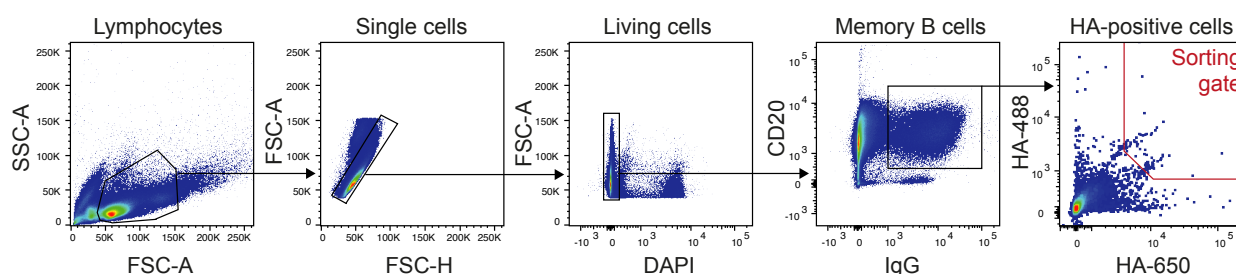


Figure 25. Gating strategy for the sorting of A/Texas/37/2024(Y98F) HA-reactive memory B cells. Antigen specific memory B cells recognizing H5N1 A/Texas/37/2024(Y98F) HA Δ TM were isolated from CD19-enriched PBMCs by flow cytometry. Lymphocytes were first gated based on forward and side scatter area (FSC-A vs. SSC-A) and doublets excluded by forward scatter height versus area discrimination (FSC-H vs. FSC-A). Dead cells were removed by excluding DAPI-positive cells. CD20⁺ IgG⁺ B cells were then gated and antigen specific cells identified as double-positive for H5N1 A/Texas/37/2024(Y98F) HA Δ TM labeled with DyLight 488 or DyLight 650. Figure taken from Daniel et al., 2026.

The highest frequency of H5-specific B cells was observed in the H5-vaccinated individual, where cells double positive for HA represented 0.96% of total IgG⁺ B cells (Figure 26A). In H5N1-naïve individuals, frequencies ranged between 0.11% and 0.43%. Sorted cells were subsequently analyzed by BCR reverse transcription PCR (RT-PCR) followed by Sanger sequencing and sequence analysis using the antibody repertoire analysis toolkit (AbRAT). After processing of 979 B cells and subsequent quality filtering, between 10 and 174 functional IgG heavy chain sequences were obtained per individual, resulting in a total of 494 heavy chain sequences (Figure 26B).

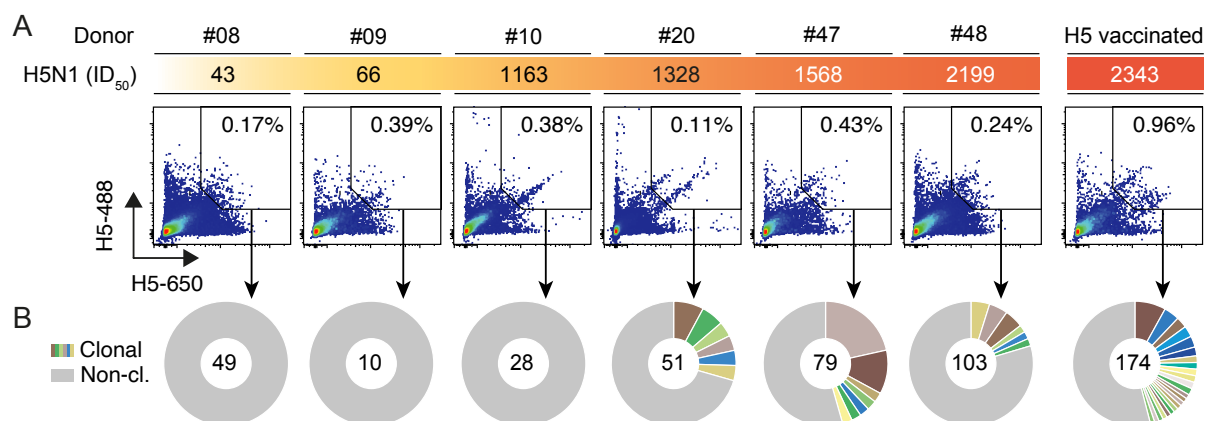


Figure 26. Clonal analysis of H5-reactive memory B cells isolated from H5N1-naïve individuals and one H5-vaccinated individual. (A) Final sorting gates of IgG⁺ CD20⁺ B cells double-positive for H5N1 A/Texas/37/2024(Y98F) HAΔTM labeled with DyLight 488 or DyLight 650 for 6 H5N1-naïve individuals and one H5-vaccinated individual. Percentages shown in the gates represent the fraction of H5 double-positive cells among the IgG⁺ B cell population. Neutralization titers against A/Texas/37/2024 pseudovirus are depicted above the plots as half-maximal inhibitory dilutions (ID₅₀). **(B)** Clonal analysis of H5-reactive B cells sorted from individual donors. The total count of evaluated sequences per donor is displayed in the center of the chart. Figure taken from Daniel et al., 2026.

Clonal relationships between B cells were inferred based on sequence similarity, revealing clonally related memory B cells in four out of the seven individuals. In the H5-naïve donors #20, #47, and #48, only 6–7 B cell clones comprising 2–17 sequences were identified. The H5-vaccinated individual showed 23 distinct clones, with clone sizes between 2 and 14 sequences, indicating a more expanded and diverse response. Overall, these results demonstrate that H5-reactive memory B cells can be isolated from H5N1-naïve individuals.

3.5.2 Isolated B cells encode for H5-neutralizing antibodies that cross-react with H1

Of the 494 amplified functional BCR sequences, between 3 and 33 mAbs per individual were recombinantly expressed, resulting in a total of 136 mAbs originating from both clonal and non-clonal B cells (Figure 27A). All antibodies were analyzed for binding and neutralization against H5N1 (A/Texas/37/2024), H1N1 (A/Hawaii/70/2019), and H3N2 (A/Hong Kong/4801/2014) by ELISA, cell-based binding assay, and pseudovirus neutralization assay.

Of the 136 generated mAbs, 55 reacted with H5 in at least one of the three assays, with 45 showing H5 pseudovirus neutralization (Figure 27B-D). These H5-reactive antibodies originated from five of the six H5N1-naïve individuals and from the H5N1-vaccinated individual. No HA-reactive mAbs were found for Donor #09, however, only three antibodies were tested from this individual. With 18 out of 30 antibodies reacting to H5, frequency of H5-reactive antibodies was highest in the H5-vaccinated individual. In the H5-naïve individuals, 1 to 14 mAbs reacted with H5 in one of the assays. Because the number of mAbs produced per donor differed, direct comparison of frequencies between individuals and vaccination groups is limited.

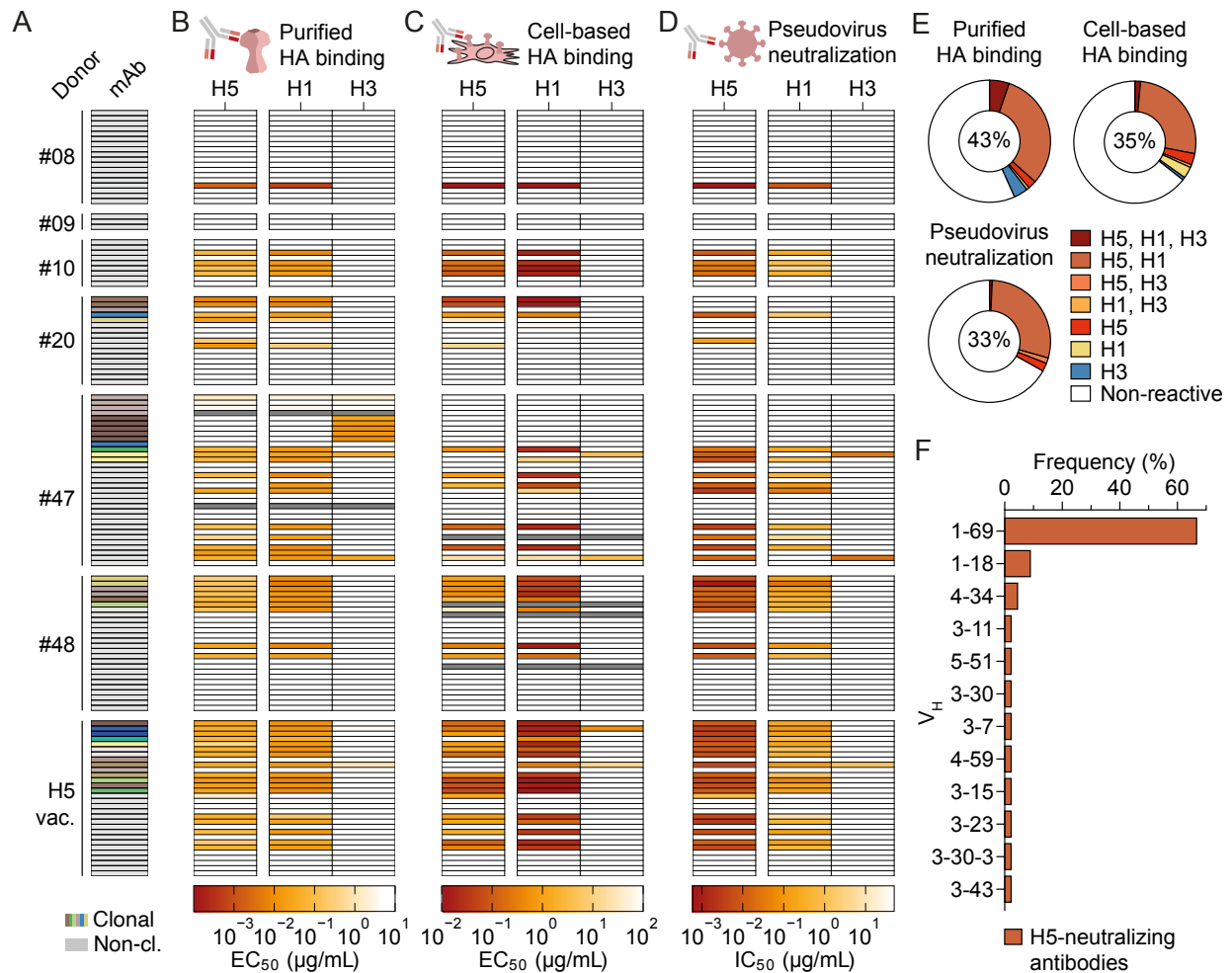


Figure 27. Binding and neutralization of H5-reactive antibodies from H5N1-naïve individuals and one H5-vaccinated individual against H5, H1, and H3. A total of 136 antibodies from the individuals and B cells presented in Figure 26, were tested for their binding and neutralizing reactivity against A/Texas/373/2024 (H5), A/Hawaii/70/2019 (H1), and A/Hong Kong/4801/2014 (H3) HA. Clonal relationships between tested antibodies are shown in (A). Heatmaps indicate half-maximal effective concentrations (EC₅₀) measured by IgG-ELISA (B) and cell-based binding (C) or half-maximal inhibitory concentrations (IC₅₀) determined by pseudovirus neutralization assay (D). (E) Antibody reactivity across the three assay systems and strains in (B)–(D) is summarized in pie charts. Percentages shown in the charts indicate the proportion of HA-reactive antibodies. (F) Heavy chain variable (V_H) gene segment usage among the H5-neutralizing antibodies. Figure taken from Daniel et al., 2026.

Most H5-reactive antibodies cross-reacted with the group 1 H1N1 variant, with 90% binding H1 in ELISA, 92% in the cell-based binding assay and 89% neutralizing H1 pseudovirus. In contrast, only 5%, 13%, and 7% of H5-reactive antibodies showed cross-reactivity with the group 2 H3N2 variant in the respective assays. Across the three assays, antibody reactivity patterns were largely consistent, with 43% of antibodies binding HA in ELISA, 35% in the cell-based assay and 33% showing neutralization in the pseudovirus assay (Figure 27E). Further sequence analysis showed that V_H 1-69 was the dominant HC V gene segment, representing 67% of all H5-neutralizing antibodies (Figure 27F). V_H 1-18 and V_H 4-34 were the next most frequently utilized segments, with 9% and 4%, respectively. These findings indicated that H5-reactive antibodies are present in both H5N1-naïve and vaccinated individuals. Most antibodies cross-reacted with H1 but rarely with H3 and V_H 1-69 dominated the heavy chain usage among H5-neutralizing antibodies.

3.6 Characterization of H5-reactive antibodies isolated from H5-naive individuals

In the next step, the 45 H5 pseudovirus-neutralizing antibodies isolated from H5N1-naive and vaccinated individuals were characterized in more detail. Their neutralization potency, epitope specificity and capacity to confer protection *in vivo* were analyzed to better understand the H5-reactive antibody response identified in the previous experiments.

3.6.1 H5-reactive antibodies from H5-naive individuals are highly potent

First, the neutralization potency of the isolated antibodies was assessed by comparison with well-characterized HA-reactive antibodies. To this end, a panel of nine HA-reactive reference antibodies was selected, some of which had already been used during the establishment and validation of the pseudovirus neutralization assay and recombinant HA protein production (see chapters 3.1.3 and 3.2.2). The panel included broadly reactive antibodies recognizing both group 1 and group 2 HA subtypes (MEDI8852, 39.29, and CR9114), as well as group- or subtype-specific antibodies (CR6261, CR8020, F045-92, 100F4, H5.3, and 5J8). A SARS-CoV-2-specific antibody was included as negative control. Five antibodies in the panel (MEDI8852, 39.29, CR9114, CR6261, and CR8020) have previously been evaluated in clinical studies, highlighting their potent and well-characterized antiviral activity (Ali et al., 2018; Beukenhorst et al., 2026; Han et al., 2021; Lim et al., 2022, 2020; Tharakaraman et al., 2014).

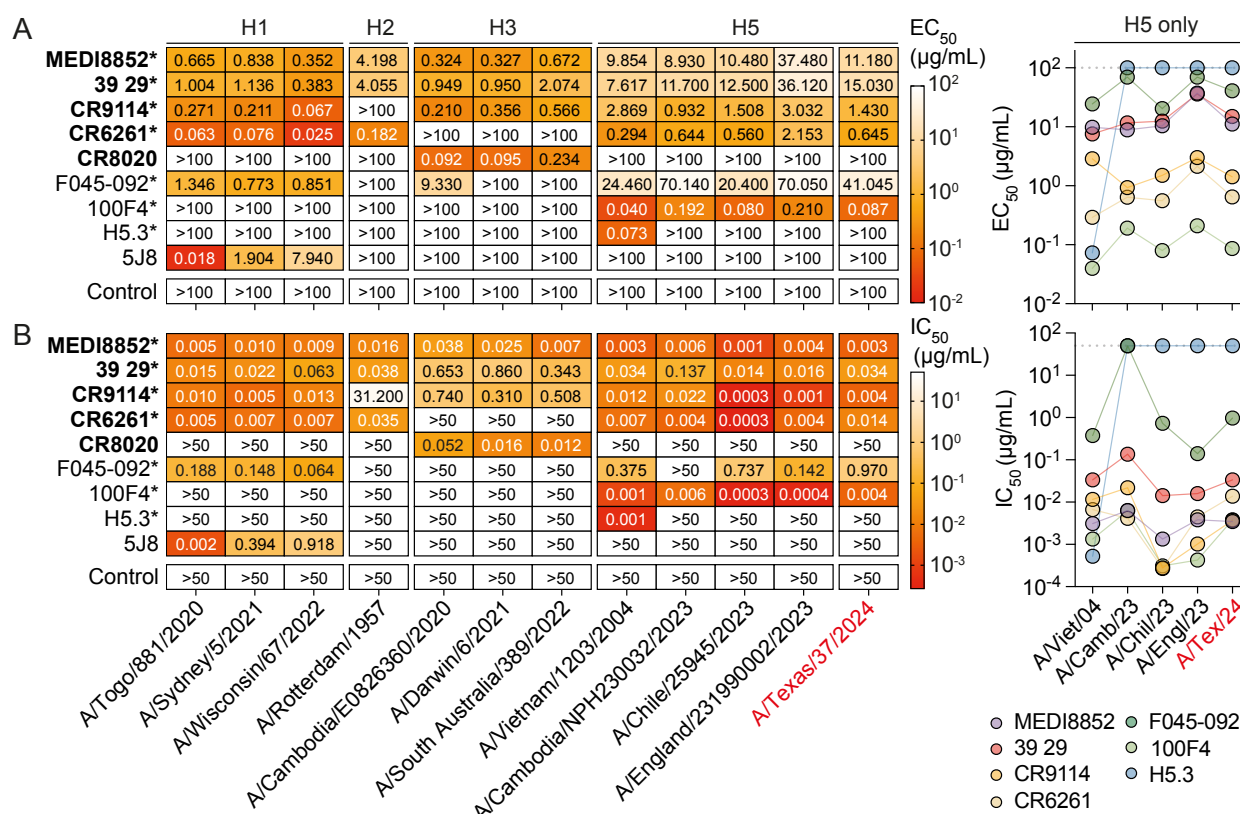
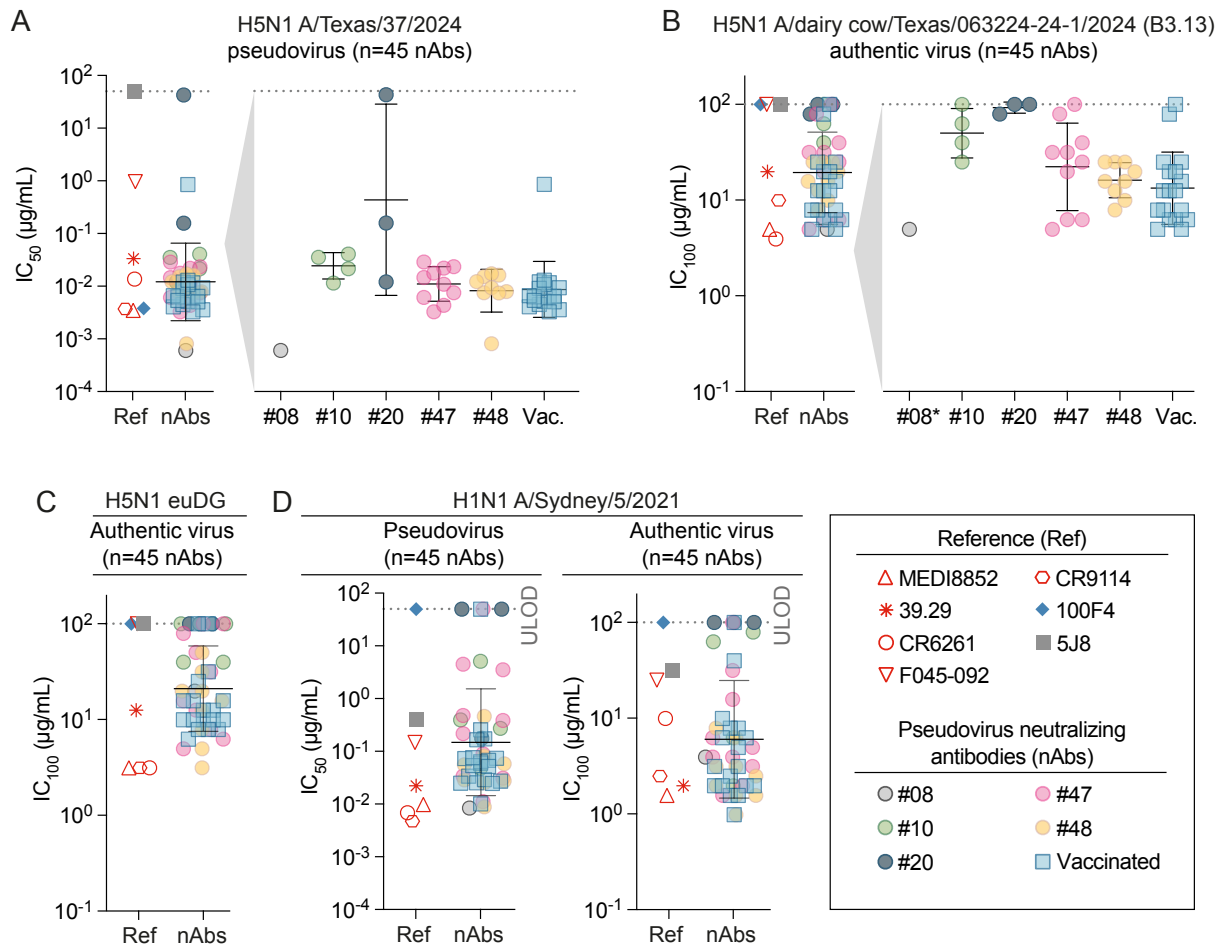


Figure 28. Binding and neutralization of reference antibodies against H1, H2, H3, and H5 strains. Heatmaps showing binding and neutralization for nine HA-reference antibodies and a SARS-CoV-2 antibody (CnC2t1p1_D6, Control) against the indicated influenza strains of the subtypes H1, H2, H3, and H5. In panel (A), half-maximal effective concentrations (EC₅₀) were measured by cell-based binding assay. In panel (B), half-maximal inhibitory concentrations (IC₅₀) were determined by pseudovirus neutralization assay. Dot plots on the right compare EC₅₀/IC₅₀ values between the seven H5-reactive reference antibodies for the tested H5 strains. Bold font marks antibodies evaluated in clinical trials. * marks antibodies previously described as H5-reactive. Figure taken from Daniel et al., 2026.

Cell-based binding and pseudovirus neutralization were evaluated for the reference antibody panel against A/Texas/37/2024 as well as a set of additional group 1 and group 2 influenza strains, including four H5N1, three H1N1, one H2N2, and three H3N2 viruses. Of the seven antibodies previously reported to neutralize H5 viruses (MEDI8852, 39.29, CR9114, CR6261, F045-92, 100F4, and H5.3), all except H5.3 bound to and neutralized A/Texas/37/2024 (Figure 28). Binding EC₅₀ values ranged from 0.087 to 41.05 μg/mL and neutralization IC₅₀ values between 0.003 and 0.97 μg/mL, demonstrating that A/Texas/37/2024 is recognized as effectively as older H5 strains, with no resistance to the well-characterized H5 antibodies. Reactivity against H1, H2, and H3 strains was consistent with binding and neutralization profiles based on previously reported breadth of the respective antibodies (summarized in Jiao et al., 2023). Together, these data defined a potency range for the subsequent benchmarking of H5-reactive antibodies isolated from H5N1-naïve and vaccinated individuals.

To this end, A/Texas/37/2024 neutralization potency of the 45 H5-neutralizing antibodies isolated from H5N1-naïve and vaccinated individuals was compared with that of the reference antibodies using the pseudovirus assay. Of the 45 antibodies, 43 showed IC₅₀ values within the range of the reference antibodies (Figure 29A). Only one antibody from Donor #20 displayed

weaker neutralization, while two antibodies from Donors #08 and #48 exhibited higher activity than antibodies from the reference panel, with IC_{50} values of 0.0006 and 0.0008 $\mu\text{g/mL}$, respectively.



When the same comparison was performed in an authentic virus assay against A/dairy cow/Texas/063224-24-1/2024 (B3.13) and the European variant A/wild goose/Germany-NW/2024AI02730/2024 (euDG), a similar outcome was observed (Figure 29B-C). Here, only 4 reference antibodies and between 36 and 40 of the 45 isolated H5 pseudovirus-neutralizing antibodies showed neutralization of B3.13 and euDG, in line with the lower sensitivity of the authentic virus assay. Nevertheless, the majority of antibodies displayed IC_{100} values within the range of the four neutralizing reference antibodies. An additional comparison showed that against the H1N1 strain A/Sydney/5/2021, neutralization activity of most isolated H5-neutralizing antibodies was also within the range of the reference antibodies (Figure 29D). In summary, the

majority of A/Texas/37/2024-neutralizing antibodies isolated from H5N1-naïve individuals showed high neutralization potencies comparable to clinically tested reference antibodies.

3.6.2 H5-reactive antibodies from H5-naïve individuals target the HA stem

To investigate the binding regions of the 45 H5 pseudovirus-neutralizing mAbs, competition ELISAs on recombinant A/Texas/37/2024 HA protein were performed. Antibodies were tested for competition with the two stem-reactive antibodies MEDI8852 and CR6261 and the H5 head-specific antibody 100F4. Antibodies were considered competing when the binding signal of the biotinylated reference antibody to HA was reduced by at least 10% in the presence of the test antibody.

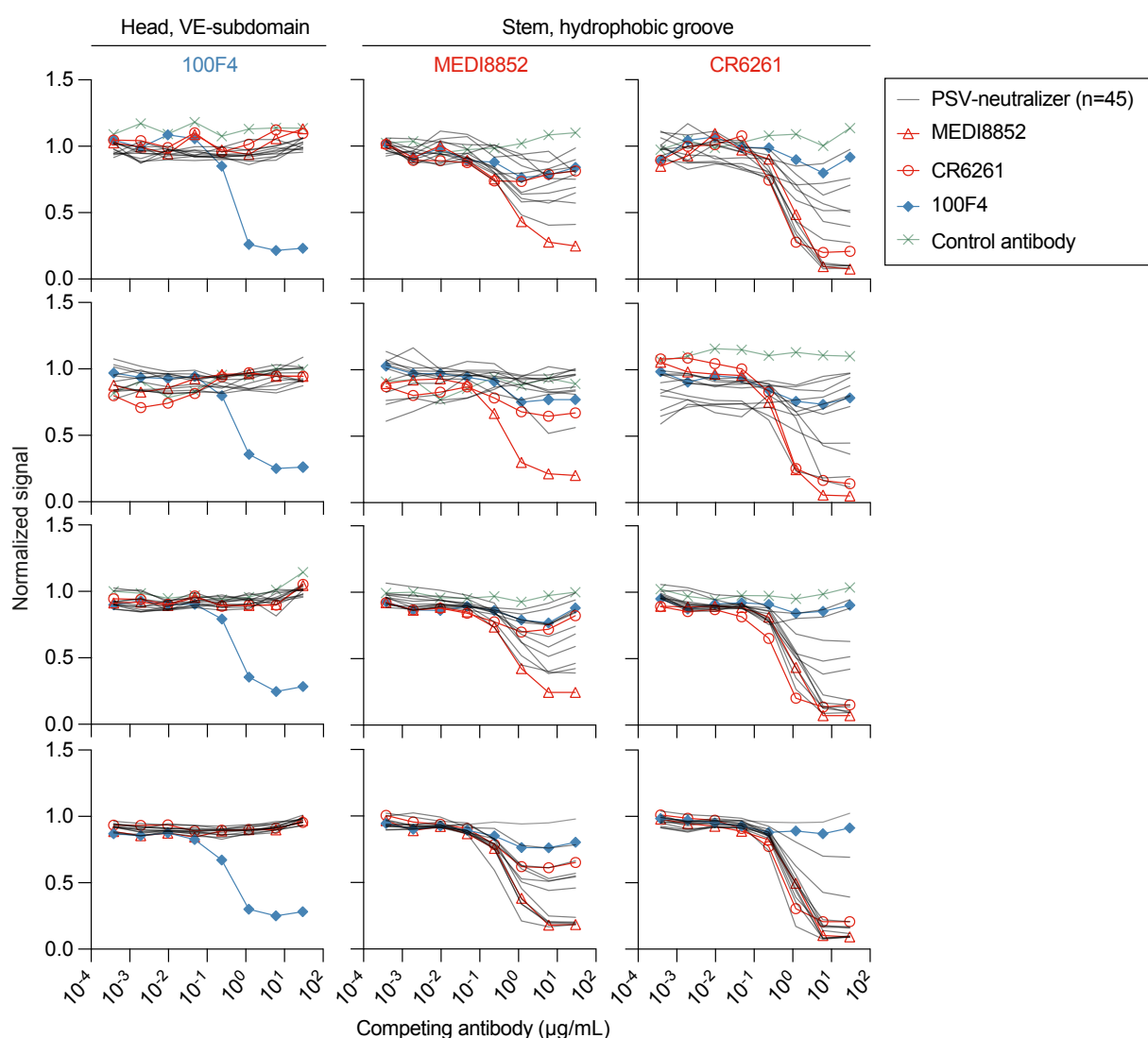


Figure 30. Binding competition of isolated antibodies with head- and stem-reactive reference antibodies. Competitive ELISA testing the 45 isolated H5 pseudovirus (PSV)-neutralizing antibodies (see Figure 27) for their ability to compete with the biotinylated reference antibodies 100F4, MEDI8852, and CR6261 for binding to HA of A/Texas/37/2024. Binding of the biotinylated antibodies was detected. Symbols represent normalized binding signals of technical duplicates. Control: SARS-CoV-2 antibody (CnC2t1p1_D6). Figure taken from Daniel et al., 2026.

Self-competition was observed for all three reference antibodies, confirming assay functionality (Figure 30). Moreover, the two stem antibodies competed with each other, consistent with overlapping stem epitopes. Of the 45 antibodies, 35 competed with both stem antibodies and four with CR6261 alone. Only one antibody showed weak competition (12%) with 100F4, although this likely does not reflect specific HA head binding, as it also competed with CR6261 (88%) and MEDI8852 (43%). Six antibodies did not compete with any of the three reference antibodies. Overall, the data suggest that most tested antibodies likely target the stem region of the HA protein.

3.6.3 H5-reactive antibodies from H5-naïve individuals protect mice from lethal H5N1 infection

As a final step to further characterize the H5-reactive antibody response, H5-neutralizing antibodies isolated from H5N1-naïve individuals were tested for their ability to protect mice from a lethal H5N1 challenge. For the analysis, two antibodies showing highly potent A/Texas/37/2025 pseudovirus neutralization (ID_{50} of 0.0008 and 0.007) from Donors #48 (48H5-1-1G9K, mAb1) and #47 (47H5-1-2B12K, mAb2) were selected. The SARS-CoV-2 antibody DZIF-10c (Halwe et al., 2021; Kreer et al., 2020b) was included as a negative control. Mice were pretreated intraperitoneally (i.p.) with 10 mg/kg of the respective antibody one day prior intranasal (i.n.) infection with a lethal dose of the bovine H5N1 virus A/dairy cow/Texas/063224-24-1/2024 (B3.13) (Figure 31A). Body weight and clinical symptoms were monitored daily for 10 days post challenge.

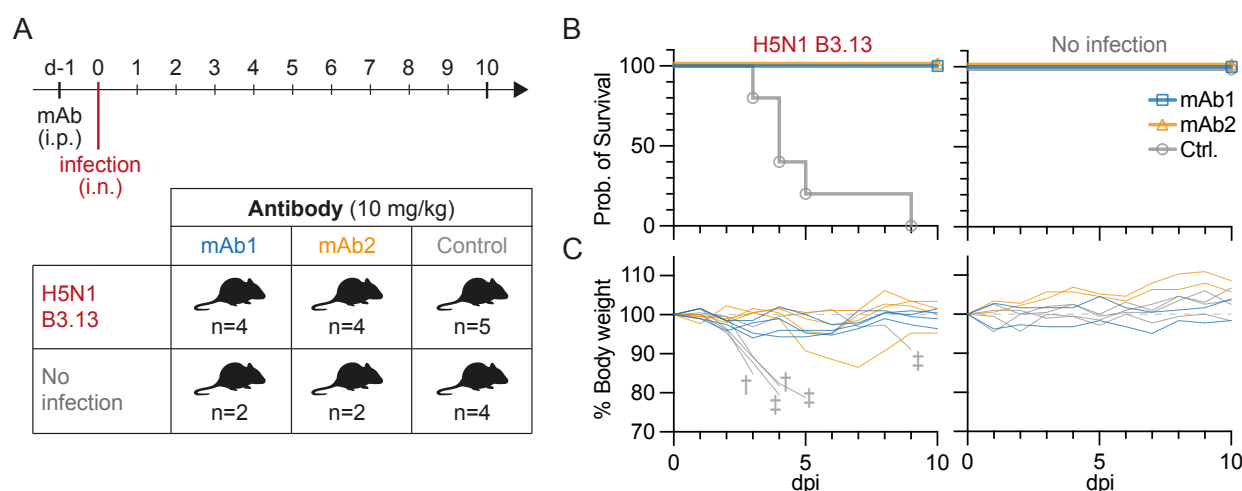


Figure 31. Evaluation of antibodies isolated from H5N1-naïve individuals in a lethal H5N1 mouse challenge model. (A) Overview of the experiment timeline and treatment groups. Mice received 10 mg/kg of either one of the H5-neutralizing antibodies from H5N1-naïve individuals 48H5-1-1G9K (mAb1) or 47H5-1-2B12K (mAb2), or the SARS-CoV-2 antibody DZIF-10c (control) by intraperitoneal injection (i.p.) one day before intranasal (i.n.) infection with a lethal dose of H5N1 (A/dairy cow/Texas/063224-24-1/2024, genotype B3.13). Mock controls received the same treatment but were not infected. (B) Probability of survival following lethal H5N1 challenge compared with non-infected controls. (C) Changes in body weight relative to day 0. † Mice died. ‡ Mice euthanized upon reaching humane endpoints, defined as a $\geq 20\%$ loss of initial body weight or severe clinical symptoms. Figure taken from Daniel et al., 2026.

All non-infected animals survived, confirming that antibody treatment caused no adverse effects (Figure 31B). In contrast, all animals receiving the SARS-CoV-2 control antibody prior to infection either died or were euthanized upon reaching humane endpoints, defined as a $\geq 20\%$ loss of initial body weight or severe clinical symptoms. Treatment with the H5-specific antibodies resulted in a complete survival of all animals after H5N1 infection. Only one of the animals treated with mAb2 lost more than 10% of its body weight. In summary, these data show that H5-neutralizing antibodies isolated from H5N1-naïve individuals confer strong protection against lethal H5N1 infection in mice.

4 Discussion

The rapid global spread of highly pathogenic avian H5N1 clade 2.3.4.4b influenza viruses and frequent outbreaks in mammalian species have raised substantial concerns about their pandemic potential. The outbreak in dairy cattle in the US, with repeated transmissions to humans, has further emphasized this concern, as it represents a shift in host range toward a species in close contact with humans and may facilitate zoonotic transmission and viral adaptation. In contrast to newly emerging pathogens, influenza viruses circulate continuously in the human population, resulting in a complex immune background shaped by repeated infections and/or vaccination. Pre-existing immunity of the human population is a key determinant of susceptibility to emerging zoonotic variants and a central parameter in pandemic risk assessment (CDC, 2024; Levine et al., 2022; Trock et al., 2015). However, at the beginning of the cattle outbreak, the extent to which such pre-existing influenza immunity can recognize the newly emerging bovine H5N1 clade 2.3.4.4b virus was not yet fully understood. Against this background, the present study assessed pre-existing humoral immunity to the human H5N1 clade 2.3.4.4b isolate A/Texas/37/2024 derived from the cattle outbreak in H5N1-naïve individuals.

Following the dairy cattle outbreak, research activity on H5N1 clade 2.3.4.4b viruses has expanded considerably, with numerous studies published between 2024 and 2026. Among these, some have also explored aspects of pre-existing immunity to clade 2.3.4.4b viruses. The following chapters discuss the results of the present study in the context of the current state of literature.

4.1 The pseudovirus neutralization assay and its experimental limitations

A central prerequisite for this study was the establishment of a lentivirus-based HA-pseudovirus neutralization assay to quantify functional antibody activity against the emerging A/Texas/37/2024 and other IAV strains. The use of a pseudovirus system offered several advantages, including reduced biosafety requirements and the ability to perform standardized analysis across diverse viral strains independent of the availability of wild-type virus isolates. The optimized assay system showed high reproducibility across independent experiments (Figure 12). In addition, neutralization signals were largely independent of virus input (Figure 13), enabling the definition of a robust working range for the assay. This range closely overlapped with the one described by Garcia et al., further supporting that neutralization signals remain stable across the tested virus input range (Garcia et al., 2010). The specificity of the assay was validated with well-characterized HA-specific antibodies as well as human sera (Figure 11), which yielded the expected neutralization patterns without evidence of off-target effects.

As pseudovirus neutralization assays are designed to mimic wild-type virus neutralization, they are frequently benchmarked against established wild-type virus-based assays to assess their validity. Previous studies have reported strong, often statistically significant correlations between pseudovirus neutralization assays and classical wild-type assays such as HAI, MN or single-radial

hemolysis assay, particularly for H5 subtype viruses (Alberini et al., 2009; Buchy et al., 2010; Du et al., 2010; Huang et al., 2025; Qiu et al., 2013, 2012; Temperton et al., 2007). However, a study by Qiu et al. suggests that the strength of correlation between assay formats may vary depending on HA subtypes and virus strains (Qiu et al., 2012). In line with these observations, the comparison of the pseudovirus neutralization assay established here with an authentic virus neutralization assay for H1N1 demonstrated strong correlation between the assays, for both serum and mAb samples (Figure 14).

Notably, the assay comparison also revealed enhanced sensitivity of the pseudovirus system, reflected by systematically higher neutralization activity compared to the authentic virus assay. Increased sensitivity of influenza pseudovirus assays relative to wild-type virus assays has been repeatedly observed, particularly for H5 viruses (Alberini et al., 2009; Buchy et al., 2010; Garcia et al., 2010; Temperton et al., 2007). Two of these studies quantified this effect, reporting increases in H5 antibody titers of approximately 32% to 200% compared to MN assays (Buchy et al., 2010; Garcia et al., 2010). The increased sensitivity likely arises from a combination of structural differences between pseudotyped and wild-type virus particles and methodological differences between the assay systems. Structurally, differences in HA density on the viral surface, which is often assumed to be lower in pseudotyped particles, have been suggested to influence epitope accessibility (Alberini et al., 2009; Oh et al., 2009). In the system established here, the absence of NA on pseudotyped particles may further contribute to altered epitope accessibility (Alberini et al., 2009). Moreover, incomplete HA cleavage can result in a proportion of noninfectious particles capable of antibody binding, potentially reducing the effective neutralization of infectious particles. However, this effect is likely negligible in the present system, given the observed independence of neutralization signals from virus input. Methodologically, in the present study, the pseudovirus assay relies on an enzymatically amplified luciferase-based readout, whereas the authentic virus assay employs a non-amplified microscopic CPE-based readout. In addition, differences in the definition of neutralizing activity (ID₅₀ vs. ID₁₀₀) and the use of distinct cell lines (MDCK-SIAT1 vs. MDCKII) may contribute to the observed variation in neutralization activity.

Overall, the pseudovirus neutralization assay provides a robust and highly sensitive platform for the comparative analysis of influenza HA-specific antibody activity that shows strong agreement with wild-type neutralization. However, its increased sensitivity may lead to overestimation of functional antibody activity and should be considered when interpreting absolute neutralization titers.

For certain other immunological assays, most prominently the HAI assay, correlates of protection have been defined, thereby providing a framework for the interpretation of anti-influenza antibody titers (Krammer et al., 2020). For example, an HAI titer of 1:40, that has been established through epidemiological and human challenge studies, is generally accepted to

correspond to 50% protection from influenza infection (Cowling et al., 2019; Hobson et al., 1972; Ng et al., 2019). This threshold is widely used in vaccine and seroprotection studies, however, studies suggest that it is not universally applicable, as higher titers may be required to achieve comparable protection in children and elderly populations (Black et al., 2011; Dunning et al., 2016). Moreover, this correlate of protection has been established only for seasonal influenza strains and does not necessarily translate to avian influenza viruses.

In contrast, standardized correlates of protection have not been defined for pseudovirus-based neutralization assays, neither for seasonal nor zoonotic influenza viruses. Although some studies have attempted to infer protective thresholds by relating pseudovirus neutralization data to alternative reference measures, these estimates vary substantially (Alberini et al., 2009; Huang et al., 2025; Wang et al., 2019). Consequently, in the absence of validated correlates of protection, neither pseudovirus nor authentic virus neutralization data obtained in the present study allow conclusions to be drawn regarding the protective capacity of the measured antibody titers.

Finally, it should be noted that most HA-pseudovirus neutralization assays, including the one used in the present study, specifically measure antibodies that interfere with HA-mediated viral entry. This includes primarily HA head-directed antibodies as well as HA stem-directed antibodies that inhibit membrane fusion. Therefore, they do not capture the full spectrum of HA-specific antibody responses, such as antibodies interfering with viral egress or those mediating Fc-dependent effector functions, which have also been proposed to be associated with influenza protection (Edgar et al., 2023; Vanderven and Kent, 2020; Yamayoshi et al., 2017). In the present study, to complement neutralization-based readouts, recombinant HA proteins were produced and validated to measure antibody binding by ELISA (Figure 16). In combination with a cell-based binding assay, this approach allowed a more comprehensive assessment of pre-existing humoral immunity against the emerging H5N1 virus.

4.2 Pre-existing H5-reactive antibodies in H5N1-naïve individuals

Pre-existing humoral immunity against the H5N1 clade 2.3.4.4b isolate A/Texas/37/2024 was first assessed on the serum and plasma level. The analysis demonstrated that most H5N1-naïve individuals displayed measurable IgG binding and neutralizing activity against A/Texas/37/2024 in plasma and serum samples. Among the 66 individuals analyzed, pseudovirus neutralization was detected in all individuals, while ELISA and cell-based binding were observed in 94% and 45%, respectively, against this novel H5 variant (Figure 17). Notably, in a subset of 20 individuals, authentic virus neutralization against the A/Texas/37/2024 virus was also assessed, but no neutralizing activity was detected. The discrepancies observed across the four assay systems are likely attributable to differences in antigen presentation, assay sensitivity, and readout systems, in line with the methodological and structural differences between pseudovirus and wild-type virus assays discussed above. The observation of pseudovirus neutralization in the absence

of detectable binding likely reflects that antibody binding measurements do not necessarily correlate with neutralization potency, as previously described for SARS-CoV-2 antibodies (Zost et al., 2020). At the same time, this finding underscores the high sensitivity of the pseudovirus neutralization assay and demonstrates that the assay can detect neutralizing activity even at antibody concentrations below the detection thresholds of the two binding assays.

Further experiments confirmed that the observed serum and plasma reactivity was mediated by H5-reactive IgG antibodies and ruled out assay artifacts and unspecific inhibition. Neutralizing activity correlated strongly between serum and purified IgG fractions (Figure 18). Consistent with previous reports, IgG depletion from plasma largely abrogated influenza reactivity (Figure 19, Corti et al., 2010). Furthermore, depletion of HA-specific antibodies from IgG fractions resulted in a complete loss of neutralizing activity against H5, H1, and H3 (Figure 20), demonstrating that HA-specific IgG antibodies are the primary drivers of the observed H5 reactivity in H5N1-naïve individuals. Residual binding and/or neutralizing activity after whole IgG or HA-specific antibody depletion likely reflects incomplete depletion or minor contributions from other antibody isotypes, such as IgA or IgM.

The detection of H5-reactive antibodies in H5N1-naïve individuals is consistent with other studies reporting H5-specific binding and/or neutralizing activity in serum or plasma samples from unexposed individuals (Alcocer Bonifaz et al., 2026; Bresson et al., 2006; Corti et al., 2010; Garcia et al., 2009; Li et al., 2026; Nefkens et al., 2007; Pichyangkul et al., 2014; Power et al., 2026; Sanz-Muñoz et al., 2024; Sui et al., 2011; Treanor et al., 2006; Wang et al., 2019; Yassine et al., 2018). While most of these studies detected H5 reactivity only in a small subset of individuals, Sui et al. observed H5-binding IgG in all 77 participants analyzed (Sui et al., 2011). Similarly, a recently published study identified antibodies against pseudoviruses expressing H5 and N1 from a clade 2.3.4.4b virus in all 44 individuals examined (Alcocer Bonifaz et al., 2026). The authors further showed a strong correlation between neutralizing activity and IgG levels against H5 HA, suggesting a major contribution of HA-directed antibodies. In contrast, other studies have failed to detect any pre-existing H5 reactivity in cohorts of H5N1-naïve individuals (Daulagala et al., 2024; Zhang et al., 2011). The discrepancies in the reported prevalence of pre-existing H5 reactivity are likely driven by differences in assay systems, including sensitivity, readout formats as well as the use of antigenically distinct H5 strains. For example, the HAI assay used by some of the studies (Bresson et al., 2006; Daulagala et al., 2024; Li et al., 2025; Sanz-Muñoz et al., 2024; Treanor et al., 2006) is limited to the detection of head-directed antibodies and may therefore underestimate broader cross-reactive responses, which are often mediated by antibodies directed against the conserved HA stem region (Sun et al., 2024).

To further characterize the widespread detection of H5-reactive antibodies in H5N1-naïve individuals, neutralization activity was compared across different human and avian influenza strains and serum sources. The highest ID₅₀ observed in an H5N1-naïve individual was 4.6- to

53.2-fold lower than those measured in cattle experimentally infected with H5N1 (Figure 22). Within the panel of 12 analyzed H5 viruses, neutralization of A/Texas/37/2024 was comparable to that of the other H5 strains, indicating no major deviation in susceptibility relative to previously circulating H5 variants. At the same time, variation in neutralization titers across different H5 clades and strains, spanning approximately one $\log_{10}$ scale, suggests that antigenic evolution of H5 viruses within the avian reservoir can influence the extent of pre-existing immunity in humans. In addition, comparison with seasonal influenza showed that in the full cohort of 66 H5N1-naïve individuals, A/Texas/37/2024-neutralization was 3.9- to 15.6-fold lower than neutralization of selected H1 and H3 strains (Figure 17). Similarly, when analyzed for a subset of 20 participants, mean neutralization of the 12 H5 strains was 4.7- and 5.8-fold lower than mean neutralization of a panel of previously circulating H1N1pdm09 and H3 variants, respectively (Figure 23). However, direct comparisons of neutralization data for pseudoviruses of human and avian influenza strains should be interpreted with caution. Avian influenza viruses, including H5, preferentially bind α 2,3-linked sialic acids. The MDCK-SIAT1 cells used for the pseudovirus assay, however, overexpress α 2,6-linked sialic acids, which are preferred by human influenza viruses (Matrosovich et al., 2003). Consequently, infection efficiency may differ between pseudoviruses of avian and human subtypes, which in turn may limit direct scalability of neutralization titers between avian and human viruses in the present system.

Overall, the serum and plasma analysis indicates that while neutralizing activity against H5N1 clade 2.3.4.4b A/Texas/37/2024 can be detected in unexposed individuals, its magnitude is consistently lower than that observed for seasonal H1 and H3 influenza viruses. As discussed above, no conclusions can be drawn regarding the protective capacity of the detected H5-reactive antibodies. Moreover, the present analysis is based on a relatively small cohort of 66 individuals from the Cologne region characterized by an elevated influenza vaccination coverage (70%), which may restrict extrapolation of the observations to other populations.

Importantly, the present study focused on pre-existing humoral immunity to the HA protein of A/Texas/37/2024, as HA-specific antibodies are the primary correlate of protection against influenza. However, additional immune parameters are known to contribute to protection and are therefore likely to play a role in pre-existing immunity to zoonotic influenza viruses. In particular, NA-specific antibodies are associated with reduced disease severity for seasonal influenza (Eichelberger et al., 2008; Krammer et al., 2020). This may be especially relevant in the context of H5N1, which shares the N1 subtype with seasonal H1N1 viruses. Accordingly, recent studies showed that NA antibodies cross-reacting with clade 2.3.4.4b viruses, including A/Texas/37/2024, are present in H5N1-naïve individuals (Daulagala et al., 2024; Li et al., 2025; Singh et al., 2026). Protectivity of antibodies against other viral proteins is less well studied. Beyond humoral immunity, cellular immune responses also represent an important component of anti-influenza immunity, and T cells cross-reacting with H5N1 clade 2.3.4.4b viruses have been detected in

H5N1-naïve individuals (Sidney et al., 2024). These studies highlight that pre-existing immunity to emerging zoonotic influenza viruses is multifaceted and not fully captured by HA-directed antibody responses alone. Further studies are needed to better understand the complex interplay of these pre-existing cross-reactive immune components and their relevance for susceptibility to and protection against emerging influenza viruses.

4.3 B cell origin and functional properties of pre-existing H5-reactive antibodies

To further characterize the underlying B cell and antibody responses driving pre-existing H5-reactivity observed at the serum level, A/Texas/37/2024 HA-reactive memory B cells were isolated from six H5N1-naïve individuals and one H5N1-vaccinated individual. As expected, the highest frequency of antigen-specific B cells was detected in the H5-vaccinated individual (Figure 26). Functional evaluation of 136 encoded monoclonal antibodies by ELISA, cell-based binding assay, and pseudovirus neutralization assay showed that 40% of these antibodies reacted with A/Texas/37/2024 H5 in at least one assays (Figure 27). This relatively low recovery rate can be explained by the sorting strategy. A Y98F-mutated HA of A/Texas/37/2024 was used as a sorting bait, as this mutation has previously been described to reduce nonspecific binding of HA to sialic acid residues on the surface of B cells (Martín et al., 1998; Whittle et al., 2014). Nevertheless, the high number of single and double positive events indicated substantial residual background. To prioritize recovery of rare H5-specific B cells, the sorting gate was deliberately relaxed. This increased overall yield but also led to the inclusion of cells that did not confirm H5 reactivity upon subsequent validation.

Subsequent characterization of the isolated H5-reactive antibodies demonstrated that most of them cross-react with H1 HA, another group 1 HA subtype, but not with group 2 H3 HA (Figure 27). Moreover, the analysis revealed that most of the antibodies recognize conserved epitopes within the HA stem region, as they compete with the HA stem antibodies MEDI8852 and CR6261 (Figure 30). Together, these findings strongly suggest that the A/Texas/37/2024 H5-reactivity detected on the serum and plasma level is largely mediated by group 1 cross-reactive antibodies directed against the HA stem. These antibodies likely originate from previous exposure to seasonal group 1 influenza viruses (H1N1 or H2N2) through infection and/or vaccination. This interpretation is consistent with previous studies that were able to isolate HA stem antibodies cross-reacting with H5 from H5N1-naïve individuals (Corti et al., 2010; Sui et al., 2011; Throsby et al., 2008; Wrammert et al., 2011; Yassine et al., 2018). Moreover, heterologous H5 vaccination has been shown to induce HA stem-specific responses, supporting their role in H5 cross-neutralization (Ellebedy et al., 2014; Nachbagauer et al., 2014).

As the present single B cell analysis only comprised six H5N1-naïve individuals, direct conclusions from the data about the prevalence of such cross-reactive memory B cells are limited. However, other studies strongly suggest wide prevalence of group 1 reactive HA stem antibodies

in the human population, with some of them even demonstrating their cross-reactivity for H5 (Ellebedy et al., 2014; Sui et al., 2011; Yassine et al., 2018).

It is well established, that antibody responses to defined antigens are often characterized by biased immunoglobulin V gene usage, reflecting convergent selection of germline genes that facilitate recognition of specific epitopes (Rao and Coelho, 2025). Such recurrent V gene usage patterns have been described across a wide range of viral infections, including for example V_H3-23-dominated responses in Zika virus infection or V_H3-15 usage in Ebola virus-directed antibody responses (Ehrhardt et al., 2019; Robbiani et al., 2017). In the present study, V_H1-69 was the predominant HC V gene among H5-neutralizing antibodies isolated from H5N1-naïve individuals, accounting for 67% of those antibodies (Figure 27). This observation aligns with previous reports describing recurrent involvement of V_H1-69 in influenza-specific antibody responses, particularly in broadly neutralizing group 1 antibodies and H5 stem-reactive antibodies (Corti et al., 2010; Pappas et al., 2014; Sui et al., 2009; Wheatley et al., 2015). In addition, V_H6-1 and V_H1-18 have been described in stem-directed antibodies with reactivity across both group 1 and group 2 influenza viruses (Joyce et al., 2016), with V_H1-18 also contributing to 9% of the isolated H5-neutralizing antibodies in the present study. Together, these findings highlight recurrent patterns of germline gene usage in cross-reactive influenza responses, which may have implications for structure-guided vaccine design aiming at conserved epitopes.

Not only the induction of broadly cross-reactive antibodies by vaccines but also therapeutic and prophylactic applications of such antibodies have been extensively investigated over the last decades. For other viral infections such as RSV, COVID-19, or Ebola, mAbs have already been licensed for therapeutic and preventive use, although in the European Union this is currently limited to COVID-19 and RSV (Casadevall and Focosi, 2025; Mulangu et al., 2022, 2019; Sun et al., 2023). Numerous bnAbs against influenza, often recognizing both group 1 and group 2 subtypes, have been isolated primarily from infected or vaccinated individuals (Corti et al., 2011; Dreyfus et al., 2012; Ekiert et al., 2011; Friesen et al., 2014; Kallewaard et al., 2016; Krause et al., 2011; Nakamura et al., 2013; Ohshima et al., 2011; Throsby et al., 2008). Several of these influenza bnAbs have entered clinical trials or are currently under clinical evaluation. Of note, a subset of these well-characterized antibodies was also used here for validation of the pseudovirus neutralization assay and HA protein production. In the present study, the binding and neutralizing capacity of nine previously described influenza antibodies was also evaluated for the novel A/Texas/37/2024 strain in comparison to other H5 viruses and seasonal influenza strains (Figure 28). These included both clinically evaluated as well as additional reference antibodies. MEDI8852, 39.29, CR9114, CR6261, F045-092, and 100F4 all showed potent binding and neutralizing activity against this new variant *in vitro* with titers comparable to those observed for older H5 variants.

Direct comparison using pseudovirus and authentic virus neutralization assay further showed that the H5-neutralizing antibodies isolated from H5N1-naïve individuals exhibit neutralization potency comparable to these clinically tested influenza antibodies (Figure 29). The high neutralizing potency was further supported by an *in vivo* challenge experiment, in which two of the isolated H5-neutralizing antibodies conferred strong protection against lethal H5N1 infection in mice (Figure 31). This is consistent with previous studies demonstrating the protective efficacy of HA stem antibodies in animal models (Corti et al., 2011; DiLillo et al., 2014; Ekiert et al., 2011; Liu et al., 2021; Yamayoshi et al., 2017). In contrast, human studies assessing the efficacy of HA stem antibodies in influenza prevention and treatment have shown variable results (Ali et al., 2018; Han et al., 2021; Hershberger et al., 2019; Lim et al., 2022, 2020; Park et al., 2018; Tan et al., 2024). A controlled influenza A(H1N1)pdm09 human challenge study, for example, evaluated pre-existing HA stem antibodies as correlates of protection (Park et al., 2018). They showed that higher baseline anti-HA stem titers were associated with reduced viral shedding but not with reduced disease severity.

Overall, further studies are needed to address the role of HA stem antibodies in humans. Despite their frequent detection and functional cross-reactivity *in vitro*, their contribution to protection against infection and modulation of disease severity remains incompletely understood. A more comprehensive understanding of their relevance in natural human infections will be essential to also clarify their potential impact in the context of emerging zoonotic influenza viruses, including H5N1.

4.4 Influence of immune history on H5 cross-reactive antibody responses

Human influenza immune responses, and thus also cross-reactive HA antibody responses, are shaped by repeated exposure through infection or vaccination. The serum analysis demonstrated that individuals who reported prior seasonal influenza vaccination had modest but significantly higher neutralization titers against A/Texas/37/2024 than unvaccinated individuals (Figure 21 and Figure 24). In the context of pandemic preparedness, the question whether currently licensed seasonal influenza vaccines can elicit cross-reactive antibody responses recognizing zoonotic influenza viruses has been investigated across multiple cohorts and vaccine platforms (Alcocer Bonifaz et al., 2026; Corti et al., 2010; Garcia et al., 2009; Li et al., 2025; Sanz-Muñoz et al., 2024; Wang et al., 2019). In line with the observation of the present work, the majority of these studies report an increase in H5 binding and/or neutralizing antibody titers following seasonal influenza vaccination. However, the magnitude and proportion of individuals showing increased titers varies considerably between the studies and two studies did not observe any effect of vaccination on H5-specific responses (Li et al., 2025; Wang et al., 2019). Notably, two other studies specifically demonstrate those increased cross-reactive responses after seasonal influenza vaccination also for clade 2.3.4.4b viruses (Alcocer Bonifaz et al., 2026; Sanz-Muñoz et al., 2024). Functional

relevance of these findings is further supported by experimental *in vivo* evidence. Sun et al. demonstrated that ferrets immunized with seasonal influenza vaccines exhibited reduced clinical disease severity and decreased viral shedding following challenge with a clade 2.3.4.4b H5N1 virus, suggesting partial heterologous protection under controlled conditions (Sun et al., 2025). However, the extent to which these observations translate into meaningful protection in humans remains uncertain and warrants further investigation.

Besides recent seasonal influenza vaccination, a person's first exposure to influenza has been proposed to influence pre-existing cross-reactive immunity to zoonotic influenza viruses through antigenic imprinting. The analysis of serum neutralization against the panel of 77 human and avian influenza strains showed clear association of neutralization levels and the participants' year of birth for the subtypes H2 and H3. Individuals born shortly before or during the period of H2N2 circulation (1957–1968) showed very high H2 neutralization and rather weak responses against group 2 H3 strains. In contrast, younger individuals displayed higher H3 neutralization, with the strongest responses detected for individuals born in the 1990s against H3 strains circulating during that decade. Together, these observations are consistent with previous studies on immune imprinting and antigenic seniority in seasonal influenza and provide further support for early-life exposure as a key determinant of long-lasting influenza immunity (Fonville et al., 2014; Francis, 1960; Gostic et al., 2019; Lessler et al., 2012; Matsuzawa et al., 2019).

For H5 neutralization, no clear association with the participants' birth year could be detected in the present study (Figure 21 and Figure 24). However, this analysis is limited by the small sample sizes of 66 or 20 individuals, respectively, and the underrepresentation of older, likely group 1 imprinted age groups within the cohort. In contrast, Garretson et al. reported increased H5N1 antibody titers in older individuals, with a stronger correlation to birth year than to age, which supports a role for imprinting in shaping pre-existing H5-reactive immunity (Garretson et al., 2025). Similarly, another recent study found that individuals imprinted with group 1 HA viruses exhibited higher levels of H5N1 cross-reactive antibodies compared to those imprinted with group 2 HA viruses (Alcocer Bonifaz et al., 2026). In addition, Gostic et al. analyzed data from all reported human cases of H5N1 and H7N9 and inferred the subtype of first influenza exposure for each birth-year cohort based on historical influenza circulation and seroprevalence data (Gostic et al., 2016). Using statistical modeling, the authors showed that childhood HA imprinting is a major determinant of disease severity for both H5N1 and H7N9. They therefore propose that the first influenza infection in life provides long-lasting protection against severe disease caused by newly emerging HA subtypes within the same phylogenetic group (e.g. H1N1 or H2N2 imprinting confers protection against severe H5N1 infection). Although the underlying mechanisms remain not fully elucidated, imprinting of HA stem-directed responses, as observed in multiple studies (Andrews et al., 2015; Arevalo et al., 2020; Sun et al., 2026), likely contributes to birth year-dependent differences in H5 cross-reactive immunity.

Further studies are needed to better define these mechanisms and the extent to which HA imprinting and overall immune history shape protection across different influenza subtypes. A more detailed understanding of this will help to identify high-risk age cohorts in future influenza pandemics and could for example guide prioritization of preventive measures.

4.5 Conclusion and outlook

In summary, this study demonstrates detectable but overall low levels of serum antibody reactivity against HA of the emerging H5N1 A/Texas/37/2024 strain in individuals without prior exposure to this influenza subtype. The isolation of H5-reactive memory B cells and characterization of their encoded antibodies strongly suggest that this reactivity is largely mediated by highly potent, group 1 cross-reactive HA stem antibodies. However, these findings do not allow conclusions regarding protective immunity.

Future studies are needed to better define the clinical relevance of cross-reactive antibodies, particularly those HA stem-directed responses, for protection against zoonotic influenza viruses. Establishing robust and standardized correlates of protection for emerging influenza variants, together with harmonized immunological assays, will be essential to improve comparability between studies and enable more reliable evaluation of antibody-mediated immunity. In this context, a better understanding of how immune history shapes these cross-reactive responses and contributes to susceptibility to zoonotic variants will be important.

At the same time, viral evolution poses a persistent challenge to humoral immunity, as even single mutations can mediate escape from pre-existing antibody responses, as previously shown for influenza and other viral pathogens (Cao et al., 2022; Doud et al., 2018; Gruell et al., 2022; Kikawa et al., 2023; Oraby et al., 2025). This reinforces the importance of continuous and globally coordinated surveillance of circulating influenza A variants. A One Health approach integrating human, animal, and environmental health will be crucial for early detection and risk assessment of emerging viruses. Ultimately, combining improved immunological understanding with expanded global surveillance strategies will be essential to strengthen pandemic preparedness for future influenza outbreaks.

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6 Declaration on the use of artificial intelligence tools

The artificial intelligence (AI)-based tool ChatGPT (OpenAI, GPT-5.3) was used during the preparation of this thesis to assist with linguistic editing and stylistic refinement of the text. No AI tools were used for data generation, figure preparation, analysis, or interpretation. All content was critically reviewed and approved by the author, who takes full responsibility for the final version of this thesis.

7 Eidesstattliche Erklärung

Hiermit versichere ich an Eides statt, dass ich die vorliegende Dissertation selbstständig und ohne die Benutzung anderer als der angegebenen Hilfsmittel und Literatur angefertigt habe. Alle Stellen, die wörtlich oder sinngemäß aus veröffentlichten und nicht veröffentlichten Werken dem Wortlaut oder dem Sinn nach entnommen wurden, sind als solche kenntlich gemacht. Ich versichere an Eides statt, dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie – abgesehen von unten angegebenen Teilpublikationen und eingebundenen Artikeln und Manuskripten – noch nicht veröffentlicht worden ist sowie, dass ich eine Veröffentlichung der Dissertation vor Abschluss des Prüfungsverfahrens der Promotion nicht vornehmen werde. Die Bestimmungen der Promotionsordnung der Mathematisch-Naturwissenschaftlichen Fakultät der Universität zu Köln sind mir bekannt. Darüber hinaus erkläre ich hiermit, dass ich die Ordnung zur Sicherung guter wissenschaftlicher Praxis und zum Umgang mit wissenschaftlichem Fehlverhalten der Universität zu Köln gelesen und sie bei der Durchführung der der Dissertation zugrundeliegenden Arbeiten und der schriftlich oder elektronisch verfassten Dissertation beachtet habe, und verpflichte mich hiermit, die dort genannten Vorgaben bei allen wissenschaftlichen Tätigkeiten zu beachten und umzusetzen.

Die Primärdaten, Analysen und Versuchsprotokolle, die dieser Dissertation zugrunde liegen, sind im Datenspeicher der Forschungsgruppe archiviert, in der die Arbeit durchgeführt wurde. Sofern sie nicht auf öffentlichen wissenschaftlichen Datenservern – wie in der Dissertation angegeben – zugänglich sind, bleiben sie dort in Übereinstimmung mit den geltenden gesetzlichen Bestimmungen gespeichert. Die Versuchsmaterialien wurden gemäß den Regelungen der Forschungsgruppe, in der die experimentellen Arbeiten durchgeführt wurden, gesichert.

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Köln, den 25.05.2026

Katharina Daniel

8 Acknowledgements

9 Appendix

Table 16. Blood donor information. Table taken from Daniel et al., 2026.

ID	Sex	Year of birth	Sampling date	Age at sampling	Any Influenza vaccination	Last vaccination date	Previous Influenza A infection	Last known infection date
1	F	1972	Aug-23	51	Yes	Oct-21	No	N/A
2	F	1986	Aug-23	36	Yes	Oct-21	No	N/A
3	F	1969	Aug-23	54	Yes	Oct-22	No	N/A
4	F	1989	Aug-23	33	No	N/A	No	N/A
5	F	1966	Aug-23	56	Yes	Nov-20	No	N/A
6	M	1970	Aug-23	53	Yes	Dec-22	No	N/A
7	F	1972	Aug-23	50	Yes	Oct-09	No	N/A
8	F	1999	Aug-23	24	No	N/A	Yes	2015
9	M	1977	Aug-23	46	No	N/A	No	N/A
10	M	1967	Aug-23	55	No	N/A	No	N/A
11	F	1988	Aug-23	34	Yes	Nov-22	No	N/A
12	F	1996	Aug-23	26	Yes	Oct-21	No	N/A
13	F	1995	Aug-23	28	Yes	Oct-22	No	N/A
14	M	1971	Aug-23	51	Yes	Oct-22	No	N/A
15	M	1992	Aug-23	31	N/A	N/A	No	N/A
16	F	1954	Aug-23	69	No	N/A	No	N/A
17	F	1961	Aug-23	61	Yes	Jul-05	No	N/A
18	F	1979	Aug-23	44	Yes	Sep-22	No	N/A
19	M	1969	Aug-23	53	Yes	Nov-22	No	N/A
20	M	1966	Aug-23	57	Yes	Nov-22	No	N/A
21	M	1987	Aug-23	36	Yes	Dec-22	No	N/A
22	F	1995	Aug-23	27	Yes	Feb-20	No	N/A
23	F	1969	Aug-23	54	Yes	Nov-12	No	N/A
24	F	1989	Aug-23	34	Yes	Oct-22	No	N/A
25	M	1981	Aug-23	42	Yes	Oct-22	No	N/A
26	F	1977	Aug-23	46	No	N/A	No	N/A
27	M	1987	Aug-23	35	No	N/A	No	N/A
28	F	1952	Aug-23	71	Yes	N/A	Yes	N/A
29	M	1993	Aug-23	30	No	N/A	No	N/A
30	F	1997	Sep-23	26	Yes	Jul-05	No	N/A
31	F	1972	Sep-23	51	Yes	Oct-22	No	N/A
32	M	1964	Sep-23	59	No	N/A	No	N/A
33	M	1968	Sep-23	54	No	N/A	No	N/A
34	F	1996	Nov-23	27	No	N/A	N/A	N/A
35	F	1984	Nov-23	39	Yes	Oct-23	No	N/A
36	M	1952	Nov-23	71	No	N/A	No	N/A
37	F	2004	Dec-23	19	No	N/A	No	N/A
38	F	1978	Dec-23	45	Yes	Oct-23	No	N/A
39	F	1994	Jan-24	29	No	N/A	No	N/A
40	F	1966	Apr-24	57	Yes	Sep-06	No	N/A
41	F	1971	Apr-24	52	Yes	>15 years	No	N/A
42	M	1966	Apr-24	58	Yes	>10 years	No	N/A
43	M	1965	Apr-24	58	Yes	Sep-23	No	N/A
44	M	1997	Apr-24	26	Yes	Nov-21	No	N/A
45	F	1966	Apr-24	57	Yes	Oct-23	No	N/A
46	F	1996	Apr-24	27	Yes	Oct-21	No	N/A
47	F	1986	May-24	38	No	N/A	Yes	Feb-24
48	F	1996	Jun-24	28	Yes	2023	No	N/A
49	M	1998	Jun-24	26	No	N/A	No	N/A
50	F	1989	Jun-24	34	Yes	Jan-24	Yes	2019
51	F	2000	Jun-24	23	No	N/A	No	N/A
52	F	1999	Jun-24	24	Yes	Nov-23	No	N/A

53	F	1994	Jun-24	29	Yes	N/A	No	N/A
54	F	1986	Jun-24	37	Yes	Nov-23	No	N/A
55	F	1987	Jun-24	36	Yes	Nov-23	No	N/A
56	F	2001	Jun-24	22	Yes	Nov-23	No	N/A
57	M	1986	Jun-24	38	Yes	Oct-23	No	N/A
58	F	1998	Jun-24	25	Yes	Oct-23	No	N/A
59	M	1993	Jun-24	30	Yes	Feb-23	No	N/A
60	F	1981	Jun-24	43	Yes	Oct-22	No	N/A
61	F	1963	Jun-24	60	No	N/A	No	N/A
62	M	1975	Jun-24	49	Yes	Nov-23	Yes	N/A
63	F	1980	Jun-24	43	Yes	Nov-22	No	N/A
64	F	1995	Jun-24	29	Yes	Nov-23	Yes	Jan-23
65	F	1994	Jun-24	29	No	N/A	No	N/A
66	F	2002	Jun-24	22	Yes	Oct-22	Yes	2010

Table 17. Influenza strain information. Table taken from Daniel et al., 2026.

#	HxNy	Strain	EPI
1	H1N1	A/Wisconsin/67/2022	EPI_ISL_15928538
2	H1N1	A/Sydney/5/2021	EPI_ISL_6424984
3	H1N1	A/Togo/881/2020	EPI_ISL_1015524
4	H1N1	A/Ghana/140/2020	EPI_ISL_959864
5	H1N1	A/Perth/41/2020	EPI_ISL_482750
6	H1N1	A/Victoria/2570/2019	EPI_ISL_548964
7	H1N1	A/Wisconsin/588/2019	EPI_ISL_404460
8	H1N1	A/Guangdong-Maonan/SWL1536/2019_C1C1	EPI_ISL_391019
9	H1N1	A/Guangdong-Maonan/SWL1536/2019_E4	EPI_ISL_392654
10	H1N1	A/Hawaii/70/2019	EPI_ISL_400916
11	H1N1	A/Brisbane/64/2019	EPI_ISL_389532
12	H1N1	A/Oman/5442/2018	EPI_ISL_347417
13	H1N1	A/Brisbane/2/2018	EPI_ISL_410633
14	H1N1	A/Brisbane/59/2007	EPI_ISL_23380
15	H1N1	A/Beijing/262/1995	EPI_ISL_22625
16	H2N2	A/Rotterdam/1957	EPI_ISL_84911
17	H2N2	A/Japan/305/1957	EPI_ISL_5869
18	H3N2	A/South Australia/389/2022	EPI_ISL_16613678
19	H3N2	A/Darwin/6/2021	EPI_ISL_3534319
20	H3N2	A/Darwin/9/2021	EPI_ISL_3100998
21	H3N2	A/Cambodia/E0826360/2020	EPI_ISL_1296487
22	H3N2	A/Bangladesh/1813/2020	EPI_ISL_882827
23	H3N2	A/Sydney/473/2019	EPI_ISL_406676
24	H3N2	A/Ghana/3532/2019	EPI_ISL_406176
25	H3N2	A/Kanagawa/159/2019	EPI_ISL_398727
26	H3N2	A/Hong Kong/2671/2019_E7	EPI_ISL_400888
27	H3N2	A/Hong Kong/2671/2019_C1S2	EPI_ISL_391201
28	H3N2	A/South Australia/34/2019_E4	EPI_ISL_400538
29	H3N2	A/South Australia/34/2019_SIAT1	EPI_ISL_346283
30	H3N2	A/Sydney/549/2017	EPI_ISL_396727
31	H3N2	A/Victoria/659/2017	EPI_ISL_396885
32	H3N2	A/Victoria/601/2017	EPI_ISL_396870
33	H3N2	A/Victoria/723/2017	EPI_ISL_289904
34	H3N2	A/Tunisia/1597/2017	EPI_ISL_262280
35	H3N2	A/Victoria/2030/2017	EPI_ISL_396820
36	H3N2	A/Victoria/2014/2017	EPI_ISL_271306
37	H3N2	A/Kansas/14/2017_E17	EPI_ISL_350966
38	H3N2	A/Kansas/14/2017_S2	EPI_ISL_292575
39	H3N2	A/Switzerland/8060/2017	EPI_ISL_294252
40	H3N2	A/Canberra/7/2016	EPI_ISL_227639

41	H3N2	A/Singapore/INFIMH160019/2016	EPI_ISL_285898
42	H3N2	A/South Australia/118/2016	EPI_ISL_239826
43	H3N2	A/Hong Kong/4801/2014	EPI_ISL_165554
44	H3N2	A/Switzerland/9715293/2013	EPI_ISL_162149
45	H3N2	A/Texas/50/2012	EPI_ISL_122006
46	H3N2	A/Victoria/361/2011	EPI_ISL_134450
47	H3N2	A/Perth/16/2009	EPI_ISL_31055
48	H3N2	A/Brisbane/10/2007	EPI_ISL_23194
49	H3N2	A/Wisconsin/67/2005	EPI_ISL_115646
50	H3N2	A/California/7/2004	EPI_ISL_113070
51	H3N2	A/Moscow/10/1999	EPI_ISL_127595
52	H3N2	A/Sydney/5/1997	EPI_ISL_29667
53	H3N2	A/Nanchang/933/1995	EPI_ISL_111174
54	H3N2	A/Netherlands/179/1993	EPI_ISL_111045
55	H3N2	A/Netherlands/620/1989	EPI_ISL_114347
56	H3N2	A/Bilthoven/1761/1976	EPI_ISL_113970
57	H3N2	A/Bilthoven/23337/1972	EPI_ISL_110819
58	H5N1	A/Texas/37/2024	EPI_ISL_19027114
59	H5N1	A/England/231990002/2023	EPI_ISL_17736649
60	H5N1	A/Chile/25945/2023	EPI_ISL_17468386
61	H5N1	A/Cambodia/NPH230032/2023	EPI_ISL_17024123
62	H5N8	A/Astrakhan/3212/2020	EPI_ISL_1038924
63	H5N6	A/Hubei/29578/2016	EPI_ISL_256213
64	H5N6	A/Guangxi/55726/2016	EPI_ISL_240704
65	H5N1	A/Egypt/N0005/2015	EPI_ISL_17767282
66	H5N1	A/Indonesia/NIHRD15028/2015	EPI_ISL_195732
67	H5N1	A/Cambodia/X0123311/2013	EPI_ISL_166636
68	H5N1	A/Vietnam/1203/2004	EPI_ISL_10656749
69	H5N1	A/Hong Kong/213/03	EPI_ISL_268
70	H7N9	A/Hebei/27401/2017	EPI_ISL_285159
71	H7N9	A/Anhui/01887/2014	EPI_ISL_285159
72	H7N7	A/Italy/3/2013	EPI_ISL_171318
73	H7N1	A/Shangdong/2/2013	EPI_ISL_17264959
74	H9N2	A/Oman/2747/2019	EPI_ISL_353983
75	H9N2	A/Anhui-Luijiang/39/2018	EPI_ISL_330737
76	H9N2	A/Bangladesh/0994/2011	EPI_ISL_140388
77	H9N2	A/Chicken/Hong Kong/G9/1997	EPI_ISL_146698

Table 18. Antibody information. Table taken from Daniel et al., 2026.

#	Antibody name	Origin	Target	In clinical development	Reference
1	MEDI8852	Blood donor in Switzerland; genetically optimized version of FY1	HA stalk	Yes, VIR2482, NCT02350751, NCT02603952	Kallewaard et al., 2016
2	39.29	2009 seasonal influenza vaccine recipient	HA stalk	Yes, Gedivumab (MHAA4549A); NCT01877785, NCT02284607, NCT01980966, NCT02293863, NCT02623322	Nakamura et al., 2013
3	CR9114	Seasonal influenza vaccine recipient	HA stalk	Yes, CTIS2023-505020-57-00	Dreyfus et al., 2012
4	CR6261	Seasonal influenza vaccine recipient	HA stalk	Yes; NCT02371668, NCT01406418	Throsby et al., 2008
5	F045-092	Apheresis donor, born in 1974	HA head	No	Ohshima et al., 2011

6	100F4	H5N1 subclade 2.3.4 convalescent donor	HA head	No	Hu et al., 2013
7	H5.3	Participants of a H5N1 (A/Vietnam/1203/2004) vaccine trial	HA head	No	Thornburg et al., 2013
8	5J8	Middle-aged woman	HA head	No	Krause et al., 2011
9	CR8020	2006/2007 Seasonal influenza vaccinated donors	HA stalk	Yes; NCT01938352, NCT01756950	Ekiert et al., 2011
10	CnC2t1p1_D6	SARS-CoV-2 convalescent donor	SARS-CoV-2 RBD	No	Kreer et al., 2020b
11	DZIF-10c (HbnC3t1 p1_F4)	SARS-CoV-2 convalescent donor	SARS-CoV-2 RBD	Yes; EudraCT Number: 2020-004448-27	Halwe et al., 2021; Kreer et al., 2020b

Table 19. Primer sequences.

Primer name	Sequence	Reference
3' IgG internal	GTTCCGGGAAGTAGTCCTTGAC	Tiller et al., 2008
3' C _κ 543	GTTTCTCGTAGTCTGCTTTGCTCA	Tiller et al., 2008
3' C _κ 494	GTGCTGTCCTTGCTGTCTGCT	Tiller et al., 2008
3' C _λ	CACCAGTGTGGCCTTGTTGGCTTG	Tiller et al., 2008
3' XhoI C _λ	CTCCTCACTCGAGGGYGGGAACAGAGTG	Tiller et al., 2008
5' Ab-sense	GCTTCGTTAGAACGCGGCTAC	Tiller et al., 2008
Cg-RT	AGGTGTGCACGCCGCTGGTC	Ozawa et al., 2006
Cm-RT	ATGGAGTCGGGAAGGAAGTC	Ozawa et al., 2006
CMV for	CGCAAATGGGCGGTAGGCGTG	N/A
HA Rev L12	GATCCTCGAGCCAGTCTTTGTACCCGGACTTCAGC	This study
HA Rev L14	GATCCTCGAGCTGGTAGATCCTGGTGCTCTCCAGC	This study
LU P4	GAGAGCCAGATCCAGATCCACTGCCAG	This study
LU P7	CATTGACGCAAATGGGCG	This study
LU P11	GTGTCAGCTTTCCATTGG	This study
LU P12	CTTGATTCTCATGGTCTGGG	This study
LU P13	GTGCCCTCATAAGGTCTG	This study
LU P27	CAATCCGCCCTCACTACAACCG	This study
LU P28	TCCCTCATCGACGCCAGAGTAG	This study
oCKc51	ATTAGCCAGAAGTCAGATGC	This study
oPR-IGHV-1_fw	ATGGACTGGACCTGGAGCATCC	Kreer et al., 2020a
oPR-IGHV-2_fw	ATGGACTGGACCTGGAGGATCCTC	Kreer et al., 2020a
oPR-IGHV-3_fw	ATGGACTGGACCTGGAGGGTCTTC	Kreer et al., 2020a
oPR-IGHV-4_fw	ATGGACTGGATTTGGAGGGTCTCTTC	Kreer et al., 2020a
oPR-IGHV-5_fw	ATGGACACACTTTGCTACACACTCCTGC	Kreer et al., 2020a
oPR-IGHV-6_fw	ACTTTGCTCCACGCTCCTGC	Kreer et al., 2020a
oPR-IGHV-7_fw	GGCTGAGCTGGGTTTTCTTGTTG	Kreer et al., 2020a
oPR-IGHV-8_fw	GGCTCCGCTGGGTTTTCTTGTTG	Kreer et al., 2020a
oPR-IGHV-9_fw	CACCTGTGGTTCTTCCTCCTGCTG	Kreer et al., 2020a
oPR-IGHV-10_fw	ATGAAACACCTGTGGTTCTTCCTCCTCC	Kreer et al., 2020a
oPR-IGHV-11_fw	ACATCTGTGGTTCTTCCTCCTGGTG	Kreer et al., 2020a
oPR-IGHV-12_fw	GCCTCTCCACTTAAACCCAGGCTC	Kreer et al., 2020a
oPR-IGHV-13_fw	ATGTCTGTCTCCTTCCTCATCTTCCTGC	Kreer et al., 2020a
oPR-IGHV-14_fw	ATGGAGTTGGGGCTGAGCTGG	Kreer et al., 2020a
oPR-IGHV-15_fw	ATGGGGTCAACCGCCATCCTC	Kreer et al., 2020a
oPR-IGKV-1_fw	ATGAGGCTCCTTGCTCAGCTTCTGG	Kreer et al., 2020a
oPR-IGKV-2_fw	ATGGAAGCCCCAGCTCAGCTTC	Kreer et al., 2020a
oPR-IGKV-3_fw	CCCAGCTCAGCTTCTCTTCCTCCTG	Kreer et al., 2020a
oPR-IGKV-4_fw	TGGTGTGTCAGACCCAGGTCTTCATTC	Kreer et al., 2020a

oPR-IGKV-5_fw	GTCCCAGGTTTCACCTCCTCAGCTTC	Kreer et al., 2020a
oPR-IGKV-6_fw	GCCATCACAACCTCATTGGGTTTCTGCTG	Kreer et al., 2020a
oPR-IGKV-7_fw	TCCCTGCTCAGCTCCTGGG	Kreer et al., 2020a
oPR-IGKV-8_fw	CCTGGGACTCCTGCTGCTCTG	Kreer et al., 2020a
oPR-IGLV-1_fw	CCCTGGGTCATGCTCCTCCTGAAATC	Kreer et al., 2020a
oPR-IGLV-2_fw	CTCTGCTGCTCCTCACTCTCCTCAC	Kreer et al., 2020a
oPR-IGLV-3_fw	ATGGCATGGATCCCTCTCTTCCTCG	Kreer et al., 2020a
oPR-IGLV-4_fw	CCTCTCTGGCTCACTCTCCTCACTC	Kreer et al., 2020a
oPR-IGLV-5_fw	ACACTCCTGCTCCCACTCCTCAAC	Kreer et al., 2020a
oPR-IGLV-6_fw	ATGGCCTGGATCCCTCTACTTCTCC	Kreer et al., 2020a
oPR-IGLV-7_fw	ATGGCCTGGGTCTCCTTCTACC	Kreer et al., 2020a
oPR-IGLV-8_fw	ATGGCCTGGACTCCTCTCTTTCTGTTC	Kreer et al., 2020a
oPR-IGLV-9_fw	ATGGCCTGGATGATGCTTCTCCTC	Kreer et al., 2020a
oPR-IGLV-10_fw	GTCCCCTCTCTTCCTCACCCTCATC	Kreer et al., 2020a
oPR-IGLV-11_fw	CTCCTCGCTCACTGCACAGG	Kreer et al., 2020a
oPR-IGLV-12_fw	CCTCTCCTCCTCACCCTCCTC	Kreer et al., 2020a
oPR-IGLV-13_fw	CTCCTCCTCACCCTCCTCACTC	Kreer et al., 2020a
oPR-IGLV-14_fw	ATGGCCTGGACCCCTCTCC	Kreer et al., 2020a
oPR-IGLV-15_fw	ATGGCCTGGACCCCACTCC	Kreer et al., 2020a
P0_NA_Mut_rev	GGCCACCGGTTCTAG	This study
P1_NA_Mut	GGCCAAGCTTGGTACCGCCACCATGAACCCCAACCA GAAGAT	This study
P13 rev	TGGCAACTAGAAGGCACAGT	This study
pCAGGS_fwd_new	CGGCAGGAAGGAAATG	This study
Texas_for	AGCTTGGTACCGCCACCATG	This study
Texas_rev	CGATTCTCGAGCTGGTAGGTGCCACGCTTTC	This study

Table 20. Composition of primer mixes for single B cell PCR.

Primer mix	Primers included
oPR-IGHV	oPR-IGHV-1_fw oPR-IGHV-2_fw oPR-IGHV-3_fw oPR-IGHV-4_fw oPR-IGHV-5_fw oPR-IGHV-6_fw oPR-IGHV-7_fw oPR-IGHV-8_fw oPR-IGHV-9_fw oPR-IGHV-10_fw oPR-IGHV-11_fw oPR-IGHV-12_fw oPR-IGHV-13_fw oPR-IGHV-14_fw oPR-IGHV-15_fw
oPR-IGKV	oPR-IGKV-1_fw oPR-IGKV-2_fw oPR-IGKV-3_fw oPR-IGKV-4_fw oPR-IGKV-5_fw oPR-IGKV-6_fw oPR-IGKV-7_fw oPR-IGKV-8_fw
oPR-IGLV	oPR-IGLV-1_fw oPR-IGLV-2_fw oPR-IGLV-3_fw oPR-IGLV-4_fw oPR-IGLV-5_fw oPR-IGLV-6_fw

	oPR-IGLV-7_fw oPR-IGLV-8_fw oPR-IGLV-9_fw oPR-IGLV-10_fw oPR-IGLV-11_fw oPR-IGLV-12_fw oPR-IGLV-13_fw oPR-IGLV-14_fw oPR-IGLV-15_fw
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