Generation of therapeutic monoclonal antibodies to treat influenza virus infections

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submitted by

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List of abbreviations

ADCC	Antibody-dependent cell-mediated cytotoxicity
ADCP	Antibody dependent cellular phagocytosis
AP	Alkaline phosphatase
BSA	Bovine serum albumin
cDMEM	Complete Dulbecco's Modified Eagle's Medium
cDNA	Complementary deoxyribonucleic acid
DEAE	Diethylaminoethyl cellulose
dH_20	Distilled water
DMEM	Dulbecco's Modified Eagle's Medium
EC_{50}	Half maximal effective concentration
E.coli	*Escherichia coli*
ELISA	Enzyme-linked Immunosorbent Assay
FBS	Fetal bovine serum
HA	Hemagglutinin
HAT	Hypoxanthine-aminopterin-thymidine
HCL	Hydrochloric Acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
High 5	BTI-TN-5B1-4 cells
(H+L)	Heavy chain + light chain
IC_{50}	Half maximal inhibitory concentration
IF	Immunofluorescence
IgG	Immunoglobulin-G
IP	Intraperitoneal
IPTG	Isopropyl β-D-1-thiogalactopyranoside
KLN6-antibody	Krammer Lab Neuraminidase 6 - antibody
KTG	Kanamycin Tetracycline Gentamycin
LASV	Lassa virus
LB	Lysogeny broth
LD_{50}	Lethal Dose, which causes death in 50% of tested animals
mAbs	Monoclonal antibodies
MDCK	Madin-Darby Canine Kidney
MEM	Minimal essential medium

MOI	Multiplicity of infection
NA	Neuraminidase
NaCl	Sodium chloride
NaH_2PO_4	Sodium dihydrogen phosphate
NI	Neuraminidase inhibition
Ni-NTA	Nickel-nitrilotriacetic acid
N6	Neuraminidase type 6
OD	Optical density
ON	Overnight
PBS	Phosphate-buffered saline
PBST	Phosphate-buffered saline with Tween
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
Pen/Strep	Penicillin/Streptomycin
PFA	Paraformaldehyde
PFU/ml	Plaque forming units per millilitre
PNA	Peanut agglutinin
Poly I:C	Polyinosinic:polycytidylic acid
PR8	A/Puerto Rico/8/1934
P0/1/2/3	Passage 0/1/2/3
RNA	Ribonucleic acid
RT	Room temperature
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SFM	Serum free medium
Sf9	ATCC CRL-1711 cells
Sigma Fast OPD	SigmaFast o-Phenylenediamine dihydrochloride
SMH4N6	A/swine/Missouri/A01727926/2015
SOC	Super Optimal Broth
SZIH5N6	A/Shenzhen/1/2016 (H5N6)
TPCK	L-1-Tosylamide-2-phenylethyl chloromethyl ketone

Abstract

The influenza virus is known to be one of the most severe pathogens in the group of respiratory illnesses accompanied with a high rate of morbidity and mortality[1]. In the past decade, the immunosubdominant surface glycoprotein neuraminidase (NA) has been proven to be effective as an antigen and vaccination can protect against infection[2]. In 2014, an avian H5N6 influenza virus successfully emerged and started to sporadically infect humans for the first time in history, which caused several deaths in the people's republic of China[3]. Little is known about the N6 in general and since it has crossed species barriers, the development of viable therapeutics against it is a crucial goal. In this study, we successfully generated cross-reactive murine monoclonal antibodies against the N6 of A/duck/Zhejiang/D9/2013 (H4N6), A/swine/Missouri/A01727926/2015 (H4N6), A/Caspian seal/Russia/T1/2012 and A/Shenzhen/1/2016 (H5N6) via hybridoma technology. We obtained a panel of 10 murine antibodies, that are exclusively reacting against the epitopes of the N6 subtype. We tested the binding activity of those antibodies via ELISA as well as IF-assay and observed that they are highly cross reactive against the N6 of different influenza viruses. Furthermore, they strongly inhibit neuraminidase activity, reduce plaque size and number *in vitro,* exhibit strong ADCC activity, and even protect mice *in vivo* against lethal infection. Through this work it is possible to show, that antibodies directed against the NA are capable to prevent infection and could be used as therapeutics for disease caused by influenza viruses of the N6 subtype.

1. Introduction

Influenza virus infections are a major health burden worldwide and can cause up to 650.000 deaths every year. Seasonal influenza virus vaccines are available on an annual basis and are formulated each year based on a predication by the WHO of virus strains that are expected to circulate [1,4]. The influenza virus belongs to the family of *Orthomyxoviridae* and can be further classified into four different genera A, B, C and D. Influenza B and C viruses are widely circulating only among humans, whereas influenza A viruses can cause infections in avian and other mammalian species as well[5]. Influenza D viruses have been described the first time in 2011 and are believed to infect mainly cattle[6].

Aquatic birds are commonly identified as reservoirs of influenza A viruses. In birds, influenza infections are usually causing mild infections and are in general reported to be low pathogenic. Nevertheless, in the past, there were a few strains that showed a high mortality rate, which led to severe outbreaks within the avian species[7].

However, it is untypical that avian influenza viruses are transmitted to humans directly. This is based on the different form of sialic acid linked receptors within the respiratory tract of humans and in avian species. In humans, upper respiratory cells are mainly presenting 2,6 sialic acid residues, whereas avian influenza viruses are binding to α-2,3 sialic acid linked receptors[8]. However, pigs are known to serve as “mixing vessels” of avian and human influenza viruses, since they possess cells inside their trachea that are presenting both types of receptors[9]. Therefore, multiple viruses can infect the same cell, leading to a reassortment of viral genes, which results in newly created viruses[10].

In general, the influenza virus is a single-stranded negative sense RNA virus and possesses two surface glycoproteins, the hemagglutinin (HA) and the neuraminidase (NA) (Figure 1)[11]. The immunodominant protein on the viral surface is the HA, which binds to the sialic acid linked receptors on the host cells and mediates the fusion of the viral envelope bilayer and the endosomal membrane of the host cell[11]. Since both processes are essential for viral infection and the generation of new virions, the HA is an attractive target for vaccine design as well as for the generation of monoclonal antibodies (mAbs).

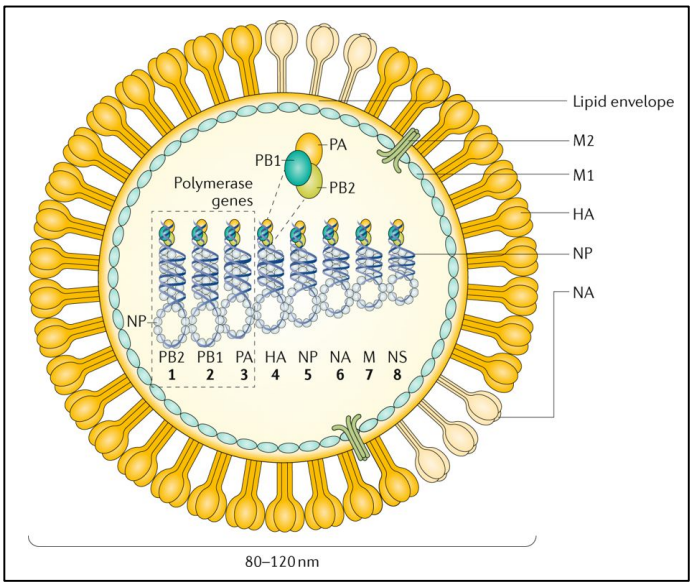

Figure 1 | Schematic figure of the Influenza virus. The virus is a single stranded negative sense RNA-virus and is approximately 80-120 nm in size. The genome is divided into 8 segments, which are all encoding for different internal as well as surface proteins. On the surface of the virus are the two major glycoproteins located, the hemagglutinin (HA) and the neuraminidase (NA). The HA is the immunodominant protein on the viral surface and mainly used as a target in vaccine design[12]. *Image taken from Krammer, F. et al. Influenza. Nat. Rev. Dis. Prim. (2018)[12].*

While the HA is clearly immunodominant, the NA also plays a critical role in infection and viral spread. The NA is a tetrameric type II transmembrane protein, with each monomer containing a 470-amino acid long residue, that encloses four different domains, a N-terminal cytoplasmic domain, a hydrophobic transmembrane domain, a hypervariable stalk domain and a globular head domain (Figure 2A)[13]. By inactivating the neuraminidase activity, the NA cannot cleave the sialic acid from glycans located on the host cell surface, called receptor-destroying activity[14]. Newly formed virus particles are then hindered from budding through the cell surface, which leads to an aggregation of those on the host cell surface (Figure 2B). This activity makes the NA to an important enzyme for the release of progeny virus particles from infected cells as well as to a crucial factor for the survival of the virus [13].

The head domain of the NA appears to be more stable and less susceptible to acquire mutations compared to the stalk domain. Furthermore, the globular head domain hosts the enzymatic active sites of the protein. Since the virus enters the host cell via the HA molecule, antibodies directed against the NA usually do not have neutralizing activity. However, since the virus depends on the NA for viral spread, antibodies can still protect against infection, as demonstrated by recent papers[15,16]. It has been shown that non-neutralizing antibodies have other function e.g. antibody-dependent cellular

cytotoxicity (ADCC), which can enable the lyse of target cells and therefore contribute to the prevention of infections[15]. The inhibition of the neuraminidase has also been shown to be effective in limiting infections and viral spreading. Therefore, various antiviral drugs are using this concept of neuraminidase inhibition successfully (e.g. Oseltamivir, Zanamivir) to lead to a reduced severity as well as to a decreased viral shedding[17].

Besides antiviral drugs, the neuraminidase is in discussion to be a potential component of influenza virus vaccines. At the moment, influenza vaccines are mostly based on the HA content, including none or a varying amount of NA. The main issue that makes it challenging to include the NA in influenza virus vaccines, is the lack of appropriate standardization methods to measure the NA content within the vaccine. By generating a vaccine that includes both, the HA and the NA component, it would be more efficient to compensate for potential variations within the HA leading to a better protection against influenza virus infections and to a reduction in epidemic outbreaks[18]. Therefore, the NA is a promising antigen in terms of vaccine development and should also be considered to be part of the universal influenza virus vaccine approach.

Influenza A virus zoonotic infections have already caused multiple, severe outbreaks within the human population. Those outbreaks were mostly based on the transmission of animal adapted influenza viruses to humans, which normally do not have immunity against those “novel” viruses[1]. The N6 subtype is usually common in avian species but can also be found in different mammalian isolates. The NA’s can be classified into group 1 (N1, N4, N5, N8) and group 2 (N2, N3, N6, N7, N9) as well as in an Influenza A-like group (N10, N11) (Figure 2C)[19].

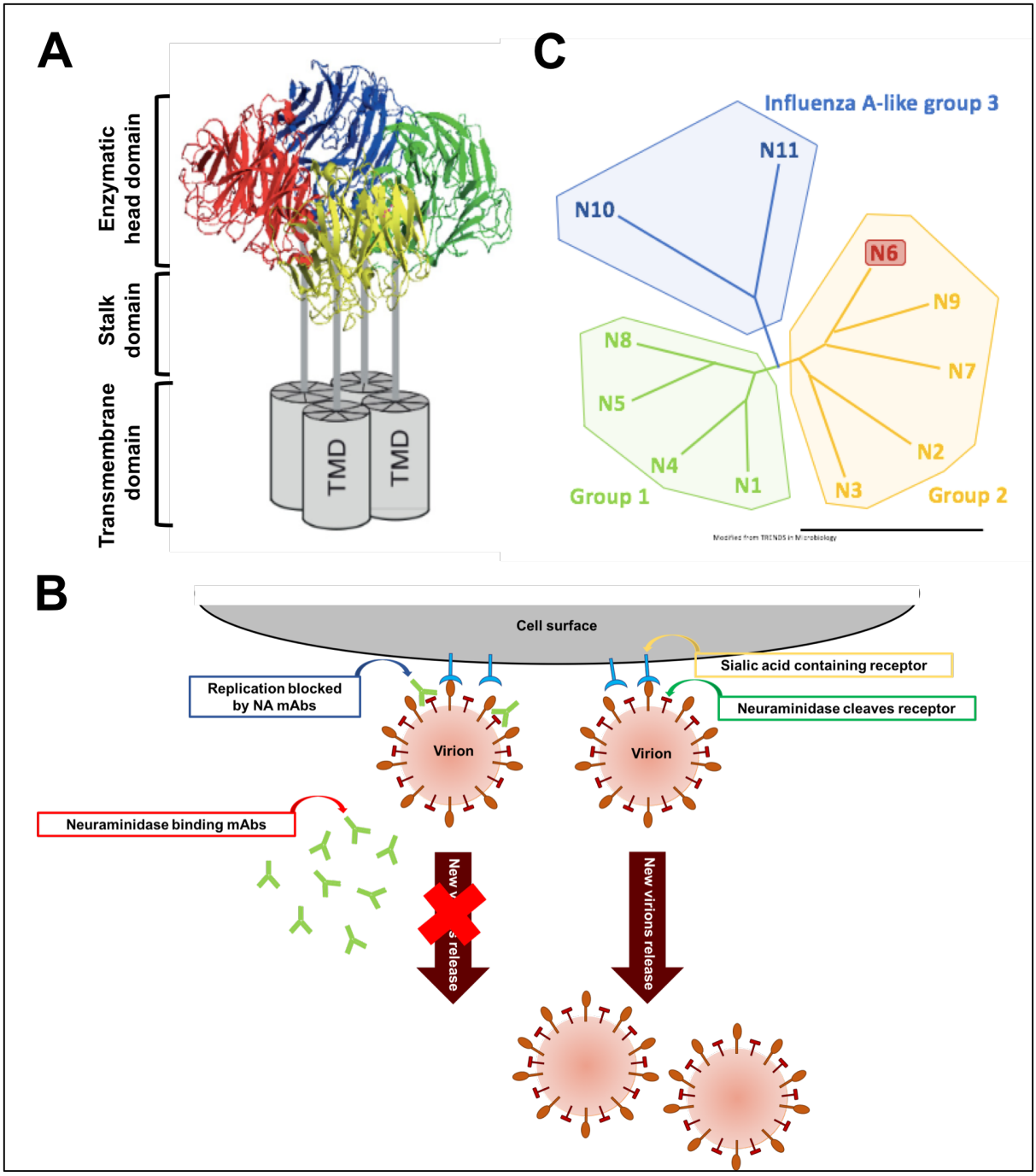

Figure 2 | Structure of the Neuraminidase, phylogenetic tree of the NA-subgroups and function of the NA. (A) Scheme of the neuraminidase structure showing the enzymatic head domain, the variable stalk domain and the transmembrane domain. *Image adapted from Air, G. M. et al.,* Influenza neuraminidase. Influenza.Other Respir.Viruses.*(2012)*[20]. **(B)** Phylogenetic tree displaying the different neuraminidase groups. *Image adapted from Wu, Y. et al., Bat-derived influenza-like viruses H17N10 and H18N11. Trends in Microbiology (2014)*[21]. **(C)** Replication inhibition of the influenza virus mediated through antibodies that are targeting the neuraminidase.

In 2014, an avian H5N6 virus successfully emerged and infected humans for the first time, which caused several deaths in the People's Republic of China (PRC)[3]. This type of N6 virus, originated from H6N6 and H5N1, is known to be a low virulent strain when

it comes to infect poultry and waterfowl [22]. However, once the virus accomplished the capability to infect humans, the reported cases were described as severe with strong influenza like symptoms. In Asia H5N6 viruses are nowadays more common in avian species than H5N1 infections and therefore raising global concerns regarding their potential as human pandemic threats[23]. In general, not much is known about the N6 and since it has crossed species barriers, it is extremely important to further study this NA and develop viable therapeutics against it[24]. Therefore, we generated murine monoclonal antibodies via hybridoma technology against the N6 of A/duck/Zhejiang/D9/2013 (H4N6), A/Caspian seal/Russia/T1/2012 (H4N6), A/swine/Missouri/A01727926/2015 (H4N6) and A/Shenzhen1/2016 (H5N6). The ten antibodies generated are highly cross-reactive and have differential binding profiles to various recombinant N6 proteins. Furthermore, six of the antibodies showed neutralizing potential at the highest antibody dilution against A/duck/Czechoslovakia/1956 (H4N6) *in vitro*. The antibodies were shown to impact the plaque formation and reduce the size of plaques in general. The antibodies successfully inhibited the activity of neuraminidase when tested *in vitro* and even displayed ADCC activity. In addition, when administrated two hours prior to infection, these antibodies protect mice from infection *in vivo*. Our work demonstrates the crucial role that NA antibodies can play against infection and these antibodies can be used as therapeutics for disease caused by influenza viruses of the N6 subtype.

2. Material and Methods

2.1 Cells, viruses and proteins

Sf9 insect cells adapted from the cell line ATCC CRL-1711 were grown in TNM-FH insect medium (Gemini Bioproducts) supplemented with 1 % Penicillin/Streptomycin (Pen/Strep), 1% Pluronic F-68 and 10% Fetal Bovine Serum (FBS). For passaging the baculoviruses in Sf9 cells 3 % TNM-FH insect medium (1% Pen/Strep, 1% Pluronic F-68, 3% FBS) was used. The BTI-TN-5B1-4 (High Five) cells were grown in serum-free SFM4-insect cell medium (HyClone) with 1% Pen/Strep. Madin-Darby Canine Kidney (MDCK) cells used for various assays were grown in complete Dulbecco's Modified Eagle's Medium (DMEM) (1% Pen/Strep, 10% FBS, 1% Hydroxyethylpiperazine Ethane Sulfonic Acid (HEPES)). For the hybridoma fusion SP2/0-Ag14 myeloma cells were grown in complete DMEM supplemented with 1% L-glutamine. The influenza viruses A/duck/Wisconsin/480/1979 (H12N6), A/redhead/Alberta/192/2002 (H3N6), A/duck/England/1956 (H11N6), A/gull/Maryland/704/1977 (H13N6), A/black-legged kittiwake/Quebec/02838-1/2009 (H13N6), A/ring-billed gull/Quebec/02434-1/2009 (H13N6), A/mallard/Alberta/125/1999 (H11N6), A/blue-winged teal/Illinois/10OS1546/2010 (H3N6) and A/blue-winged teal/Wisconsin/402/1983 (H4N6) were obtained from Biodefense and Emerging Infections Research Resources Repository (BEI Resources) and were then grown in 8-10 day-old embryonated chicken eggs (Charles River Laboratories). The eggs were grown for 2 days at 37°C. The allantoic fluid was harvested, aliquoted and stored at -80°C for further usage. The titer was determined by performing standard plaque assays on MDCK cells.

2.2 Animals

Female 6-8-week-old BALB/c mice (The Jackson Laboratory) were immunized intraperitoneal with 10^5 PFU/ml of A/swine/Missouri/A01727926/2015 followed by an intranasal infection after 21 days with a sublethal dose of 10^5 PFU/ml of the same virus. Three weeks later, a third infection was administrated with a sublethal dose of 10^3 PFU/ml of A/Shenzhen/1/2016, a low pathogenic virus rescued in a PR8 backbone (6:2). After 21 days, one mouse was boosted intraperitoneal with 100 µg of recombinant N6 protein of A/duck/Zhejiang/D9/2013 (H4N6) adjuvanted with 10 µg of poly I:C (Invivogen). For performing LD_{50} studies as well as for testing the prophylactic

efficiency of the N6 antibodies, female 6-8-week-old DBA/2J mice (The Jackson Laboratory) were used.

2.3 Generation of mouse mAbs

Three days after the final boost, the mouse was sacrificed, and the spleen sterile removed. The spleen was washed with phosphate-buffered saline (PBS) and then flushed with serum-free DMEM (Gibco; 1% Pen/Strep) using a 10ml syringe with a 20-gauge needle to obtain the splenocytes. The isolated splenocytes were centrifuged for 5 min at 400 x g. The supernatant was removed and the splenocytes washed three times with DMEM. The cells were counted using a Thoma cell counting chamber and then mixed gently with SP2/0-Ag14 myeloma cells in a ratio of 5 : 1. The cell mixture was centrifuged for 10 min at 400 x g. To fuse the splenocytes with the SP2 cells, 1ml of preheated polyethylene glycol (PEG) was dropwise added to the cell mixture. The cells were gently mixed after every drop and then incubated for 1 min at room temperature. Afterwards, DMEM was added and the mixture centrifuged for 10 min at 400 x g. The supernatant was removed, the cells resuspended in 25 ml of complete DMEM and then moved to a T175 flask. The cells were incubated at 37°C, 5 % CO_2 overnight. The following day, the cells were harvested for 10 min at 400 x g. The supernatant was aspirated and the pellet resuspended in 10 ml of cDMEM. The cell suspension was transferred into one 90 ml bottle of hybridoma – semi solid selection & cloning medium with hypoxanthine-aminopterin-thymidine (HAT, Molecular Devices) and mixed by gently shaking. The suspension was incubated for 20 min at room temperature and then spread into petri dishes using a 10-ml syringe with a 15-gauge Luer Stub adapter (Becton Dickinson). The plates were incubated for 10-14 days at 37°C with 5% CO_2.

Single colonies were picked and transferred to a 96-well plate containing Clonacell-HY Medium E (Molecular Devices). After 5 days, the supernatants of the individual hybridoma clones were used as primary antibody in an enzyme-linked immunosorbent assay (ELISA) to screen against reactivity to the recombinant N6 protein of A/duck/Zhejiang/D9/2013 (H4N6). Reactive clones were isotyped using the Pierce rapid antibody isotyping kit (Life Technologies). Only IgG heavy-chain subgroups were continuously expanded. The selected hybridoma clones were first expanded in Clonacell-HY Medium E and then constantly switched to Hybridoma SFM media (Gibco) supplemented with 1% Pen/Strep. The cells were grown to a final volume of

400 ml culture and then incubated for 10 days at 37°C, 5% CO_2. The cells were then spun down at 4000 x g for 15 min at 4°C. The supernatant was filtrated using a 0.22 µm pore size sterile filtration unit (Millipore) and the antibodies then purified by using a gravity flow column packed with protein G-Sepharose 4 Fast Flow beads (GE Healthcare). The antibodies bound to the resins were washed three times with PBS and were then eluted with 45 ml of 0.1 M Glycine-HCl buffer (pH=2.7). The eluted antibodies were neutralized by adding 5 ml of 2 M Tris-HCl buffer (pH=10) and were then concentrated by using 30 kDa Amicon filter units (Merck Millipore). The filter membrane was washed three times with PBS and the antibodies then resuspended in PBS to a final volume of 3 ml. The concentration was determined by using a Nanodrop (ThermoFisher Scientific).

2.4 ELISA to verify binding of mAbs against recombinant N6 proteins

To screen the hybridoma supernatants for reactivity against recombinant N6 protein, 96-well, flat bottom, non-sterile Immulon 4 HBX plates (ThermoFisher Scientific) were coated with 50 µl/well of 2 µg/ml recombinant protein in 1x coating buffer (Seracare) at 4°C ON. The coating solution was removed and the plate blocked with 100µl/well of 3% milk/Phosphate Buffered Saline with Tween (PBST, 0.1 % Tween) for 1h at room temperature. Afterwards, the blocking solution was removed and 50 µl of each hybridoma supernatant was added to the plate to act as primary antibody. The supernatant was incubated on the plate for 1h at room temperature. The plate was then washed three times with PBST and incubated for 1h at room temperature with anti-mouse secondary antibody (Anti-mouse IgG (H&L) Antibody Peroxidase Conjugated, Rockland) diluted 1:3000 in 1% milk/PBST (100µl/well). The plate was washed three times with PBST and 100 µl/well of SigmaFast o-Phenylenediamine dihydrochloride (OPD) developing solution (Sigma Aldrich) were added. The reaction was stopped after 10 min incubation at room temperature with 50 µl/well of 3M HCl. The plate was read with a Synergy H1 hybrid multimode microplate reader (BioTek) at an OD of 490 nm. To test the binding activity of the antibodies to different recombinant N6 proteins, the purified antibody was added to the plate at a start concentration of 30µg/ml and 1:3 serial dilutions in 1% milk/PBST (0.1% Tween) were performed.

2.5 Generation of recombinant N6 expression constructs

For the generation of the recombinant N6 expression constructs, the vector pFastBacDual containing an ampicillin resistance cassette was used. For the amplification of the insert, the RNA of the respective viruses was extracted (Direct-zol RNA MiniPrep Plus Kit, Zymo Research) and a reverse transcription (SuperScript™ III Reverse Transcriptase, Invitrogen) performed to obtain cDNA. The insert was then amplified by using the KOD-polymerase kit (Novagen). Afterwards, the PCR product was purified with the QIAquick PCR Purification Kit (Qiagen). The insert as well as the pFastBacDual vector were digested with the restriction enzymes XbaI and HindIII overnight at 37°C. The digest was loaded on a 1 % agarose gel and the vector as well as the insert were purified by using the QIAquick Gel Extraction Kit (Qiagen). The insert was ligated with the vector in a molecular ratio of 7:1 by using the T4 ligase (New England Biolabs Inc.) for 3 h at room temperature. Afterwards, 3 µl of the ligation mix were added to 50 µl of chemocompetent XL10 Gold *E. coli* cells and incubated on ice for 30 min. The cells were heat shocked for 45 seconds at 42°C and then incubated on ice for 1 min. For recovering 500 µl of SOC (Corning) were added to the bacteria and incubated shaking at 37°C for one hour. The bacteria were platted on LB-Ampicillin plates (imMedia™ Growth Medium, Invitrogen) and incubated overnight at 37°C.

Clones were screened by colony PCR and the positive ones inoculated in 4 ml of LB-media ON. The plasmid was extracted by mini-prep (QIAprep Spin Miniprep Kit, Qiagen) and afterwards sequenced. Plasmids that contain the desired insert, were re-transformed into DH10bac *E.coli* by adding 3 µl of the plasmid to 100 µl of chemocompetent DH10bac. After 20 min incubation on ice, the bacteria were heat shocked at 42° C for 45 sec and 500 µl of SOC media were added for recovery. The bacteria were incubated for 4h at 37°C on the shaker and were then platted on KTG plates (imMedin Kanagar (Invitrogen), gentamycin, 0.1 M Isopropyl β-D-1-thiogalactopyranoside (IPTG), tetracyclin, X-Galactose). The plates were incubated at 37°C for 48h. White colonies were picked and the bacmid DNA obtained by midi-prep (PureLink HiPure Plasmid Midiprep Kit, Invitrogen).

2.6 Transfection of N6-expression constructs into Sf9 cells

Sf9 cells were seeded at a density of $2x10^5$ cells/well in a 6-well plate in TNM-FH insect medium (1% Pen/Strep). For the transfection 2 µg of bacmid DNA were mixed

with 100 µl of TNM-FH insect medium and 6 µl of cellfectin (Invitrogen) with 100 µl of TNM-FH in a separate tube. The DNA as well as the cellfectin were incubated for 20 min at room temperature. The volumes of both tubes were combined and dropwise added to the cells. After 6h of incubation at 27°C without CO_2, the medium was changed to 3% TNM-FH (1% Pen/Strep, 1% Pluronic F-68, 3% FBS) and the plates incubated for 5-7 days under the same conditions. The cells were then spun down at 1000 x g for 5 min at 4°C. The supernatant was collected (=P0) and the cells used to verify protein expression by western blot. The P0 was then used for further passaging in Sf9 cells. Therefore, 5 x 10^6 Sf9 cells were seeded in a T175 flask with 3% TNM-FH media. The cells were infected with 300 µl of the previous passage and then incubated at 27°C without CO_2 for 5-7 days. The cells were harvested at 1000 x g and the supernatant (=P1) stored at 4°C. This procedure was repeated until P3, which was used as a working stock for protein expression.

2.7 Verification of protein expression by Western blot

The cell pellet obtained after the bacmid transfection into Sf9 cells, was used to verify protein expression. Therefore, the cell pellet was washed twice with PBS and was then resuspended in 100 µl PBS. An aliquot of 10 µl was mixed with 10 µl of
Sodium dodecyl sulfate (SDS)-loading dye (2x Laemmli buffer, Biorad; 5% β-mercaptoethanol) and then boiled for 15 min at 98°C. The sample was loaded on a 4-20% acrylamide gel (4–20% Mini-PROTEAN® TGX™ Precast Protein Gels, Biorad) and ran at 200V for 30 min. The gel was transferred to a nitrocellulose membrane (Amersham protran premium 0.45 NC, GE Healthcare Life Sciences) by semi-dry transfer at 0.18 A for 20 min. The membrane was blocked in 3% milk/PBST (0.1% Tween) for 1h at room temperature. Since all the proteins have a C-terminal hexahistidine tag, an anti-his primary antibody (Takara, 1:3000 in 1% milk/PBST) was used for detection. The membrane was incubated shaking for 1h at room temperature and was then washed three times with PBST. The secondary anti-mouse antibody (Anti-mouse IgG (H&L) Antibody Peroxidase Conjugated, Rockland) was diluted 1:3000 in 1% milk/PBST and incubated with the membrane for 1 h shaking at room temperature. The membrane was washed three times with PBST and then developed with the alkaline phosphatase (AP) conjugate substrate kit (Biorad).

2.8 Expression of N6 proteins in High Five cells

For the expression of one N6 protein, 8x T175 flasks of fully confluent High Five cells were harvested at 1000 x g for 10 min. The supernatant was removed and the cell pellets combined in 8 ml of P3 working stock. The baculovirus was incubated with the cells for 20 min inside the hood and then transferred to 500 ml of serum free SFM4 insect cell medium in a large Erlenmeyer-flask. The flask was incubated on the shaker for 3 days at 27°C. The culture was spun down at 4000 x g for 10 min at 4°C. The supernatant was collected and incubated with 8 ml of PBS washed nickel-nitrilotriacetic acid (Ni-NTA) beads (Qiagen)The Erlenmeyer-flask was washed with dH_2O to remove remaining cells before transferring the supernatant/beads solution back to the flask. The supernatant was incubated for 3-4 hours at 27°C on the shaker and then purified by gravity-flow chromatography using polypropylene columns (Qiagen). The flow through was discarded and the beads washed 4x with Ni-wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM Imidazole). The protein was eluted on ice by adding 4x 2ml of Ni-elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM Imidazole) for 5 min per elution step. Amicon filter units (Merck Millipore) were equilibrated with PBS for 10 min at 4000 x g. The eluted proteins were transferred to the equilibrated filter units and spun at 4000 x g until the fluid went through. The flow-through was discarded and the filter membrane washed three times with PBS. The protein was then resuspended by washing the filter membrane several times with 200 µl of PBS. The protein concentration was measured by Bradford and the aliquoted proteins then stored at -80°C. To check the protein integrity, a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was run and stained with Coomassie-blue (SimplyBlue SafeStain, Novex).

2.9 Plaque assay

To determine the viral concentration in plaque forming units per ml (PFU/ml), the virus was titrated in MDCK cells. Therefore, 3 x 10^5 cells/well were seeded in a 12-well plate and incubated overnight. On the following day, the virus was diluted in minimal essential medium (MEM) from 1:10 to 1:1.000.000. The cells were washed with PBS and 250 µl of the virus dilution were added for 1h at 37°C. Afterwards, an overlay was added consisting out of minimal essential medium (MEM), 2% Oxoid agar, 1% Diethylaminoethyl cellulose (DEAE) as well as L-1-Tosylamide-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin (1 µg/ml). The plates were incubated for

2 days at 37°C and then fixed with 3.8% of paraformaldehyde (PFA). The plaques were stained with crystal violet solution (Sigma Aldrich) and then counted to determine the PFU/ml.

2.10 Immunofluorescence assay

MDCK cells were seeded at a density of 50.000 cells/well in a sterile 96-well cell culture plate by using complete DMEM media and were then infected the next day with a multiplicity of infection (MOI) of 1.0 overnight for 16 h. The cells were fixed with 100 µl of 3.8 % paraformaldehyde for 1 h at room temperature. The plate was then blocked with 3% milk/PBS for 1 h at room temperature. The antibodies were diluted to a concentration of 30 µg/ml in 1% milk/PBS and 100 µl was added per well for 1 h at room temperature. The cells were then washed twice with 1x PBS and then incubated for 1 h with goat anti-mouse IgG heavy plus light chain (H+L)–Alexa Fluor 488 antibody (Abcam) diluted 1:1000 in 1% milk/PBS. Afterwards, the plate was washed three times with PBS and then kept in PBS during immunofluorescence microscopy (Olympus IX-70).

2.11 NA assay

To determine the EC_{50} (50% of maximal effective concentration) of the N6 viruses, a 96-well, flat bottom, non-sterile Immulon 4 HBX plate (ThermoFisher Scientific) was coated with 150µl/well of fetuin (Sigma Aldrich) at a concentration of 50µg/ml overnight at 4°C. On the following day, the virus dilutions were prepared in a separate plate. Therefore, the respective virus was 1:2 serially diluted, beginning with 300 µl in the first well. The dilutions were prepared in duplicates and then incubated shaking for 1h 40 min at room temperature. During this time, the fetuin coated plates were washed 6x with PBST and then blocked with 5% bovine serum albumin (BSA)/PBS for at least 1h at room temperature. Afterwards, the plates were washed 6x with PBST and 100 µl of the virus dilution were added for 2h at 37°C. The plates were washed another time and then incubated with 5 µg/100µl/well of peanut agglutinin (PNA, Sigma Aldrich) in the dark for 1h 45 min. The plates were washed again and developed by using 100µl/well of Sigmafast OPD solution (Sigma Aldrich). The reaction was stopped after 7 minutes in the dark by adding 50 µl of 3M HCL solution and detected at an absorbance of 490 nm with the Synergy H1 hybrid multimode microplate reader (BioTek). The EC_{50}-values were calculated by analysing the data with GraphPad prism.

2.12 Neuraminidase inhibition assay

For the performance of neuraminidase inhibition assays 96-well, flat bottom, non-sterile Immulon 4 HBX plates (ThermoFisher Scientific) were coated with 150 µl/well of fetuin (Sigma Aldrich) at a concentration of 50 µg/ml overnight at 4°C. The N6 antibodies were serially diluted 1:3 in PBS with a start concentration of 300 µg/ml in a separate plate. The respective viruses were diluted in PBS to 2x the 50% effective concentration (EC_{50}), which was determined in a NA-assay beforehand. The virus (75 µl/well) was then added to the mAbs dilution and incubated for 1h 40 min shaking at room temperature. During this time, the fetuin coated plates were washed 6x with PBST and then blocked with 5% BSA/PBS for at least 1h at room temperature. The plates were washed and 100 µl of the virus/antibody mixture were added for 2h at 37°C. Afterwards, the plates were washed again and were then incubated with 5µg/100µl/well of PNA for 1h 45 min in the dark. The plates were washed and then developed by using 100 µl/well Sigmafast OPD solution (Sigma Aldrich). After 7 minutes of incubation in the dark, the reaction was stopped by adding 50 µl of 3M HCL and the plates read at an absorbance of 490nm using a Synergy H1 hybrid multimode microplate reader (BioTek). The IC_{50} values were calculated by using GraphPad prism.

2.13 Antibody-dependent cell-mediated cytotoxicity reporter assay

To determine potential ADCC activity of our mAbs the ADCC reporter bioassay kit from Promega was used. Therefore, 5 x 10^4 MDCK cells/well were seeded in a white, flat bottom 96- well cell culture plate (Corning) the day before. The cells were washed with PBS and then infected with an MOI of 1 with the respective virus at 37°C for 16h. On the following day, antibody dilutions were prepared using a start concentration of 100 µg/ml. The antibodies were 1:3 serial diluted and then added in duplicates to the cells. The human derived monoclonal antibody CR9114 was included as a positive and an irrelevant anti-Lassa virus antibody was used as a negative control. Afterwards, 7.5 10^4 effector cells were added to the plate and incubated for 6 h at 37°C. To develop the plates 75 µl/well of Bio-Glo Luciferase Assay reagent (Promega) were added and the luminescence immediately measured using a Synergy Hybrid Reader (BioTek).

2.14 Plaque reduction neutralization assay

Plaque reduction neutralization assays were performed on MDCK cells, which were seeded at a density of 3 x 10^5 cells/well in a 12-well plate the day before. On the next

day, the antibodies were serially diluted 1:3 in 1x MEM and 50 µl of A/duck/Czechoslovakia/1956 (1000 PFU/ml) were added to each dilution and incubated shaking at room temperature for 1h. Afterwards, the cells were washed with PBS and the antibody-virus mixture was added for 1h at 37°C. The mixture was then aspirated and the cells overlaid with agar consisting out of minimal essential medium (2xMEM), 2% Oxoid agar, 1% DEAE, TPCK-treated trypsin as well as the respective antibody. The plates were incubated at 37°C for 2 days and the cells then fixed with 3.8% paraformaldehyde. The plaques were visualized by immunostaining. Therefore, the agar-overlay was removed and the plates blocked with 3% milk/PBS for 1h at room temperature. Afterwards, an antibody-cocktail made out of all 10 N6-antibodies diluted 1:3000 in milk/PBS was added and incubated for 2h at room temperature. The plates were washed and incubated with secondary antibody (Anti-mouse IgG (H&L) Antibody Peroxidase Conjugated, Rockland) diluted 1:3000 in 1% milk/PBS for 1 h. The plates were washed again before developing by using Trueblue reagent (KPL). The plaque number as well as the plaque size was determined for each antibody dilution.

2.15 Determination of the LD_{50} for N6 viruses

For determining the LD_{50} value of A/duck/Czechoslovakia/1956 (H4N6) and A/Shenzhen/1/2016 (H5N6), 6-8-week-old DBA/2J mice (The Jackson Laboratory) were intranasally infected with 10^2, 10^3, 10^4 and 10^5 PFU of the respective virus (n=4 per group). Their weight loss as well as survival was monitored over 14 days. Mice were sacrificed if they were dropping under 75% of their initial body weight. The LD_{50} was then calculated by using the Reed and Muench method[25].

2.16 Evaluation of the prophylactic efficacy in mice

To test the prophylactic efficiency of the N6 mAbs, female 6-8-week-old DBA/2J mice (The Jackson Laboratory) received an antibody dose of 5mg/kg intraperitoneal (n=5 per group). An irrelevant anti-Lassa antibody KL-AV-IA12 was given to one group as a negative control. Two hours after antibody administration, the mice were anesthetized by using 100 µl/per mouse of a ketamine-xylazine-H_2O (1:1:4.3) mixture and then intranasally challenged with 5x LD_{50} of A/duck/Czechoslovakia/1956 (H4N6) or low pathogenic A/Shenzhen/1/2016 (H5N6). Both viruses were rescued 6:2 in a PR8 backbone beforehand, containing the internal genes of A/Puerto Rico/8/1934 and the HA and NA of the respective virus. Their weight was monitored over 14 days and mice, which lost more than 25% of their initial body weight were sacrificed.

3. Results

3.1 Specific antibodies against the N6 subtype were generated through hybridoma fusion

To generate murine hybridoma clones, a naïve single BALB/c mouse was intraperitoneal immunized with A/swine/Missouri/A01727926/2015 (H4N6) to induce a first immune response against both surface glycoproteins. The mouse was given the same virus through a different route three weeks later, to enhance this response. For the third time, the human derived virus A/Shenzhen/1/2016 (H5N6) was administrated, which contained a different hemagglutinin but the same NA subtype as used before. Through this concept, a broader antibody response against the N6 should be induced. For the final boost, recombinant N6 protein of A/duck/Zhejiang/D9/2013 was given intraperitoneal, to enhance the immune response towards the N6 subgroup. Afterwards, the mouse was sacrificed by cervical dislocation and the spleen removed to generate N6 specific hybridoma clones (Figure 3).

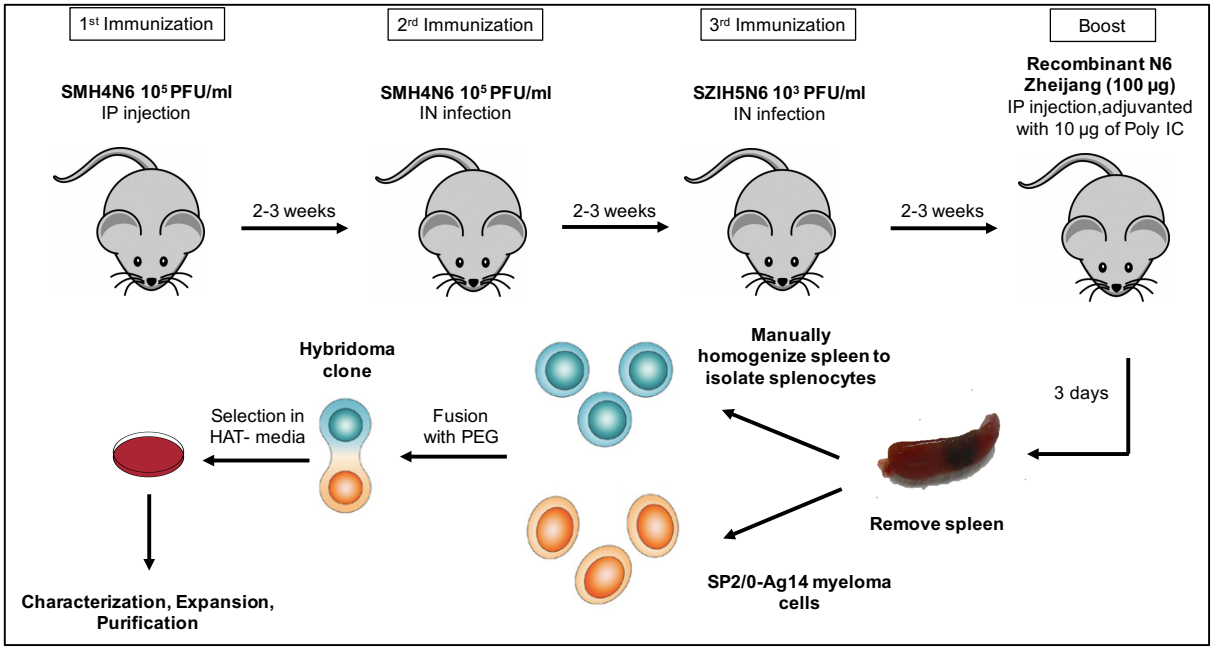

Figure 3 | Generation of murine monoclonal antibodies against the N6 influenza subtype. A naïve female 6-8 weeks old BALB/c mouse was intraperitoneal immunized with 10^5 PFU/ml of A/swine/Missouri/A01727926/2015 (SMH4N6) to induce a first immune response. After 2-3 weeks, the mouse was given the same virus intranasally with a sublethal dose of 10^5 PFU/ml. For the third immunization, 10^3 PFU/ml of the low pathogenic A/Shenzhen/1/2016 (SZIH5N6) were used to enhance the immune response towards the N6. For the final boost, 100 µg of recombinant N6 protein expressed through the baculovirus system were intraperitoneal administrated and adjuvanted with 10 µg of poly I:C. After three days, the mouse was sacrificed and the spleen removed and homogenized. The isolated splenocytes, were fused with SP2/0-Ag14 myeloma cells, using polyethylenglycol (PEG) to obtain

hybridoma clones. The clones were selected in HAT-media and then further characterized, expanded and purified.

3.2 Antibodies are showing strong cross reactivity against various N6 viruses

By using hybridoma technology it was possible to generate 31 monoclonal antibodies, which bound to N6 of A /duck/Zhejiang/D9/2013. By further characterization, a panel of 10 antibodies were identified to have an IgG heavy chain, and were therefore used for the following experiments. Various recombinant N6 proteins were expressed within the baculovirus system and used for quantitative ELISA to show binding activity of those mAbs *in vitro*. Usually, the structure of neuraminidases contains a N-terminal cytoplasmic domain, a transmembrane domain and a hypervariable stalk domain, which is more susceptible for mutations than the globular head domain. The C-terminal head domain contains the unique enzymatic active sites as well as a universally conserved sequence, which is the same through all types of NA regardless of their group (Figure 4A). However, for the expression of recombinant N6 proteins, the constructs were designed starting at their globular head domain, missing the other three preceding (smaller) domains. This design leads to the obtainment of soluble proteins, which can be directly purified from the cell supernatant. Otherwise, the glycoproteins would be expressed on the surface of the cells, leading to difficulties during purification as well as to folding and stability issues. However, in ELISA it was possible to see that all 10 antibodies were showing high cross-reactivity against the purified recombinant N6 proteins of A /duck/Zhejiang/D9/2013, A/Caspian seal/Russia/T1/2012 and A/swine/Missouri/A01727926/2015 (Figure 4B-D). An anti-his antibody was included as a positive control, since all recombinant proteins expressed are tagged on their C-terminus with a hexahistidine tag. However, by testing the reactivity to the more distant N6 of A/Shenzhen/1/2016 only KLN6-3F10, KLN6-3B5 and KLN6-3E1 were binding (Figure 4E). To see if the antibodies are cross-reacting to other NAs within group 2 NAs, recombinant N9 of A/Anhui/1/13 (H7N9) was used. None of the antibodies was showing reactivity against the N9, which is the closest related NA to N6 (Figure 4F). This indicates, that all 10 antibodies are highly specific for the N6 subgroup.

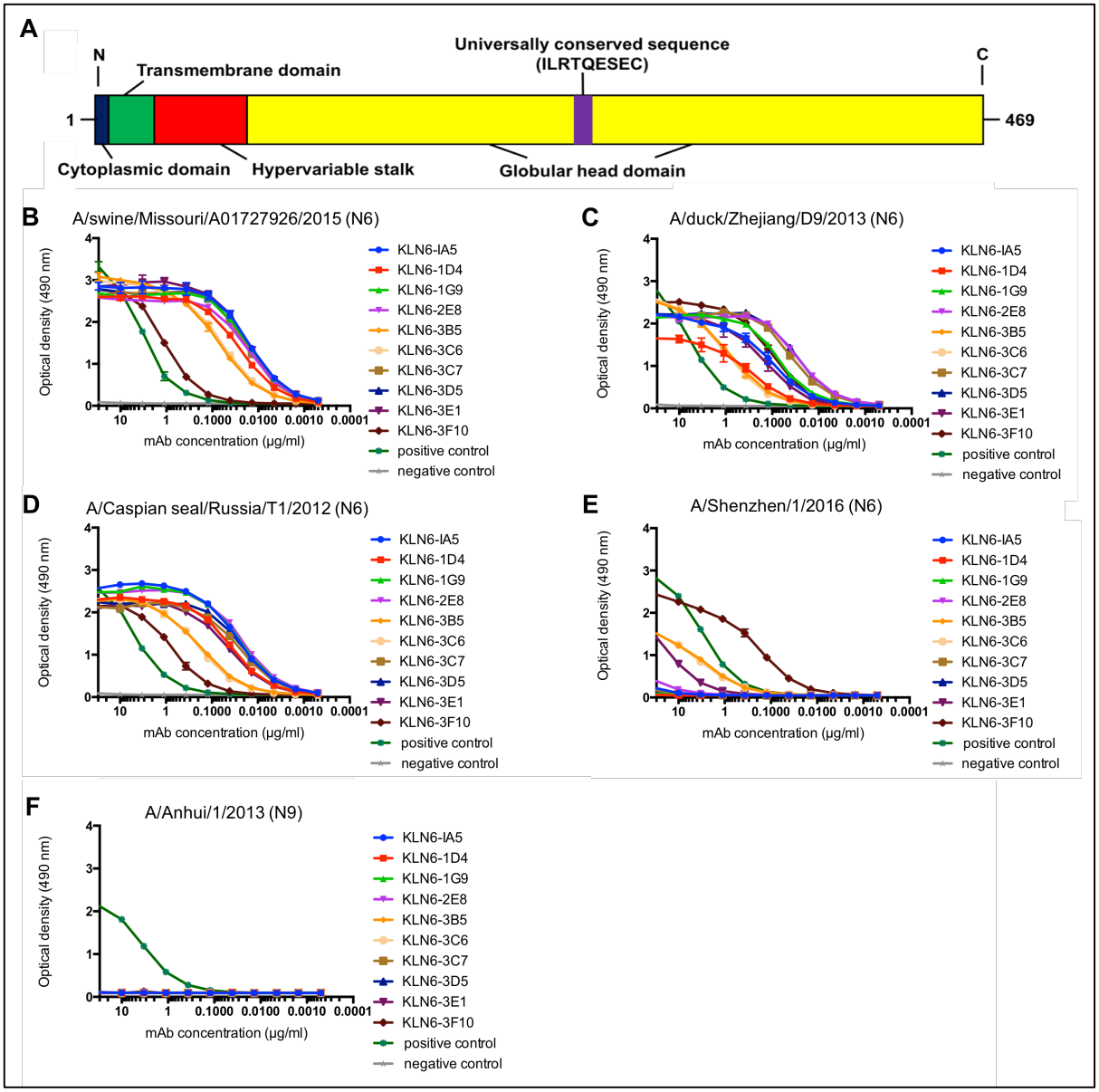

Figure 4 | N6 antibodies are showing high cross-reactivity through different species (A) Schematic structure of the neuraminidase glycoprotein showing the N-terminal cytoplasmic domain, the transmembrane domain, the hypervariable stalk domain and the globular head domain as well as the universal conserved sequence[13]. **(B-E)** The generated murine mAbs are showing high cross-reactivity against the recombinant NA proteins of various N6 influenza viruses. The ELISA plates were coated with 2µg/ml of recombinant N6 protein. The antibody starting concentration was 30µg/ml followed by a 1:3 dilution. An anti-Lassa virus KL-AV-IA12 antibody was used as a negative control and an anti-histidine antibody included as positive control. **(F)** The N6 reactive antibodies are not binding to other NAs within the same group. The reactivity of the antibodies was tested against recombinant N9 protein of A/Anhui/1/13 (H7N9). The controls were the same as mentioned before.

By looking at the NA sequences of these viruses, it was possible to see that the N6s of A /duck/Zhejiang/D9/2013 and A/Caspian seal/Russia/T1/2012 share an amino acid similarity of 94%, whereas A/duck/Zhejiang/D9/2013 and A/swine/Missouri/ A01727926/2015 as well as A/Caspian seal/Russia/T1/2012 and A/swine/Missouri/

A01727926/2015 have 90% similarity. The NA of A/Shenzhen/1/2016 appears to be more different compared to A/ swine/Missouri/ A01727926/2015 with a similarity of 85% but is closer to A/Caspian seal/Russia/T1/2012 and A /duck/Zhejiang/D9/201 with 92% similarity. In general, the sequence analysis showed that the N6 appears to be pretty conserved among avian, mammalian and human species. However, it is possible to see that besides the high amino acid similarity the viruses selected for this analysis were clustering in two groups, whereas the A/Shenzhen/1/2016 appears to be more distinct (Figure 5).

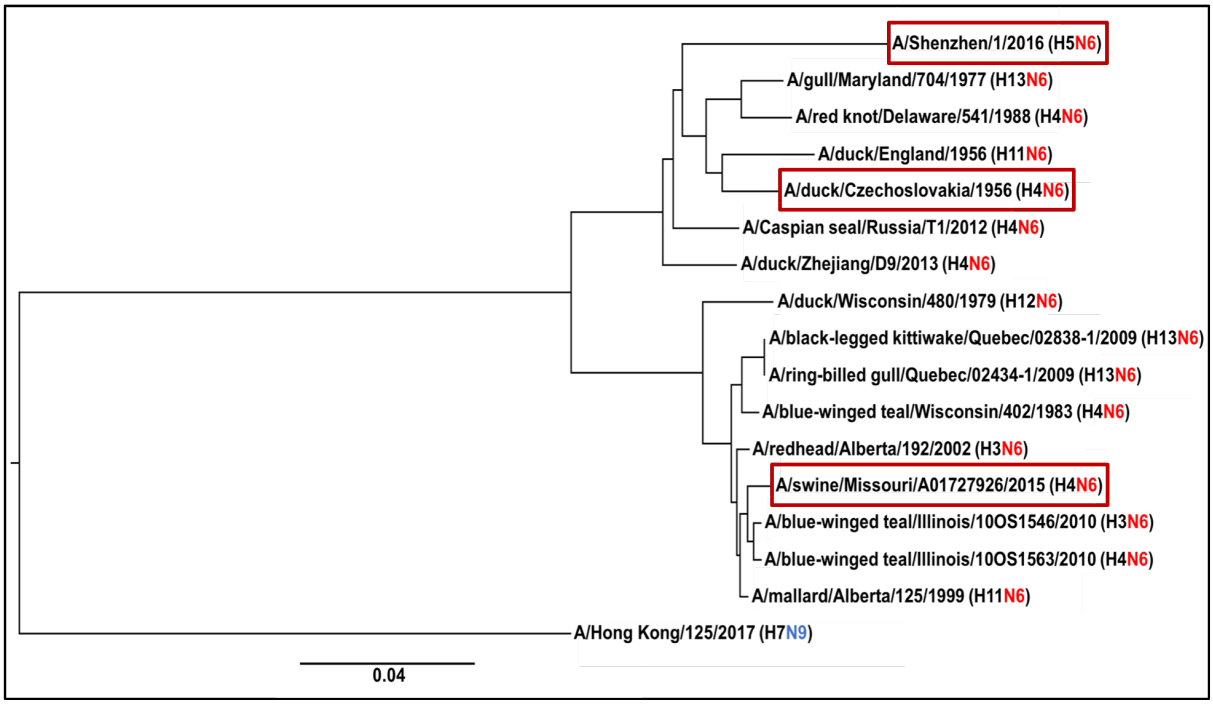

Figure 5 | Phylogenetic tree of selected viruses within the N6 subgroup. N6 influenza viruses are conserved across avian, mammalian and human species. The selected influenza A viruses used for the sequence analysis showed high amino acid similarity compared to each other (> 90%), except the more distinct human A/Shenzhen/1/2016 (H5N6) virus, which showed only 85% similarity to A/swine/Missouri/ A01727926/2015. The viruses used for further experiments, are highlighted in red squares.

However, since recombinant proteins were used to asses binding activity via ELISA, the binding of the N6-mAbs was furthermore tested in an immunofluorescence assay to detect binding to N6 proteins in their native conformation presented on the virus. Therefore, MDCK-cells were infected with a multiplicity of infection (MOI) of 1 with the respective virus. The cells were stained with the isolated N6-mAbs. The human antibody CR9114 was used as a positive control and an irrelevant anti-LASV antibody KL-AV-IA12 as a negative control. It was possible to see that the antibodies were showing broad binding activity to all 14 different N6 viruses included in this assay

(Figure 6). Since the viruses cover a broad time-span from 1956 (A/duck/Czechoslovakia/1956) to 2013 (A/duck/Zhejiang/D9/2013), it appears that the N6 protein did not undergo drastic changes over the years. The best and broadest binding through all viruses was seen for KLN6-2E8, KLN6-3C7 and KLN6-3F10. Those three antibodies were also showing the highest binding to the human isolate of A/Shenzhen/1/2016. Compared with the ELISA data, only KLN6-3F10 showed binding in both assays for A/Shenzhen/1/2016. However, the N6-viruses received from BEI resources were obtained in their wildtype from and are therefore known the be less infectious in mice accompanied with a low morbidity rate at a high viral titer. Therefore, the viruses A/Shenzhen/1/2016 (H5N6), A/duck/Czechoslovakia/1956 (H4N6) and A/swine/Missouri/ A01727926/2015 (H4N6) were selected for further experiments, since they were rescued in a PR8 backbone beforehand. This backbone contains all the internal genes from A/Puerto Rico/8/1934 as well as the neuraminidase and hemagglutinin from the respective virus, which leads to a 6+2 reassortant strain. This strategy was chosen, since the backbone of A/Puerto Rico/8/1934 is known to increase the infectiousness in the DBA/2J mice model, is safe in humans and commonly used as a vaccine backbone.

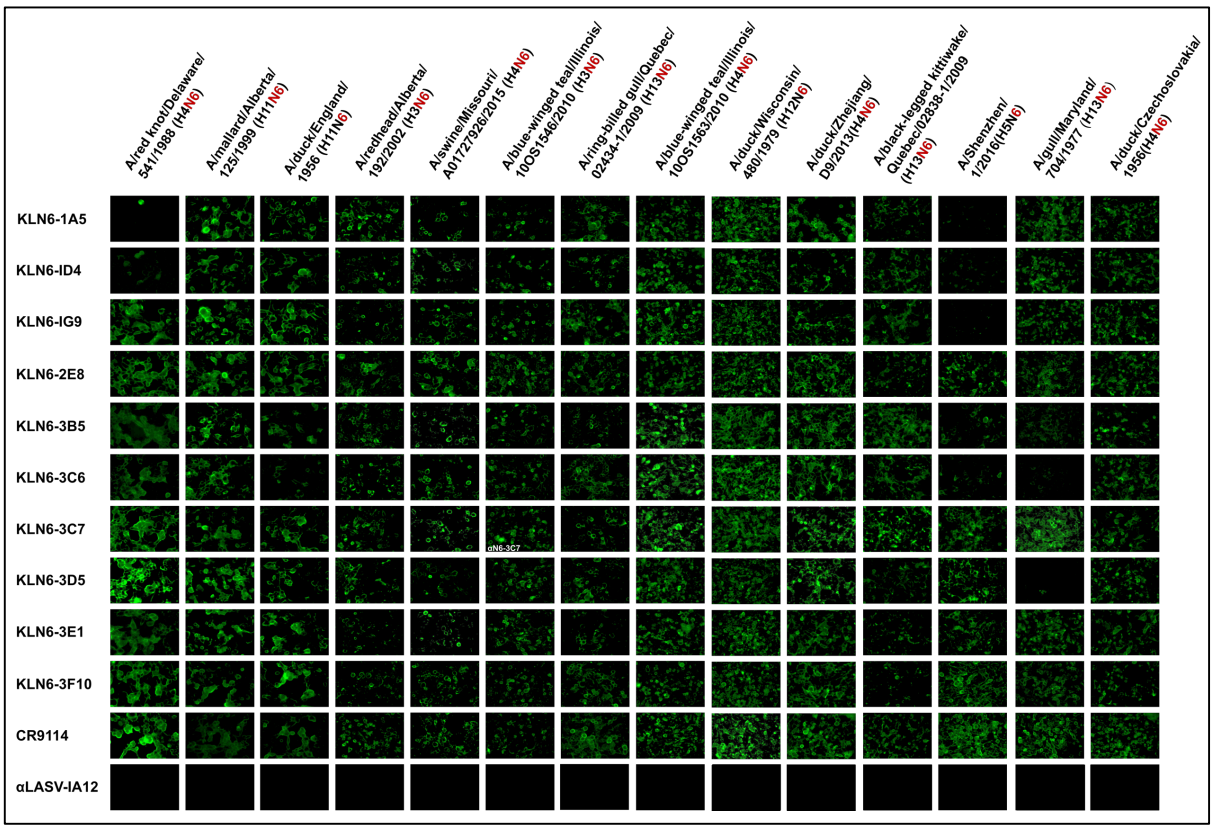

Figure 6 | Immunofluorescence-assay of different N6-expressing influenza viruses. The isolated monoclonal antibodies are binding to cells infected with various N6-influenza viruses. The cells were

infected with an MOI of 1 overnight with 14 different influenza viruses, that are all expressing N6. The cells were stained with 30 µg/ml of the respective mAbs. CR9114 was used as a positive control and KL-AV-IA12 as a negative control. Antibodies are showing high binding activity against all viruses except A/Shenzhen/1/2016. The highest binding was seen for KLN6-2E8, KLN6-3C7 and KLN6-3F10. Especially for the A/Shenzhen/1/2016 virus, only the antibodies KLN6-2E8 to KLN6-3F10 show intense binding.

3.3 Anti-N6 antibodies demonstrate NI as well as plaque reducing activity against various viruses

To determine if the isolated N6-antibodies are mediating neuraminidase inhibition activity, a fetuin-based neuraminidase inhibition assay was performed. Through this assay, it is possible to detect if our antibodies prevent the enzymatic ability of the neuraminidase to cleave sialic acids from newly formed viruses, by binding to either the active site or simply by steric hindrance. All 10 antibodies were tested regarding their NI-activity against the viruses A/Shenzhen/1/2016 (H5N6), A/duck/Czechoslovakia/1956 (H4N6) and A/swine/Missouri/ A01727926/2015 (H4N6). In this assay, CR9114 was included as a positive control. Even though, this antibody binds to the stalk domain of the hemagglutinin, it can still mediate neuraminidase inhibition activity most likely through steric hindrance. As a negative control KL-AV-IA12 was used. For the mammalian isolate A/swine/Missouri/ A01727926/2015 (H4N6), all 10 antibodies showed 100% NI-activity in the highest antibody dosage of 300 µg/ml (Figure 7C). However, when comparing the IC_{50} values it was possible to see that the antibodies KLN6-1A5, KLN6-1G9, KLN6-2E8, KLN6-3C7, KLN6-3D5 had the lowest values, all of which were < 0.05 µg/ml indicating their high effectiveness. The antibodies KLN6-3B5 and KLN6-3C6 displayed IC_{50} values of 6.9 µg/ml and 7.5 µg/ml. Almost the same was true for the avian isolate A/duck/Czechoslovakia/1956 (H4N6), where the 8 antibodies KLN6-1A5, KLN6-1D4, KLN6-1G9, KLN6-2E8, KLN6-3C6, KLN6-3D5, KLN6-3E1 and KLN6-3F10 displayed an inhibition activity between 93%-99%. The best IC_{50}-values were obtained through, KLN6-2E8 (IC_{50} = 0.028 µg/ml), KLN6-3C6 (IC_{50} = 0.026 µg/ml) and KLN6-3D5 (IC_{50} = 0.023 µg/ml). The antibodies KLN6-3B5 (IC_{50} = 14.84 µg/ml) and KLN6-3C7 (IC_{50} = 32.80), showed less inhibition activity of approximately 77% (Figure 7A). For both strains, the positive control showed over 50% maximum inhibition, which underlines that the antibody, even though as a HA-stalk binder has measurable NI-activity, which can be displayed in an IC_{50}-value.

For the human isolate A/Shenzhen/1/2016, the positive control had the highest inhibition value of around 89%, indicating that the NI-activity of CR9114 could be more effective in some groups of HA's compared to others. However, KLN6-3F10, showed the highest NI-activity of all 10 N6 antibodies, with a maximum inhibition of 84% (Figure 7B). This antibody also had the best IC_{50} value of 16,56 µg/ml followed by KLN6-3B5 (IC_{50} = 48.32), whereas the other antibodies didn't have detectable IC_{50}'s, since an inhibition of 50% could never been reached, not even at the highest possible antibody concentration.

Furthermore, the neutralizing activity of the N6 antibodies was determined through a plaque-reduction neutralization assay. It is commonly known, that NA antibodies do not prevent against infection in the first place, but can inhibit the spread of newly formed viruses. This can be seen, in the size of the resulting plaques as well as in the number of plaques. For the determination of plaque reduction activity, we used the avian A/duck/Czechoslovakia/1956 (H4N6) virus. The antibodies KLN6-1A5, KLN6-1D4, KLN6-1G9, KLN6-3B5, KLN6-3D5 and KLN6-3E1 showed no plaques in the highest antibody concentration of 100 µg/ml. The antibodies KLN6-2E8, KLN6-3C6 and KLN6-3C7, showed plaques already in the first antibody dilutions with a stronger increase of plaque size until the lowest antibody dilution (Figure 7D). The same antibodies, KLN6-2E8, KLN6-3C6 and KLN6-3C7, were also showing less reduction in the number of plaques. Whereas the remaining 6 antibodies, reduced the amount of plaques rapidly, indicating that they could have neutralizing potential (Figure 7E).

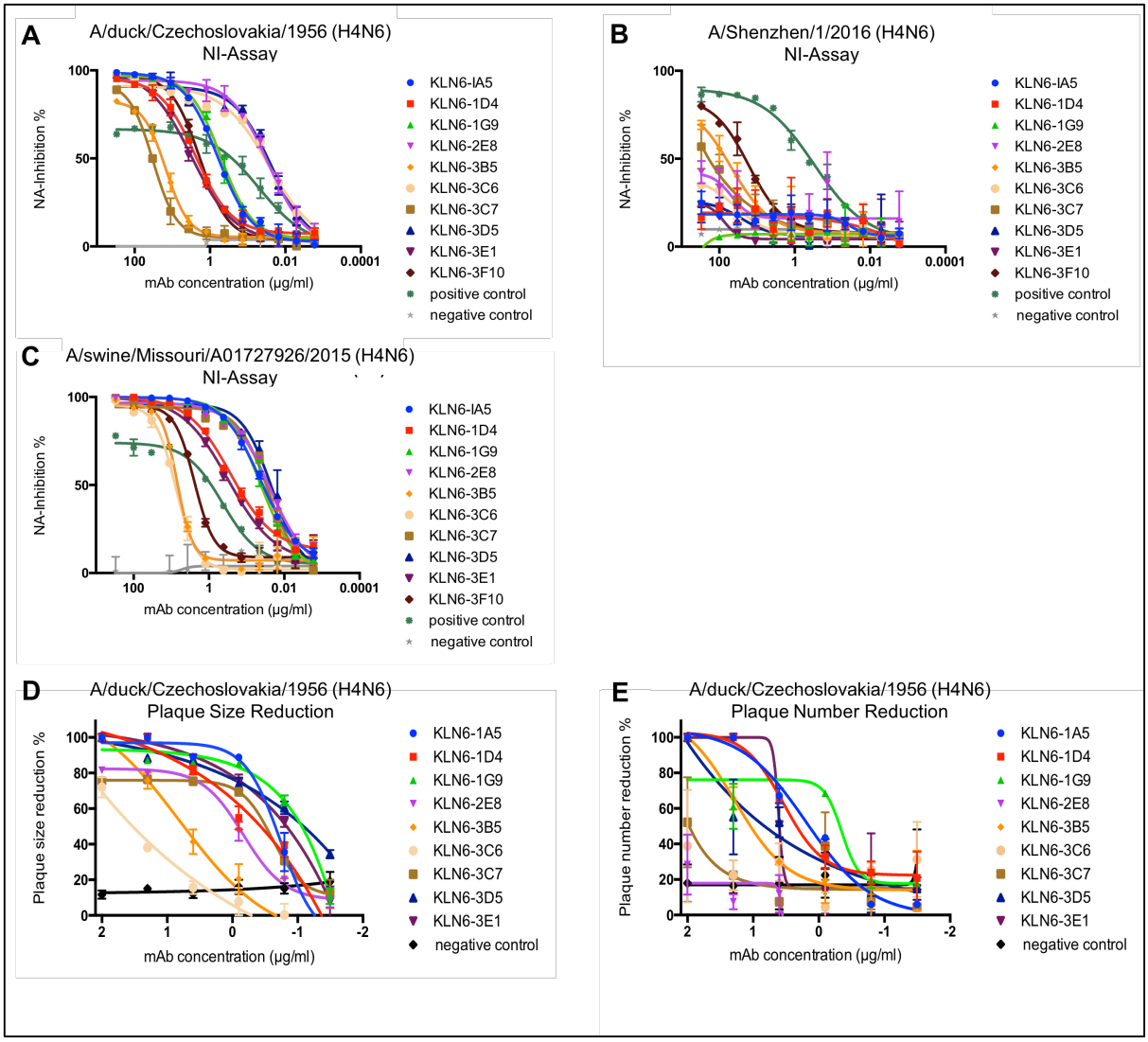

Figure 7 | Neuraminidase inhibition activity and neutralization activity of the isolated N6 antibodies. (A-C) Antibodies are showing strong neuraminidase inhibition activity against all three selected virus strains. The activity was tested in a fetuin-based NI-assay, with an antibody starting concentration of 300 µg/ml. The human antibody CR9114 was used as a positive control and KL-AV-IA12 was used as a negative control. For A/swine/Missouri/ A01727926/2015 **(C)** all 10 antibodies showed 100% inhibition at the highest antibody concentration of 300 µg/ml. In case of A/duck/Czechoslovakia/1956 **(A)** 8 antibodies (KLN6-1A5, KLN6-1D4, KLN6-1G9, KLN6-2E8, KLN6-3C6, KLN6-3D5, KLN6-3E1 KLN6-3F10) showed inhibition between 93%-99%, whereas the remaining two antibodies (KLN6-3B5, KLN6-3C7) displayed around 77% inhibition activity. For A/Shenzhen/1/2016 **(B)** the maximum inhibition was reached at 80% mediated through the antibody KLN6-3F10. **(D-E)** Antibodies are showing reduction in plaque size as well as in plaque number. The virus was incubated with the respective mAb for 1 h, before the mixture was added to MDCK cells. After one hour, the mixture was removed and an agar-overlay containing the respective antibody was added to the cells. The assay was incubated for 48 h, to determine plaque size and plaque number.

3.4 Anti-N6 antibodies show distinct pattern of antibody-dependent cell mediated cytotoxic activity

Neuraminidase antibodies are known to be potentially protective *in vivo* through different cell mediated functions like ADCC and ADCP[15]. To determine if the N6-antibodies are showing ADCC activity against the three selected strains an ADCC reporter assay was performed. Against A/swine/Missouri/ A01727926/2015 (H4N6) all 10 antibodies showed ADCC activity (Figure 8A). However, KLN6-3B5 and KLN6-3C6, showed the highest activity with more than 20-fold induction. The antibodies KLN6-2E8 and KLN6-3F10 had the lowest activity of under 10-fold induction. For A/duck/Czechoslovakia/1956 and A/Shenzhen/1/2016, the activity was lower with an overall maximum of around 12-fold-induction for the A/Shenzhen/1/2016 and around 10-fold induction for the A/duck/Czechoslovakia/1956 (Figure 8B-C). The highest activity, was again mediated through the antibodies KLN6-3B5 and KLN6-3C6. However, for A/Shenzhen/1/2016, KLN6-3F10 also almost showed a 10-fold induction and KLN6-1A5 and KLN6-2E8 had approximately a 5-fold induction of activity. For the remaining antibodies, no ADCC reporter activity could be detected. In case of A/duck/Czechoslovakia/1956 all antibodies showed an ADCC activity ranging from 5 to 12-fold induction. As a positive control the human antibody CR9114 was used and showed the highest ADCC activity through all three virus strains. The negative control KL-AV-IA12, did not show any activity.

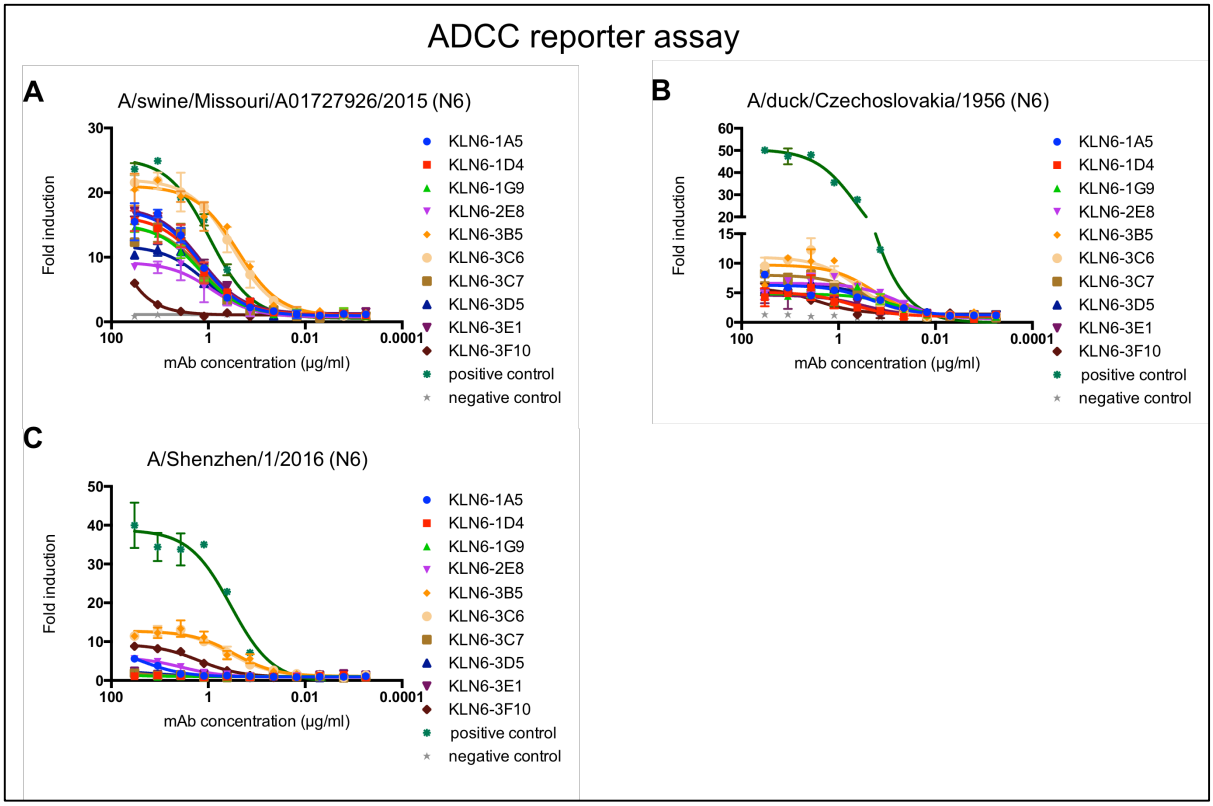

Figure 8 | Neuraminidase antibodies against the N6 subtype are showing ADCC activity *in vitro*. MDCK cells were infected overnight with the respective viruses. On the following day, mAb dilutions as well as the effector cells were added to the infected MDCK cells. The ADCC activity was obtained, through luminescence measurement at the end of the assay. The stalk binding human antibody CR9114 was used as a positive control and KL-AV-IA12 as a negative control.

3.5 Antibodies reveal *in vivo* protection against avian N6 virus

For the preparation of an *in vivo* antibody protection study in the DBA/2J mouse model, the murine lethal dose 50 (LD_{50}) for A/duck/Czechoslovakia/1956 (H4N6) was determined. For this, the mice were intranasally infected with the virus, which was rescued (6:2) in a PR8 backbone. The weight loss as well as the survival were monitored over 14 days (Figure 9A-B). The virus showed morbidity from 10^5 to 10^3 PFU. The mice started to drop under 75% of their body weight on day 6 post infection. Therefore, it was possible to observe, that the virus could be used in further experiments, since it was capable of infect mice and result in morbidity and mortality. The calculated LD_{50} value appeared to be 2.9 x 10^4 PFU/50µl.

To test if the N6-antibodies have a protective effect in an *in vivo* challenge model, we administrated 5mg/kg of the respective antibody 2 h prior to infection. The mice were then intranasally challenged with 5xLD_{50} dose of A/duck/Czechoslovakia/1956 (H4N6). After challenge, the mice were monitored regarding weight loss and survival for 14 days (Figure 9C-D). It was possible to see, that all antibodies except KLN6-3E1,

KLN6-3D5 and KLN6-3C7, were completely protective in the DBA/2J mouse model. Furthermore, the antibodies KLN6-1A5, KLN6-3C6 and KLN6-3F10 even protected from weight loss. The antibodies KLN6-3C7 and KLN6-3D showed 80% and KLN6-3E1 60% protectiveness *in vivo*. The antibodies KLN6-1D4 and KLN6-1G9 displayed a higher weight loss, but mice started to recover on day 6 post challenge. The same was true for KLN6-3B5 and KLN6-2E8, where mice started to gain weight after day 8. Mice treated with an irrelevant KL-AV-IA12 antibody, showed rapid weight loss and died by day 9 post challenge.

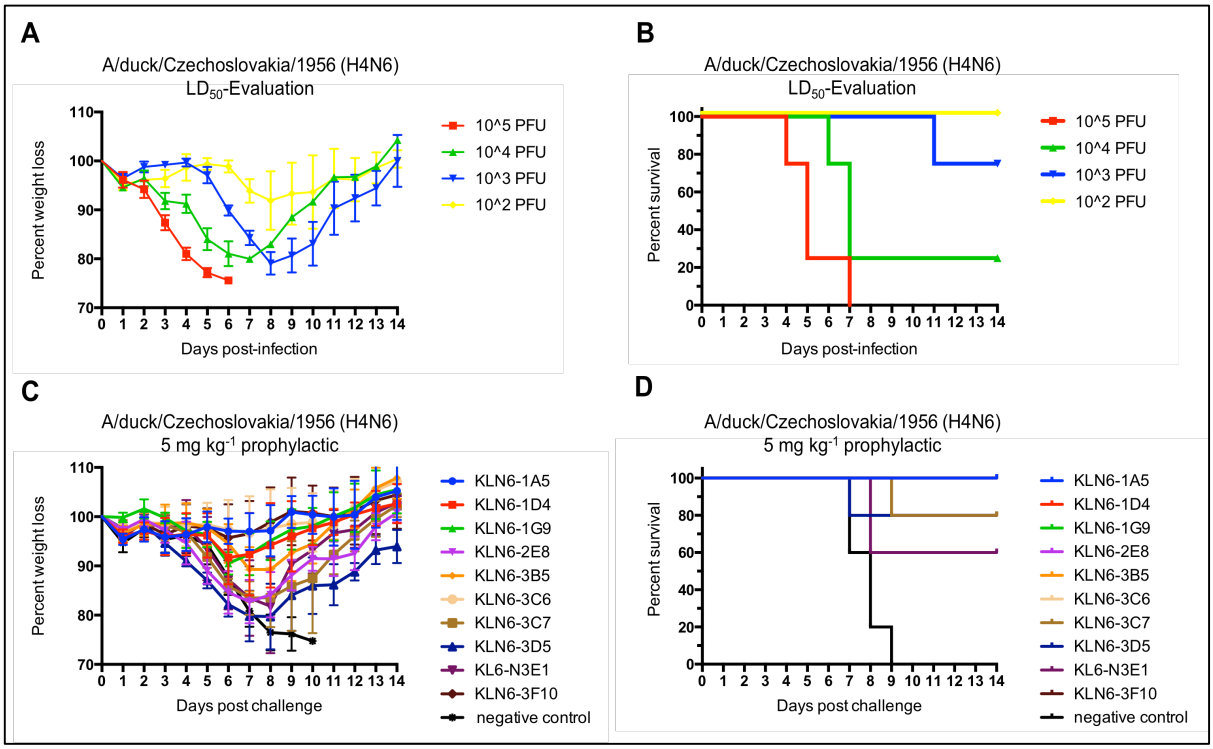

Figure 9 | Determination of the murine lethal dose (LD_{50}) of the A/duck/Czechoslovakia PR8 6:2 reassortant virus and prophylactic efficacy in the DBA/2J mouse model. **(A)** Weight loss was monitored over 14 days in 6-8-week-old female DBA/2J mice (n=4 per group), which were intranasally infected with A/duck/Czechoslovakia (H4N6). A weight loss of over 25% was defined as the human endpoint. **(B)** Survival curves showing the survival in percent for different viral dosages from 10^2 to 10^5 PFU. **(C)** Weight loss curve in a prophylactic setting. 5 mg/kg of antibody was administrated to 6-8-week-old female DBA/2J mice (n=5 per group) 2 h prior to challenge. The mice were challenged with 5xLD_{50} dose and the weight monitored over 14 days. KL-AV-IA12 was used as an irrelevant antibody. **(D)** Survival curves showing the survival in percent after prophylactic treatment.

4. Discussion

The aim of this study was to further investigate the antigenicity, immunogenicity and protective potential of the mainly uncharacterized N6 protein in different influenza virus strains as a potential target for antiviral approaches or as a component for influenza virus vaccines.

Therefore, we have successfully generated a panel of 10 broadly-binding murine monoclonal antibodies that target avian, mammalian and human influenza virus NAs of the N6 subtype. In general, it was possible to observe that all of our antibodies are showing a high binding activity against the N6 protein expressed on the viruses A/swine/Missouri/A0172 7926/2015 (H4N6), A/Caspian seal/Russia/T1/2012 (H4N6) and A/duck/Zhejiang/D9/2013 (H4N6) (Figure 4B-D). Especially, the antibodies KLN6-3B5, KLN6-3C6 and KLN6-3E1 showed the highest binding profile amongst all different N6 proteins. However, KLN6-1A5 had the strongest binding activity against the mammalian isolates of A/swine/Missouri/A01727926/2015 (H4N6) and A/Caspian seal/Russia/T1/2012 (H4N6), whereas KLN6-3F10 was the most efficient one against the human isolate of A/Shenzhen/1/2016 (Figure 4E). Furthermore, it was possible to observe that those antibodies are only reactive against the N6 subgroup and not to other NA proteins within the group 2 NAs (Figure 4F).

Through sequence alignment of different N6 influenza strains, we observed an amino acid similarity of 94% between A /duck/Zhejiang/D9/2013 and A/Caspian seal/Russia/T1/2012 and 90% between A/duck/Zhejiang/D9/2013 and A/swine/Missouri/ A01727926/2015 as well as /Caspian seal/Russia/T1/2012 and A/swine/Missouri/ A01727926/2015. The NA of A/Shenzhen/1/2016 was most different to A/ swine/Missouri/ A01727926/2015 and had a similarity of 85%.

By comparing the N6 subtype with the N9 protein of A/Hong Kong/125/2017, which is also a member of the group 2 NA's, it was possible to obtain an average identity of 60% (Figure 5). This result is mostly based on the conserved regions within the globular head domain of all NA subgroups.

To see if our antibodies are binding with the same efficiency to N6 proteins in their native confirmation, we performed an immunofluorescence assay of 14 different N6-influenza viruses (Figure 6). The antibodies KLN6-2E8, KLN6-3C7 and KLN6-3F10 displayed the strongest binding affinity to all 14 viruses. However, only 5 out of 10 antibodies (KLN6-2E8, KLN6-3C7, KLN6-3D5, KLN6-3E1, KLN6-3F10) showed

binding to the human isolate A/Shenzhen/1/2016. Interestingly, the antibodies were not the same ones, that showed binding in ELISA beforehand. The antibodies KLN6-3E1 and KLN6-3F10, showed binding in both assays. Since the recombinant proteins expressed through the baculovirus system are including only the globular head domain and are missing the other three preceding domains, the folding of the proteins might be different as expressed on the influenza virus or infected cells (Figure 4A). Based on this, some antibodies might be hindered to target their epitopes, once the protein occurs in its native conformation resulting in a loss of binding. On the opposite, some epitopes might be more exposed and therefore more easily accessible for antibodies, which results in a detectable signal in ELISA. An important factor for the effectiveness of antiviral therapies, is to inhibit the virus in its replication and spread. Since it is known that once the neuraminidase is inhibited, the newly formed virions are unable to detach from the host cell and aggregating on the outer membrane, the NA has become an attractive target in various antiviral approaches[13].

To determine if our antibodies possess neuraminidase inhibition activity, we performed a NI assay using the viruses A/duck/Czechoslovakia/1956 (H4N6), A/swine/Missouri/A01727926/2015 (H4N6) and A/Shenzhen/1/2016 (H5N6). All 10 antibodies showed a detectable neuraminidase inhibition activity against the mammalian and avian isolate (Figure 7A-C). This could be a useful characteristic for the generation of antiviral therapeutics, to prevent severe disease outcome after a zoonotic N6 influenza virus infection.

However, neuraminidase antibodies are known to be non-neutralizing, since the virus enters the cell through the hemagglutinin, which leads to at least one infected cell before the NA-antibodies can perform their function[13]. Nevertheless, six of our N6-antibodies showed no visible plaques in the highest antibody dilution of 100 µg/ml in a plaque reduction neutralization assay. Through the high amount of antibody, it is possible that the virus is overloaded with antibody, leading to a steric hindrance of the hemagglutinin, which would limit the HAs ability to attach to the host cell (Figure 7D-E).Another attribute, that makes the neuraminidase to an attractive target in vaccine design, is the NA-based immunity through ADCC and ADCP mediated functions[15].

Therefore, we tested our antibodies regarding their ADCC-activity and found that all of our antibodies have a high ADCC activity against A/swine/Missouri/A01727926/2015 (H4N6) (Figure 8A). For A/duck/Czechoslovakia/1956 (H4N6), the activity was lower but still present for all 10 antibodies (Figure 8B). The ADCC-activity

in response to A/Shenzhen/1/2016 was only detectable in 5 out of 10 antibodies (KLN6-1A5, KLN6-2E8, KLN6-3B5, KLN6-3C6, KLN6-3F10), the highest caused through KLN6-3F10 (Figure 8C).

Since the NI as well as ADCC-results were promising for A/duck/Czechoslovakia/1956, we decided to investigate the prophylactic effect of our antibodies *in vivo* using the DBA/2J mouse model, which appears to be more susceptible for avian influenza viruses [26,27]. It was possible to observe that 7 out of 10 antibodies (KLN6-1A5, KLN6-1D4, KLN6-1G9, KLN6-2E8, KLN6-3B5, KLN6-3C6, KLN6-3F10) prevented against mortality and 3 out of these 7 (KLN6-3B5 and KLN6-3C6) even prevented against morbidity (9C-D). For the other 4 antibodies, the mice experienced higher morbidity, indicated through thin fur, loss of weight and activity but recovered after 8 days post challenge.

The results collected in this study, underline the efficiency of neuraminidase antibodies to prevent a severe disease outcome after influenza infections. Especially, since the cases of humans infected with N6-viruses were described as severe with a high morbidity rate, these antibodies could be used as potential therapeutics as well as preventive medication for high risk groups[23]. Since the neuraminidases in general appear to be more stable and less susceptible to acquire mutations, they should be considered to be part of the seasonal influenza vaccine and potentially also for a universal influenza virus vaccine.

5. References

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6. List of figures