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An influenza A virus (H7N9) anti-neuraminidase monoclonal antibody protects mice from morbidity without interfering with the development of protective immunity to subsequent homologous challenge

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Abstract

The emergence of A(H7N9) virus strains with resistance to neuraminidase (NA) inhibitors highlights a critical need to discover new countermeasures for treatment of A(H7N9) virus-infected patients. We previously described an anti-NA mAb (3c10-3) that has prophylactic and therapeutic efficacy in mice lethally challenged with A(H7N9) virus when delivered intraperitoneally (i.p.). Here we show that intrananasal (i.n.) administration of 3c10-3 protects 100% of mice from mortality when treated 24 h post-challenge and further characterize the protective efficacy of 3c10-3 using a nonlethal A(H7N9) challenge model. Administration of 3c10-3 i.p. 24 h prior to challenge resulted in a significant decrease in viral lung titers and deep sequencing analysis indicated that treatment did not consistently select for viral variants in NA. Furthermore, prophylactic administration of 3c10-3 did not inhibit the development of protective immunity to subsequent homologous virus re-challenge. Taken together, 3c10-3 highlights the potential use of anti-NA mAb to mitigate influenza virus infection.

Keywords

Influenza; Neuraminidase; A(H7N9); Monoclonal antibody; Antiviral; Avian influenza; Passive transfer; Immunotherapeutic

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Conflicts of interest

J.R.W, Z.G, J.S and I.A.Y are listed as inventors on a patent application that discloses the therapeutic potential of mAb 3c10-3.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.virol.2017.08.016.

1. Introduction

Since the emergence of low pathogenic avian influenza (LPAI) A(H7N9) virus infection of humans in 2013 (Gao et al., 2013), mainland China has experienced annual waves of epidemics spanning five years. These annual epidemics have culminated in 1580 laboratory-confirmed human infections and resulted in 609 fatal cases as of 13 July 2017; a case-fatality rate of 38% (Food and Agriculture Organization of the United Nations, 2016). Although some limited human-to-human spread has occurred, there has been no evidence of sustained human-to-human transmission of A(H7N9) despite these viruses having the ability to bind both avian and human-like receptors (Shi et al., 2013; Yang et al., 2013; Zhou et al., 2013). The U.S. Centers for Disease Control and Prevention (CDC) has reported that A(H7N9) virus has the highest pandemic risk potential of any novel influenza A virus that has been assessed using the Influenza Risk Assessment Tool (Wang et al., 2014).

Because most human infections have been associated with direct exposure to live poultry, local Chinese governments have implemented control measures to decrease the prevalence of A(H7N9) virus in live-poultry markets such as environmental sampling of poultry markets and laboratory testing to identify if A(H7N9) virus is present (Kang et al., 2015; Wang et al., 2015). Live-bird markets have been closed in a number of cities, a measure that has been effective in reducing the risk for A(H7N9) virus transmission to humans (Wu et al., 2014). Active surveillance is important because most A(H7N9) viruses lack a hemagglutinin (HA) polybasic cleavage site that is typical of highly-pathogenic avian influenza (HPAI) viruses, cause little or no disease in poultry, allowing undetected circulation. Despite these control measures, the number of human cases identified during the fifth wave (fall 2016 to spring 2017) are the highest reported (> 700) (Food and Agriculture Organization of the United Nations, 2016). Epidemiologic assessment of the human A(H7N9) cases identified during the fourth epidemic in China indicate that the geographic range of the virus is continuing to spread with an increasing proportion of those infected living in rural areas, making surveillance an even more daunting task (Xiang et al., 2016). Alarmingly, sequence analysis of fifth wave isolates has revealed that some virus isolates from both humans and environmental samples contain a polybasic amino acid insert (RKRT) in the cleavage site of HA, which is typical of highly pathogenic avian influenza (HPAI) viruses.

Monoclonal antibodies (mAbs) are recognized as a major class of biotherapeutics that are increasingly being developed as potential tools to fight viral infection. For influenza, since the discovery of the mAb C179 (Okuno et al., 1993), many broadly neutralizing mAb have been described that target the conserved stem or receptor binding region of HA (reviewed (Air, 2015)). Currently there are seven anti-influenza mAb in clinical development according to ClinicalTrials.gov and the WHO International Clinical Trials Registry Platform (ICTRP) as of January 1st, 2017 (Sparrow et al., 2016). Six of these mAbs are directed against various epitopes contained within the HA surface protein while one targets the matrix protein 2 (M2). In addition to the HA and M2, anti-neuraminidase (NA) mAb have also proven to be protective in the mouse model but, to our knowledge, have yet to enter clinical development (Doyle et al., 2013; Jiang et al., 2015; Shoji et al., 2011; Wan et al., 2013; Wohlbold et al., 2016; DiLillo et al., 2016). One potential concern regarding mAb as antivirals include the possibility that their use may drive the evolution of viral escape

mutations which could be resistant not only to the mAb but to natural or vaccine-induced immunity. As well, therapeutic use of mAb could provide a clinically favorable outcome from the initial infection but may prevent treated patients from developing effective immunity to subsequent natural infections.

Previously, we described an anti-NA mAb (3c10-3) that has prophylactic and therapeutic efficacy in mice lethally challenged with A(H7N9) virus (Wilson et al., 2016). Here we further characterize the protective efficacy of 3c10-3 in vivo using a sublethal challenge model and show that prophylactic administration of this anti-NA mAb significantly reduces A(H7N9) virus lung titers while still allowing the development of immune responses capable of fully protecting mice from morbidity upon subsequent virus re-challenge.

2. Materials and methods

2.1. Viruses and animals

The wild-type LPAI A/Anhui/1/2013 (H7N9) (Anhui/1) virus, isolated from a fatal human case, was propagated in the allantoic cavity of 10-day-old embryonated chicken eggs as previously described (Belser et al., 2013). IDCDC-RG32A (RG32A) is a reverse-genetics reassortant virus containing the HA and NA from A/Shanghai/2/2013 (H7N9), which is antigenically similar to Anhui/1, with the 6 internal genes from A/Puerto Rico/8/1934 (H1N1) (PR8) generated as previously described (Ridenour et al., 2015). All studies involving the use of animals were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at the CDC in an Association for the Assessment and Accreditation of Laboratory Animal Care accredited facility. All work employing RG32A was performed at Biosafety Level 2-enhanced containment while Anhui/1 challenge studies were performed at Animal Biosafety Level 3-enhanced containment.

2.2. Mouse challenge studies

To examine the therapeutic efficacy of 3c10-3 when delivered i.n. in a lethal challenge model, six- to eight- week old female BALB/c mice (The Jackson Laboratory, N = 5 per group) were challenged with 12,500 plaque forming units (PFU) of Anhui/1 virus by i.n. instillation (50 μl volume) and treated 24 or 48 h later with a single administration of 3c10-3 (i.n.) at the indicated dose. Control mice received a single dose of 2 mg/kg isotype matched control mAb (m-IgG1/Kappa, Crown Bioscience Inc., targeting Hen Egg Lysozyme) 1 day post-infection. Mice were monitored daily for weight for 14 days post-challenge. Mice were anaesthetized by isoflurane inhalation for all i.n. instillations. Mice reaching ≥ 25% weight loss from pre-inoculation levels were humanely euthanized.

For our sublethal challenge model, six- to eight- week old female BALB/c mice (N = 15 per group) were passively immunized by i.p. injection with the indicated dose of 3c10-3 or isotype-matched control mAb (m-IgG1/Kappa, Crown Bioscience) in a final volume of 200 μl. Twenty-four h post transfer, mice were challenged with 5,000 PFU of Anhui/1 virus by i.n. instillation as described above. On day 3 and day 6 post-inoculation, five mice per group were sacrificed for collection of lung tissue which was immediately frozen at −80 °C until processing. The remaining mice (n = 5 per group) were cheek bled on d 33 for sera

characterization and re-challenged on d 35 with 25,000 PFU of Anhui/1 by i.n. instillation. Mice were monitored daily for body weight for 14 days post-challenge, when the experiment was terminated.

2.3. Tissue homogenization and virus titration

To determine viral load in mAb- or control-treated mice, whole lungs were thawed and homogenized in 1 ml of cold PBS, clarified by centrifugation, and serially titrated for infectious virus by standard plaque assay in Madin-Darby Canine Kidney (MDCK) cells as previously described (Zeng et al., 2007). The limit of virus detection was 10 PFU. Concurrently, clarified tissue homogenate was placed in AVL Buffer (Qiagen) to extract RNA for next generation sequence (NGS) analysis of the viral population.

2.4. Deep sequencing analysis of viral genomes

We used the Illumina platform to determine the whole genome consensus sequence and to identify intra-host single nucleotide variants (SNVs), including minor variant populations, from lung homogenates of A(H7N9) challenged mice day 3 and day 6 post infection. RNA from 70 μl of inactivated A(H7N9) (A/Anhui/01/2013)-infected cells were extracted using a custom QIAamp DNA kit (Qiagen) in a high-throughput automated liquid handler- QIAcube HT (Qiagen). A(H7N9) viral genes were then selectively amplified through multisegment-RTPCR (M-RTPCR) as previously described (Zhou and Wentworth, 2012). The resulting amplicons were then quantified using Quant-iT dsDNA High Sensitivity Assay (Invitrogen) and normalized to 0.2 ng/μl. Seven μl aliquots of the 0.2 ng/μl amplicons were analyzed by the QIAxcel Advanced System (Qiagen) for size confirmation and presence of amplicon segments. DNA libraries were produced using the Nextera XT Sample Prep kit (Illumina) and Nextera XT Index kit v2 (Illumina). Library preparation followed the manufacturer's protocol except that half-volumes were used. Paired-end libraries were purified with 0.8× AMPure XP beads (Beckman Coulter Inc.) using a Zephyr Compact Liquid handling workstation (Perkin Elmer). Library molarities were calculated using Quant-iT dsDNA to measure concentration and based on library sizes determined on the QIAxcel. Libraries were then normalized to 2.0 nM and combined into one single pooled library. Six pM of the pooled libraries, including 5% PhiX, was loaded into a MiSeq v2 300 cycle kit and sequencer.

Genome assemblies were generated and minor variant frequency detection was performed using IRMA software as described (Shepard et al., 2016). Post-assembly thresholds of 100× average coverage depth were applied and full-length reading frames were verified. For NGS minor variants detected, frequencies reported from IRMA were considered within coding regions above a 5% minimum threshold. Nucleotide positions were recalculated according to start codon sites, and HA mature amino acid numbering was employed. Interpretations of the IRMA minor variants detected across sample conditions were performed using custom perl scripts. The NGS data sets contributing to genome assemblies have been deposited in the NCBI Sequence Read Archive under accession number SRP111744. As a targeted amplification strategy was employed, reads that did not classify as influenza were deemed artifacts and discarded prior to submission to SRA. Analysis of selection on the isolates was

performed with SNPgenie (Nelson et al., 2015) using a threshold of 1% and a sliding window length of 50 nucleotides.

2.5. Hemagglutination inhibition assay

Day 33 sera (Fig. 2) were tested for the ability to inhibit hemagglutination of turkey red blood cells as previously described (Organization, 2011). Sera were treated with receptor-destroying enzyme (Seiken) overnight at 37 °C followed by heat inactivation for 30 min at 56 °C. Sera were diluted 2-fold starting at a concentration of 1:10. Four hemagglutinating units (HAU) of RG32A were used to agglutinate 0.5% turkey red blood cells.

2.6. Neuraminidase inhibition assay

Day 33 sera (Fig. 2) were pooled for each group and screened for the ability to inhibit neuraminidase activity using the Enzyme-Linked Lectin Assay (ELLA) as previously described (Couzens et al., 2014). The pooled sera was serial two-fold diluted starting at a concentration of 1:20 and an H6N9 reassortant virus bearing the NA of A/Shanghai/2/2013 (Sh/2) and an unrelated HA was used. End-point NAI titers were calculated as the reciprocal of the highest dilution with at least 50% inhibition.

2.7. Enzyme linked immunosorbent assay

For detection of NA and HA reactivity Enzyme linked immunosorbent assays (ELISA) were performed as previously described (Wilson et al., 2014). For serological analysis, day 33 sera (Fig. 2) were RDE treated and used at a starting dilution of 1:200. Sera were diluted two-fold and endpoint titers were determined as a > two-fold increased OD450 over negative control. Purified 3c10-3 was used as a positive/negative control. Recombinant NA (recN9) from Anhui/1 and recombinant H7 (recH7) from Sh/2 viruses were used as test antigens. To screen 3c10-3 for reactivity toward contemporary A(H7N9) isolates, endpoint titers were determined against recNA representing A/Hong Kong/125/2017 (HK/125), A/Taiwan/1/2017 (TW/1) and A/Guandong/17SF003/2016 (GD/17SF003), as indicated above.

2.8. MUNANA-based neuraminidase inhibition assay

3c10-3 was screened for the ability to inhibit the enzymatic activity of HK/125, TW/1 and GD/17SF003 recNA by MUNANA assay as previously described (Wilson et al., 2016).

3. Results

3.1. Intranasal therapeutic administration of 3c10-3 protects mice from lethal A(H7N9) infection

We have previously demonstrated the ability of the anti-N9 mAb 3c10-3 to protect mice from lethal A(H7N9) infection when administered up to 72 h post-challenge via the i.p. route (Wilson et al., 2016). Recently it has been shown that local administration of a broadly neutralizing anti-HA stalk mAb (Leyva-Grado et al., 2015) or an anti-HA1 mAb (He et al., 2013) showed improved therapeutic efficacy compared to animals treated via the i.p. route. Here we sought to determine if i.n. delivery of an anti-NA mAb could also protect mice from a lethal A(H7N9) challenge in a therapeutic setting. Mice challenged with a lethal dose of

Anhui/1 virus were treated 24 or 48 h post-infection with a single i.n. dose of 3c10-3 (either 2 or 0.4 mg/kg) while control mice received 2 mg/kg of IgG1 isotype mAb 24 h post-infection. Local administration of a single dose of 2 or 0.4 mg/kg 24 h post-lethal challenge prevented weight loss in a dose dependent manner and protected 100% of mice from mortality while all control mice succumbed to infection by day 7 post-infection (Fig. 1A & B). This appears to be an increase in efficacy at the 0.4 mg/kg dose since, in our previous study, we observed only 20% survival when 3c10-3 was delivered i.p. at 24 h post-lethal challenge (Wilson et al., 2016). Mice treated i.n. with either 2 or 0.4 mg/kg of 3c10-3 at 48 h post-infection were poorly protected as all showed substantial weight loss and only 20% survival (Fig. 1A & B). This is in contrast to our previously observed 80% and 20% protection when mice were administered the same respective dose of 3c10-3 at 48 h post-challenge via the i.p. route of administration (Wilson et al., 2016). These data indicate that delivery of 3c10-3 by the i.n. route has improved therapeutic efficacy when administered early during an infection but i.p. may be the most beneficial delivery route for extended therapeutic protective efficacy.

3.2. Prophylactic administration of 3c10-3 significantly reduces viral lung titers after sublethal challenge

To gain a better understanding of how 3c10-3 provides protection in vivo we used a sublethal challenge model (Fig. 2). Mice were pre-treated with the indicated dose of 3c10-3 or isotype-matched control mAb (day −1) followed 24 h later by a sublethal challenge with Anhui/1 virus. Mice pre-treated with 3c10-3 were protected from weight loss in a dose-dependent manner (Fig. 3A). Compared to isotype-matched control mice, 3c10-3 treated mice showed a significant difference in weight loss on day 8 for the low dose group and on days 5 through 11 for the high dose group ($p < 0.05$). Furthermore, on day 3 and day 6 post-challenge, mice pretreated with 3c10-3 had significantly lower viral lung titers ($p < 0.05$) than did mice treated with isotype-matched control mAb (Fig. 3B). These data support that 3c10-3 effectively protects mice from morbidity following virus challenge, and leads to reduced viral load compared with untreated mice.

3.3. Prophylactic treatment of mice with 3c10-3 does not consistently select for drift variants

A potential concern with immunotherapeutic treatment of rapidly-mutating pathogens such as influenza virus is the emergence of escape mutations in treated patients, rendering the treatment ineffective as a therapeutic for further use in patients subsequently infected with the resistant variants. To evaluate if 3c10-3 selected for drift variants, whole lung homogenates harvested on day 3 and day 6 after Anhui/1 virus challenge were analyzed by next generation deep sequencing of full genomes of the resident viral lung population. As expected for influenza viruses, a number of single nucleotide variants (SNVs) were detected throughout the genome of the challenge virus as well as the lungs of mice treated with either isotype control mAb or 3c10-3 on day 3 and day 6 p.i. (Supplemental Table 1). Signatures of positive selection ($dN/dS > 1$) were identified in NA and to a lesser extent in HA (Supplemental Table 2), but the selection was similar in the stock virus, and in viruses isolated from mice treated with isotype control antibody and with 3c10-3; the positive selection presumably reflected adaptation to replication in eggs and/or mice. The regions of

NA under positive selection did not encompass the antibody footprint of 3c10-3 (Supplemental Table 2). Focusing on NA, no unique SNVs that surpassed 10% of the resident lung viral population were detected in 3c10-3 treated mice on day 3, while there were four instances where a day 6 3c10-3 treated mouse contained SNVs that surpassed 10% of the virus population and that were not also present in isotype control mice (Table 1). An individual mouse in the day 6 p.i. 2 mg/kg 3c10-3 treated group contained a virus encoding SNVs at either C32T (19%), G1196A (25%) or A1270G (36%) in NA. Each was a nonsynonomous change resulting in a S11L, S399N or I424V change in the NA protein, respectively. The fourth instance was observed in a single mouse from the day 6 p.i. 0.4 mg/kg 3c10-3 treated group which contained SNVs at C152T (11%) in NA (Table 1) resulting in a T51I change. Again, these variants were only observed in isolation and are not directly within the defined epitope on NA that is critical for 3c10-3 binding (Wilson et al., 2016). These data indicate that prophylactic treatment of mice with 3c10-3 does not consistently select for sequence variants in NA (Table 1), or any other influenza gene segment (Supplemental Table 1, Supplemental Table 2).

3.4. Prophylactic treatment of mice with 3c10-3 does not inhibit the development of an anti-A(H7N9) protective immune responses to subsequent homologous challenge

From a clinical standpoint, the use of passive immunotherapy would protect individuals from morbidity/mortality induced by influenza infection while still allowing the development of adaptive immune responses capable of providing protection from future viral exposure. To see if 3c10-3 treated mice develop adaptive immune responses capable of providing protection from subsequent homologous re-infection, mice that had recovered from Anhui/1 virus challenge (Figs. 2 and 3A) were re-challenged with Anhui/1 virus 35 days after the initial challenge. Mice that received 3c10-3 (36 days prior), but were not infected, served as a negative control and these mice exhibited significant weight loss compared with previously infected mice ($p < 0.05$) (Fig. 4). Mice that had been infected after pre-treatment with 3c10-3 (high or low dose), or with isotype control mAb, were fully protected from weight loss compared to the 3c10-3-treated/uninfected group (Fig. 4). Since passive protection was no longer present, these mice must have been protected by acquired immunity, indicating that prophylactic use of 3c10-3 protects mice from morbidity without disrupting the development of a protective antiviral response rendering mice protected against weight loss after subsequent homologous virus challenge. Consistent with this observation, ELISA and HAI analysis of the day 33 sera (2 days prior to re-challenge) indicated that 3c10-3 pre-treated and isotype control mice develop similar levels of anti-HA antibody responses, confirming that passive immunization of mice with 3c10-3 does not affect the development of the anti-HA antibody responses during the initial virus challenge (Fig. 5A and C). Interestingly, the anti-NA antibody response was considerably reduced in the mice pre-treated with 3c10-3 compared to isotype-control mice in both ELISA and NAI assays (Fig. 5B and D). These data indicate that prophylactic use of 3c10-3 may act as a shield, blunting the recognition of NA by the immune response.

3.5. 3c10-3 cross-reacts with the recNA of contemporary fifth wave A(H7N9) isolates to varying degrees in vitro

Phylogenetic analysis of A(H7N9) virus isolates has shown continuous evolution over the five pandemic waves in the NA gene segment (Su et al., 2017). To see if 3c10-3 maintained the ability to bind contemporary N9 variants, we screened for in vitro binding and functional activity by ELISA and MUNANA, respectively, against the recNA representing three recently identified candidate vaccine viruses (CVVs) signifying both LPAI and HPAI lineages. By ELISA, 3c10-3 bound the LPAI recNA of HK/125 at a 32-fold higher concentration than that of Sh/2 while binding the HPAI isolates TW/1 and GD/17SF003 at 8- and 64-fold higher concentration, respectively (Table 2). A similar but less drastic trend was also observed for the inhibition of recNA enzymatic activity by MUNANA analysis as HK/125, TW/1 and GD/17SF003 enzyme activity was reduced 8-, 4.5- and 6.5-fold respectively. These data indicate that 3c10-3 maintains the ability to bind and inhibit the functional activity of the NA of representative contemporary A(H7N9) viruses, albeit to varying degrees. Further studies are underway to determine if 3c10-3 maintains the ability to provide in vivo protection against these viruses.

4. Discussion

There has recently been a rise in interest in the clinical development of mAb based immunotherapies targeting influenza viral proteins to serve as new alternative countermeasures to the currently available anti-virals. It is becoming clear that mAb passive immunization has great potential to serve as pre- or post-exposure prophylaxis to prevent or treat influenza infection. As A(H7N9) viruses continue to circulate in fowl in China and cause annual human epidemics, the pandemic potential of this virus continues to pose a significant public health threat. Antigenic characterization of human and environmental isolates from the most recent epidemic has identified antigenic drift as well as HPAI virus variants, leading the World Health Organization (WHO) Influenza Surveillance and Response System (GISRS) to recommend two additional candidate vaccine viruses to serve as stockpiles for pandemic preparedness purposes (Iuliano et al., 2017). Furthermore, several antiviral resistant isolates have been identified from patients treated with the currently available anti-NA drugs (Richt et al., 2017). As A(H7N9) viruses are currently seen as a potential pandemic threat, the development of new anti-viral therapies is vital. We have previously described the anti-N9 mAb, 3c10-3, that has prophylactic and therapeutic efficacy in mice lethally challenged with A(H7N9) virus (Wilson et al., 2016). Here we further explored the antiviral activity of 3c10-3 by gaining a better understanding of the in vivo protective properties of this mAb.

mAb 3c10-3 binds to an epitope that spans the NA enzyme active site causing inhibition of NA enzymatic activity and, although not directly virus-neutralizing, results in a reduction of in vitro cell-to-cell spread of virus particles likely due to inhibition of viral egress (Wilson et al., 2016). To gain a further understanding of the protective mechanism of 3c10-3 in vivo we looked at the impact of 3c10-3 passive immunization on A(H7N9) virus replication in the lung. On day 3 and day 6 post-infection, virus lung titers were significantly reduced compared to mice treated with an isotype-matched control antibody. Our previous in vitro

data leads us to conclude that 3c10-3 inhibition of cell-to-cell spread or egress, at least in part, contributes to its protective properties, although the IgG1 fragment constant (Fc) region engagement of Fcγ receptor (FcγR) and subsequently mediated effector functions may also contribute to protection. It has previously been shown that some anti-HA stem and anti-NA broadly neutralizing and non-neutralizing mAb can require activation of Fc-FcR mediated effector functions to provide protection in vivo (DiLillo et al., 2016, 2014). Of note, these studies were carried out with the mAb in the IgG2a Fc background, a domain that preferentially engages activating FcγRs to drive ADCC activity compared to IgG1 (Guilliams et al., 2014). Recently it has been reported that anti-M2e mAb on the IgG2a background can better protect mice against influenza infection through engagement of each of the three activating FcγRs rather than anti-M2e mAb in the IgG1 background which relies solely on FcγRIII for protection (Van den Hoecke et al., 2017). Since 3c10-3 contains the IgG1 Fc, research is ongoing to evaluate if generating a version of 3c10-3 that contains the murine IgG2a Fc can increase the protective efficacy of this mAb.

The error-prone RNA-dependent RNA polymerase (RdRP) of influenza viruses leads to high mutational frequencies, so that selective pressure can lead to the rapid emergence of drift variant viruses capable of escaping the pressures of the antibody or antiviral therapy. However, in some cases escape mutants may arise slowly, such as when evading the selection pressure itself leads to loss of fitness. Deep sequencing makes it possible to observe low-frequency viral variants with high precision. Here, we used deep sequencing to monitor the impact of prophylactic use of 3c10-3 on the genetic diversity of the resident lung population of A(H7N9) virus. Analysis of synonymous vs non-synonymous nucleotide changes indicated that similar selection was present on NA in the presence and absence of 3c10-3. Four independent instances were identified that could represent escape mutations, i.e. where a non-synonomous change occurred in the NA of a 3c10-3 treated mouse that was present in > 10% of the viral sequences within a mouse lung, and that was also not observed in the stock virus or control mice. Importantly, these changes were only observed in isolation (a single mouse out of 20 that received 3c10-3), indicating that these changes are likely not due to selective pressure from 3c10-3, although further investigation is required to definitively rule this out.

Two of the NA mutations (S399N and I424V), identified independently from a respective day 6 lung sample of a 3c10-3 treated mouse, affected a residue that is near the putative binding footprint of 3c10-3. During our previous epitope mapping screen we looked at the effect of a W398K change on 3c10-3 binding activity (Wilson et al., 2016). Although this change did not decrease the binding activity of the 3c10-3/NA complex beyond 50% that of the wild-type binding cutoff (the bar we set as significant), binding was reduced by 48%. However, 399 N and 424 V were not evaluated directly for an impact on 3c10-3 binding to NA. Since neither of these residues (unlike 398) are surface-exposed, based on the 3-dimensional structure of N9 (PDB accession code 4MWJ), they are less likely to affect binding directly. Of the 1705 N9 sequences available for review in the GISAID database, 1704 and 1703 encode S399 and I424, respectively. None of the N9 sequences encode 399 N while one has the 424 V, suggesting that changes at these positions may not be well tolerated.

Mice therapeutically treated via i.n. instillation with 3c10-3 24 h post-lethal challenge resulted in 100% protection from lethality compared to only 20% survival when delivered 48 h post-challenge for both doses tested (2 or 0.4 mg/kg). Although this appears to be an improved efficacy compared to our previously observed therapeutic study when 3c10-3 was delivered i.p. 24 h post-challenge (Wilson et al., 2016), it does not reach the extended therapeutic coverage observed for broadly neutralizing anti-HA stalk directed antibodies delivered i.n. at similar doses, as long as 72 h post-lethal challenge (Leyva-Grado et al., 2015). It is possible that intrinsic differences exist in the protective capacity of different mAbs that target the same antigen (Tsouchnikas et al., 2015) as well as different influenza antigens (HA vs NA) or even the challenge virus. Here we only tested the therapeutic efficacy of 3c10-3 when administered at very low dose (≤ 2 mg/kg). It is conceivable that i.n. delivery of 3c10-3 at an increased dose may provide improved protection in a therapeutic setting and thus warrants further investigation.

A concern with using mAb for treatment of influenza infection is that the treatment may block the onset of adaptive immune responses, leaving patients susceptible to repeat infection after recovery from the initial infection. Ideally, therefore, passive immunotherapy should protect individuals from morbidity and mortality induced by influenza infection, while still allowing the development of protective adaptive immune responses. Mice treated prophylactically with 3c10-3 were found to establish protective memory responses to A(H7N9) virus infection. Both ELISA and HAI analyses showed that the levels of antibodies targeting the viral surface protein HA were similar in mice that received 3c10-3, or isotype control mAb, following recovery from the initial infection and re-challenge showed that both groups of mice were equally well protected. This is likely a result of this mAb's mechanism of action. Because 3c10-3 does not directly neutralize virus infectivity but acts by limiting virus egress and subsequent cell-to-cell spread, as indicated by our in vitro data (Wilson et al., 2016) and the currently reported reduction of virus lung titers in vivo, an active but attenuated infection is likely established. This allows recognition and subsequent development of effective adaptive immune responses that are capable of protecting against homologous infection and is similar to a study which found that passive immunity with ovine polyclonal antibodies targeting influenza not only protected mice from primary infection but also preserved the development of antiviral adaptive immunity to subsequent viral challenge (Stevens et al., 2016). Interestingly, we observed a reduced anti-NA antibody response as indicated by both ELISA and NAI assays in the 3c10-3 treated mice prior to re-challenge. This reduction may be a result of 3c10-3 shielding its epitope on NA, decreasing the availability of NA to be presented as antigen to the host immune system and potentially altering the fine specificity of the polyclonal antibody responses to NA without impairing the induction of antibodies to HA. Despite the observed reduction in antibodies targeting NA, 3c10-3 treated mice were still fully protected from virus re-challenge.

In light of the ongoing annual human A(H7N9) epidemic and the pandemic potential that these viruses possess it is vital that we develop alternative therapeutics to the currently available anti-viral drugs. The use of passive immunotherapy has the potential to fill this void and 3c10-3 further demonstrates the potential that targeting NA with mAb have as an alternative therapy to currently licensed anti-viral drugs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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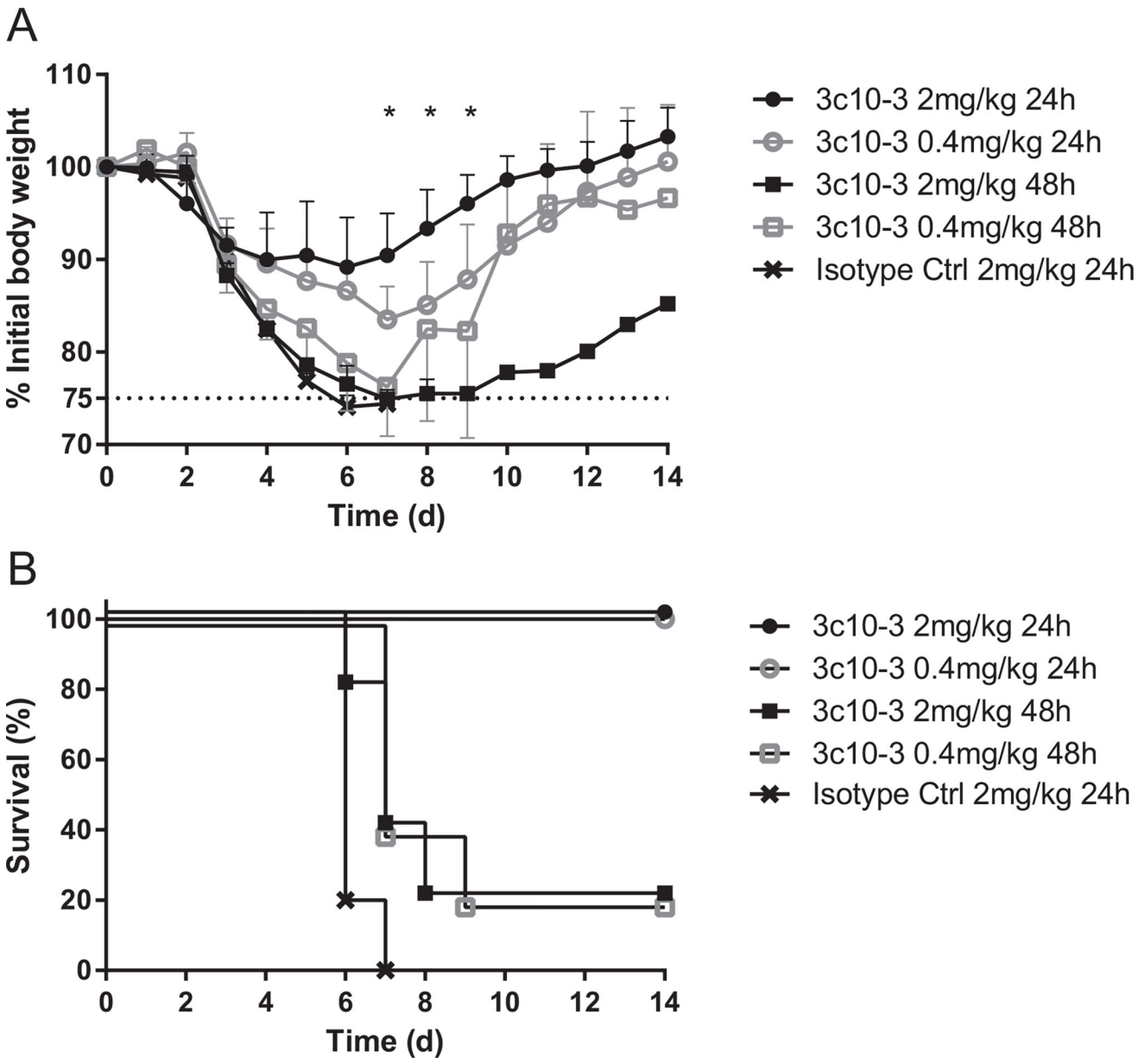

Fig. 1.

Intranasal delivery of 3c10-3 therapeutically protects mice from A(H7N9) lethal challenge. Six- to eight- week old female BALB/c mice (n = 5 per group) were infected with a lethal dose of Anhui/1 virus and later treated by i.n. instillation at 24 or 48 h post-challenge with the indicated dose of 3c10-3. Control mice received 2 mg/kg of isotype-matched antibody (IgG1/Kappa) 24 h post-challenge. A) The mean percentage of initial body weight (+ or − SD). (*, $p < 0.05$ 3c10-3 2 mg/kg 24 h compared to 3c10-3 0.4 mg/kg 24 h using student *t*-test). B) Percent survival.

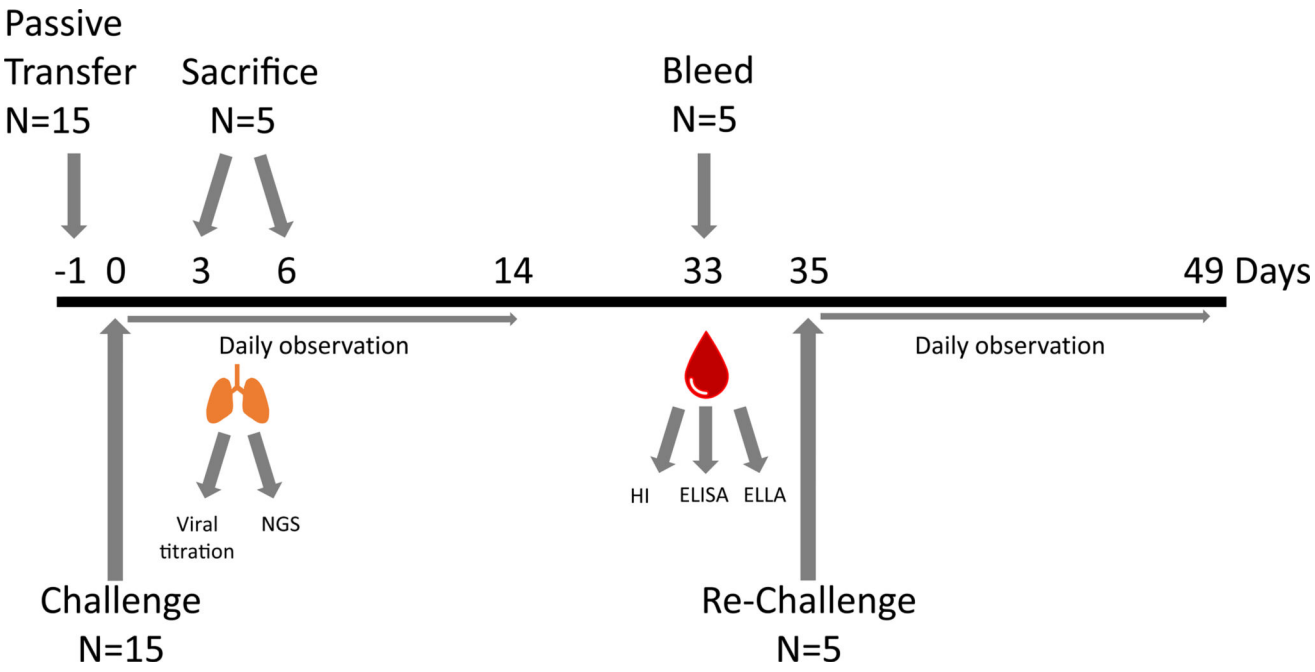

Fig. 2.

Sublethal challenge experimental design. Fifteen six- to eight- week old BALB/c mice were injected (i.p.) with 2 or 0.4 mg/kg of 3c10-3 or isotype control mAb (2 mg/kg) and 24 h later inoculated with a sub-lethal dose of Anhui/1. Five mice were also injected with 2 mg/kg 3c10-3, but not challenged, to serve as a negative control for the day 35 re-challenge. Lungs were harvested from mice (n = 5) on day 3 and day 6 post-inoculation for virus titration and deep sequence analysis of viral genomes. Weight was monitored daily for 14 days following virus inoculation. On day 33, all remaining mice were cheek bled to obtain sera for ELISA, HI and ELLA analysis. On day 35 post-infection mice were re-challenged with Anhui/1 and monitored for weight loss for 14 days. Control mice for the re-challenge experiment were the above mentioned mice that received 2 mg/kg 3c10-3 on day −1 but were not inoculated with virus on day 0.

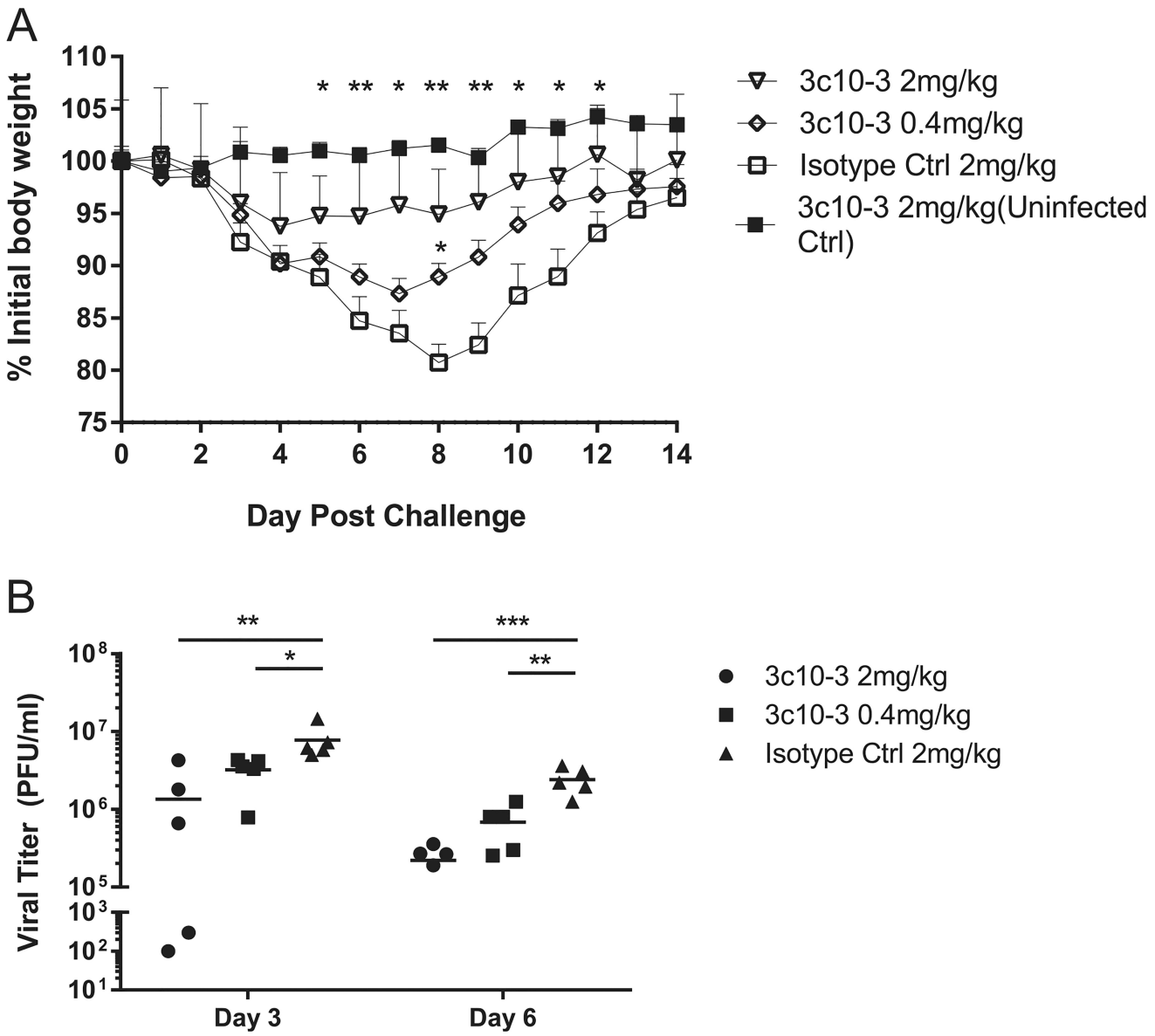

Fig. 3.

Pre-treatment with 3c10-3 reduces virus lung titer in a dose dependent manner. Six- to eight-week old female BALB/c mice (n = 5 or more per group) were injected i.p. with the indicated amount of mAb and 24 h later infected with a sublethal dose (5000 PFU) of Anhui/1 virus. Day 0 – 3n = 15 per group, day 4 – 6n = 10 per group, day 7 – 14n = 5 per group. (A) The mean percentage of initial body weight (+ SD) are shown. (B) Lung tissue collected on the indicated day post Anhui/1 challenge (n = 5 per group) were titered for the presence of infectious virus by standard plaque assay. (***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$ compared to Isotype control mice by student *t*-test).

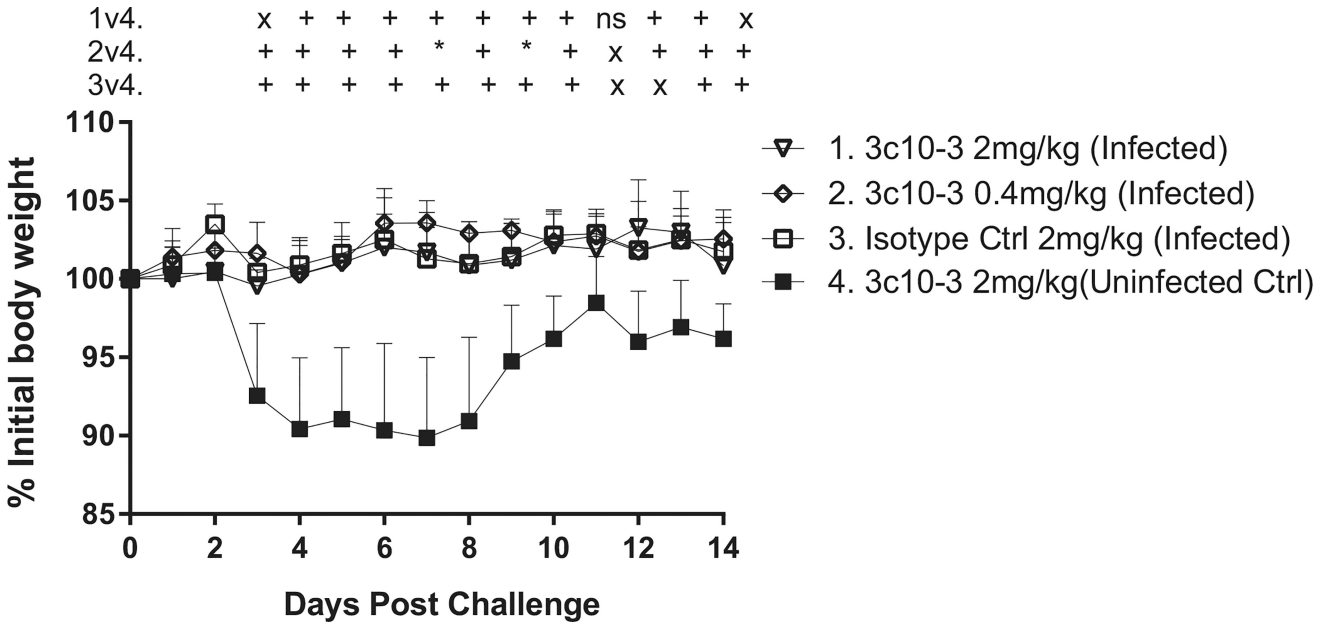

Fig. 4.

Administration of 3c10-3 prior to infection does not affect the development of protective immunity to subsequent homologous challenge. Mice from the sublethal challenge experiment (n = 5) were re-challenged with A(H7N9) virus (25000 PFU) on day 35 post initial challenge. The mean percentage of initial body weight (+ SD) is shown. (x, $p < 0.05$; +, $p < 0.001$; *, $p < 0.0001$ compared to previously uninfected 3c10-3 treated control mice by student *t*-test).

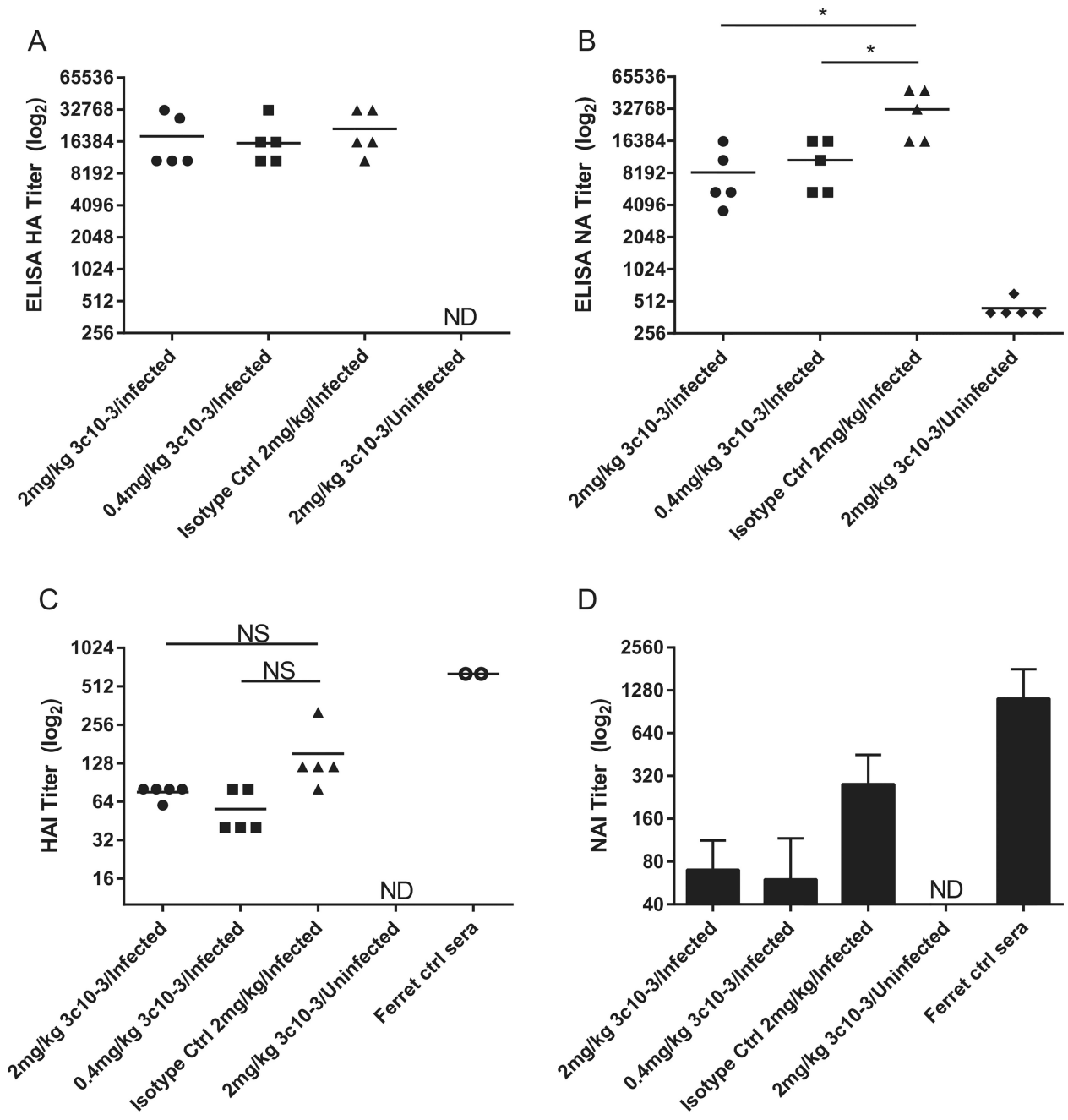

Fig. 5.

Serum ELISA, HAI and NAI titers. A & B) Sera anti-HA and NA ELISA antibody endpoint titers were determined on day 33 post initial A(H7N9) challenge. The results represent the average of two independent assays. C) HAI titers were determined on day 33 post initial A(H7N9) challenge. The results represent the average of two independent assays run with 4 HA units of RG32a. ND; not detected (titer < 1:10). D) NAI titers were determined for pooled sera from day 33 post initial A(H7N9) challenge by ELLA assay. ND; not detected (titer < 1:20). Data represent the average (+ SD) of two independent assays run in duplicate. Hyperimmune ferret antisera served as a positive control for the HI and NAI analysis. (NS,

not significant, $p > 0.05$; *, $p < 0.05$ versus Isotype Ctrl 2 mg/kg/Infected mice by student *t*-test).

Table 1

Prophylactic treatment of mice with 3c10-3 does not consistently select for NA drift variants.

		NT	29	32	40	41	66	152	591	808	825	1130	1196	1217	1221	1270	1379
	Anhui/1 Stock virus		C/T*|75/25	C	G/A|90/10 †	C	A	C	T	A	C/T|91/9	A	G	T	C	A	A/G|56/44
Day 3	Isotype control 2mg/kg	1	C/T|50/50	C	G/A|90/10	C	A	C	T	A	C/T|95/5	A	G	T	C	A	A/G|85/15
		2	T/C|61/39	C	G/A|90/10	C	A	C	T	A	C	A	G	T	C	A	A/G|84/16
		3	T/C|66/34	C	G/A|89/11	C	A	C	T	A	C	A	G	T	C	A	A/G|88/12
		4	T/C|61/39	C	G/A|89/11	C	A	C	T	A	C	A	G	T	C	A	A/G|76/24
		5	T/C|70/30	C	G/A|89/11	C	A	C	T	A	C	A	G	T	C	A	A/G|83/17
	3c10-3 0.4mg/kg	1	T/C|59/41	C	G/A|90/10	C/T|92/8	A	C	T	A	C	A	G	T	C	A	A/G|89/11
		2	C/T|72/28	C	G	C	A	C	T	A	C	A	G	T	C	A	A/G|85/15
		3	C/T|50/50	C	G	C	A	C	T	A	C	A	G	T	C	A	A/G|83/17
		4	C/T|56/44	C	G/A|92/8	C	A	C	T	A	C	A/G|94/6	G	T	C	A	A/G|88/12
		5	T/C|64/36	C	G/A|92/8	C	A	C/T|93/6	T	A	C	A	G/T|94/5	T	C	A	A/G|73/27
	3c10-3 2mg/kg	1	Y	C	G	C	A	C	T	A	C	A	G	T	C	A	R
		2	C/T|63/37	C	G	C	A	C	T	A	C/T|95/5	A	G	T	C	A	G/A|50/50
		3	C/T|93/7	C	G	C	A	C	T	A	C	A	G	T	C	A	A/G|95/5
		4	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
		5	T/C|57/43	C	G/A|71/29	C	A	C	T	A	C	A	G	T	C	A	A/G|94/6
Day 6	Isotype control 2mg/kg	1	C/T|61/39	C	G/A|62/38	C	A	C	T	A	C	A	G	T	C	A	A/G|91/9
		2	C/T|62/38	C	G/A|95/5	C	A	C	T	A	C	A	G	T	C/T|91/8	A	A/G|94/6
		3	C/T|63/37	C	G/A|87/13	C	A	C	T	A	C	A	G	T	C	A	A/G|93/7
		4	C/T|55/45	C	G/A|93/7	C	A	C	T	A	C	A	G	T	C	A	A/G|87/13
		5	T/C|57/42	C	G/A|89/10	C	A	C	T	A	C	A	G	T	C	A	A/G|91/9
	3c10-3 0.4mg/kg	1	T/C|63/37	C	G/A|84/16	C	A	C	T	A	C	A	G	T	C	A	A/G|92/8
		2	C/T|63/37	C/T|91/8	G	C	A	C/T|89/11	T	A	C	A	G	T	C	A	A/G|88/12
		3	T/C|67/32	C	G/A|90/10	C	A	C	T	A	C	A	G	T	C	A	A/G|93/7
		4	C/T|63/37	C/T|93/7	G	C	A	C	T	A	C	A	G	T	C	A	A
		5	T/C|59/41	C	G/A|86/14	C	A	C	T	A	C	A	G	T	C	A	A/G|84/16

	3c10-3 2mg/kg	1	C/T|61/39	C	G	C	A	C	T	A	C	A	G	T	C	A	A/G|88/12
		2	C/T|88/12	C	G	C	A	C	T/A|91/9	A/G|92/8	C	A	G/A|71/25	T	C	A	A
		3	C/T|68/31	C/T|81/19	G/A|87/13	C	A	C	T	A	C	A	G	T	C	A	A/G|94/6
		4	C/T|84/16	C	G	C	A	C	T	A	C	A	G	T/C|91/9	C	A	A
		5	T/C|81/19	C	G	C	A/G|92/8	C	T	A	C	A	G	T	C	A/G|64/36	A/G|90/10
		AA change	**I10T or T10I**	**S11L**	**A14T**	**A14V**	**Synonymous**	**T51I**	**N197K**	**K270E**	**Synonymous**	**N377S**	**S399I or S399N**	**M406T**	**Synonymous**	**I424V**	**K460R or R460K**

Abbreviation: ND; not detected, R; A or G, Y; C or T

*; nucleotide(s) detected

†; ratio of nucleotides observed

Table 2

In vitro activity of 3c10-3 toward the recNA of respective contemporary (fifth wave) A (H7N9) virus isolates.

	Sh/2	HK/125	TW/1[a]	GD/17SF003[a]
ELISA (ng/ml)[b]	18.31	585.9 (32)	146.8 (8)	1171.88 (64)
MUNANA (μg/ml)[c]	6.447	53.5 (8)	29.3 (4.5)	42.98 (6.5)

() The respective fold reduction compared to Sh/2 endpoint titer.

[a]HPAI fifth wave isolate.

[b]The minimum 3c10-3 concentration required to produce a 2-fold signal above negative control. Data represents the average of two experiments run in duplicate.

[c]The minimum 3c10-3 concentration required to inhibit 50% of recNA enzyme activity. Data represents the average of three experiments.