M2e-specific monoclonal antibody cocktails against influenza A virus are superior to individual monoclonal antibody treatments, universally effective, and viral escape mutant resistant

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CONFLICT OF INTEREST STATEMENT

The authors have declared that no conflict of interest exists.

ABSTRACT

Influenza virus has pandemic potential, seasonal epidemics burden the human population, and viral resistance has developed to all available treatment options. A universally effective, escape mutant resistant therapeutic agent is desperately needed. We previously described extensive analyses of seven Matrix Protein 2 ectodomain-specific monoclonal antibodies (M2e-mAbs) as the basis for an effective and universal treatment for influenza A virus (IAV) infection. On the strength of these findings, we developed an effective prophylactic cocktail agent using three M2e-mAbs distinct in their M2e epitopes. This cocktail protected mice challenged with laboratory and pandemic IAV strains at a low dose significantly better than single M2e-mAb treatments. Notably, no viral escape mutants developed in immunocompetent and immunodeficient mice after viral passaging in the presence of single, cocktail, or alternating M2e-mAb treatments. Our study reveals the superiority of M2e-mAb cocktails, their universal effectiveness, and resistance to viral escape. It will critically shape future influenza-therapeutic development.

INTRODUCTION

Influenza viruses are rapidly mutating RNA viruses and the causative agent of about one billion annual respiratory virus infections and 290,000-650,000 deaths worldwide [1]. The influenza A genome contains eight genes encoding 11 proteins [2] and is named after protein subtypes expressed on the virion's surface: hemagglutinin (H) and neuraminidase (N). IAVs can infect many different species. In contrast, influenza B viruses only infect humans and are limited to Victoria and Yamagata's two lineages. Based on this terminology, presently circulating influenza viruses in humans include influenza A H1N1, influenza A H3N2, and influenza B. Upon natural influenza virus infection, surviving humans develop robust immunity. However, antigenic drift, defined as mutations occurring within a serotype, and antigenic shift, the recombination of two different serotypes, continuously create new variants. Novel transmissible strains resulting from antigenic shift have pandemic potential as there is limited pre-existing immunity in the human population. Amongst influenza viruses of public health concern are two "Highly Pathogenic Avian Influenza A" (HPAI) strains, H5N1 and H7N9. Both occasionally spill into the human population, where infections generally result in a 40-65% mortality rate [3].

Seasonal influenza vaccines are available, but current vaccination rates for US children and adults are generally well under 50 percent [4]. Also, vaccine efficacy varies yearly, as annual vaccine production depends on strain predictions based on circulating strains. Once strains have been selected for seasonal vaccine production, it takes at least six months to manufacture and distribute a seasonal influenza virus vaccine [5]. Consequently, should a pandemic strain develop after seasonal vaccine efforts have begun, vaccine availability can lag peak infection rates by several

months, as during the most recent H1N1 pandemic in 2009 [6-9].

There are currently six FDA-approved treatments for influenza A virus (IAV) infection [2]. However, viral escape mutants have been reported to each in clinical trials and/or during seasonal or pandemic outbreaks [10-18]. Should another influenza virus pandemic occur, viral resistance to common IAV treatments would be especially detrimental during the time required for pandemic influenza vaccine development. Considering the high dependence of current vaccine efforts on predictions of antigenic drift, low vaccination rates, the emergence of viral resistance against IAV therapies, and the high mortality rate resulting from IAV infections annually, it is imperative to explore other treatment options for severe influenza virus disease. Ideally, such a treatment would be a safe, effective, and universally protective "off the shelf" therapy, and resistant to viral immune escape. Such an agent would be a precious treatment option during seasonal epidemics and potential pandemics.

Influenza encoded matrix protein 2 (M2) proton channel is essential for viral replication. M2 is required to disassemble influenza's viral core, virus assembly, and budding [19]. Also, the extracellular region of the M2 proton channel (M2e) is highly conserved across different IAV serotypes. Based on these considerations, M2's ectodomain has long been considered an excellent target for an IAV vaccine or therapy [8, 20]. These efforts include developing M2e-specific monoclonal antibody (M2e-mAb) treatments. As demonstrated by passive immunizations in mouse models, both M2e-specific-antibody-containing immune sera and purified M2e-mAbs protect from severe IAV disease [8, 21]. However, previously reported experimental M2e-mAb treatments were either serotype-specific, thus lacking universality [22], or initially universally

protective but susceptible to viral immune escape ([23, 24]. Also, a human M2e-mAb, TCN-032 [22], went through phase 1/2 clinical trials. However, while TCN-032 improves disease severity with a 35% reduction in symptoms, TCN-032 overall did not achieve its goal of significantly decreasing the number of subjects with moderate to severe symptoms of influenza infection [25]. These results are nevertheless encouraging, considering they demonstrate the safety and potential efficacy of an M2e-mAb. Also encouraging is that the trial found no evidence for developing viral resistance [25].

We have previously published an extensive analysis of seven M2e-mAbs derived from the AuNP-M2e-CpG vaccination in BALB/c mice [26]. Our data demonstrate that several of these antibodies are highly protective *in vivo* and may be universally protective against IAVs, as they recognize a range of M2e sequences in multiple IAV serotypes. However, the potential development of viral escape mutants is a critical concern when developing an IAV therapy. Theoretically, combinatorial antibody administration can reduce the risk of viral escape. However, this has not yet been demonstrated for influenza A viruses. This study takes advantage of our antibodies' differential and yet cross-protective binding to produce a triple M2e-mAb cocktail superior to individual mAb treatments at lower doses, universally effective against IAV challenge, and viral escape mutant resistant in immunocompetent and immunodeficient mice. Our study establishes M2e-specific mAb cocktails as viable candidates for developing a universal “off the shelf” influenza virus therapy.

RESULTS

M2e-specific antibodies bind to M2e competitively.

We previously reported generating seven influenza M2e-mAbs recognizing M2e encoded by eight different IAV strains, including clinically relevant and potentially pandemic strains [26]. We used inactivated influenza virions to perform competition assays to determine which antibodies compete for binding. We compared clones 472, 522, 602, 770, and 1191 (**Supplemental Table 1**) when binding to H1N1 A/PR/8/34 (PR8), pH1N1 A/CA/07/2009 (CA07), A/FM/1/1947 (FM1), A/Vietnam/1203/2004 (VN1203), A/Anhui/1/2013 (Anhui1), A/sw/NE/A01444614/2013 (swNE), A/sw/TX/A01049914/2011 (swTX), and A/sw/MO/ A01444664/2013 (swMO) (**Table 1**). To perform this assay, we added M2e-mAbs sequentially to plates coated with the indicated inactivated virions, first an unlabeled competitor antibody and then a biotinylated monitored antibody for detection. A reduction of the absorbance indicates interference by the competing antibody with the monitored antibody's binding.

Looking first at mouse attenuated PR8, a commonly used laboratory IAV, it is evident that clone 770 has limited to no interference with the other M2e-mAbs, suggesting its distinct binding compared to the other antibodies. Clone 770 interfered most with clone 1191 binding, then clone 602 binding, followed by slight interference with clone 522 binding and no interference with clone 472 binding. However, nearly every other antibody showed interference with clone 472 binding and with the other antibodies. One exception is clone 1191, which did not interfere with the binding of clone 522. However, clone 522 did interfere with the binding of clone 1191, suggesting that clone 522 binds with higher affinity than clone 1191 and outcompetes clone 1191 for shared

binding sites (**Figure 1A and C**). Our data indicate that clone 770 has the least competitive and most dissimilar epitope from the other antibodies. These findings are consistent with our previously published work [26].

Similar trends in relative binding emerged when we tested M2e-mAb binding to mouse attenuated CA07, the 2009 H1N1 pandemic virus. Most notably, while clone 522 still bound to CA07, clone 1191 competed more effectively. This indicates that one or more of the mutations in CA07 affected the binding of one or both antibodies to make clone 1191 more competitive with clone 522. These data add new insight to our previous findings, which showed that these two antibodies had slightly altered binding to CA07 from PR8. We now know that IAV strain-specific changes in the M2e sequence increase the relative binding efficiency of clone 1911 over clone 522. Clone 770 also was less inhibited by the competing antibodies in this assay, consistent with the decreased binding and protection provided by clone 770 against CA07 (**Figures 1B and C**).

Next, we tested various recently circulating strains and the two HPAI strains, H5N1 and H7N9. The results of our competitive binding assays were similar to FM1 (**Figure 1C, Supplemental Figure 1A**), VN1203 (**Figure 1C, Supplemental Figure 1B**), Anhui1 (**Figure 1C, Supplemental Figure 1C**), swNE (**Figure 1C, Supplemental Figure 1D**), swTX (**Figure 1C, Supplemental Figure 1E**), and swMO (**Figure 1C, Supplemental Figure 1F**). As expected, generally, a given antibody most strongly competed with itself. However, another antibody, with presumably higher affinity for a shared epitope, outcompeted the original antibody at a level higher than self-interference in a few instances. Specifically, clones 522 and 602 did outcompete 1191 in binding to PR8, and clone 522 did outcompete clone 602 in binding to VN1203. Finally, one of the most

interesting results is the competition between clones 770 and 602 when binding to Anhui1. These antibodies did not show competitive binding to any other viruses, except a slight competition in binding to FM1. However, they strongly competed against each other for binding against Anhui. Overall, we conclude that the binding of our antibodies to the M2 ectodomain differs between antibodies and IAV strains having distinct M2e sequences.

We have previously reported clones 472, 522, and 602 as the most universal of our antibodies at binding to eight different strains of IAV [26]. They are also the most effective at protecting against four different strains of IAV. Having learned that our M2e-specific mAb clones bind differentially and competitively, depending on the IAV strain, we tested whether clones 472, 522, and 602 prophylaxis or therapy drive influenza virus escape mutants with the long-term goal of developing a therapeutic M2e-mAb cocktail.

M2e-specific monoclonal antibodies do not develop escape mutants in vivo.

Viral escape mutants have been reported to all FDA-approved influenza virus therapies, including those targeting M2 function [27]. In humans, escape mutants to influenza therapies have arisen as early as 48 hours after treatment [10, 13]. It is established that IAV can develop escape mutations to M2e-mAbs. The first discovery of viral resistance to protective mechanisms of M2e-specific antibodies was reported in 1989 after in vitro culture with the M2e-mAb 14C2 [23]. Studies of passaging IAV in the presence of other M2e-mAbs in immunodeficient mice have shown the development of viral escape mutants [24, 28]. While the sequence of M2e is highly conserved, these studies demonstrate that with selective pressure from M2e-mAbs strains IAV can develop escape mutations in M2e, which may result in the development of IAV strains resistant to clinical

administration of M2e-mAbs. However, it is possible to develop M2e-mAbs that do not drive the development of viral resistance (e.g., TCN-032), albeit none have proven both effective treatments and resistant to escape mutants to date [25]. To determine if treatment with our most effective M2e-mAbs resulted in viral escape mutants, we utilized an established *in vivo* passaging model [24, 28]. We passaged PR8 in the presence of 472, 522, 602, or PBS control in immunocompetent (WT) and T and B cell-deficient Recombinase Activating Gene 2 knock out (RAG2-KO) mice (**Supplemental Figure 2A and B**). We tested RAG2-KO mice to mimic progression through immunocompromised patients, which comprise about ~3% of the US population [29]. RAG2-KO mice lack T and B cells, cannot produce antibodies or mount an adaptive immune response, and develop chronic influenza virus infections [30]. However, they have innate immune cells capable of responding to antibodies and, like immunocompromised people [31], are known to rapidly develop viral escape mutants to therapies, specifically M2e-mAbs [10, 24]. We also tested control and M2e-mAb treated WT mice for viral escape mutants to simulate passage through the general, mostly healthy population.

PR8 virus was passaged seven times between WT or three times between RAG2-KO mice (4-8 mice/passage) for three and a half weeks. We passaged the virus by collecting lungs from one set of IAV infected passage mice, bulk isolating the virus through lung homogenization, and blind passaging by infecting the next set of mice with the isolated bulk virus. For WT mice, viral passaging was performed twice a week (every 3 or 4 days) for 24 days (**Supplemental Figure 2A**). We chose to passage the virus at days three or four after IAV infection, as this timeframe is well established to be the peak of viral replication for PR8 in WT mice. For RAG2-KO mice, viral passaging was performed once a week (every seven days) for 21 days (**Supplemental Figure 2B**).

We chose this timeframe as influenza virus infections in immunocompromised mice and humans resemble chronic infection with more minor inflammation [30]. We treated all mice with M2e-mAb or isotype-matched controls mAbs twice a week to subject the virus to selective pressure. Twenty-one (Rag-KO mice) or 24 days later (WT mice), we isolated viral RNA from the lung tissues of individual mice and generated M gene segment-specific cDNA, which was subjected to Sanger sequencing. Excitingly, comparisons of the M sequences revealed that no mutant escape viruses developed in any of our treatment groups compared to the M2-sequence of our initial (day 0) PR8 virus despite constant selective and immune pressure from M2e-mAb treatment for 24 days (**Table 2, Supplemental Figure 3**). Likewise, we did not see the outgrowth of escape mutants in any of our treatment groups after 21 days of passaging through RAG2-KO mice (**Table 2, Supplemental Figure 3**). While other M2e-mAbs have been reported to develop escape mutants in mice [23, 24, 32, 33], we do not see evidence of escape mutations with our antibody treatments. Our data is consistent with data obtained with the highly universal mAb TCN-032, which did not develop escape mutants in clinical trials [25].

M2e-specific antibodies are more protective against lethal influenza virus challenge as a cocktail.

Given influenza's mutation rate and ability to undergo antigenic shift and drift, an effective therapeutic agent must remain effective against influenza strains that may arise in the future. Therefore, to prevent the development of viral escape mutants and improve the universality of a potential IAV therapeutic, we combined three high-affinity M2e-mAbs (clones 472, 522, and 602) that differ in their affinities for known M2e sequences encoded by specific IAV strains for further testing. Our experimental rationale is based on the following important considerations: First,

clones 472, 522, and 602 are protective against PR8, CA07, VN1203, and Anhui and bind strongly to infected cells and virions, as demonstrated by ELISAs [26]. Second, clones 472, 522, and 602 bound to PR8, CA07, VN1203, and Anhui virions with somewhat different and complementary relative binding affinities (**Figure 1, Supplemental Figure 1**), and we have already established that all three antibodies are highly protective against these strains *in vivo* [26]. Third, these antibodies are the most cross-protective of our seven options [26]. Fourth, we did not detect viral escape mutants in response to therapy when clones 472, 522, and 602 were tested individually (**Table 2**). Finally, all three M2e-mAb clones are effective antiviral isotypes (IgG1 and IgG2a) [26, 34]. We have demonstrated that these clones bind to a highly conserved region of M2e; however, they bind to different viruses with different affinities. These data lead us to hypothesize that clones 472, 522, and 602 would interact synergistically. Thus, a mutation in M2e that decreases or escapes the binding of one mAb would not necessarily escape the binding of another.

To assess the potential of an M2e-monoclonal antibody cocktail treatment, we prophylactically treated mice with either a 60 μg dose, composed of equal parts 472, 522, and 602 (20 μg/each), or a dose of 30 μg of the mixture (10 μg/each), and challenged mice with a $5XLD_{50}$ dose of PR8. Compared to isotype control mice, all mice (100%) treated with 60 μg of the 472/522/602 cocktail were fully protected from lethal IAV challenge. Further, 30 μg of the mixture protected 60% of the mice (**Figure 2A**). Thus, the 472/522/602 cocktail was more effective against lethal PR8 challenge than single antibody treatments [26], where the most effective M2e-mAb, clone 472, was 100% protective at 100 μg and 25% protective at 25 μg. We also tested the 60 and 30 μg doses of the 472/522/602 cocktail against a lethal ($10xLD_{50}$) dose of CA07. The 60 μg dose protected 70% of the mice, while the 30 μg dose protected 60% (**Figure 2B**). In our previous study with

CA07 [26], single antibody treatments with individual M2e-mAb clones protected between 80 and 100% of mice at 100 μg. Our data demonstrate that the 472/522/602 cocktail is highly protective at low doses against PR8 and CA07 and more effective than the single mAb treatment.

Testing 60 μg and 30 μg 472/522/602 cocktail doses against a lethal challenge with VN1203, we again found that the antibody cocktails were highly protective at both doses (**Figure 2C**). For comparison, while 60 μg of the triple cocktail is 90% effective here, our previous study showed that only 100 μg of the most effective single antibody treatment against VN1203, clone 934, prevented death in all mice [26]. Thirty μg of the 472/522/602 cocktail provided 50% protection, similar to the protection seen with 100 μg of any component antibody (472: 50%, 522: 70%, and 602: 70%) [26]. Finally, we tested the 472/522/602 cocktail against Anhui1. We used the same experimental design, testing 30 and 60 μg doses of the 472/522/602 cocktail prophylactically against a $10XLD_{50}$ dose of Anhui1. Again, 100% of the mice in both groups were protected from the lethal challenge (**Figure 2D**). These results are similar to the high protection demonstrated by the individual antibodies against this strain at 25 μg [26]. Importantly, prophylactic treatment with the 472/522/602 cocktail also significantly improved weight loss in three of the four IAV challenges (**Figure 2**), indicating reduced disease severity. We conclude that the M2e-specific antibody cocktail comprised of 472/522/602 is highly effective against the four strains of IAV tested, protecting against lethality and improving clinical signs. The cocktail is also more effective at a low dose than most single M2e-specific antibodies against PR8, CA07, and VN1203 and remains highly effective against Anhui1. These and our previously published data [26] strongly support developing our novel M2e-mAb cocktail as an "off the shelf" universal therapeutic against influenza A virus infection.

Consistent with our previous report of individual mAbs [26], the triple cocktail does not significantly decrease viral titers from CA07, VN1203, or Anhui1 (Supplemental Figure 4).

M2e-specific antibody triple cocktail is more effective than its component pairs.

To determine whether all three M2e-mAbs were needed and contributed equally to the protection by the cocktail, we tested to see if double cocktails composed of the component pairs of the triple cocktail were as effective as the triple cocktail against lethal PR8 challenge. We prophylactically treated mice with a 30 μg dose of the triple cocktail (472 IgG2a/522 IgG1/602 IgG2a), or the 472 IgG2a/522 IgG1, 472 IgG2a/602 IgG2a, or 522 IgG1/602 IgG2a double cocktail. Each cocktail was composed equally of its component antibodies (10 μg each antibody for the triple cocktail and 15 μg each antibody in the double cocktails). While the 472/522/602 triple cocktail protected 88% of the mice, protection mediated by all three double cocktails was lower and only protected 33 to 44% of mice from a lethal challenge (**Figure 2E**). Further, only the 472/522/602 cocktail was significantly protective when comparing overall percent weight loss. The double cocktails improved weight loss slightly, with four days each of significant differences in percent weight compared to the isotype control group. However, this was far less than the 472/522/602 cocktail with 11 days of significant weight loss differences (**Figure 2E**). These results indicate that the protection provided by the triple cocktail is not attributable to a single monoclonal antibody or the effect of two together. The increased protection is also not due to a combination of the IgG1 and IgG2a isotypes. Instead, using the triple cocktail has an additive effect, increasing protection from lethal IAV infection at low doses.

Of note, we attempted to add antibody 770 to the triple cocktail to see if increasing the number of

clones further improves protection. However, contrary to our expectations, 770 reduced the 472/522/602 cocktail protection at low doses (**Supplemental Figure 4A**). Further, while 770 did not affect the 472/522/602 cocktail at the standard 60 μg dose, 770 decreased protection from 522 (**Supplemental Figure 4B**). These results demonstrate a synergistic effect of the 472/522/602 cocktail independent of an increased number of clones in the mixture.

472/522/602 Antibody Cocktail also avoids escape mutants.

We next evaluated whether a low dose 472/522/602 cocktail therapy results in the outgrowth of viral escape mutants. To do so, we performed the same WT and RAG2-KO passages described above, this time using the 472/522/602 cocktail and a treatment regimen where individual cocktail components are alternated as a therapeutic treatment (**Supplemental Figure 2**). We found that neither the cocktail nor the alternating treatment resulted in the outgrowth of escape mutations (**Table 2, Supplemental Figure 3**), even when the PBS injection allowed time for potentially uncontrolled viral outgrowth after selective pressure. Collectively, our data demonstrate that the 472/522/602 cocktail is equally effective at avoiding escape mutants as the individual antibodies.

Of note, IAV generally increases in virulence against mice with additional passages as the virus adapts to the mouse host. However, we found that passaging with all antibody treatments mitigated virulence compared to the PBS passage (**Table 3**). Interestingly, the effect on the virus's virulence correlated with the efficacy of the individual mAbs against PR8: 522<602<472. Also, notably, the 472/522/602 cocktail maintained robust effectiveness against both the PBS-passaged and the 472/522/602 cocktail-passaged virus (**Figure 3**). As such, our sequencing and *in vivo* data demonstrate the virus's failure to escape from our highly effective antiviral therapy. These data are

important as they exclude the possibility that mutations outside of the M region allow viral escape, a viral escape mechanism demonstrated by others [23, 28].

M2e-specific antibodies as a triple cocktail or alternating treatments improve protection compared to the least effective component antibody.

Theoretically, future mutations in influenza virus-encoded M2e may reduce the efficacy of individual antibody clones contained in our cocktail. Due to this possibility, we experimentally examined whether the 472/522/602 triple cocktail would remain effective if one of its M2e-mAbs failed to protect from lethality. As WT mice survive a lethal challenge when prophylactically treated with clone 472, 522, or 602 [26], we used Rag-KO mice for this study. In contrast to WT-mice, Rag-2-KO mice do not benefit from a prophylactic infusion with clone 522, while clones 472 and 622 significantly prolong survival after IAV challenge (**Figure 4**). To evaluate whether the 472/522/602 triple cocktail will remain effective despite clone 522's failure to mediate significant protection, we infected RAG2-KO mice with a BALB/c 5xLD50 dose of PR8. We treated Rag-2-KO mice prophylactically on day -1 and twice a week therapeutically throughout the experiment (**Figure 4A**). We tested six treatment options: individually, clones 472, 522, 602 (60 μg each), the 472/522/602 cocktail (60 μg total), a PBS control, or an alternating treatment (between clones 472, 522, 602, and PBS; 60 μg total). We predicted that, at a minimum, the 472/522/602 triple cocktail and the alternating treatments would increase protection past the protection of the least effective antibody.

522 was not significantly protective in the RAG2-KO model compared to controls. However, groups of RAG2-KO mice treated with 60 μg total antibody per dose comprised either of clones

472 or 602, the 472/522/602 triple cocktail, or alternating M2e-mAb clones and PBS saw significantly delayed lethality (**Figure 4**). Thus, administering the cocktail, or providing more effective antibodies as part of alternating treatments, provided protection leading to delayed lethality compared to treatments with clone 522 alone. We conclude that our M2e-mAb cocktail is not only more effective than its individual components, but in the case of a universal therapeutic, a cocktail would allow for increased protection even if one component M2e-mAb becomes less effective or ineffective.

DISCUSSION

Seasonal influenza virus epidemics burden the human population and kill about half a million people annually, including about 50,000 people in the US. Besides its annual disease burden, influenza virus infections cost the US economy an estimated 11.2 billion US dollars [35]. There are currently six FDA-approved treatments for IAV [2, 36]. However, viral escape mutants have been found to each [10-13, 17, 18], some already during clinical trials [17], with some mutations increasing the transmissibility of resistant viruses [15]. Two of these treatments, amantadine, and rimantadine, are matrix protein 2 (M2) channel blockers now rendered ineffective due to widespread resistance. By 2009, all H3N2 and H1N1 isolates tested were resistant to adamantane treatment [10]. To better prepare for seasonal and pandemic IAVs, a safe, effective, and universally protective "off the shelf" treatment option is needed.

Influenza A virus-encoded M2 protein is a suitable target for such a therapy. M2 assembles into a highly conserved proton channel expressed on influenza virions and infected cells. The 5-prime coding region is shared between M1 and M2 proteins [8]. Consequently, its N-terminus is highly conserved, and antibodies specific to this conserved region seem more resistant to escape mutants [25]. We have previously published an analysis of seven M2e-specific monoclonal antibodies derived from the AuNP-M2e-CpG vaccination in BALB/c mice [26]. Our data identified several clones to be broadly protective against IAV and recognizing a range of M2e sequences encoded by different influenza A subtypes (**Table 1**). Our data suggest that our antibodies bind epitopes within M2e's highly conserved N-terminal region, albeit this requires further epitope mapping. Notably, despite IAV's recognized ability to rapidly develop escape mutations to M2e-mAbs *in vitro* [23] and in mice [23, 24, 32, 33], viral passaging of PR8 in the presence of the triple cocktail,

or each one of its component antibodies in wild type (WT) and RAG2-KO mice did not result in the development of viral resistance. Thus, it is possible to target IAVs with M2e-mAbs specific to an M2 region that is not easily mutated by selective pressure. To our knowledge, we are the first to develop universally protective and viral escape mutant resistant M2e-mAbs antibodies and an M2e-mAb cocktail.

M2 is essential for viral core disassembly, assembly, and budding [19]. However, most M2e-specific antibodies do not neutralize IAVs [20, 26, 33, 37-40], and those who do neutralize certain IAV strains do so at the cost of universality [23, 32, 40-43]. It is important to note that neutralization, while effective, is not a requirement for protection from lethal IAV challenge, as demonstrated by non-neutralizing but protective antibodies to M2e-mAbs and influenza's HA stalk region [33, 44, 45]. Our M2e-mAbs are also not neutralizing, albeit clone 472 significantly, but incompletely, reduced viral replication *in vivo* [26]. Therefore, we are currently exploring potential Fc receptor-mediated antiviral functions elicited by our M2e-mAbs that may mediate the observed protection from severe disease [26] and **Figures 2-4**.

While combinatorial antibody administration can reduce the risk of viral escape, this theory has not yet been demonstrated for influenza A viruses. Instead, in vitro and in vivo antibody treatments are established methods to map out influenza virus escape variants important for modeling antigenic drift [46, 47]. Therefore, to enable protection at lower doses and increase universality and resistance to viral immune escape, we tested double and triple M2e-mAb cocktails composed of individual mAb components that each protect mice from lethal IAV challenge. While low dose combinations of two M2e-mAb pairs fail to protect lethally IAV challenged mice completely, our

triple cocktail is effective at low doses, universally protective, and does not elicit viral escape mutants in immunocompetent and immunocompromised mice. These characteristics are all desirable for an "off the shelf" IAV therapeutic, as they ensure rapid availability, consistent protection between strains, and prevention of viral resistance. In comparison, our 60 μg dose is approximately 3.7 mg/kg and 100% protective against H1N1 PR8 when administered prophylactically, while TCN-032 was 60% protective when administering three 24.5 mg/kg doses on days 1, 3, and 5 post-infection [22]. Estimates based on our data for a starting prophylactic dose for a human trials would be 0.3 mg/kg (based on Nair and Jacob's method described in 2016), a dose over 130 times lower than the therapeutic dose used in the TCN-032 clinical trial [22, 48]. Further, the protection provided by the triple M2e-mAb cocktail is not the result of a dominant effect by one or two of the component antibodies. Instead, all three M2e-mAbs contributed to producing the additive protection seen with the triple cocktail.

Influenza virus mutation is mainly unpredictable, so we considered that our antibodies might not protect against a future IAV strain. We tested whether the combined effect of the cocktail would compensate for decreased protection by one of the component antibodies. Excitingly, our triple M2e-mAb cocktail remained highly efficient even if one of its component mAbs became inefficient (**Figure 4**). Furthermore, no escape mutants were observed upon this treatment, even in immunocompromised mice. Thus, our data suggest that this cocktail would consistently treat infection with IAVs having diverse M2e sequences. Consequently, the development of escape mutant viruses with clinical M2e-mAb triple cocktail administration is unlikely even when a single mAb within the cocktail becomes less effective. The concept of combining multiple therapies or combining epitopes has been suggested previously [8, 49]. However, to our knowledge, this is the

first study demonstrating a combination of mAbs for IAV treatment to be more effective than individual mAb therapies and viral escape mutant resistant.

In summary, our study establishes a triple cocktail of cross-protective M2e-mAbs to be 1) efficacious at preventing IAV lethality at low doses, 2) consistently and universally protective between IAV strains, and 3) resistant to viral immune escape. Overall, these data indicate that the M2e-mAb clone 472/522/602 triple cocktail is a strong candidate for developing a universal IAV treatment. To our knowledge, these data are the first demonstration of a successful universally protective and viral escape mutant resistant influenza A virus specific cocktail. We will now use the knowledge gained from our robust prophylactic studies to develop the most effective and universally protective IAV therapeutic agent. Our study will critically shape future M2e-mAb-based influenza-therapeutic development.

METHODS

Animals

At Baylor College of Medicine (BCM), BALB/c and RAG2-KO mice were ordered from or bred internally from breeders obtained from Charles River Laboratories. Mice were cared for in the animal facilities of the Center for Comparative Medicine at BCM and Texas Children's Hospital. At Scripps Research Institute, BALB/c and RAG2-KO mice were ordered from Charles River Laboratories. Mice were cared for in the animal facilities of the Immunology Building at The Scripps Research Institute, La Jolla, CA. At the University of Georgia Athens, female BALB/c mice were purchased from Envigo RMS, Inc., Indianapolis, Indiana, USA. Mice were cared for in

the animal facilities of the University of Georgia Athens. Mice were 6-8 weeks at the start of experiments. All protocols involving the use of experimental animals in this study were approved by Baylor College of Medicine's, The Scripps Research Institute's, and the University of Georgia, Athens' Institutional Animal Care and Use Committees and were consistent with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Viruses

Prior to use in this study all viruses were obtained, passaged, isolated, and quantified as previously described [26]. All experiments using H7N9 or highly pathogenic H5N1 avian influenza virus were reviewed and approved by the institutional biosafety program at the UGA and were conducted in biosafety level 3 enhanced containment. Work with highly pathogenic avian influenza virus followed guidelines for the use of Select Agents approved by the CDC. All viruses are wildtype (WT; not PR8 background), and M2e-sequence is confirmed within two passages.

Biotinylation of M2e-specific mAbs

M2e-specific mAbs were biotinylated using EZ-Link Hydrazide Biocytin (ThermoFisher Scientific) according to the manufacturer's instructions for labeling glycoproteins with hydrazide biocytin. Biotinylated mAb was separated from non-reacted material by dialysis (10kd MWCO; ThermoFisher Scientific) in 1X PBS for 12 hours. Samples were removed from dialysis cassettes, aliquoted, and stored at 4ºC.

Competition ELISAs

Nunc Maxisorp Flat-Bottom plates (ThermoFisher Scientific) were coated overnight at four °C

with 100 µl/well of purified inactivated influenza virus at 0.5 µg/mL in bicarbonate buffer (pH 9.6). After washing 3 times with PBS/T, the plates were blocked with 100 µl/well of 1% BSA in PBS for 2 hours. Plates were washed 3x with PBS/T and 50 µl/well of biotinylated M2e-specific mAb at 2 µg/mL (the concentration resulting in approximately 50% saturation in the assay) were added to plates and incubated for 1 hour at 37ºC. M2e-specific mAbs were added as the competing antibody in 4-fold dilutions and incubated for 1 hour at 37ºC. After washing, 50 µl/well of a 1:10,000 Streptavidin-HRP (Vector Laboratories) was added to plates and incubated for 1 hour at 37ºC. Plates were washed with PBS/T, and 50 µl/well of TMB substrate was added for 10-15 minutes for the color change, and the reaction was stopped with the addition of 50 µl/well H2SO4. Absorbance was measured at OD450 nm.

M2e-escape mutant development experiments in BALB/c mice

For the experiment in BALB/c, the mice were injected intraperitoneally (i.p.) with 60 µg of the indicated M2e-mAbs on day -1. On day 0, the mice were anesthetized with isoflurane and oxygen and infected intranasally (i.n.) with the 5X 50% lethal dose (LD_{50}) of PR8. The mice were sacrificed with isoflurane overdose alternating between day 3 and day 4 post-infection. The virus was isolated from the lungs and pooled, and 20 µl of the isolated virus was passaged to a new naïve mice group that had been treated with the indicated M2e-mAbs on the previous day. The virus was passaged 6 times through 7 sets of the mice group. 4 mice per group were used for the first 6 sets of the mice, and 8 mice per group were used for the last set of the mice. Isolated virus was aliquoted and stored at -80℃ for sequencing. See **Supplemental Figure 2**.

M2e-escape mutant development experiments in RAG2-KO mice

For the viral passages in RAG2-KO mice, the mice were injected with the indicated M2e-mAbs twice a week (on day-1 and day3), infected i.n. with $5XLD_{50}$ of PR8 on day 0, and sacrificed with isoflurane overdose on day 7 to isolate the virus from the lungs. The 20 µl of the isolated virus was passaged to new mice groups. Virus was passaged twice through 3 sets of mice. 4 mice per group were used for the first 2 sets of the mice, and 8 mice per group were used for the last set of the mice. The isolated virus was aliquoted and stored at -80℃ for sequencing.

Viral Isolation for Viral Passages

Virus was isolated as previously described [26] using Ultracel-100 tubes (Amicon Ultra-15 Centrifugal Filter Unit; Ultracel-100 regenerated cellulose membrane) as directed.

IAV M2e sequencing

The M2 sequences of the PR8 strain (LC120394.1) are obtained from GenBank. We extracted total RNA from the viruses isolated after several passages in BALB/c or RAG2-KO mice using QIAamp Viral RNA Mini Kit (Qiagen). The cDNA was synthesized through the extracted total RNA with M2-2 primer (5'-GCGAAAGCAGGTAGATATTG-3'), which binds to a 3' noncoding region of influenza vRNA7, using Omniscript RT Kit (Qiagen). The cDNA was amplified via PCR using KAPA HiFi HotStart ReadyMix (Roche) with the M2-2 and SEQ7 (5'-ATATCGTCTCGTATTAGTAGAAACAAGGTAG-3') primers. SEQ7 binds to a 5' noncoding region of influenza vRNA7. The size of the PCR product was 1,042 bp. This product was sequenced by Genewiz, Inc using M2SeqN1 (5'-ATGTTATCTCCCTCTTGAGC-3') and SEQ7 primers to resolves M2e sequences, and the results compared to the original M2e sequences [50]. M2SeqN1 was annealed to 331-351 of M1 cDNA, and SEQ7 was annealed to a 5' noncoding

regions of cDNA.

Protection Studies

As previously described [26], mice were given an IP injection of the specified mAb at a specified dose 24 hours before virus challenge with 5X or 10X 50% lethal dose (LD_{50}) of the specified virus. PR8 challenge virus was administered in 20 μL of PBS intranasally to mice anesthetized with isoflurane. The CA07 and VN1203 challenge viruses were administered in 30 μL of PBS intranasally to mice anesthetized with Ketamine/xylazine. The Anhui1 virus was administered intranasally to mice anesthetized with 2,2,2-tribromoethanol in tert-amyl alcohol (Avertin; Aldrich Chemical Co). Each challenge with CA07, VN1203, and Anhui1 viral inoculum was back-tittered on MDCK-ATL cells to confirm the dose. If specified, a subset of mice was humanely euthanized, and tissues collected for virus titer 3 days post-infection (dpi). All animals were monitored for body weight and humane endpoints for euthanize. Survival and weight loss were monitored for up to 21 dpi or until all animals recovered to at least 90% starting body weight.

Viral Titer

Plaque assays were performed as previously described [26].

Statistics

Graphpad Prism 7 was used for all statistical analyses. A one-way ANOVA with a Tukey's multiple comparisons test was utilized to compare overall percent weight loss. A two-way ANOVA with a Dunnett's multiple comparisons test was utilized for daily percent weight loss comparisons after condensing data through mean and SD analysis. A Mantel-Cox log rank test was

used for survival analysis. All statistics are indicated in the figure legends. **** $p<0.0001$, *** $p<0.001$, ** $p<0.01$, * $p<0.05$.

Supplemental Material

Supplemental Table 1: M2e-specific Monoclonal Antibodies

Supplemental Figure 1: M2e-specific antibodies bind to M2e competitively.

Supplemental Figure 2: M2e-specific monoclonal antibodies do not develop escape mutants in vivo.

Supplemental Figure 3: Sequencing chromatograms for WT and RAG2-KO passages with single and cocktail antibody therapies.

Supplemental Figure 4: The 472/522/602 triple cocktail does not decrease viral load with CA07, VN2103, of Anhui1.

Supplemental Figure 5: Adding 770 to the 472/522/602 triple cocktail loses efficacy at low doses.

Study approval

All institutions, each institutions' Animal Care and Use Committees approved all protocols for animal experiments, and all institutions follow the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research.

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AUTHOR CONTRIBUTIONS: SP and SMT conceptualized the project. SP, SMT, LB, and TK developed methodologies and designed experiments. LB, SLR, TK, AYS, SKJ, and CAJ performed experiments. SP, SMT, LB, SLR, TK, AYS, SKJ, and CAJ analyzed data. LB, TK, and SLR visualized data. SP and SMT acquired funding for the study and administered and supervised

the project in their respective laboratories. LB and SP wrote the original manuscript draft, SP, SMT, and LB edited the manuscript. For determining the order for co-authors, as LB contributed to writing the manuscript, LB was listed first.

Virus	Subtype	Strain Abbreviations	Amino Acid Sequence																							
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
Consensus sequence			M	S	L	L	T	E	V	E	T	P	I	R	N	E	W	G	C	R	C	N	D	S	S	D
A/PR/8/1934	H1N1	PR8	M	S	L	L	T	E	V	E	T	P	I	R	N	E	W	G	C	R	C	N	G	S	S	D
A/CA/07/2009	pH1N1	CA07	M	S	L	L	T	E	V	E	T	P	T	R	S	E	W	E	C	R	C	S	D	S	S	D
A/FM/1/1947 (WT & MA)	H1N1	FM1	M	S	L	L	T	E	V	E	T	P	T	K	N	E	W	E	C	R	C	S	D	S	S	D
A/Vietnam/1203 /2004	H5N1	VN1203	M	S	L	L	T	E	V	E	T	P	T	R	N	E	W	E	C	R	C	S	D	S	S	D
A/Anhui/1/2013	H7N9	Anhui1	M	S	L	L	T	E	V	E	T	P	T	R	T	G	W	E	C	N	C	S	G	S	S	E
A/sw/NE/A01444614/2013	H1N1	swNE	M	S	L	L	T	E	V	E	T	P	T	R	N	G	W	E	C	K	C	N	D	S	S	D
A/sw/TX/A01049914/2011	H3N2	swTX	M	S	L	L	T	E	V	E	T	P	T	R	S	E	W	E	C	R	C	S	D	S	S	D
A/sw/MO/A01444664/2013	H1N2	swMO	M	S	L	L	T	E	V	E	T	P	T	R	N	G	W	E	C	K	C	N	D	S	S	D

Table 1: M2e sequences of influenza A viruses used in this study compared to the consensus sequence. M2e sequences of viruses used in experiments, including viruses from humans, birds, and swine for the M2e sequence [8]. The consensus is derived from seasonal influenzas viruses circulating since 1957 (H1N1, H2N2, and H3N2; shown). Mutations from the consensus sequence are shown in blue.

Mouse Strain	Passaging mAb	Nucleotide Sequence
WT	M2e Coding Region 1:	G T T T C G A C C T C G G T T A G A A G A C T C A T
	472	- -
	522	- -
	602	- -
	472/522/602	- -
	ALT	- -
	PBS	- -
	M2e Coding Region 2:	A T C A C T T G A A C C G T T G C A T C T G C A C C C C C A T T C G T T T C T G A T A G G C
	472	- - - - - - - - - - - - - - - - - - -

Virus	Passaging Antibody	LD50 (PFU/mL)	5XLD50 (PFU/mL)
PR8	PBS	3.05	15.25
	472	12.62	63.1
	522	4.25	21.25
	602	9.4	47
	472/522/602	5.99	29.95

Table 3: Passaging in PR8 in WT mice with M2e-mAbs affects virulence. LD50 was calculated using the Reed-Muench method[51] based on viral challenge of BALB/c mice with 3 to 4 doses between 0.24 and 30 PFU with specified virus post passage.

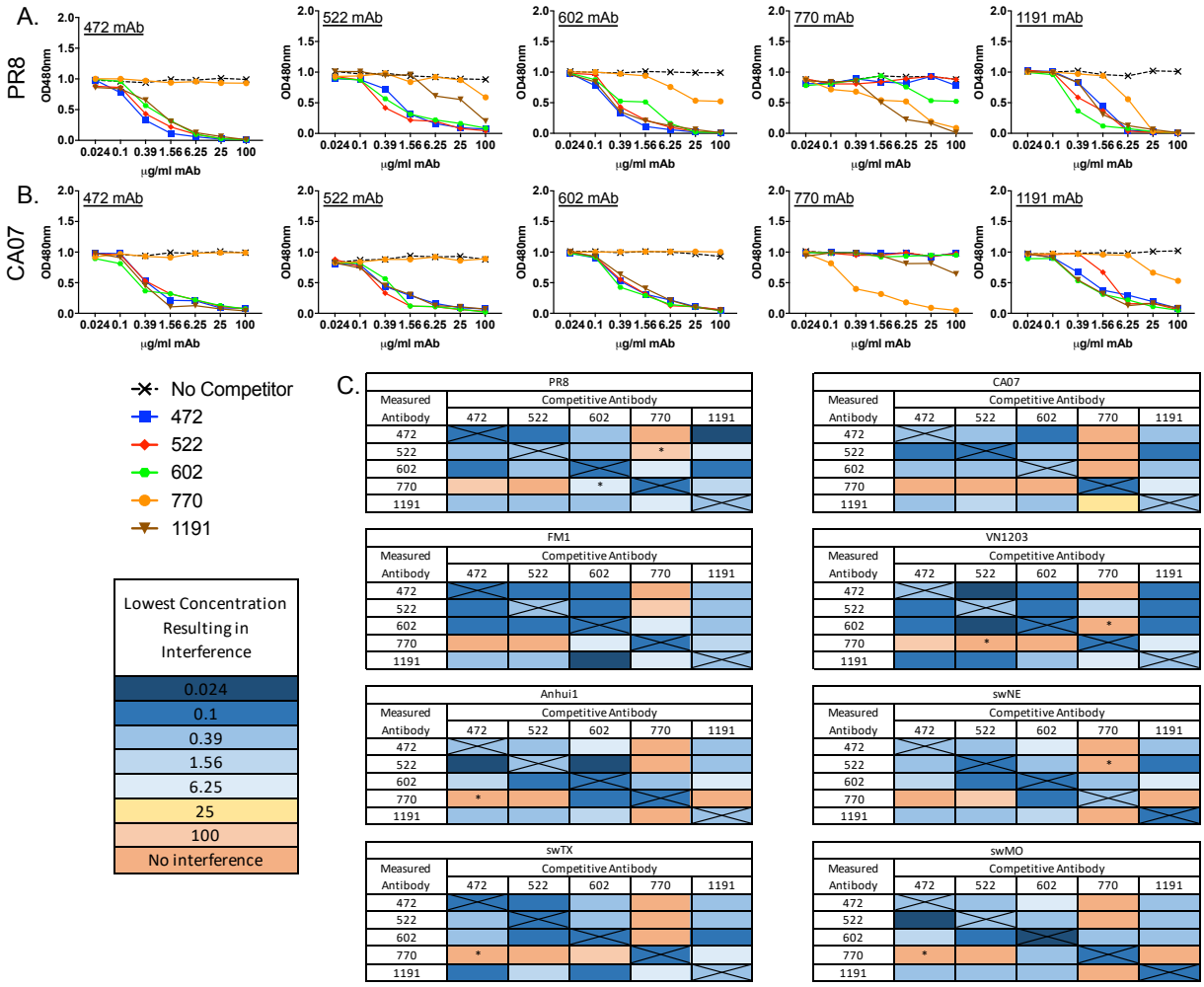


Figure 1: M2e-specific antibodies bind to M2e competitively. Complete results from (A) PR8 and (B) CA07 competition ELISAs. Inactivated virions were used as coating antigen. The measured biotinylated antibody was added at a standard concentration of 2 μg/mL to the wells. The competitive antibody was added at 4-fold dilutions from 100 μg/mL. Absorbance was measured with a biotin binding secondary. (C) Representative results of competition ELISAs shown in more detail in **Supplemental Figure 1**. The figures above were produced by noting the concentration at which the absorbance dropped 0.1 below the average absorbance of the no competitor control (specific to the antibody and virus). * indicates variability, one or more lower dilutions going below the indicated cut off with higher dilutions still above the cutoff.

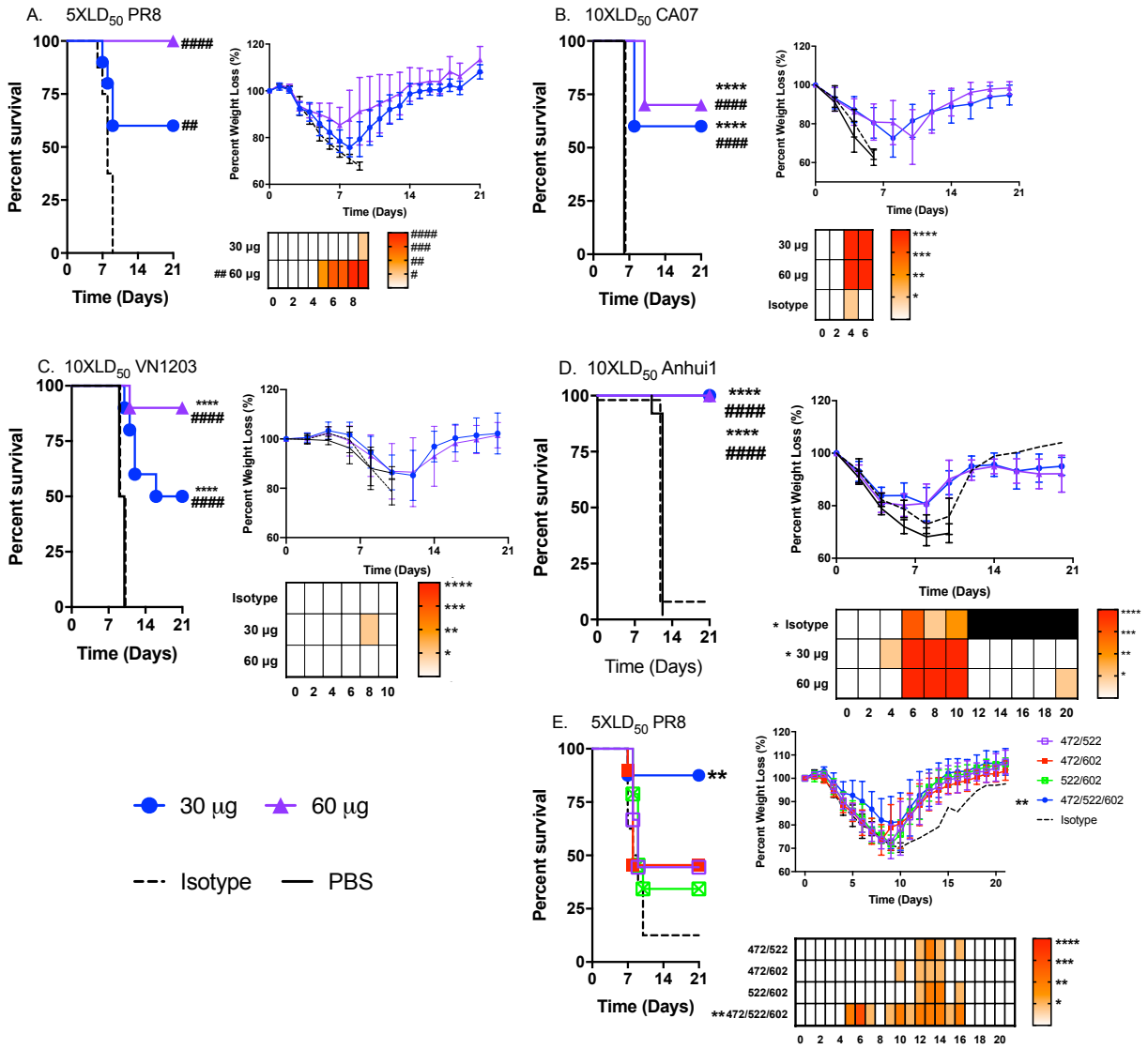


Figure 2: M2e-specific antibodies are more protective against lethal challenge with PR8 as a cocktail. (A-D) BALB/c mice were treated with indicated dose of M2e-mAb 472/522/602 cocktail one day prior to infection with (A) PR8, (B) CA07, (C) VN1203, or (D) Anhui1. (E) BALB/c mice were treated with a 30 µg dose of the indicated M2e-mAb cocktail, containing 2 or 3 M2e-specific antibodies in equal parts, one day prior to infection with a 5XLD$_{50}$ PR8. (A-E) Data for each challenge includes a survival analysis, percent weight loss data analysis, and a heatmap is

indicative of significantly different percent weight loss from isotype control group each day. (A-D) n=10 or (E) n=8-9. **** $p<0.0001$, *** $p<0.001$, ** $p<0.01$, * $p<0.05$, log-rank analysis. * indicates significance compared to PBS control, # indicates significance compared to isotype control. Percent weight loss data for survival analysis is shown to the right of each graph. Heatmap below each weight loss curve is indicative of significantly different weight loss from control group on each day. For heatmap, * indicates significance compared to PBS control, unless unavailable for that timepoint, in which case treatment groups were compared to isotype control. Black indicates the isotype control has become the control group for the comparison as all PBS animals have died.

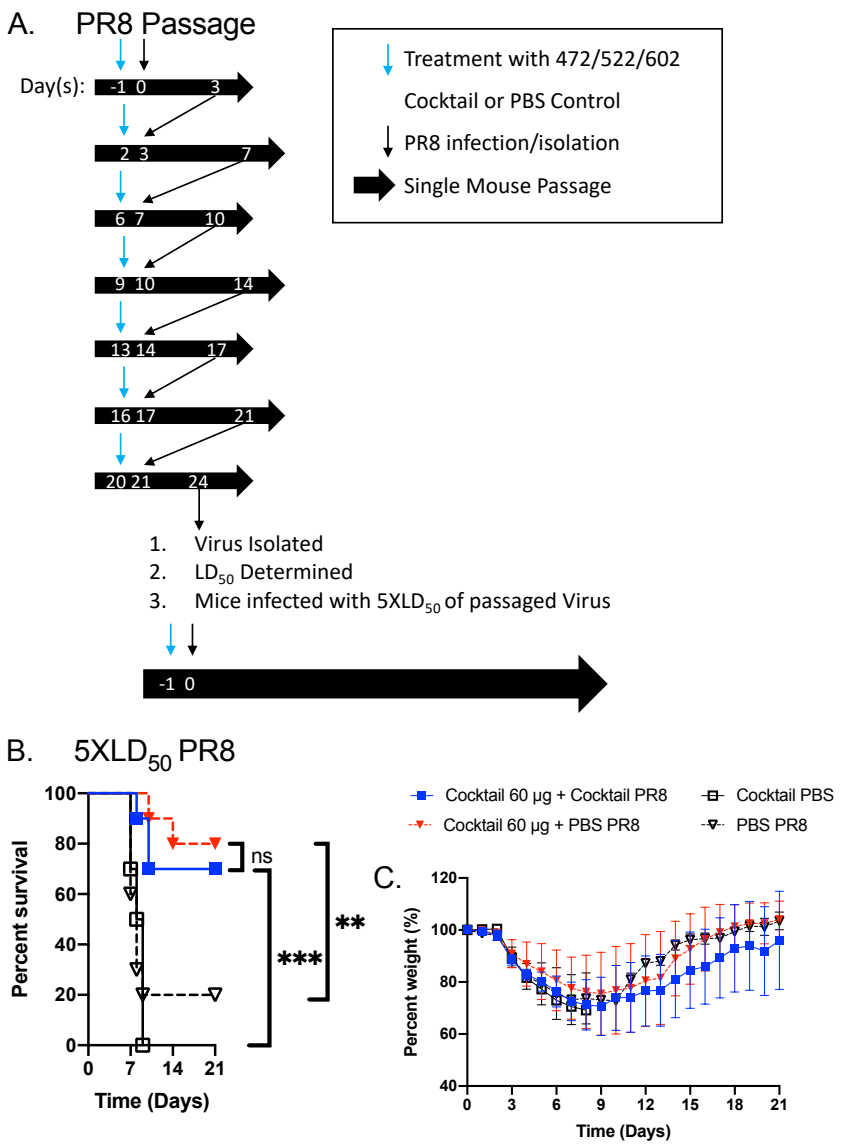


Figure 3: M2e-specific antibody triple cocktail maintains effectivity after virus has been passaged with the triple cocktail treatment. BALB/c mice were treated with 60 µg dose of 472/522/602 M2e-mAb cocktail one day prior to infection with a $5XLD_{50}$ of PR8 that has been passaged in WT mice with PBS control or 472/522/602 cocktail (LD_{50} for each virus specified in **Table 3**). (A) Diagram of timepoints for viral passaging in WT mice, (B) Survival analysis and (C) percent weight loss data analysis; n=10. **** $p<0.0001$, *** $p<0.001$, ** $p<0.01$, * $p<0.05$, log-rank analysis.

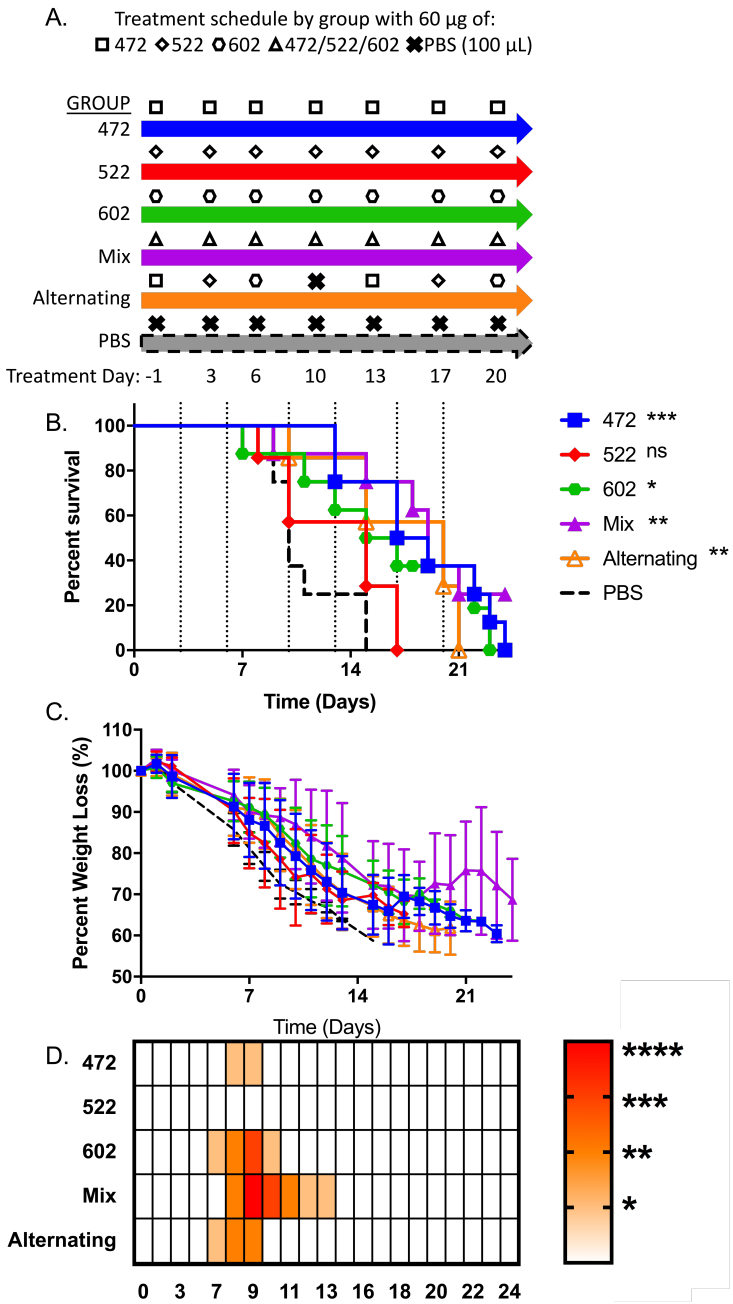
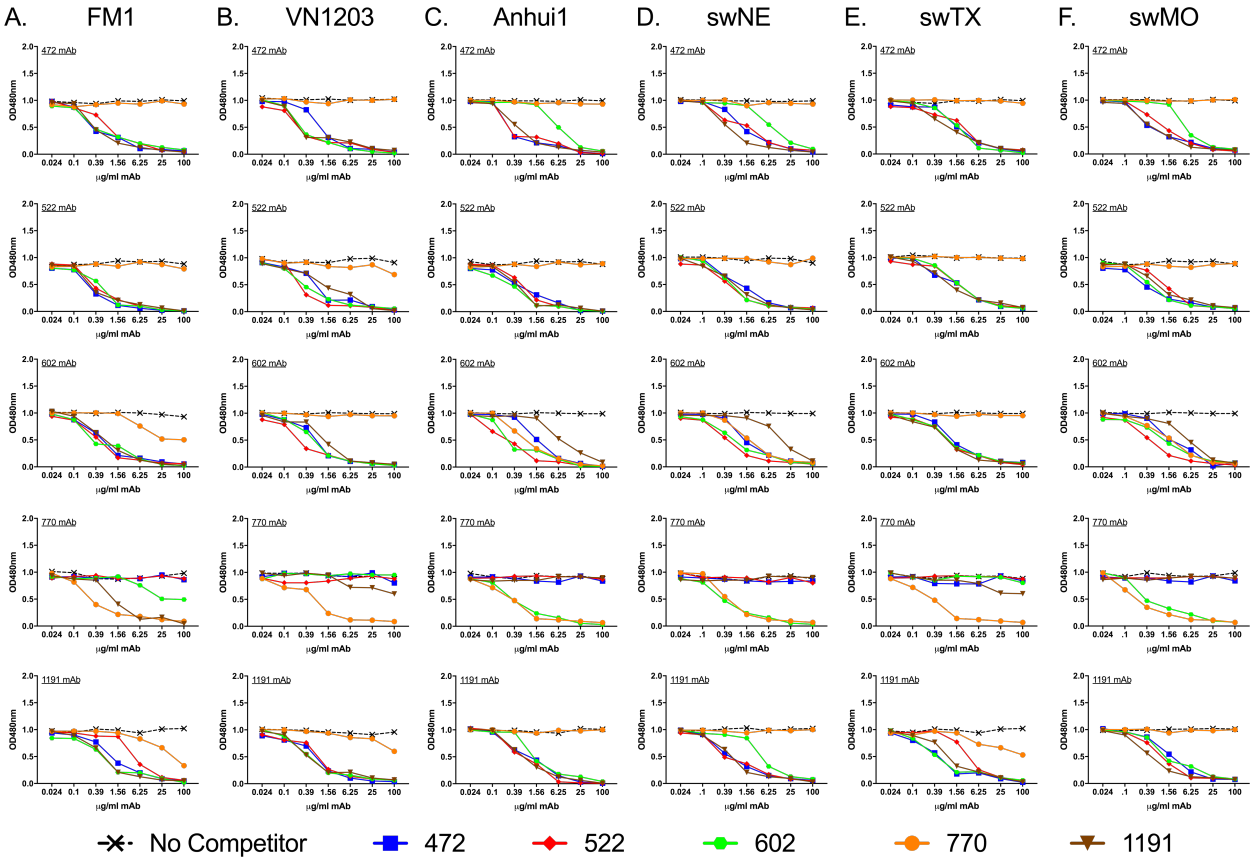


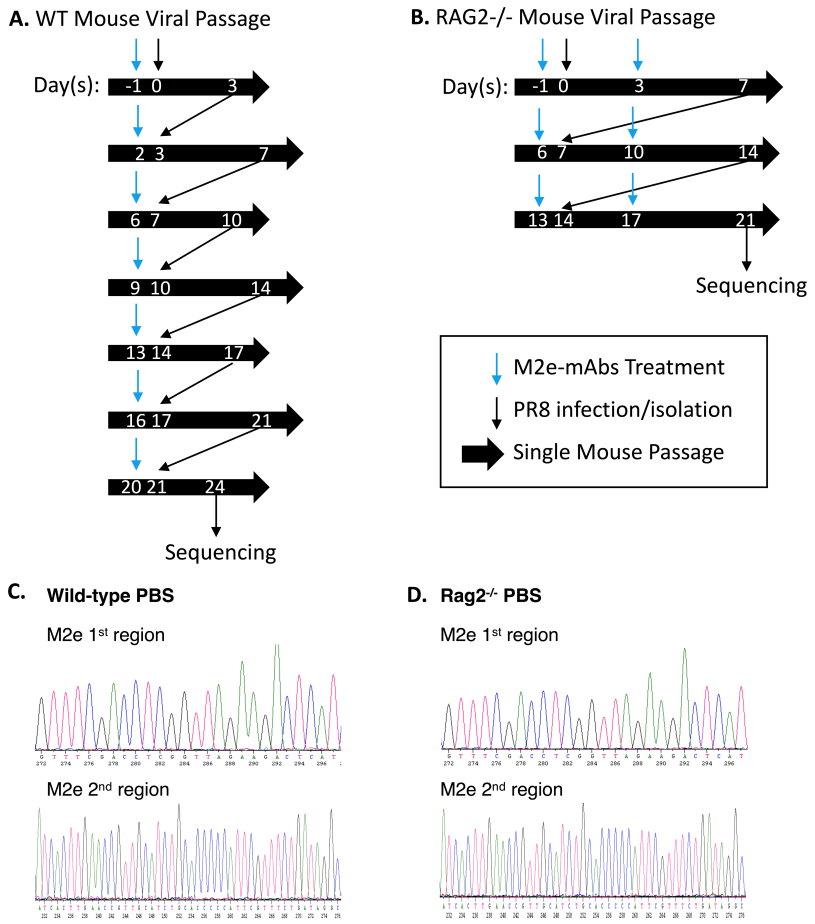
Figure 4: RAG2-KO mice are protected by M2e-specific antibodies as a triple cocktail or alternating treatments. (A) RAG2-KO mice were treated with indicated a 60 µg dose of the indicated M2e-mAb or cocktail, one day prior to infection with a LD_{50} PR8. Additional treatments of 60 µg were administered 2 times a week, alternating 3 and 4 days apart. Treatments post infection are indicated by dotted lines on the graph. The alternating treatment rotated treatments between 472, 522, 602, and PBS, in order and was repeated. (B) Survival analysis, (C) percent weight loss data analysis, and (D) a heatmap is indicative of significantly different percent weight loss from isotype control group each day. n=7-8. ** $p<0.005$, * $p<0.05$, log-rank analysis.

Clone Name	IgG Subclass
391	IgG1
472	IgG2a
522	IgG1
602	IgG2a
770	IgG2a
1191	IgG2b

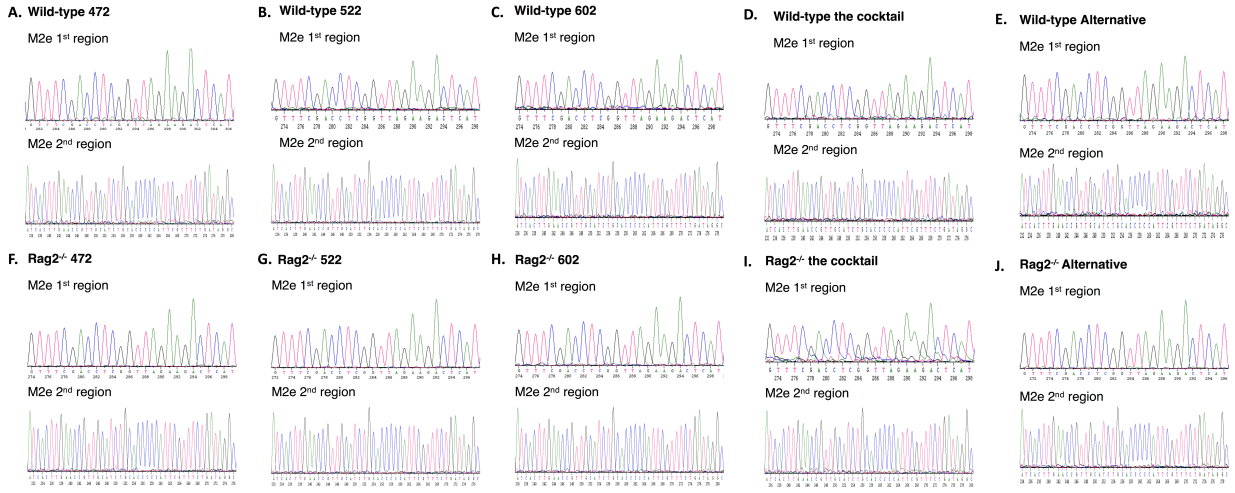
Supplemental Table 1: M2e-specific Monoclonal Antibodies. M2e-mAbs used in this study with their IgG subclass. Antibodies included in cocktail mixture shown in blue.



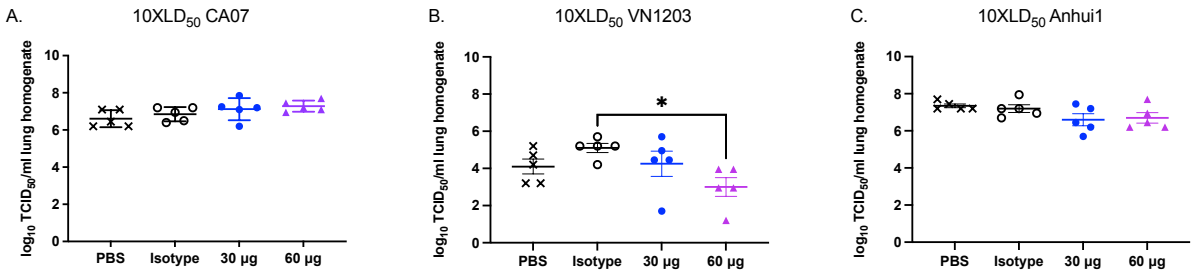
Supplemental Figure 1: M2e-specific antibodies bind to M2e competitively. (A-E) Complete results from (A) FM1, (B) VN1203, (C) Anhui1, (D) swNE, (E) swTX, or (F) swMO competition ELISAs. Inactivated virions of specified virus were used as coating antigen. The measured biotinylated antibody was added at a standard concentration of 2 μg/mL to the wells. The competitive antibody was added at 4-fold dilutions from 100 μg/mL. Absorbance was measured with a biotin binding secondary.



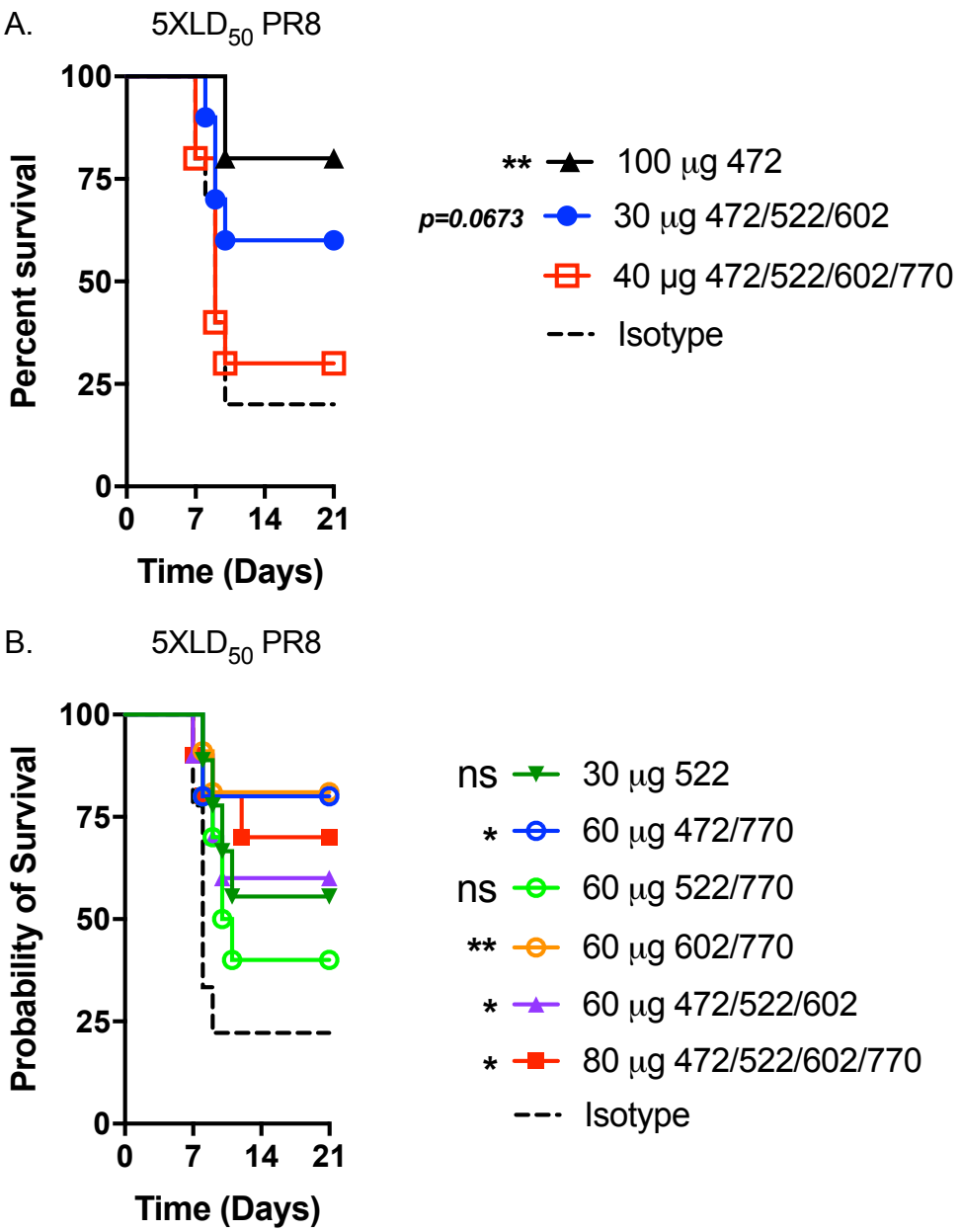
Supplemental Figure 2: M2e-specific monoclonal antibodies do not develop escape mutants in vivo. (A, B) Diagram of timepoints for viral passaging in WT and RAG2-KO mice. PR8 stock virus was passaged through (A) 7 groups of WT or (B) 4 groups of RAG2-KO mice for 24 or 21 days, respectively. 4 mice per group were used for each passage, except for the final passage in both genotypes of mice, which contained 8 mice. Mice receiving each treatment were caged only with mice receiving the same treatment to avoid cross contamination. IP Injections of M2e-mAbs treatments of single mAbs: 472, 522, or 602; the 472/522/602 cocktail; alternating treatments of 472, 522, 602, and PBS (in order); or PBS control are indicated by blue arrow. Passaged occurred once every 3 or 4 days in WT and once every 7 days in RAG2-KO mice. At each passage, virus from all 4 mice receiving the same treatment was isolated, pooled, and used to infect the next passage mice receiving that treatment intranasally. The final viral isolate was sequenced. (C, D) Example sequencing chromatograms from PBS group passaging in (C) WT or (D) RAG2-KO mice. Results for all groups reported in **Table 2** and chromatograms for remaining groups in **Supplemental Figure 3**.



Supplemental Figure 3. Sequencing chromatograms for WT and RAG2-KO passages with single and cocktail antibody therapies. Sequencing chromatograms from passaging experiment outlined in **Supplemental Figure 2** with results reported in **Table 2**. Viral sequences from virus passaged with specified antibody in (A-E) WT or (F-J) RAG2-KO mice.



Supplemental Figure 4: The 472/522/602 triple cocktail decreased viral load for VN1203, but not CA07 and Anhui1. (A-C) BALB/c mice were treated with indicated dose of M2e-mAb 472/522/602 cocktail one day prior to infection with (A) CA07, (B) VN1203, or (C) Anhui1. Lungs for viral titers removed on day 3 post infection. Viral titers measured via plaque assay. One-way ANOVA with a Tukey's multiple comparison test. n=5. **** $p<0.0001$, *** $p<0.001$, ** $p<0.01$, * $p<0.05$.



Supplemental Figure 5: Adding 770 to the 472/522/602 triple cocktail loses efficacy at low doses. (A and B) BALB/c mice were treated with the indicated dose of the indicated M2e-mAb treatment, containing 1 to 3 M2e-specific antibodies in equal parts, one day before infection with a $5XLD_{50}$ PR8. Survival analysis: (A) n=10 and (B) n=9-10. **** $p<0.0001$, *** $p<0.001$, ** $p<0.01$, * $p<0.05$, log-rank analysis.

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