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A single-shot vaccine approach for the universal influenza A vaccine candidate M2e

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Influenza, commonly referred to as "flu," is a major global public health concern and a huge economic burden to societies. Current influenza vaccines need to be updated annually to match circulating strains, resulting in low take-up rates and poor coverage due to inaccurate prediction. Broadly protective universal flu vaccines that do not need to be updated annually have therefore been pursued. The highly conserved 24–amino acid ectodomain of M2 protein (M2e) is a leading candidate, but its poor immunogenicity has been a major roadblock in its clinical development. Here, we report a targeting strategy that shuttles M2e to a specific dendritic cell subset (cDC1) by engineering a recombinant anti-Clec9A monoclonal antibody fused at each of its heavy chains with three copies of M2e. Single administration in mice of 2 μg of the Clec9A–M2e construct triggered an exceptionally sustained anti-M2e antibody response and resulted in a strong anamnestic protective response upon influenza challenge. Furthermore, and importantly, Clec9A–M2e immunization significantly boosted preexisting anti-M2e titers from prior flu exposure. Thus, the Clec9A-targeting strategy allows antigen and dose sparing, addressing the shortcomings of current M2e vaccine candidates. As the cDC1 subset exists in humans, translation to humans is an exciting and realistic avenue.

Clec9A | M2e | universal influenza A vaccine | single shot

Seasonal influenza epidemics afflict between 13 and 100 million individuals annually, including 3 to 5 million cases of severe illness and 300,000 to 600,000 deaths worldwide, representing a top global public health concern and an extraordinary economic burden to all societies (1). Pandemics are less frequent but are generally more severe and pose a greater threat. Over the past century there have been at least four devastating pandemics caused by influenza A virus that took the lives of hundreds of millions of individuals.

Influenza is an enveloped, single-stranded, negative-sense RNA virus that belongs to the *Orthomyxoviridae* family (2). Influenza A viruses can be subtyped according to the two major glycoproteins present on their viral surface, namely hemagglutinin (HA) and neuraminidase (NA) (2). These glycoproteins are the target of host neutralizing antibodies (3), and immune pressure exerted by the host results in the emergence of drift variants with differing antigenic properties, leading to yearly regional epidemics (4). Less frequently, genetic reassortment between cocirculating influenza A subtypes can lead to the emergence of novel HA (and to a lesser extent NA) subtypes, occasionally causing deadly pandemics.

Vaccination arguably represents the most effective way to prevent influenza (1), but the current vaccination strategy suffers from certain limitations. Existing inactivated influenza vaccines rely on neutralizing anti-HA antibodies, which confer protection against homologous strains but are ineffective against antigenically distinct, heterologous viruses (5). Because of the high degree of seasonal antigenic variation (6), vaccines need to be updated annually in order to match the circulating strain (7). Despite surveillance programs, past experiences have highlighted the sheer unpredictability of both seasonal epidemics and pandemic flu events, thus making current flu vaccination approaches vastly inadequate and leaving societies extremely vulnerable. Furthermore, long and complicated production cycles of the flu vaccines with limited production capacity represent another bottleneck that precludes their use in the face of pandemics that demand mass vaccination in a short time frame (8). To address these shortcomings, the development of broadly protective "universal" flu vaccines has been explored (9). Based on highly conserved antigens across all influenza A strains, these vaccines are expected to confer basal pan-flu protection that will boost the effectiveness of seasonal strain-specific flu vaccines (10). They are also expected to confer protection against outbreaks or pandemics due to newly emerging flu viruses.

Significance

Although the need for a universal influenza vaccine has long been recognized, only a handful of candidates have been identified so far, with even fewer advancing in the clinical pipeline. The 24–amino acid ectodomain of M2 protein (M2e) has been developed over the past two decades. However, M2e-based vaccine candidates have shortcomings, including the need for several administrations and the lack of sustained antibody titers over time. We report here a vaccine targeting strategy that has the potential to confer sustained and strong protection upon a single shot of a small amount of M2e antigen. The current COVID-19 pandemic has highlighted the importance of developing versatile, powerful platforms for the rapid deployment of vaccines against any incoming threat.

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The M2 viral protein has garnered much interest as a potential universal vaccine candidate for influenza A. M2 is a tetrameric type III membrane viral protein, which contains 97 amino acids and acts as a viroporin (11). The protein consists of an intracellular C-terminal domain (positions 47 to 97), a transmembrane domain (positions 24 to 46), and an extracellular N-terminal domain (M2e; positions 1 to 23) (11). The first nine amino acids in M2e are remarkably conserved among all known human influenza A strains that have been in circulation since 1918, including H17N10 and H18N11 viruses that were isolated from bats (12, 13). M2e has been pursued as a potential flu universal candidate since it was first described by Lamb et al. (14). While M2 protein is scarce on the virion itself, flu-infected cells surface express the protein in copious amounts, with M2e protruding (15) and therefore readily accessible to antibodies. Consistently, M2e-mediated protective immunity was found to primarily rely on nonneutralizing antibodies (16) that bind to infected cells and engage Fcγ-mediated effector mechanisms, such as antibody-dependent cell cytotoxicity (ADCC) or antibody-dependent cell phagocytosis (17, 18). Alveolar macrophages play an important role in mediating protection via elimination of virus-infected cells as part of M2e-based protective immunity (17). The role of natural killer (NK) cells in mediating ADCC, however, has remained unclear, with conflicting findings (19, 20). Complement-dependent cytolysis also contributes to the reduction of lung viral titers upon influenza infection in M2e-vaccinated mice (21).

However, upon natural influenza A infection, poor serum antibody responses against M2e are detected as the antibody and cellular immune responses are mainly directed at immunogenic HA and NA proteins (22–24). Furthermore, because of its small size, M2e is poorly immunogenic per se. To overcome this issue various, carriers and delivery systems have been used; however, typically, any M2e vaccination regimen so far reported has involved at least two injections (20, 25–32). Furthermore, a rapid decline in M2e-specific antibody titers has been observed with these vaccines, as illustrated in a recent clinical trial with the ACAM-FLU-A vaccine candidate (an M2e–hepatitis B virus core fusion protein) (33). These shortcomings therefore represent a serious roadblock in the clinical development and deployment of M2e-based vaccines, and novel approaches are needed.

Shuttling antigens to specific dendritic cell (DC) subsets represents a promising approach to improve the protective potential of poorly immunogenic subunit vaccine candidates (34). This targeting strategy consists of fusing the antigen of interest with a monoclonal antibody specific to a molecule that is surface expressed by a specific DC subset. Pioneer work was described with chimeric monoclonal antibodies targeting the endocytic receptor DEC205 that is expressed by a conventional DC1 subset.

Subsequently, our group reported shuttling antigens to Clec9A, a C-type lectin-like molecule that is selectively expressed by the mouse and human DC subset cDC1 (mouse $CD8^+$; human $CD141^+$) and at low level by plasmacytoid DCs (35–38). The restricted expression of Clec9A on limited DC subsets allows the vaccine construct to persist in the systemic circulation, resulting in prolonged and sustained antigen presentation over time (36, 39). The subunit vaccine candidates are genetically fused at the C-terminal extremities of the heavy chains of a Clec9A-specific monoclonal antibody (39–41). Our previous studies have shown that targeting prototype antigens such as nitrophenol or ovalbumin to Clec9A led to potent follicular $CD4^+$ T helper (Th) cell generation that in turn resulted in potent and prolonged antibody production upon a single administration (36, 39). More recently, we applied the Clec9A-targeting strategy to subunit vaccine candidates against infectious diseases (41). Specifically, we reported that the single administration of a Clec9A–M2e construct induced a significant anti-M2e antibody response.

Building on these promising observations, here, we have explored further the potential of the Clec9A-targeting approach to confer M2e-mediated protective immunity. Our results indicate that targeting M2e to Clec9A-expressing DCs in a single administration regimen represents a promising vaccine strategy that addresses the shortcomings of current M2e vaccine candidates.

Results

Single Administration of Clec9A–M2e Induces High M2e-Specific Antibody Titers. Previously, we reported that the antibody response induced upon single administration of the Clec9A–M2e construct was enhanced in the presence of CpG adjuvant (41). Class B CpG oligodeoxynucleotides is a Toll-like receptor-9 (TLR9) agonist, known to induce strong and prolonged B cell activation (42). Here, we investigated the suitability and potency of TLR3 agonist polyinosinic–polycytidylic acid (poly I:C). TLR3 is indeed expressed at high levels by cDC1 cells (43), and, consistently, poly I:C was found to effectively activate $CD8^+$ cDCs (44). Furthermore, poly I:C has been included in cancer vaccine formulations currently undergoing human trials, suggesting that this adjuvant will likely be approved for human use soon (45). We therefore compared the antibody responses triggered upon single administration of the Clec9A–M2e construct or the nontargeting isotype control construct GL117–M2e adjuvanted with either CpG, poly I:C, or a combination of both. Enzyme-linked immunosorbent assay (ELISA) data performed on the individual sera collected 1 mo postadministration indicated that mice vaccinated with Clec9A–M2e + CpG and Clec9A–M2e + poly I:C had significantly higher M2e-specific immunoglobulin G (IgG) antibody titers than the groups immunized with the nontargeting construct (Fig. 1*A*). However, the presence of both adjuvants in the GL117–M2e formulation led to increased antibody titers compared with the singly adjuvanted construct formulations and reached levels that were similar to those measured with the Clec9A–M2e formulations. In contrast, mixing both adjuvants with Clec9A–M2e did not result in further enhancement of the antibody titers compared with the singly adjuvanted construct. Furthermore, while all the groups that received Clec9A–M2e had comparable anti-M2e IgG titers regardless of the adjuvant, the isotype profile differed, with the CpG-adjuvanted formulation displaying more pronounced IgG2a and IgG1 responses than the poly I:C–adjuvanted group (Fig. 1 *B–D*).

To further evaluate the qualitative antibody response produced in the vaccinated mice, we analyzed the ability of the immune sera to bind to flu-infected cells based on the rationale that flu-infected cells surface express M2 (15). H1N1/PR8-infected Madin–Darby Canine Kidney (MDCK) cells were incubated with individual immune sera (or a monoclonal anti-M2e antibody as a positive control), followed by incubation with a labeled anti-mouse secondary antibody. The percentage of positive cells was then determined by flow cytometry (*SI Appendix*, Fig. S1). Since combining CpG and poly I:C in the vaccine formulation did not show any synergistic effect in term of antibody titers, we omitted this group in the analysis. The data indicated that the immune sera from Clec9A–M2e-

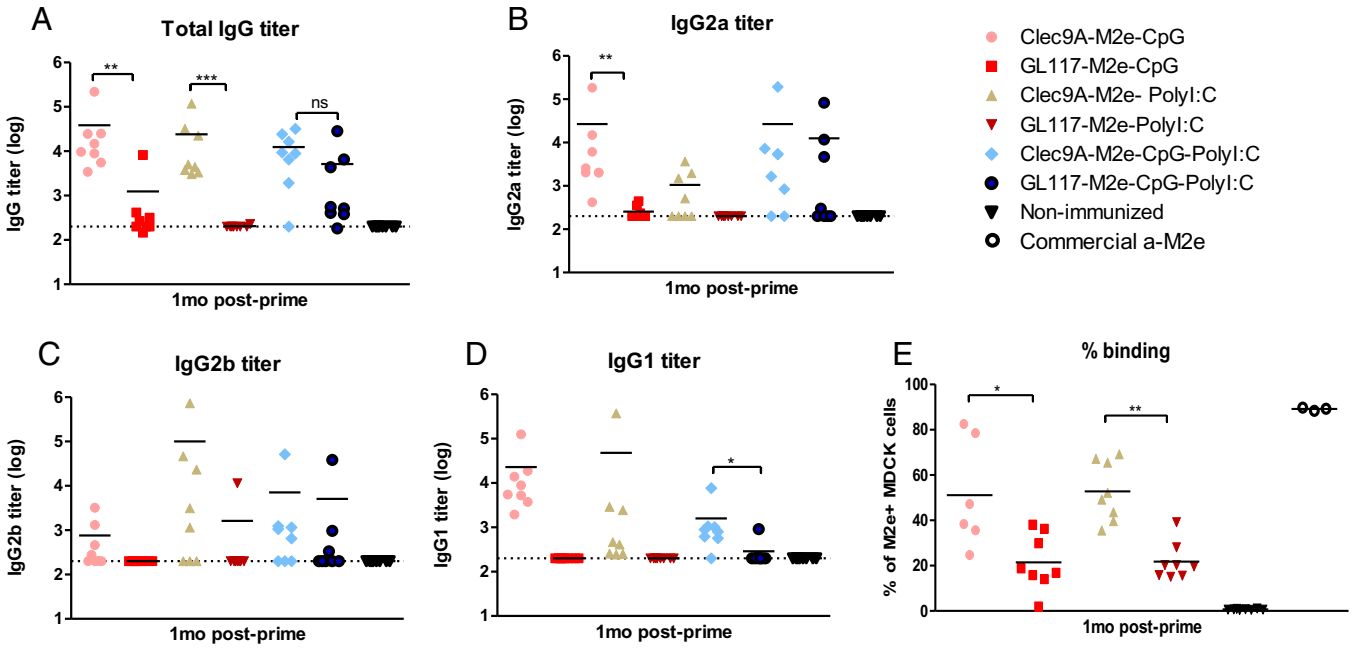

Fig. 1. Anti-M2e IgG antibody responses upon single administration of Clec9A–M2e or GL117–M2e. (*A–D*) Antibody titers. Adult mice (n = 6 to 8) were immunized once with 2 μg of Clec9A–M2 or GL117–M2e construct adjuvanted with CpG, poly I:C, or a combination of both, as indicated. One month later, their systemic anti-M2e IgG titers were determined by ELISA. The dashed line represents the lowest dilution of the sera, and a nominal titer of 200 was assigned to samples with a titer below the lowest dilution. Log-transformed M2e-specific IgG titers were compared, and statistical significance was assigned to the differences. A Mann–Whitney U test was used for statistical analysis between the Clec9A–M2e groups and their respective GL117–M2e groups (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$). Horizontal bars represent the mean of six individual serum samples. (*E*) Binding efficacy of immune sera to flu-infected cells. MDCK cells were infected with H1N1/PR8 at a multiplicity of infection of 10 and coincubated with heat-inactivated immune sera at a dilution of 1:20 or with commercial anti-M2e monoclonal antibody. Samples were analyzed by flow cytometry. A Mann–Whitney U test was used for statistical analysis between the Clec9A–M2e groups and their respective GL117–M2e groups (*$P \leq 0.05$, **$P \leq 0.01$). These experiments were performed twice independently, and one representative set is shown.

vaccinated groups gave comparable percentages of positive cells, which were greater than the percentages obtained with the immune sera from mice vaccinated with the nontargeting construct (Fig. 1*E*).

Together, these results were consistent with our previous work and indicated that targeting M2e to Clec9A does potentiate the antigen-specific antibody responses and reduces the need for strong adjuvants. Furthermore, adjuvanting with CpG or poly I:C led to comparable M2e-specific IgG levels, although with a different isotype profile signature.

Single Administration of Clec9A–M2e Significantly Protects against Lethal Flu Challenge. We next investigated the in vivo protection efficacy of the vaccine formulations. One month after immunization, mice were infected with a lethal dose of H1N1/PR8 virus. Their survival rate and weight loss were monitored daily for 2 wk postchallenge. Results indicated that mice immunized with the Clec9A–M2e formulations displayed protection rates that ranged between 62.5 and 87.5% (Fig. 2*A*). In contrast, groups vaccinated with the nontargeting construct had no protection or a protection rate as low as 12.5% (Fig. 2*A*). These data thus further confirmed the superior protective efficacy of the Clec9A-targeting strategy. Interestingly, upon analyzing the body weight loss profile of the animals upon challenge, mice that received the poly I:C–adjuvanted construct displayed the least body weight loss compared with mice that received the construct adjuvanted with CpG or CpG + poly I:C (Fig. 2*B*). This observation was even more striking when analyzing the body weight loss profile of the survivors only, where mice vaccinated with the poly I:C–adjuvanted vaccine experienced minimal body weight loss, and mice vaccinated with Clec9A–M2e adjuvanted with CpG only or CpG + poly I:C lost more than 20% of their original body weight (Fig. 2*C*). This observation thus suggested a more robust protection afforded by the formulation containing poly I:C alone.

Furthermore, viral loads in the lungs of infected mice were determined. No significant reduction in viral loads were measured for all the vaccinated groups at day 4 postinfection compared with nonvaccinated controls (Fig. 2*D*). However, at day 7 postinfection, mice that received the Clec9A–M2e constructs (regardless of the adjuvant) displayed approximately 1 log reduction of viral loads compared with GL117–M2e-immunized and nonimmunized groups (Fig. 2*D*).

To further characterize the protection observed in the two Clec9A–M2e-vaccinated groups adjuvanted with CpG or poly I:C, we examined hematoxylin and eosin (H&E)–stained lung sections harvested at day 4 and 7 postinfection (Fig. 3*A*). Inflammation, bronchiolar necrosis, and edema in blinded samples were scored by a veterinarian pathologist (Fig. 3*B*). Comparable lung inflammation was seen at day 4 postinfection among all the vaccinated groups. At day 7 postinfection, slightly greater inflammation, as evidenced by greater cellular infiltration, was seen in the lungs from mice vaccinated with the CpG-adjuvanted constructs than in mice that received the poly I:C–adjuvanted formulations. More striking was the increased bronchiolar necrosis observed in the lungs at 7 days postinfection in mice that were immunized with Clec9A–M2e + CpG. In addition, increased severity of pulmonary edema was also seen with this group at day 4 postinfection. The histological data thus indicated more severe lung pathology in mice immunized with the CpG-adjuvanted construct, which correlated with the greater body weight loss observed in these mice and is likely the result of immune-mediated pathology rather than virus-mediated cytopathy. It thus appears that immunization

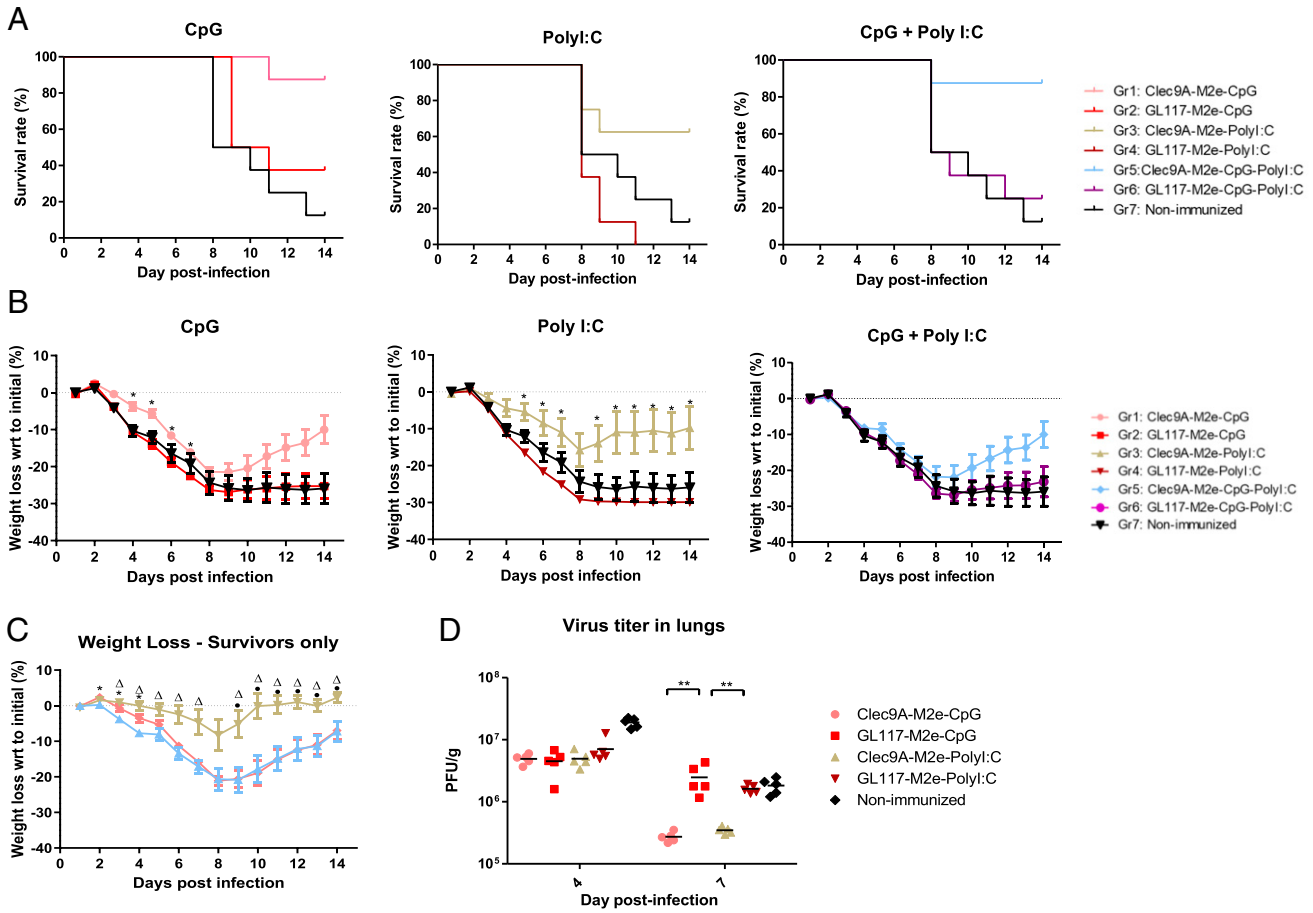

Fig. 2. Influenza challenge. Adult mice (n = 8 to 10) were immunized once with 2 μg of the vaccine candidates adjuvanted with poly I:C, CpG, or both as indicated and were challenged 1 mo later with a lethal dose of H1N1/PR8 virus. Body weight was monitored daily for 14 d postinfection, and mice were euthanized according to the National University of Signapore Institutional Animal Care and Use Committee guidelines when they had lost 30% of initial body weight. (*A*) Survival rate. (*B*) Body weight loss profile of all the mice. A multiple t test was used to evaluate significance between 10B4–M2e-vaccinated mice and GL117–M2e-vaccinated mice and corrected for multiple comparisons using the Holm–Sidak method (*$P \leq 0.0.5$). (*C*) Body weight loss profile of survivors only. A two-way ANOVA was used, followed by post hoc analysis using Tukey's multiple comparison test (the delta symbol [Δ] signifies statistical significance between group (Gr) 3 and group 5, the asterisk (*) signifies statistical significance between group 1 and group 5, and the bullet symbol (●) signifies statistical significance between group 1 and group 3; $P \leq 0.0.5$). (*D*) Lung viral loads. At days 4 and 7 postchallenge, mice (n = 5) were euthanized, and their lungs were harvested and processed for viral titer determination by plaque assay. A Mann–Whitney U test was used for statistical analysis between the Clec9A–M2e groups and their respective GL117–M2e group (**P = 0.0079). Horizontal bars represent the mean. These experiments were performed twice independently, and one representative set is shown.

with the CpG-adjuvanted vaccine construct could effectively reduce viral titers in the lungs but failed to protect against influenza-induced hyperinflammation, supporting that adjuvanting with poly I:C is a better option.

We also compared the immunogenicity and protective efficacy of Clec9A–M2e when adjuvanted with either poly I:C or AddaVax, a squalene-based oil-in-water adjuvant. AddaVax is an MF59 similar (46), the adjuvant present in the trivalent inactivated seasonal flu vaccine FLUAD (47) and in the H5N1 vaccine developed by Seqirus (48). A previous study has also shown the superior efficacy of AddaVax-adjuvanted H7N9 vaccine formulations compared with Alum-adjuvanted and nonadjuvanted formulations (49). However, we found that adjuvanting Clec9A–M2e with AddaVax led to lower levels of anti-M2e total IgG titers than adjuvanting with poly I:C, with undetectable IgG2a levels (*SI Appendix*, Fig. S2*A*). Consistently, the AddaVax-adjuvanted formulation failed to confer significant protection against lethal flu challenge (*SI Appendix*, Fig. S2 *B* and *C*). Together, these data suggested that MF59 similar AddaVax is not a suitable adjuvant for the Clec9A-targeting vaccine approach.

Finally, earlier studies have demonstrated the cross-protective potential of M2e-based vaccine candidates against various influenza A virus subtypes (25, 30, 31). Consistently, sera from mice immunized with the Clec9A–M2e construct displayed significant binding to H3N2-infected cells (*SI Appendix*, Fig. S3*A*). Furthermore, single administration of Clec9A–M2e + poly I:C conferred protection against H3N2 challenge as evidenced by improved body weight loss profile and reduced lung viral loads upon H3N2 challenge compared with nonimmunized mice as well as mice immunized with the nontargeting construct (*SI Appendix*, Fig. S3 *B* and *C*).

Two Micrograms of Clec9A–M2e is the Optimal Dose. The data generated so far were obtained upon administration of 2 μg of the constructs. We asked whether a higher amount of Clec9A–M2e would increase further the M2e-specific antibody titers. In addition, we investigated lower doses of 0.5 and 0.2 μg of the construct. Results showed that comparable anti-M2e IgG titers were obtained between mice that received 20 μg and 2 μg of the construct, suggesting that there is no further benefit in administering a higher amount (*SI Appendix*, Fig. S4*A*). When comparing 2, 0.5, and 0.2 μg, a dose-dependent response was observed, whereby administration of 2 μg of the construct triggered the highest anti-M2e total IgG titers (*SI Appendix*, Fig. S4*A*), the highest survival rate (62.5%; *SI Appendix*, Fig. S4*B*), and the lowest viral loads (*SI Appendix*, Fig. S4*C*). We thus concluded that 2

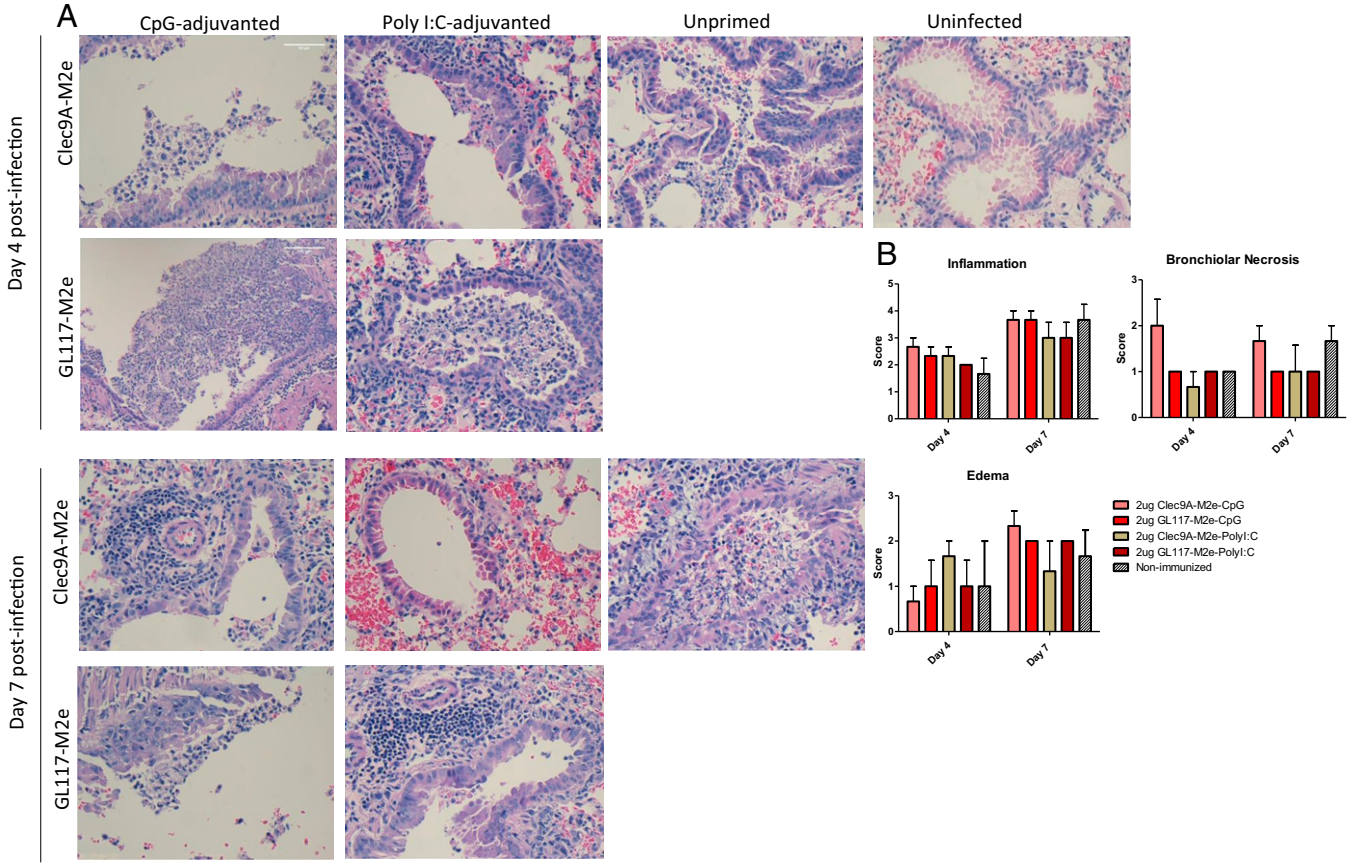

Fig. 3. Lung histology. Adult mice (n = 3) were immunized once with 2 μg of the vaccine candidates adjuvanted with poly I:C or CpG as indicated and were challenged 1 mo later with a lethal dose of H1N1/PR8 virus. At days 4 and 7 postinfection, the animals were euthanized, and lungs were harvested and processed for H&E staining. (*A*) Representative images for each group. Observations were made at 40× magnification. (*B*) Lungs were scored according to severity of inflammation, bronchiolar necrosis, and edema, where 0 indicates no abnormalities detected, 1 indicates minimal abnormalities (5 to 20% lung area), 2 indicates mild abnormalities (20 to 40% lung area), 3 indicates moderate abnormalities (40 to 60% lung area), 4 indicates marked abnormalities (60 to 80% lung area), and 5 indicates severe abnormalities (80 to 100% lung area). These experiments were performed twice independently, and one representative set is shown.

μg represents the minimum amount of Clec9A–M2e construct that induced optimal protective immunity. It is interesting to note that mice immunized with 0.5- and 0.2-μg doses displayed improved survival rates compared with the nonvaccinated group, although their viral loads were not significantly different from the control (*SI Appendix*, Fig. S4*C*). This observation may suggest that these lower vaccine doses are still able to protect from influenza-induced hyperinflammation.

Single Administration of Clec9A–M2e Induces Sustained M2e-Specific Antibody Responses. We next monitored over a 17-mo period the M2e-specific antibody response induced upon single administration of the Clec9A–M2e construct. Remarkably, sustained anti-M2e IgG titers were measured up to 13 mo postimmunization, and titers began to slowly decline from 15 mo onward (Fig. 4*A*). Furthermore, at 1 mo and 17 mo postimmunization, the number of M2e-specific B cells was quantified in the spleen and bone marrow of the mice, respectively. Significantly higher amounts of M2e-specific B cells were measured in mice vaccinated with Clec9A–M2e than in mice immunized with the nontargeting construct and in nonimmunized mice (Fig. 4 *B* and *C*). Thus, these data supported that targeting M2e to Clec9A effectively induces the generation of long-lived plasma cells for a sustained M2e-specific humoral response. Lastly, since the formation of plasma cells depends on the CD4 Th cell environment, we sought to characterize the M2e-specific T cell response induced upon vaccination. M2e has been reported to contain an $H2^d$-restricted CD4 T cell epitope but no CD8 T cell epitope (27). Splenocytes from mice vaccinated with Clec9A–M2e or GL117–M2e were therefore restimulated with M2e, and the number of interferon–γ (IFN–γ)-expressing and interleukin–4 (IL–4)-expressing T cells was determined by enzyme-linked immunospot (ELISpot) assay. Data indicated significantly higher number of IFN–γ-producing and IL–4-producing cells in mice vaccinated with Clec9A–M2e than in animals vaccinated with the nontargeting construct and in unvaccinated mice (Fig. 4*D*). The IFN-γ^+/IL-4^+ ratio was greater than 1 for samples from Clec9A–M2e-vaccinated mice, reflecting Th1-skewed immune responses upon Clec9A–M2e immunization (Fig. 4*E*). This was not observed with samples from the GL117–M2e-vaccinated group, which had an IFN-γ^+/IL-4^+ ratio lower than 1. Interestingly, restimulation of splenocytes harvested from mice infected with a sublethal dose of H1N1 virus led to low levels of M2e-specific IFN-γ^+ T cells, confirming prior observations that natural flu infection generates low levels of M2e-specific immune responses (22). Together, the data further support that immunization with Clec9A–M2e induces a strong M2e-specific $CD4^+$ Th1 immune response that likely contributes to the high and sustained antibody levels.

Flu Exposure after Clec9A–M2e Vaccination Boosts the Anti-M2e Antibody Titers. We next asked how exposure to flu post-vaccination would affect the M2e-specific antibody titers. To

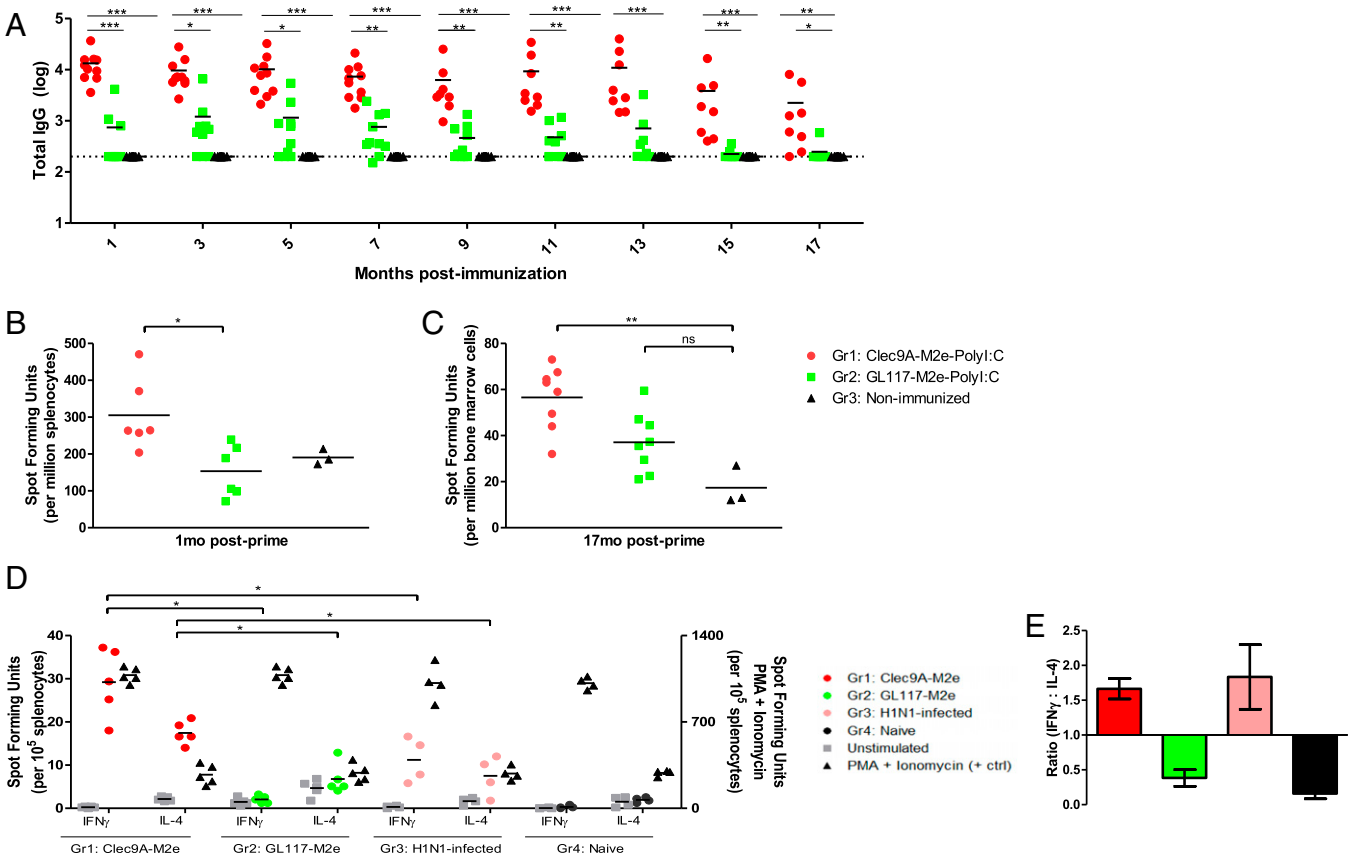

Fig. 4. Analysis of the anti-M2e antibody, B cell, and T cell responses. (*A*) Systemic anti-M2e IgG titers monitored over time. Adult mice (n = 8 to 10) were immunized once with 2 μg of Clec9A–M2e or GL117–M2e (+poly I:C). Systemic anti-M2e IgG titers were determined by ELISA as described in the legend of Fig. 1. A nonparametric one-way ANOVA (Kruskal–Wallis) was performed for statistical analysis (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$). (*B* and *C*) M2e-specific B cells. The spleen (*B*) and bone marrow (*C*) from immunized mice were harvested 1 mo (*B*) or 17 mo (*C*) postimmunization, and a B cell ELISpot was performed to enumerate the frequency of M2e-specific antibody–secreting cells. (*D*) M2e-specific T cells. Spleens from immunized mice were harvested 7 d postvaccination or post-H1N1 infection and restimulated with M2e peptide. IFN-γ^+ and IL-4$^+$ cell numbers were determined by ELISpot. Horizontal bars represent the mean; phorbol myristate acetate. (*E*) Ratio of IFN-$\gamma^{+/}$IL-4$^+$ cells. Error bars represent SEM. A nonparametric one-way ANOVA (Kruskal–Wallis) was performed for statistical analysis (**$P \leq 0.01$). These experiments were performed twice independently, and one representative set is shown.

answer this question, mice were immunized with Clec9A–M2e or GL117–M2e and sublethally infected with either H1N1/PR8 or H3N2/Aichi 1 mo later. ELISA results indicated a significant increase of approximately 1 order of magnitude in anti-M2e IgG titers after flu infection in mice that were vaccinated with Clec9A–M2e compared with nonvaccinated mice (Fig. 5 *A* and *B*). This boost effect was not seen in mice vaccinated with the nontargeting GL117–M2e construct, which displayed titers comparable to the noninfected controls (Fig. 5). These observations thus supported that single immunization with Clec9A–M2e has the potential to induce a strong anamnestic anti-M2e antibody response upon flu exposure that may confer robust protection to subsequent flu exposure. Furthermore, measurement of M2e-specific antibody-secreting cells in the bone marrow 3 mo after flu infection further confirmed the superior ability of Clec9A–M2e immunization to prime the immune system and allow generation of long-lived plasma cells compared with the nontargeting construct (*SI Appendix*, Fig. S5).

Clec9A–M2e Vaccination Boosts the Anti-M2e Titers in Mice with Preexisting Flu Immunity. During their lifetime, individuals have been exposed several times to influenza infection (50, 51) and have developed moderate levels of M2-specific immunity (22). Therefore, we were interested in studying how M2-specific preexisting immunity could influence and shape the M2e-specific antibody response upon immunization with Clec9A–M2e. To this end, mice were infected sequentially with a sublethal dose of H1N1, followed by sublethal infection with H3N2 1 mo later. The control group was not exposed to influenza infection. All mice were then vaccinated with 2 μg of Clec9A–M2e. Results show that preexisting immunity to influenza greatly enhanced the M2e titers upon Clec9A–M2e vaccination that were 2 logs in magnitude higher than the titers measured in vaccinated only (no prior flu infection) animals (Fig. 6*A*). Furthermore, such high antibody titers were sustained for at least 4 mo postvaccination.

Since preexposure to flu boosts the anti-M2e antibody response upon Clec9A–M2e vaccination, we hypothesized that perhaps a smaller vaccine dose may be sufficient. To test this hypothesis, mice were sequentially infected with sublethal doses of H1N1/PR8 and H3N2 as above, followed 1 mo later by vaccination with either 2 μg or 0.2 μg of Clec9A–M2e. ELISA results indicated that vaccination with either dose boosted significantly the anti-M2e antibody titers (Fig. 6*B*). However, greater variability in the antibody titers was seen in the group of mice that received 0.2 μg of Clec9A–M2e than in mice vaccinated with 2 μg, indicating that 0.2 μg may represent a suboptimal dose. These observations therefore supported that vaccination with 2 μg of Clec9A–M2e in the context of preexisting flu immunity can boost the anti-M2e antibody titers to levels that are likely to confer robust pan-flu protection.

Clec9A–M2e Immunization in Young and Old Mice. Children and the elderly are two vulnerable demographic groups due to

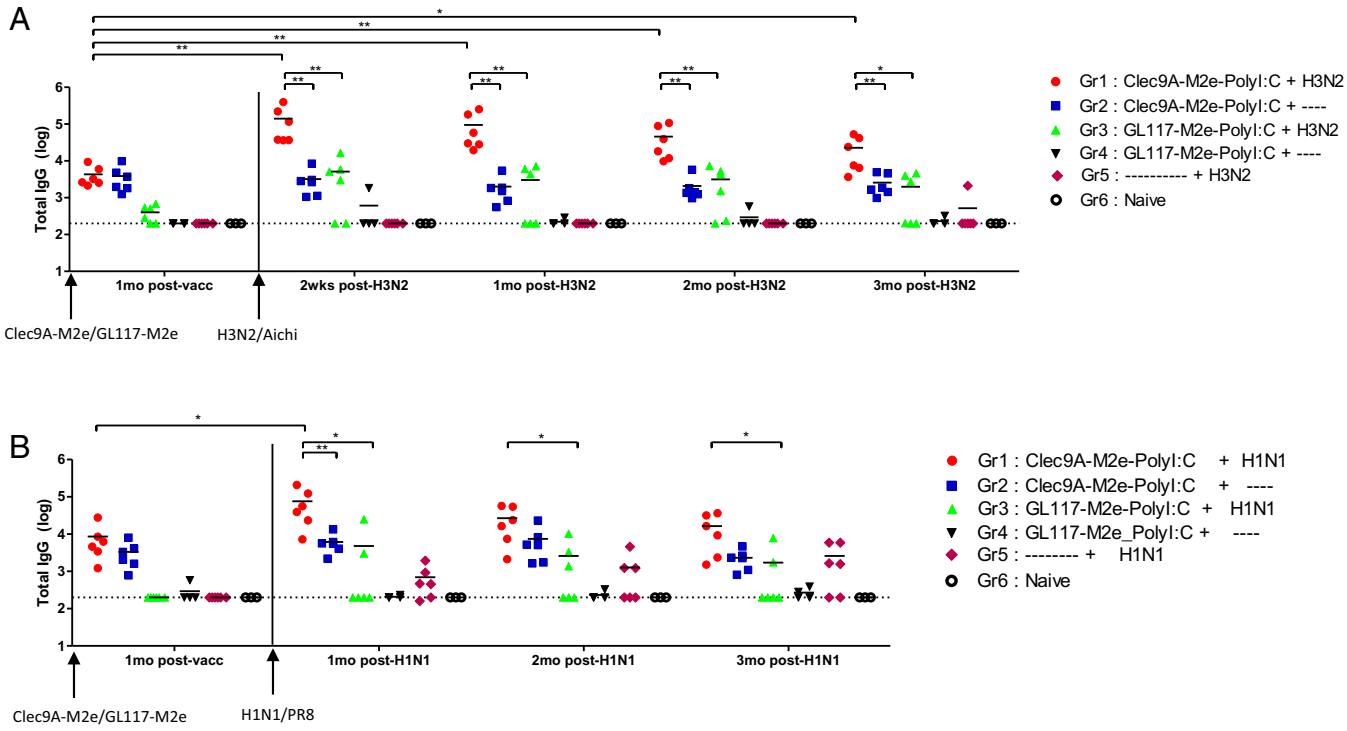

Fig. 5. Vaccination with Clec9A–M2e before exposure to sublethal influenza infection. (*A* and *B*) Adult mice (n = 6) were immunized once with 2 μg of Clec9A–M2e or GL117–M2e (+poly I:C) and subsequently infected with a sublethal dose of H3N2/Aichi (*A*) or H1N1/PR8 (*B*). Control groups include mice that were immunized only but not infected and mice that were not immunized but infected. Systemic anti-M2e IgG titers were determined by ELISA as described in the legend of Fig. 1. A Mann–Whitney t test was conducted between the indicated groups for statistical analysis (*$P \leq 0.05$, **$P \leq 0.01$). Horizontal bars represent the mean. These experiments were performed twice independently, and one representative set is shown.

suboptimal immune responses to infection and vaccination (52, 53). We therefore evaluated the efficacy of Clec9A–M2e immunization in both age groups. We first compared the number of Clec9A-expressing DCs in the spleens from juvenile (3- to 4-wk–old), young adult (5- to 6-wk–old), and old (12-mo–old) mice and found comparable numbers (*SI Appendix*, Fig. S6), thus suggesting that the Clec9A vaccine-targeting strategy is likely to prime effectively the murine immune system regardless of age. Consistently, 3- to 4-wk–old mice (juvenile) developed significant M2e-specific IgG titers upon a single injection of Clec9A–M2e

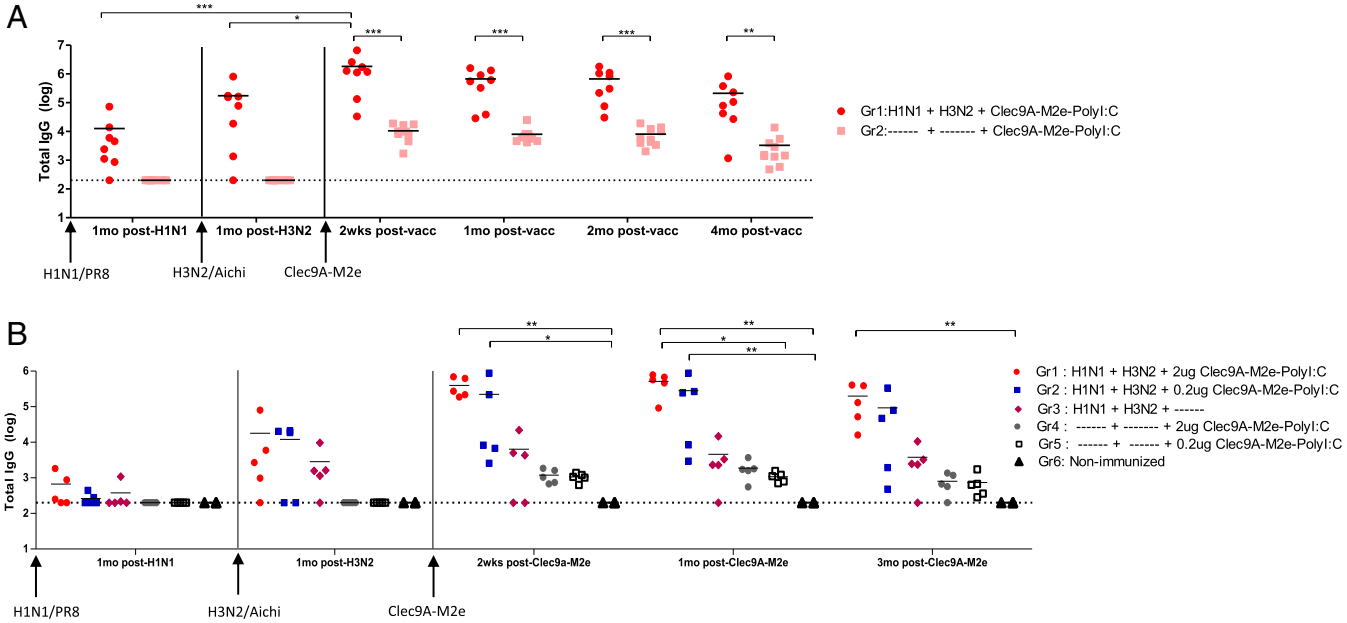

Fig. 6. Vaccination with Clec9A–M2e in the context of preexisting flu immunity. Adult mice (n = 5 to 8) were sequentially infected with a sublethal dose of H1N1/PR8 and H3N2/Aichi 1 mo apart or left uninfected. (*A*) One month after the last flu infection, mice were immunized once with 2 μg of Clec9A–M2e (+poly I:C), and the anti-M2e antibody IgG titers were monitored over time. Systemic anti-M2e titers were determined by ELISA as described in the legend of Fig. 1. A Mann–Whitney U test was used for statistical analysis between indicated groups (*$P \leq 0.05$, **$P \leq 0.01$). Horizontal bars represent the mean. (*B*) One month after the last flu infection, mice were immunized with 2 μg or 0.2 μg of Clec9A–M2e or GL117–M2e (+poly I:C), and the anti-M2e IgG titers were measured at the indicated time points. A nonparametric one-way ANOVA (Kruskal–Wallis) was used for statistical analysis (*$P \leq 0.05$, **$P \leq 0.01$). These experiments were performed twice independently, and one representative set is shown.

and displayed a 50% survival rate upon lethal influenza challenge (Fig. 7*A*). This was in sharp contrast with vaccination with the nontargeting construct that led to undetectable anti-M2e IgG titers and complete lack of protection (Fig. 7*A*).

We also explored the efficacy of Clec9A–M2e vaccination in aged mice. However, since the elderly human population has likely been exposed to multiple influenza infections over their lifetime, we sought to mimic this situation by exposing first 12-mo–old mice to sequential sublethal influenza infections before immunizing them with either 2 or 0.2 μg of Clec9A–M2e. The anti-M2e IgG titers were visibly increased upon vaccination, particularly with the 2-μg vaccine dose, although it did not reach statistical difference (Fig. 7*B*). This observation thus suggested that the Clec9A-targeting vaccination strategy can boost preexisting M2e immunity in older mice upon a single shot.

Discussion

M2e-based vaccines have been proposed to either act as standalone vaccines that can provide basal immunity to vulnerable groups against novel pandemic strains or to complement seasonal flu vaccination in case of significant mismatch between the circulating strains and the seasonal flu vaccine strains (20, 54, 55).

Our work indicates that targeting M2e to Clec9A-expressing DCs in a single administration regimen elicited strong, sustained, and protective M2e-specific IgG titers when adjuvanted with poly I:C. Interestingly, we observed that mice vaccinated with a CpG-adjuvanted formulation experienced significant body weight loss and lung inflammation upon flu challenge. In contrast, vaccination with a poly I:C–adjuvanted formulation led to minimal body weight loss and limited lung inflammation upon flu challenge. The high level of expression of TLR3 in $CD8^+$ DCs may explain this observation, facilitating codelivery of both vaccine candidate and adjuvant to the same target DC subset (43). The fine balance between protection and pathology during influenza infection is underpinned by the quality and extent of the proinflammatory cytokine response engendered. Lung damage resulting from cytokine storm induced by highly virulent influenza viruses results in high mortality, especially in vulnerable groups (56, 57). Therefore, a vaccine formulation that is able to limit cytokine storm–mediated lung damage is highly desirable. Adjuvants have important immunomodulatory effects and significantly influence the Th1/Th2 balance (58). Surprisingly, we did not find AddaVax to be a suitable adjuvant for Clec9A–M2e vaccination, in sharp contrast with previous studies that have reported AddaVax or its equivalent MF59 as an efficacious adjuvant for influenza vaccines (47, 59, 60). Absence of IgG2a antibodies in mice vaccinated with AddaVax-adjuvanted Clec9A–M2e may explain the observed lack of protection. Previous studies have indeed underscored the importance of this isotype in M2e-mediated protective immunity (16–18), in line with the fact that IgG2a antibodies bind to Fcγ receptors I (FcγRI), III, and IV (18), which are found on neutrophils, NK cells, macrophages, and monocytes (61), whereas IgG1 antibodies only bind to FcγRIII (18). Therefore, a broader Fc receptor–binding range allows for the activation of a broader range of antiviral effector mechanisms.

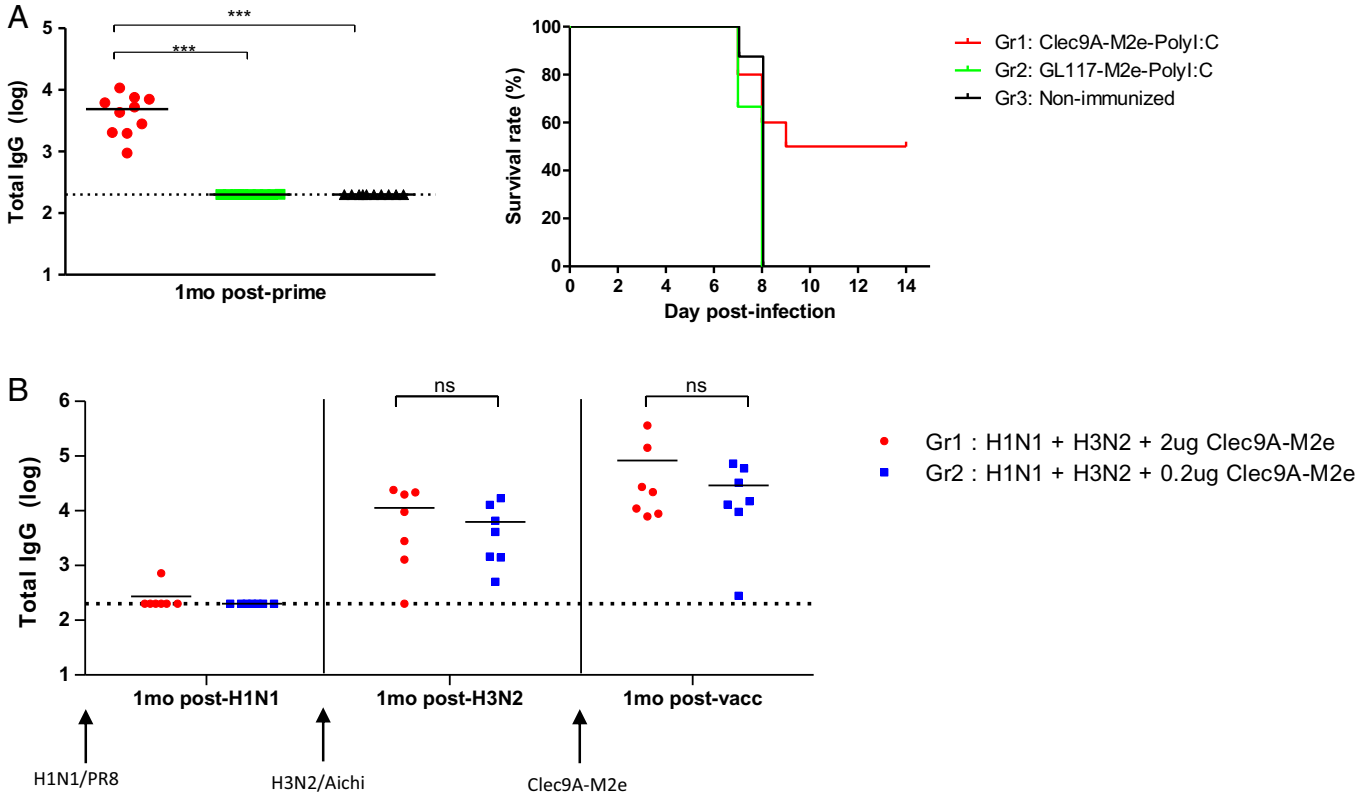

Fig. 7. Immunogenicity of Clec9A–M2e vaccination in juvenile and old mice. (*A*) Three- to 4-wk–old mice (n = 6) were immunized once with 2 μg of Clec9A–M2e or GL117–M2e (+poly I:C) and were lethally challenged with H1N1/PR8 1 mo postvaccination. Body weight was recorded daily, and mice were euthanized when they had lost 30% of initial body weight. A nonparametric one-way ANOVA (Kruskal–Wallis) was used for statistical analysis (***$P \leq 0.001$). (*B*) Twelve-mo–old mice (n = 6) were exposed to sequential sublethal H1N1/PR8 and H3N2/Aichi infection at a 2-mo interval. One month after the last infection, mice were vaccinated either with 2 μg or 0.2 μg of Clec9A–M2e (+poly I:C). Systemic anti-M2e IgG titers were monitored by ELISA as described in the legend of Fig. 1. A Mann–Whitney U test was used for statistical analysis between the indicated groups. Horizontal bars represent the mean. These experiments were performed twice independently, and one representative set is shown.

Our work also showed that as little as 2 μg of the Clec9A–M2e construct (corresponding to 0.2 μg of M2e antigen) is sufficient to confer significant protection against lethal flu challenge in both juvenile and young adult mice. The pediatric population tends to experience the highest annual attack rates (62), with the Centers for Disease Control and Prevention estimating that children under 2 y old are particularly at high risk for developing flu-related complications (63). Of further concern is that this group may be the primary transmitters of influenza within a community (64, 65), underscoring the importance of protecting this demographic group to achieve effective epidemic containment. Importantly, we found that a single dose of Clec9A–M2e vaccine given to naive animals (i.e., that have not been previously exposed to flu before) primed the immune system effectively, which led to significantly boosted anti-M2e antibody titers upon subsequent flu infection. This finding thus further supports the usefulness of vaccinating the younger, flu-naive population with Clec9A–M2e prior to any exposure to flu.

A previous study indicated that in humans, the cDC1 subset remains detectable and constant throughout life (from birth to 80 y) in most of the organs and tissues analyzed (66). Similarly, we found comparable numbers of cDC1 in the spleens from juvenile, young adult, and old mice. Consistently, Clec9A–M2e vaccination in 15-mo–old mice preexposed to flu significantly boosted the anti-M2e antibody titers. We did not explore the efficacy of Clec9A–M2e vaccination in flu-naive old mice, which represents an unlikely scenario in the human population. Indeed, adult individuals are regularly exposed to influenza viruses, especially healthcare workers who have high incidence rates of influenza (67). Sequential exposure to flu has been reported to lead to moderate anti-M2e antibody levels (68). A study that analyzed M2e-specific seroconversion during the 2009 H1N1 pandemic in 118 individuals reported incremental seroprevalence of M2e-specific antibodies with age (22). It was observed that infection with the 2009 H1N1 virus boosted the preexisting M2e-specific antibody response, whereas those with no preexisting M2e-specific antibodies prior to the pandemic infection had low M2e-specific seroconversion (22). Therefore, it is expected that Clec9A–M2e vaccination in the adult and elderly populations will result in significantly boosted anti-M2e titers that may confer robust protection against subsequent flu exposure.

Beyond the ability to prime or boost the anti-M2e antibody response, single-dose vaccination with Clec9A–M2e led to remarkably sustained anti-M2e titers with generation of long-lived plasma cells. Since Clec9A is restricted in expression to rare DCs, the anti-Clec9A–antigen construct persists for an extended period in the circulation, leading to prolonged antigen presentation, and driving extensive follicular helper T cell generation and memory. We also observed a strong M2e-specific Th1 response induced upon Clec9A–M2e vaccination, which may contribute to the high and sustained antibody levels. Furthermore, we recently reported that when the antigen is targeted to Clec9A, DCs surface display the native antigen for B cell activation, leading to a tripartite interaction between DCs, B cells, and T cells that results in a high and persistent antibody response involving germinal center formation and affinity maturation (69).

Sustained, long-term protective immunity is an important attribute that fulfills one of the criteria set as part of the World Health Organization's strategic priority to focus on next-generation influenza vaccines that elicit a durable, cross-protective immunity across influenza A strains. Furthermore, the antigen-sparing nature of the Clec9A–M2e vaccination approach represents an equally critical attribute, as it is expected to greatly ease the burden of vaccine production. Furthermore, monoclonal antibodies are biologics that have been used routinely to treat diseases such as cancer (immune check point inhibitors), inflammatory diseases (e.g., anti-TNF therapy), and more recently infectious diseases (Ebola and coronavirus disease 2019). Therefore, the procedures and protocols for large-scale good manufacturing practice production have been established and are available worldwide.

Together, our work supports that the Clec9A-targeting approach addresses the major roadblocks currently encountered with existing M2e-based vaccine candidates that have reached preclinical and clinical development, including the need for several administrations of high amounts of antigen combined with strong adjuvants (which are associated with adverse reactions) and the lack of sustained antibody titers over time (33). More specifically, prior work has demonstrated the superiority of Clec9A targeting compared with targeting other DC surface molecules, including the gold standard DEC205 (39). Consistently, in a recent study that reported targeting M2e to DEC205, three injections were required to reach protective antibody titers (70).

There are currently several universal flu vaccine candidates in late trials (71). Recently, Biondvax Pharmaceuticals Ltd. announced disappointing results from their pivotal phase 3 clinical trial of M-001, a stand-alone universal flu vaccine candidate that failed to demonstrate a statistically significant difference between vaccinated and placebo groups in reducing flu illness (72). This underscores the need to keep feeding the research and development pipeline with novel vaccine candidates and platforms.

Beyond influenza, the Clec9A-targeting technology is a versatile plug-and-go platform that has great potential for wide application to both infectious and noninfectious diseases, the latter including cancer. Given that the equivalent cDC1 subpopulation exists in humans (66), and Clec9A targeting has been previously successfully reported in nonhuman primates (73) and humanized mice (74), translation to humans is a realistic and exciting avenue.

Materials and Methods

Referenced details of the materials and methods, including mammalian cell lines, virus strains, culture conditions, mouse experiments, and in vitro/ex vivo assays are provided in the *SI Appendix*. The described animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) from National University of Singapore (NUS) under protocols number R13-4447 and R17-0275, and carried out in the AAALAC-accredited animal facility at NUS. H1N1/PR8 virus stocks were prepared in embryonated eggs, while mouse-adapted H3N2 virus was obtained after successive passages in murine lungs. Virus titers were determined by plaque assay in MDCK cells. Juvenile (3- to 4-wk–old), adult (5- to 6-wk–old), and older (12- to 15-mo–old) BALB/c mice were immunized with Clec9A–M2e or GL117–M2e construct (adjuvanted with poly I:C, CpG, poly I:C + CpG, or Addavax) via the intravenous or subcutaneous route. Viral infections were performed via the intratracheal route. Serum antibody titers were measured by ELISA using M2e as coating antigen. The ability of immune sera to bind flu-infected MDCK cells was assessed by fluorescence-activated cell sorting (FACS). M2e-specific IgG-secreting B cells were quantified in the bone marrow from immunized mice by B cell ELISpot. M2e-specific IFN-γ- and IL-4-producing T cells were quantified by ELISpot.

Data Availability. All study data are included in the article and/or supporting information.

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