


ORIGINAL ARTICLE

Repeated influenza vaccination provides cumulative protection from distinct H3N2 viruses

Niloufar Kavian^{1,2,3,4}, Asmaa Hachim^{1,2}, Benjamin J Cowling² & Sophie A Valkenburg^{1,2} ¹HKU-Pasteur Research Pole, School of Public Health, The University of Hong Kong, Pokfulam, Hong Kong SAR, China²World Health Organization Collaborating Centre for Infectious Disease Epidemiology and Control, School of Public Health, The University of Hong Kong, Pokfulam, Hong Kong SAR, China³Université Paris Descartes, Sorbonne Paris Cité, Faculté de Médecine, Assistance Publique–Hôpitaux de Paris, Hôpital Universitaire Paris Centre, Centre Hospitalier Universitaire Cochin, Service d'Immunologie Biologique, Paris, France⁴Institut Cochin, INSERM U1016, Université Paris Descartes, Sorbonne Paris Cité, Paris, France**Correspondence**SA Valkenburg, HKU Pasteur Research Pole,
School of Public Health, The University of
Hong Kong, 5 Sassoon Rd, Pokfulam,
Hong Kong SAR, China
E-mail: sophiev@hku.hk

Received 30 March 2021;

Revised 20 May 2021;

Accepted 21 May 2021

doi: 10.1002/cti2.1297

Clinical & Translational Immunology
2021; 10: e1297**Abstract**

Objectives. Current inactivated influenza vaccines provide suboptimal protection against antigenic drift, and repeated annual vaccinations shape antibody specificity but the effect on protection from infection is not well understood. **Methods.** We studied the effects of cumulative and staggered vaccinations in mice to determine the effect of influenza vaccination on protection from infection and immune quality. **Results.** We found that the timing of vaccination and antigenic change impacted the quality of immune responses. When mice received two different H3N2 strains (A/Hong Kong/4801/2014 and A/Singapore/INFIMH-16-0019/2016) by staggered timing of vaccination, there were higher H3HA antibody and B-cell memory responses than four cumulative vaccinations or when two vaccinations were successive. Interestingly, after challenge with a lethal-drifted H3N2 virus (A/Hong Kong/1/1968), mice with staggered vaccination were unable to produce high titres of antibodies specific to the challenge strain compared to other vaccination regimens because of high levels of vaccine-specific cross-reactive antibodies. All vaccination regimens resulted in protection, in terms of viral loads and survival, from lethal challenge, while lung IL-6 and inflammation were lowest in staggered or cumulative vaccination groups, indicating further advantage. **Conclusion.** Our findings help justify influenza vaccination policies that currently recommend repeat vaccination in infants and annual seasonal vaccination, with no evidence for impaired immunity by repeated seasonal vaccination.

Keywords: antibodies, cross-protection, immune imprinting, influenza virus, repeated vaccination

INTRODUCTION

Inactivated influenza vaccines (IIV) are our most effective tool for combating seasonal influenza circulation in the community¹. IIV are the most widely used vaccines in the world with vaccination campaigns worldwide using over 500 million doses annually, and seasonal influenza epidemics can infect up to 20% of the population.² Older adults over 65 years of age are most susceptible to complications from infection, accounting for > 75% of influenza-associated mortality, while vaccination of children can reduce disease in the community.³ In many countries, annual influenza vaccination is prioritised for high-risk individuals, such as older adults and health care workers, and in the United States, universal vaccination is recommended for everyone from 6 months of age and older without contraindications.⁴ Annual vaccination is recommended because of continual antigenic drift necessitating vaccine updates and because of decline in vaccine-induced antibody titres.⁵ It is estimated that vaccine-mediated protection declines by 6 months post-vaccination⁶ because of waning of haemagglutinin (HA) inhibition antibodies.⁷ Also, young children, under 8 years of age, and especially under 2 years of age, are particularly susceptible to complications from influenza virus infection. Therefore, the first vaccination of infants under 2 years of age is recommended a prime-boost regime because of their naïve status. Infants are given two-dose vaccination from 6 months of age and within at least 4 weeks for adequate protection.⁸

Tropical and subtropical locations typically choose either the Northern Hemisphere (NH) or Southern Hemisphere (SH) formulation based on their local epidemiology, and strain changes can occur between seasons; therefore, twice-annual vaccination is being considered in these regions, such as Hong Kong⁹ and Singapore,¹⁰ to maintain high titre of HAI antibodies for year-round protection and to match circulating strains. Twice-annual vaccination occurred for the first time in 2015 in Hong Kong in some older adults because of antigenic mismatch of H3N2 virus in the 2014/2015 NH vaccine which contained A/Texas/50/2012 that did not match the circulating H3N2 A/Switzerland/9715293/2013-like strain.¹¹ Twice-annual vaccination resulted in elevated haemagglutination inhibition (HAI) titres in the second round of vaccination, but reduced influenza-specific CD4⁺ T cell responses.⁹ Similarly,

twice-annual vaccination in tropical Singapore in SH 2016 and NH 2016/2017¹² showed an increase in H1N1 HAI titres and a lower incidence of influenza-like illness in the following 6 months.

There are disparate reports about the effect that repeated immunisation plays on the quality of the vaccine immune response and subsequent protection from influenza virus infection^{13,14} and disease. Repeated once-annual vaccination with surface HA proteins which are relatively similar can limit antibody boosting, known as the antigenic distance hypothesis.¹⁵ Repeated vaccination may even reduce seasonal vaccine efficacy, with reports of higher rates of protection in individuals that were *not* vaccinated in the previous year compared to those who were,^{16,17} especially when the vaccine strains are maintained between yearly vaccine formulations and only a minor antigen drift has occurred in circulating strains, as this can impact the ability of the individual to respond to new strain during infection.

However, repeated once-annual vaccination can also reportedly benefit the quality of the immune response. In older adults who received 3–4 years of annual and repeated IIV vaccination, rather than single vaccination, the memory CD4⁺ T cells had a higher response magnitude, long-term durability and multifunctional quality.¹⁸ Whereas HAI titres and memory B cells were boosted after each immunisation, these responses plateaued by the final season of vaccination. Recently, a ferret study found increased viral shedding and delayed recovery from influenza infection in ferrets who received IIV twice compared to once only.¹⁹ This has led to further questions about the quality of antibody responses generated from repeated and cumulative IIV vaccination, which are difficult to deconvolute in the human population because of high pre-existing immunity and in ferrets because of the limited availability of reagents. Therefore, we employed the influenza mouse IIV vaccination model using standard seasonal IIV that recapitulates the prime boost recommended for infants, and once- and twice-annual vaccination programmes being investigated in Hong Kong and Singapore as clinical trials. Since the A(H3N2) virus undergoes antigenic drift more rapidly compared to A(H1N1) virus subtypes and has acquired egg-adapted mutations which affect vaccine efficacy,²⁰ H3N2 poses a more significant hurdle for vaccine-mediated protection,²¹ we therefore focused our study on assessing

responses against A(H3N2). Vaccinated mice were challenged with a drifted H3N2 influenza virus to determine the effect of cumulative and staggered repeat influenza vaccination for protection from lethal influenza challenge and the impact on antibody quality.

RESULTS

Staggered vaccination elicited the largest H3HA IgG titres

To determine the effect of the repeated use of IIV, we used a panel of vaccines which are being studied separately in older adults for twice-annual vaccination in Hong Kong in our ongoing clinical trial (NCT ClinicalTrials.gov NCT02957890). We used IIV from Northern Hemisphere and Southern Hemisphere formulations over four seasons 2017 to 2019 (that contained as H3HA strain either from H3-HK-2014 or from H3-Singapore-2016) and used them as vaccines in mice (Figure 1b). Mice received either 1 (Group 1) vaccine dose or 2 (Group 2), 3 (Group 3), 4 (Group 4) vaccine doses at 3-week intervals, while Group 5 had a longer staggered 9-week interval between its 2-dose vaccination first containing H3HA HK-2014 and then H3HA Singapore-2016 (Figure 1a and b).

The magnitude of the total antibody responses was determined by endpoint titrations for binding by enzyme-linked immunosorbent assay (ELISA) and neutralisation to vaccine strains and drifted H3N2 virus (Figure 2). The various vaccine regimens induced significant antibody titres to the H3 vaccine antigens as shown by the endpoint titrations of H3HA HK-2014 and Singapore-2016 (Figure 2a–d). As expected, mice from Group 1 and Group 2 that received only the H3HA Singapore-2016 as vaccination had the lowest levels of H3HA HK-2014-specific IgG (Figure 2d). Surprisingly, the largest antibody titres were found in Group 5 for vaccine and drifted H3N2 strains. Group 5 had received two-dose staggered vaccination 9 weeks apart for H3HA HK-2014 and H3HA Singapore-2016 and had the highest titres determined by area under the curve (AUC) for ELISA for H3HA HK-2014 (Figure 2a and b), H3HA Singapore-2016 (Figure 2c and d) and H3HA HK-1968 (Figure 2e and f), compared to other vaccine groups and mock PBS, leading to the highest cumulative antibody response (Figure 2g). Strikingly mice from Groups 1 and 2 that were only vaccinated with IIV containing H3N2

Singapore-2016 virus had the lowest levels of H3HA Singapore-2016-specific IgG (Group 1: 4.157 AUC \pm 0.9481, Group 2: 3.732 AUC \pm 1.603) (Figure 2c and d), demonstrating a boosting and broadening effect of a prior vaccination with H3HA HK-2014 in the Groups 3, 4 and 5 (Group 3 6.298 AUC \pm 1.941, Group 4: 6.665 AUC \pm 1.342, Group 5: 9.266 AUC \pm 1.354).

We measured IgG levels to the H3HA protein from the H3N2 HK-1968 strain, representing over 46 years of difference with the H3N2 vaccine strains (15% amino acid difference, Figure 1c). Vaccinated mice had some level of cross-reactive HK-1968 IgG (Figure 2e and f) that was lower than vaccine strains HK-2014 IgG and Singapore-2016 IgG (Figure 2g). Again, the highest HK-1968 HA IgG levels were observed in mice from Group 5 (Figure 2e and f). While Group 1 and Group 2, which had only been vaccinated with one H3N2 type (Singapore-2016), again had the lowest responses to the distantly related H3HA HK-1968 (Figure 2f). Groups 1 and 2 had comparable antibody titres to all 3 H3HA tested; therefore, there was no booster effect upon homologous second vaccination in Group 2. This demonstrates the positive effect of the heterologous prime boost (H3N2 Singapore-2016 and HK-2014) vaccination for cross-reactive H3HA HK-1968-specific IgG in Groups 3, 4 and 5.

We next investigated the levels of neutralising antibodies to H3N2 viruses which are more strain-specific than responses for antibody binding measured by ELISA. HK-2014 and Singapore-2016 Virus-neutralising antibody (VNA) titres were highest in Group 3, 4 and 5 mice with sequential vaccination to the two closely related HK-2014 and Singapore-2016 H3N2 strains compared to Group 1 and 2 mice exposed only to Singapore-2016 (Figure 2h), which was consistent with IgG binding by ELISA. As expected, neutralisation titres to the drifted strain HK-1968 were drastically reduced in all groups compared to the titres observed for HK-2014 and Singapore-2016 H3N2 viruses (Figure 2h).

Heterologous and cumulative immunisation boosted B memory and long-lived B cells

Memory B cells are a critical arm of the vaccine-generated response for early B-cell recall upon infection. In the vaccination site draining inguinal lymph node (iLN), total memory B-cell responses were highest in Group 4 and Group 5 mice

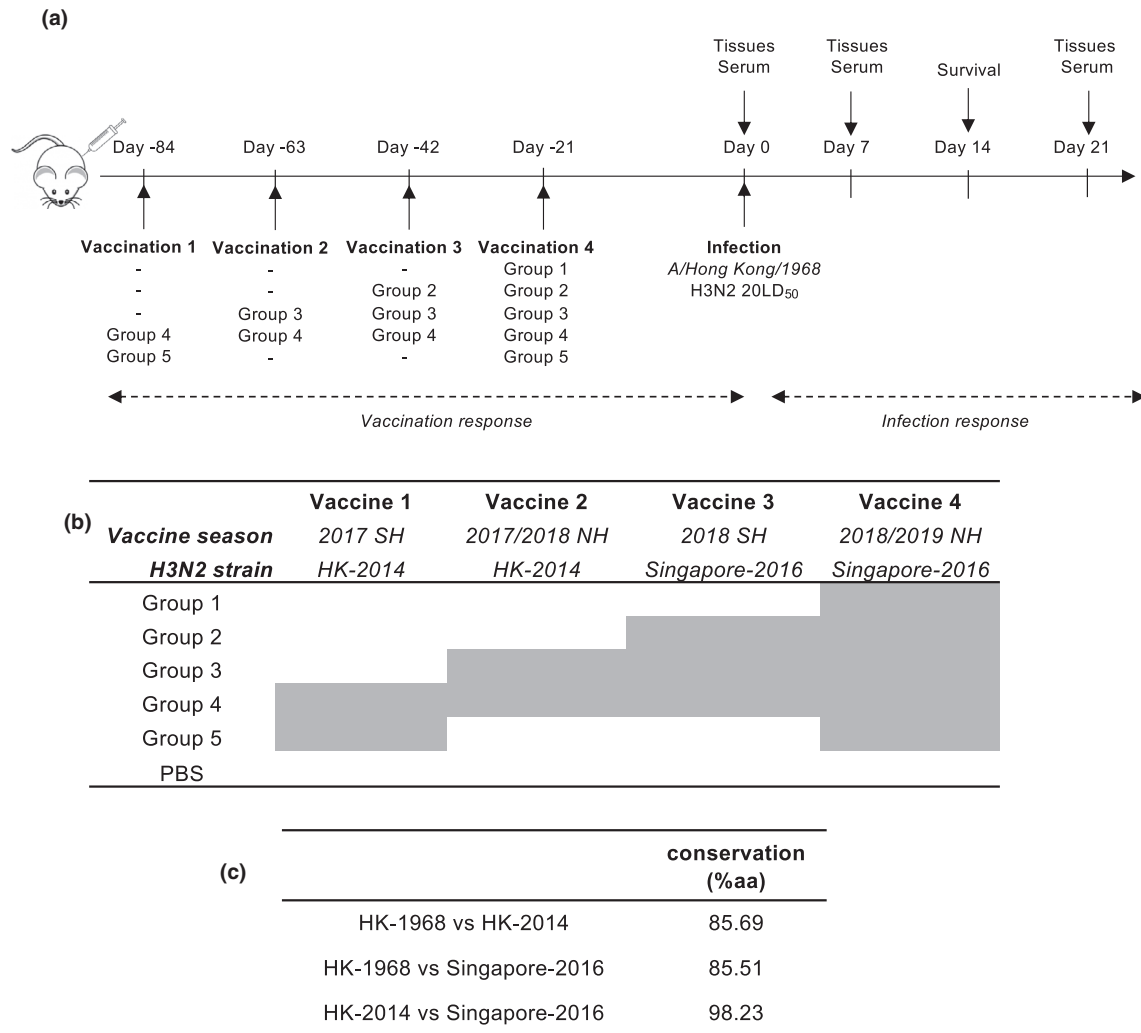


Figure 1. Mouse vaccination and challenge regimen. **(a)** Mice were vaccinated 1 to 4 times at 21-day intervals, challenged with H3N2 HK-1968 virus, in a successive (group 1–4) or staggered vaccination regime (group 5) or mock PBS. **(b)** Summary of H3N2 vaccine strains received per each group. **(c)** HA amino acid sequence conservation of H3N2 virus strains for vaccination and challenge.

compared to mock PBS mice (Figure 3a). Increased memory B cells in Group 4 and Group 5 corresponded to increased mRNA transcripts for Blimp1 (Figure 3b), CXCR4 (Figure 3c) and CD21 (Figure 3d), which are associated with antibody production, activation-induced differentiation and long-term B-cell survival in B cells. Furthermore, in Group 5, antibody-secreting B cells, which reside in the bone marrow and form long-term memory responses, had the highest H3HA HK-2014 and Singapore-2016 antibody production capacity upon restimulation with vaccine antigens (Figure 3e) and drifted H3HA HK-1968 (Figure 3f). Group 3 and 4 had some notable antibody-secreting B-cell

function above background, while Group 1 and 2 formed no response (Figure 3e and f).

Cumulative vaccination leads to protection from lethal challenge by increased IgA and reduced lung inflammation

Mice were challenged with a lethal dose of drifted H3N2 virus, and all vaccinated mice survived and had significantly lower weight loss than the unvaccinated mice (Figure 4a and b). Viral loads were determined by RT-PCR of the influenza M gene and showed that Group 4 mice had the lowest viral loads of all vaccine groups

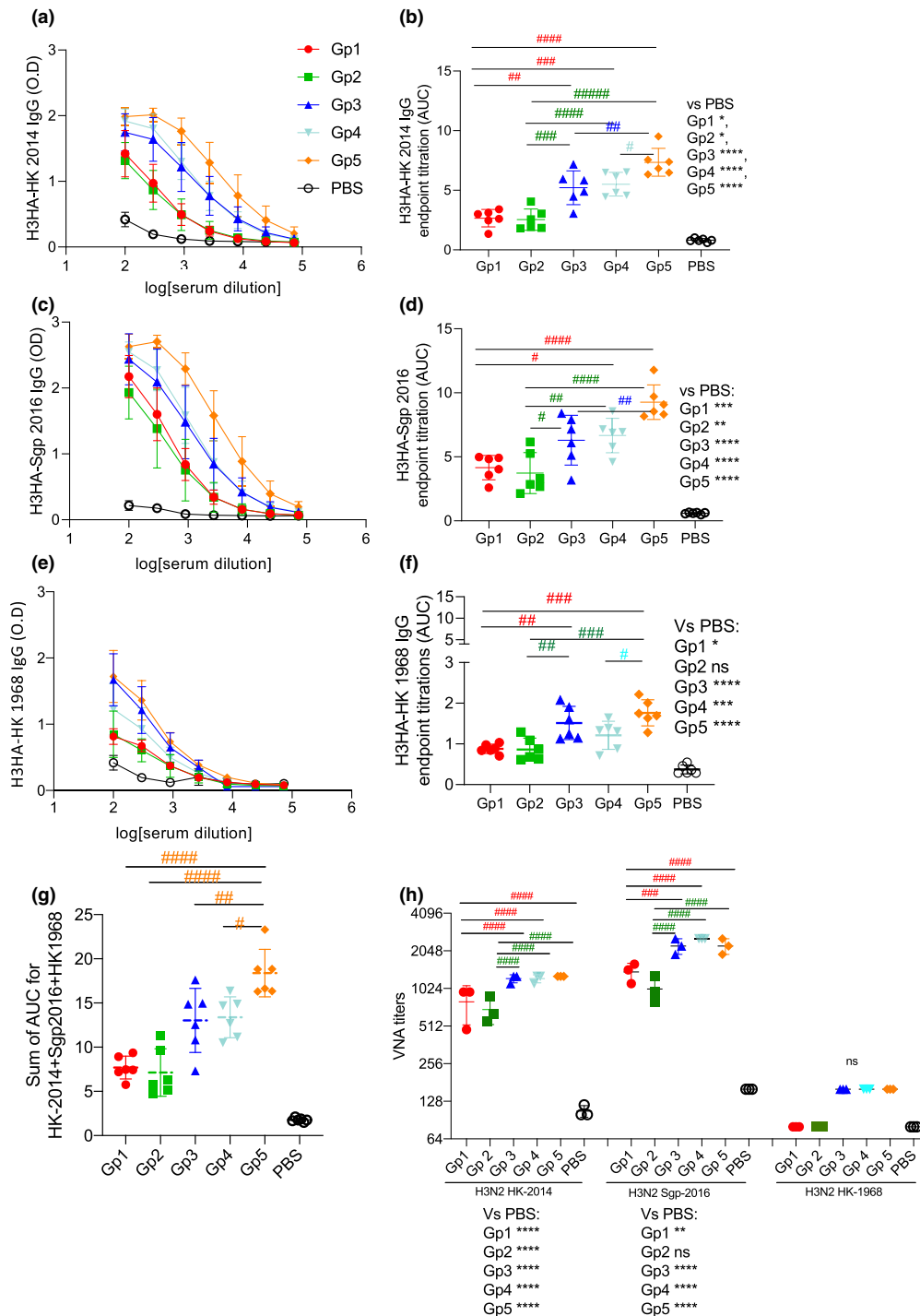


Figure 2. Antibody responses to vaccine and drifted H3N2 viruses. IgG endpoint titration curves (**a, c, e**) and area under the curve (AUC) of endpoint titration (**b, d, f**) to H3HA from the vaccination strains Hong Kong-2014 (HK-2014) (**a, b**), Singapore-2016 (Sgp-2016) (**c, d**) and the drifted strain Hong Kong-1968 (HK-1968) (**e, f**). Sum of the AUC (**g**) for 3 antibody responses to HK-2014, Sgp-2016 and HK-1968. (**h**) VNA titres to H3N2 virus HK-2014, Sgp-2016 and HK-1968. Antibody responses to H3 vaccine antigens and a H3-drifted strain at vaccination time point (d21 post-boost) were assessed by ELISA ($n = 6$ mice per group, **a-g**) and virus neutralisation assay (VNA, $n = 3$ per group, **h**). Data represent the individual mice and group mean \pm SD. (Black) * shows statistical significance versus PBS unvaccinated group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$, **** $P < 0.0001$. (Coloured) # shows statistical significance between vaccinated groups. # $P < 0.05$, ## $P < 0.01$, ### $P < 0.005$. Experiments were repeated twice.

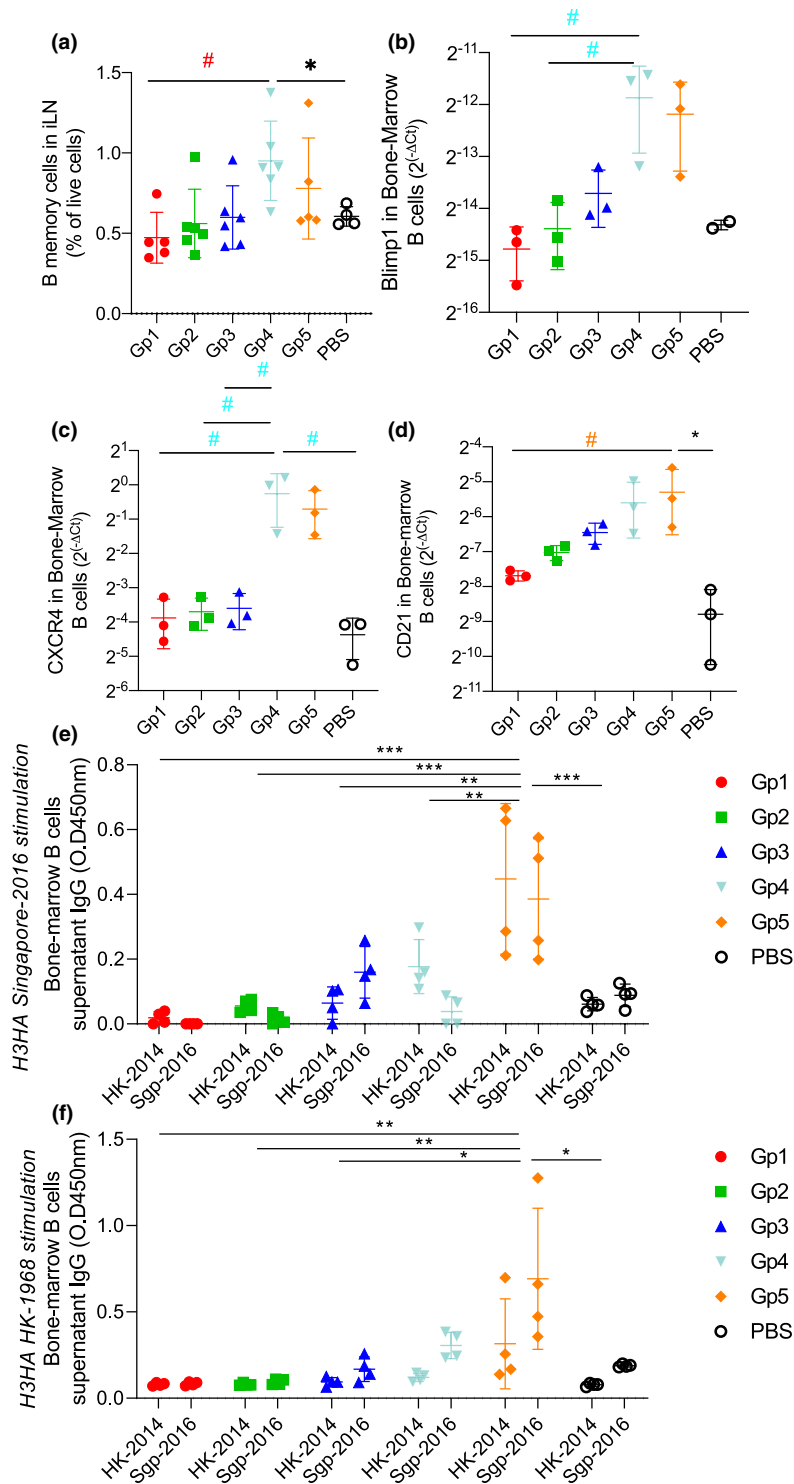


Figure 3. Memory B-cell responses are increased by cumulative or staggered heterologous vaccination. Inguinal lymph nodes (iLN, $n = 6$ per group) and bone marrow ($n = 4$ per group) were harvested at day 21 after the last vaccination. **(a)** Percentage of B memory cells in iLN measured by flow cytometry. Blimp1 **(b)**, CXCR4 **(c)**, CD21 **(d)** mRNA levels by RT-PCR in bone marrow B cells. **(d, e)** H3HA HK-2014 and H3HA Singapore-2016 IgG levels in supernatant from bone marrow B cells stimulated with H3HA Singapore-2016 **(e)** and with H3HA HK-1968 **(f)**. Data represent the individual mice and group mean \pm SD. (Black) *shows statistical significance versus PBS unvaccinated group. $*P < 0.05$, $**P < 0.01$, $***P < 0.005$. (Coloured) #shows statistical significance between vaccinated groups. $\#P < 0.05$. Experiments were repeated twice.

($P < 0.0001$ versus PBS, Figure 4c). We next assessed markers of lung local inflammation, defined by IL-6 and total protein concentration, which were also the lowest in Group 4 and 5 compared PBS (Figure 4d). H3HA HK-1968-specific secretory IgA in the lung was increased in all vaccinated groups compared to PBS group, and the highest IgA levels were detected in Group 2 and 4, while Group 5 had significantly lower IgA responses ($P < 0.0001$ for vaccinated groups versus PBS, Figure 4e).

Mice with staggered vaccination do not produce IgG to drifted H3N2 early after virus challenge

As a result of the differences of vaccine antibodies which may impact the generation of new *de novo* responses or recall of memory at infection, we assessed the antibody responses at the time of H3N2-1968 challenge. Vaccine-specific antibodies for HK-2014 and Singapore-2016 followed a similar trend at day 7 challenge (Figure 5a and b, and Supplementary figure 1), as vaccination time points (Figure 2a, b, d and e), with elevated responses in Groups 4 and 5, and to some extent Group 3. However, Group 5 had a distinct day 7 H3HA HK-1968 IgG response with markedly lower IgG responses than other vaccine groups ($P = 0.0020$, $P = 0.0143$ and $P = 0.0002$ for Group 5 versus Groups 1, 2 and 3) and was comparable to mock-vaccinated mice ($P = ns$, Figure 5c), while Group 4 had an intermediate level of H3HA HK-1968 IgG, which was not significantly different to other vaccine groups, but was still significantly higher than mock-vaccinated mice (Figure 5c).

B cells from lung draining mediastinal lymph nodes were stimulated with HK-2014 and Singapore-2016 vaccine antigens, to determine the capacity of vaccine-specific B cells to produce cross-reactive HK-1968 antibodies (Figure 5d). Local B-cell responses showed Group 4 had the highest local production of cross-reactive H3HA HK-1968 IgG ($P = 0.0007$ vs PBS), while B cells from Groups 1, 2 and 5 had poor performance for *de novo* H3HA HK-1968 IgG production with responses that were comparable to mock. T follicular helper (Tfh) cells are a cornerstone for the development of an early and mature antibody response. Activation (by Ox40 and CD25 upregulation) of H3HA HK-1968 specific Tfh was also significantly elevated in Groups 3 and 4

compared to mock PBS mice, while Groups 1, 2 and 5 did not show increased Tfh activation compared to mock ($P = 0.0012$ and $P = 0.0061$ for Group 3 and 4, respectively, versus PBS, Figure 5e). Furthermore, the HK-2014 N2-NA-specific antibody responses are only significantly increased in Group 4 at day 7 of infection compared to mock PBS (AUC of 3.534 ± 1.553 for Group 4 versus AUC of 1.121 ± 0.3027 for PBS, $P = 0.0270$, Figure 5f). Similarly, the H3-stem response was only significantly increased in Group 4 at day 7 post-infection (AUC of 4.613 ± 1.371 for Group 4 versus AUC of 3.042 ± 0.9991 for PBS, $P = 0.0467$, Figure 5g).

Repeated and staggered vaccinations induce diverse maturation of the antibody response 21 days after challenge

Maturation of the antibody response was assessed following recovery from H3N2-1968 infection (day 21 post-challenge), to determine the recruitment of vaccine memory versus the establishment of a *de novo* response to H3N2-1968 virus challenge. A neutralising antibody response was detectable from day 7 and further increased by day 21 in all groups (Figure 6a). Unvaccinated survivor PBS mice developed the highest neutralising antibody titres at day 21 over all vaccinated groups (Figure 6a, $P < 0.0001$ versus all vaccinated groups), showing that recovery from natural infection with no prior vaccination is highly efficient at inducing neutralising antibodies. While the abundance of H3HA HK-1968 IgG by ELISA was significantly higher in all vaccinated groups compared to PBS, indicating the high levels of cross-reactive antibodies triggered by all vaccine regimens (Figure 6b–d).

Antibodies targeting the stem of HA are highly cross-reactive, immune correlate of protection²² and goal for universal vaccine design²³. Group 2 HA-stem-specific antibodies were therefore measured to determine the impact of repeated vaccination on the ability to generate HA-stem-specific antibodies at infection and recovery (Figures 5f and 6e, Supplementary figure 1e). Mice from Groups 1 and 2 vaccinated only with one strain of H3HA (Singapore-2016) developed higher levels of HA-stem antibodies compared to groups immunised with both H3HA HK-2014 and Singapore-2016 (Figure 6e). This resulted in the total cumulative antibody response in vaccinated mice showing distinct patterns of specific

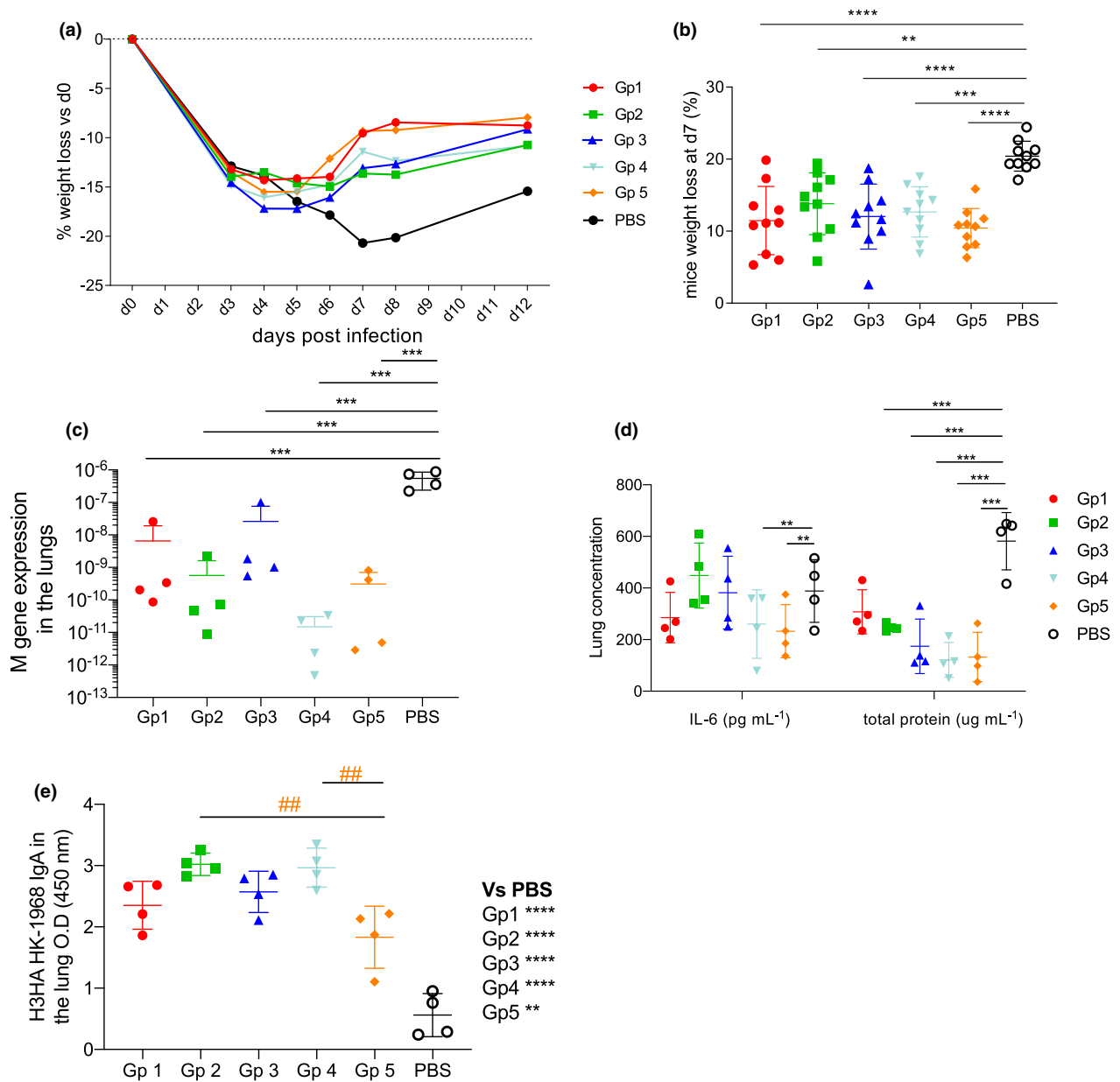


Figure 4. Improved cross-protection against H3N2 HK-1968 by cumulative or staggered heterologous vaccination. Mice ($n = 10$) were challenged 21 days after the last vaccination; 4 mice were culled at day 7 post-challenge to assess immune responses to challenge. Lungs and bronchoalveolar lavage fluids were collected. **(a)** Mice weight loss curve after challenge ($n = 10$). **(b)** Percentage weight loss in mice at day 7 after challenge. **(c)** Expression of the influenza M gene in the lungs of mice ($n = 4$) at day 7 post-challenge measured by absolute quantitative RT-PCR. **(d)** IL-6 and total protein concentrations in the lung at day 7 post-challenge. **(e)** H3HA HK-1968 secretory IgA in the lungs by ELISA. (Black) *shows statistical significance versus PBS unvaccinated group. $*P < 0.05$, $**P < 0.01$, $***P < 0.005$, $****P < 0.0001$. (Coloured) #shows statistical significance between vaccinated groups. $##P < 0.01$. Experiments were repeated twice.

antibody responses at vaccination and challenge time points (Figure 6f), whereby Group 5 had the largest vaccination response but poorer *de novo* H3HA HK-1968 response at infection (Figure 6g). The kinetics of recall of a memory response versus the generation of a *de novo* H3HA HK-1968

antibody response was derived from the difference of IgG response magnitude by AUC from vaccination to early challenge response (day 7) or at recovery (day 21 post-challenge) versus the early challenge response (day 7; Figure 6g). At day 7, Groups 1, 2 and 3 had a significantly larger

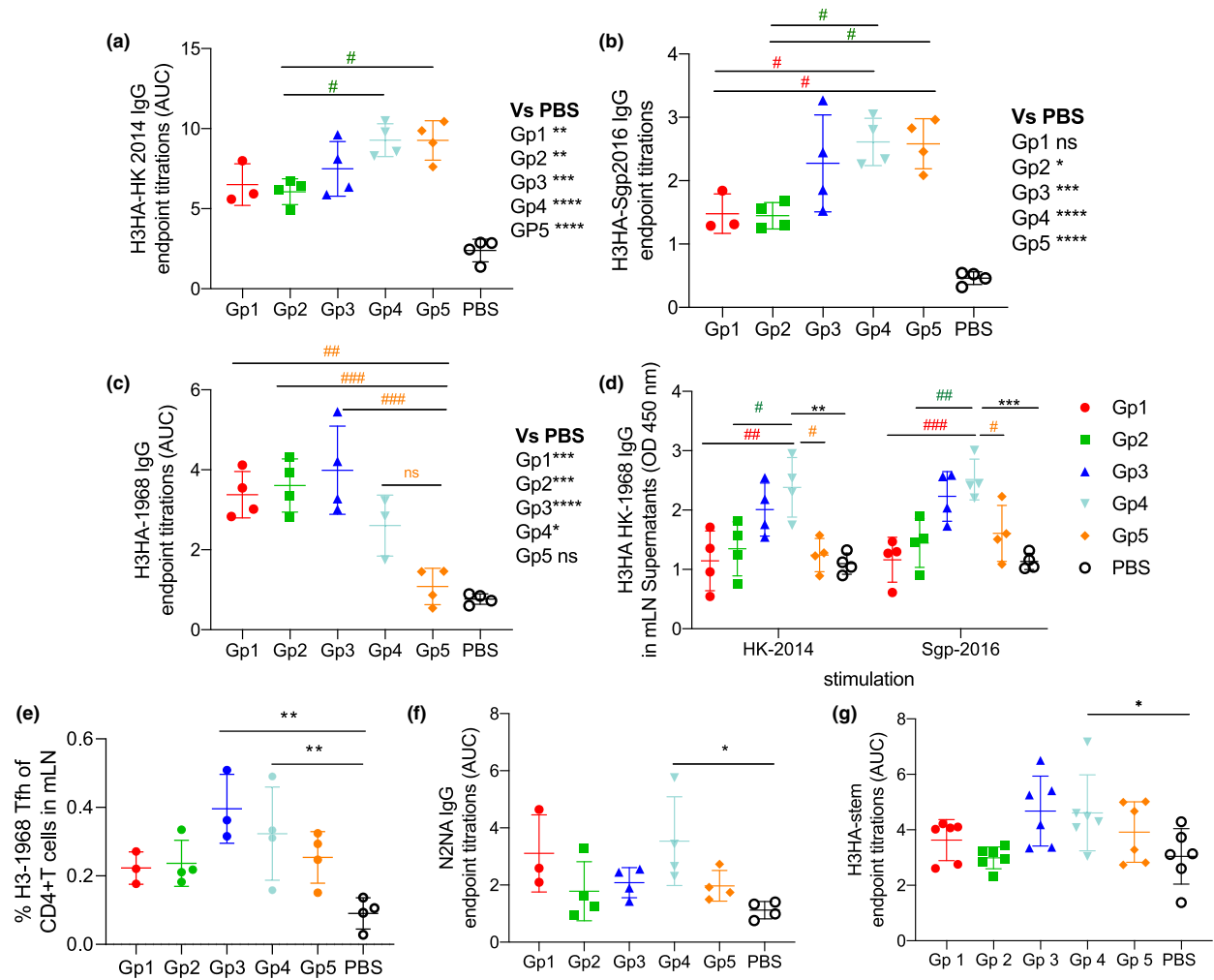


Figure 5. Early antibody responses at viral challenge (d7 post-infection). Mice ($n = 10$) were challenged 21 days after the last vaccination; 4 mice per group were culled at day 7 post-challenge to assess immune responses. Serum was collected and antibody responses were assessed by ELISA, as well as mediastinal lymph nodes (mLN) for cellular responses and secreted antibody. AUC of serum IgG endpoint titration curves to H3HA from the vaccination strains HK-2014 (a), Sgp-2016 (b) and HK-1968 (c). (d) Stimulation of mLN with H3HA HK-2014 and H3HA Singapore-2016 for H3HA-1968-IgG. (e) Percentage of HK-1968-specific activated (Ox40⁺ CD25⁺) T follicular helper (Tfh) of CD4⁺ T cells in mLN. AUC of serum IgG endpoint titration curves to N2NA from H3N2 HK-2014 (f) and H3HA-stem (g). (Black) *shows statistical significance versus PBS unvaccinated group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$, **** $P < 0.0001$. #shows statistical significance between vaccinated groups. (Coloured) # $P < 0.05$, ## $P < 0.01$, ### $P < 0.005$. Experiments were repeated twice.

change in the antibody response magnitude from vaccination compared to Group 5 or PBS, while Group 4 had intermediate early recall. While by day 21 recovery, Group 5 and PBS mice had increased responses, Groups 1–4 had contracted their antibody responses.

DISCUSSION

There is a conflicting body of evidence for the immunological effects of repeated annual vaccination for influenza, and its impact of

protection from influenza virus infection is unclear. A growing number of studies conducted in humans suggest that repeated vaccination either yearly or twice annually provides increased vaccine efficacy; however, results are heterogeneous with some years reporting better protection in repeated vaccinees over first-time vaccinees, whereas in other years protection is reduced.^{15,24} We used four clinically relevant seasonal influenza vaccines representing the 2017 to 2019 NH and SH vaccine formulations to mimic the vaccination regimen possibilities in a subtropical region like Hong Kong.

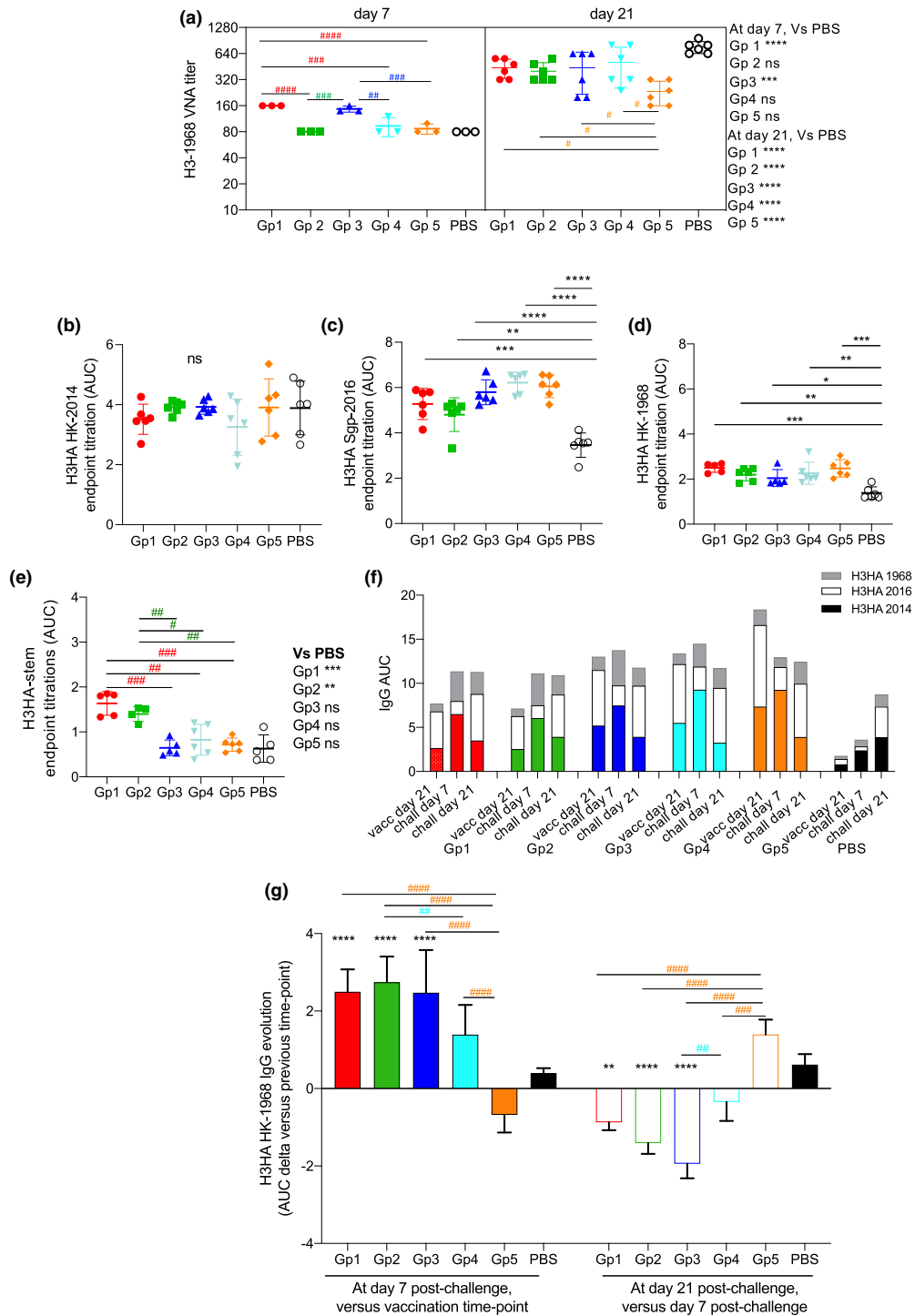


Figure 6. Maturation of the antibody response at recovery. **(a)** H3N2 HK-1968 VNA titres at day 7 and day 21 post-challenge. **(b–e)** AUC from serum IgG endpoint titration curves (from Supplementary figure 1) for H3HA IgG from HK-2014, Sgp-2016, HK-1968 and H3-stem. **(f)** AUC of H3HA HK-2014, Sgp-2016, HK-1968 IgG at each time point per group (AUC). **(g)** Evolution of H3HA HK-1968 IgG with time. The delta change for the AUC at different time points was calculated as follows: (delta 1) AUC at day 7 post-challenge - AUC at vaccination time point; and (delta 2) AUC at day 21 post-challenge - AUC at day 7 post-challenge. *shows statistical significance versus PBS unvaccinated group. (Black) * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$, **** $P < 0.0001$. (Coloured) #shows statistical significance between vaccinated groups. # $P < 0.05$, ## $P < 0.01$, ### $P < 0.005$, #### $P < 0.0001$. Experiments were repeated twice.

The mouse model allowed us to study several immune parameters and determine protection from lethal viral challenge. The study of repeat vaccination in humans can be confounded by the immunocompetence of the vaccinee, diverse and unknown previous influenza immunisations and virulence and transmissibility of influenza virus strains.^{12,16,25} Our study focused on the immune responses to repeated H3N2 exposure which has been circulating since 1968 and has evolved since then.²⁶ It is of particular interest as recent influenza seasons have shown increased severity of H3N2 dominated seasons and lower H3N2 vaccine efficacy. A recent study by Gouma *et al.*²⁷ reported that an individual's ability to generate protective antibody titres upon infection of circulating H3N2 strains is dependent on the childhood first exposure strains of H3N2. Similarly, early imprinting shows suboptimal immunity for H3N2 infection compared to H1N1 infection with differing rates of waning and subsequent residual protection.²⁸ Increasing age and exposure to influenza by infection or vaccines over a lifetime may affect the outcome of repeated vaccination in older individuals.²⁹ Large serological surveys are needed with donors of multiple vaccination backgrounds to determine the impact of age on the H3N2-specific antibody landscape, yet the impact on infection is inferred, hence the advantage of mouse vaccination and challenge studies.

Our mouse model allowed us to assess the immunological impact of homologous versus heterologous repeat vaccination, the timing of repeat vaccination and the effect of antigenic drift (H3HA HK-2014 and Singapore-2016) followed by infection with an antigenically drifted H3 strain (H3N2 HK-1968). We used a classic 3-week prime-boost interval between each immunisation, as an extended timeline of vaccinating mice every 6 months or 1 year is not feasible because of their limited lifespan and introduces issues of age effects rather than antigenic distance and vaccine immunogenicity which were under investigation in this study. We also did not assess the long-term effects of these repeated vaccination regimen for immune waning or H1N1 and influenza B viruses. There is, however, a clear effect within our study on the timing and doses of influenza vaccines on the vaccine and infection-specific antibody response.

Our mouse vaccination data show that mice that received three or four vaccinations displayed

a higher cumulative IgG production at vaccination compared to mice that received only 1 or 2; however, alteration of the timing and antigenic content can affect the vaccine and infection response, as evident in Group 5 mice. The vaccination regime has 2 parameters which are difficult to deconvolute, that Group 2 and Group 5 had a different timing (3 weeks versus 9 weeks between vaccinations) and vaccine antigens (Group 2 had homologous vaccination with H3HA Singapore-2016, and Group 5 had heterologous vaccination with H3HA Singapore-2016 and HK-2014). However, the regimes of Groups 2 and 5, repeated vaccination versus staggered delayed vaccination, were designed to mimic a vaccination with the varying duration between doses and compare once-annual versus twice-annual type of vaccination with an antigenic change that did occur during vaccination in Hong Kong. Group 1 and Group 2 vaccine groups performed similarly, while Groups 4 and 5 had an immunological advantage at vaccination and infection.

The staggered vaccination regimen at 3 weeks after final vaccination of Group 5 elicits the highest antibody titres for the H3N2 vaccination strains HK-2014, Singapore-2016 and also for drifted strain HK-1968 not included in the vaccines. However, at challenge with the HK-1968 strain, we observed that mice vaccinated with staggered vaccination were not capable of producing an early high titre *de novo* response for HK-1968-specific IgG by ELISA or VNA compared to mice vaccinated with the 4 other regimens. This absence of an early *de novo* response in Group 5 could be because of the presence of high levels of cross-reactive antibodies in this group after vaccination (Figure 1) that neutralise the virus prior to the induction of a specific HK-1968 response. However, weight loss and viral loads indicate infection of Group 5 was comparable to other groups and high levels of pre-existing antibodies did not fully block infection. The production of high levels of HK-2014 and Sgp-2016 IgG after stimulation of bone marrow B cells from vaccination with HK-1968 HA supports this hypothesis of cross-reactivity. Data from day 21 post-challenge VNA and ELISA show that Group 5 is indeed producing HK-1968-specific IgG with delayed kinetics (Figure 6). In addition, levels of IL-6, total proteins and IgA at challenge show that Group 5 had a protective immune response with reduced local inflammation.

Groups 3 and 4 (with 3 and 4 repeated vaccinations, respectively) showed cumulative maturation of memory B cells and antibody titres. Importantly, regardless of the *de novo* response at infection, all mice had reduced viral loads and weight loss compared to PBS mice, but Group 5 also had reduced viral titres and lung inflammation and therefore attained a greater level of immune protection by vaccination.

In our model, increasing the number of repeated vaccinations did not lead to a ceiling effect on antibody levels nor to a reduced vaccine efficacy at viral challenge. Indeed, 4 repeated vaccinations in Group 4 had increased IgG titres and higher B-cell activation which led to an overall increased antibody response with no disadvantage at vaccination and at challenge compared to groups that received 3, 2 or 1 vaccinations.

The benefit of vaccination in Group 4 over Group 5, that is cumulative vaccinations versus staggered heterologous vaccination, is only evident by considering the rapid *de novo* response at infection in Group 4 but not in Group 5, which supports repeated annual influenza vaccination. Furthermore, from vaccination Group 1 and 2 had a less matured B-cell response and at infection were therefore able to make HA-stem-specific responses which may be important for outcomes at subsequent reinfection, that were not present in the PBS recovered group despite high neutralisation. Therefore, a balance exists in the influenza vaccine response, where there are advantages in each scenario of vaccination. Cumulative vaccination drives B-cell maturation which has the immediate benefit for reduced viral titres and inflammation at infection; however, a less mature B-cell responses (with no repeated vaccinations) are able to generate HA-stem-specific antibodies which may play a role in protection from diverse sources of infection. Therefore, immune responses to vaccination programmes and schedules should not be studied in isolation but in the context of current infection and future influenza encounters.

METHODS

Vaccination and infection of mice

To assess immunogenicity of repeat vaccination and vaccine-mediated protection, female 6-week-old BALB/c mice were vaccinated once to up to four times at 21 days apart (Figure 1a and b) via the intramuscular (i.m.) route with a third of a human dose of FluQuadri (Sanofi Pasteur,

Lyon, France) in 100 μ L of 2017 SH [A/Michigan/45/2015 (H1N1)pdm09-like virus, A/Hong Kong/4801/2014 (H3N2)-like virus (clade 3C.2a), B/Brisbane/60/2008-like virus and B/Phuket/3073/2013-like virus], 2017/2018 NH (same antigen content as 2017 SH), 2018 SH [A/Michigan/45/2015 (H1N1)pdm09-like virus, A/Singapore/INFIMH-16-0019/2016 (H3N2)-like virus (clade 3C.2a1), B/Brisbane/60/2008-like virus and B/Phuket/3073/2013-like virus], 2018/2019 NH (same antigen content as 2018 SH) or PBS. A/Hong Kong/4801/2014 (clade 3C.2a, HK-2014) and A/Singapore/INFIMH-16-0019/2016 (clade 3C.2a1, Singapore-2016) share 98.23% amino acid homology for the HA protein by CLUSTALW (Figure 1c). For assessment of vaccination responses, mice were culled at day 21 after final vaccination (equivalent to day 0 of virus challenge experiments).

To assess whether the breadth of vaccine-mediated protection was impacted by repeat vaccination for H3N2 strains that are substantially different to the vaccine, mice were challenged with a 'drifted' H3N2 strain [A/Hong Kong/1/1968 (HK-1968)], which circulated 46 years earlier of the vaccine strains. This represents a 'heterologous' challenge, with HA homology with vaccine strains at only 85.69% versus HK-2014, and 85.59% versus Singapore-2016. Vaccinated mice were challenged with HK-1968 virus at 21 days after the final vaccination. Mice were anaesthetised and then infected intranasally with a lethal dose (20LD₅₀) of HK-1968 (1×10^4 pfu per 25 μ L), and mice were culled at day 7 or day 21 after virus challenge (Figure 1a).

Blood was collected by cardiac puncture and clotted (MiniCollect, Greiner Bio-one, Kremsmünster, Austria), and serum was harvested after centrifugation and aliquoted and heat-inactivated, at 30 min at 56°C, before all *in vitro* experiments. To quantify virus replication, antibody and cellular responses, the lungs, BAL, lymphoid organs (mLN for challenged mice or iLN for vaccinated mice) and bone marrow were processed as previously described.³⁰ Lung viral titres were determined from homogenates by M gene quantification using quantitative RT-PCR.³⁰ All experimental procedures were conducted in accordance with the standards approved by the Committee on the Use of Live Animals in Teaching and Research, the University of Hong Kong.

Antibody quantification by enzyme-linked immunosorbent assay

To assess influenza-specific binding antibodies from vaccination or infection, protein-specific ELISAs were performed on mice heat-inactivated sera. Commercial proteins to represent challenge viruses and vaccine strains included the following: HK-2014 HA (MyBiosource, San Diego, CA, USA) and NA (Sinobiological, Beijing, China; A/Hong Kong/4801/2014), Singapore-2016 HA (A/Singapore/INFIMH-16-0019/2016; MyBiosource), HK-1968 HA (A/Hong Kong/1/1968) (Sinobiological). The H3-stem (A/Aichi/2/1968) protein (from Raghavan Varadarajan, Indian Institute of Science) was made as previously described.^{23,31} Recombinant HA proteins (at 80 ng mL⁻¹) and HA-stem protein (at 800 ng mL⁻¹) were coated on 96-well flat-bottom immunosorbent plates (Nunc-Immuno MaxiSorp, Roskilde, Denmark), in 100 μ L coating buffer (PBS with 53% Na₂CO₃ and 42% NaHCO₃, pH 9.6) at 4°C overnight. An

additional plate coated with a non-specific protein (blocking buffer, PBS with 5% FBS) was used to measure the background binding of each individual mouse serum and used as a background subtraction from protein-specific responses. Following FBS blocking and thorough washing, diluted serum samples (starting at 1:100), followed by 1:3 subsequent dilutions for the endpoint titrations, were bound for 2 h, further washed and then detected by a secondary anti-mouse IgG-HRP (Invitrogen, Carlsbad, CA, USA). TMB/peroxide was used as substrate, and the reaction was stopped by addition of sulfuric acid (R&D systems, Minneapolis, MN, USA) and optical density (O.D.) absorbance read at 450 nm (Tecan Life Sciences, Mannedorf, Switzerland).

Single-cell suspensions from bone marrow or mediastinal lymph nodes (pooled from four per group) were harvested from mice and stimulated for 72 h with H3HA proteins ($5 \mu\text{g mL}^{-1}$) in complete RPMI [10% FBS, 1% NEAA, 1% sodium pyruvate, 1% L-glutaMAX, 1% Pen-Strep, 1% HEPES, 0.05 mM 2-mercaptoethanol, in RPMI (all Invitrogen)], and supernatants were collected. The presence of H3HA Hong Kong-2014 or Singapore-2016-specific IgG was then assessed by ELISA on 1:2 diluted supernatants as described above. Protein-specific ELISAs were performed on lungs supernatants to assess the presence of secretory IgA. Lungs were collected at day 7 of virus challenge and homogenised (Omni, Kennesaw, GA, USA). After clarification by centrifugation, supernatants were used at a 1:10 dilution for ELISA using the same protocol as above, with HRP anti-mouse IgA (Invitrogen) as the secondary antibody.

Virus-neutralising antibody assays

Influenza-specific VNA was measured by standard micro-neutralisation (MN) assay. Twofold serial dilutions from 1:5 to 1:3200 of mouse serum samples were prepared in virus medium (MEM, 100 U mL⁻¹ penicillin and 100 $\mu\text{g mL}^{-1}$ streptomycin). An equal volume of 200TCID₅₀ 35 μL^{-1} of influenza viruses: H3N2-2014, H3N2-2016 and H3N2-1968 was added to the sample dilutions (final serum dilution 1:10 to 1:1280) and incubated for 2 h. Then, 35 μL of antibody/virus was added to MDCK cells and incubated for 72 h. Visualisation of cytopathic effect was used to measure reciprocal titres of antibodies for virus inhibition.

Memory B-cell profiling by flow cytometry

To quantify and profile memory B cells from vaccination, single-cell suspensions were prepared from inguinal lymph nodes, as previously described.³² Cells were stained with Zombie live/dead (Biolegend, San Diego, CA, USA), FcR-blocked (anti-CD16/CD32, BD Bioscience, Franklin Lakes, NJ, USA) and stained with a cocktail containing anti-mouse B220-PECy7, CD38-PE, IgD-APC, CD95 (Fas)-BV605, IgM-FITC, IgG-APCCy7 (all Biolegend), for 30 min on ice in FACS buffer (PBS, 1% FBS, 0.5% NaN₃). Cells were finally fixed with 100 μL of 4% PFA for 20 min on ice. B memory cells were defined as B220⁺ CD38^{+/−} Fas^{low} IgD[−] IgM⁺ or IgG⁺. Samples were acquired by flow cytometry on a FACS Attune (Invitrogen) and analysed with FlowJo software (BD). Representative FACS plots are shown in Supplementary figure 2a.

Activation-induced markers of T follicular helper responses

Single suspensions from mLN were prepared as described above. Cells were then stimulated with H3HA HK-1968 ($5 \mu\text{g mL}^{-1}$ in 250 μL) or vehicle control with BSA ($5 \mu\text{g mL}^{-1}$) in 96-well plates, and anti-CD154-PE mAb (Biolegend) for 18 h at 37°C in the dark. Stimulated cells were then washed and surface-stained with an antibody cocktail as follows: live-dead Zombie-Violet, B220-PECy7, CD3-BV605, CD4-APCCy7, CXCR5-PerCPCy5.5, PD1-FITC, CD25-BV510, Ox40-BV711, ICOS-APC for 30 min at 4°C in the dark. Cells were washed, fixed and acquired on a FACS Attune and analysed with FlowJo software. Representative FACS plots are shown in Supplementary figure 2b.

Inflammation and damage by total protein in BAL

BAL samples were harvested in a total of 3 mL MEM and supernatant for measuring total protein and cytokine concentrations. Total protein was measured on BAL fluid with the BCA Protein Assay Kit (Thermo-Fisher Scientific, Waltham, MA, USA) with a twofold standard range using BSA, according to the manufacturer's instructions. Optical densities were read on a plate reader at 562 nm. For the measurement of IL-6 in the BAL fluid, the Quantikine ELISA mouse kit (R&D systems) was used following the manufacturer's instructions.

Quantitative RT-PCR of B cell genes associated with antibody regulation

Quantitative RT-PCR (qRT-PCR) was performed for the murine Blimp1, CXCR4, CD21, on B cells isolated from bone marrow using magnetic B cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany), as previously described.³⁰ RNA was extracted using RNeasy mini kit (Qiagen, Hilden, Germany), and a reverse transcription using Oligo dT primers with PrimeScript RT reagent kit (TAKARA, Kusatsu, Japan) was performed. The cDNA was then used in the qPCR (Fast SYBR green, ABI, St. Louis, MO, USA) with gene-specific primers (Supplementary table 1), then run and analysed in the LightCycler480 (Roche, Basel, Switzerland). To quantify the gene expression, relative qRT-PCR was performed by normalising the data to GAPDH gene expression.

Statistical analysis

Results represent the individual result, mean \pm SEM of 4–6 mice per group, unless indicated otherwise. Statistical significance was compared between vaccinated groups 1–5 versus PBS (indicated in black as * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.0001$) and when relevant and indicated within vaccinated groups (indicated in colour for the comparator group as # $P < 0.05$, ## $P < 0.01$ and ### $P < 0.0001$) using a one-way ANOVA (unless indicated) on GraphPad Prism Software v8 (San Diego, CA, USA). To compare the endpoint titrations of IgG between groups, the area under

the curve (AUC) was calculated for each individual mouse serum and groups AUC was compared using a *one-way ANOVA* on GraphPad Prism Software v8. In Figure 6h, the delta change for the AUC at different time points was calculated as follows: delta 1 representing AUC at day 7 post-challenge – AUC at vaccination time point; and delta 2 representing AUC at day 21 post-challenge – AUC at day 7 post-challenge.

ACKNOWLEDGMENTS

The authors thank Raghavan Varadarajan (Indian Institute of Science) for providing HA-stem proteins. This work was supported by the National Institute of Allergy and Infectious Diseases (Centers of Excellence for Influenza Research and Surveillance [CEIRS] contract number HHSN272201400006C), the General Research Fund of the University Grants Committee of Hong Kong (17113718) and the Theme-based Research Scheme of the Research Grants Council of the Hong Kong SAR Government (Project No. T11-712/19-N).

AUTHOR CONTRIBUTIONS

NK, AH and SAV designed the study and wrote the first draft of the manuscript. NK, AH, and SAV performed the experiments. NK, AH, SAV and BJC provided analysis and interpretation of experiments. All authors critically revised the paper. BJC was the consulting statistician.

CONFLICT OF INTEREST

BJC has received honoraria from Sanofi and Roche for advisory committees. The authors report no other potential competing interests.

REFERENCES

- Barberis I, Myles P, Ault SK, Bragazzi NL, Martini M. History and evolution of influenza control through vaccination: from the first monovalent vaccine to universal vaccines. *J Prev Med Hyg* 2016; **57**: E115–E120.
- Iuliano AD, Roguski KM, Chang HH et al. Estimates of global seasonal influenza-associated respiratory mortality: a modelling study. *Lancet* 2018; **391**: 1285–1300.
- Charu V, Viboud C, Simonsen L et al. Influenza-related mortality trends in Japanese and American seniors: evidence for the indirect mortality benefits of vaccinating schoolchildren. *PLoS One* 2011; **6**: e26282
- Lambert M. Influenza vaccination recommendations for 2017–2018: updates from ACIP. *Am Fam Physician* 2017; **96**: 545–546.
- Advisory Committee on Immunization P, Smith NM, Bresee JS et al. Prevention and control of influenza: recommendations of the Advisory Committee on Immunization Practices (ACIP). *MMWR Recomm Rep* 2006; **55**: 1–42.
- Young B, Sadarangani S, Jiang L, Wilder-Smith A, Chen MIC. Duration of influenza vaccine effectiveness: a systematic review, meta-analysis, and meta-regression of test-negative design case-control studies. *J Infect Dis* 2017; **217**: 731–741.
- Kissling E, Nunes B, Robertson C et al. I-MOVE multicentre case-control study 2010/11 to 2014/15: Is there within-season waning of influenza type/subtype vaccine effectiveness with increasing time since vaccination? *Euro Surveill* 2016; **21**. <https://doi.org/10.2807/1560-7917.ES.2016.21.16.30201>
- Flu & Young Children. Center of Disease Control and Prevention. Available from: <https://www.cdc.gov/flu/highrisk/children.htm>
- Tam YH, Valkenburg SA, Perera R et al. Immune responses to twice-annual influenza vaccination in older adults in Hong Kong. *Clin Infect Dis* 2018; **66**: 904–912.
- Young B, Sadarangani S, Yew HS et al. The immune response to 6-monthly versus annual standard dose inactivated trivalent influenza vaccination in older people: study protocol for a randomised clinical trial. *Trials* 2017; **18**: 67.
- Xie H, Wan XF, Ye Z et al. H3N2 Mismatch of 2014–15 northern hemisphere influenza vaccines and head-to-head comparison between human and ferret antisera derived antigenic maps. *Sci Rep* 2015; **5**: 15279.
- Young B, Sadarangani S, Haur SY et al. Semiannual versus annual influenza vaccination in older adults in the tropics: an observer-blind, active-comparator-controlled. Randomized Superiority Trial. *Clin Infect Dis* 2019; **69**: 121–129.
- Ramsay LC, Buchan SA, Stirling RG et al. The impact of repeated vaccination on influenza vaccine effectiveness: a systematic review and meta-analysis. *BMC Med* 2019; **17**: 9.
- Belongia EA, Skowronski DM, McLean HQ, Chambers C, Sundaram ME, De Serres G. Repeated annual influenza vaccination and vaccine effectiveness: review of evidence. *Expert Rev Vaccines* 2017; **16**: 1–14.
- Smith DJ, Forrest S, Ackley DH, Perelson AS. Variable efficacy of repeated annual influenza vaccination. *Proc Natl Acad Sci USA* 1999; **96**: 14001–14006.
- Kwong JC, Chung H, Jung JK et al. The impact of repeated vaccination using 10-year vaccination history on protection against influenza in older adults: a test-negative design study across the 2010/11 to 2015/16 influenza seasons in Ontario, Canada. *Euro Surveill* 2020; **25**: e1900245.
- Skowronski DM, Chambers C, De Serres G et al. Serial vaccination and the antigenic distance hypothesis: effects on influenza vaccine effectiveness during A (H3N2) epidemics in Canada, 2010–2011 to 2014–2015. *J Infect Dis* 2017; **215**: 1059–1099.
- Trieu M-C, Zhou F, Lartey S et al. Long-term maintenance of the influenza-specific cross-reactive memory CD4⁺ T-cell responses following repeated annual influenza vaccination. *J Infect Dis* 2017; **215**: 740–749.
- Music N, Tzeng WP, Liaini Gross F et al. Repeated vaccination against matched H3N2 influenza virus gives less protection than single vaccination in ferrets. *NPJ Vaccines* 2019; **4**: 28.
- Zost SJ, Parkhouse K, Gumina ME et al. Contemporary H3N2 influenza viruses have a glycosylation site that alters binding of antibodies elicited by egg-adapted vaccine strains. *Proc Natl Acad Sci USA* 2017; **114**: 12578–12583.

21. Bhatt S, Holmes EC, Pybus OG. The genomic rate of molecular adaptation of the human influenza A virus. *Mol Biol Evol* 2011; **28**: 2443–2451.
22. Ng S, Nachbagauer R, Balmaseda A *et al.* Novel correlates of protection against pandemic H1N1 influenza A virus infection. *Nat Med* 2019; **25**: 962–967.
23. Valkenburg SA, Mallajosyula VV, Li OT *et al.* Stalking influenza by vaccination with pre-fusion headless HA mini-stem. *Sci Rep* 2016; **6**: 22666.
24. Beyer WE, de Bruijn IA, Palache AM, Westendorp RG, Osterhaus AD. Protection against influenza after annually repeated vaccination: a meta-analysis of serologic and field studies. *Arch Intern Med* 1999; **159**: 182–188.
25. Linderman SL, Ellebedy AH, Davis C *et al.* Influenza immunization in the context of preexisting immunity. *Cold Spring Harbor Perspect Med* 2020; a040964.
26. Allen JD, Ross TM. H3N2 influenza viruses in humans: Viral mechanisms, evolution, and evaluation. *Hum Vaccin Immunother* 2018; **14**: 1840–1847.
27. Gouma S, Kim K, Weirick ME *et al.* Middle-aged individuals may be in a perpetual state of H3N2 influenza virus susceptibility. *Nat Commun* 2020; **11**: 4566.
28. Hoa LNM, Sullivan SG, Mai LQ *et al.* Influenza A(H1N1) pdm09 but not A(H3N2) virus infection induces durable seroprotection: results from the Ha Nam cohort. *J Infect Dis* 2020: jiaa293. Online ahead of print.
29. Cowling BJ, Thompson MG, Ng TWY *et al.* Comparative Reactogenicity of enhanced influenza vaccines in older adults. *J Infect Dis* 2020; **222**: 1383–1391.
30. Kavian N, Hachim A, Li AP *et al.* Assessment of enhanced influenza vaccination finds that FluAd conveys an advantage in mice and older adults. *Clin Transl Immunology* 2020; **9**: e1107.
31. Mallajosyula VV, Citron M, Ferrara F *et al.* Hemagglutinin sequence conservation guided stem immunogen design from influenza A H3 subtype. *Front Immunol* 2015; **6**: 329.
32. Valkenburg SA, Li OTW, Li A *et al.* Protection by universal influenza vaccine is mediated by memory CD4 T cells. *Vaccine* 2018; **36**: 4198–4206.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.



This is an open access article under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.