

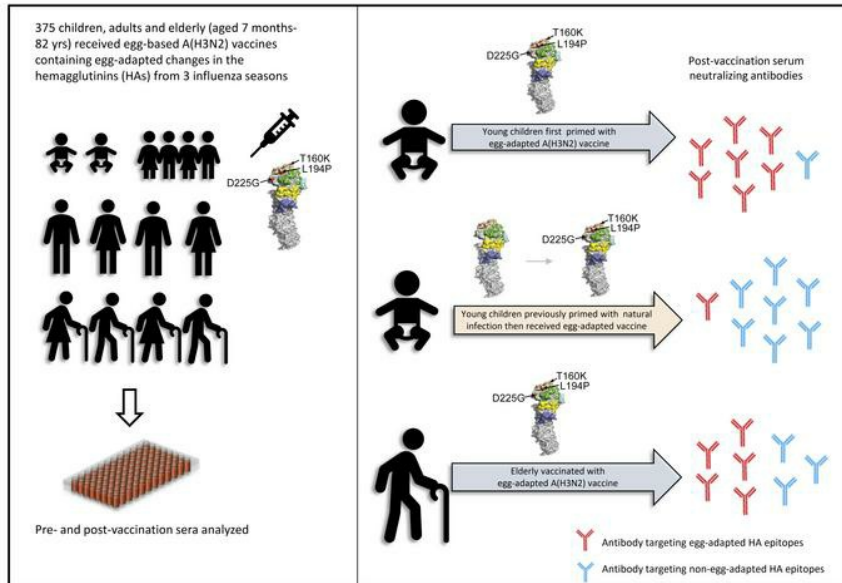
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Graphical abstract



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Age-specific effects of vaccine egg adaptation and immune priming on A(H3N2) antibody responses following influenza vaccination

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A(H3N2) influenza vaccine effectiveness (VE) was low during the 2016–19 seasons and varied by age. We analyzed neutralizing antibody responses to egg- and cell-propagated A(H3N2) vaccine and circulating viruses following vaccination in 375 individuals (aged 7 months to 82 years) across all vaccine-eligible age groups in 3 influenza seasons. Antibody responses to cell- versus egg-propagated vaccine viruses were significantly reduced due to the egg-adapted changes T160K, D225G, and L194P in the vaccine hemagglutinins. Vaccine egg adaptation had a differential impact on antibody responses across the different age groups. Immunologically naive children immunized with egg-adapted vaccines mostly mounted antibodies targeting egg-adapted epitopes, whereas those previously primed with infection produced broader responses even when vaccinated with egg-based vaccines. In the elderly, repeated boosts of vaccine egg-adapted epitopes significantly reduced antibody responses to the WT cell-grown viruses. Analysis with reverse genetic viruses suggested that the response to each egg-adapted substitution varied by age. No differences in antibody responses were observed between male and female vaccinees. Here, the combination of age-specific responses to vaccine egg-adapted substitutions, diverse host immune priming histories, and virus antigenic drift affected antibody responses following vaccination and may have led to the low and variable VE against A(H3N2) viruses across different age groups.

Introduction

Influenza viruses continue to cause high morbidity and mortality annually. Amid the current pandemic caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) viruses and influenza/SARS-CoV-2 cocirculation, influenza vaccination has become especially important. Vaccination is the most effective public health measure to combat influenza, however, the constant genetic and antigenic drift of influenza viruses requires annual updates of seasonal influenza vaccine components. In the United States, evaluation of seasonal influenza vaccine effectiveness (VE) based on the “test-negative” design has been conducted annually since the 2004–05 influenza season (1). Decreased VE can occur when predominantly circulating viruses have antigenically drifted from the vaccine viruses, such as the low VE reported for A(H3N2) during the 2014–15 season (2). Currently, most licensed influenza vaccines are still produced in chicken eggs, which can introduce substitutions in the hemagglutinins (HAs) of the viruses as a result of egg adaptation. Egg-adapted substitutions occurred in multiple HA epitopes of recent egg-based A(H3N2) vaccines. These included substitutions from threonine (T) to lysine (K) at HA amino acid position 160 (T160K), and from leucine (L) to proline (P) at position 194 (L194P) at antigenic site B, which can alter the antigenicity of these vaccines (2–8). Levine et al. reported that during the 2017–

18 influenza season, adult serum antibody titers against circulating viruses, but not egg-adapted A(H3N2) vaccines, correlated with protection against influenza infections (9). Thus, egg-adapted changes in HA are thought to be another form of “antigenic mismatch” between vaccine virus and circulating strains (6).

In recent years, it has also become apparent that, even within the same influenza season for the same subtype of viruses, VE can still vary greatly among different age groups. This is likely due to the complex exposure history to influenza infection or vaccination in humans, including initial childhood immune priming (10–15). Immune priming can play significant roles in shaping an individual’s antibody responses to newer influenza viruses later in life and affect vaccine responses (11–14). Birth cohort effects on age-specific VE have been reported for both A(H1N1)pdm09 and A(H3N2) (13, 16, 17). Additional factors or a compounding effect of multiple factors may also contribute to the differences in observed VE. For example, compared with the 2016–17 influenza season, in 2017–18, even though the A(H3N2) vaccine remained unchanged and there was no clear antigenic drift of circulating A(H3N2) viruses, a higher hospitalization rate due to A(H3N2) infections was reported, and a reduced VE was observed in elderly groups, but not in very young children (4, 18). Further studies are needed to fully understand the age-related differences in VE in order to design effective vaccination strategies for different age groups, especially for those who are at higher risk of influenza illness.

In the 2016–17 to 2018–19 influenza seasons, A(H3N2) vaccine viruses were antigenically similar, but the reported VE varied between seasons and across different age groups (4, 7, 19). This offered an opportunity to investigate the underlying immune

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Table 1. Characteristics of age cohorts of participants from 3 influenza seasons in the study

	Age groups ^a	n	Birth years	Median age	Sex		Egg-based A(H3N2) vaccine strain in QIV
					Male n (%)	Female n (%)	
2016–17	<3 yr	23	2014–15	22 mo	8 (35%)	15 (65%)	A/Hong Kong/4801/2014-like virus
	18–49 yr	20	1969–97	34 yr	7 (35%)	13 (65%)	
	50–64 yr	23	1953–66	58 yr	8 (35%)	15 (65%)	
	≥65 yr	24	1938–51	67 yr	11 (46%)	13 (54%)	
2017–18	<3 yr	21	2015–17	16 mo	10 (48%)	11 (52%)	A/Hong Kong/4801/2014-like virus
	3–8 yr	30	2010–14	6 yr	N.A	N.A	
	9–17 yr	33	2000–2007	14 yr	12 (36%)	21 (64%)	
	18–49 yr	26	1968–99	26 yr	11 (42%)	15 (58%)	
	50–64 yr	21	1954–66	57 yr	8 (38%)	13 (62%)	
	≥65 yr	21	1932–52	68 yr	6 (29%)	15 (71%)	
2018–19	<3 yr	22	2015–17	24 mo	14 (64%)	8 (36%)	A/Singapore/INFIMH-16-0019/2016-like virus
	3–8 yr	24	2010–13	7 yr	N.A	N.A	
	9–17 yr	22	2002–9	12 yr	16 (73%)	6 (27%)	
	18–49 yr	21	1971–2000	25 yr	10 (48%)	11 (52%)	
	50–64 yr	22	1953–68	57 yr	7 (32%)	15 (68%)	
	≥65 yr	22	1936–53	70 yr	9 (41%)	13 (59%)	
Total	7 mo–82 yr	375	1936–2017	20 yr	137 (43%) ^b	184 (57%) ^b	

^aAge at the time of the enrollment. ^b3–8 years was not included in proportion, as the sex information was unknown.

mechanism that may have contributed to the difference in VE. Here, we analyzed pre- and post-vaccination sera collected over 3 influenza seasons from a wide age range (from 7 months to 82 years) of cohorts who received quadrivalent egg-based, inactivated (QIV) vaccines. We compared age-related preexisting immunity and its impact on vaccine-induced antibody responses across 6 age groups. We also constructed reverse genetic (RG) viruses that had individual egg-adapted substitutions to explore the immunodominance of HA epitopes that could shape the antibody responses among different age groups.

Results

Egg adaptation in A(H3N2) vaccines in the 2016–19 influenza seasons resulted in lower neutralizing antibody responses to WT cell-grown vaccine viruses across all age groups. We analyzed sera collected from a total of 375 individuals ranging in age from 7 months to 82 years in the 2016–17, 2017–18, and 2018–19 influenza seasons for their neutralizing antibody responses to A(H3N2) viruses before and after influenza vaccination (Table 1). Participants from the 2016–17 and 2017–2018 influenza seasons received vaccines containing A/Hong Kong/4801/2014-like virus (HK/14, 3C.2a), and participants from the 2018–19 season received an updated A(H3N2) vaccine containing A/Singapore/INFIMH-16-0019/2016-like virus (Singapore/16, 3C.2a1). We were able to collect sera from individuals in all age groups who were eligible to receive influenza vaccines, including children (<3 years old, 3–8 years old, and 9–17 years old), adults (18–49 years old), older adults (50–64 years old), and elderly individuals (≥65 years old). Here, we used the same age groupings as those used for most seasonal influenza vaccine licensure and VE estimates (2, 3, 7, 20). The participants' birth years ranged from 1932 to 2017; the birth years of the 2 oldest cohorts (older adults and elderly) predated the emergence of the A(H3N2) in 1968 and thus overlapped with periods when seasonal A(H1N1) and A(H2N2) circulated (Figure 1).

Upon immunization with an egg-based QIV, most participants mounted robust neutralizing antibody responses to the egg-propagated A(H3N2) vaccine viruses, however, antibody titers against their cell-propagated counterparts that represented WT circulating viruses were significantly lower ($P < 0.05$) across all 3 seasons in all age groups (Figure 2). In addition, pre-vaccination geometric mean titers (GMTs) against the cell-propagated WT vaccine virus were also significantly lower ($P < 0.05$) than those against the corresponding egg-propagated vaccine viruses in all age groups except among the very young children, who were under 3 years of age (Figure 2). The fold rise in post-vaccination microneutralization (MN) antibody titers against egg-adapted A(H3N2) vaccine virus was also higher than the MN antibody titers against their cell-propagated counterparts in most age groups ($P < 0.05$, Supplemental Figure 1; supplemental material available online with this article; <https://doi.org/10.1172/JCI146138DS1>). When analyzed on the basis of sex, we found no significant difference ($P > 0.05$) between male and female participants with regard to their pre- and post-vaccination MN GMTs against either egg- or cell-propagated A(H3N2) vaccine viruses in all age groups across all 3 seasons (Supplemental Figures 2–4).

We then examined the difference in HA sequences between egg- and cell-propagated A(H3N2) vaccine viruses for these 3 seasons. Multiple egg-adapted substitutions occurred in each egg-propagated A(H3N2) vaccine virus. Among those, T160K and L194P substitutions were in both egg-propagated HK/14 and Singapore/16 viruses (Figure 3 and Table 2). The T160K egg-adapted change resulted in a loss of a glycosylation site at HA position 158, leading to the exposure of additional epitopes. Furthermore, additional egg-adapted substitutions were introduced in the high-growth reassortant candidate vaccine viruses (CVVs) for vaccine production. Here, the 2 CVVs used in QIVs (X-263B for HK/14 and NIB-104 for Singapore/16) had an additional egg-adapted

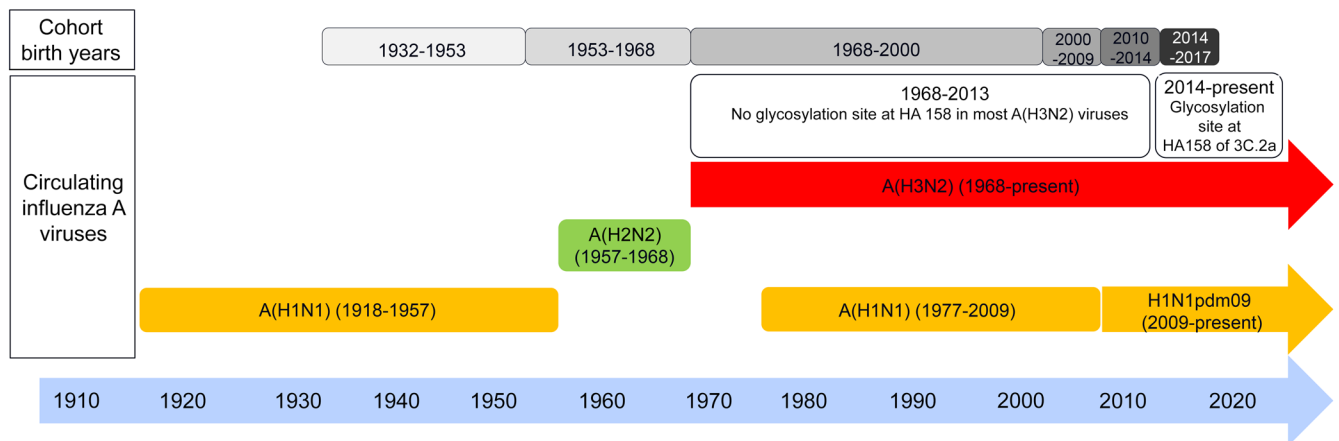


Figure 1. Diagram of cohort birth years and circulation of seasonal influenza A viruses.

substitution from aspartic acid (D) to glycine (G) at position 225 (D225G), near the receptor binding site. The 2018–19 season egg-propagated vaccine virus Singapore/16 used in the current study had a D/G mixture at position 225, and further mutated to a complete D225G substitution in the egg-propagated CVV NIB-104 (Figure 3). The combinations of these egg-adapted changes on the HA resulted in the reduced antibody responses to cell-propagated vaccine viruses.

Differential antibody responses to vaccination in young children (<3 years old) primed with natural infection versus those primed with egg-adapted A(H3N2) vaccines. The youngest pediatric group (<3 years old) provided an opportunity to investigate the impact of egg adaption on immune priming and the subsequent response to vaccination. Here, we specified that children younger than 3 years of age who provided sera in this age group had not received prior influenza vaccinations and did not have laboratory-confirmed influenza through reporting by a parent or guardian. We were able to further stratify these young children into 2 groups on the basis of their pre-vaccination MN titers in each season: (a) “unprimed” children were those with no preexisting MN titers (<40 against both egg- and cell-propagated vaccine viruses) and immunologically naive, thus the vaccination would be their first exposure to influenza antigens (first immune priming in life); and (b) “primed” children were those who had pre-vaccination MN titers (≥ 40 against cell-propagated WT vaccine viruses) suggesting prior exposure to a probable asymptomatic influenza natural infection (Figure 4). Of note, since a few young children had pre-existing egg/cell titer ratios higher than 4, we could not rule out the possibility that they may have received prior vaccination, but this was not reported by their parent or guardian. Therefore, they were excluded from the “primed” group for this analysis.

In the unprimed children, vaccine induced antibody responses mostly to egg-propagated vaccine viruses (GMT: 99–494 across 3 seasons, $n = 34$), with little responses to cell-propagated WT vaccine viruses (GMT: 12–32, 8- to 28-fold reductions compared with their egg-propagated counterparts; Figure 4, A, C, and E), suggesting that the majority of their antibody responses were targeting the egg-adapted epitopes absent on the WT viruses. In contrast, following vaccination with the same egg-based QIV, children who

were probably first primed by natural infection were able to mount similar MN antibody titers against egg- and cell-propagated WT vaccine viruses ($P > 0.05$), and titers for both of these vaccine viruses were significantly higher than those in the unprimed children ($P < 0.01$, Figure 4, B, D, and F). These primed children had a post-vaccination GMT of 1076 or higher against egg-propagated vaccine viruses and a GMT of 640 or higher against cell-propagated WT vaccine viruses across all 3 seasons (<3 years old, group B, Table 3). These data suggest that priming with natural infection induced antibodies targeting HA epitopes presented on both egg- and cell-propagated WT vaccine viruses, whereas priming with egg-adapted A(H3N2) vaccines in the 2016–19 seasons mostly induced antibodies focused on egg-adapted epitopes that were absent on the cell-propagated WT viruses.

Preexisting immunity to egg- versus cell-propagated A(H3N2) vaccine viruses impacts vaccine responses. Participants from each age group were then stratified by their pre-vaccination neutralizing antibody titers against egg- versus cell-A(H3N2) vaccine viruses to define their preexisting immunity: (a) those with no preexisting MN titers (group A, <40 against both egg and cell vaccine viruses, Table 3); (b) those with pre-vaccination MN titers of 40 or higher against egg virus and an egg/cell titer ratio below 4 (group B, with dominant preexisting MN antibodies targeting non-egg-adapted epitopes); and (c) those with pre-vaccination MN titers of 40 or higher against egg vaccine virus and an egg/cell ratio of 4 or higher (group C, with dominant preexisting MN antibodies targeting egg-adapted epitopes; Supplemental Figures 5–7). In all age groups of children (<3, 3–8, and 9–17 years of age), following vaccination, those without preexisting MN titers (group A) tended to mount lower antibody responses to egg- and/or cell-propagated WT vaccine viruses than did those with preexisting MN titers (groups B and C, Table 3). However, this trend was less pronounced in the adult and elderly groups (18–49, 50–64, and ≥ 65 years of age). Children without preexisting MN titers more likely mounted de novo responses following vaccination, whereas in adults, even in those without preexisting MN titers, vaccination probably boosted preexisting memory B cells rather than causing de novo responses. Across all age groups in the 3 seasons, individuals with either no preexisting MN titers (group A), or those with preexisting MN antibodies most-

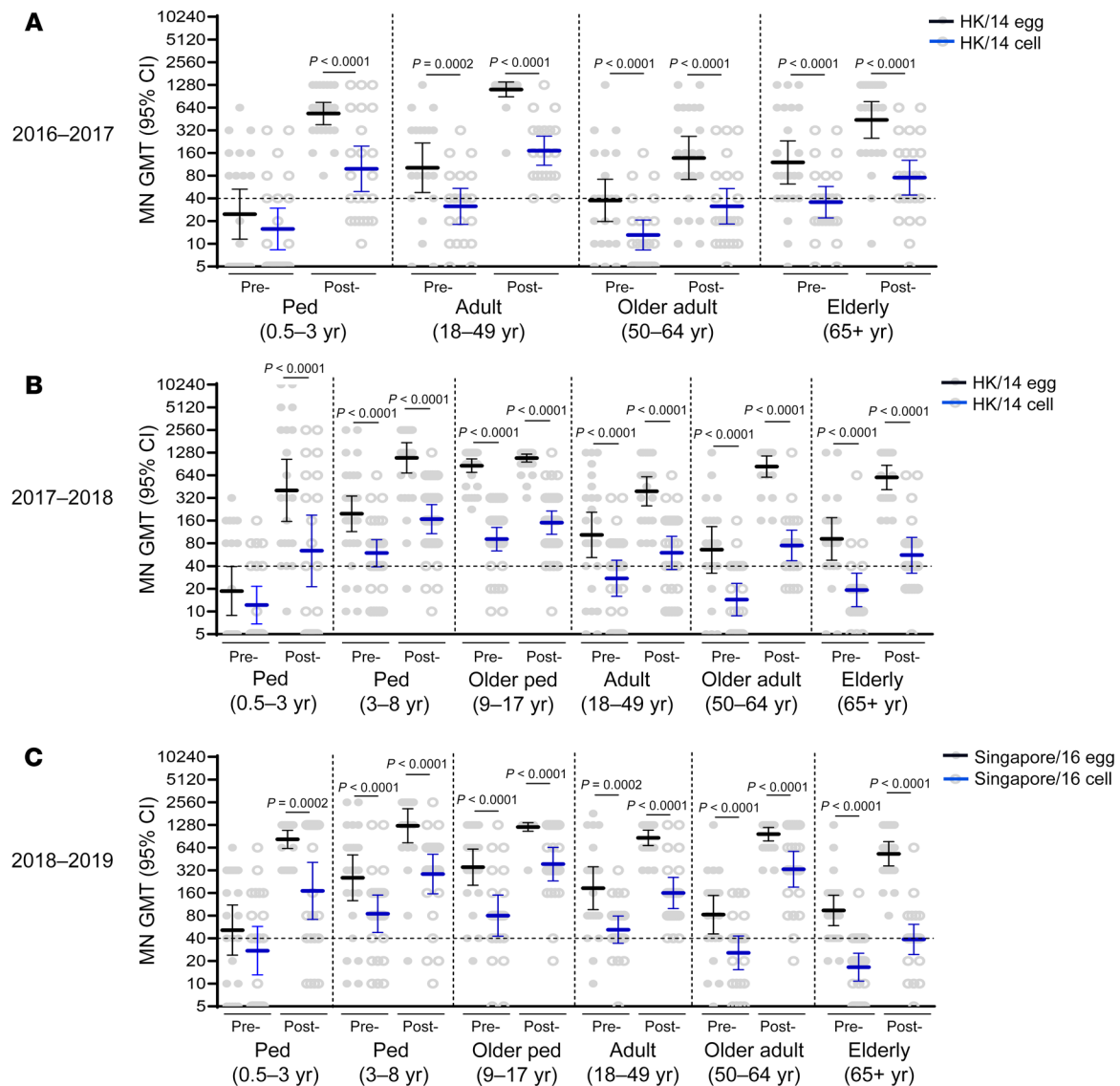


Figure 2. Neutralizing antibody responses to egg- versus cell-propagated A(H3N2) WT vaccine viruses in individuals from 6 age groups who received egg-based QIV in 2016–17, 2017–18, and 2018–19 influenza seasons. (A) 2016–17 season, (B) 2017–18 season, (C) 2018–19 season. MN antibody titers for each individual are shown in the y axis. Bars represent the geometric mean titers with a 95% CI. Dashed line denotes a MN titer of 40. Pre- and post-vaccination MN GMTs between egg-propagated A(H3N2) vaccine virus (solid circles) and cell-propagated A(H3N2) WT vaccine virus (open circles) in each season were compared for each age cohort by a Wilcoxon matched-pairs, signed-rank test. *P* values are indicated where there was statistical significance ($P < 0.05$). Ped, pediatric group.

ly targeting egg-adapted epitopes (group C) had the highest egg/cell titer ratios following vaccination (bolded ratio for each group in Table 3), suggesting that most of the vaccine responses targeted egg-adapted epitopes in these individuals. Collectively, these results indicated that preexisting immunity to seasonal A(H3N2) vaccine virus varied across different age groups and can affect the vaccine responses targeting egg-adapted epitopes.

A(H3N2) vaccine egg adaptation differentially affected age cohorts across 3 seasons, with the highest impact on the elderly group. Next, we used the egg/cell ratio of post-vaccination MN antibody titers against the vaccine virus as a proxy to quantify the effect of vaccine egg adaptation on antibody responses in each age group. With the exception of the very young children (<3 years old), many individ-

uals aged 3 years or older had significantly higher pre-vaccination MN titers against egg-propagated A(H3N2) viruses than cell-propagated A(H3N2) viruses (Figure 2), suggesting antibody targeting HA egg-adapted sites preexisted. Moreover, when HK/14 was the A(H3N2) vaccine strain for 2 consecutive seasons, the egg/cell ratio of post-vaccination MN antibody titers increased from the 2016–17 season to the 2017–18 season in several age groups (Figure 5). Most notably, in older adults (50–64 years old) and elderly individuals (≥ 65 years old), the egg/cell titer ratios in the 2017–18 season were significantly higher ($P < 0.05$) than those in the 2016–17 season. In the 2017–18 season, the average egg/cell titer ratios in older adults and elderly individuals were also higher, although not statistically significant, than those in the younger age groups. In the 2018–19

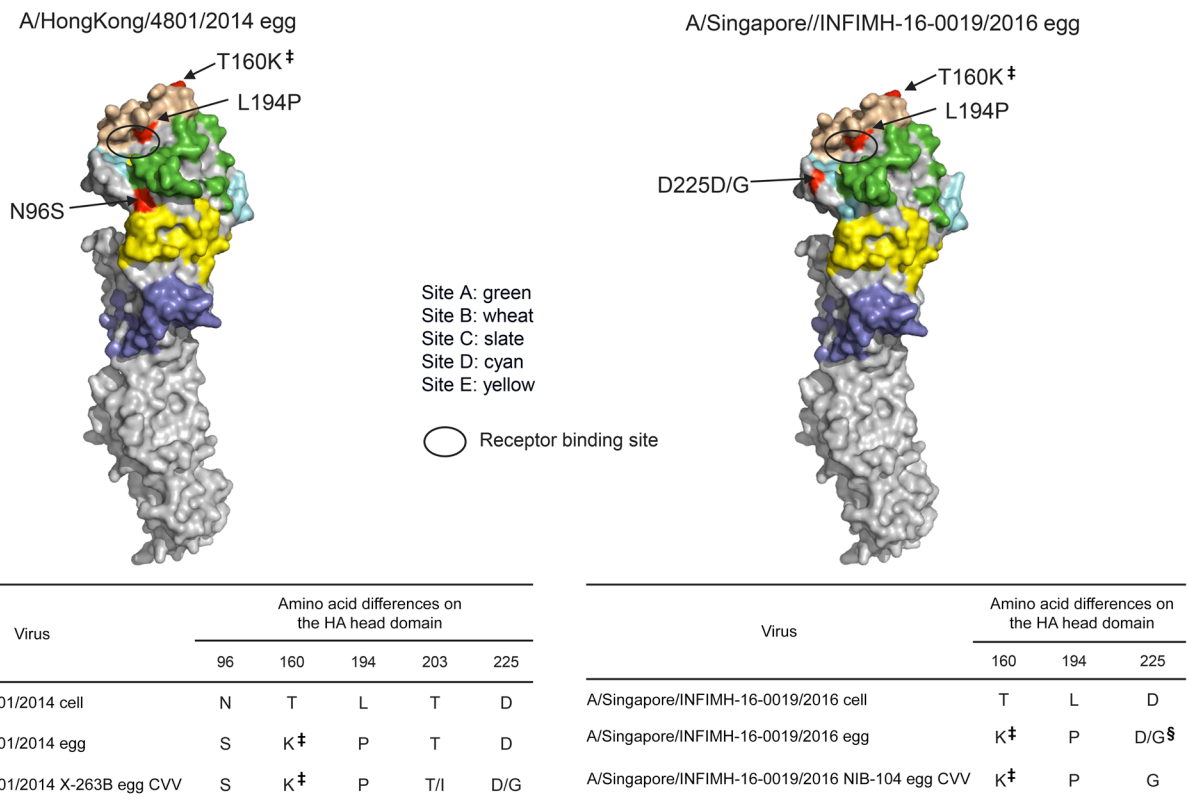


Figure 3. 3D structures of the HA monomer and the egg-adapted substitutions in HK/14 and Singapore/16 A(H3N2) egg-propagated vaccine viruses. HA modeling was based on the A/Victoria/361/2011 A(H3N2) HA structure (Protein Data Bank [PDB] code 4WE8). Five conventional HA antigenic sites are color coded, with the receptor-binding site indicated by an oval outline. CVVs listed were the A(H3N2) components formulated in the inactivated QIVs the study participants received. [‡]Loss of glycosylation. [§]Mix, 78.05% D, 21.95% G.

season, when the A(H3N2) vaccine strain was updated to the Singapore/16 strain, the elderly group (≥65 years old) continued to have significantly higher egg/cell titer ratios when compared with those of all other age groups ($P < 0.05$, Figure 5).

The preferential imprinting that targeted HA egg-adapted epitopes in young children (<3 years old) following egg-based vaccination (Figure 4) prompted us to further examine the sequences of historic A(H3N2) viruses that individuals may have been exposed to earlier in their life. We analyzed the HA sequences of representative seasonal A(H3N2) strains that circulated between 1968 and 2019 (Table 2). Most historical A(H3N2) viruses that circulated prior to 2014 lacked a glycosylation motif at 158–160 of HA, and therefore not glycosylated, most bore 160K, which is the same as the HA egg-adapted substitution in both HK/14 and Singapore/16 egg-based vaccines (Figure 3). It is therefore likely that early life exposure to A(H3N2) viruses could have imprinted memory B cells targeting the unglycosylated HA 158–160 motif in older-aged populations, which was then repeatedly boosted when these individuals were vaccinated with egg-based A(H3N2) vaccines bearing the same motif. For individuals born before 2014, it is very likely that they had A(H3N2) priming by a strain carrying the unglycosylated HA 158–160 motif (Table 2 and Figure 1).

Low neutralizing antibody responses to cell-propagated WT A(H3N2) vaccine virus in the 2018–19 season were mostly related to vaccine egg-adapted substitutions on the HA 160 and 225 sites. Most

individuals in the 2018–19 season mounted significantly lower (≥4-fold reduction) post-vaccination neutralizing antibody titers against cell- versus egg-propagated Singapore/16 viruses (low responders), with proportions ranging from 55% in young children (<3 years old) to 100% in the elderly group (≥65 years old, Table 4). The reduction of antibody titers against cell-propagated viruses was likely due to the combination of 3 egg-adapted changes in the HA head, T160K, L194P, and D225G, of the Singapore/16 vaccine virus. To determine which substitution was responsible for the difference in antibody responses between egg- versus cell-propagated WT vaccine viruses in the 2018–19 season, we generated 3 RG viruses bearing a single egg-adapted substitution (T160K, L194P, or D225G) in the background of the cell-propagated Singapore/16 vaccine virus. Among those, the L194P RG virus failed to propagate, probably because of poor viral fitness.

The MN assays used in the current study mainly detect HA head-specific antibodies. In 2018–19, post-vaccination MN GMT titers against cell-propagated Singapore/16 were significantly lower than the MN GMTs against RG viruses carrying a single egg-adapted substitution of either T160K or D225G in all the age groups tested (Supplemental Figure 8). Among the low responders to cell-propagated viruses (≥4-fold reduction in titers by the Singapore/16 egg/cell ratio, Table 4, column A), a single substitution of either T160K or D225G in RG viruses was able to recover antibody titers to levels similar to those seen with egg-propagated Singapore/16 (with-

Table 2. Potential imprinting sites on the HA head domain associated with egg-adapted changes in the 2016–19 seasonal influenza A(H3N2) vaccine strains

H3N2 viruses	Passage	Genbank or GISAID accession nos.	Amino acid position on HA head domain ^A					RBS
			D 96	B 158	B 159	B 160	B 194	
A/Aichi/2/1968 ^B	Egg	CY121117	N	G	S	T	L	G
A/England/42/1972	Egg	CY009356	N	G	S	T	L	G
A/Port Chalmers/1/1973	Egg	CY009348	N	G	S	A	L	G
A/Victoria/03/1975	Egg	V01098	N	G	S	T	L	G
A/Texas/1/1977	Egg	AF450246	N	E	S	T	L	G
A/Bangkok/1/1979	Egg	J02092	N	E	S	K	L	G
A/Philippines/2/1982	Egg	U08858	N	E	S	K	L	G
A/Mississippi/1/1985	Egg	AF008893	N	E	S	K	L	G
A/Leningrad/360/1986	Egg	DQ508849	N	E	Y	K	L	G
A/Shanghai/11/1987	Egg	AF008886	N	E	Y	K	L	G
A/Sichuan/2/1987	Egg	AF008884	N	E	Y	K	L	G
A/Shanghai/16/1989	Egg	AF008668	N	E	H	K	L	G
A/Beijing/353/1989	Egg	U97740	N	E	Y	K	L	G
A/Beijing/32/1992	Egg	U26830	N	E	Y	K	L	G
A/Shandong/9/1993	Egg	AF008820	N	E	Y	K	L	G
A/Johannesburg/33/1994	Egg	AF008774	N	E	Y	K	L	G
A/Wuhan/359/1995	Egg	AF008722	N	E	Y	K	I	G
A/Nanchang/933/1995	Egg	AF008725	N	E	Y	K	L	G
A/Sydney/05/1997	Egg	AJ311466	N	K	Y	K	I	G
A/Panama/2007/1999	Egg	DQ508865	N	K	Y	K	I	G
A/Fujian/411/2002	Cell	CY112933	N	K	Y	K	L	D
A/Wyoming/03/2003	Egg	DQ865946	N	K	Y	K	L	D
A/California/07/2004	Egg	EU103820	N	K	F	K	L	D
A/Wisconsin/67/2005	Egg	CY034116	N	K	F	K	L	N
A/Brisbane/10/2007	Egg	EU199366	N	K	F	K	L/P	N
A/Perth/16/2009	Egg	GQ293081	N	N	F	K	L	N
A/Victoria/361/2011	Egg	KJ942680	N	N	F	K	L	N
A/Texas/50/2012	Egg	KC892952	N	N	F	K	L	N
A/Switzerland/9715293/2013	Cell	EPI814528	N	N	S	K	L	D
A/Switzerland/9715293/2013	Egg	EPI540526	N	N	S	K	L	D
A/Hong Kong/4801/2014	Cell	EPI653201	N	N^C	Y	I	L	D
A/Hong Kong/4801/2014^D	Egg	EPI578430	S	N	Y	K	P	D
A/Singapore/INFIMH-16-0019/2016	Cell	EPI1106235	N	N^C	Y	I	L	D
A/Singapore/INFIMH-16-0019/2016^D	Egg	EPI1047604	N	N	Y	K	P	D/G
A/Kansas/14/2017	Cell	EPI1653968	N	N	S	K	L	D

^AB and D denote the H3 HA1 antigenic sites B and D, respectively; RBS, receptor-binding site. ^BThe A/Aichi/2/1968 virus was used as a reference for HA amino acid alignment. ^CAddition of glycosylation motif at position 158–160. ^DEgg-propagated A(H3N2) vaccine-like viruses used in this study, amino acids in red indicate egg-adapted mutations.

in 2-fold of titers against the egg-propagated Singapore/16 virus) in only half (58%) of the young children (<3 years old), but in most (95%) of the older children aged 9–17 years (Table 4, column B). In contrast, in adults, older adults, and elderly individuals, the T160K substitution alone could recover antibody titers similar to Singapore/16 egg virus titers in 82%–100% of the low responders, whereas the D225G substitution could only recover titers in 53%–77% of the low responders in the same age groups (Table 4, column B). Moreover, in smaller proportions of low responders, both T160K and D225G substitutions could not recover antibody titers to levels similar to those of the Singapore/16 egg virus (Table 4), suggesting that these individuals may have mounted antibodies targeting the

third egg-adapted substitution: L194P (Table 4 and Figure 3). Taken together, these data indicated that the impact of these egg-adapted substitutions (T160K, D225G, and L194P) varied by age.

Focused antibody response to HA egg-adapted epitopes, antigenic drift, and low A(H3N2) VE in the 2016–19 seasons. The estimated VE against A(H3N2) viruses was low during the 2016–17, 2017–18, and 2018–19 seasons when similar egg-adapted changes occurred in the vaccines. Further breakdown of the VE by age groups also indicated variability by age (refs. 3, 7 and Supplemental Table 1). HK/14 (3C.2a) was the vaccine virus for both 2016–17 and 2017–18 seasons, with no apparent antigenic drift in the circulating viruses, but focused antibody responses to HA egg-adapted epitopes

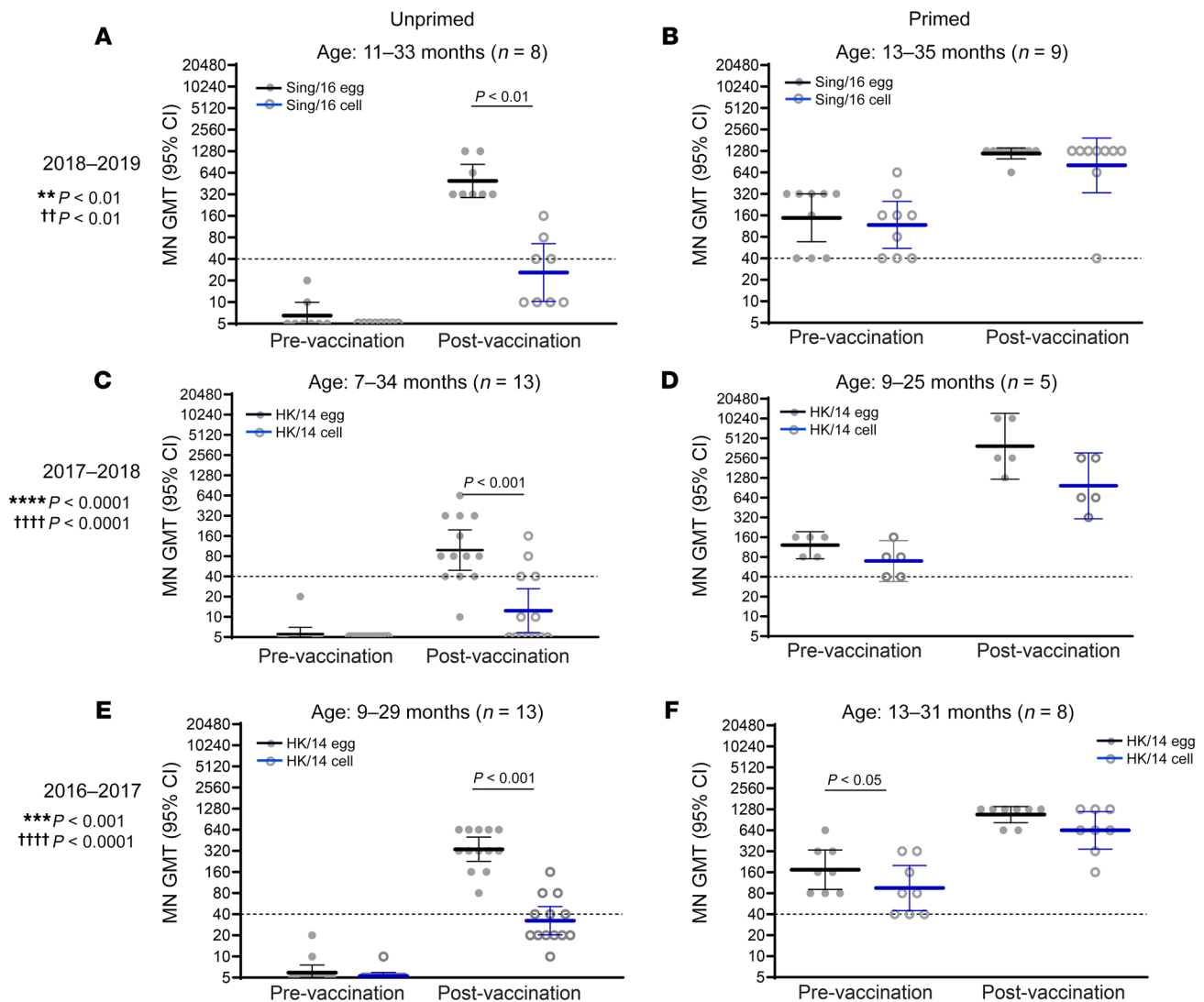


Figure 4. Neutralizing antibody responses to egg- versus cell-propagated A(H3N2) WT vaccine viruses among primed and unprimed young children (<3 years old) in 3 influenza seasons (2016–19). Young children (<3 years old) in each season were grouped according to the following pre-vaccination MN titers: “unprimed” (MN <40 against both egg- and cell-propagated A(H3N2) WT vaccine viruses) versus “primed” (MN ≥40 against cell-propagated WT vaccine virus). (A and B) Antibody responses of unprimed (A) and primed (B) children in the 2018–19 season. (C and D) Antibody responses of unprimed (C) and primed (D) children in the 2017–18 season. (E and F) Antibody responses of unprimed (E) and primed (F) children in the 2016–17 season. The y axis shows MN antibody titers for each individual. Bars reflect the MN GMTs (95% CI) against egg-propagated (solid circles) and cell-propagated (open circles) vaccine viruses. Dashed lines denote a MN titer of 40. P values are indicated where there was statistical significance ($P < 0.05$). P values shown at the top of the graphs represent a comparison of titers against egg- versus cell-propagated vaccine viruses (Wilcoxon matched-pairs, signed-rank test). P values shown on the side of the graphs are a comparison of post-vaccination MN GMTs between unprimed and primed children in each season (Mann-Whitney U unpaired t test). ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$, comparing post-vaccination GMTs with egg virus in unprimed versus primed children. †† $P < 0.01$ and †††† $P < 0.0001$, comparing post-vaccination GMTs with cell virus in unprimed versus primed children.

differed across age groups, as indicated by the egg/cell ratio that increased significantly ($P < 0.05$) between seasons in older adults and elderly individuals (Figure 5), suggesting a repeated boost of the focused antibody responses to egg-adapted epitopes.

In 2018–19, the A(H3N2) vaccine was updated to Singapore/16, a 3C.2a1 virus, however, it still bore similar egg-adapted substitutions including T160K and L194P that were likely further boosted in a third season. In addition, at the end of the 2018–19 season, the influenza A(H3N2) 3C.3a virus predominated in the US (3, 21). We therefore also analyzed Singapore/16 vaccine sera with a cell-propagated representative 3C.3a WT virus from 2018–

19. Neutralizing antibody titers against the circulating 3C.3a virus A/Kansas/14/2017 were significantly reduced ($P < 0.05$) compared with either egg- or cell-propagated Singapore/16 WT vaccine viruses in almost all age cohorts, indicating a clear antigenic drift (Figure 6) that likely further contributed to the reduced VE against A(H3N2) viruses across most age groups in the 2018–19 season (Supplemental Table 1).

Discussion

Variability in influenza vaccine-induced immunity and causality for reduced VE are likely multifactorial. In the current study, we exam-

Table 3. MN antibody responses to seasonal influenza A(H3N2) egg- and cell-propagated vaccine-like viruses by preexisting immunity in different age cohorts from the 2016–17 to 2018–19 seasons

Age groups (birth yr) and preexisting immunity conditions	2018–19 season ^a post-vac GMT				2017–18 season ^b post-vac GMT				2016–17 season ^b post-vac GMT			
	n (%)	Egg virus	Cell virus	Egg/cell ^f	n (%)	Egg virus	Cell virus	Egg/cell	n (%)	Egg virus	Cell virus	Egg/cell
<3 yr (2014–2017)												
A: Unprimed ^d	8 (36%)	494 ^f	37 ^f	13.5	13 (62%)	99 ^{f,g}	12 ^{f,g}	8	13 (56%)	338 ^f	32 ^f	10
B: Pre-vac ≥40 to egg virus and egg/cell <4 (primed)	10 (45%)	2079	1522 ^h	1.4	5 (24%)	4200	1050	4	8 (35%)	1076	640 ^h	1.7
C: Pre-vac ≥40 to egg virus and egg/cell ≥4 (primed)	4 (19%)	1076	174	6.2	3 (14%)	4064	806	5	2 (9%)	640	80	8
3–8 yr (2010–2014)												
A: Pre-vac <40 to both egg and cell virus	4 (17%)	135 ^{f,g}	20 ^{f,g}	6.8	4 (13%)	190 ^{f,g}	40 ^f	4.8	NA	NA	NA	NA
B: Pre-vac ≥40 to egg virus and egg/cell <4	7 (29%)	2319	861 ^h	2.7	9 (30%)	1280	320	4	NA	NA	NA	NA
C: Pre-vac ≥40 to egg virus and egg/cell ≥4	13 (54%)	1763	356	5	17 (57%)	1507	167	9	NA	NA	NA	NA
9–17 yr (2000–2009)^e												
A: Pre-vac <40 to both egg and cell virus	1 (5%)	453 ^{f,g}	28 ^f	16.2	0	NA	NA	NA	NA	NA	NA	NA
B: Pre-vac ≥40 to egg virus and egg/cell <4	4 (18%)	2153	761	2.8	2 (6%)	1076	453	2.4	NA	NA	NA	NA
C: Pre-vac ≥40 to egg virus and egg/cell ≥4	17 (77%)	2360	277	8.5	31 (94%)	1082	140	6.3	NA	NA	NA	NA
18–49 yr (1968–2000)												
A: Pre-vac <40 to both egg and cell virus	1 (5%)	1280	640	2	5 (20%)	160 ^{f,g}	20 ^f	11.3	4 (20%)	1280	113	11
B: Pre-vac ≥40 to egg virus and egg/cell <4	7 (33%)	1103	476 ^h	2.3	6 (24%)	570	127	4.5	7 (35%)	861	238	3.6
C: Pre-vac ≥40 to egg virus and egg/cell ≥4	13 (62%)	975	122	8	14 (56%)	1327	187	7.1	9 (45%)	1280	160	8
50–64 yr (1953–1968)												
A: Pre-vac <40 to both egg and cell virus	7 (32%)	1280	205	6.2	5 (24%)	735	30	14	5 (28%)	106 ^f	23	4.6
B: Pre-vac ≥40 to egg virus and egg/cell <4	5 (23%)	1689	970 ^h	1.9	4 (19%)	761	160	4.8	3 (17%)	640	160	4
C: Pre-vac ≥40 to egg virus and egg/cell ≥4	10 (45%)	1114	117	9.5	12 (57%)	905	67	13	10 (55%)	299	43	7
65+ yr (1932–1953)^e												
A: Pre-vac <40 to both egg and cell virus	2 (9%)	320	20	16	3 (14%)	403	50	8	2 (9%)	80 ^f	14 ^f	5.7
B: Pre-vac ≥40 to egg virus and egg/cell <4	0	NA	NA	NA	4 (19%)	453	135	3.4	8 (35%)	349	80	4.4
C: Pre-vac ≥40 to egg virus and egg/cell ≥4	20 (91%)	651	50	13	14 (67%)	1647	124	13	13 (56%)	881	116	7.6

^aH3N2 vaccine component used in 2018–19 season is Singapore/2016 egg virus. ^bH3N2 vaccine component used in 2017–18 and 2016–17 seasons is Hong Kong/2014 egg virus. ^cRatios between GMT to egg- and cell-propagated H3N2 vaccine-like virus (the highest ratio is indicated in bold). ^dS1<40 to both egg- and cell-propagated H3N2 vaccine-like viruses. ^eOne-way ANOVA corrected for multiple comparisons (Tukey's test) was used for statistical analysis except for 65-year-old+ cohort in 2018–19 season, and 9- to 17-year-old cohort in 2017–18 season, where a Mann-Whitney *U*, unpaired *t* test was used, as there was 1 group with a case number of 0. ^fPost-vaccination GMT in preexisting immunity, A versus B: $P < 0.05$ or $P < 0.01$. ^gA versus C: $P < 0.05$ or $P < 0.01$. ^hB versus C: $P < 0.05$ or $P < 0.01$.

ined the age-related impact of vaccine egg adaptation, immune priming, antigenic drift, and sex on antibody responses following A(H3N2) vaccination in 3 recent influenza seasons (2016–19). The 2 influenza vaccines used during our study period (HK/14 and Singapore/16) bear similar egg-adapted substitutions including T160K, L194P, and D225G. Egg-adapted substitutions on HA can cause altered antigenicity (6, 13, 22). Here, we provided serologic evidence of the dominant neutralizing antibody responses targeting egg-adapted substitutions in the HA of A(H3N2) egg vaccine viruses in 3 influenza seasons. Coincidentally, these egg-adapted substitutions, particularly the unglycosylated HA 158–160 motif and 225G were naturally prevalent in historic A(H3N2) viruses that circulated in the previous decades. Our analysis suggested that antibodies targeting the unglycosylated HA 158–160 motif and 225G in older individuals resulted from prior exposures and could be preferentially boosted by the recent A(H3N2) egg-based vaccines that acquired a similar motif as a result of egg adaptation.

Antibodies targeting HA epitopes imprinted in childhood can have a long-lasting effect with subsequent influenza expo-

sure over an individual's lifespan (13, 23–26). In the very young pediatric cohort (<3 years old), we observed clear evidence of the profound effect of the “first influenza exposure in life,” namely immune priming, on the antibody responses following influenza vaccination. Those who were likely first primed by natural infection with the A(H3N2) virus mounted antibody responses to epitopes that are shared with cell-propagated viruses, even when they received egg-based vaccines containing multiple egg-adapted substitutions. This may offer a partial explanation for the relatively higher VE observed among young children compared with older-aged cohorts in the 2016–19 seasons (ref. 4 and Supplemental Table 1). In contrast, when children received the egg-adapted vaccines as their first priming antigen, they largely mounted antibody responses targeting the egg-adapted epitopes that were absent on the WT viruses (Figure 4). This could in turn also direct their antibody responses toward the undesirable egg-adapted epitopes following future influenza vaccines. Our finding has important implications in the design of optimal vaccination strategies for children. As the first vaccination can be their first “immune imprinting” for

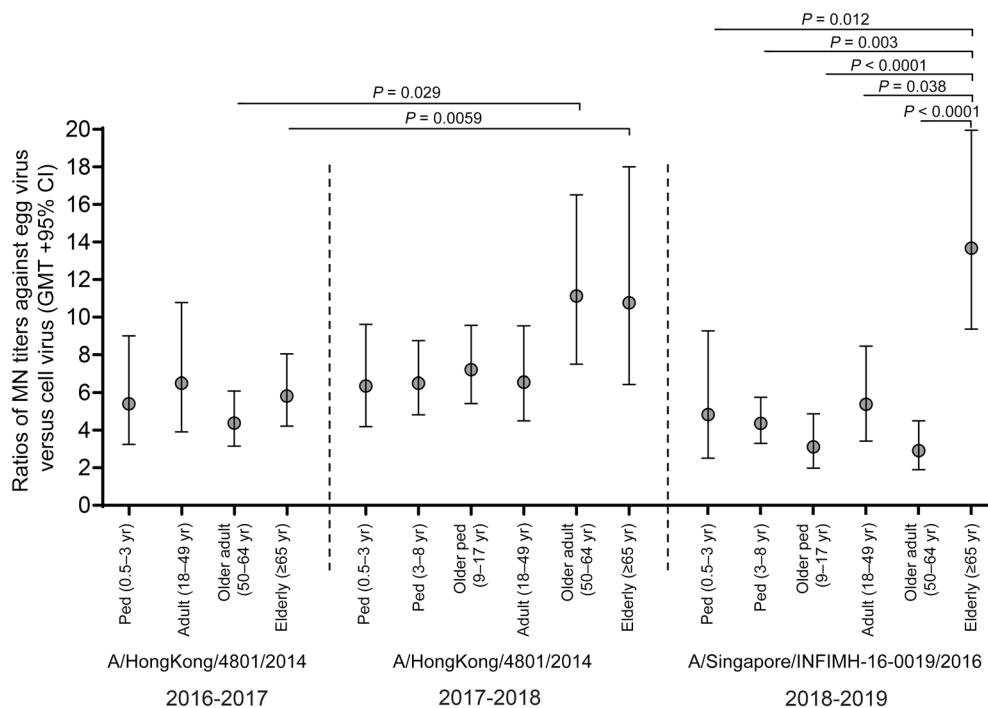


Figure 5. Fold reduction of post-vaccination MN antibody titers against cell-propagated A(H3N2) WT vaccine virus (egg/cell ratio) in the 2016–19 seasons across age cohorts. Fold reduction of post-vaccination MN antibody titers against cell-propagated vaccine virus is expressed as the ratio of egg/cell virus titers for each season. Circles represent the geometric mean fold change in titer reduction for each age cohort with a 95% CI. Comparisons of age groups in each season were analyzed by 1-way ANOVA with Tukey's multiple-comparison correction. Comparisons between the 2016–17 and 2017–18 seasons of individuals in the same-aged groups who received the same A(H3N2) egg vaccine virus were analyzed by Mann-Whitney *U* unpaired *t* test. *P* values are indicated where there was a statistically significant difference ($P < 0.05$).

influenza, it may be beneficial for young children to receive their first dose of influenza vaccine without egg-adapted mutations (such as cell or recombinant vaccines), or a vaccine that can elicit broader immune responses (such as adjuvanted vaccines). To date, some of these alternative vaccines are still not yet licensed for the youngest children (i.e., 6 months old) in the US. Optimal vaccination strategies for very young children still remain to be further investigated.

Egg adaptations can alter the antigenicity of the vaccines (27–30) and immune responses in different age groups (13, 22, 31, 32). Recently, Zost et al. reported that the HA T160K substitution in the A(H3N2) egg vaccine used in the 2016–17 season resulted in poor neutralization of WT vaccine virus–like A(H3N2) strains that carried 160T (6). Our study further demonstrated the accumulative effects of vaccines containing the same egg-adapted substitutions in multiple seasons. Across 3 consecutive seasons, we found increased egg/cell titer ratios in vaccine responses among all age groups. The elderly group (≥65 years old) had the most notable increase over time, from an egg/cell titer ratio of 5.8 in 2016–17, to 10.8 in 2017–18 and 13.7 in 2018–19 (Figure 5), suggesting repeated boosting of antibodies targeting the T160K, L194P, and D225G epitopes. In the 2016–17 and 2017–18 seasons, the post-vaccination egg/cell titer ratios in the elderly groups increased significantly (Figure 5), although the vaccine virus (HK/14) remained the same in both seasons, and there was no apparent antigenic drift of the circulating viruses. VE in this age group was 21% (95% CI: –15% to 45%) in 2016–17 but only 10% (95% CI: –32% to 38%) in 2017–18 (Supplemental Table 1), suggesting that the repeated boosting of the focused antibody responses to egg-adapted epitopes may have contributed to lower VE. Elderly individuals also had significantly higher egg/cell ratios than did other age groups ($P < 0.05$) in the 2018–19 season (Figure 5), probably because they had the highest frequencies of prior exposures to the unglycosylated 158–160

epitopes (Table 2). Collectively, these data may provide a partial explanation of the lower VE observed in the elderly group (Supplemental Table 1) and underscore the potential benefit of vaccines without egg-adapted mutations for this high-risk age population.

Our results suggested that the unglycosylated 158–160 HA motif was an immunodominant epitope in vaccine responses in the 2018–19 seasons in many individuals with preexisting immunity (Table 3, group C, and Table 4). This motif is a part of the 7 sites identified by Koel et al. (33) that can determine major antigenic changes during influenza A(H3N2) virus evolution. Our study also suggested that D225G was another major target of A(H3N2) egg-adapted substitutions that can alter vaccine responses in different age groups (Table 4). It is important to note that not all egg-adapted substitutions in the HA of influenza viruses are immunodominant in humans. For example, our previous study of A(H1N1) viruses revealed that the Q223R egg-adapted mutation in A(H1N1)pdm09 viruses only led to reduced antibody responses to circulating 223Q A(H1N1) viruses in 10% of the 281 adult vaccinees studied (13). Whereas in the current study, in the 2018–19 season, we found that 53%–100% of vaccinees across all age cohorts mounted antibody responses targeting either the T160K and/or D225G egg-adapted substitutions (Table 4). Furthermore, multiple egg-adapted substitutions often coexist. In this study, the HK/14 CVV had 2 additional egg-adapted substitutions at HA sites 96 and 203 (Figure 3). An exploration of the immunodominance of HA epitopes would be necessary to improve the antigenic characterization of the influenza virus for vaccine strain selection (34). It would also be important to do this in the context of the human immune system using human sera, since naive animal models (such as with ferret antisera) may not detect antibody responses to certain HA epitopes that are otherwise recognized by humans (13).

Low A(H3N2) VE was observed in the 2016–19 seasons, even in 2016–17 and 2017–18, when there was no clear antigenic drift

Table 4. Lower MN antibody responses to cell-propagated A/Singapore/INFIMH-16-0019/2016 A(H3N2) virus are related to egg-adapted changes on the HA head domain

Age groups	Age	Birth years	N	A No. (%) of individuals with ≥ 4 -fold reduction in post-vac MN titers against Sing/16 cell virus ^a	B No. (%) of individuals among A with recovered post-vac MN titers to Sing/16 cell RG virus ^b		
					T160K	D225G	T160K + D225G ^c
					Pediatrics	<3 yr	2015–17
Older pediatric individuals	9–17 yr	2002–09	22	19 (86%)	18/19 (95%)	18/19 (95%)	17/19 (89%)
Adults	18–49 yr	1971–2000	21	15 (71%)	15/15 (100%)	8/15 (53%)	8/15 (53%)
Older adults	50–64 yr	1953–68	22	15 (68%)	13/15 (87%)	11/15 (73%)	9/15 (60%)
Elderly	65+ yr	1936–53	22	22 (100%)	18/22 (82%)	17/22 (77%)	15/22 (68%)

^aFold reduction of MN titers against Singapore/16 (Sing/16) cell virus is expressed as the ratio of post-vaccination (post-vac) MN titers: Singapore/16 Egg/Cell-propagated viruses. ^bRecovered post-vaccination MN titers were defined as within 2-fold of post-vaccination MN titers against the Singapore/16 egg vaccine virus. Reversion to egg-adapted amino acid substitutions T160K and D225G were respectively introduced into the HA sequence of the Singapore/2016 cell-propagated virus. Post-vaccination MN titers against each of the RG viruses were compared with egg-propagated Singapore/2016 virus titers. ^cIndividuals showed recovered post-vaccination MN titers against both Singapore/16 cell T160K and D225G RG viruses simultaneously when compared with the Singapore/16 egg virus.

of the circulating viruses (refs. 3, 4 and Supplemental Table 1). Moreover, age-specific VE appears to differ across seasons and cannot be explained by vaccine egg adaptation alone. Immune priming of the influenza virus in childhood can affect the performance of influenza vaccines received later in life (13, 35). In a previous study, we demonstrated that immune priming can affect the specificity of the antibodies elicited by newer A(H1N1)pdm09 vaccines (13). Recently, researchers in Canada and Europe proposed an “imprint-regulated effect of vaccine (I-REV)” hypothesis to interpret the negative VE observed against 3C.3a A(H3N2) viruses during the 2018–19 season, especially in cohorts born between 1964 and 1986 (17, 36). Gouma et al. also found that individuals born in the 1960s and 1970s possess non-neutralizing antibodies against contemporary 3C.2a A(H3N2) viruses and that HA amino acid similarity between the imprinting A(H3N2) strain and the 3C.2a viral strain could affect the specificity of antibody responses to 3C.2a A(H3N2) viruses (37). Furthermore, Gostic et al. demonstrated through statistical modeling that childhood H1 or H3 HA imprinting is crucial to provide substantial protection against severe disease for group-matched novel influenza pandemic strains like H5 (group 1) or H7 (group 2), although not necessarily against infection (38). They also suggested that imprinting with one HA group via natural infection in childhood could influence subsequent exposures to another HA group. In our study, the elderly group (birth years: 1932–53) was likely first imprinted by A(H1N1) viruses, and for older adults (birth years: 1953–68), the first exposure to influenza would likely be to an A(H2N2) strain (circulated during 1957–68), as the A(H3N2) virus did not start circulating in humans until 1968 (Figure 1). Likewise, individuals born after 1977 could have been imprinted by either A(H3N2) or A(H1N1) when these 2 subtypes of viruses cocirculate (Figure 1). Phylogenetically, H1 and H2 belong to HA group 1, whereas H3 belongs to group 2. It is not fully understood yet how the imprinting with A(H1N1) or A(H2N2) viruses could impact the antibody responses to A(H3N2) viruses. Nonetheless, here, older adults and elderly individuals possessed neutralizing antibodies targeting the unglycosylated HA158-160 and 225G epitopes, consistent

with early priming with older A(H3N2) viruses that have circulated since 1968. Our study provided a plausible immune basis on which to support the birth cohort effect on the effectiveness of A(H3N2) influenza vaccines used in the 3 seasons. The unglycosylated HA158-160 motif was prevalent in seasonal A(H3N2) viruses from 1968 to 2014; historic A(H3N2) viruses from 1968 to 1999 also bear 225G in their HAs (Table 2). Both epitopes could be imprinted in early childhood and were then repeatedly boosted because of their prolonged prevalence in historically circulating A(H3N2) viruses. Neutralizing antibodies targeting the unglycosylated HA158-160 and 225G epitopes were less efficient in neutralizing the circulating A(H3N2) strains carrying the glycosylated HA158-160 and 225D epitopes (9, 37). Vaccination with egg-based vaccines can further boost responses to these epitopes, and the magnitudes of boosting may reflect the frequency of past exposures to these epitopes (Tables 2 and 3). Both immune priming and egg adaptation of the A(H3N2) vaccines have probably contributed to the low and variable VE in some age groups. Last, we also assessed the impact of sex on vaccine responses (8) and found no significant difference between males and females.

Our study has several limitations. First, although we were able to assess vaccine responses among individuals across a broad age range and 3 influenza seasons, the number of participants within each age group per season was relatively small and may not represent the whole population. Second, we only analyzed the neutralizing antibody responses; other vaccine-induced immunity, such as that resulting from neuraminidase antibodies and cell-mediated immunity, may also correlate with protection against influenza infections. We recognize that other immunologic, virologic, and host factors may also have contributed to the variability of VE observed between seasons and among the different age groups.

After decades of efforts to improve influenza vaccines, the rapid evolution of influenza viruses and complex human immune background continue to pose challenges to achieving optimal influenza VE. Our study demonstrated a scenario in which the effectiveness of A(H3N2) influenza vaccines can be affected by the combination of age-specific vaccine responses to HA egg-adapted

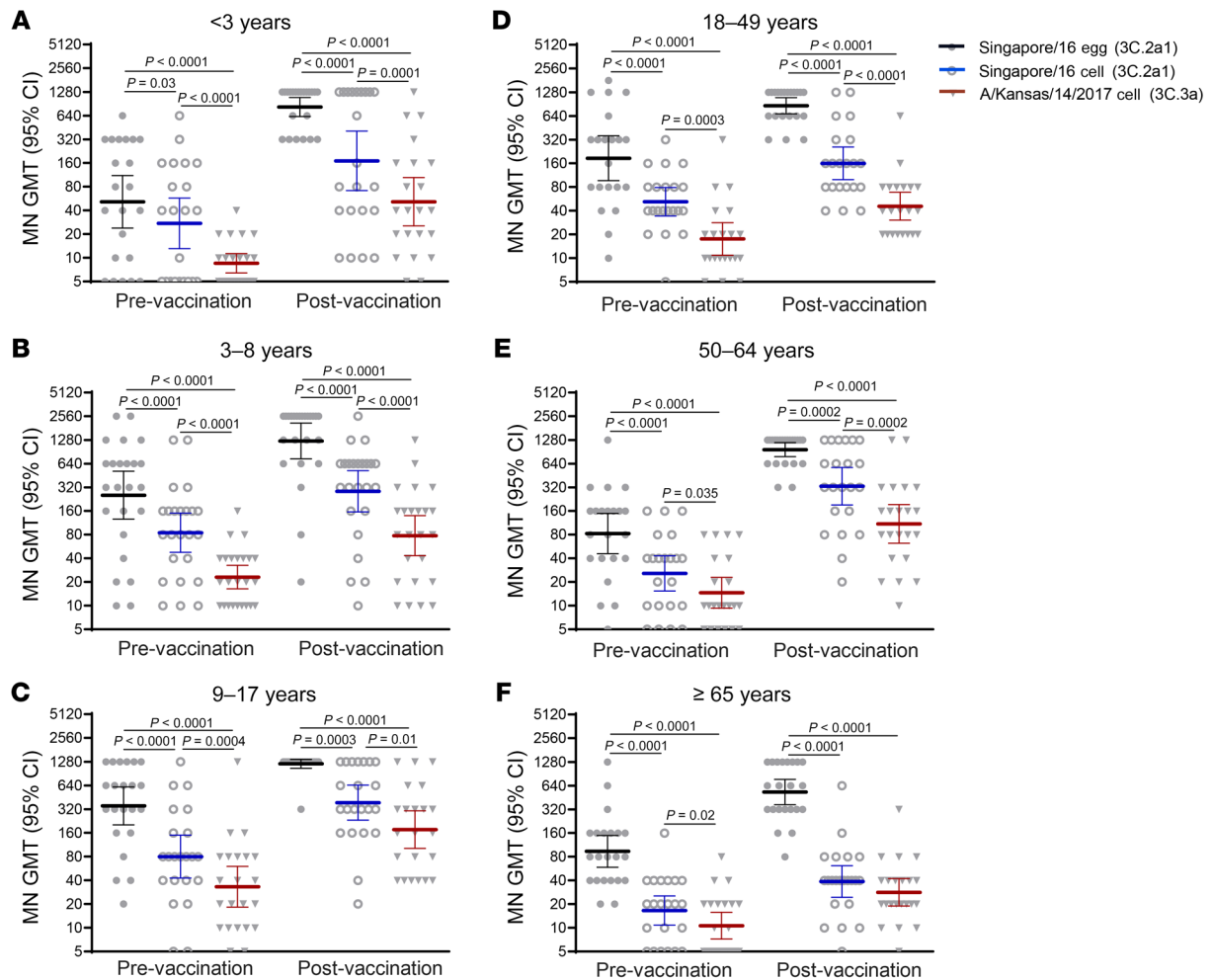


Figure 6. Pre- and post-vaccination neutralizing antibody responses to egg- and cell-propagated WT vaccine viruses and cell-propagated circulating 3C.3a virus among all 6 age groups in the 2018–19 season. Pre- and post-vaccination age groups: (A) under 3 years of age, (B) 3–8 years of age, (C) 9–17 years of age, (D) 18–49 years of age, (E) 50–64 years of age, and (F) 65 years of age or older. The y axis shows MN antibody titers for each individual. Bars reflect the MN GMTs (with 95% CI) against egg-propagated Singapore/16 virus (solid circles), cell-propagated Singapore/16 virus (open circles), and cell-propagated A/Kansas/14/2017 3C.3a virus (triangles). Statistical comparisons of pre- and post-vaccination MN GMTs in each age group were analyzed by 1-way ANOVA with Tukey's multiple-comparison correction. *P* values are indicated where there was a statistically significant difference ($P < 0.05$).

substitutions, preexisting host immunity, and viral antigenic drift. It also provided evidence for the utility of designing age-specific vaccination strategies for high-risk age cohorts (such as pediatric and elderly populations). More studies are needed to further explore age-related VE. Ultimately, the development and licensure of non-egg-based vaccines and enhanced vaccines that can offer broader and long-lasting immunity are needed to combat influenza infections across all age groups.

Methods

Viruses. The influenza A(H3N2) viruses used in this study were propagated either in allantoic cavities of 9- to 11-day-old embryonated eggs or in Madin-Darby Canine Kidney cells stably transfected with cDNA of human 2,6-sialyltransferase (MDCK-SIAT1) following previously published procedures (39).

In addition, 2 RG-engineered MDCK-SIAT1-propagated viruses were generated containing the HA and neuraminidase genes from A/Singapore/INFIMH-16-0019/2016 cell-propagated A(H3N2) virus

and 6 internal genes from A/Puerto Rico/8/1934, including A/Singapore/INFIMH-16-0019/2016-PR8-T160K (RG T160K), and A/Singapore/INFIMH-16-0019/2016-PR8-D225G (RG D225G). RG virus containing L194P was also rescued but failed to propagate. Details of RG virus generation are included in the Supplemental Methods.

Sera. Three hundred seventy-five anonymous pre- and 21- to 28-day post-vaccination sera samples collected from individuals from 6 age cohorts and 3 influenza seasons (2016–17, 2017–18, and 2018–19) were analyzed. For children 8 years of age or younger who had not had prior vaccination received 2 doses, sera were collected before vaccination and 21–28 days after the second dose. All vaccinees received egg-based, inactivated QIVs (Table 1).

MN assays. MN assays were performed using MDCK-SIAT1 cells to measure neutralizing antibody responses following previously described procedures (39, 40). Two-fold serial dilution sera were mixed with 100 TCID₅₀ (50% tissue culture infective doses) of each virus and incubated at 37°C with 5% CO₂ for 1 hour. The mixture of virus and serum was used to infect 1.5×10^4 MDCK-SIAT1 cells/well and incubated at

37°C for 18–20 hours. The presence of viral protein was determined by ELISA with monoclonal antibodies specific to influenza A virus nucleoprotein. MN titers were defined as the reciprocal of the highest dilutions of serum that gave 50% neutralization. MN titers below 10 (initial sera dilution) were assigned a value of 5 for the analysis.

HA sequence and structure modeling. All viruses used in this study were sequenced and analyzed. HA sequence analysis was performed using BioEdit, version 7.0.9.0. 3D structures of HAs were generated by SWISS-MODEL (<http://swissmodel.expasy.org>). All structure figures were generated using PyMOL software.

Statistics. Geometric mean pre- and post-vaccination titers and antibody titer ratios between egg- and cell-propagated WT vaccine viruses (egg/cell ratio) were compared. The Wilcoxon matched-pairs, signed-rank test, Mann Whitney *U* unpaired *t* test, and 1-way ANOVA were used for statistical comparisons. A *P* value of less than 0.05 was considered significant. GraphPad Prism 8 software (GraphPad Software) was used for statistical analyses.

Study approval. Anonymized serum samples used in the study were obtained from a contract organization (Navitas Clinical Research Inc., Rockville, Maryland, USA). Written informed consent was received from participants prior to inclusion in the study. The use of sera was approved by the National Center for Immunization and Respiratory Diseases, CDC Human Subject Research Determination Review.

Author contributions

MZL conceived the study. FL and MZL designed the experiments. FL, FLG, CH, SNJ, YHB, LW, and BZ performed experiments. FL and MZL wrote the manuscript. All authors reviewed the manuscript.

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