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Comparison of neutralizing antibody and cell-mediated immune responses to pandemic H1N1 2009 influenza virus before and after H1N1 2009 influenza vaccination of elderly subjects and healthcare workers

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SUMMARY

Background: The recent H1N1 pandemic virus that emerged in 2009 resulted in high morbidity rates mainly in younger individuals, albeit with relatively low mortality. We investigated both humoral and cellular immune responses against the pandemic H1N1 2009 virus before and after immunization with inactivated H1N1 2009 vaccine.

Methods: We obtained paired blood specimens from a cohort of participants from nursing homes ($n = 108$) and a public hospital ($n = 60$) in Singapore. Serum samples were tested for neutralizing antibodies against H1N1 2009 using microneutralization assays, while peripheral blood mononuclear cells were subjected to interferon-γ enzyme-linked immunosorbent spot (ELISPOT) assays for whole virus-specific T-cell responses.

Results: We observed significant increases in geometric mean titers of neutralizing antibodies after H1N1 2009 vaccination (from 23.6 pre-vaccination to 94.7 post-vaccination). Approximately 77% and 54% of the cohort exhibited ≥2-fold and ≥4-fold increases in neutralizing antibody titers following vaccination; 89.9% of the cohort had a post-vaccination antibody titer of ≥32. Adjusted for gender, participants aged ≥60 years were less likely to have a ≥4-fold increase in antibody titers after vaccination than those aged <60 years (0.48; 95% confidence interval (95% CI) 0.32–0.71, $p = 0.007$). There was a 1.4-fold elevation in H1N1 2009-specific T-cell responses after vaccination ($p < 0.05$). Adjusted for gender, age ≥60 years was positively associated with a greater increase in T-cell response ($\beta = 4.9$, 95% CI 1.58–8.29, $p = 0.018$). No significant correlation was observed between humoral and cellular immune responses.

Conclusions: Influenza vaccination elicits significant neutralizing antibody and T-cell responses to pandemic H1N1 2009 influenza virus. However, in response to vaccination, increases in neutralizing antibody titers were comparatively lower but T-cell responses were higher in older participants. Therefore, our study suggests that memory T-cells may play a crucial role in protecting older individuals against pandemic H1N1 2009 infection.

1. Introduction

Epidemics caused by influenza viruses result in the highest number of vaccine-preventable deaths worldwide annually. Influenza viruses have also caused several pandemics in the last century, including the 1918 H1N1 Spanish flu pandemic that resulted in up to 40 million deaths, the 1957 H2N2 Asian flu pandemic with a death toll of 1.5 million worldwide, and the 1968 H3N2 Hong Kong flu pandemic with about 1 million deaths.[1–3] Influenza pandemics occur when a population with low herd immunity encounters a newly emerged influenza virus that can be transmitted efficiently. In April 2009, the World Health Organization (WHO) announced the emergence of a novel influenza that originated from Mexico and spread around the world. This was subsequently declared as the first influenza pandemic of the 21st century in June 2009. The 2009 pandemic influenza virus (pH1N1 2009) originated from a triple-reassortant virus that had been circulating in swine during the preceding 10 years.[4,5] The pandemic virus caused a high morbidity rate amongst younger age groups, but a relatively low overall mortality.[6] Pre-existing

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cross-reactive antibodies to pH1N1 2009 have been found to exist in elderly individuals over 65 years of age, but none or very low levels of antibodies were observed in adults and children.[7] These observations could potentially be explained by cross-reactivity between pandemic H1N1 2009 and older H1N1 strains circulating before 1950.[8,9]

Influenza epidemics and pandemics are usually caused by antigenic drift and shift, respectively.[10] Influenza vaccines confer protection by triggering memory B-cells to rapidly produce antibodies and specific T-cell responses upon infection.[11] However, vaccines need to be updated annually to address the antigenic drift culminating from the high mutation rates of influenza viruses.[12] In addition, antigenic shift is largely unpredictable, rendering seasonal influenza vaccines relatively ineffective for pandemic influenza strains. For instance, the 2008–2009 seasonal influenza vaccines (inactivated and live, attenuated influenza vaccines) were found to induce little or no cross-reactive antibody responses to pH1N1 2009 in any age group.[7] However, the antibody response is only one aspect of adaptive immunity, since influenza virus infection also activates cellular immunity in the human host. Antibodies are produced by B-cells to neutralize the virus, while cytotoxic T-cells kill infected cells by binding to epitopes presented by antigen-presenting cells (APCs).[13,14] After killing cells that present foreign antigens, the immune system will 'remember' the infection through memory cells, which will recognize and react to any future infection by related strains. By simultaneously measuring both humoral and cellular responses before and after vaccination, the efficacy of the relatively new pH1N1 2009 vaccine in triggering these responses during an infection can be assessed. The objective of this study was to assess both humoral and cellular responses to pH1N1 2009 vaccination, identify any differences in pre-existing immunity, and examine the responses following vaccine challenge by key demographic characteristics, namely age and gender.

2. Methods

2.1. Study design and sources of samples

Two sets of blood samples were collected from participants, one prior to pH1N1 2009 vaccination and the other at 4–6 weeks after vaccination. To include subjects from a wide range of ages, we recruited consenting participants from four groups of individuals who were offered pH1N1 2009 vaccination between November 16, 2009 and March 11, 2010. Participants were residents from three nursing homes in Singapore (Jamiyah Home, Bukit Batok Home, and Christalite Methodist Home) and healthcare staff of Tan Tock Seng Hospital, who provided paired samples for the isolation of serum and peripheral blood mononuclear cells (PBMCs).

Serum samples and PBMCs were then prepared from the blood samples for the microneutralization assay and the interferon gamma (IFN-γ) enzyme-linked immunosorbent spot (ELISPOT) assay, respectively. Microneutralization assays were performed to measure the level of neutralizing antibodies in serum as an indicator of the humoral immune response. ELISPOT assays were used to detect the number of spots formed by IFN-γ-producing influenza-specific T-cells present within the PBMCs as a measure of the cellular immune response.

2.2. Processing of serum and PBMC samples

Serum from the test participants was heat-inactivated at 56 °C for 30 min and stored at −80 °C until use in the microneutralization assay. For the isolation of PBMCs, Ficoll-Paque PLUS solution at a density of 1.077 g/ml (Amersham Biosciences, Uppsala, Sweden) was used to underlay the whole blood samples, which were subsequently centrifuged at 900 *g* at 18–20 °C for 30 min. After centrifugation, the whole blood was separated into its respective components, and the buffy coat containing the PBMCs was extracted. The PBMCs were then washed twice with phosphate-buffered saline and resuspended in freezing medium comprising RPMI-1640, 20% fetal bovine serum (FBS), and 10% dimethylsulfoxide. PBMC samples were aliquoted and stored at −80 °C until use in the ELISPOT assays.

2.3. Influenza virus and vaccine

Influenza H1N1 A/Singapore/GP2651/2009 virus strain isolated in Singapore during the H1N1 2009 pandemic was used for both the microneutralization and ELISPOT assays. This virus is highly homologous to the vaccine strain, and was propagated in Madin–Darby canine kidney (MDCK) cells using Eagle's minimum essential medium (EMEM). Study participants were immunized with the Panvax inactivated split H1N1 2009 influenza vaccine derived from the A/California/7/2009 (H1N1) virus-like strain propagated in eggs (CSL, Melbourne, Australia).

2.4. Microneutralization assay

MDCK cells were first seeded into 96-well flat bottom plates and incubated at 37 °C with 5% CO_2 for 24 h to obtain confluent monolayers. Serial two-fold dilutions of each serum sample were prepared using EMEM as diluent starting with the 1:8 dilution. Equal volumes of 100 $TCID_{50}$ (median tissue culture infective dose) of H1N1 A/Singapore/GP2651/2009 virus were incubated with the diluted serum samples at 35 °C with 5% CO_2 for 2 h.

The cells were washed three times before adding serum-free medium containing 3 μg/ml of tosyl phenylalanyl chloromethyl ketone (TPCK)–trypsin, then inoculated with 50 μl of the virus–serum mixtures and incubated at 35 °C with 5% CO_2 for 72 h before observing for cytopathic effect (CPE) with an inverted microscope to assess any residual viral replication. The neutralizing antibody titer was defined as the reciprocal of the highest dilution of the serum at which the infectivity of 100 $TCID_{50}$ of the virus for MDCK cells was completely neutralized in 50% of the wells.[8]

2.5. IFN-γ ELISPOT assay

The secretion of IFN-γ was detected by ELISPOT assay. Multiscreen 96-well plates (Millipore, Billerica, MA, USA) were coated overnight at 4 °C with 5 μg/ml of anti-human IFN-γ capture antibody (Mabtech, Nacka Strand, Sweden). Plates were blocked with AIM-V medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS for 2 h at room temperature. Cryopreserved PBMCs were thawed, washed, and added to the plates in triplicate at 10^5 per well in AIM-V medium supplemented with 2% human serum, and then incubated at 37 °C for 5 h. Concanavalin A (Sigma-Aldrich, St. Louis, MO, USA) at 5 μg/ml was included as the positive control to ensure the viability of the PBMCs tested, while filtered pandemic H1N1 A/GP2651/2009 strain was added to the test wells at a multiplicity of infection of 3 before further incubation at 37 °C for 16 h. Plain medium with PBMCs served as negative control. Medium control wells contained supernatant obtained from flasks in which H1N1 2009 was passaged in MDCK cells. This was to test whether the medium would give rise to non-specific production of IFN-γ.

Plates were washed and incubated for 2 h with 2 μg/ml of biotinylated anti-IFN-γ secondary detection antibody (BD Biosciences, San Jose, CA, USA), followed by 1 h incubation with streptavidin–horseradish peroxidase enzyme and substrate (BD Biosciences) at room temperature. 3-Amino-9-ethylcarbazole (AEC) chromogen in substrate solution (BD Biosciences) was

added to each well for spot development and the plates were then read by an automated ELISPOT reader (Cellular Technology, Shaker Heights, OH, USA). One spot formed in a well reflects one IFN-γ-producing cell, and the size of the spot is proportional to the amount of IFN-γ produced.[15,16] Mean spot-forming units (SFU) were calculated after subtracting the number of spots in the negative well from the test well. Samples were considered to be positive for a T-cell response if the number of H1N1-specific IFN-γ-producing spots was above five when the negative control was zero, or at least two times the mean SFU of the negative control when the negative control was above zero.[16,17]

2.6. Statistical analyses

For humoral immunity, the geometric mean titer (GMT) and fold-change were analyzed. For calculation of the GMT, antibody titers less than 8 were assigned a value of 4, while titers more than 1024 were assigned a value of 2048. The fold-change was then calculated by dividing the post-vaccination antibody titer by the pre-vaccination antibody titer. A paired sample *t*-test was employed to compare antibody titers and T-cell responses before and after vaccination. Using the Chi-square test, we assessed if age and gender were associated with pre-vaccination and post-vaccination titers ≥32, as well as with a ≥4-fold change in antibody titers (the cut-off neutralizing antibody titer of ≥32 is similar to that used in other pH1N1 2009 vaccine studies).[18] Univariate and multivariate logistic regression were used, with robust standard errors computed, the grouping variable being the respective institutions of the participants.

We compared T-cell responses elicited pre- and post-vaccination, with the change response being the post-vaccination minus the pre-vaccination T-cell response. In addition, using the unpaired sample *t*-test, we assessed whether age and gender were associated with pre-vaccination and post-vaccination T-cell responses. As there are no established cut-off points for T-cell responses, the relationship between age and gender, pre- and post-vaccination T-cell ELISPOT responses, and changes in T-cell ELISPOT values were analyzed on a continuous scale using multivariate linear regression, again using robust standard errors.

We also utilized Spearman correlation to assess the relationship between neutralizing antibody titers and T-cell responses. All statistical analyses were performed using STATA software version 10 (StataCorp, College Station, TX, USA). Standard confidence intervals, *p*-value intervals, robust standard errors and associated *p*-values in the regression analyses are presented where appropriate. In all analyses, two-tailed *p*-values of <0.05 were considered to be statistically significant.

3. Results

3.1. Study cohort characteristics

A total of 168 participants were recruited, including 36 residents from each of three nursing homes and 60 staff from Tan Tock Seng Hospital. Table 1 shows the demographic data for the study cohort; there were similar numbers of men and women. The age of participants ranged from 22 to 93 years, with a mean of 58.0 years and median of 64 years. For our regression analyses, we divided the cohort into two age groups (<60 and ≥60 years); 56.5% of participants were aged ≥60 years.

3.2. Pre- and post-vaccination changes in neutralizing antibody and T-cell responses

The pre- and post-vaccination H1N1 2009 influenza-specific antibody responses are shown in Figure 1A. More than half (52.4%) of the cohort had a pre-vaccination antibody titer of ≥32, and this proportion increased to 89.9% post-vaccination. The geometric mean titer increased from 23.6 pre-vaccination to 94.7 post-vaccination ($p < 0.001$, paired *t*-test), a mean rise of about 4-fold. Of the cohort, 53.6% displayed a ≥4-fold increase, while 77.4% exhibited a ≥2-fold increase in neutralizing antibody titers against the pandemic virus.

The pre- and post-vaccination H1N1 2009-specific T-cell responses are shown in Figure 1B. Furthermore, 68.5% of the participants revealed a positive T-cell response before vaccination. The mean SFU per 10^5 PBMCs increased significantly from 9.2 SFU pre-vaccination to 13.8 SFU post-vaccination ($p < 0.001$, paired *t*-test), with an average fold increase of 1.39.

Table 1
Demographic data of the study cohort.

N = 168	*n* (%) or mean (SD)
Participant type, No (%)	
Bukit Batok Home residents	36 (21.4)
Christalite Methodist Home residents	36 (21.4)
Jamiyah Home residents	36 (21.4)
Tan Tock Seng Hospital staff	60 (35.7)
Gender	
Male	80 (47.6)
Female	88 (52.4)
Age group	
<60 years	73 (43.5)
≥60 years	95 (56.5)
Age in years, mean (SD)	58.0 (20.4)

3.3. Differences in neutralizing antibody and T-cell responses by age and gender

There were significant differences in the prevalence of pre- and post-vaccination neutralizing antibody and T-cell responses, as

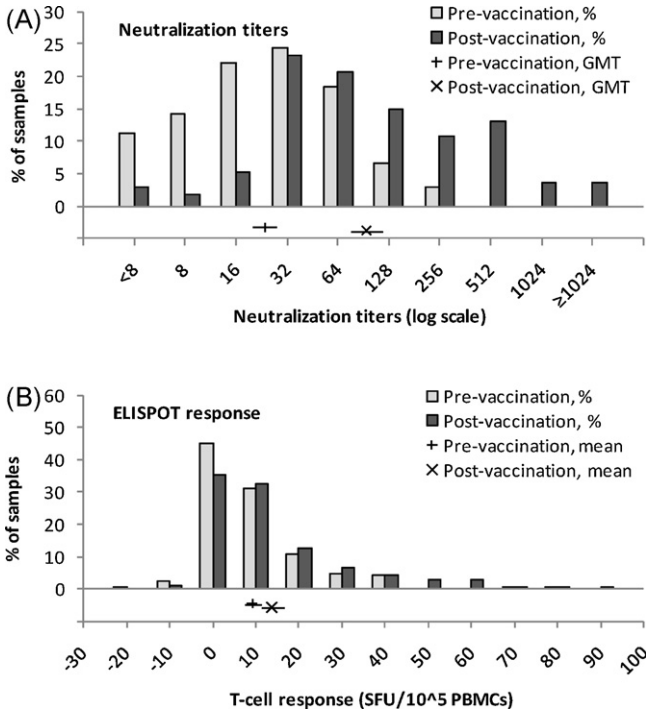

Figure 1. Distribution of pre- and post-vaccination antibodies and T-cell responses (*N* = 168). Columns show the proportion of participants; geometric mean titers (GMT) are indicated below (with 95% confidence intervals as error bars). (A) Antibody responses as measured by H1N1-specific neutralizing antibodies. (B) Interferon-gamma-producing T-cell responses as measured by ELISPOT assays.

Table 2
Differences by age and gender in pre-vaccination, post-vaccination virus neutralization titers and T-cell responses, including changes in antibody titers and T-cell responses.

	Microneutralization results, %			Mean ELISPOT results, SFU/10^5 PBMCs (95% CI)		
	Pre-vaccination titers ≥32	Post-vaccination titers ≥32	≥4-fold increase in titers	Pre-vaccination response	Post-vaccination response	Change in response
Age group						
<60 years	28.8	82.2	67.1	6.8 (4.0, 9.5)	8.5 (5.4, 11.6)	1.7 (−1.3, 4.7)
≥60 years	70.5	95.8	43.2	11.1 (8.3, 13.8)	17.8 (14.1, 21.5)	6.7 (3.2, 10.2)
p-Value	<0.001[a]	0.004[a]	0.002[a]	0.033[b]	<0.001[b]	0.040[b]
Gender						
Male	65.0	92.5	45.0	11.4 (8.1, 14.6)	18.3 (14.0, 22.6)	6.9 (2.7, 11.1)
Female	40.9	87.5	61.4	7.2 (4.9, 9.6)	9.6 (6.9, 12.4)	2.4 (0.0, 4.8)
p-Value	<0.002[a]	0.283[a]	0.034[a]	0.041[b]	<0.001[b]	0.062[b]

ELISPOT, enzyme-linked immunosorbent spot assay; SFU, spot-forming units; PBMC, peripheral blood mononuclear cells; CI, confidence interval.
[a] Chi-square test.
[b] Unpaired *t*-test.

well as the change in antibody titers and T-cell responses following vaccination, between age and gender groups (Table 2). There was a higher percentage of participants with pre- and post-vaccination titers of ≥32 in the older age group compared with the younger age group (pre-vaccination: 70.5% in those ≥60 years old vs. 28.8% in those <60 years old, $p < 0.001$; post-vaccination: 95.8% vs. 82.2% in the older vs. younger age group, $p = 0.004$). On the other hand, a higher proportion of the younger age group displayed a ≥4-fold increase in antibody titer after vaccination compared to the older age group (67.1% vs. 43.2%, $p = 0.002$). The older age group also revealed significantly higher pre- and post-vaccination T-cell responses (expressed as SFU per 10^5 PBMCs) compared to the younger age group (pre-vaccination: 11.1 vs. 6.8, $p = 0.033$; post-vaccination: 17.8 vs. 8.5, $p < 0.001$). Unlike the antibody responses, the older age group showed significantly greater increases in T-cell responses after vaccination compared to the younger age group (6.7 vs. 1.7, $p = 0.040$). Some gender differences were also observed, with males having a significantly higher prevalence of

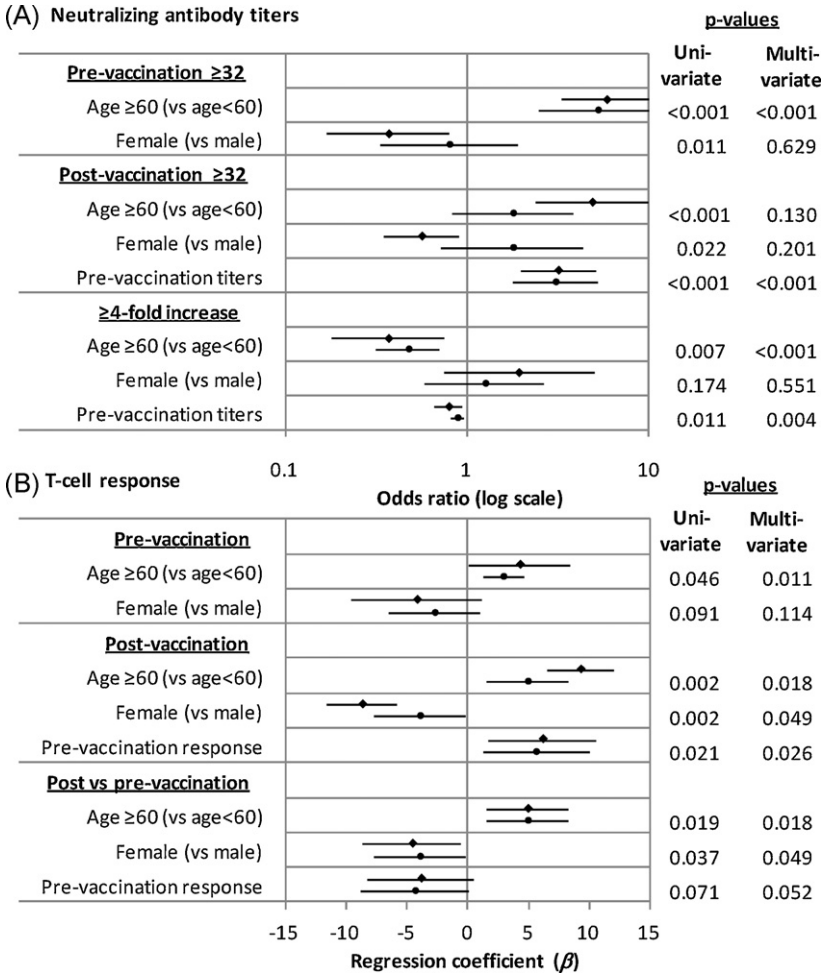

Figure 2. Factors associated with neutralizing antibodies and T-cell responses on univariate (diamonds) and multivariate (circles) regression analysis. Error bars denote 95% confidence intervals computed using robust standard errors to account for possible group level clustering of results, with corresponding *p*-values provided in the text. (A) Pre- and post-vaccination titers ≥32, and ≥4-fold increase in titers following vaccination; pre-vaccination titers as an explanatory variable is modeled on a continuous log scale for every two-fold difference in pre-vaccination titer. (B) Pre- and post-vaccination T-cell responses, and changes in T-cell response following vaccination; pre-vaccination responses as an explanatory variable is modeled on a continuous scale for every 10 SFU per 10^5 PBMC difference in pre-vaccination response.

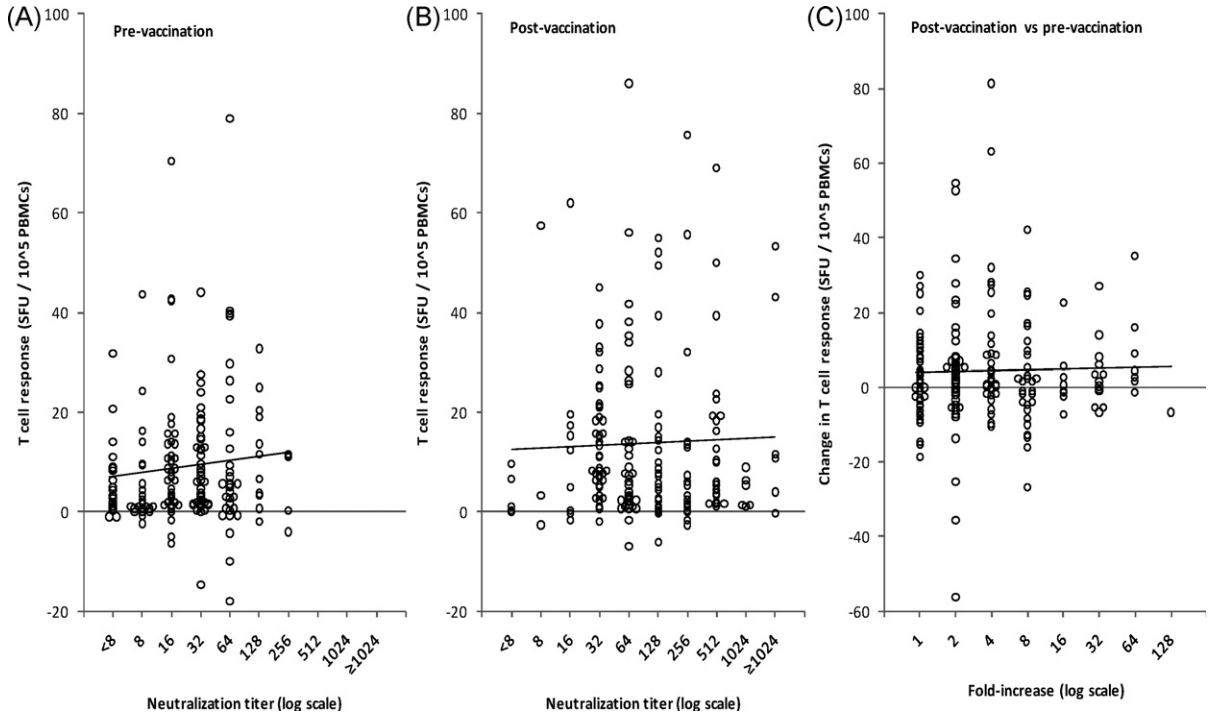

Figure 3. Correlation between humoral and cellular immune responses ($N = 168$). (A) Pre-vaccination neutralization titers and T-cell responses. (B) Post-vaccination neutralization titers and T-cell responses. (C) Fold-change in neutralization titers (log scale) and absolute change in T-cell responses. Overlapping data points have been offset laterally.

pre-vaccination antibody titers ≥32, lower probability of achieving a ≥4-fold increase in titers, and higher pre- and post-vaccination T-cell responses.

We assessed the extent that these differences could be explained by confounding in multivariate regression models. On multivariate analysis, older age remained significantly associated with higher pre-vaccination titers ≥32 (adjusted odds ratio (OR) 5.34, $p < 0.001$) (Figure 2A). However, older age was not associated with higher post-vaccination titers after accounting for the effect of higher pre-vaccination titers (adjusted OR 1.80, $p = 0.130$). On the other hand, we found that older individuals were still less likely to exhibit a ≥4-fold increase in titers (adjusted OR 0.48, $p < 0.001$), even after adjusting for the effect from higher pre-vaccination antibody titers. None of the associations observed with gender remained significant in the multivariate analyses, while pre-vaccination titers were independently associated with higher post-vaccination titers and with less likelihood of a ≥4-fold increase in titers.

On multivariate regression, older age remained significantly associated both with higher pre- and post-vaccination T-cell responses (adjusted $\beta = 3.01$, $p = 0.011$, and adjusted $\beta = 4.93$, $p = 0.018$, respectively), as well as a greater increase in T-cell responses following vaccination (adjusted $\beta = 4.93$, $p = 0.018$) (Figure 2B). Females showed significantly lower post-vaccination T-cell responses and a smaller change in T-cell responses following vaccination. Higher pre-vaccination T-cell responses were also positively associated with post-vaccination T-cell responses (adjusted $\beta = 5.67$, $p = 0.026$), but were negatively associated with the change in T-cell responses (adjusted $\beta = -4.33$, $p = 0.052$).

3.4. Lack of correlation between humoral and cellular immunity to H1N1 2009

We also examined the correlation between humoral and cellular immunity to H1N1 2009 as measured by the microneutralization and ELISPOT assays. There was no correlation between antibody titers and T-cell responses for either the pre-vaccination or post-vaccination response (Figure 3A, pre-vaccination: $\beta = 0.81$, $p = 0.137$, $R^2 = 0.009$; Figure 3B, post-vaccination: $\beta = 0.29$, $p = 0.620$, $R^2 = 0.0011$). Moreover, there was also no significant correlation between the change in antibody titers and change in T-cell responses induced by H1N1 2009 vaccination ($\beta = 0.18$, $p = 0.803$, $R^2 = 0.0004$) (Figure 3C).

4. Discussion

In this study, we simultaneously studied humoral and cellular immune responses following stimulation with a monovalent inactivated H1N1 2009 pandemic virus vaccine. The pH1N1 2009 vaccine (Panvax) used in our study was a purified, inactivated, monovalent, split virion vaccine containing antigens of A/California/7/2009 (H1N1-like virus). In concordance with other vaccine studies,[5,19] we found that more than half of the cohort exhibited a ≥4-fold increase in antibody titers, with close to 90% achieving neutralization titers of ≥32 following vaccination. Compared to live attenuated influenza vaccines, it is generally thought that inactivated influenza vaccines are inefficient in inducing strong cytotoxic T-cell responses.[20,21] Our study revealed significant increases in T-cell responses after vaccination. However, there was no concordance between the antibody and T-cell responses, a result that concurs with a previous finding in individuals vaccinated with trivalent inactivated influenza vaccine in 2008, suggesting that vaccination does not trigger equivalent humoral and cellular responses.[20,22]

Moreover, there were important age-related differences in the T-cell responses compared to the antibody responses to vaccination, with older individuals showing both higher pre-vaccination antibody titers and T-cell responses to pH1N1 2009. The association between older age and higher pre-vaccination antibody titers was documented by Hancock et al.[7] The existence of such cross-reactive antibodies may be explained by prior exposure to the more closely related H1N1 strains, especially those that

share similar T-cell epitopes, most likely during their childhood years when strains antecedent to the H1N1 2009 pandemic were circulating.[11] Furthermore, there is antigenic similarity between the hemagglutinin (HA) epitopes of pH1N1 1918 and pH1N1 2009 viruses, while antibody obtained from 1918 H1N1 pandemic survivors has high affinity to pH1N1 2009 viral HA.[9] The higher pre-vaccination T-cell responses could similarly be attributed to previous exposure to related viruses, so that older individuals are more likely than younger adults to harbor memory B-cells that produce cross-reactive antibodies, as well as memory T-cells capable of recognizing epitopes on the pH1N1 2009 virus. However, it is interesting that we found that older individuals were less likely to achieve ≥4-fold increases in antibody titers than younger adults, even after adjusting for their higher pre-vaccination titers which are known to reduce the probability of observing a further increase in titers,[12] while at the same time exhibiting stronger T-cell responses than younger adults following stimulation with inactivated pH1N1 2009 vaccine.

Previously, others have also observed that older age groups have a lower likelihood of seroconverting after seasonal influenza vaccination,[10,23–25] and Woodland and Blackman have postulated that humoral responses decrease with age.[26] However, naïve T-cell responses decrease with age, so that memory T-cell responses become the main T-cell responses in the elderly.[20] We suggest the following explanation for our findings. Firstly, the observed antibody increase is likely due to the vaccine stimulating predominantly naïve B-cells in both the young and the old, with the humoral responses being stronger in younger individuals.[26] However, because T-cell epitopes are relatively more conserved than B-cell epitopes,[27] the pH1N1 2009 strain on which the vaccine was based was better able to stimulate memory T-cell responses compared to memory B-cell responses. Older individuals were probably primed with previously circulating H1N1 strains, and hence harbored relevant memory T-cell responses. The reason why older age was independently associated with the changes in pre- and post-vaccination T-cell responses may be attributed to the effect of memory T-cell responses in the older age group outweighing the naïve T-cell responses in the younger age group.

The above interpretation of our findings, if correct, has several implications both for understanding the epidemiology of pH1N1 2009 influenza and for the use and design of future seasonal and pre-pandemic vaccines. Firstly, we suggest that memory T-cells in particular may be playing a larger role in protecting against symptomatic infection than is commonly thought.[28] This would explain why older adults had substantially lower symptomatic infection and seroconversion rates than younger adults, despite the fact that the majority of these individuals in the community did not have antibodies to pH1N1 2009 at levels that are considered protective.[29] Secondly, with regards to influenza vaccines, there is at least a theoretical concern that the widespread use of inactivated vaccine formulations that prevent natural infection but which do not adequately stimulate naïve T-cell responses in younger individuals may lead to more severe outcomes in future pandemics, should related influenza subtypes be re-introduced into the population (as was the case for pH1N1 2009), particularly since T-cell epitopes tend to be relatively more conserved.[21,27] Given that there is substantial evidence that individuals who received seasonal influenza vaccines had an increased risk of medically attended influenza during the early waves of pH1N1 2009 infections, such findings may also be related to inadequate T-cell-mediated immunity.[30] What is needed therefore is to further clarify the role of T-cell immunity in protection against influenza, possibly through cohort studies measuring T-cell responses against specific strains prior to influenza outbreaks to see if certain correlates of protection can be ascertained. Quantifying the role of T-cell immunity in protecting against influenza may also yield improved strategies for the development of pre-pandemic vaccines targeted at relatively well-conserved T-cell epitopes,[19,31] since it is difficult for pre-pandemic vaccines to be cost-effective without a reasonably high degree of cross-protection.[32]

Our study has several limitations. Firstly, our inclusion of the four groups of participants was opportunistic. Ideally, we would have liked to have performed the study in groups of individuals who were similar in all aspects other than age, but we had to recruit a mix of healthcare staff and residents from long-term care facilities to assemble a cohort with the range of age groups necessary for meaningful analyses. Notably, the observed associations between immunity and gender on univariate analyses likely resulted from our choice of study population, since the healthcare staff were largely younger females while the residents comprised older individuals of both genders. Some of the observed pre-vaccination immunity may be due to natural infection with pH1N1 2009 during the epidemic in Singapore from June to September 2009, although it should be noted that estimated infection rates in the long-term care facilities and the healthcare institution included in our study were reasonably low, i.e. less than 10% in our previous study.[29] Secondly, unlike conventional ELISPOT, the whole virus was used in our experiments instead of peptides to stimulate the PBMCs.[33,34] The use of whole virus in ELISPOT allows us to mimic actual influenza infection in vivo, where actively replicating viruses interact directly with immune cells. Since the objective of this study was to ascertain whether individuals vaccinated against pH1N1 2009 were able to generate humoral and cellular immune responses, we believe this is a valid approach. Due to the limited quantity of blood sample and PBMC number obtained from each subject, the CD4 and CD8 T-cell populations were not analyzed separately. Hence, further studies may be helpful for distinguishing the contributions of the responses of the two T-cell subsets, and for identifying the relevant specific T-cell epitopes.

In conclusion, our study has revealed that there was a substantial prevalence of pre-vaccination antibodies as well as T-cell responses to the pH1N1 2009 virus, particularly in individuals aged 60 years and above. The pH1N1 2009 vaccine (Panvax) stimulated both antibody production and the T-cell response. While we observed no correlation between humoral and cellular immunity, we found that there were important age-related differences in the changes observed in humoral and cellular immunity following vaccination, with individuals born before 1950 having lower seroconversion rates but more robust increases in T-cell responses. We suggest this may be due to stimulation of memory T-cells in the elderly, and propose that memory T-cells may play an important role in affording protection for older persons against influenza infections.

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Conflict of interest: The authors declare no conflicts of interest.

Ethical approval: Our study design was approved by the ethics review boards of the National University of Singapore and the National Healthcare Group, Singapore.

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