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Recombinant quadrivalent influenza vaccine (RIV) induces robust cell-mediated and HA-specific B cell humoral immune responses among healthcare personnel

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Author contributions

FSD, MT, MZL, ALN, MG. SG. SK, MW, SB, SS, MJP, SD, and SS designed the study, recruited subjects, collected, and processed the clinical materials. RV, JS, JC, and PC provided key reagents. WC, ZE, SDS, SDR, RK, AK, US, CDB, PR, and MM tested the clinical samples. PRK provided technical support.

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Margarita Mishina: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis. **Weiping Cao:** Project administration, Investigation, Formal analysis. **Zachary Ende:** Methodology, Investigation. **Suresh S. Sharma:** Methodology, Investigation. **Sean D. Ray:** Methodology, Investigation. **Rashmi Kumari:** Methodology, Investigation. **Amrita Kumar:** Methodology, Investigation. **Uma Shanmugasundaram:** Methodology, Investigation. **Caitlin D. Bohannon:** Methodology, Investigation. **Priya Ranjan:** Methodology, Investigation. **Jessie Chang:** Methodology. **Paul Carney:** Methodology. **James Stevens:** Methodology. **Min Z. Levine:** Writing – review & editing, Project administration, Methodology, Investigation. **Sara Kim:** Project administration, Funding acquisition. **Meredith Wesley:** Project administration, Funding acquisition. **Sarah Ball:** Project administration, Methodology, Funding acquisition. **Marcelo Jorge Pando:** Methodology, Investigation. **Sheila Dobin:** Methodology, Investigation. **Paul R. Knight:** Supervision, Resources. **Raghavan Varadarajan:** Methodology. **Mark Thompson:** Project administration, Funding acquisition, Conceptualization. **Fatimah S. Dawood:** Writing – review & editing, Project administration, Investigation, Conceptualization. **Allison L. Naleway:** Writing – review & editing, Project administration, Conceptualization. **Manjusha Gaglani:** Writing – review & editing, Project administration, Investigation, Conceptualization. **Shivaprakash Gangappa:** Writing – review & editing, Methodology, Investigation, Conceptualization. **Suryaprakash Sambhara:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Methodology, Investigation, Conceptualization.

Disclaimer

The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the Centers for Disease Control and Prevention or the Agency for Toxic Substances and Disease Registry, US Department of Health and Human Services.

Ethical review

The study protocol was reviewed and approved by the institutional review boards (IRBs) of the two study sites and Abt Associates, which provided oversight and data management support. The IRB of the Centers for Disease Control relied on the single IRB review from the BSWH IRB. The main trial is registered on [ClinicalTrials.gov](https://clinicaltrials.gov) under the number [NCT03722589](https://clinicaltrials.gov/ct2/show/study/NCT03722589).

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: A. L. N. received funding from Pfizer for an unrelated study. M. G. received funding from CDC with Ambulatory US Flu VE Network and HAIVEN (Hospitalized Adult Influenza Vaccine Effectiveness Network), outside the submitted work. M. G. received support from MedImmune/Astra-Zeneca for ICILE study, FDA required post-marketing LAIV4 effectiveness study in children from 2013 to 2016, outside the submitted work. All other authors report no potential conflicts. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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Abstract

Egg-free influenza vaccines, specifically cell culture-based inactivated influenza vaccine (ccIIV) and recombinant influenza vaccine (RIV), represent a significant advancement over traditional egg-based inactivated influenza vaccines (IIV), particularly for populations with extensive vaccination histories. This comprehensive immunological study investigated the comparative efficacy of ccIIV, IIV, and RIV in healthcare personnel (HCP) with repeated vaccination histories, examining both cellular and humoral immune responses through multiple analytical approaches.

Our investigation employed a multi-faceted analytical framework, combining serological assessments via hemagglutination inhibition (HI) and microneutralization (MN) assays with detailed cellular immune response analysis. We utilized advanced flow cytometry techniques with recombinant hemagglutinin (HA) probes to evaluate both circulating T follicular helper cells (cTfh) and HA-specific B cells, providing a comprehensive view of vaccine-induced immune responses.

The results revealed RIV's superior immunogenicity profile, demonstrating significantly elevated levels of both cTfh and HA-specific B cells compared to ccIIV and IIV. RIV's enhanced performance was particularly evident in its response to influenza A components, with notably higher immunogenicity against both A(H3N2) and A (H1N1) strains. This superiority was reflected in elevated HI titers and markedly increased HA-specific B cell induction. While RIV also demonstrated enhanced HA-specific B cell responses against influenza B components compared to ccIIV, interestingly, HI titers remained comparable across all vaccine groups for these strains.

These findings underscore the critical importance of comprehensive immune response evaluation in vaccine assessment. The disparity between cellular and serological responses, particularly for influenza HA-specific B cells, highlights that traditional serological measures alone may not fully capture the breadth and depth of vaccine-induced immunity. This study provides compelling evidence for the inclusion of cellular immunity assessments in vaccine evaluation protocols, offering crucial insights into vaccine immunogenicity that may be missed by conventional serological analysis alone.

Keywords

Influenza; Healthcare personnel; Vaccine; HA-tags; Circulating follicular T helper cells (cTfh); Recombinant influenza vaccine (RIV); Egg-derived inactivated influenza vaccine (IIV); Cell culture derived inactivated influenza vaccine (ccIIV); Quadrivalent influenza vaccine (QIV)

1. Background

Influenza represents a significant public health challenge, causing substantial morbidity and mortality worldwide, with annual vaccination remaining the most cost-effective strategy for preventing its complications [1]. Despite this approach, traditional egg-based inactivated influenza vaccines (IIVs) offer only moderate protection, with their effectiveness contingent upon multiple factors: the match between vaccine and circulating strains, the specific influenza virus subtype, and the immunological characteristics of vaccine recipients [2]. Healthcare personnel (HCP) represent a particularly vulnerable population due to their heightened occupational exposure to influenza. While annual vaccinations are strongly recommended for this group, mounting evidence suggests diminished vaccine effectiveness among individuals with frequent vaccination histories [3–5]. Studies have demonstrated that repeated vaccinations can attenuate antibody responses to crucial viral components, including hemagglutinin (HA) and neuraminidase (NA) [6–8]. This phenomenon underscores the critical need for both enhanced vaccine immunogenicity and a deeper understanding of newly licensed vaccine alternatives for this high-risk population.

The limitations of conventional egg-based IIVs extend beyond effectiveness concerns. The production process can induce egg-adaptive mutations in HA, creating antigenic disparities between vaccine strains and circulating wild-type viruses [9–14]. To address these challenges, two alternative vaccines have emerged in the United States: cell culture-based inactivated influenza vaccine (ccIIV, Flucelvax™, 15 µg of HA per strain by Seqirus Inc) and recombinant influenza vaccine (RIV, Flublok™, 45 µg of HA per strain by Sanofi Pasteur). ccIIV, produced using chemically inactivated influenza viruses grown in Madin-Darby Canine Kidney (MDCK) cells, minimizes egg-adaptation risks and has demonstrated comparable humoral responses to traditional IIV, with potentially improved effectiveness in adult populations [15–17] and may improve vaccine effectiveness in adults [18,19]. RIV, utilizing recombinant viral vector technology, eliminates adaptation-related concerns entirely while enhancing antigen characteristics and purity [20,21]. RIV has demonstrated superior immunogenicity relative to ccIIV and IIV in healthy adults aged 18–49 (humoral and CD4 T cell responses tested) [22] and in HCP aged 18–64 (humoral response tested) 23–25. Moreover, RIV demonstrated better protection in adults compared to standard dose IIV [18,26,27]. The quadrivalent influenza vaccine contains four critical viral components: two influenza A strains (H1N1 and H3N2) and representatives from both influenza B virus lineages (Victoria and Yamagata). This comprehensive formulation targets the predominant circulating influenza viruses responsible for seasonal epidemics. Individual immune responses to each component vary significantly based on immunological history—previous natural infections create unique “imprinting” patterns, while vaccination history shapes memory B and T cell populations.

HA-specific immunity plays a pivotal role in protection against influenza [28]. As the virus's primary surface glycoprotein, HA comprises head and stem domains, with conventional vaccines primarily inducing neutralizing antibodies against the receptor-binding site (RBS) in the head domain. While hemagglutination inhibition (HI) titers are considered as a widely accepted standard for correlate of protection for IIVs [29], the immune response to the more conserved HA stem region, though typically subdominant and inadequately stimulated by seasonal vaccines, represents a promising target for universal vaccine development due to its cross-subtype conservation [30] and stem-specific antibodies tend to neutralize viruses by inhibiting membrane fusion and provide *in vivo* protection through Fc-mediated functions [31,32].

Contemporary A(H3N2) influenza viruses present unique challenges for traditional serological assessment due to significant evolutionary adaptations in their HA glycoprotein. These viruses have undergone substantial antigenic drift, resulting in altered receptor-binding characteristics that fundamentally compromise the reliability of HI assays. Specifically, mutations in the receptor-binding domain have reduced avidity for sialic acid receptors on red blood cells, rendering the HI assay—once the gold standard for influenza serology—increasingly problematic for accurate antigenic characterization.

In response to these limitations, virus neutralization (VN) assays have emerged as the superior methodological approach for evaluating immunological responses to contemporary A(H3N2) strains. These cell culture-based assays directly measure the functional capacity of antibodies to prevent viral infection, providing a more physiologically relevant assessment of protective immunity. The transition to VN testing represents a critical advancement in influenza surveillance and vaccine effectiveness evaluation, enabling more precise antigenic characterization of circulating strains and more accurate measurement of population immunity against these rapidly evolving pathogens [33,34].

Current research reveals influenza protection involves sophisticated B and T cell networks beyond traditional antibody measures. This complexity has driven a paradigm shift in vaccine assessment, with researchers now developing expanded immunological markers—including cellular immunity, mucosal antibodies, ADCC, and cytokine profiles—in addition to HI and VN titers. This comprehensive approach enables more accurate prediction of vaccine efficacy and guides development of next-generation influenza vaccines offering broader, more durable protection [35,36]. The complexity of the antibody response involves mature B cells, both memory and naive subsets, which upon activation differentiate into antibody-secreting cells (ASCs) and memory B cells (MBCs) [37]. T follicular helper cells (Tfh) are crucial orchestrators of this process, facilitating B cell activation, differentiation, class-switching, and affinity maturation through cytokine production and costimulatory signaling [38]. The discovery that human blood CXCR5⁺ CD4⁺ T cells share functional properties with Tfh cells and represent a circulating memory compartment (cTfh) has provided valuable insights, with multiple studies establishing correlations between cTfh induction and post-vaccination antibody levels [39–43].

Building upon these foundations, our study expands a previous trial focused on serological responses [23] to comprehensively evaluate cTfh and HA-specific B cell responses. Through

this investigation, we aim to compare the cellular and humoral immune responses elicited by conventional standard-dose IIV against novel alternatives, specifically ccIIV and RIV, in a cohort of healthcare personnel with extensive vaccination histories.

2. Results

2.1. Participant characteristics

For the current study 101 participants were selected from a larger clinical study ($n = 727$) that compared the serological responses to traditional egg-based [standard-dose IIV (Fluzone® Quadrivalent by Sanofi Pasteur or Fluarix Quadrivalent by GSK, 15 µg of HA per strain)] vs egg-free influenza vaccines [ccIIV (Flucelvax™ Quadrivalent by Seqirus, Inc., 15 µg of HA per strain) or RIV (Flublok® Quadrivalent by Sanofi Pasteur, 45 µg of HA per strain)] during 2018–19 influenza season in the repeatedly vaccinated HCP [23]. The 101 participants provided blood sample for additional testing for peripheral blood mononuclear cells (PBMC) to assess cell-mediated immune (CMI) responses and the focus of the current paper is B cell and T cell response to vaccination. Among the 101 participants in this study, the demographics across the three vaccine arms-ccIIV ($n = 37$), IIV ($n = 41$), and RIV ($n = 23$), were comparable in terms of age and gender. Specifically, 86–88 % of participants were female, and 49–52 % were within the 45–64 age range (Table 1).

To quantify HA-specific B cell responses to the vaccines via flow cytometry, we employed fluorescently labeled recombinant trimeric H1 and H3 HA (HAΔSA) and HA stem probes and validated them prior to their use (please see methods section). To assess HA-specific B cell responses to the vaccines, we focused on IgG+ B cells, which represent the majority of class-switched memory B cells in circulation, play a crucial role in recall responses [44] and are essential for the protection conferred by inactivated influenza vaccines [28].

The vaccine strains included in the 2018–19 influenza vaccines were A/Michigan/45/2015 (H1N1)-like, A/Singapore/INFIMH-16-0019/2016 (H3N2)-like, B/Colorado/06/2017 (Victoria)-like, and B/Phuket/3073/13 (Yamagata)-like [45]. We used the multiplexing approach to study the responses to all components of the quadrivalent vaccines (H1N1, H3N2 and both IBV lineages), when there was limitation of the clinical material and need to interrogate the high number of cells for detection of low frequency HA-specific cells. The individual immune response to each influenza vaccine component varies markedly based on host-specific factors—particularly immunological history with influenza viruses, pre-existing antibody titers, and demographic characteristics—as well as vaccine-specific properties including antigen design. When optimal conditions converge, B lymphocyte activation triggers two critical protective mechanisms: the rapid differentiation of antibody-secreting cells (ASCs) that produce class-switched, high-affinity neutralizing antibodies providing immediate protection, and the establishment of durable memory B cell populations that enable accelerated responses to subsequent viral encounters. We studied HA-specific B cell response in the context of serological immunity specific for each vaccine component.

2.2. A(H1N1)-specific response to vaccines

Pre-vaccination hemagglutination inhibition (HI) geometric mean titers (GMTs) against the H1N1 cell-grown virus were as follows: ccIIV (27.4, 95 % CI: 16.5–45.7), IIV (46.6, 95 % CI: 29.4–73.7), and RIV (64.6, 95 % CI: 37.7–110.7) (Table 2; Fig. 1A). At one-month post-vaccination (m1), RIV GMTs (147.3, 95 % CI: 101.8–213.2) were significantly higher than those of ccIIV (47.6, 95 % CI: 27.8–81.3) and IIV (61.7, 95 % CI: 38.7–98.5), with statistical significance specifically for RIV versus ccIIV. No significant differences in GMT fold rise (FR) were observed among the vaccine groups at m1 or month six (m6) (Table 2; Fig. 1B). At m1, RIV induced a higher H1 HA-specific IgG+ MBC response (FR 2.5, 95 % CI: 1.7–3.3) compared to IIV (FR 1.5, 95 % CI: 1.2–1.7), with $P = 0.004$ (Fig. 2A). The responses to Group 1 HA stem-specific B cells were comparable across all vaccine types (Fig. 2B).

2.3. A(H3N2)-specific response to vaccines

Pre-vaccination microneutralization (MN) GMTs against the H3N2 cell-grown virus showed no significant differences among the vaccine arms: ccIIV (72.9, 95 % CI: 49.7–107.0), IIV (112.1, 95 % CI: 80.4–156.4), and RIV (90.7, 95 % CI: 55.3–148.8). At m1 and m6 post-vaccination, RIV GMTs were significantly higher than those of ccIIV or IIV (Table 2; Fig. 1A). The GMT FR from pre- to post-vaccination for H3N2 was 8.5 (95 % CI: 4.1–12.9) for RIV, while it was 3.5 (95 % CI: 1.3–5.7) for ccIIV and 1.2 (95 % CI: 1.0–1.5) for IIV (Table 2; Fig. 1B). The RIV group consistently demonstrated a greater induction of H3N2 HA-specific B cells compared to ccIIV and IIV at all time points, except for the RIV versus IIV comparison at day 7 (d7). Specifically, for RIV, the fold rise in cell percentage was 1.6 (95 % CI: 1.2–2.1) at d7, 1.7 (95 % CI: 1.3–2.2) at m1, and 1.4 (95 % CI: 1.1–1.7) at m6 (Fig. 2A). In contrast, ccIIV and IIV showed only slight increases in HA-specific B cells (1.2 with 95 % CI: 0.1–1.4 and 1.3 with 95 % CI: 1.1–1.4, respectively) at d7 post-vaccination. There were no significant differences in B cell responses to Group 2 HA stem among the vaccine groups (Fig. 2B).

2.4. IBV(Victoria)-specific response to vaccines

Pre-vaccination hemagglutination inhibition (HI) geometric mean titers (GMTs) against the B(Victoria) cell-grown virus were similar across the vaccine arms: ccIIV (49.3, 95 % CI: 33.8–71.9), IIV (72.0, 95 % CI: 51.3–100.9), and RIV (60.1, 95 % CI: 45.3–79.6) (Table 2; Fig. 1A). At one-month post-vaccination (m1), GMTs remained comparable among the groups. However, by month six (m6), ccIIV GMTs (17.6, 95 % CI: 10.9–28.4) were significantly lower than those of IIV (50.4, 95 % CI: 35.9–70.8) and RIV (57.5, 95 % CI: 39.9–82.8). Despite these differences in absolute titers, the fold rise (FR) in GMTs from pre- to post-vaccination was comparable across all vaccine types at both m1 and m6 (Table 2; Fig. 1B).

Notably, RIV induced a higher level of B(Victoria) HA-specific B cells at m1 (FR 1.9, 95 % CI: 1.6–2.2) compared to ccIIV (FR 1.6, 95 % CI: 1.4–1.8) (Fig. 2A). In contrast, the frequency of IBV stem-specific B cells in the ccIIV group decreased at d7, m1, and m6 compared to baseline levels (Fig. 2B). Both IIV and RIV demonstrated low-level induction

of IBV stem-specific B cells, with a significant difference observed between the IIV and ccIIV groups at m1.

2.5. IBV(Yamagata)-specific response to vaccines

Pre-vaccination HI GMTs against the B(Yamagata) cell-grown virus were similar across the vaccine arms: ccIIV (98.8, 95 % CI: 71.2–137.2), IIV (100.8, 95 % CI: 73.1–139.0), and RIV (81.3, 95 % CI: 59.9–110.4) (Table 2; Fig. 1A). GMTs and FRs at both m1 and m6 were comparable among the three vaccine types (Table 2; Fig. 1A & B). However, RIV induced a significantly higher level of B(Yamagata) HA-specific B cells at m1 (FR 2.3, 95 % CI: 1.8–2.9) compared to ccIIV (FR 1.4, 95 % CI: 1.1–1.7) (Fig. 2A).

2.6. cTfh response to vaccines

Overall, RIV induced higher HA-specific B cell response and/or HI or MN antibody response in HCP compared to ccIIV and IIV. The magnitude of RIV response was dependent on the vaccine component. H3N2 response demonstrated much higher difference between RIV vs inactivated vaccines (ccIIV and IIV) in B cell and antibody responses. H1N1-specific response followed the pattern of H3N2-specific response but with less significant response of RIV. The HA-specific B cell induction was higher for RIV, however, HI response to IBV components of vaccines was comparable among the vaccine groups. Additionally, we assessed the response of circulating T follicular helper (cTfh) cells (CXCR5+ PD1+ CD4+ B cells) to vaccination, which reflects the overall immune response to all vaccine components (Supplementary Fig. 1B; Supplementary Table 1B). Consistent with the B cell responses findings, RIV elicited a higher cTfh cell response at m1 (FR 1.4, 95 % CI: 1.1–1.8) compared to IIV (FR 1.0, 95 % CI: 0.8–1.1) and ccIIV (FR 1.0, 95 % CI: 0.8–1.2), with the difference reaching statistical significance when comparing RIV to IIV (Fig. 3).

2.7. Correlation between humoral, CMI, and serological responses

Using the HA probes we focused on memory recall B cells expressing HA-specific antibodies and examined the correlation between MBC, cTfh and serological responses. In the RIV group, d7/d0 H3 HA-specific B cell FR correlated with H3N2-specific MN GMTs at m1 and m6 (Fig. 4A–B) ($p = 0.0006$, $r = 0.67$ and $p = 0.0006$, $r = 0.72$), although lower MN titers were observed at m6. Additionally, d28/d0 H3 HA-specific B cell FR correlated with H3N2-specific MN GMTs at m1 ($p = 0.018$, $r = 0.05$). However, no correlations were found between B cell FR and GMTs for other vaccine groups or components, as well as no correlation between cTfh and B cell/serological responses was detected (data not shown).

3. Discussion

Our study compared immune responses in repeatedly vaccinated healthcare personnel (HCP) to three quadrivalent influenza vaccines: egg-free cell culture-derived inactivated influenza vaccine (ccIIV), recombinant influenza vaccine (RIV), and conventional egg-based inactivated influenza vaccine (IIV). The analysis focused on both cell-mediated immunity (CMI) and humoral immunity, specifically examining vaccine-stimulated circulating T follicular helper (cTfh) cells and hemagglutinin (HA)-specific B cells.

One month post-vaccination, we observed distinct patterns in antibody responses across vaccine groups. RIV demonstrated the most robust response compared to other vaccine production platforms, showing significant induction of hemagglutination inhibition (HI) and microneutralization (MN) geometric mean titers (GMTs) across H1N1, H3N2, and B(Victoria) components (Table 2). RIV demonstrated remarkable immunogenicity against multiple viral strains, particularly in generating robust microneutralization geometric mean titers (MN GMTs) against cell-propagated A/H3N2 reference viruses and substantially elevated hemagglutination inhibition geometric mean titers (HI GMTs) against A/H1N1 strains compared to those observed with traditional manufacturing platforms. In contrast, ccIIV showed moderate effectiveness, primarily inducing H1N1 and H3N2 antibody titers, while IIV's response was limited to H1N1 HI GMTs.

RIV demonstrated superior performance over IIV and ccIIV in several key areas. The vaccine showed enhanced HA-specific B cell induction for H1N1, H3N2, B(Victoria), and B(Yamagata), and was unique in its ability to induce CXCR5 + PD1+ CD4+ circulating memory Tfh population. Additionally, RIV exhibited longer persistence of immune response for H3N2, detected at 6 months, and extended response to B lineage components compared to ccIIV. Regarding stem-specific responses, all vaccine platforms showed comparable induction of group 1 and group 2 stem-specific memory B cells, though IIV demonstrated superior IBV stem-specific memory B cell responses compared to ccIIV.

Several factors contribute to RIV's superior immunogenicity. Research suggests the enhanced immune response stems from the quality of recombinant HA rather than antigen quantity. Previous studies showed high-dose IIV (60 µg HA/strain) and standard-dose IIV (15 µg HA/strain) elicited similar responses, while RIV (45 µg HA/ strain) demonstrated superior performance [22,25]. The manufacturing process offers distinct advantages, as RIV uses no inactivation agents, thereby preserving native HA conformation [20] [46].

In contrast, IIV vaccines (Fluzone or Fluarix) and ccIIV (Flucelvax) are split vaccines, produced using different inactivation methods: formaldehyde for IIV [47] and β -propiolactone for ccIIV [48]. The chemical modifications introduced during the inactivation process can affect HA binding and fusion capacities, with effects that may vary by strain [49]. Additionally, RIV consists solely of HA proteins, whereas IIV and ccIIV contain HA along with other viral proteins, such as neuraminidase (NA), nucleoprotein (NP), and matrix protein 1 (M1) [50]. It has been shown that influenza IIV can induce NP-, NA-, and M-specific antibody responses and memory B cells [51]. The immune response to RIV is more "HA-focused," while IIV and ccIIV elicit a more "diffused" response due to the presence of multiple antigens. Perhaps the number of antigens in the vaccines may determine the magnitude of anti-HA response, as shown in the case of Pertussis vaccines [52].

Henry et al. found that RIV-vaccinated individuals produce a higher proportion of B cells that generate antibodies targeting the receptor binding domain compared to those vaccinated with ccIIV [53]. However, both vaccines were poor at inducing HA-stem-specific responses.

In our study, the induction of group 1 and group 2 stem-specific memory B cells was comparable between vaccine groups, although IBV stem-specific memory B cell responses were higher in the IIV group than in the ccIIV group. The accessibility of the stem region in vaccine formulations may be influenced by influenza strain or type [49].

Memory B cell response is a crucial aspect of adaptive immunity, providing a “second wall” of protection following the breach of the “first wall” of pre-existing antibodies produced by long-lived plasma cells. Memory B cells are typically faster to respond and are more efficient than primary responses due to their unique transcriptional profiles, characterized by increased expression of activation-related genes. These cells are clonally expanded, class-switched, and affinity-matured, allowing for rapid adjustments to heterologous antigens. Somatic hypermutations (SHM) in memory B cells can accumulate through repeated exposure to evolving antigens, resulting in a broader repertoire of antigen specificity compared to plasma cells.

Discordance between humoral immunity (serological responses) and memory B cell responses has been documented, with various underlying mechanisms proposed [54,44]. In our study, we observed a correlation between humoral responses (GMT titers) and B-cell responses (fold rise in HA-specific B cells) only for the H3N2 component of RIV which demonstrated a robust B cell and antibody induction compared with other vaccine component/platform combinations. There was no correlation between HI/MN GMTs and corresponding B cell fold rises for any components of ccIIV or IIV. Evaluating the induction of antigen-specific memory B cells in circulation, alongside antibody responses, offers a comprehensive view of vaccine immunogenicity and effectiveness. Further investigation is needed to understand the relationship between CMI memory B cells and serological responses, elucidate molecular mechanisms governing plasma cell survival and functionality, and develop comprehensive evaluation methods combining traditional serological assays with broader immune response measures. These insights will be crucial for developing next-generation influenza vaccines with enhanced effectiveness. The findings underscore the importance of considering multiple aspects of immune response when evaluating vaccine efficacy, suggesting that future vaccine development should focus not only on antibody production but also on generating robust and lasting cellular immunity.

4. Methods

4.1. Clinical samples

This study included a subset of participants from a larger randomized comparative immunogenicity trial evaluating the recombinant influenza vaccine (RIV), cell culture-derived inactivated influenza vaccine (ccIIV), and conventional egg-based inactivated influenza vaccine (IIV) among healthcare personnel (HCP) aged 18–64 years (NCT03722589) [23]. Participants who consented to the main trial were randomized by age stratum (18–49 years and 50–64 years) to receive a single dose of the Northern Hemisphere RIV (45 µg of HA per strain, Flublok™ Quadrivalent by Sanofi Pasteur), ccIIV (15 µg of HA per strain, Flucelvax™ Quadrivalent by Seqirus, Inc.), or IIV (15 µg of HA per strain, either Fluzone® Quadrivalent by Sanofi Pasteur or Fluarix Quadrivalent by GSK) during the 2018–19 influenza season. Following randomization, participants were invited to enroll in

this sub study, which included peripheral blood mononuclear cell (PBMC) collection, until we reached a total of 101 participants, evenly distributed across age strata and vaccine arms (Table 1).

Serum samples were collected just prior to vaccination and at approximately one, and, six months post-vaccination to assess serological immune responses. Whole blood was also collected pre-vaccination and at approximately seven days, one month, and six months post-vaccination to evaluate humoral and CMI responses via flow cytometry. PBMCs were isolated using the BD Vacutainer CPT method (BD Biosciences) and cryopreserved until analysis.

4.2. Microneutralization (MN) assay for human sera

MN assay was performed against H3N2 virus only as HI assay became unreliable due to altered binding characteristics of receptor binding site to the red blood cells. The assay has been described previously [23,34]. Microneutralization assays were performed by Southern Research Institute using CDC protocols after technicians had passed CDC proficiency panels. Sera were heat inactivated at 56 °C for 30 min. Two-fold serial diluted sera were incubated with 100 tissue culture infectious dose per 50 µl of A/Singapore/INFIMH-16-0019/2016(H3N2) virus and incubated at 37 °C 5 % CO₂ for 1 h. The virus-sera mixture was used to infect Madin-Darby Canine Kidney (MDCK) cells, incubated for 18–20 h at 37 °C with 5 % CO₂, and then fixed using cold 80 % acetone in phosphate buffered saline. An ELISA using influenza A nucleoprotein monoclonal antibodies was used to quantify influenza viral proteins. MN titers were defined as the reciprocal of the highest dilutions of serum that resulted in 50 % virus neutralization. The virus was propagated in MDCK-SIAT1 (Millipore Sigma, USA, Cat 05071502). The limit of detection was ≥10 [55].

4.3. Hemagglutination Inhibition (HI) Assay for human sera

The assay has been previously described [23,56]. Hemagglutination inhibition assays were conducted at Battelle Eastern Science and Technology Center, Aberdeen, MD using CDC protocols after technicians had passed CDC proficiency panels for H1N1 and B viruses. Serum samples were treated with receptor destroying enzyme, tested for non-specific agglutinins and adsorbed as needed using washed 0.5 % turkey erythrocytes and cell-grown strains A/Michigan/45/2015 (H1N1), B/Colorado/06/2017 (Victoria lineage), and B/Phuket/3073/2013 (Yamagata lineage) propagated in MDCK-SIAT1 (Millipore Sigma, USA, Cat 05071502). Two-fold serial dilutions were performed starting at 1:10 and the HI titer was defined as the reciprocal of the last dilution of serum that completely inhibited hemagglutination. Serum from all time points was tested on the same plate. All samples were tested in duplicate and the final titer was the geometric mean of the duplicate titers. Samples with results <10 were assigned a titer of 5. If initial duplicate titers were not within one two-fold serial dilution of each other, all test samples from the same individual were repeated in duplicate. The limit of detection was ≥1:10 dilution of sera [57]. HI assays were performed against H1N1 and IBVs.

4.4. Design and construction of recombinant HA probes (except IBV HA stem probe)

The rec HAs expressed as RBS-mutated hemagglutinin (ectodomain or H1 domain) or HA stem domain were codon optimized and produced in the baculovirus-insect cell expression system as trimers (with C-terminal foldon and His-tag) as previously described ([58] (Supplementary Table 2). Secreted soluble trimeric rec HA protein was recovered from the cell culture supernatant by tangential flow filtration through a 30 kDa (HA ectodomain or HA1 domains) or 10 kDa (HA stems) molecular weight cut-off membrane, metal affinity chromatography, and gel filtration chromatography.

Rec H1N1 HAs with Tyr91Phe (Y91F) substitution ([59]) and rec H3N2 HAs with the equivalent Tyr98Phe (Y98F) substitution were expressed as ectodomain to generate HAΔSA probes [42,60–62]. For the IBV HAs we performed a structural analysis with various residues selected for mutation. We expressed and purified B/Colorado/06/2017 constructs and assessed their glycan binding. Microarrays were printed and supplied under a contract with James Paulson at the Scripps Research Institute (La Jolla, CA). We prepared rec HA-antibody pre-complexes of wt and F95A IBV proteins using IBV HA1, a primary mouse anti-penta-His antibody (Qiagen), and a secondary anti-mouse-IgG-Alexa Fluor antibody (ThermoFisher Scientific) in a 4:2:1 M ratio. After 1 h of incubation on ice, we subjected these prepared mixtures to glycan array analysis as described previously ([63]). We found that the F95A substitution abrogated binding to glycan microarrays (Supplementary Fig. 2), and we also introduced it into the B/Phuket/3073/13 construct. We expressed the rec IBV HAs as HA1 trimers.

We designed A/Michigan/45/2015 (group 1) HA stem and A/Singapore/INFIMH-16–0019/2016 (group 2) HA stem based on the GEN-4 construct [64]. We labeled 500 µg of each protein following the manufacturer's protocol. We chemically labeled the probes at primary amines with DyLight dyes 488, 550, 650 (ThermoFisher Scientific, USA, Cat 53024, 84530, 84535) or PerCP-Cy5.5 (Abcam, USA, Cat ab 102911).

4.5. Recombinant IBV HA stem probe

We used a single IBV HA stem probe because of the similarity between the HA stems of Victoria and Yamagata lineages. We prepared an IBV HA stem probe from the B/Brisbane/60/2008 (Victoria lineage) HA-based stem construct BHA10F (Supplementary Table 2) that was based on an earlier design for an H1 mini-stem [65]. We used the native connectivity of HA for the design, and the construct BHA10F contained residues 151–361 of HA1, a four-residue GSAG linker, followed by 3021–3341 of HA1, a seven residue GSAGSAG linker followed by 3842–4632 stretch of HA2 (rcsb.org/structure/4fqm). We incorporated previously characterized [66], low-pH conformation destabilizing mutations, M4172D and L4202D, into the design. To promote the formation of a trimer, BHA10F had the globular, β-rich trimerization motif 'foldon' [67].

The *E. coli* codon-optimized BHA10F were synthesized (GenScript, USA) and cloned into the expression vector pET-28a (+) (Novagen) between *Nde*I and *Hind*III restriction sites. The protein sequence was in-frame with the N-terminal His-Tag from the vector pET-28a (+). Gen-Script USA, Inc. prepared rec IBV HA stem protein by over-expressing in *E.*

coli cells and obtaining from the soluble fraction of the cell lysate using Ni-NTA resin. Endotoxin was measured (4.6 EU/mg (LAL Endotoxin Assay Kit, GenScript, USA). We stored the protein in a buffer (50 mM Tris-HCl, 10 % glycerol, 150 mM NaCl, pH 8.0) at -80°C . 500 mg of protein was chemically labeled at primary amines with DyLight dyes 550 (ThermoFisher Scientific, USA, Cat 84530) following the manufacturer's protocol.

4.6. Immunization of BALB/c mice and sample processing for HA probe validation

To study the effect of RBS mutation on the antigenic characteristics of rec HAs, we immunized 6–8-week-old female BALB/c mice (Jackson Laboratories, Bar Harbor, ME) with A/California/07/09(H1N1) MIV (GSK, 10 $\mu\text{g}/\text{mouse}$) or HA wt or HA Y91F (HA Δ SA) (10 μg , and 1 μg of HA per mouse), as well as PBS (negative control). We immunized animals four times every four weeks. We tested the response by HI against the A/California/07/09 virus three weeks after each vaccination. We purified splenocytes four weeks after the last vaccination by manually grinding the spleen in the complete Roswell Park Memorial Institute (RPMI) medium. We passed the cells through a cell strainer, treated them with 2 ml RBC lysis buffer for 3 min, and neutralized with up to 30 ml of total volume with RPMI supplemented with 10 % Fetal Bovine Serum (FBS). Finally, the cells were collected, resuspended in PBS to detect HA-specific B cells by flow cytometry. To study the binding of sera from MIV-vaccinated mice to Fluorescent-tagged or unaltered HAs, we immunized BALB/c mice with A/California/07/09 MIV(H1N1) MIV (GSK, 10 $\mu\text{g}/\text{mouse}$) or A/Texas/50/2012(H3N2) MIV (GSK, 10 $\mu\text{g}/\text{mouse}$), boosted four weeks later and sera were collected three weeks post-booster immunization for ELISA analysis.

4.7. Assays to validate the probes

The HA Δ SA probes (H1 HA Y91F, H3 HA Y98F and IBV HA F95A Victoria and Yamagata) were validated by different experimental approaches using human PBMCs as well mouse model. We performed flow cytometric analysis of PBMCs stained with wt HA or mutated HA proteins. The RBS mutations effectively minimized non-specific binding of HA probes to B cells (Supplementary Fig. 3). To demonstrate that the RBS Y91F mutation did not alter antigenicity of rec HAs, BALB/c mice were immunized with wild type (wt) HA, HA Δ SA or monovalent influenza vaccine (MIV) of the same viral strain (as positive control) and PBS as negative control. The serological response measured by HI assay and HA-specific B cell response were comparable between wt and mutant HAs (Supplementary Fig. 4). The results were consistent with those of the previous publications [42,62] which demonstrated that H3N2 HA Y98F and the corresponding H1N1 HA Y91F mutations did not alter the antigenicity of rec HA. The HA probes were chemically labeled with fluorescent DyLight dyes. To ensure that fluorescent dye conjugated to HA did not change antigenicity of rec HAs, we compared binding of sera from mice vaccinated with H1N1 or H3N2 to conjugated HA Δ SA vs unconjugated HA Δ SA by ELISA. There was no difference in the binding based on conjugation status (Supplementary Fig. 5). Hence, fluorochrome conjugation did not impact HA protein antigenicity.

For detecting HA stem-specific B cells, we utilized fluorescently labeled group 1 and group 2 HA stem probes [64]. These HA stem probes were tested by Bio-Layer Interferometry (BLI) for binding with the previously characterized stem-specific cross-binding monoclonal

antibodies (mAbs): CR6261 (group 1 HA stem-specific) [68], CR8020 (group 2 HA stem-specific) [69], and CR9114 (group 1 and group 2 HA stem-specific) [70] and Fi6v3 (group 1 and group 2 HA stem-specific) [71] (Supplementary Fig. 6). HIV-specific VRC-01 mAb [72] was used as negative control. The HA stem probes demonstrated the expected affinity, binding only the relevant mAbs. Due to the structural similarities between the HA stems of the Victoria and Yamagata lineages a single HA stem probe was used for detection of IBV HA stem-specific B cell response (see methods section). Testing for probe specificity through dual probe staining (using two HAΔSA of the same specificity conjugated to different fluorochromes) [73] revealed significantly higher percentage of double positive HA-specific cells in IgG+ B cell subset (72.7, 95 %CI: 41.9–103.5) compared to naïve CD27-IgG- B cells (28.5, 95 % CI 5.1–51.9) (Supplementary Fig. 7), consistent with the findings of Koutsakos et al. [42].

Dual HAΔSA probe staining [73] confirmed the specificity of the corresponding HA probes used for staining the clinical samples, with 76–90 % of double-stained IgG+ B cells for H1N1, H3N2, and IBV HAs, and 69–88 % for the HA stem probes (Supplementary Fig. 8). Two antibody panels were developed for staining HA-specific IgG+ B cells: one panel included H1N1, H3N2, and both IBV HAΔSA probes (Panel 1), and the second panel included HA stem probes (group 1, group 2, and both IBV HA stems) (Panel 2) (Supplementary Fig. 1, Supplementary Table 1 A).

4.8. Flow cytometry

Cryopreserved PBMCs were thawed, counted, and stained for flow cytometric analysis. The HA-specific B cell response was assessed through co-staining with recombinant RBS-mutated hemagglutinin probes (HAΔSA) derived from the 2018–19 influenza vaccine viruses (using cell staining panel 1) and HA stem probes (using cell staining panel 2) (Supplementary Table 1 A; Supplementary Table 2; Supplementary Fig. 1 A). T cell responses were evaluated using multi-parametric flow cytometry, including markers for circulating T follicular helper cells (cTfh, CXCR5+ PD1+ CD4+) (Supplementary Table 1B; Supplementary Fig. 1B). The target cell numbers were 2–3 million for each B cell panel and 1–3 million for T cell panel.

Data acquisition was performed using either a BD LSRFortessa or a BD FACSymphony A5, with analysis conducted using BD FACS Diva Software and FlowJo v9. B and T cell responses were presented as fold rise (FR) in the percentage of cells at each time point compared to baseline levels at day 0. To ensure the quality control, we set a cut-off of fewer than 5 HA-specific cells for excluding samples from analysis, addressing potential outliers arising from factors such as the rarity of HA-specific memory B cells in baseline samples, inadequate cell numbers, and variability in HA probe specificity and conjugated fluorochrome.

4.9. ELISA

We coated the Immulon 2 HB 96-well microtiter plates (Thermo Fisher Scientific, USA) with 100 µl of 5 µg/mL labeled or unlabeled A/California/07/09(H1N1) or A/Texas/50/2012(H3N2) rec HAs in phosphate-buffered saline (PBS) at 4 °C overnight. We

washed the plates with wash buffer (PBS-Tween 0.05 %) and blocked them with 4 % bovine serum albumin (BSA) in wash buffer for 1 h at 37 °C. We added a 10-fold serially diluted mouse serum of the corresponding specificity to the plates. After 2 h of incubation at 37 °C, we washed plates and added anti-mouse IgG conjugated to HRP (Invitrogen, USA, Cat 62–6520) at 1:1000 in 100 µl wash buffer. After 1-h incubation at room temperature, we washed the plates. We added 50 µl TMB substrate solution (Thermo Scientific™, USA, Cat ENN301) to wells followed by 50 µl of TMB stop solution (Thermo Scientific™, Cat N600) and immediately read at 450 nm on Bio Tek Synergy 4 (Agilent).

4.10. Viruses

We propagated influenza A/California/07/2007(H1N1) stock in the allantoic cavity of 10-day-old embryonated chicken eggs. We pooled allantoic fluid, clarified by centrifugation, sequenced, titered, aliquoted, and stored at –80 °C until use.

4.11. Hemagglutination Inhibition (HI) assay with mouse sera

We treated the sera collected from immunized mice with receptor-destroying enzyme (RDE, Denka Seiken) at 37 °C overnight. We tested sera against influenza viruses by the standard HI assay using 1 % turkey red blood cells (RBCs). Briefly, we added 25 µl of 1× PBS to wells of a 96-well V-bottom plate (Corning). Next, we added 50 µl of RDE-treated sera to each column and then serially diluted. Then, we added 25 µl of 4 HA units of influenza virus to each well and incubated at room temperature (RT) for 30 min. Finally, we added 50 µl of 1 % standardized turkey RBCs in PBS to each well and incubated at RT for exactly 30 min. We recorded the reciprocal serial dilutions of the sera that showed complete inhibition of hemagglutination as the HI titer.

4.12. Bio-Layer Interferometry (BLI)

The Octet R8 system (Sartorius, NY, USA) was used for to measure binding kinetics of mABs to group 1 or group 2 HA stem rec proteins. HIigK biosensors (Sartorius, Cat 18–5122) were equilibrated with Octet Kinetic Buffer [KB] (Sartorius, Cat 18–1105) for 10 min and loaded with 10 µg/ml of HA stem protein for 200 s followed by baseline in KB for 140 s. The association with 30 µg/ml of mABs was performed for 100 s following by dissociation for 300 s. For negative control, HIV-specific VRC-01 mAb [72] was used. Data were analyzed with Octet Analysis Studio (Sartorius, NY, USA).

4.13. Statistical analysis

Statistical analyses were performed using GraphPad Prism. We utilized one-way ANOVA with Tukey's multiple comparisons test and paired *t*-tests as indicated in the figure legends. Results are reported as geometric means or means with 95 % confidence intervals (CI), and correlation was assessed using Pearson correlation coefficients (*r*).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data availability

Data will be made available on request.

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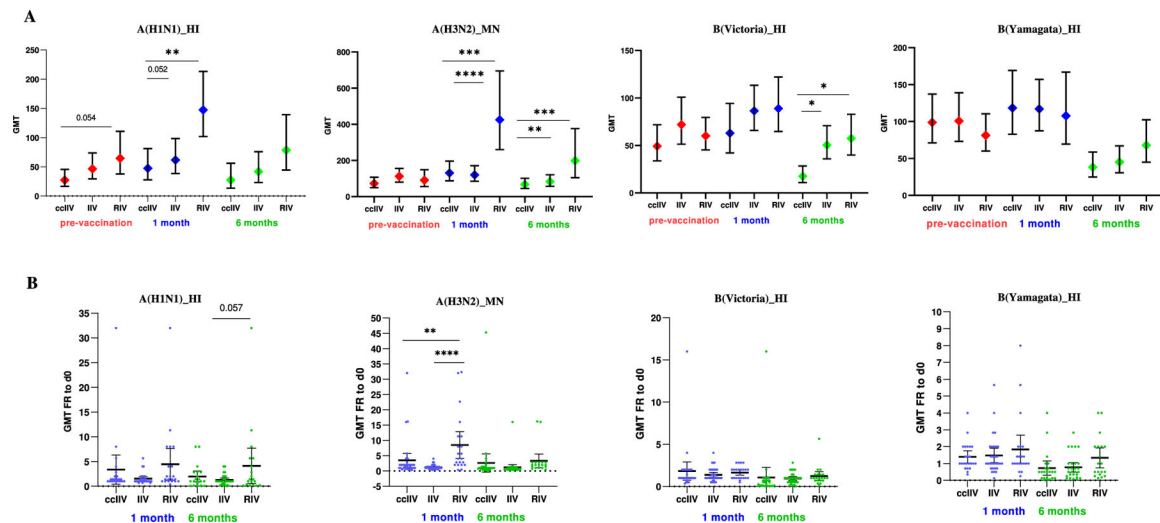


Fig. 1. Antibody response to vaccination by hemagglutination inhibition (HI) or microneutralization (MN) assays.

A. Geometric mean titers (GMTs) and 95 % confidence intervals prior to vaccination, at 1 month and 6 months post vaccination by hemagglutination inhibition (HI) and microneutralization (MN) against the cell-grown vaccine reference virus A/Michigan/45/2015, A/Singapore/INFIMH-16–0019/2016, B/Colorado/06/2017(Victoria), B/Phuket/3073/13(Yamagata). Vaccine types included ccIIV (quadrivalent cell culture-based influenza vaccine, 37 subjects), IIV (egg-based quadrivalent inactivated influenza vaccine, 41 subjects), and RIV (quadrivalent recombinant influenza vaccine, 23 subjects). **B.** The fold rise (FR) in HI GMT and MN GMT means and 95 % confidence intervals at 1 month and 6 months compared to baseline. Statistical analyses were performed using one-way ANOVA with Tukey's multiple comparison test. Statistically significant differences are indicated as $P < 0.05$, $**P < 0.01$, $***P < 0.001$, and $****P < 0.0001$. The limit of detections for HI and MN assays were 1:10.

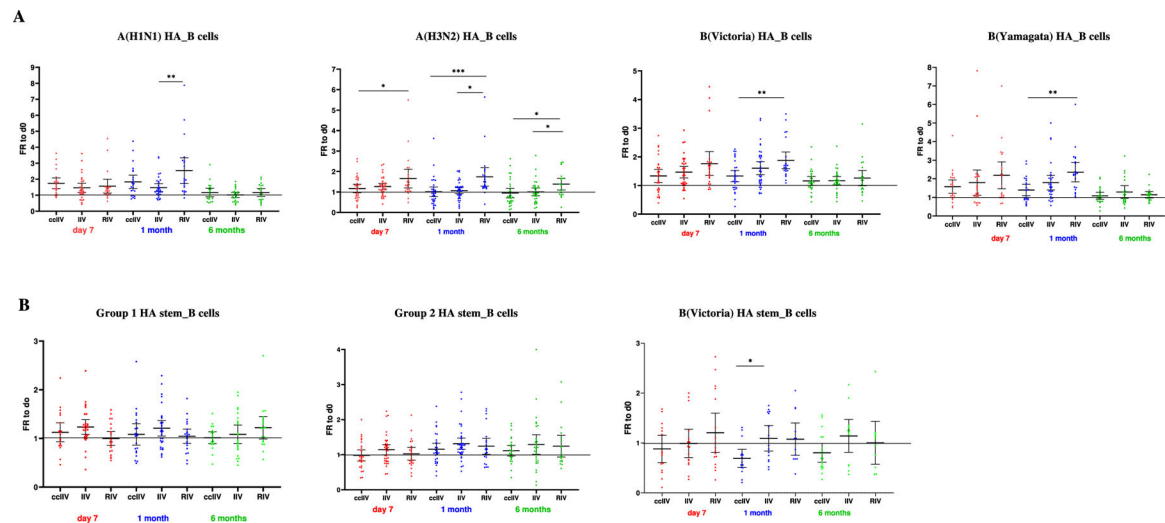


Fig. 2. Influenza virus hemagglutinin (HA)-specific B cell response.

A. HA-specific IgG+ B cell FR means, and 95 % confidence intervals measured by flow cytometry with HA probes at days 7, 1 month and 6 months compared to baseline. **B.** HA stem-specific IgG + B cell FR means, and 95 % confidence intervals measured by flow cytometry with HA stem probes at days 7, 1 month and 6 months compared to baseline. A single IBV HA stem probe was used because of the similarity between the HA stems of Victoria and Yamagata lineages. Statistical analyses were conducted with one-way ANOVA and Tukey's multiple comparison test. Statistically significant differences are indicated as * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Minimum detection limit is set at 5 HA-specific B cells.

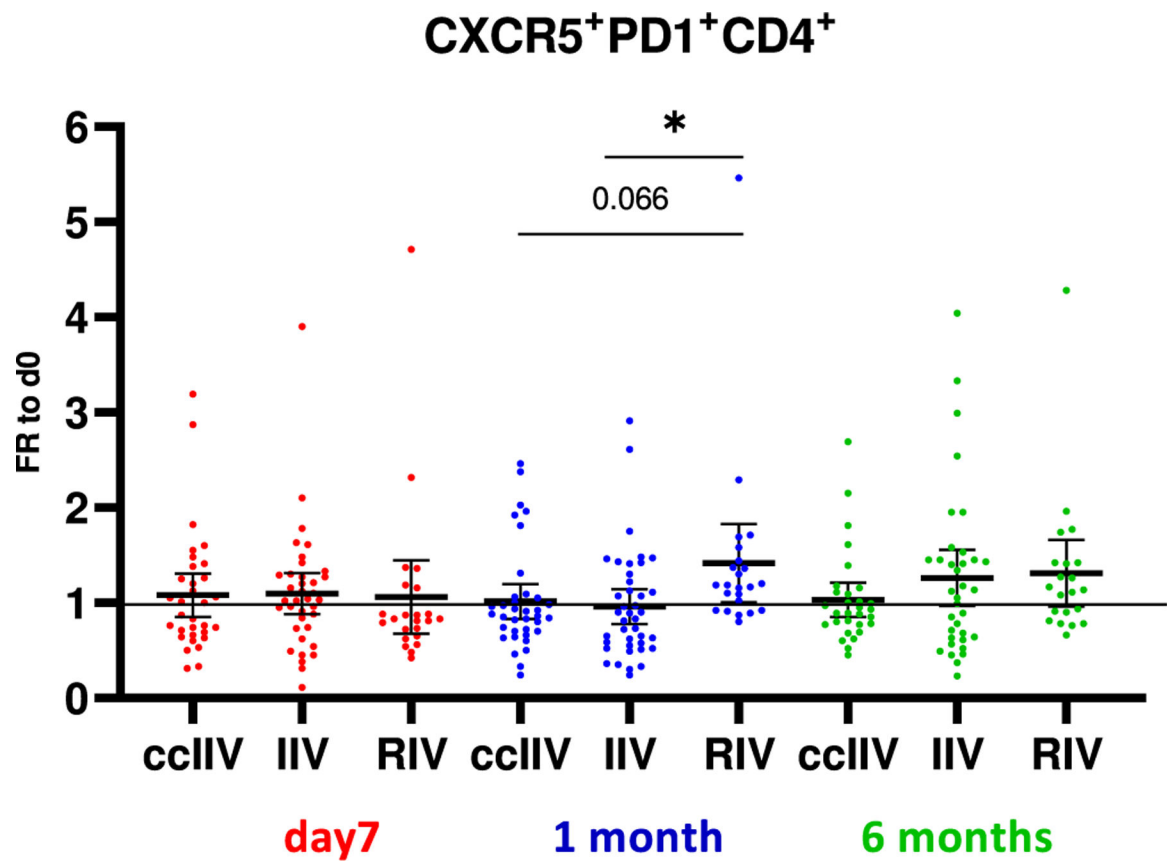


Fig. 3. cTfh response to vaccination.

The fold rise (FR) of circulating T follicular helper cells (cTfh, CXCR5 + PD1+ CD4+) and 95 % confidence intervals at days 7, m1, and m6 compared to pre-vaccination levels are presented. Statistical significance was determined using one-way ANOVA with Tukey's multiple comparison test, with significant differences indicated as $*P < 0.05$.

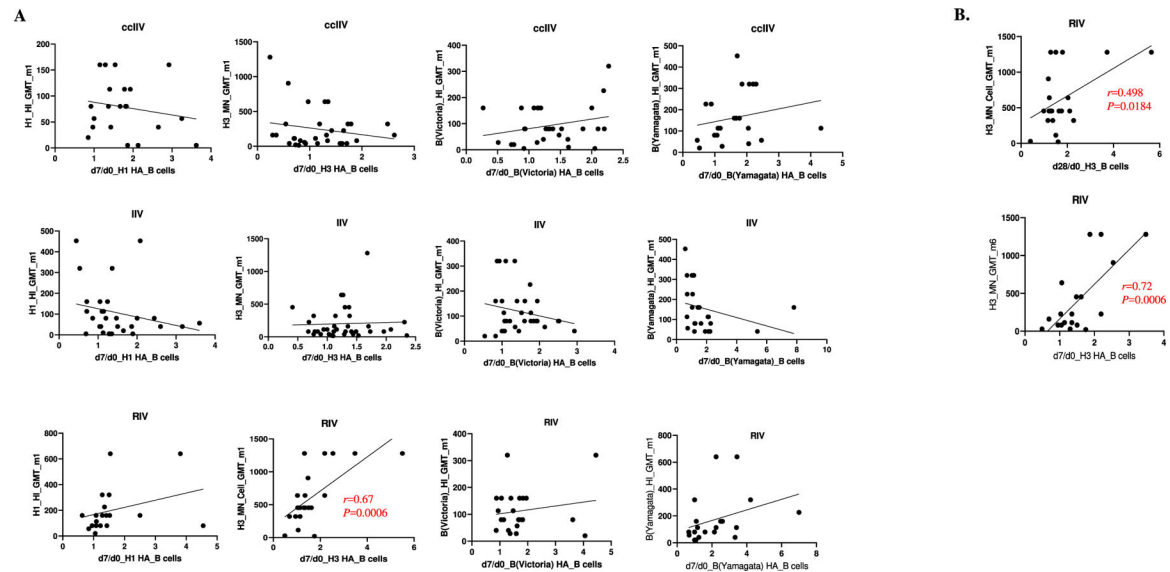


Fig. 4. Correlation between serological and B cell responses.

A. Correlation between hemagglutination inhibition (HI) or microneutralization (MN) geometric mean titers (GMTs) against the cell-grown vaccine reference viruses [A/Michigan/45/2015, B/Colorado/06/2017(Victoria), B/Phuket/3073/13(Yamagata) were used for HI assay and A/Singapore/INFIMH-16-0019/2016 was used for MN assay] at 1 month post vaccination (m1) and the fold rise in the corresponding strain HA-specific B cell at day 7 compared with baseline (d7/d0). **B.** Correlation between MN GMTs against the cell-grown vaccine reference virus A/Singapore/INFIMH-16-0019/2016 at 1 month(m1) or 6 months (m6) post vaccination and the induction of the corresponding strain HA-specific B cell at day 7 or day 28 compared with baseline (fold rise d7/d0 or d28/d0). Correlations are for ccIV, IIV and RIV vaccine groups. Statistical analyses were conducted using simple linear regression. Pearson correlation coefficient, r is indicated for the statistically significant correlations.

Table 1 Characteristics of the study participants.

The participants received a single dose of ccIIV (Flucelvax™ Quadrivalent by Seqirus, Inc., 15 µg of HA per strain) versus standard-dose IIV (Fluzone® Quadrivalent by Sanofi Pasteur or Fluarix Quadrivalent by GSK, 15 µg of HA per strain) versus RIV (Flublok® Quadrivalent by Sanofi Pasteur, 45 µg of HA per strain) during 2018–19 season. The vaccines included A/Michigan/45/2015 (H1N1)-like, A/Singapore/INFIMH-16–0019/2016 (H3N2)-like, B/Colorado/06/2017(Victoria lineage)-like and the B/Phuket/3073/13(Yamagata Lineage)-like virus components.

	<u>ccIIV(n = 37)</u>		<u>IIV (n = 41)</u>		<u>RIV (n = 23)</u>	
	n	%	n	%	n	%
age group, years						
18–44	18	49	21	51	11	48
45–64	19	51	20	49	12	52
age (years), mean (SDEV)	41(12)		43(11)		43(13)	
female	32	86	36	88	20	87

Table 2 Serological response to vaccines.

Antibody levels at baseline, m1 and m6 post-vaccination by HI or MN tests against cell-grown vaccine reference viruses A/Michigan/45/2015(H1N1), A/Singapore/INF16H-16-0019/2016 (H3N2), B/Colorado/06/2017 (Victoria lineage) and B/Phuket/3073/2013 (Yamagata lineage) among recipients of ccIIV, IIV and RIV. One-way ANOVA with Tukey's multiple comparison test to compare GMTs and GMT FR between the vaccines simultaneously. T-test to compare GMT for the same vaccine at different time points. Abbreviations: GMT Geometric mean titer; FR Fold Rise in GMT; CI confidence interval; N/A not applicable.

	ccIIV				IIV				RIV				P-value (Ordinary one-way ANOVA/ Tukey's multiple comparisons test)					
	P-value (t-test)				P-value (t-test)				P-value (t-test)				d0		1 m		6 m	
	1 m	6 m	1 m vs d0	6 m vs d0	1 m	6 m	1 m vs d0	6 m vs d0	1 m	6 m	1 m vs d0	6 m vs d0	IIV vs ccIIV	IIV vs RIV	IIV vs ccIIV	IIV vs RIV	IIV vs ccIIV	IIV vs RIV
Influenza A (H1N1), HI n	22	20			29	21			19	21								
GMT	47.6	27.3	0.003	0.23	42.0	64.6	0.022	0.16	78.6	147.3	0.002	0.62	0.299	0.531	0.054	0.470	0.005	0.212
Lower 95 % CI of GMT	27.8	13.3			23.2	37.7			44.3	101.8								
Upper 95 % CI of GMT	81.3	56.0			75.9	110.7			139.2	213.2								
GMT FR	3.4	2.0	N/A	N/A	1.3	4.4	N/A	N/A	4.1	4.1	N/A	N/A	N/A	N/A	0.123	0.827	0.057	0.244
Lower 95 % CI of GMT FR	0.4	0.9			0.9	1.2			0.6	1.2								
Upper 95 % CI of GMT FR	6.3	3.1			1.7	7.6			7.7	7.6								
Influenza A (H3N2), MN n	34	31			36	22			19	22								
GMT	72.9	131.0	0.014	0.93	83.1	90.7	0.44	0.19	198.9	424.8	<0.0001	0.010	0.361	0.847	0.798	0.894	<0.0001	0.0004
Lower 95 % CI of GMT	49.7	87.2			57.1	55.3			105.0	259.6							0.908	0.001
Upper 95 % CI of GMT	107.0	196.7			120.9	148.8			376.7	695.2								

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	ccIIV				IIV				RIV				P-value (Ordinary one-way ANOVA/ Tukey's multiple comparisons test)											
	P-value (t-test)				P-value (t-test)				P-value (t-test)				d0				1 m				6 m			
	d0	1 m	6 m	1 m vs d0	d0	1 m	6 m	1 m vs d0	d0	1 m	6 m	1 m vs d0	IIV vs ccIIV	IIV vs RIV	IIV vs ccIIV	IIV vs RIV	IIV vs ccIIV	IIV vs RIV	IIV vs ccIIV	IIV vs RIV	IIV vs ccIIV	IIV vs RIV	IIV vs ccIIV	IIV vs RIV
GMT FR		3.5	2.6	N/A	N/A	1.2	1.2	N/A	N/A	8.5	3.3	N/A	N/A	N/A	N/A	N/A	<0.0001	0.0088	0.556	0.368	0.895			
Lower 95 % CI of GMT FR		1.3	-0.3			1.0	0.3			4.1	1.1													
Upper 95 % CI of GMT FR		5.7	5.5			1.5	2.1			12.9	5.5													
B/ Victoria, HI n	30	29	27			36	33			23	21													
GMT	49.3	63.0	17.6	0.081	0.002	72.0	50.4	0.937	0.042	60.1	57.5	0.004	0.157	0.169	0.995	0.561	0.997	0.676	0.024	0.997	0.043			
Lower 95 % CI of GMT	33.8	42.1	10.9			51.3	35.9			45.3	39.9													
Upper 95 % CI of GMT	71.5	94.3	28.4			100.9	70.8			79.6	82.8													
GMT FR		1.8	1.1	N/A	N/A	1.4	0.9	N/A	N/A	1.6	1.2	N/A	N/A	N/A	N/A	0.578	0.844	0.930	0.967	0.838	0.945			
Lower 95 % CI of GMT FR		0.8	-0.1			1.1	0.7			1.3	0.7													
Upper 95 % CI of GMT FR		2.9	2.3			1.7	1.2			2.0	1.7													
B/ Yamagata, HI n	23	23	22			30	28			21	19													
GMT	98.8	118.4	38.2	0.073	0.0003	100.8	45.3	0.800	0.01	81.3	67.9	0.083	0.727	0.285	0.742	0.995	0.941	0.972	0.916	0.277	0.168			
Lower 95 % CI of GMT	71.2	82.9	24.8			73.1	30.6			59.9	45.1													
Upper 95 % CI of GMT	137.2	169.1	58.7			139.0	67.0			110.4	102.4													
GMT FR		1.4	0.7	N/A	N/A	1.5	0.8	N/A	N/A	1.8	1.3	N/A	N/A	N/A	N/A	0.969	0.611	0.508	0.984	0.129	0.115			

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	P-value (Ordinary one-way ANOVA/ Tukey's multiple comparisons test)											
	ccHIV				IIV				RIV			
	P-value (t-test)				P-value (t-test)				P-value (t-test)			
	1 m	6 m	1 m vs 6 m	d0	1 m	6 m	1 m vs 6 m	d0	1 m	6 m	1 m vs 6 m	d0
Lower 95 % CI of GMT FR	1.0	0.3			1.1	0.5			1.0	0.7		
Upper 95 % CI of GMT FR	1.8	1.2			1.9	1.1			2.7	1.9		