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Priming Vaccination With Influenza Virus H5 Hemagglutinin Antigen Significantly Increases the Duration of T cell Responses Induced by a Heterologous H5 Booster Vaccination

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Background. Influenza A(H5N1) virus and other avian influenza virus strains represent major pandemic threats. Like all influenza A virus strains, A(H5N1) viruses evolve rapidly. Innovative immunization strategies are needed to induce cross-protective immunity.

Methods. Subjects primed with clade 1 H5 antigen, with or without adjuvant, and H5-naive individuals were boosted with clade 2 H5 antigen. The impact of priming on T cells capable of both proliferation and cytokine production after antigen restimulation was assessed.

Results. Subjects previously vaccinated with clade 1 H5 antigen developed significantly enhanced clade 2 H5 cross-reactive T cell responses detectable 6 months after vaccination with clade 2 H5 antigen. Priming dose (15 µg vs 45 or 90 µg) had no effect on magnitude of heterotypic H5 T cell responses. In contrast, age at priming negatively modulated both the magnitude and duration of heterotypic H5 T cell responses. Elderly subjects developed significantly less heterotypic H5 T cell boosting, predominantly for T cells capable of cytokine production. Adjuvant had a positive albeit weaker effect than age. The magnitude of $CD4^+$ interferon–γ producing T cells correlated with H5 antibody responses.

Conclusions. H5 heterotypic priming prior to onset of an A(H5N1) pandemic may increase magnitude and duration of immunity against a newly drifted pandemic H5 virus.

Keywords. H5N1; influenza; vaccine; prime/boost; human; clade.

Influenza remains a major world public health problem [1]. Vaccines are the most effective preventive strategies, but new vaccines are required each year because of antigenic drift. Antigenic shifts due to reassortment of viral genomes between human and animal influenza viruses pose major pandemic threats to global health. Therefore, there is interest in developing vaccines that induce broadly cross-reactive immunity.

Rarely, some individuals develop broadly cross-neutralizing antibodies after natural influenza virus infection [2–5]. However, many broadly cross-protective antibodies detected to date show evidence of extensive somatic hypermutation, predicting the need for 50–100 repeated antigenic stimulations for generation. It is unclear whether such antibodies could be induced by conventional vaccination strategies.

T cells can recognize epitopes widely expressed among influenza A viruses. In murine models, T cells provide at least partial protection against widely diverse influenza A virus strains [6–11]. Epidemiological data implicate T cells in protection against pandemic influenza [12–16]. $CD4^+$ T cells release interferon γ (IFN-γ) involved in protective immunity against intracellular pathogens, enhance production of neutralizing antibodies by B cells, and facilitate development of protective $CD8^+$ T cells. $CD8^+$ T cells can directly recognize and destroy virus-infected cells, limiting infection. Therefore, optimal influenza vaccines should target induction of broadly cross-reactive T cells.

The focus of this study was to determine whether priming with an avian hemagglutinin (HA) vaccine derived from an earlier avian influenza H5 strain could enhance T cell responses reactive with heterologous avian influenza H5 strains encountered >1 year later. To explore this possibility, healthy subjects were primed with a clade 1 H5 vaccine and boosted with a clade 2 H5 vaccine. In a separate article, we demonstrated that

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heterotypic priming enhanced hemagglutination inhibition (HAI) antibody responses after boosting with a newly drifted, potentially pandemic strain [17]. In this report, we demonstrate that (1) both heterotypic cross-reactive $CD4^+$ and $CD8^+$ T cell responses were stimulated, (2) the prime-boost regimen led to increased duration of H5-specific T cell responses, (3) the dose of clade 1 H5 priming was not important for heterotypic T cell boosting, (4) age of ≥65 years was associated with a reduced magnitude and duration of heterotypic T cell boosting, (5) inclusion of alum adjuvant during clade 1 H5 priming enhanced heterotypic IFN-γ–producing $CD8^+$ T cell boosting, and (6) T cell boosting correlated with heterotypic cross-reactive antibody responses.

MATERIALS AND METHODS

Vaccinations and Human Samples

Figure 1 presents a Consolidated Standards of Reporting Trials diagram illustrating the Division of Microbiology and Infectious Diseases (DMID) 07/0022/08-0030 trial design (previously described by Winokur et al [17]). Subjects included healthy adults who had participated in 1 of 9 studies of clade 1 H5 priming (DMID-04-063, DMID-05-0090, DMID-04-076, DMID- 05-0015, DMID-05-0127, DMID-05-0141, DMID-06-0089, DMID-04-062 and DMID-06-0052) [18–25]. Subjects in priming studies received 2–3 intramuscular injections 1–6 months apart, containing purified H5 in doses from 3.75 to 90 µg prepared by either Sanofi (subvirion) or Novartis (purified surface antigen) from a clade 1 A(H5N1) virus (A/H5N1/Vietnam/1203/2004), with or without Alhydrogel adjuvant. A subset of volunteers was given placebo in a double-blinded randomized fashion. Subjects who completed the clade 1 trials were recruited, and 517 subjects who had given informed consent were enrolled into the DMID-07-0022 heterotypic H5 booster trial. (364 received Sanofi clade 1 H5 priming, 120 received Novartis clade 1 H5 priming, and 33 received placebo). Subjects in vaccine arms of the priming studies were randomized to receive a single vaccination with 15 or 90 µg of H5 derived from the clade 2 A/H5N1/Indonesia/05/05 virus. Placebo recipients from the clade 1 H5 trials received 2 doses of 15 or 90 µg of clade 2 H5, 1 month apart.

Peripheral blood mononuclear cells (PBMCs) from volunteers enrolled in the clade 2 study were harvested for a cell-mediated immunity substudy (DMID 08-0030), on days 0 (immediately before the first clade 2 vaccination), 28 (1 month after the first clade 2 vaccination, immediately before the second clade 2 vaccination, if given), and 180 (6 months after the last clade 2 vaccination). For the studies reported here, we used a convenience sample of 176 of the 517 subjects, for whom we had matched sets of frozen PBMCs collected on days 0, 28, and 180 to represent different groups based on age, dose of clade 1 priming, inclusion or not of alum adjuvant with the clade 1 priming, and dose of clade 2 boosting. The median time since the last dose of vaccine in the initial clade 1 H5 priming study was 2.72 years (range, 2.25–3.37 years). Supplementary Table 1 presents details of sample sizes for all groups.

T cell Assays of Proliferation and IFN-γ Production

Antigen-specific proliferation and production of IFN-γ by T cells were measured using a CFSE dilution, intracellular cytokine staining (ICS) assay as previously described [26]. CFSE-stained PBMCs were stimulated with purified full-length glycosylated recombinant H5 HA proteins from influenza virus A/Indonesia/05/05 (clade 2 H5) and A/Vietnam/1203/04 (clade 1 H5) that were produced in Sf9 insect cells, using a baculovirus expression vector system (2 µg/mL), FluMist (2010-2011 formulation; 20 µL/mL), a pool containing a combination of 35 class I peptides and 16 class II peptides (peptide pools I and II, respectively) details previously described [26] shown to be highly conserved among influenza A strains (5 µg/mL/each peptide), or rested in medium only for 1 week at 37°C with 5% CO_2. Interleukin 2 (20 U/mL) was added on the fourth day of incubation. After 1 week of in vitro expansion, T cell subsets that had undergone antigen-specific proliferation and produced IFN-γ were assessed by flow cytometry.

After exclusion of dead cells, absolute numbers of $CD4^+$ and $CD8^+$ T cells that were CFSE-low and IFN-γ positive were determined by multiplying viable cell counts after expansion by T cell subset percentages. Figure 2 shows representative dot plot data for $CD4^+$ and $CD8^+$ T cells stimulated with clade 2 H5.

Statistical Analyses

Antigen-specific normalized expansion indices (EIs) were determined by dividing the absolute number of stimulated T cells by the absolute number of rested (unstimulated) T cells. Before normalization, cell counts of 0 were imputed with a value of 0.5. A 2-sided *t* test was used to compare geometric mean log-normally distributed EIs (GMEIs) between naive and primed subjects at baseline. Analysis of covariance (ANCOVA) type III fixed-effects models were used to compare log-transformed EIs (log EIs) between naive and primed subjects on days 28 and 180, with adjustment for baseline log EI. To evaluate effects within the primed group, ANCOVA models with fixed effects for age (19–64 years vs ≥65 years), clade 1 priming dose (15 µg vs 45 or 90 µg), clade 1 priming adjuvant (with alum vs without alum), and interaction terms between all 3 main effects were separately fitted for days 28 and 180, with adjustment for baseline log EI and time in years since the last clade 1 priming vaccination. Models were re-run without the significant interaction terms. Adjusted GMEIs and corresponding 95% confidence intervals (CIs) were used for visualizing effects. The Somers Dxy nonparametric rank correlation measure (mRMRe R package) was used to evaluate associations between T cell expansion index and left-censored antibody response (limit of detection, a titer of 10). For all tests, an individual α

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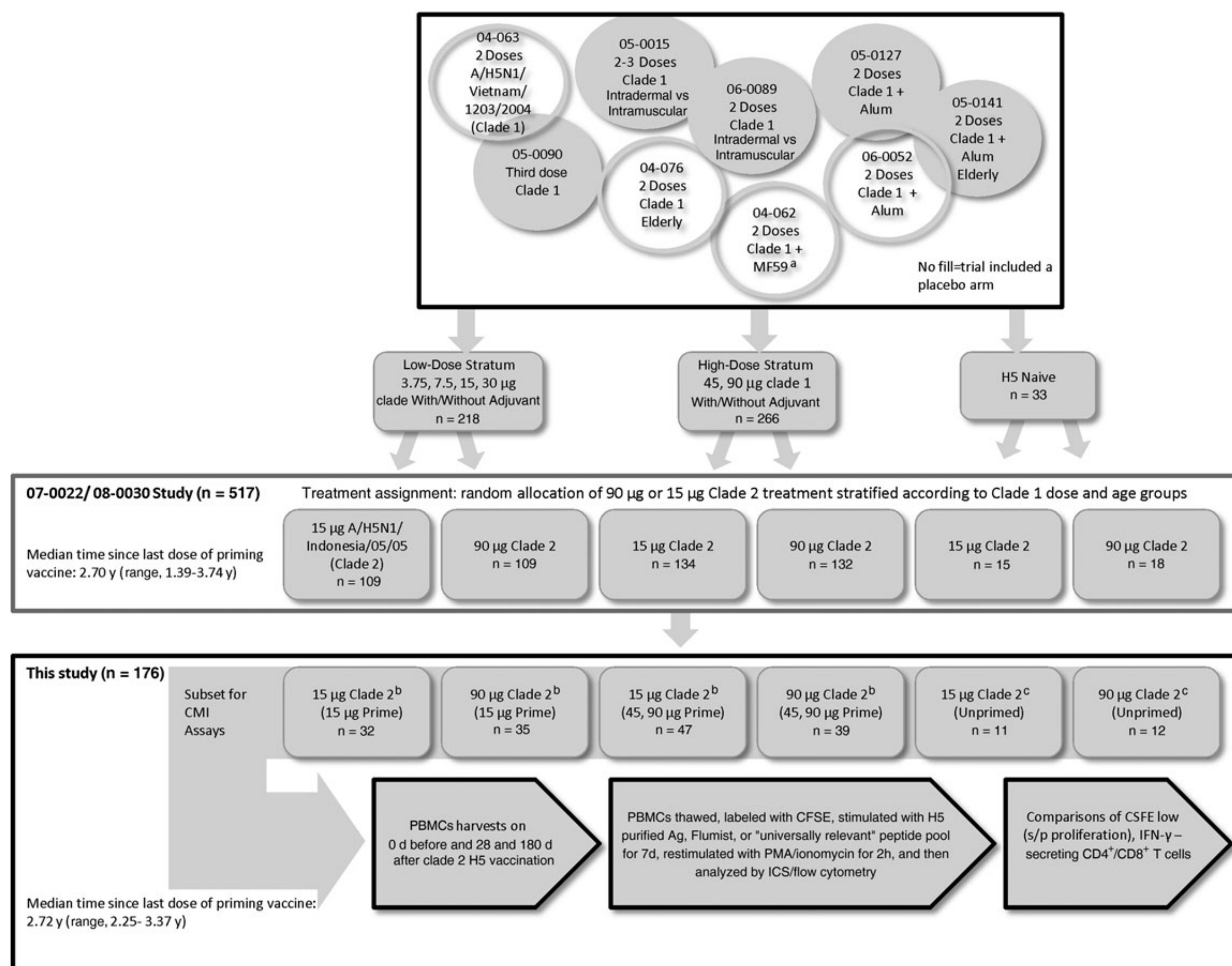

Figure 1. Consolidated Standards of Reporting Trials diagram of study design and experimental tests performed. Subjects who previously participated in one of the 9 listed Division of Microbiology and Infectious Diseases–sponsored clade 1 H5 priming studies were recruited to participate in a clade 2 H5 booster study. Peripheral blood mononuclear cells (PBMCs) were obtained on days 0 (immediately before the first clade 2 vaccination), 28 (1 month after the first clade 2 vaccination), and 180 (6 months after the last clade 2 vaccination). Interferon γ (IFN-γ)–secreting $CD4^+$ and $CD8^+$ T cell responses were evaluated after in vitro stimulation with purified clade 1 H5, clade 2 H5, FluMist (the 2010-2011 seasonal live intranasal influenza vaccine), or a pool of 51 highly conserved influenza virus peptides predicted to be $CD4^+$ and $CD8^+$ T cell epitopes. [a]Subjects in the MF59 clade 1 groups were not included in the CMI subset studied. [b]One clade 2 vaccination (day 0). [c]Two clade 2 vaccinations (day 0 and day 28). Abbreviations: Ag, antigen; CMI, cell-mediated immunity; ICS, intracellular cytokine staining.

level of 5% was used. ANCOVAs adjusting for baseline levels showed that the clade 2 vaccination dose (15 µg vs 90 µg) had no significant effect on T cell responses (Supplementary Figure 1). Thus, low and high clade 2 dose groups were combined for all presented analyses. (Supplementary Data).

RESULTS

Effects of Clade 1 H5 Priming on Clade 2 H5 Boosting of H5-Specific T Cell Responses

We first evaluated cross-reactive heterotypic clade 2 H5-specific T cell responses induced by clade 1 H5 priming by comparing all adult volunteers (aged 19–64 years) given any dose of clade 1 H5 priming to those given placebo prior to clade 2 H5 vaccination. We focused on this age group because of the small number of older subjects (n = 3) who were not primed, coupled with the fact that we found age differences in the primed group. Descriptive statistics of absolute numbers of clade 2–stimulated and rested (unstimulated) T cells by cell type, priming group, and time point are summarized in Supplementary Table 2. GMEIs are provided in Supplementary Table 3. Prior to clade 2 vaccination (day 0), clade 1 H5–primed individuals had 1.7–1.8-fold higher $CD4^+$ and 1.3–1.5 fold higher $CD8^+$ GMEI T cell responses as compared to unprimed individuals, although these differences did not achieve statistical significance. These results indicate that heterotypic H5-specific T cell responses remained increased for 2–3 years after clade 1 H5 priming. To control for T cell responses detectable prior to vaccination with clade 2 H5, we compared day 28 and 180 responses following clade 2

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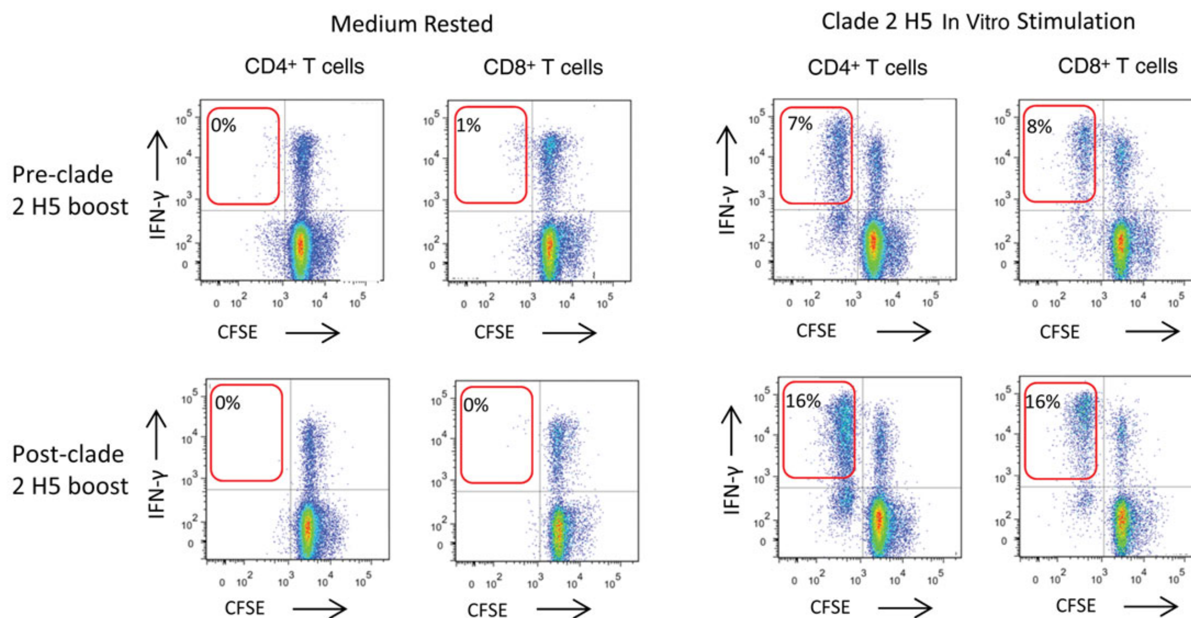

Figure 2. Representative fluorescence-activated cell-sorting dot-plot data from CFSE-dilution, intracellular cytokine staining, cell-mediated immunity assay. $CD4^+$ and $CD8^+$ T cells are shown following 7 day in vitro incubation in medium alone (left column) or stimulation with purified clade 2 H5 (right column). Prevaccination results are shown in the top row, and postvaccination results are shown in the bottom row. The dots and percentages shown in the upper left quadrant of each image (outlined in red) represent $CD4^+$ or $CD8^+$ T cells that have proliferated (as indicated by CFSE dilution) and have produced interferon γ (IFN-γ).

boosting, after adjustment for baseline values, using ANCOVA models. Adjusted GMEIs and 95% CIs for both $CD4^+$ and $CD8^+$ T cells are shown in Figure 3. For $CD4^+$ T cells, at day 28 after clade 2 H5 vaccination, clade 1–unprimed GMEI responses were higher than in the primed group, but the difference was not statistically significant. Most impressively, at day 180 after clade 2 H5 vaccination, the GMEI for both $CD4^+$ (the IFN-γ–producing subset) and $CD8^+$ (the total proliferating and IFN-γ–producing subsets) T cell responses were significantly higher (by 2.5-, 2.3-, and 3.5-fold, respectively; $P < .05$, by ANCOVA) in volunteers who had received clade 1 H5 priming 2–3 years earlier (Table 1).

Factors That Modulate the Effect of Clade 1 H5 Priming on Clade 2 H5 Boosting

Next, we assessed the impact of age at baseline (19–64 years vs ≥65 years), adjuvant given with clade 1 priming vaccine (alum vs none), and clade 1 priming vaccine dose level (15 µg vs 45 or 90 µg) on modulating heterotypic T cell priming effects. ANCOVA results for the primed group, adjusted for baseline T cell responses and time since last clade 1 priming vaccination, showed no statistically significant interactions between age, adjuvant, and clade 1 priming dose effects for day 28 or day 180 following clade 2 vaccination. After refitting the models without interaction terms, age had a significant effect on heterotypic T cell responses 180 days after clade 2 boosting, with significantly lower $CD4^+$ and $CD8^+$ T cell responses ($P < .05$, by ANCOVA) in the older age group (Figure 4 and Supplementary Table 4). The effect was more pronounced for $CD4^+$ and $CD8^+$ T cells that proliferated and produced IFN-γ ($P < .01$, by ANCOVA), with 2.7- and 2.9-fold lower adjusted GMEIs, respectively, for the older age group.

In contrast to age, the use of alum adjuvant had a positive impact on T cell responses. In all cases, adjusted GMEIs of T cell responses were higher in subjects primed with alum adjuvant than in those who did not receive an adjuvant (Supplementary Figure 2). On day 180 after heterotypic clade 2 H5 boosting, the inclusion of alum adjuvant during clade 1 H5 priming had a statistically significant enhancing effect ($P = .033$, by ANCOVA) on $CD8^+$ T cells that proliferated and produced IFN-γ with a 2-fold higher GMEI (Supplementary Table 4). The difference in adjusted GMEIs for IFN-γ–producing $CD4^+$ T cells also was marginally statistically significant ($P = .0503$, by ANCOVA), with 1.9-fold higher levels in the adjuvanted group.

We next explored whether the priming dose of clade 1 H5 was important for maximal heterotypic T cells against clade 2 H5. Adjusted GMEIs of T cell responses were similar for the clade 1 low and high dose priming for both $CD4^+$ and $CD8^+$ T cell responses (Supplementary Figure 3). This finding was consistent with the corresponding ANCOVA results showing no significant effects of clade 1 dose on heterotypic boosting (Supplementary Table 4).

Effects of Clade 1/Clade 2 H5 Prime/Boosting on Universally Relevant Influenza Virus–Specific T Cells

We next investigated $CD4^+$ and $CD8^+$ T cell responses in the clade 1 H5–primed and naive groups (aged 19–64 years)

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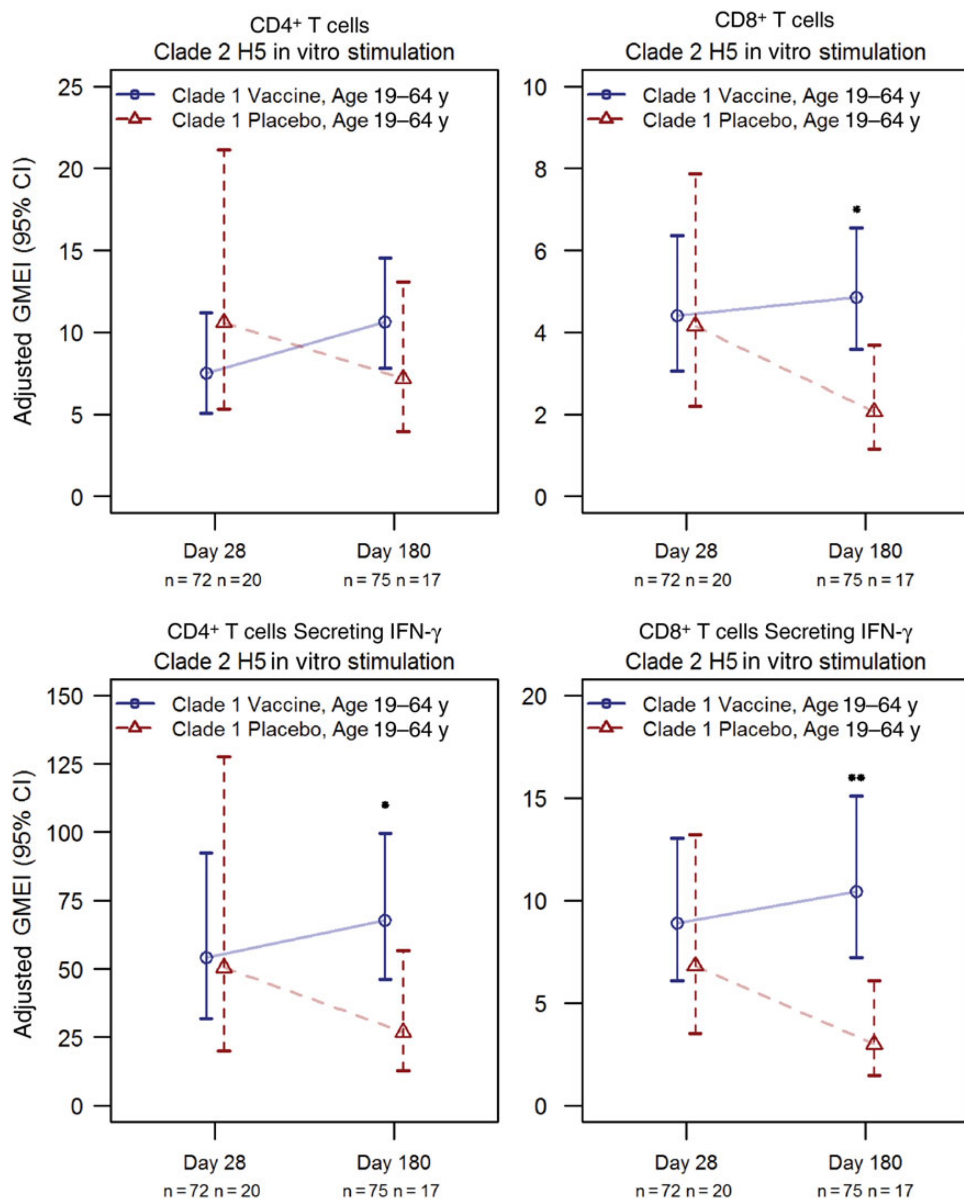

Figure 3. Clade 1 H5 priming resulted in significantly increased cross-reactive heterotypic $CD4^+$ and $CD8^+$ T cell responses to in vitro clade 2 H5 stimulation 6 months after clade 2 H5 boosting. Comparisons of $CD4^+$ and $CD8^+$ T cell analysis of covariance (ANCOVA)–adjusted geometric mean expansion indices (GMEI) between subjects 19–64 years of age who were or were not primed with clade 1 H5 to in vitro stimulation with purified clade 2 H5 are shown. GMEIs for all T cells that proliferated in response to in vitro clade 2 H5 stimulation are shown at the top and those that proliferated and produced interferon γ (IFN-γ) at the bottom for days 28 and 180 after clade 2 H5 vaccination. GMEIs for each postvaccination day and group were adjusted for baseline T cell responses, using ANCOVA. *$P < .05$ and **$P < .01$, by ANCOVA, for differences between groups that did and those that did not receive clade 1 H5 priming. Abbreviation: CI, confidence interval.

directed against highly conserved epitopes present in almost all influenza A virus strains. Before clade 2 boosting, peptide pool I and II responses in IFN-γ–producing $CD4^+$ T cells were significantly higher ($P = .034$, by the t test) for the clade 1 primed group as compared to the naive group, with a 2.6-fold higher GMEI for the primed group. The increase in baseline levels for the other primed T cells ranged from 1.08-fold to 1.6-fold as compared to the naive group, but these differences were not statistically significant. ANCOVA results accounting for baseline T cell responses did not show any significant differences between primed and naive groups on days 28 or 180 after clade 2 boosting (Supplementary Figure 4).

Correlations Between Heterotypic Antibody and T cell Responses

Antibody responses induced by this clade 1/clade 2 H5 prime/boost trial are reported separately [17]. Overall, clade 1 priming was shown to enhance both HAI and microneutralization (MN) responses specific for the clade 2 H5–expressing viruses. We

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Table 1. Significant Analysis of Covariance Results to Evaluate Effects of Clade 1 Priming on Clade 2 H5 Boosting of H5-Specific T cell Responses

Cell Type	Time After Clade 2 H5 Boost, d	T	*P*	Clade 1 H5, Any Dose,[a] Age 19–64 y		Clade 1, Placebo, Age 19–64 y	
				No.	Adjusted GMEI[b] (95% CI)	No.	Adjusted GMEI[b] (95% CI)
IFN-γ producing $CD4^+$ T cells, CFSE low	180	2.18	.0320	75	67.7 (46.1–99.6)	17	26.8 (12.7–56.6)
$CD8^+$ T cells, CFSE low	180	2.60	.0110	75	4.9 (3.6–6.6)	17	2.1 (1.2–3.7)
IFN-γ producing $CD8^+$ T cells, CFSE low	180	3.10	.0027	75	10.4 (7.2–15.1)	17	3 (1.5–6.1)

Abbreviation: IFN-γ, interferon γ.

[a] Clade 1 H5 priming doses; 15 µg vs 45 µg or 90 µg.

[b] Data denote geometric mean expansion indices (GMEIs) of H5-specific T cells elicited after stimulation with clade 2 H5 in peripheral blood mononuclear cells collected 180 days after clade 2 H5 vaccination.

investigated whether T cell responses reported for primed subjects (aged 19–64 years) were correlated with the magnitude of the antibody responses. Statistically significant correlations between T cell and antibody responses were observed on day 180 (Figure 5). IFN-γ–secreting $CD4^+$ T cell responses were weakly (d_{xy}, 0.34–0.38) but significantly ($P < .05$) positively correlated with findings of MN assays done using clade 1 H5–expressing viruses. Antibodies against clade 2 H5–expressing viruses also showed a trend for positive correlation with $CD4^+$ IFN-γ responses (d_{xy}, 0.4–0.42) but did not reach statistical significance ($P = .055–.108$; Figure 5). Interestingly, only the $CD4^+$ IFN-γ T cells induced by in vitro stimulation with seasonal live attenuated influenza vaccine (FluMist) and the $CD4^+$ IFN-γ T cells induced by the highly conserved, universally relevant T cell epitope pool (peptide pools I and II) were significantly correlated with H5-specific MN responses. There were no significant correlations detected between the T cell responses induced in vitro by purified H5 antigens and either HAI or MN responses at day 28 or 180.

DISCUSSION

Earlier reports demonstrated that priming with clade 1 H5 enhanced the HAI and MN antibody responses against both viruses [17]. We found that heterotypic H5 prime/boosting also enhanced cross-reactive $CD4^+$ and $CD8^+$ T cells. Responses in the primed group were most reproducibly enhanced 6 months after boosting. The fact that the primed group received a single dose of clade 2 vaccine, while unprimed subjects received 2 doses, could potentially confound interpretations of the effects of priming on T cell responses. However, this difference would result in a bias against seeing effects due to priming and therefore strengthens our conclusions that clade 1 priming enhances T cell responses to clade 2 vaccine.

The priming dose of the clade 1 H5 vaccine was not important for clade 2 H5–specific boosting effects on either T cell or antibody responses. This lack of dose dependency on priming would allow for potential antigen-sparing effects should this strategy be used for influenza pandemic preparedness. The enhanced cross-reactive $CD4^+$ T cells may have contributed helper effects for both protective antibody and $CD8^+$ cytotoxic T-lymphocyte (CTL) responses, allowing them to develop more rapidly after the H5 clade 2 boost, both of which could be key for more-rapid protection against a newly drifted pandemic strain. Influenza virus strain–specific antibody responses are well known to protect against initial viral infection, as well as secondary viral spread. $CD8^+$ CTL responses could identify infected cells and prevent further amplification of viral infection, limiting morbidity and mortality associated with infection.

Immunosenescence has been shown to occur after the age of 65 years [24–27] and has been reported to affect both T and B cells. Therefore, it is not surprising that ANCOVA analyses identified significant age effects on boosted H5-specific T cell responses, particularly for IFN-γ–producing subsets. These results suggest that H5 prime/boost regimens are likely to be less effective in elderly populations. We were unable to directly address this conclusion because only 3 subjects ≥65 years of age received placebo instead of clade 1 priming. Even if immunosenescence impairs heterotypic T cell prime/boosting, multiple vaccine priming doses and/or adjuvants may compensate.

The mechanisms of adjuvant function include prolonging antigen presentation by more-sustained release and activation of inflammatory pathways sensed as danger by the immune system, resulting in increased antigen processing and presentation, as well as immune-enhancing cytokine production [27, 28]. However, the aluminum hydroxide adjuvant used with clade 1 H5 priming in this trial has been shown to primarily induce $CD4^+$ T-helper type 2 (Th2) cells producing IL-4, rather than $CD4^+$ Th1 cells producing IFN-γ [29–31]. Th2 cells help antibody responses develop but are usually not associated with direct protective effects against intracellular pathogens, and therefore it is somewhat surprising that the alum adjuvant was associated with significant enhancement of IFN-γ–producing $CD8^+$ T cells known to protect against intracellular pathogens. It is possible that this prime/boost strategy focuses on the enhancement of cross-reactive memory T cells that have already developed an imprinted Th1/Tc1 differentiation program. At least in murine models, the induction of $CD4^+$ Th1 cells has been associated with a stable Th1 memory profile capable of responding years

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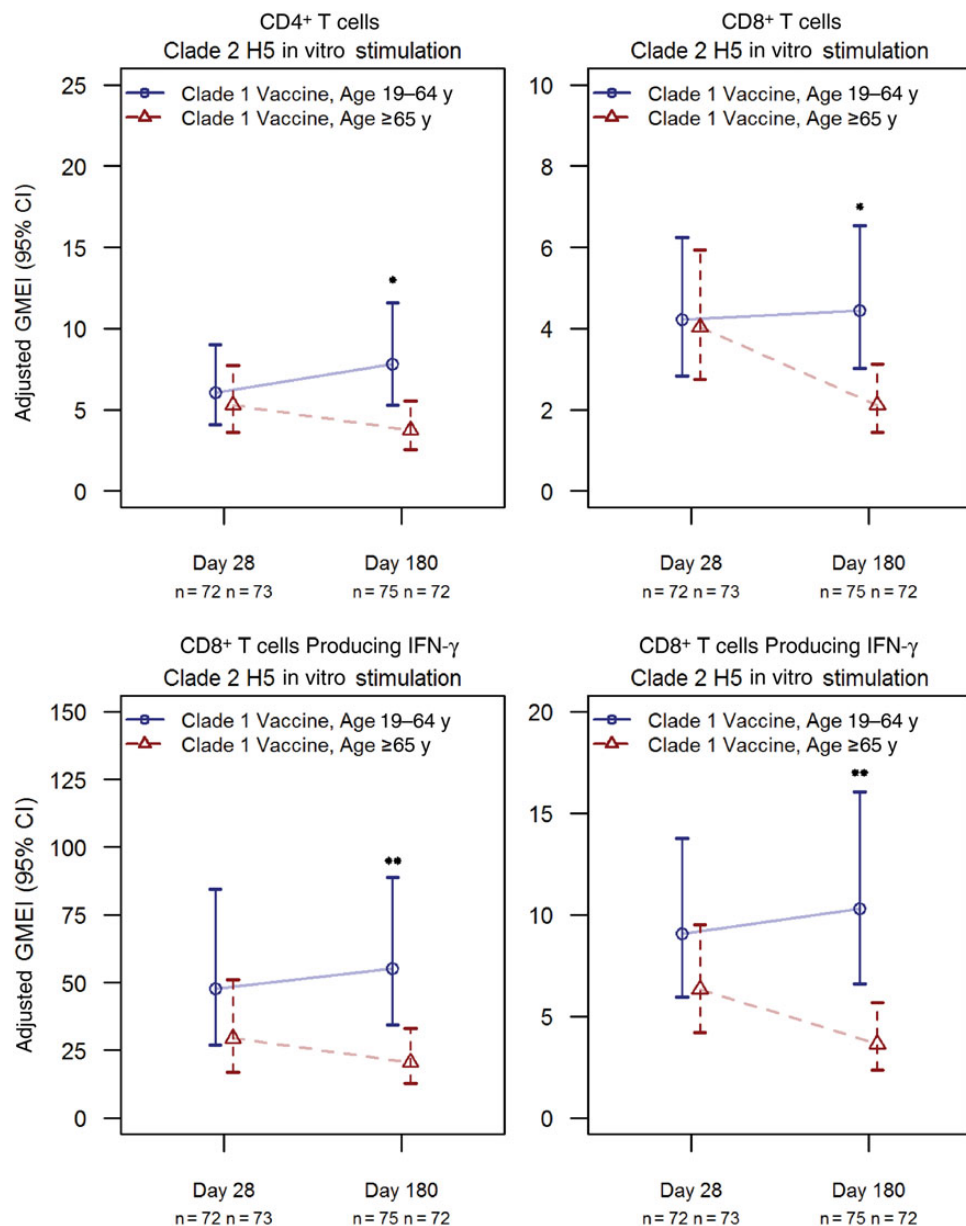

Figure 4. Effect of age on CD4+ and CD8+ T cell responses to in vitro clade 2 H5 stimulation following clade 1/clade 2 H5 prime/boosting. Comparisons of analysis of covariance (ANCOVA)–adjusted CD4+ and CD8+ T cell geometric mean expansion indices (GMEIs) elicited by purified clade 2 H5 in subjects ≥65 years of age versus subjects 19–64 years of age who were primed with clade 1 H5 are shown. GMEIs for T cells that proliferated in response to in vitro clade 2 H5 stimulation are shown at the top and values for those that proliferated and produced IFN-γ at the bottom for days 28 and 180 after clade 2 H5 vaccination. GMEIs for each postvaccination day and group were adjusted for baseline T cell responses, time since last clade 1 priming dose, clade 1 adjuvant, and clade 1 dose, using ANCOVA. *$P < .05$ and **$P < .01$, by ANCOVA, for differences between age groups. Abbreviation: CI, confidence interval.

after the initial T cell induction [32]. It is highly likely that other, more-classical Th1 and CD8+ T cell–inducing adjuvants (eg, MF59 or ASO3) would be even more effective in future heterotypic influenza prime/boosting strategies.

The major antigenic target for prime/boosting of immune responses in this trial focused on inducing antibody and T cell responses shared between heterotypic H5 antigens derived from clade 1 and clade 2 A(H5N1) viruses. However, we thought it was of interest to also determine whether this strategy could induce T cells directed against highly conserved influenza virus epitopes present in internal antigens from virtually all influenza A viruses. Indeed, 2–3 years after priming with clade 1 H5, IFN-γ–producing CD4+ T cells reactive with a pool of highly conserved T cell epitopes encoded by the influenza virus nucleoprotein and matrix peptides were significantly increased (by 2.6-fold) prior to clade 2 vaccination. After clade 2 vaccination, no further clade 1 priming effects for CD4+ or CD8+ T cell responses were observed. Bioinformatic analyses predicted that

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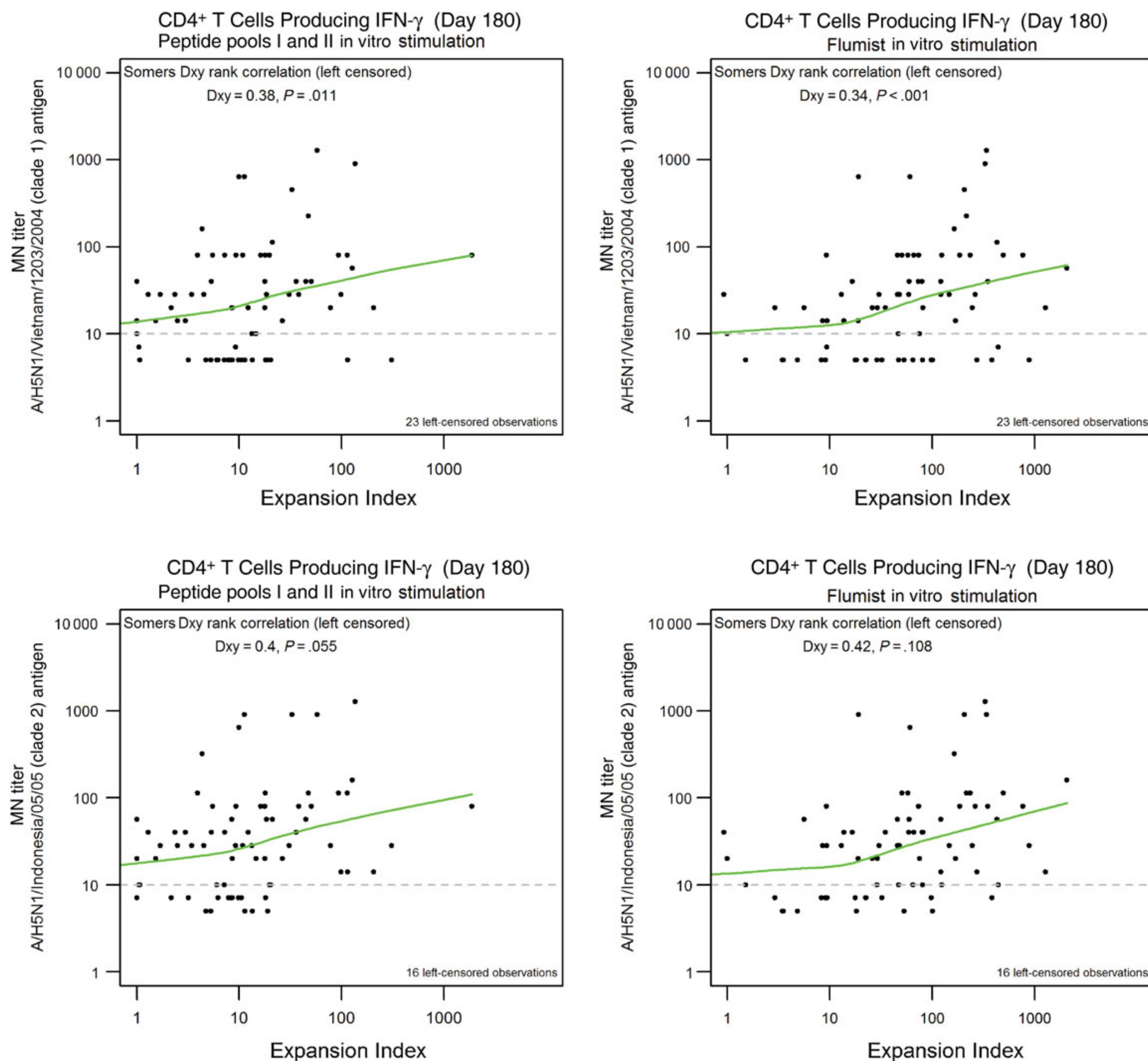

Figure 5. Correlation between interferon γ (IFN-γ)–producing CD4+ T cell responses and neutralization titers to clade 1 and clade 2 antigens 6 months after clade 2 boost. Figure 5 shows correlations between IFN-γ–producing CD4+ T cell responses and microneutralization (MN) assay responses for specimens collected 180 days after clade 2 H5 vaccination. The left panels show proliferating CD4+ and IFN-γ–producing T cell responses induced by highly conserved epitopes (peptide pools I and II) in vitro versus MN responses reactive with H5N1 (clade 1 and 2 in top and bottom panels, respectively). The panels on the right show CD4+ T cell responses (both proliferating and IFN-γ producing) induced by FluMist in vitro versus MN responses reactive with H5N1 (clade 1 and 2 in top and bottom panels, respectively). Results of Somers Dxy nonparametric rank correlation analysis between T cell responses and left-censored MN responses are shown at the top left of each panel. The dashed lines indicate the limits of detection (LODs) for the microneutralization assays (ie, a titer of 10). Antibody titers represent geometric means of technical replicates after imputing half the LOD for replicate values below the LOD. The dashed lines indicate the cutoff for left-censored observations (those for which the geometric mean of technical replicates is less than the LOD). The green lines represent locally weighted regression fits.

these peptides could be the targets of T cell immunity in virtually all human populations, regardless of major histocompatibility complex allele expression [26]. These results support our current efforts to develop T cell–based vaccines using these highly conserved T cell epitopes which have the potential to induce at least partial protection against future influenza pandemics. However, the mechanism responsible for enhancing T cells against these epitopes is unknown. The H5 vaccines were prepared with whole inactivated virus and are enriched for HA antigen. Variable levels of contamination with internal viral proteins occur. Therefore, either or both of the clade 1 and clade 2 H5 vaccines may have contained the nucleoprotein and matrix antigens capable of inducing recall responses in broadly reactive memory T cells. Perhaps the H5 immunological naiveté biased for induction of memory T cells reactive with these minor internal contaminants because they were the major

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targets of previously induced influenza virus–specific memory T cells. Another potential mechanism is bystander enhancement of memory T cells due to T cell activating cytokines produced by H5-specific T cells. A similar phenomenon, termed "epitope spreading," has been reported during autoimmune disease progression [33–35]. These possible mechanisms are not mutually exclusive but remain speculative at this point, requiring future investigation.

As shown in Figure 5, we identified significant correlations between the T cell and antibody responses against clade 1 H5–expressing viruses induced in these trials. Surprisingly, H5-specific antibody responses were found to correlate with T cells reactive with conserved epitopes present within internal influenza virus proteins, as well as whole live attenuated viruses, but not purified H5. These results may be at least partially due to mechanisms similar to those proposed above to explain the enhanced universally relevant T cell responses. These correlations could represent a parallel development of broadly reactive antibody and T cells, directed against highly conserved HA stem epitopes (antibody responses) and contaminating internal proteins (T cell responses), respectively. Antibody responses induced by H5 clade 1 and 2 heterotypic prime/boost regimens should be studied for HA head-specific versus stem-specific reactivity to address this possibility.

Investigators at Baylor also studied T cell responses in subjects from the same study [36]. Instead of using a CSFE dilution/ICS, they used ELISPOT and cytokine secretion assays. Overall, results of the 2 studies are similar in demonstrating that the H5 heterotypic prime/boosting strategy enhanced cross-reactive T cell responses. However, their analyses did not find enhancing effects of alum adjuvant or negative effects of advanced age [36]. The 7-day restimulation period used in our study is optimized to detect memory T cells with the capacity for both proliferation and IFN-γ production. The direct ex vivo cytokine secretion assays identify actual resting frequencies of antigen-specific memory T cells but do not address proliferative capacity, which is critically important for long-term immunity induced by prophylactic vaccines. Relevant populations of protective T cells ideally must expand in response to pathogenic challenge to provide optimal immunity. Therefore, the CSFE dilution/ICS assay may provide a more sensitive assessment of the overall protective capacity of memory T cells, and it is possible that the expansion capacity of T cells was more affected by age and adjuvant.

Our results provide additional support for the rationale for heterotypic HA prime/boosting designed to more rapidly induce protection against novel pandemic influenza virus strains. However, the true importance of the cross-reactive T cell responses induced can only be determined in phase 3 efficacy studies, where it will be possible to look for correlations between T cell responses and protection against influenza virus infection and disease.

Supplementary Data

Supplementary materials are available at http://jid.oxfordjournals.org. Consisting of data provided by the author to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the author, so questions or comments should be addressed to the author.

Notes

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Potential conflicts of interest. R. B. B. has served as a consultant to MedImmune and speaker for MedImmune, Sanofi and Merck. All other authors report no potential conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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