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High-dose influenza vaccine favors acute plasmablast responses rather than long-term cellular responses

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Abstract

High-dose (HD) influenza vaccine shows improved relative efficacy against influenza disease compared to standard-dose (SD) vaccine in individuals ≥ 65 years. This has been partially credited to superior serological responses, but a comprehensive understanding of cell-mediated immunity (CMI) of HD vaccine remains lacking. In the current study, a total of 105 participants were randomly administered HD or SD vaccine and were evaluated for serological responses. Subsets of the group (n=12–26 per group) were evaluated for B and T cell responses at days 0, 7, 14 and 28 post-vaccination by flow cytometry or ELISPOT assay. HD vaccine elicited significantly higher hemagglutination inhibition (HI) titers than SD vaccine at d28, but comparable titers at d365 post-vaccination. HD vaccine also elicited higher vaccine-specific plasmablast responses at d7 post-vaccination than SD vaccine. However, long-lived memory B cell induction, cytokine-secreting T cell responses and persistence of serological memory were comparable regardless of vaccine dose. More strategies other than increased Ag amount may be needed to improve CMI in older adults.

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Introduction

Adults ≥ 65 years of age suffer the most from seasonal influenza-related hospitalizations and death [1, 2]. Although influenza vaccination is recommended to reduce disease burden in

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this population, a steady and systemic degeneration of the immune system with increasing age, termed ‘immunosenescence’ [3], reduces vaccine effectiveness (VE) [4]. As a strategy to increase immunogenicity of influenza vaccines, FDA approved high-dose (HD) influenza vaccine that contained 4- times (60μg HA/strain) more hemagglutinin (HA) than the standard-dose (SD) vaccine (15μg HA/strain) for people aged ≥ 65 years in 2009 [5]. This was based on a study in 2006, demonstrating significantly higher antibody (Ab) responses (seroconversion and seroprotection rates) by HD vaccine without an increase in clinically relevant adverse reactions [6]. Subsequently, a large randomized-control trial demonstrated a clinical benefit of HD vaccine in reducing laboratory-confirmed influenza illness by 24.2% [95% confidence interval (CI) 9.7–36.5] as compared to SD vaccine during the 2011–2013 influenza seasons [7]. In addition, a retrospective cohort analysis with US Medicare beneficiary records showed the relative effectiveness of HD vaccine in reducing influenza infections, defined by receipt of rapid influenza test followed by dispensing oseltamivir, and influenza-related hospital care [8]. HD vaccine also significantly reduced cardio-respiratory adverse events associated with influenza-related hospitalization [9]. Therefore, HD influenza vaccine is a more effective alternative to SD vaccines in individuals aged ≥65 years.

Neutralizing Abs remain the primary target of vaccine development and the standard assessment of immunogenicity for licensure of inactivated influenza vaccines [10]. However, clinical contribution of cell-mediated immunity (CMI) in reducing influenza disease symptoms has been recently demonstrated in healthy adults [11, 12]. For individuals aged ≥ 65 years in particular, the frequency of IFNγ-secreting T cells, rather than HI titers, correlates better with clinical protection following vaccination [13, 14]. Many studies have shown that HD vaccine elicits superior HI titers [6, 7, 15], but whether HD vaccine elicits superior CMI is unknown. Therefore, a comprehensive understanding of the scope of immunogenicity conferred by HD vaccines, encompassing humoral and cellular immunity, is important for the evaluation of the clinical benefits of existing HD vaccines and the development of better influenza vaccines for older adults. This study represents the first comprehensive evaluation of the induction of vaccine-specific, cytokine-secreting CD4 or CD8 T cell responses as well as B cell responses of healthy volunteers aged ≥65 years receiving HD or SD influenza vaccines.

Material and Methods

Study Design, participants and specimens

A total of 105 community-dwelling adults (SD, n=52 and HD, n=53) ≥ 65 years were enrolled, prior to the 2010–11 influenza season in Nashville, TN. Participants were not excluded due to any medical conditions except for prior allergic reaction to influenza vaccine or a history of Guillain-Barré syndrome. Written informed consent was received from study participants, who were then randomized to receive either SD or HD vaccine by intramuscular injection. Vaccine allocation was blinded to participants and researchers except for a designated un-blinded nurse who performed vaccination. All further steps including sample preparation, data analyses and evaluation were performed by blinded staff members and researchers. Peripheral blood mononuclear cells (PBMCs) were collected at days 0, 7, 14 and 28 post-vaccination and stored at −80°C until shipment to CDC for CMI

analysis. Serum samples were collected at days 0, 28 and 365 post-vaccination and stored at −80°C until shipment to Battelle (Columbus, OH) for HI assay. Procedures, informed consent documents and data collection forms were reviewed and approved by the Institutional Review Boards at Vanderbilt University and Centers for Disease Control and Prevention.

Influenza virus and vaccines

Influenza viruses, A/California/08/2009 (H1N1) and A/Perth/16/2009 (H3N2), were propagated for 2 days in the allantoic cavity of 10–11 days old embryonated chicken eggs (Hy-line, Mansfield, GA) and clarified by centrifugation. Pooled allantoic fluid was used for stimulation of PBMCs, as previously described [16]. Influenza vaccines (Sanofi Pasteur) contained 2010–2011 trivalent inactivated influenza viruses; A/California/7/2009 (H1N1), A/Perth/16/2009 (H3N2), and B/Brisbane/60/2008.

Hemagglutinin inhibition (HI) assay

HI assay was performed in duplicate against the influenza vaccine strains in the 2010–11 Northern Hemisphere influenza vaccine components according to the standardized protocol by the world health organization [17]. Seroprotection was defined as an HI titer of ≥40; seroconversion was defined as a four-fold rise in HI titers at post-influenza vaccination compared to pre-vaccination, or ≥ 40 if pre-vaccination titer was <10.

Assessment of CMI

CMI was assessed using PBMCs collected at days 0, 7, 14 and 28 days post-vaccination as previously described [16]. Level of activated, cytokine-expressing T cells was expressed as %cytokine/total $CD4^+CD69^+$ or $CD8^+CD69^+$ T cells. The limit of detection was 0.05 %.

Assessment of B cell responses

Frequencies of plasmablasts ($CD3^-CD20^-CD38^{hi}CD27^{hi}$) and memory B cell subsets (class switched memory B cells:$CD20^+IgD^-CD27^+$ and IgM memory B cells:$CD20^+IgD^+CD27^+$) were measured by flow cytometry using paired d0 and d7 PBMCs. Activation status of memory B cells was simultaneously evaluated by flow cytometry and shown as mean fluorescence intensity (MFI) of cell surface markers CD69, CD86 or cells expressing $CD69^{hi}$ or $CD86^{hi}$. Vaccine-specific plasmablasts (antibody-secreting cells) were measured *ex vivo* by ELISPOT as described [16] without *in vitro* stimulation. Induction of long-lived memory B cells were measured by ELISPOT as described [16].

Statistical Analysis

Calculation of $\log_2$-transformed HI titers for geometric mean titers (GMT), $\log_{10}$-transformed percentages (B and T cell responses), and antigen-specific B cells (ELISPOT) for geometric mean percentage (GMP) have been described [16]. Cell-surface activation markers were calculated as geometric mean value (GMV). Means and differences in means were estimated using repeated measures linear mixed models [18, 19]. Back-transforming model-estimated means yielded GMT/GMP/GMV and back-transforming differences between days at post-vaccination and day 0 means yielded GMT/GMP/GMV fold-rises.

Fold-rise >1 indicates significant induction of vaccine responses. HD/SD ratios were calculated from estimated GMT/GMP/GMV of HD or SD vaccine at each time point to determine if the HD vaccine induced greater responses than SD vaccine. HD/SD >1 indicates that HD vaccine induces significantly higher responses than SD vaccine. Chi-square tests or Wilcoxon rank-sum tests were performed appropriately for comparisons between treatment groups and categorical or continuous variables respectively. Serological responses at d28 post-vaccination were evaluated using logistic regression models. Multiple linear regression models were conducted using post-vaccination CMI as outcome and age, vaccine dose, and pre-existing CMI level as covariates. Comparison between stimulated (A/Cal(H1) or A/Perth(H3)) vs. unstimulated cells shown in Table 2 was analyzed by paired *t*-test. All analyses were performed using SAS software version 9.3 (SAS Institute Inc., Cary, NC) and R version 3.1.3 (www.cran.r-project.org).

Results

Study participants Characteristics

A total of 105 participants were randomly grouped into SD (n=52) and HD (n=53) groups (Supplemental Figure 1). Demographic information was comparable between the two groups (Supplemental Table 1). Subjects selected for CMI assessment (26 HD and 22 SD) were also similar in age with a mean age (± standard deviation) of 72.9 years ± 6.4 (HD) and 72.7 years ± 6.6 (SD), high-risk medical conditions and body mass index. The HD group had 13 samples from females (50%) and SD had 8 (36%) (p=0.34).

Summary of serological responses

Both vaccine groups induced geometric mean titer (GMT) fold-rise >1 at days 28 and 365 post-vaccination for all vaccine components (Figure 1A, Supplemental Figure 2A). HD vaccine elicited significantly higher Ab responses than SD vaccine at d28 post-vaccination, as shown by seroconversion and seroprotection rates (Supplemental Table 2) and the HD/SD ratio > 1 for all vaccine components (Figure 1B). At d365 post-vaccination, Ab responses were comparable between the two groups (Figure 1B). Significant cross-reactive responses against H3 and B strains of the prior (2009–2010) influenza vaccine were also induced in both vaccine groups (Figure 1C), but the HD/SD ratio was greater than 1 only at d28 post-vaccination (Figure 1D). Pre-existing HI titers were significantly correlated with seroprotection and seroconversion rates regardless of vaccine dose (Supplemental Table 3). However, vaccine dose, not age, strongly predicted the seroprotection and seroconversion rates at d28, but again, not at d365 post-vaccination. Therefore, while HD elicited superior Ab responses, they were comparable to SD at 1 year post-vaccination.

HD vaccine significantly increased plasmablast responses at d7 post-vaccination

Plasmablast (antibody-secreting cells; ASCs) responses and activation of memory B cell subsets at d7 post-vaccination are summarized in Table 1. The geometric mean percent (GMP) fold-rise of plasmablast frequency was higher than 1 in the HD group (Figure 2A). The HD/SD ratio of plasmablasts was higher than 1 at d7 post-vaccination, suggesting that HD induced higher plasmablast responses than SD vaccine (Figure 2A). Both vaccine groups significantly induced vaccine-specific IgG-secreting ASCs for all vaccine

components (Figure 2B–D). Estimated HD/SD ratios of d7 ASCs were higher than 1 for all vaccine components, but did not reach statistical significance due to high variability of observations (Figure 2B–D).

HD vaccine induced a significant increase in $CD69^{hi}$ or $CD86^{hi}$-expressing class-switched memory B cells (CS-MBCs) (GMP fold-rise >1), but the HD/SD ratios were not different from 1 (Supplemental Figure 3A–B). The overall CD69 MFI on CS-MBCs and $CD69^{hi}$-expressing IgM-MBCs were significantly induced in both vaccine groups, but HD/SD ratios were not different from 1 (Supplemental Figure 3C–D).

Induction of long-lived memory B cells was comparable regardless of vaccine dose

Induction of long-lived memory B cells at d28 post-vaccination are summarized in Table 1. Both vaccines induced significant IgG^+ memory B cells for all vaccine components; however, no statistical differences in the d28 HD/SD ratios were found (Supplemental Figure 4).

Development of CMI was comparable between the HD and SD group

Virus-specific, cytokine-secreting T cell responses at days 0, 7, 14 and 28 post-vaccination are summarized in Table 2. Both vaccine groups showed detectable pre-existing T cell responses to viruses (d0; unstimulated vs. virus-stimulated). Both vaccine groups showed moderate induction in GMP fold-rises of IFNγ or TNFα-secreting CD4 T cells (Figure 3A–B). However, no consistent statistical significance between groups (HD/SD ratios) was found. GMP fold-rise of $CD8^+$TNFα T cells were significant at all time-points in both vaccine groups, but again, no differences between HD and SD-induced responses were detected (Figure 3C). In contrast to TNFα secretion, IFNγ-secretion by CD8 T cells was moderately induced, yet the post-vaccine responses were also comparable between HD and SD subjects (Figure 3D). Overall, both HD and SD vaccines modestly induced T cell activation yet higher vaccine Ag amount did not proportionally increase CMI.

Pre-existing CMI was a significant predictor for post-vaccine CMI regardless of vaccine dose

Given the comparable T cell responses between HD and SD groups, *Post hoc* analyses were used to identify potential covariates associated with the differential T cell responses by HD vaccine. Age, whether overall age spectrum or divided into 65–74 vs. ≥75 bracket, was not correlated with cytokine (IFNγ, TNFα, IL-2)-secreting T cells regardless of vaccine dose or strain (Supplemental Figure 5). However, pre-existing T cell responses were significantly correlated with post-vaccine T cell responses for $IFN\gamma^+$CD4 T cells and $TNF\alpha^+$CD8 T cells in stimulation with A/Cal(H1) or A/Perth(H3) virus, regardless of vaccine dose (Figure 4A, Supplemental Figure 6A–D). However, pre-existing T cell responses were not correlated with the GMP fold-rise (d28/d0) (Supplemental Figure 7).

Given the role of CD4 T cells for promoting Ab responses [20], $IFN\gamma^+$CD4 T cell responses may better support Ab responses in the HD group than in the SD group. The HD group showed a moderate correlation between CD4 T cells and Ab responses, whereas the SD group showed no correlation (Figure 4B). However, the correlation was detected only for A/

Cal(H1)-specific responses, but not for A/Perth(H3)-specific responses (Figure 4B). In addition, the regression slopes of A/Cal(H1)-specific correlations at days 7, 14 and 28 were comparable between HD and SD groups, whereas the slopes of A/Perth(H3)-specific responses of the HD group were significantly lower than those of the SD group at days 14 ($p=0.007$) and 28 ($p=0.03$) post-vaccination (Figure 4A, Supplemental Figure 6).

Discussion

HD influenza vaccine has been shown to be clinically beneficial for adults ≥ 65 years due to better effectiveness, clinical efficacy, and comparable safety profile to SD vaccine [7–9, 15, 21]. While the superior immunogenicity of HD vaccines has been characterized by serological parameters [6, 15, 22] the increasingly recognized clinical relevance of CMI in this population warrants a comprehensive understanding of HD-mediated immunogenicity. Our current findings are therefore timely in providing data on the impact of HD vaccine on the cellular compartment.

HD vaccine preferentially impacted acute, short-lived plasmablast responses (Figure 2), but had little, if any, impact on the long-term memory B cell responses including activation of pre-existing memory B cells (Supplemental Figure 3) and *de novo* induction of newly generated memory B cells (Supplemental Figure 4). At a single B cell level, the B cell receptor (BCR) may encounter increased amounts of vaccine Ag, resulting in increased avidity. Thus, ensuing BCR signaling events may mimic those derived from T-independent type 2 Ag, an organized and highly repetitive epitope. Since, BCR cross-linking by T-independent Ag can induce B cell proliferation and short-lived Ab secretion [23], HD vaccine may induce signaling pathways that favors short-lived Ab secretion rather than long-lived Abs or memory B cells. In support of this idea, the HI titers of HD and SD subjects were comparable at 1 year post-vaccination (Figure 1B). Further, a recent study found that although HD subjects had significantly higher GMT for all vaccine components at d28 post-vaccination, only H3N2-specific GMT remained higher at 6 month post-vaccination during the 2011–2013 seasons [24]. Therefore, these data collectively suggest a HD vaccine's preferential impact on the acute B cell responses.

In contrast to serological and plasmablast responses, T cell responses were comparable between HD and SD vaccine recipients. Multiple mechanisms are possible for the similar T cell responses to SD and HD vaccine in older adults. Although studies have shown that Ag dose-dependent activation of T cells can occur when Ag is not a limiting factor [25, 26], split influenza vaccine dose range resulting in dose-dependent CMI responses is unknown to this date. Thus, despite 4-times higher vaccine Ag, the Ag amount in the HD vaccine could still be insufficient to proportionally induce CMI. Alternatively, ongoing immunosenescence may compromise dose-dependent CMI. Direct age-related deterioration in T cell responses was not evident in the current study (Supplemental Figure 5) nor in a recent study [16]. However, other alterations in the T cell compartment such as progressive induction of anergic T cells or regulatory T cells [27] or in innate immunity such as impaired phagocytic and Ag cross-presentation capacity of dendritic cells (DCs) [28–30] may render effector T cells insensitive to higher amounts of Ag. Regardless of the mechanisms, comparable CD4 T cell responses between HD and SD vaccine may have resulted in induction of long-lived

memory B cells at a similar level (Supplemental Figure 4). Our current findings on T cell responses, along with results from earlier studies examining T cell IFNγ secretion of young (18–40 years) vs. elderly (≥65 years) receiving SD or HD vaccine [31], demonstrate challenges in improving vaccine-induced CMI in individuals aged ≥65 years.

HD vaccine may have differentially affected CMI in a strain-specific manner. Most study participants received 2009–2010 seasonal influenza vaccine containing A/Bris59 (H1N1), A/Bris10 (H3N2), and B/Bris60, and more than 50% of study participants (63% of SD, 55% of HD) received the 2009 monovalent pandemic H1N1 vaccine in the prior season (Supplemental Table 1). Therefore, >50% of the HD group currently encountered the A/Cal(H1) strain for the second time with 4-times higher dose than the SD group, whereas all subjects encountered the A/Perth(H3) strain for the first time. Only the HD group demonstrated correlations between the d28 $CD4^+IFN\gamma^+$ and HI titers for A/Cal(H1)-specific responses (Figure 4B). No such correlations were observed for the SD group regardless of vaccine strains. Further, when the correlation between the d28 $CD4^+IFN\gamma^+$ and HI titers of HD vaccine responses were further analyzed based on prior 2009 pdmH1N1 monovalent vaccine receipt, only those who received prior pdmH1N1 vaccine maintained significant correlation, whereas individuals without prior 2009 pdmH1N1 monovalent vaccine receipt or individuals with 1^{st} time A/Perth(H3) exposure did not show a correlation (Supplemental Figure 8). A direct impact of repeated vaccination on influenza-specific CMI is difficult to assess as pre-existing neutralizing Abs could potentially hamper development of CD8 T cell immunity [32]. However, computational modeling or clinical observational studies have found that repeated vaccination in the previous seasons is significantly correlated with reduced vaccine effectiveness [33, 34]. Our study is not designed to test this hypothesis, yet our current findings imply that HD vaccine may support the Ab response through IFNγ-secreting CD4 T cells for A/Cal(H1)-specific responses, but only in individuals previously primed with the A/Cal(H1) vaccine component. It is important to investigate the long-term protective functions of these T cells when the serological memory wanes.

There are a few limitations in the current study. The sample size for each T and B cell assay was relatively small, potentially making small effects difficult to detect. However, the variability and standard deviation of the data is comparable to findings from our previous study (16), indicating that the chance of type II error (failure to detect an effect due to small sample size) is low. PBMC samples beyond d28 post-vaccination, such as 6 months and/or 1 year post-vaccination would be useful to evaluate the long-term responses CMI stimulated by HD vaccine. It is important to note that in contrast to the well-accepted serological endpoints (HI titers), no clear standards to define CMI-responders vs. non-responders, or consensus on the phenotype or magnitude of T cell responses as correlates of protection have been established. Thus, it remains that T cell function(s) other than the frequencies of cytokine-secreting T cells as measured in the current study, may demonstrate an impact of HD vaccine. Systemic and longitudinal studies designed for assessment of CMI and surveillance over multiple influenza seasons would be necessary to address these challenges.

In conclusion, the only differences detected in CMI outcome from HD vs. SD vaccine in the current study setting was acute plasmablast responses. Presently, it remains unclear whether the lack of concomitant increase of memory B and T cell responses has influenced the

relative efficacy of the HD vaccine. Given the protective role of CMI in older adults, the current relative efficacy of HD vaccine may be further improved by increasing memory B cell and T cell responses. Fundamental innovation or additional strategies to increase memory T and B cell responses may be considered for better protection of this vulnerable population, in addition to increasing vaccine Ag dose. Engaging the innate arm of immunity may need be to be considered in order to effectively activate CMI. To this end, combining HD vaccine with alternative vaccine delivery routes such as HD intradermal vaccine, or adjuvanted vaccine formulation may represent potential strategies. Meanwhile, as the HD vaccine continues to be routinely administered, the long-term benefit or outcomes as well as the effect of repeated HD vaccination need to be studied.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- High-dose influenza vaccine induced higher antibody responses in individuals aged ≥65 years.
- High-dose influenza vaccine induced higher plasmablast responses.
- Cell-mediated immunity and memory responses were similar between high and standard-dose vaccine.

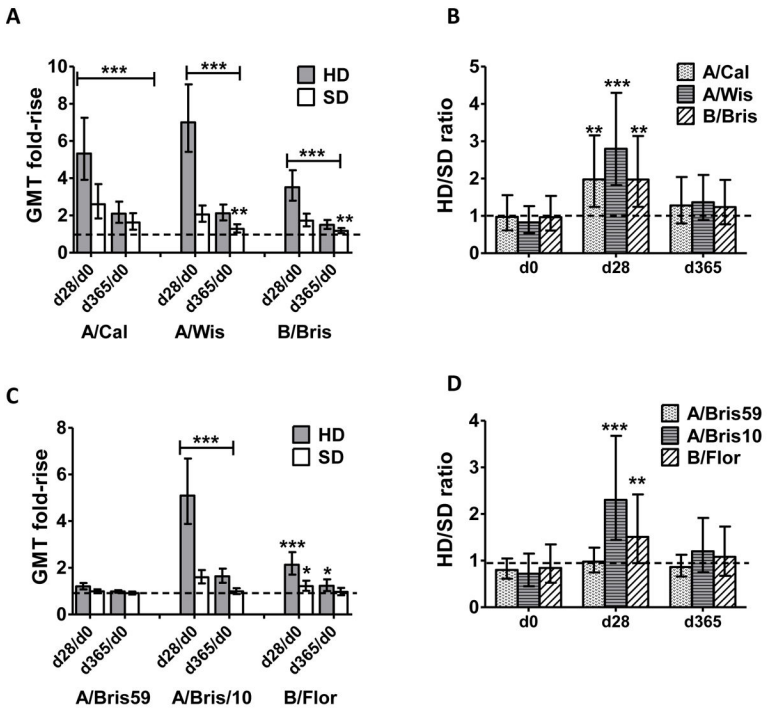

Figure 1. HD induced significantly higher serological responses than SD vaccine against all vaccine components, but did not persist up to a year

(A) GMT fold-rise (d28/d0 and d365/d0) was estimated to determine the vaccine-associated changes in Ab responses within HD vaccine recipients (d0, N=51; d28, N=49; d365, N=49) or SD vaccine recipients (d0, N=52; d28, N=51; d365, N=51) for all vaccine components. A GMT fold-rise of 1 (dotted line) is indicative of no vaccine-induced change. (B) The difference in responses between HD and SD group was estimated by HD/SD ratio at each time-point. A HD/SD ratio of 1 is indicative of no difference between HD and SD-induced Ab responses. (C) GMT fold-rises (d28/d0 and d365/d0) were estimated to determine the vaccine-mediated boost responses for the 2009–2010 influenza vaccine components (A/Bris59 (H1N1), A/Bris10 (H3N2) and B/Flor). (D) HD/SD ratio was estimated to determine the difference in boost responses between HD and SD group. Error bars indicate 95% confidence intervals. Statistical significance is shown as *p ≤ 0.05, **p ≤ 0.01, *** p ≤ 0.001.

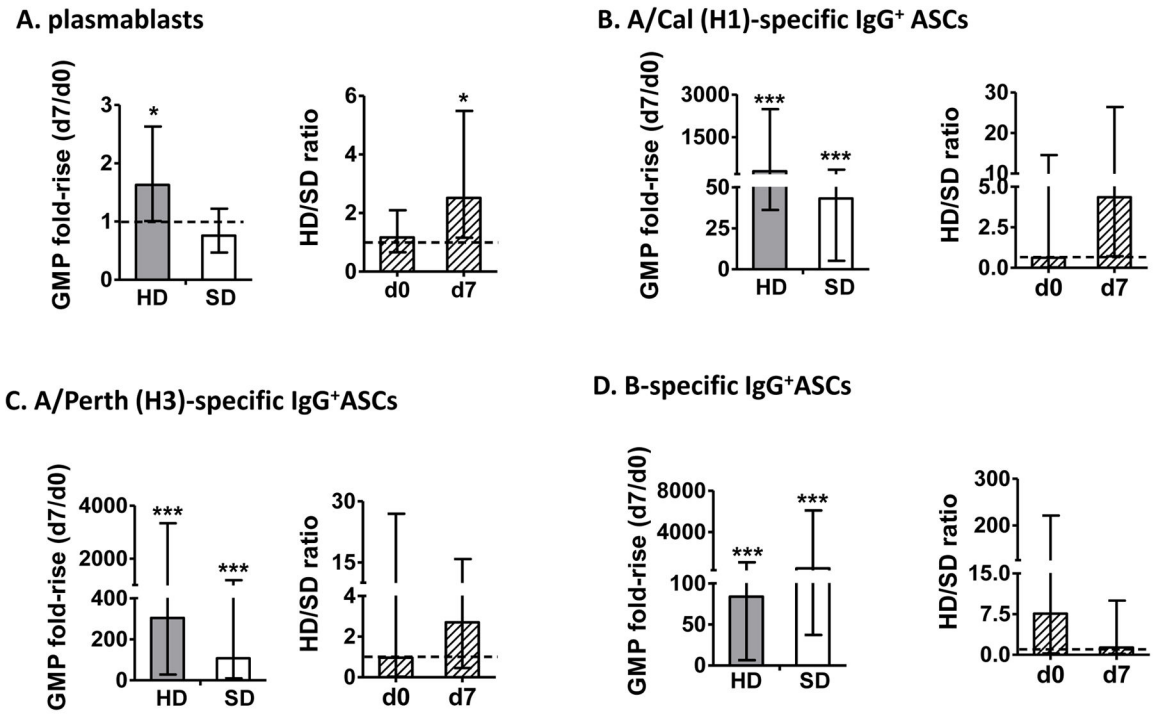

Figure 2. HD induced significantly higher plasmablast responses at d7 post-vaccination

Plasmablast responses were analyzed by flow cytometry and ELISPOT assay using PBMCs (N=12) randomly selected from each vaccine group at d0 and d7 post-vaccination. (A) Plasmablasts were identified as $CD20^{-}CD3^{-}CD27^{+}CD38^{+}$ cells by flow cytometry and their percent in PBMCs were calculated to determine geometric mean percent (GMP) at each time-point. GMP fold-rise (d7/d0) was estimated to determine vaccine-induced responses. A GMP fold-rise of 1 (dotted line) is indicative of no vaccine-induced change. Estimated GMP values from each time-point were compared to determine the difference in response between HD vs. SD group. (B–D) Ab-secreting cells (ASCs) specific to A/Cal (H1), A/Perth (H3), or B (Bris) were measured by ELISPOT assay for IgG-secreting cells. GMP fold-rise (d7/d0) were estimated to determine vaccine-induced responses. Difference in responses between HD vs. SD group were determined by HD/SD ratio at each time-point. Error bars indicate 95% confidence intervals. Statistical significance is shown as *$p \leq 0.05$, **$p \leq 0.01$, *** $p \leq 0.001$ or stated.

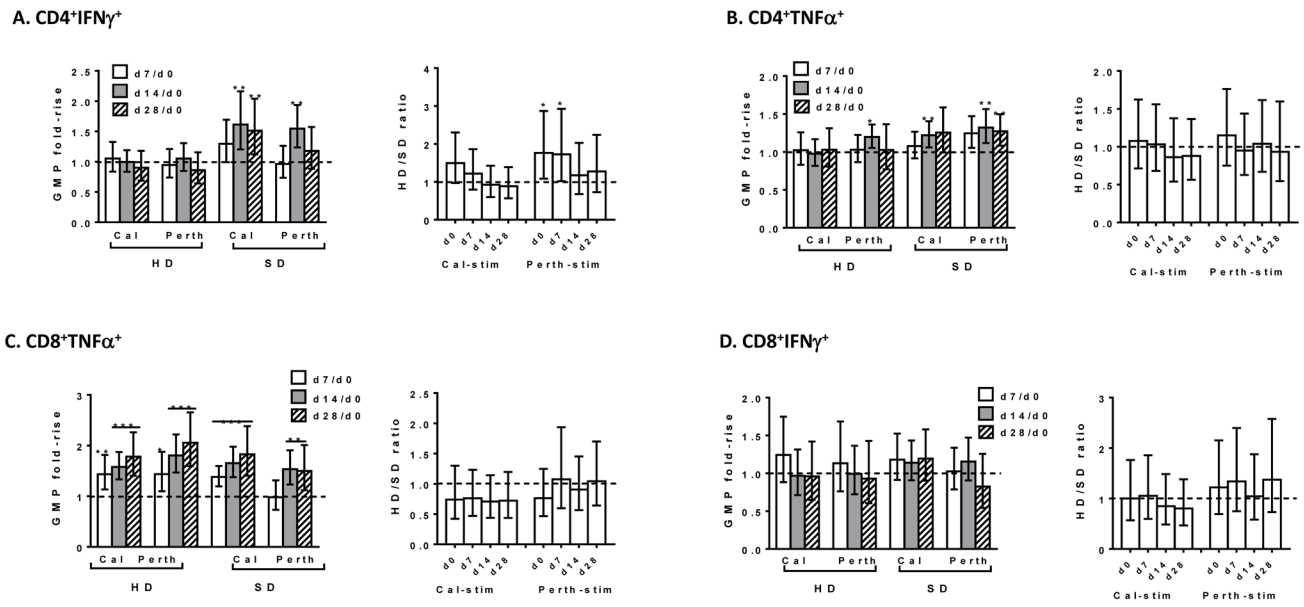

Figure 3. Cell-mediated immunity was comparable between HD and SD group

Paired sets (d0/d7/d14/d28) of PBMCs randomly selected from HD (n=26) and SD (n=22) were stimulated *in vitro* with MOI of 1 A/Cal (H1) or A/Perth (H3) wild-type virus or left unstimulated (unstim; u.s.) overnight and cytokine secretion from CD4 or CD8 T cells was analyzed by flow cytometry. GMP of activated ($CD69^+$), $IFN\gamma^+$(A) or $TNF\alpha^+$(B) CD4 T cells or $IFN\gamma^+$(C) or $TNF\alpha^+$(D) CD8 T cells were estimated at days 0,7,14 and 28 post-vaccination. GMP fold-rises (d7/d0, d14/d0, d28/d0) were estimated to determine vaccine-induced changes and shown by virus subtype and vaccine dose. Comparison of HD and SD-induced CMI were estimated by HD/SD ratios at days 0, 7, d14 and d28. A GMP fold-rise of 1 (dotted line) is indicative of no vaccine-induced change, and a HD/SD ratio of 1 (dotted line) is indicative of no difference between HD and SD-induced T cell responses. Error bars indicate 95% confidence intervals. Statistical significance is shown by *$p \leq 0.05$, **$p \leq 0.01$, *** $p \leq 0.001$.

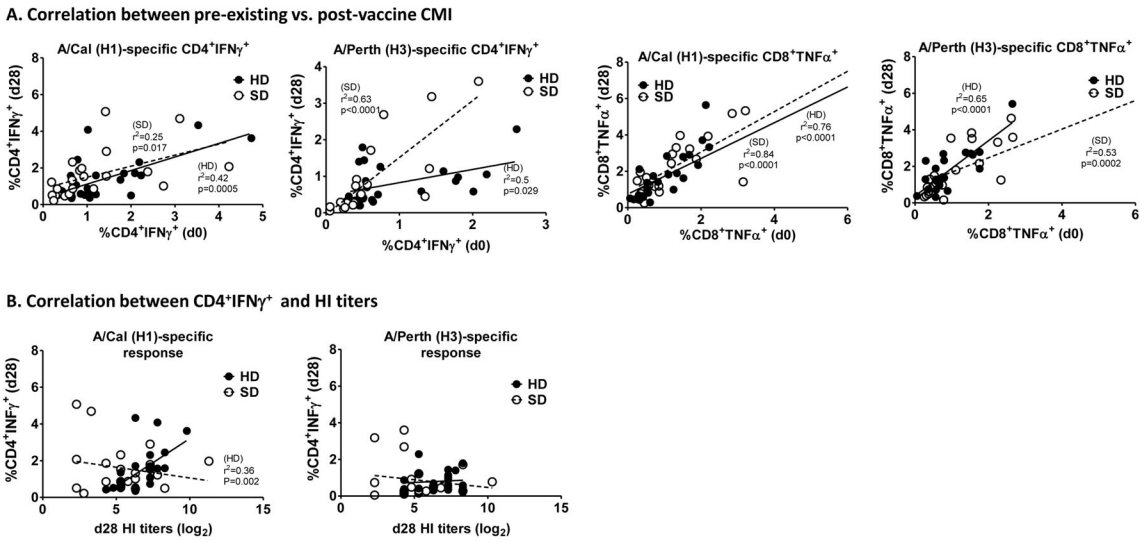

Figure 4. Pre-existing CMI level correlated with post-vaccination CMI regardless of vaccine dose

The pre-existing CMI of HD and SD group was tested for correlations with their corresponding post-vaccination responses including CMI and Ab responses using a linear mixed model. (A) Pre-existing (d0) A/Cal (H1) or A/Perth (H3)-specific $CD4^+IFN\gamma^+$ or $CD8^+TNF\alpha^+$ T cell responses were tested for correlations with d28 post-vaccine responses. (B) Post-vaccine (d28) A/Cal (H1) or A/Perth (H3)-specific $CD4^+IFN\gamma^+$ T cell responses were tested for correlations with d28 HI titers. r^2 indicates correlation coefficient. Solid or dotted lines represent regression lines of HD or SD vaccine-induced responses, respectively.

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Table 1

Summary of B cell responses.

Vaccine group	**SD**		**HD**	
Plasmablast response	d0, GMP (CIs)	d7, GMP (CIs)	d0, GMP (CIs)	d7, GMP (CIs)
%plasmablasts ($CD20^{-}CD3^{-}CD27^{hi}CD38^{hi}$)	0.13 (0.09, 0.2)	0.1 (0.06, 0.18)	0.13 (0.09, 0.2)	0.26 (0.15, 0.44)
%A/Cal (H1)-specific ASCs (IgG^{+})	0.18 (0.02, 1.69)	7.88 (2.2, 28.14)	0.11 (0.01, 1.06)	34.36 (9.62, 122.77)
%A/Perth(H3)-specific ASCs (IgG^{+})	0.04 (0.00, 0.45)	4.58 (1.31, 15.99)	0.04 (0.00, 0.43)	12.37 (3.55, 43.17)
%B-specific ASCs (IgG^{+})	0.02 (0.00, 0.27)	11.67 (2.82, 48.32)	0.19 (0.02, 2.02)	15.62 (3.77, 64.7)
B cell activation status	d0, GMP (CIs)	d7, GMP (CIs)	d0, GMP (CIs)	d7, GMP (CIs)
class-switched memory B cells				
$\%CD69^{hi}$	16.69 (12.6, 22.13)	20.95 (17.04, 25.75)	15.15 (11.43, 20.08)	25.42 (20.68, 31.25)
CD69 MFI	282.26 (228.06, 349.35)	348.66 (291.08, 417.62)	251.78 (203.43, 311.62)	369.65 (308.6, 442.76)
$\%CD86^{hi}$	7.9 (5.7, 10.9)	10.0 (7.3, 13.8)	10.83 (7.79, 15.05)	14.73 (10.74, 20.19)
IgM memory B cells				
$\%CD69^{hi}$	31.61 (25.84, 38.66)	40.65 (34.81, 47.46)	36.64 (29.96, 44.82)	46.54 (39.86, 54.34)
CD69 MFI	520.56 (436.25, 621.17)	658.12 (558.24, 775.86)	586.59 (491.59, 699.96)	762.25 (646.57, 898.62)
$\%CD86^{hi}$	3.0 (1.9, 4.7)	2.6 (1.7, 3.9)	4.79 (3.07, 7.47)	4.11 (2.68, 6.28)
Memory B cell (MBC) induction	d0, GMP (CIs)	d28, GMP (CIs)	d0, GMP (CIs)	d28, GMP (CIs)
A/Cal (H1)-specific IgG^{+} MBC	0.42 (0.12, 1.50)	3.63 (1.83, 7.20)	0.82 (0.29, 2.31)	2.65 (1.51, 4.63)
A/Perth (H3)-specific IgG^{+} MBC	0.44 (0.12, 1.63)	2.00 (1.26, 3.18)	0.65 (0.22, 1.89)	2.75 (1.88, 4.02)
B-specific IgG^{+} MBC	0.23 (0.07, 0.75)	3.29 (1.72, 6.31)	0.52 (0.2, 1.37)	2.19 (1.29, 3.72)

SD: standard-dose, HD: high-dose influenza vaccine, GMP: geometric mean percent, CIs: 95% confidence intervals. ASCs: antibody-secreting cells %plasmablast responses and B cell activation status were measured by flow cytometry. %vaccine strain-specific ASCs were measured by ELISPOT assay. Memory B cell induction was measured by ELISPOT following *in vitro* polyclonal stimulation.

Table 2

Summary of T cell responses.

stimulation Vaccine group	unstimulated		A/Cal (H1)-stimulated		A/Perth (H3)-stimulated	
	SD, GMP (CIs)	HD, GMP (CIs)	SD, GMP (CIs)	HD, GMP (CIs)	SD, GMP (CIs)	HD, GMP (CIs)
CD4 T cells						
$CD4^+CD69^+IFN\gamma^+$						
d0	0.16 (0.15, 0.17)	0.14 (0.11, 0.19)	0.79 (0.53, 1.17)*	1.18 (0.93, 1.48)†	0.41 (0.26, 0.62)*	0.72 (0.54, 0.95)†
d7	0.17 (0.13, 0.21)	0.15 (0.11, 0.20)	1.02 (0.71, 1.46)*	1.24 (0.95, 1.61)†	0.39 (0.24, 0.64)*	0.68 (0.51, 0.90)†
d14	0.18 (0.14, 0.24)	0.17 (0.11, 0.26)	1.27 (0.88, 1.82)*	1.17 (0.89, 1.54)†	0.63 (0.37, 1.07)*	0.75 (0.59, 0.96)†
d28	0.26 (0.18, 0.39)	0.14 (0.11, 0.19)	1.19 (0.84, 1.68)*	1.06 (0.78, 1.44)†	0.48 (0.29, 0.80)	0.62 (0.46, 0.83)†
$CD4^+CD69^+TNF\alpha^+$						
d0	0.16 (0.14, 0.18)	0.13 (0.10, 0.16)	0.68 (0.50, 0.93)*	0.73 (0.55, 0.98)†	0.41 (0.31, 0.55)*	0.48 (0.34, 0.66)†
d7	0.18 (0.15, 0.22)	0.14 (0.10, 0.19)	0.74 (0.56, 0.96)*	0.75 (0.54, 1.03)†	0.51 (0.38, 0.70)*	0.49 (0.36, 0.66)†
d14	0.19 (0.15, 0.24)	0.15 (0.11, 0.21)	0.83 (0.61, 1.13)*	0.72 (0.50, 1.02)†	0.55 (0.40, 0.75)*	0.57 (0.41, 0.78)†
d28	0.26 (0.18, 0.38)	0.14 (0.10, 0.19)	0.86 (0.66, 1.11)*	0.76 (0.53, 1.08)†	0.53 (0.38, 0.74)	0.49 (0.32, 0.74)†
CD8 T cells						
$CD8^+CD69^+IFN\gamma^+$						
d0	0.18 (0.16, 0.19)	0.13 (0.10, 0.17)	1.55 (0.97, 2.47)*	1.55 (1.08, 2.23)†	0.81 (0.50, 1.33)*	0.99 (0.70, 1.40)†
d7	0.19 (0.16, 0.23)	0.16 (0.10, 0.25)	1.83 (1.11, 3.00)*	1.93 (1.37, 2.70)†	0.84 (0.47, 1.49)*	1.13 (0.86, 1.47)†
d14	0.14, (0.11, 0.17)	0.18 (0.11, 0.29)	1.77 (1.10, 2.84)*	1.50 (1.06, 2.12)†	0.94 (0.53, 1.66)*	0.99 (0.74, 1.31)
d28	0.21, (0.13, 0.34)	0.20 (0.13, 0.31)	1.85 (1.18, 2.92)*	1.49 (1.06, 2.09)†	0.67 (0.36, 1.25)	0.92(0.70, 1.22)†
$CD8^+CD69^+TNF\alpha^+$						
d0	0.14 (0.12, 0.16)	0.10 (0.07, 0.14)	1.03 (0.69, 1.55)*	0.76 (0.51, 1.14)†	0.92 (0.62, 1.38)*	0.70 (0.51, 0.97)†
d7	0.19 (0.15, 0.25)	0.17 (0.12, 0.23)	1.42 (0.99, 2.05)*	1.09 (0.79, 1.51)†	0.90 (0.53, 1.55)*	1.01 (0.76, 1.35)†
d14	0.25 (0.19, 0.33)	0.19 (0.13, 0.28)	1.70 (1.18, 2.44)*	1.20 (0.86, 1.68)†	1.41 (0.9, 2.21)*	1.27 (1.00, 1.62)†
d28	0.30 (0.21, 0.41)	0.21 (0.14, 0.31)	1.88 (1.29, 2.75)*	1.36 (0.95, 1.93)†	1.38 (0.9, 2.12)*	1.45 (1.09, 1.93)†

SD: standard-dose, HD: high-dose influenza vaccine, GMP: geometric mean percent, CIs: 95% confidence intervals. %activated ($CD69^+$) and cytokine-secreting CD4 or CD8 T cells were measured by flow cytometry following *in vitro* stimulation with wild-type virus.

*denotes the significant difference ($P<0.05$) between A/Cal (H1) or A/Perth (H3)-stimulated cells vs. unstimulated cells of SD group at each time-point.

†denotes the significant difference ($P<0.05$) between A/Cal (H1) or A/Perth (H3)-stimulated cells vs. unstimulated cells of HD group at each time-point.