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Th1 MEMORY DIFFERENTIATES RECOMBINANT FROM LIVE HERPES ZOSTER VACCINES

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Abstract

The adjuvanted varicella-zoster virus glycoprotein E (VZV gE) subunit herpes zoster vaccine (HZ/su confers higher protection against HZ than the live attenuated zoster vaccine (ZV). To understand the immunologic basis for the different efficacies of the vaccines, we compared immune responses to the vaccines in adults 50 to 85-year old. gE-specific T cells were very low/undetectable before vaccination when analyzed by FluoroSpot and flow cytometry. Both ZV and HZ/su increased gE-specific responses, but at peak memory response (PMR) after vaccination (30 days after ZV or after the 2nd dose of HZ/su) gE-specific CD4+ and CD8+ T-cell responses were ≥10-fold higher in HZ/su compared with ZV recipients. Comparing the vaccines, T cell memory responses, including gE- and VZV-IL2+ spot-forming cells (SFC), were higher in HZ/su recipients and cytotoxic and effector responses were lower. At 1 year after vaccination, all gE-Th1 and VZV-IL2+ SFC remained higher in HZ/su compared to ZV recipients. Mediation analyses showed that IL2+ PMR were necessary for the persistence of Th1 responses to either vaccine and VZV-IL2+ PMR explained 73% of the total effect of HZ/su on persistence. This emphasizes the biological importance of the memory responses, which were clearly superior in HZ/su compared with ZV participants.
Introduction

Herpes zoster (HZ) occurs when varicella-zoster virus (VZV) latent in sensory ganglia reactivates and replicates to cause dermatomal pain and a vesicular rash (1, 2). These events follow when some essential component(s) of VZV-specific cell-mediated immunity (CMI) falls below a critical level, which typically happens when VZV-specific CMI is compromised by disease, medical treatment, or aging (3-7). The live attenuated zoster vaccine (ZV) boosts VZV-specific CMI in elderly vaccinees, thereby explaining the efficacy of the vaccine (8, 9). However, efficacy against HZ is limited to 51% in vaccinees older than 60 years of age (yoa), and is lower as the age at the time of vaccination increases (9, 10). Moreover, the protection provided by ZV declines significantly at 6 to 8 years after vaccination (11). The magnitude and duration of protection have been confirmed by effectiveness studies (12-14).

An alternative approach for prevention of HZ is the recently approved recombinant subunit glycoprotein E (gE) vaccine (HZ/su), which contains the AS01b adjuvant consisting of MPL (lipid A of bacterial lipopolysaccharide, a TLR4 agonist) and QS21 (a triterpene plant derivative in the family of saponins) packaged into liposomes (15). HZ/su provides 97% protection against HZ in vaccinees ≥50 yoa, including 89% efficacy in those ≥80 yoa, indicating that the efficacy of HZ/su is minimally affected by the age of the vaccinee (16, 17). Moreover, this strong protective effect persisted for the 3.8 years of follow-up reported. HZ/su-induced immune responses remained robust for the duration of the pivotal trials and have been readily detected at 6-9 years after vaccination in long-term follow-up studies (18-20).

These very favorable clinical responses to HZ/su are uniquely better compared to responses to other vaccines administered to older individuals (21, 22). It is likely that overcoming immune senescence derives from the inclusion in HZ/su of AS01b (15, 23-25). The current report
compares the immune responses elicited by ZV or HZ/su in participants 50-59 and 70-85 yoa who had never received a HZ vaccine, and also compares immune responses to the two vaccines in an additional cohort of participants 70 to 85 yoa who had received ZV ≥5 years prior to enrollment. The primary objective was to determine immunologic responses that best differentiated the two vaccines in individuals receiving a HZ vaccine for the first time. Other objectives were to compare the responses elicited by HZ/su in participants who had received ZV ≥5 years previously with responses of individuals receiving HZ/su for the first time, and to identify CD8+ T cell responses generated by HZ/su.
Results

Demographic Characteristics

The study enrolled 160 participants (Table 1). The mean age was 70 years; 86 (54%) were women, 152 (97%) white, and 156 (98%) non-Hispanic. The demographic characteristics were similar between the two vaccine groups in each of the 3 subgroups: 1st time immunized 50 to 59-year old (young primary), 1st time immunized 70 to 85-year old (older primary), and 70 to 85-year old individuals who received ZV ≥5 years before enrollment (older boosted).

VZV- and gE-Specific Th1 Responses to HZ/su and ZV Measured by FluoroSpot

VZV- and gE-IL2+, -IFNγ+, and -IL2+IFNγ+ double positive (DP) Th1 responses were measured before vaccination, 30 days after ZV or the first HZ/su dose, 30 days after the second HZ/su dose, and at 1 year after each vaccine (Figure S1). The primary immunogenicity outcome measures specified in the protocol were Th1 responses at the peak memory response (PMR) time point, which occurred at 30 days after ZV (26), and at 30 days after the second dose of HZ/su (Figure 1 and Figure S2). At baseline, participants had robust VZV-Th1 CMI [e.g. VZV-IL2 mean (SEM) =168 (17) SFC/10^6 PBMC], but very low or undetectable gE-Th1 CMI [gE-IL2 = 25 (4) SFC/10^6 PBMC]. At PMR, ZV recipients reached 323 (24) VZV-IL2 SFC/10^6 PBMC and 35 (6) gE-IL2 SFC/10^6 PBMC, while HZ/su recipients reached 426 (30) VZV-IL2 and 475 (36) gE-IL2 SFC/10^6 PBMC. It is important to note that after a single dose of HZ/su, responses were much lower compared with PMR [226 (23) VZV-IL2 and 128 (12) gE-IL2 SFC/10^6 PBMC]. In fact, VZV-IL2 responses after the 1st dose of HZ/su were lower than those of ZV recipients, underscoring the importance of the 2nd dose for the immunogenicity of HZ/su.
Baseline VZV- and gE-Th1 responses had a significant positive effect on their respective PMR to either vaccine, but age, gender or prior administration of ZV did not (data not shown). After adjusting for baseline responses, VZV-IL2 PMR was higher in HZ/su compared with ZV recipients [false discovery rate (FDR)-adjusted p=0.01; Figure 1], but there were no differences in VZV-IFNγ or -DP responses, which indicated that the type of vaccine had a significant effect only on VZV-IL2 among all VZV-Th1 PMR tested. Adjusted gE-Th1 PMR were significantly higher in HZ/su compared to ZV recipients (FDR-p<0.0001; Figure 1), indicating that the type of vaccine affected all gE-Th1 PMR.

T Cell Differentiation in Response to HZ/su and ZV

In a subset of 60 participants equally distributed between the two vaccines and across the three age/treatment groups in each vaccine arm (demographics in Table S2), we analyzed gE- and VZV-CD4+ and CD8+ T-cell differentiation profiles by flow cytometry at PMR. After ex-vivo restimulation with gE peptide pools, replication competent VZV, or mock stimulation, we identified CD4+ and CD8+ central memory (Tcm; CCR7+CD27+CD45RO+), effector memory (Tem; CCR7-CD27+CD45RO+), differentiated effectors (Teff; CCR7-CD27-CD45RO+), intermediate effectors (T ei; CCR7-CD27+CD45RO-) and terminally differentiated effectors (T ed; CR7-CD27-CD45RO-) and confirmed their specificity to the stimulating antigen by IFNγ production (gating strategy in Figure S3A). It is important to note that both gE peptide pools and replication competent VZV allow T cell epitope presentation in the context of MHC class I and II. The comparison of the baseline-adjusted PMR showed that HZ/su generated significantly higher gE-specific CD4+ Tcm and Tem and lower CD4+ Teff compared to the VZV-specific responses generated by ZV (FDR-p<0.05; Table 2). An alternative sensitivity analysis, in which the effector and memory subsets were expressed as percentages of the gE- and VZV-IFNγ+ T cells also
showed lower CD4+ Teff, CD4+ Tei and CD8+ Tei in HZ/su compared with ZV recipients (both FDR-p=0.01, 0.047 and 0.06, respectively; Figure S3B).

**CD4+ and CD8+ proliferative PMR to gE and VZV**

To determine individual contributions of CD4+ and CD8+ T cells to the immunologic memory generated by HZ/su and ZV, we measured T cell proliferation by flow cytometry after ex-vivo restimulation with gE peptide pools or replication competent VZV in a subset of 94 participants equally distributed among vaccines (N=15/primary subgroups and 17/boosted; demographic characteristics in Table S3; gE-specific results in Figure 2 and VZV-specific results in Figure S4). Both HZ/su- and ZV-recipients showed increases in gE- and VZV-CD4+ and CD8+ proliferation after vaccination. Peak VZV-CD4+ and -CD8+ proliferation adjusted for baseline was similar in HZ/su and ZV recipients, but gE-CD4+ and -CD8+ proliferation was higher in HZ/su compared with ZV recipients (FDR-p<0.001).

We further investigated if the CD8+ ex vivo proliferation represented CD8+ memory formation in response to vaccination or a bystander effect of the strong CD4+ responses to vaccination. We and others have previously demonstrated that ELISPOT measures primarily CD4+ T-cell responses (27, 28). We used this property to determine if CD8+ T-cell proliferation depended on IL2 production by CD4+ T-cells. The data did not show significant associations between PMR gE-IL2 SFC and -CD8+ proliferation (p=0.13; Figure S5). In contrast gE-IL2 SFC PMR significantly correlated with -CD4+ proliferation (p<0.0001; Figure S5). This suggested that CD8+ proliferation at PMR represented CD8+ memory responses that were independent of the stimulation provided by CD4+ via ex vivo IL2 secretion.

**Flow Cytometric Analysis of CD4+ and CD8+ gE- and VZV-T Cell Profiles at PMR**
Responses generated by the vaccines were also characterized using functional Teff, regulatory (Treg) and immunologic checkpoint (Tcheck) markers after gE, VZV, and mock ex-vivo restimulation in a subset of 30 HZ/su- and 30 ZV-recipient equally distributed across the three age and immunization subgroups (demographics in Table S2). Of the 126 flow parameters measured (Table S4), we chose to eliminate 59 parameters because the median ratio of gE- or VZV-stimulated divided by the mock-stimulated responses were <1.1 in both vaccine groups. Figure 3 shows heat maps of unsupervised clusters of the subsets remaining in the analysis. The VZV-specific CD4+ and CD8+ Teff (IFNγ+, TNFα+, CD107a+, and/or CXCR3+) clustered together with VZV-specific Treg (CD25+, CD127- and/or FOXP3+) and Tcheck (LAG3+ and TIM3+). gE-specific Teff and Tcheck also clustered together.

Regression analyses of PMR adjusted for baseline showed that compared with ZV, HZ/su recipients had significantly higher CD4+ VZV-Treg, CD8+ VZV-Tcheck and CD4+ and CD8+ gE-Teff and -Tcheck (Table 4). CD8+CD107a+ cytolytic VZV-Teff were significantly higher in ZV compared with HZ/su recipients, but only before adjustment for multiple comparisons. Age, gender and booster status did not affect the differential effect of the two vaccines on immune responses. However, compared with the primary HZ/su groups, the boosted group had lower CD4+ and/or CD8+ VZV-Teff, -Treg, and -Tcheck and CD8+ gE-Tcheck (Table S5). This was not observed in ZV recipients.

**Aggregate Results Highlighting the Differences between CMI Responses to HZ/su and ZV**

To select the best candidates for immune correlates with the superior efficacy of HZ/su compared with ZV, we built a forest plot of the top parameters which differentiated between vaccine responses (Figure 5). Responses that were higher in ZV compared with HZ/su
recipients included VZV-CD4+ Teff and -CD8+ CTL PMR. HZ/su recipients had higher gE-Th1, -Teff, -Tcheck and -CD4+ and -CD8+ proliferative PMR and -Th1 persistent responses, which was consistent with the higer amount of gE in HZ/su compared with ZV. However, HZ/su also had higher VZV-IL2 and -CD4+ Treg PMR and -IL2 persistent responses at year 1 compared with ZV recipients.

**Post-Hoc Mediation Analysis of the Difference in Persistence of Th1 Responses Between the Two Vaccines**

The difference in persistence of VZV- and gE-Th1 responses at 1 year between the two vaccines was analyzed by multivariate regression, including the factors that had a significant effect on persistence in univariate analyses: vaccine type, baseline Th1 responses and PMR Th1. The multivariate analysis showed that VZV-Th1 baseline and PMR, but not vaccine type, had independent significant effects on VZV-Th1 persistence, whereas vaccine type and gE-Th1 PMR, but not baseline gE-Th1, had independent significant effects on gE-Th1 persistence (data not shown). Upon noting that IL2 PMR represented the common denominator among factors with independent effects on persistence of both gE- and VZV-Th1, we hypothesized that the VZV- and gE-IL2 PMR after vaccination represented the immunologic mechanism necessary and sufficient for VZV- and gE-Th1 persistence after vaccination. To test this new hypothesis, we performed post-hoc mediation analyses (Table 4). The mediation analysis measured the average controlled mediated effect (ACME), which represents the estimated effect of the vaccines on persistence of Th1 responses attributed to their effects on IL2 PMR; and the average direct effect (ADE), which represents the estimated effect of the vaccines on persistence of Th1 responses that does not act through the IL2 PMR pathway (diagram below Table 2). The data showed nonsignificant ADE (p>0.1) and highly significant ACME (p≤0.01) both for gE- and VZV-Th1 1-year responses, indicating that IL2 PMR mediated the effect of vaccines on persistence of Th1 responses to vaccination.
Discussion

The primary objective of this study was to identify immune responses that may explain the superior protection against HZ conferred by HZ/su compared with ZV. Immune responses that clearly distinguished the two vaccines were the higher gE- and VZV-specific memory Th1 responses generated by HZ/su, including peak CD4+ Tcm% and Tem%, gE- and VZV-IL2 SFC, and CD4+ and CD8+ gE-memory measured by proliferation. The predominance of memory responses in HZ/su recipients may explain the sustained protection against HZ of ≥87% up to 4 years after HZ/su administration compared to ~40% protection by ZV after a similar interval (16, 17, 29, 30). Higher VZV-Treg% and gE-Tcheck% at PMR in HZ/su compared to ZV recipients are also probably related to the higher memory responses in HZ/su. The Treg and Tcheck may play a role in the Th1 differentiation by directing the immune response from effector to memory (31-33). Alternatively, they may signal that Teff are being quenched. In contrast to HZ/su, the immune response to ZV was characterized by higher VZV-CD4+ and -CD8+ effectors at PMR. This may be due to the nature of this live virus vaccine that includes an agent capable of multiple cycles of replications, which sustain the Teff for a longer period. This is in agreement with the findings of our previous study in which we used VZV DNAemia after ZV administration as an indicator of vaccine viral replication and found that DNAemia positively correlated with longer persistence of VZV-Teff in the circulation and with delayed increase of Th1 memory responses after ZV (34).

The very low or absent gE-Th1 responses before HZ/su administration, even in those who had received ZV ≥5 years before entering the study, suggest that T cell responses to gE are not dominant after wild or attenuated VZV infection and that some individuals do not mount responses to gE or lose these responses over time. In fact, after the 1st dose of HZ/su responses to gE were very low, and responses to VZV were lower than those of ZV recipients.
This finding is in agreement with previously published data showing gE-specific CD4+ Th1 responses by flow cytometry in only 20% of vaccinees after the 1st dose of HZ/su (35). Sei et al. also showed that other VZV gene products, including IE 63, IE 62, gB and ORF 9, were targeted more frequently than gE by CD4+ and CD8+ T cells in response to ZV administration. Taken together, these observations underscore two important points: 1) the 2nd dose of HZ/su is essential for the immunogenicity and, consequently, efficacy of this vaccine (this difference is not explained by ZV being administered as a single dose, since providing two doses of ZV does not significantly alter the immune response) (36, 37); 2) biologically significant gE-Th1 responders T cells may arise from naïve cells. Whether drawing responses from the naïve T cell pool may be advantageous for the host because these cells have undergone less cycles of replication than memory cells and/or are less exhausted and, thereby, may generate longer lasting memory or more efficient killing is not known. Akondy et al. showed that ZV also draws Th1 responders from the naïve T cell pool, but those responders died quickly and did not contribute to persistent immunity (38). The role of de novo responses to HZ/su in its efficacy warrants further investigation, because this factor may have important implications for the design of other vaccines for older adults.

The gE- and VZV-IL2 PMR to HZ/su and ZV not only independently contributed to the persistence of Th1 responses after vaccination, but mediated the effect of the vaccines on persistent Th1, indicating that IL2 PMR was necessary for the persistence of Th1 responses after vaccination. Our findings contrast with a previous study in which persistence of IFNγ responses to ZV was not predicted by the magnitude of the VZV-IFNγ PMR (39). The difference underscores the importance of IL2 as a predictor of immunogenicity. Currently, there is no mechanistic immune correlate of protection conferred by ZV or HZ/su. gE- and/or VZV-IL2 PMR
are strong candidates to fill this gap, which we are planning to verify in studies in which HZ is an endpoint, such as in immune compromised hosts.

CD8+ T cells have a prominent role in protection against herpesviruses (40). Increased VZV-CD8+Teff have been described during convalescence from chickenpox and HZ and after exogenous exposure to VZV or reactivation of VZV (41-43). gE-specific CD8+ Teff responses to HZ/su have not been previously demonstrated in humans, although the QS21 component of AS01B is known to promote antigen cross-presentation by dendritic cells (44, 45). CD8+ T cell responses to antigens co-formulated with AS01B was observed in mouse vaccination models and in in vitro human studies (44, 45). Here we demonstrated that HZ/su generated gE- and VZV-specific CD8+ T cell proliferative PMR independent of the CD4+ T cell IL2 production. Furthermore, sorted proliferating CD8+ T cells from HZ/su recipients respond with IFNγ and/or IL2 production when stimulated by autologous lymphoblastoid cells infected with gE-containing vaccinia virus vectors and when restimulated with gE peptide pools as previously described (46, 47). We also showed that gE- and VZV-specific CD8+ T cells generated by HZ/su produced less Th1 cytokines and cytotoxicity markers compared to CD8+ T cells generated by ZV. In contrast, HZ/su generated higher CD8+ Tcheck and Treg PMR. The upregulation of immunologic checkpoints may quench the Teff function of CD8+ T cells, thereby explaining the difficulty in demonstrating gE-specific CD8+ Teff responses after HZ/su administration. Alternatively, the CD8+ T cells generated in response to HZ/su may use cytotoxicity mediators that we did not study.

This study was the first to compare immune responses to HZ/su between older adults who previously received ZV or did not (48). The FluoroSpot responses of individuals immunized with HZ/su were similar regardless of prior ZV administration, which was confirmed by a recent publication (49). However, some Teff and Tcheck PMR were lower in HZ/su recipients who had
prior ZV. While our study was powered for the FluoroSpot outcome measure, the Teff and Tcheck studies were part of an exploratory analyses and need to be confirmed.

The immunologic responses to HZ/su may also provide insight into the immunologic mechanism(s) responsible for preventing HZ. Latent VZV is present only in sensory neurons of dorsal root ganglia (50). Current models suggest that latency is maintained either by: 1) VZV-T cells that synapse with latently infected neurons to provide signals required to maintain latency; or 2) VZV-CMI that limits replication of reactivated virus prevents symptomatic disease, for which there is growing evidence (51-53). The second model is supported by our findings, since latently infected neurons do not express gE, and yet this remarkably efficacious vaccine relies on memory gE responses for its protective effect (54-56). This implies that protection against HZ is conferred by surveillance for and rapid resolution of sporadic VZV reactivation.

There are limitations in this study. These include the small sample size, especially for certain measures of VZV-specific responses and the inability to relate the immune responses to clinical endpoints.

The high efficacy of HZ/su is exceptional among vaccines given to older adults and among investigational vaccines against herpesviruses. Compared to ZV, HZ/su is distinguished by robust and persistent memory responses. The AS01B adjuvant is critical for the magnitude of the Th response to HZ/su as previously shown (24, 35, 57) and probably plays a role in its persistence. ASO1B may be of value with other subunit antigens in older adults and in other herpesvirus vaccines.
Methods

Study Design. This study enrolled 160 participants in good health except for treated chronic illnesses typical of the age of the vaccinees. All had prior varicella or resided in the USA at least 30 years; none had prior HZ. Exclusions from the study were immune suppression and recent blood products or other vaccines. Arms A and B (Figure S1 and Table S1), which contained 90 total participants who had not previously had ZV, were randomly assigned to receive either ZV followed by placebo or 2 doses of HZ/su at days 0 and 60. Arms A and B were further stratified by age (50-59 years; N=22 or ≥70-85 years; N=23). Arms C and D contained an additional 70 participants who were ≥70-85 yo and had received ZV ≥5 years previously. They were randomly assigned to receive either an additional dose of ZV followed by placebo (Arm C) or 2 doses of HZ/su (Arm D). All vaccinations were blinded from the participant. Blood was obtained for immunologic assessment on Days 0, 30, 90, and 356 from all participants. Additional blood was drawn for Arm A on day 7 and from Arm B on days 7 and 67. Peripheral blood mononuclear cells (PBMC), plasma and serum were cryopreserved within 4 hours of acquisition (58, 59).

Flow cytometric enumeration of VZV and gE-specific T cell subsets. Thawed PBMC were cultured as above at 2.5*10^6 cells/mL in growth medium in the presence of infectious VZV (60,000 PFU/mL), gE peptide pools as above (2.5 µg/mL), or mock stimulation. CD28 (Mabtech FSP-0102-10) and CD49D (BD 340976) monoclonal antibodies (mAb) were added at 1ug/mL. Brefeldin A (Sigma, 5ug/mL), Monensin (Sigma, 5ug/mL) and anti-CD107a (clone H4A3; BD 328609) were added for the last 16h. gE-stimulated and mock-stimulated were incubated for 18h, while wells with infectious VZV were incubated 42h. At the end of the incubation PBMC were washed and incubated with zombie yellow viability stain (Biolegend). PBMC were then washed in 1% BSA (Sigma) in PBS (Mediatech) (stain buffer), divided into 3 panels and incubated with antibodies against the following markers: CD3 (Ax700; clone UCHT1; BD
557943; all panels), CD4 (PC5.5; clone 13B8.2; Beckman Coulter B16491; all panels), CD45RO (PE-CF594; clone UCHL1; BD 562327; panel 1), CCR7 (APC; clone 3D12; BD 353213; panel 1), CD27 (PE-Cy7; clone M-T271; BD 302837; panel 1), CD103 (PE; clone Ber-ACT8; Biolegend 350205; panel 1), CD57 (FITC; clone NK-1; BD 561906; panel 2), CD127 (PE-CF594; clone HIL-7R-M21; BD 562397; panel 2), PD1 (BV421; clone EH12.2H7; Biolegend 329919; panel 2), CLA (FITC; clone HECA-452; BD 561987; panel 3), LAG3 (PE; clone 3DS223H; eBioscience 12-2239-41; panel 3), TIM3 (PE-CF594; clone 7D3; BD 565561; panel 3), CD39 (PE-Cy7; clone A1; Biolegend 328211; panel 3), CTLA4 (APC; clone L3D10; Biolegend 349907; panel 3), CXCR3 (APC-Cy7; clone G025H7; Biolegend 353721; panel 3), and KLRG1 (BV421; clone 2F1/KLRG1; Biolegend 138413; panel 3). Intracellular staining was performed with antibodies against IL10 (PE-Cy7; clone JES3-9D7; Biolegend 501419; panel 2), TGFβ (APC; clone TW4-2F8; Biolegend 349607; panel 2), TNFα (APC-Cy7; clone MAb11; Biolegend 502943; panel 1), IFNγ (BV421; clone B27; BD 502531; panel 1), and FoxP3 (PE; clone 259D/C7; BD 560082; panel 2) as appropriate. Unbound antibodies were removed by washing with staining buffer and fixed in 2% paraformaldehyde (Electron Microscopy Sciences) in PBS. ≥200,000 events were acquired with the Gallios (Beckman Coulter) instrument and analyzed using FlowJo (Tree Star) software. The gating strategies are shown in Figure S8. Figure S9 shows the Treg specificity of FOXP3+CD25+ marker combination as verified during staining optimization assays.

T cell proliferation measured by flow cytometry. Thawed PBMC stained with Cell Trace Violet (BioLegend) were cultured in the presence of infectious VZV, gE peptide pools as above, or mock stimulation for 5 days at 10^6 PBMC/mL as above. On day 3, 3.3 IU/mL rhIL2 (R&D Systems) were added to mock and VZV wells. At the end of the incubation, PBMC were washed with PBS and stained with zombie yellow viability stain. PBMC were then washed, stained with
anti-CD3-Ax700, anti-CD4-PC5.5 and anti-CD8-PE-CF594 (clone RPA-T8; BD 562282) and analyzed as above. Proliferation was assessed by cell trace\textsuperscript{dim} populations.

**Primary and Secondary Outcomes:** The primary outcome was IFN\textgreek{y}/IL2 FluoroSpot results at 30 days after the last dose of vaccine in each group. The effects of vaccine, of age and of prior ZV administration on the primary outcome were pre-specified objectives. Secondary outcomes were flow cytometric enumeration of memory, effector, regulatory and exhausted CD4+ and CD8+ T cells and identifying responses that clearly differentiated the two vaccines. Description of adverse events was also a secondary objective.

**Statistical Analysis:** Frequencies (%) or means and standard deviations were calculated for baseline patient demographics. To evaluate associations between peak response and 1-year FluoroSpot and vaccine, linear regression models adjusting for baseline values were constructed. Age, gender and booster status were evaluated as covariates and excluded from the models if not found significant (p<0.05). A post-hoc mediation analysis was conducted to determine whether the effect of vaccine on persistence of Th1 responses at 1 yr were mediated through PMR responses. For each mediation analysis, two regression models were estimated per the methods outlined in Tingley, et. al (60). The average direct effect (ADE) was estimated directly from the regression coefficient describing the PMR adjusted relationship between vaccine and persistence of Th1. The average controlled mediated effect (ACME) was estimated using the product of coefficients method and testing using bootstrapping. The gE- and VZV-specific CD4+ and CD8+ T cell differentiation profiles were compared at peak response between gE- and ZV-stimulation using a tobit regression model (R function vglm from package VGAM)(61), to account for the lower detection limit in the flow cytometry data. T cell differentiation profiles were log-transformed and models were adjusted for baseline response, with the threshold was set at 0.005 for CD4 and 0.01 for CD8, reflecting their detection
thresholds. Similar to FluoroSpot, linear regression models were used to characterize peak response proliferation to vaccine, using log-transformed values and adjusting for baseline. Flow cytometry data were expressed as the ratio of counts in VZV- or gE- over mock-stimulated wells; ratios were log transformed prior to analysis. For observations where the mock-stimulated result was 0, the participant’s lowest mock cell percentage from an alternative visit was imputed, and if all mock-stimulated cell percentages were 0, the lowest observed value from that participant was imputed. Similarly, for observations where a stimulated cell percentage was 0, the lowest cell count for that participant was imputed. For parameters where more than 3% of the data were reported as 0’s, sensitivity analyses were conducted to evaluate the impact of imputation and compared to the imputed results. Flow parameters with a median VZV- or gE-to mock-stimulated ratio below 1.1 were not considered for analyses; these parameters were considered to have too large of a signal to noise ratio to be important in differentiating vaccine responses. To evaluate associations between flow parameters and vaccine group, linear regression models were constructed for each parameter of interest, adjusting for baseline value. Age, gender and booster status were evaluated as covariates and excluded from the models if not found significant (p<0.05). To account for multiple comparisons, False Discovery Rate (FDR) corrections were implemented for each outcome, within cell type (CD4 and CD8) and for each stimulant (VZV and gE); unadjusted and adjusted p-values are reported.

**Sample Size Justification:**

The primary statistical hypothesis for non-inferiority tests (Arm A vs. B and Arm C vs. D) regarding VZV- and gE-specific ELISPOT responses between the two vaccines (Vaccine 1 = HZ/su and Vaccine 2 = ZV) is H0: R1/R2 ≤0.5 versus H1: R1/R2 >0.5, where R1 is the fold-rise ratio of post-vaccine response to baseline level for Vaccine 1 and R2 is the fold-rise ratio for Vaccine 2 at 30 days after Vaccine 2. A ratio of 0.5 corresponds to a 2-fold decrease of fold-rise ratio in Vaccine 1 compared with Vaccine 2. Rejecting the null hypothesis (H0) at the 1-sided α=0.025 level
corresponds to the lower bound of the 2-sided 95% CI on the fold-rise ratio (Vaccine 1/Vaccine 2) being >0.5 and would lead to the conclusion that the response to live zoster vaccine is noninferior to the response to recombinant gE Vaccine.

With 45 subjects enrolled in each arm A and B and an assumed 10% drop-out rate, 40 subjects in each arm will be available for analysis and can achieve over 90% power to detect non-inferiority (Arm A vs. B) using a one-sided two-sample t-test with a significance level (alpha) of 0.025. With 35 subjects enrolled in each arm C and D and an assumed 10% drop-out rate, 31 subjects in each arm will be available for analysis and can achieve over 80% power to detect non-inferiority (Arm C vs. D) using a one-sided two-sample t-test with a significance level (alpha) of 0.025. The assumptions used in the power calculation are: (1) 10% drop-out rate; (2) common standard deviation (SD) of 0.41 (assumed an extra variability) on the log-transformed scale in each group based on the data from unpublished experiments administering the live herpes zoster vaccine to subjects 60-70 years of age; (3) the non-inferiority margin is 2-fold with respect to fold-rise ratio; (4) the true ratio (Vaccine 1/Vaccine 2) for fold-rise ratio is 1.0.

Study Approvals

This study in humans (NCT02114333) was reviewed and approved by the Colorado Multiple Institutions Review Board, University of Colorado School of Medicine, Aurora, CO. Subjects provided informed consent prior to participation in the study.

Author contributions: AW and MJL designed the study, analyzed the data and wrote the manuscript. MJL oversaw the clinical trial performance. AW oversaw the laboratory experiments. MEK, AH and DR performed the statistical analysis and wrote sections of the
manuscript. MJJ performed immunologic assays and wrote sections of the manuscript. NL performed the clinical activities. All authors reviewed the manuscript.

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References


52. Papaevangelou V, M. Quinlivan, J. Lockwood, O. Papaloukas, G. Sideri, E. Critselis, I.


Figure 1. Kinetics of Th1 responses to HZ/su and ZV measured by FluoroSpot. Data were derived from 158 participants equally distributed between ZV, administered at day 0, and HZ/su, administered in 2 doses at days 0 and 60. The graphs show mean±SEM SFC/10^6 PBMC over time in all ZV recipients (squares and interrupted lines) and HZ/su recipients (circles and continuous lines). The ordinates are on ln scale. Right column shows responses to VZV and left column to gE ex vivo restimulation. Upper row shows IL2, middle row IFN-γ and lower row double positive (DP) responses. Regression analyses adjusted for baseline and for multiple comparisons showed significantly higher VZV-IL2 and gE-IL2, -IFN-γ and DP responses 30 days after the last dose of vaccine in HZ/su compared with ZV recipients (FDR-adjusted p ≤ 0.01). For individual age/treatment groups, please see Figure S2.
Figure 2. gE-specific CD4+ and CD8+ T cell PMR proliferation. Data were derived from 18 HZ/su young, 17 HZ/su old, 20 HZ/su boosted, 6 ZV young, 9 ZV old and 9 ZV boosted. Panel A shows the gating strategy. Panel B shows the summary of proliferation in each age and treatment group. % proliferating cells of the parent indicates that proliferating CD4+ or CD8+ T cells are expressed as a percent of total CD4+ or CD8+ T cell parent population, as appropriate. Asterisks show the significance of differences compared with baseline using RMANOVA adjusted for multiple comparisons. In addition, baseline-adjusted PMR regression analysis between vaccine groups had FDR-adjusted p < 0.0001. Abbreviations: FS-A= forward scatter area; FS-H= forward scatter height; FS-W= forward scatter width; SS-A= side scatter area.
Figure 3. Conventional and regulatory T cell responses in HZ/su and ZV recipients at PMR. PMR was day 30 for ZV and 90 for HZ/su recipients. Data were derived from 60 participants equally distributed across vaccination and age groups. The heatmap T cell responses to VZV ex vivo restimulation were grouped by unbiased hierarchical clustering. Each
column represents a T cell subset and each row individual a participant (A = ZV primary group, B = HZ/su primary group, C = ZV boosted group, D = HZ/su boosted group). The rectangles identifies T cell clusters.
Figure 4. Conventional and regulatory T cell responses in HZ/su and ZV recipients at PMR. PMR was day 30 for ZV and 90 for HZ/su recipients. Data were derived from 60 participants equally distributed across vaccination and age groups. The heatmap T cell responses to VZV ex vivo restimulation were grouped by unbiased hierarchical clustering. Each column represents a T cell subset and each row individual a participant (A = ZV primary group, B = HZ/su primary group, C = ZV boosted group, D = HZ/su boosted group). The rectangles identifies T cell clusters.
Figure 5. Hierarchical presentation of T cell responses that significantly differentiate the two vaccines. Data were derived from 158 participants for ELISPOT, 94 for proliferation, 60 for T cell differentiation and functional PMR. The plot shows means estimated for the fold-differences of ZV/HZ/su results and 95% CI for significantly different parameters (95% CI does not overlap the null effect, i.e. equivalence, indicated by the vertical dotted). All other parameters are shown in Figure S7. The stimulant and T cell responses are indicated on the coordinate. Means <1 indicate higher responses in the HZ/su group and >1 indicate higher responses in the ZV group.
Table 1. Demographic characteristics of study participants*

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>HZ/su</th>
<th>ZV</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>70.0 (9.7)</td>
<td>69.5 (9.7)</td>
</tr>
<tr>
<td><strong>Sex</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>38 (48)</td>
<td>34 (43)</td>
</tr>
<tr>
<td>F</td>
<td>41 (52)</td>
<td>45 (57)</td>
</tr>
<tr>
<td><strong>Race</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>W</td>
<td>77 (97.5)</td>
<td>75 (95)</td>
</tr>
<tr>
<td>NW</td>
<td>2 (2.5)</td>
<td>4 (5)</td>
</tr>
<tr>
<td><strong>Ethnicity</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H</td>
<td>1 (1)</td>
<td>2 (2.5)</td>
</tr>
<tr>
<td>NH</td>
<td>78 (99)</td>
<td>77 (97.5)</td>
</tr>
</tbody>
</table>

*79 of 80 in each vaccine group completed all study visits

Abbreviations: W=white; NW=non-white; H=Hispanic; NH=non-Hispanic
Table 2. T cell responses to the HZ vaccines have distinct differentiation profiles.

<table>
<thead>
<tr>
<th>Differentiation stage</th>
<th>Means of ZV/HZ/su results</th>
<th>95% CI of the mean</th>
<th>p value</th>
<th>FDR p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Effector CD4</td>
<td>2.07</td>
<td>1.56; 2.76</td>
<td>5.16E-07</td>
<td>7.44E-06</td>
</tr>
<tr>
<td>Effector Memory CD4</td>
<td>0.55</td>
<td>0.40; 0.74</td>
<td>0.0001</td>
<td>0.007</td>
</tr>
<tr>
<td>Central Memory CD4</td>
<td>0.76</td>
<td>0.63; 0.92</td>
<td>0.005</td>
<td>0.02</td>
</tr>
</tbody>
</table>

The data were derived from 60 participants equally distributed across vaccination and age groups. Means and 95% CI were estimated by logistic regression. Means <1 indicate higher responses in the HZ/su group and >1 indicate higher responses in the ZV group. The p values were adjusted for multiple comparisons using FDR correction.

Effector CD4 = CD4+CD45RO+CCR7-CD27-; Effector memory CD4 = CD4+CD45RO+CCR7+CD27-; central memory CD4 = CD4+CD45RO+CCR7+CD27+
<table>
<thead>
<tr>
<th>Specificity</th>
<th>Subset (% of parent)</th>
<th>Mean of ZV/HZ/su results</th>
<th>95% CI</th>
<th>p-value</th>
<th>FDR p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>VZV</td>
<td>CD4+FOXP3+CD25+</td>
<td>0.85</td>
<td>0.79, 0.92</td>
<td>0.0003</td>
<td>0.01</td>
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<tr>
<td></td>
<td>CD8+CD127-</td>
<td>0.76</td>
<td>0.65, 0.88</td>
<td>0.001</td>
<td>0.02</td>
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<tr>
<td></td>
<td>CD8+CD107a+</td>
<td>1.43</td>
<td>1.09, 1.89</td>
<td>0.01</td>
<td>0.17</td>
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<tr>
<td>gE</td>
<td>CD4+TNFα+</td>
<td>0.38</td>
<td>0.30, 0.47</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td></td>
<td>CD8+LAG3+</td>
<td>0.40</td>
<td>0.31, 0.51</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
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<tr>
<td></td>
<td>CD8+CXCR3+LAG3+</td>
<td>0.44</td>
<td>0.36, 0.55</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
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<tr>
<td></td>
<td>CD8+TIM3+</td>
<td>0.76</td>
<td>0.70, 0.82</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
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<tr>
<td></td>
<td>CD4+CXCR3+LAG3+</td>
<td>0.64</td>
<td>0.56, 0.74</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
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<tr>
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<td>CD8+LAG3+TIM3+</td>
<td>0.47</td>
<td>0.37, 0.60</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
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<tr>
<td></td>
<td>CD4+TNFα+IFNγ+</td>
<td>0.25</td>
<td>0.16, 0.38</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
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<tr>
<td></td>
<td>CD4+CD107a+TNFα+</td>
<td>0.37</td>
<td>0.27, 0.52</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td></td>
<td>CD4+LAG3+</td>
<td>0.69</td>
<td>0.60, 0.79</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
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<tr>
<td></td>
<td>CD4+CD017a+IFNγ+TNFα+</td>
<td>0.26</td>
<td>0.16, 0.43</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
</tr>
</tbody>
</table>

Data were derived from 60 participants equally distributed between groups. Means and 95% CI were estimated by logistic regression for the ratios of ZV/HZsu results. Means <1 indicate higher responses in the HZ/su group and >1 indicate higher responses in the ZV group. The p values were adjusted for multiple comparisons using FDR correction. Shown are all the significant differences in VZV-specific responses and 10 gE-specific responses with the largest estimates.
Table 4 Results of the mediation analysis of the PMR IL2 on the persistent persistence of the Th1 responses to vaccination.

<table>
<thead>
<tr>
<th></th>
<th>Estimate of the IL2 PMR-mediated effect (ACME) or of the vaccine direct effect (ADE)</th>
<th>95% CI</th>
<th>p-value</th>
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</thead>
<tbody>
<tr>
<td><strong>VZV-IL2 PMR mediation on effect of treatment on persistent VZV-IL2</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>ACME</strong></td>
<td>-0.22</td>
<td>-0.40, -0.0</td>
<td>0.008</td>
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<tr>
<td><strong>ADE</strong></td>
<td>-0.12</td>
<td>-0.32, 0.07</td>
<td>0.20</td>
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<tr>
<td><strong>VZV-IL2 PMR mediation on effect of treatment on persistent VZV-IFN(\gamma)</strong></td>
<td></td>
<td></td>
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<tr>
<td><strong>ACME</strong></td>
<td>-0.14</td>
<td>-0.26, -0.04</td>
<td>0.004</td>
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<tr>
<td><strong>ADE</strong></td>
<td>-0.02</td>
<td>-0.21, 0.15</td>
<td>0.76</td>
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<tr>
<td><strong>VZV-IL2 PMR mediation on effect of treatment on persistent VZV-DP</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>ACME</strong></td>
<td>-0.14</td>
<td>-0.26, -0.05</td>
<td>0.01</td>
</tr>
<tr>
<td><strong>ADE</strong></td>
<td>-0.05</td>
<td>-0.23, 0.14</td>
<td>0.57</td>
</tr>
<tr>
<td><strong>gE-IL2 PMR mediation on effect of treatment on persistent gE-IL2</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>ACME</strong></td>
<td>-2.04</td>
<td>-2.53, -1.55</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>ADE</strong></td>
<td>-0.39</td>
<td>-0.99, 0.11</td>
<td>0.10</td>
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<tr>
<td><strong>gE-IL2 PMR mediation on effect of treatment on persistent gE-IFN(\gamma)</strong></td>
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<td></td>
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<tr>
<td><strong>ACME</strong></td>
<td>-1.59</td>
<td>-2.06, -1.17</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>ADE</strong></td>
<td>-0.25</td>
<td>-0.70, 0.22</td>
<td>0.28</td>
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<tr>
<td><strong>gE-IL2 PMR mediation on effect of treatment on persistent gE-DP</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>ACME</strong></td>
<td>-1.65</td>
<td>-2.19, -1.19</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>ADE</strong></td>
<td>-0.34</td>
<td>-0.77, 0.17</td>
<td>0.18</td>
</tr>
</tbody>
</table>

Abbreviations: ACME=average controlled mediated effect. ADE=average direct effect.
The ACME represents the effect attributed IL2 PMR to vaccines on persistent (year 1) outcomes; an effect size of 0 would indicate no IL2 PMR-mediated effect on persistent outcomes. The ADE represents the effect of vaccines on persistent outcomes that does not act through the IL2 PMR pathway; an effect size of 0 would indicate that vaccines have no effect on persistent outcomes outside of the IL2 PMR pathway. See diagram in Figure S6.