Th1 memory differentiates recombinant from live herpes zoster vaccines

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The adjuvanted varicella-zoster virus (VZV) glycoprotein E (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 years 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 second dose of HZ/su), gE-specific CD4^{+} and CD8^{+} T cell responses were 10-fold or more higher in HZ/su compared with ZV recipients. Comparing the vaccines, T cell memory responses, including gE–IL-2^{+} and VZV–IL-2^{+} spot-forming cells (SFCs), were higher in HZ/su recipients and cytotoxic and effector responses were lower. At 1 year after vaccination, all gE-Th1 and VZV–IL-2^{+} SFCs remained higher in HZ/su compared with ZV recipients. Mediation analyses showed that IL-2^{+} PMR were necessary for the persistence of Th1 responses to either vaccine and VZV–IL-2^{+} 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 an essential component or components of VZV-specific cell-mediated immunity (CMI) fall 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, which explains 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 glycoprotein E (gE) subunit herpes zoster vaccine (HZ/su), which contains the AS01$_B$ adjuvant consisting of MPL (lipid A of bacterial lipopolysaccharide, a TLR4 agonist) and QS21 (a triterpene plant derivative in the family of saponins) pack-

aged into liposomes (15). HZ/su provides 97% protection against HZ in vaccinees 50 yoa and older, including 89% efficacy in those 80 yoa and older, 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 to 9 years after vaccination in long-term follow-up studies (18–20).

These very favorable clinical responses to HZ/su are uniquely better compared with 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 AS01$_B$ (15, 23–25). The current report compares the immune responses elicited by ZV or HZ/su in participants 50 to 59 and 70 to 85 yoa who had never received HZ vaccine and also compares immune responses to the 2 vaccines in an additional cohort of participants 70 to 85 yoa who had received ZV 5 years or more prior to enrollment. The primary objective was to determine immunologic responses that best differentiated the 2 vaccines in individuals receiving HZ vaccine for the first time. Other objectives were to compare the responses elicited by HZ/su in participants who had received ZV 5 or more 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 (52%) were women, 152 (97%) were white, and 156 (98%) were non-Hispanic. The demo-

Table 1. Demographic characteristics of study participants

Characteristic		HZ/su	ZV
Age, mean (SD)		70.0 (9.7)	69.5 (9.7)
Sex, N (%)	M	38 (48)	34 (43)
	F	41 (52)	45 (57)
Race, N (%)	W	77 (97.5)	75 (95)
	NW	2 (2.5)	4 (5)
Ethnicity, N (%)	H	1 (1)	2 (2.5)
	NH	78 (99)	77 (97.5)

In each vaccine group, 79 of 80 completed all study visits. W, white; NW, nonwhite; H, Hispanic; NH, non-Hispanic.

graphic characteristics were similar between the 2 vaccine groups in each of the 3 subgroups: first time–immunized 50- to 59-year-old (young primary), first time–immunized 70- to 85-year-old (older primary), and 70- to 85-year- old individuals who received ZV 5 or more years before enrollment (older boosted).

VZV- and gE-specific Th1 responses to HZ/su and ZV measured by FluoroSpot. VZV-IL-2+ and gE–IL-2+, VZV–IFN-γ+ and gE–IFN-γ+, and VZV–IL-2+IFN-γ+ and gE–IL-2+IFN-γ+ 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 (Supplemental Figure 1; supplemental material available online with this article; https://doi.org/10.1172/JCI121484DS1). 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 Supplemental Figure 2). At baseline, participants had robust VZV-Th1 CMI (e.g., VZV–IL-2 mean ± SEM =168 ± 17 spot-forming cells [SFCs]/10⁶ peripheral blood mononuclear cells [PBMCs]), but very low or undetectable gE-Th1 CMI (gE–IL-2 = 25 ± 4 SFCs/10⁶ PBMCs). At PMR, ZV recipients reached 323 ± 24 VZV–IL-2 SFCs/10⁶ PBMCs and 35 ± 6 gE–IL-2 SFCs/10⁶ PBMCs, while HZ/su recipients reached 426 ± 30 VZV–IL-2 and 475 ± 36 gE–IL-2 SFCs/10⁶ PBMCs. It is important to note that responses were much lower after a single dose of HZ/su than the PMR that occurred after the second dose (226 ± 23 VZV–IL-2 and 128 ± 12 gE–IL-2 SFCs/10⁶ PBMCs). In fact, VZV–IL-2 responses after the first dose of HZ/su were lower than those of ZV recipients, underscoring the importance of the second dose for the immunogenicity of HZ/su.

Figure 1. Kinetics of Th1 responses to HZ/su and ZV measured by FluoroSpot. Data were derived from 158 participants equally distributed between those receiving ZV, administered at day 0, and those receiving HZ/su, administered in 2 doses, at days 0 and 60. The graphs show mean ± SEM SFCs/10⁶ PBMCs over time in all ZV recipients (squares and dotted lines) and HZ/su recipients (circles and lines). The ordinates are on a natural logarithmic scale. Right column shows responses to VZV and left column to gE ex vivo restimulation. Upper row shows IL-2, middle row shows IFN-γ, and bottom row shows DP responses. Regression analyses adjusted for baseline and for multiple comparisons showed significantly higher VZV–IL-2 and gE–IL-2, gE–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, see Supplemental Figure 2.
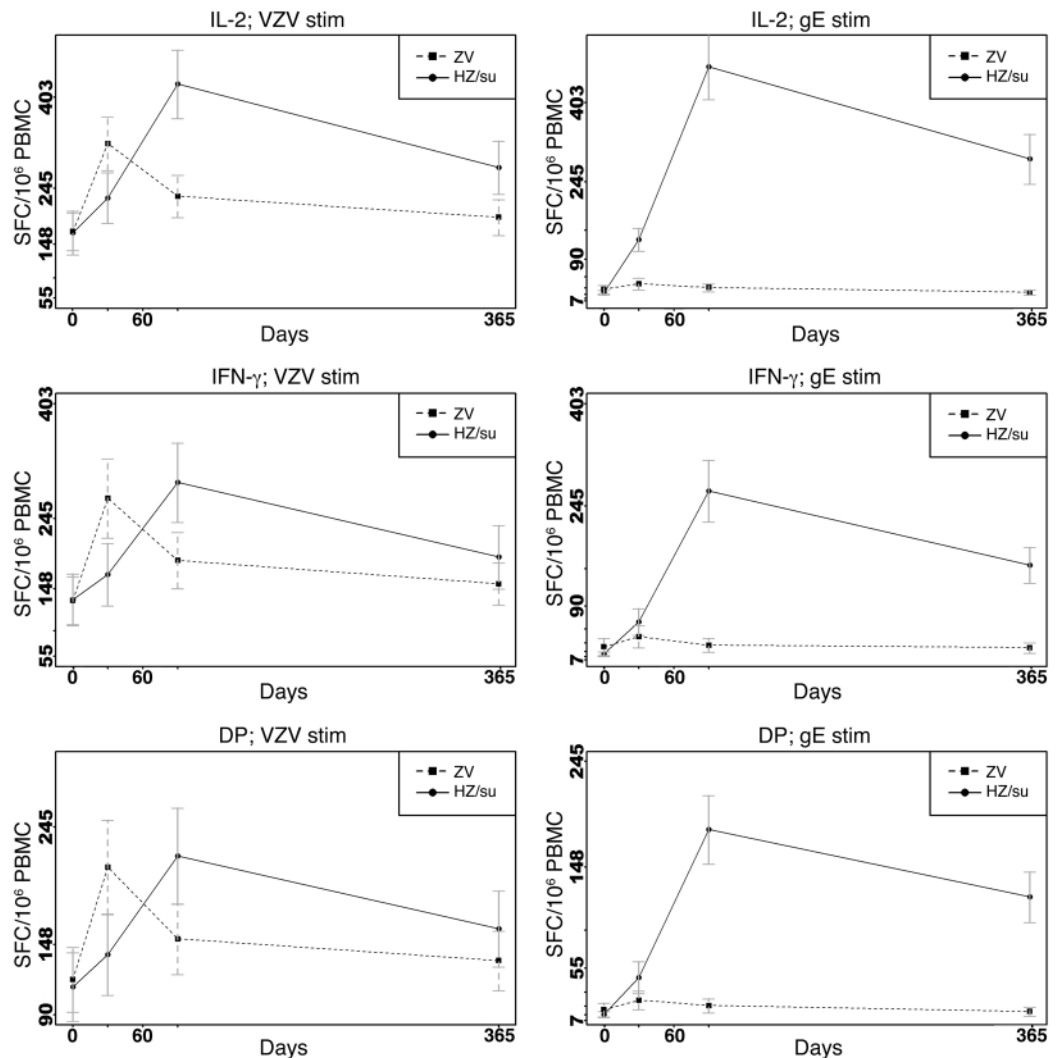
Table 2. T cell responses to the HZ vaccines have distinct differentiation profiles

Differentiation stage	Mean of ZV/HZ/su results	95% CI of the mean	P value	FDR-adjusted P value
Effector CD4	2.07	1.56; 2.76	5.16×10^{-07}	7.44×10^{-06}
Tem cells CD4	0.55	0.40; 0.74	0.0001	0.007
Tcm cells CD4	0.76	0.63; 0.92	0.005	0.02

Data were derived from 60 participants equally distributed across vaccination and age groups. Means and 95% CI were estimated by logistic regression. Means of less than 1 indicate higher responses in the HZ/su group, and those greater than 1 indicate higher responses in the ZV group. P values were adjusted for multiple comparisons using FDR correction. Effector CD4, CD4$^+$CD45RO$^+$CCR7-CD27$^-$; Tem cells CD4, CD4$^+$CD45RO$^+$CCR7$^+$CD27$^-$; Tcm cells CD4, CD4$^+$CD45RO$^+$CCR7$^+$CD27$^+$.

generated significantly higher gE-specific CD4$^+$ Tcm cells and Tem cells and lower CD4$^+$ Teff cells compared with 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-IFN-γ^+ T cells, also showed lower CD4$^+$ Teff cells, CD4$^+$ Tei cells, and CD8$^+$ Tei cells in HZ/su compared with ZV recipients (both FDR P=0.01, 0.047, and 0.06, respectively; Supplemental Figure 3B).

Baseline VZV-Th1 and gE-Th1 responses had a substantial positive effect on their respective PMR to either vaccine, but age, sex, or prior administration of ZV did not (data not shown). After adjusting for baseline responses, VZV–IL-2 PMR was higher in HZ/su compared with ZV recipients (FDR-adjusted P=0.01; Figure 1), but there were no differences in VZV–IFN-γ or VZV-DP responses, which indicated that the type of vaccine had a substantial effect only on VZV–IL-2 among all VZV-Th1 PMR tested. Adjusted gE-Th1 PMR were significantly higher in HZ/su compared with 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 2 vaccines and across the 3 age/treatment groups in each vaccine arm (demographics in Supplemental Table 2), we analyzed gE-CD4$^+$ and VZV-CD4$^+$ and gE-CD8$^+$ and VZV-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) cells (CCR7$^+$CD27$^+$CD45RO$^+$), effector memory (Tem) cells (CCR7$^-$CD27$^+$CD45RO$^+$), differentiated effector (Teff) cells (CCR7$^-$CD27$^-$CD45RO$^+$), intermediate effector (Tei) cells (CCR7$^-$CD27$^+$CD45RO$^-$), and terminally differentiated effector (Ted) cells (CR7$^-$CD27$^-$CD45RO$^-$) and confirmed their specificity to the stimulating antigen by IFN-γ production (gating strategy shown in Supplemental Figure 3A). It is important to note that both gE peptide pools and replication-competent VZV allow T cell epitope presentation in the context of MHC classes I and II. The comparison of the baseline-adjusted PMR showed that HZ/su

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 Supplemental Table 3; gE-specific results in Figure 2 and VZV-specific results in Supplemental Figure 4). Both HZ/su and ZV recipients showed increases in gE- and VZV-CD4$^+$ and gE- and CD8$^+$ proliferation after vaccination. Peak VZV-CD4$^+$ and VZV-CD8$^+$ proliferation adjusted for baseline was similar in HZ/su and ZV recipients, but gE-CD4$^+$ and gE-CD8$^+$ proliferation was higher in HZ/su compared with ZV recipients (FDR $P < 0.001$).

We further investigated whether 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

Table 3. Comparative effects of ZV and HZ/su on baseline-adjusted PMR T cell profiles

Specificity	Subset (% of parent)	Mean of ZV/HZ/su results	95% CI	P value	FDR-adjusted P value
VZV	CD4$^+$FOXP3$^+$CD25$^+$	0.85	0.79, 0.92	0.0003	0.01
	CD8$^+$CD127$^-$	0.76	0.65, 0.88	0.001	0.02
	CD8$^+$CD107a$^+$	1.43	1.09, 1.89	0.01	0.17
gE	CD4$^+$TNF-α^+	0.38	0.30, 0.47	<0.0001	<0.0001
	CD8$^+$LAG3$^+$	0.40	0.31, 0.51	<0.0001	<0.0001
	CD8$^+$CXCR3$^+$LAG3$^+$	0.44	0.36, 0.55	<0.0001	<0.0001
	CD8$^+$TIM3$^+$	0.76	0.70, 0.82	<0.0001	<0.0001
	CD4$^+$CXCR3$^+$LAG3$^+$	0.64	0.56, 0.74	<0.0001	<0.0001
	CD8$^+$LAG3$^+$TIM3$^+$	0.47	0.37, 0.60	<0.0001	<0.0001
	CD4$^+$TNF-α^+IFN-γ^+	0.25	0.16, 0.38	<0.0001	<0.0001
	CD4$^+$CD107a$^+$TNF-α^+	0.37	0.27, 0.52	<0.0001	<0.0001
	CD4$^+$LAG3$^+$	0.69	0.60, 0.79	<0.0001	<0.0001
	CD4$^+$CD017a$^+$IFN-γ^+TNF-α^+	0.26	0.16, 0.43	<0.0001	<0.0001

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 less than 1 indicate higher responses in the HZ/su group, and means greater than 1 indicate higher responses in the ZV group. 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.
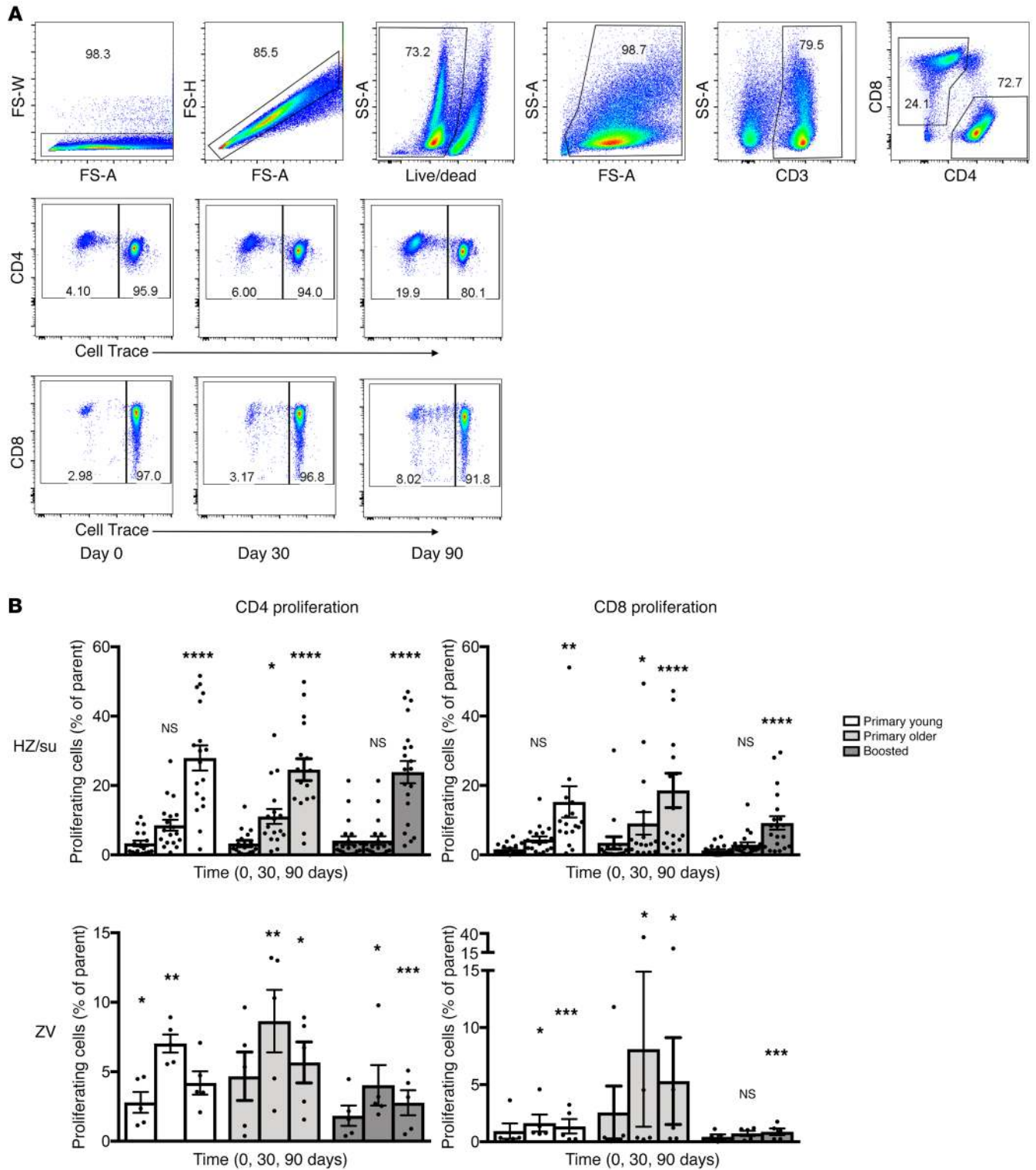
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Figure 2. gE-specific CD4+ and CD8+ T cell PMR proliferation. Data were derived from 18 HZ/su young, 17 HZ/su old, 20 HZ/su boosted, 6 ZV young, 9 ZV old, and 9 ZV boosted. (**A**) Gating strategy. (**B**) Summary of proliferation in each age and treatment group. Percentage of proliferating cells of the parent indicates that proliferating CD4+ or CD8+ T cells are expressed as a percentage of the 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. *$P < 0.05$ and > 0.01, **$P < 0.01$ and > 0.001, ***$P < 0.001$, ****$P < 0.0001$. In addition, baseline-adjusted PMR regression analysis between vaccine groups had FDR-adjusted $P < 0.0001$. FS-A, forward scatter area; FS-H, forward scatter height; FS-W, forward scatter width; SS-A, side scatter area.

Table 4. Results of the mediation analysis of the PMR IL-2 on the persistent persistence of the Th1 responses to vaccination

	Estimate of the IL-2 PMR-mediated effect (ACME) or of the vaccine direct effect (ADE)	95% CI	P value
VZV–IL-2 PMR mediation on effect of treatment on persistent VZV–IL-2			
ACME	−0.22	−0.40, −0.0	0.008
ADE	−0.12	−0.32, 0.07	0.20
VZV–IL-2 PMR mediation on effect of treatment on persistent VZV-IFN-γ			
ACME	−0.14	−0.26, −0.04	0.004
ADE	−0.02	−0.21, 0.15	0.76
VZV–IL-2 PMR mediation on effect of treatment on persistent VZV-DP			
ACME	−0.14	−0.26, −0.05	0.01
ADE	−0.05	−0.23, 0.14	0.57
gE–IL-2 PMR mediation on effect of treatment on persistent gE–IL-2			
ACME	−2.04	−2.53, −1.55	<0.001
ADE	−0.39	−0.99, 0.11	0.10
gE–IL-2 PMR mediation on effect of treatment on persistent gE-IFN-γ			
ACME	−1.59	−2.06, −1.17	<0.001
ADE	−0.25	−0.70, 0.22	0.28
gE–IL-2 PMR mediation on effect of treatment on persistent gE-DP			
ACME	−1.65	−2.19, −1.19	<0.001
ADE	−0.34	−0.77, 0.17	0.18

ACME represents the effect attributed to the IL-2 PMR to vaccines on persistent (year 1) outcomes; an effect size of 0 would indicate no IL-2 PMR-mediated effect on persistent outcomes. ADE represents the effect of vaccines on persistent outcomes that does not act through the IL-2 PMR pathway; an effect size of 0 would indicate that vaccines have no effect on persistent outcomes outside of the IL-2 PMR pathway. See diagram in Supplemental Figure 6.

used this property to determine whether CD8$^+$ T cell proliferation depended on IL-2 production by CD4$^+$ T cells. The data did not show significant associations between PMR gE–IL-2 SFCs and gE-CD8$^+$ proliferation (P = 0.13; Supplemental Figure 5). In contrast, gE–IL-2 SFC PMR significantly correlated with gE-CD4$^+$ proliferation (P < 0.0001; Supplemental Figure 5). This suggested that CD8$^+$ proliferation at PMR represented CD8$^+$ memory responses that were independent of the stimulation provided by CD4$^+$ via ex vivo IL-2 secretion.

Flow cytometric analysis of CD4$^+$ and CD8$^+$ gE–T cell and VZV–T cell profiles at PMR. Responses generated by the vaccines were also characterized using functional Teff cells, Tregs, and immunologic checkpoint (Tcheck) markers after gE, VZV, and mock ex vivo restimulation in a subset of 30 HZ/su and 30 ZV recipients equally distributed across the 3 age and immunization subgroups (demographics shown in Supplemental Table 2). Of the 126 flow parameters measured (Supplemental Table 4), 59 parameters were eliminated because the median ratio of gE- or VZV-stimulated divided by the mock-stimulated responses were less than 1.1 in both vaccine groups. Figures 3 and 4 show heatmaps of unsupervised clusters of the subsets remaining in the analysis. The VZV-specific CD4$^+$ and CD8$^+$ Teff cells (IFN-γ$^+$, TNF-α$^+$, CD107a$^+$, and/

or CXCR3$^+$) clustered together with VZV-specific Tregs (CD25$^+$, CD127$^-$, and/or FOXP3$^+$) and Tcheck (LAG3$^+$ and TIM3$^+$). gE-specific Teff cells 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 cells and gE-Tcheck (Table 3). CD8$^+$CD107a$^+$ cytolytic VZV-Teff cells were significantly higher (P = 0.01) in ZV compared with HZ/su recipients, but only before adjustment for multiple comparisons. Age, sex, and booster status did not affect the differential effect of the 2 vaccines on immune responses. However, compared with the primary HZ/su groups, the boosted group had lower CD4$^+$ and/or CD8$^+$ VZV-Teff cells, VZV-Tregs, and VZV-Tcheck and CD8$^+$ gE-Tcheck (Supplemental Table 5). This was not observed in ZV recipients.

Aggregate results highlighting the differences between CMI responses to HZ/su and to 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 that differentiated between vaccine responses (Figure 5). Responses that were high-

er in ZV compared with HZ/su recipients included VZV-CD4$^+$ Teff cells and VZV-CD8$^+$ CTL PMR. HZ/su recipients had higher gE-Th1, gE-Teff cells, gE-Tcheck, and gE-CD4$^+$ and gE-CD8$^+$ proliferative PMR and gE-Th1 persistent responses, which were consistent with the higher amount of gE in HZ/su compared with ZV. However, HZ/su also had higher VZV–IL-2 and VZV-CD4$^+$ Treg PMR and VZV–IL-2 persistent responses at year 1 compared with ZV recipients.

Post hoc mediation analysis of the difference in persistence of Th1 responses between the 2 vaccines. The difference in persistence of VZV-Th1 and gE-Th1 responses at 1 year between the 2 vaccines was analyzed by multivariate regression, including the factors that had a significant (Table 4) 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 (Table 4) effects on VZV-Th1 persistence, whereas vaccine type and gE-Th1 PMR, but not baseline gE-Th1, had independent effects on gE-Th1 persistence (data not shown). Upon noting that IL-2 PMR represented the common denominator among factors with independent effects on persistence of both gE-Th1 and VZV-Th1, we hypothesized that the VZV–IL-2 and gE–IL-2 PMR after vaccina-
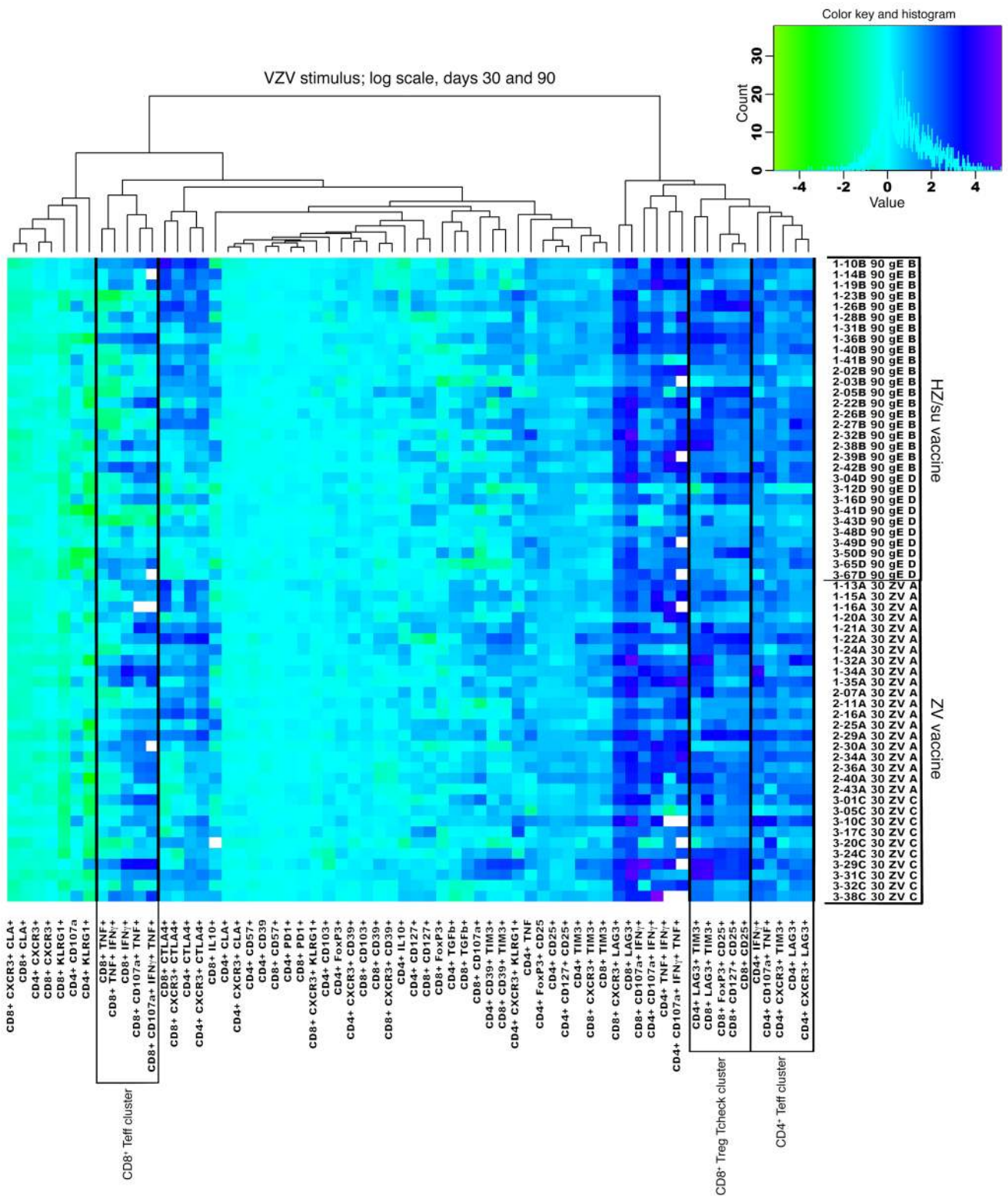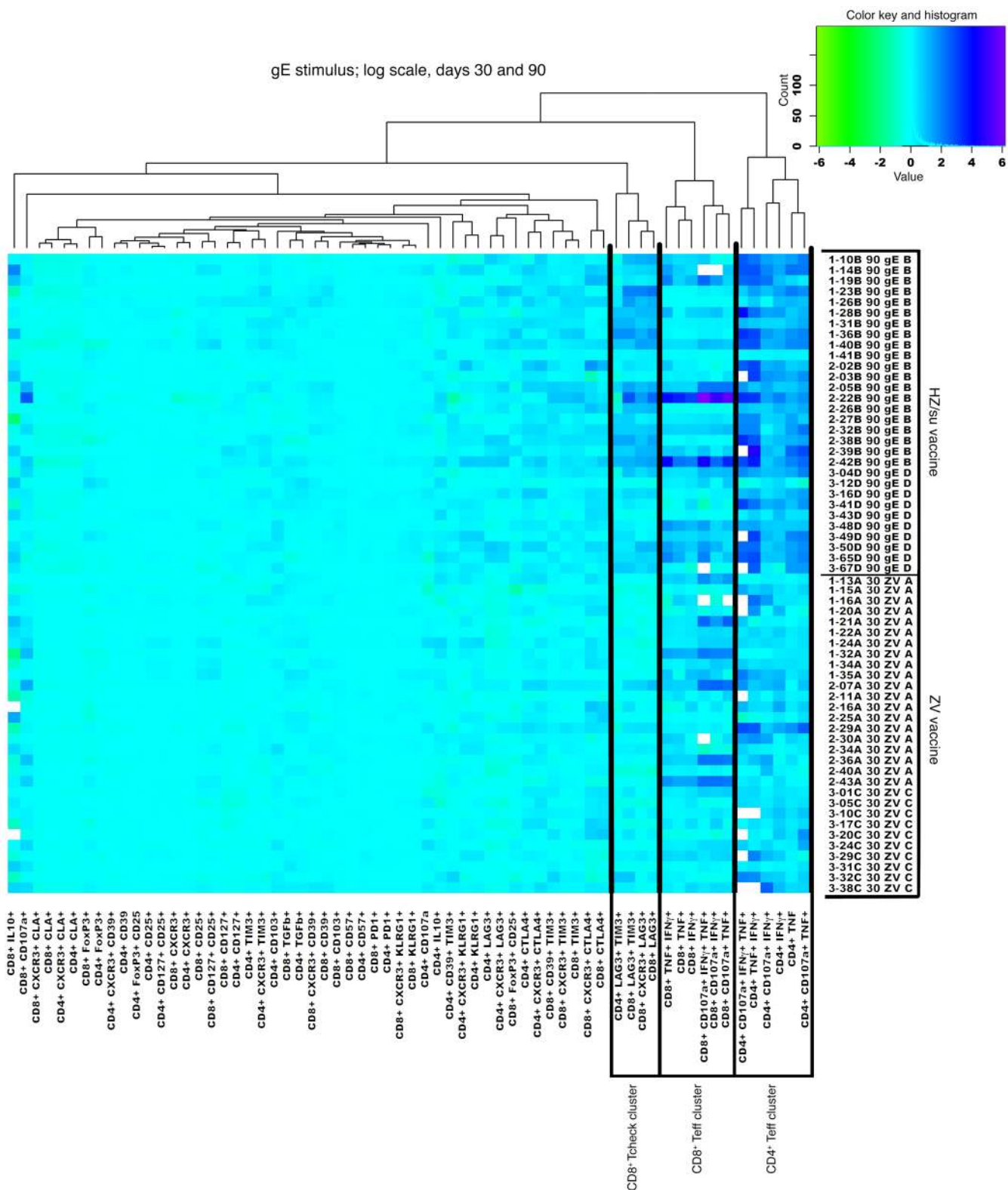
Figure 3. Conventional and regulatory T cell responses in HZ/su and ZV recipients at PMR. PMR was day 30 for ZV and 90 for HZ/su recipients. Data were derived from 60 participants equally distributed across vaccination and age groups. The heatmap T cell responses to VZV ex vivo restimulation were grouped by unbiased hierarchical clustering. Each column represents a T cell subset and each row an individual participant. A, ZV primary group; B, HZ/su primary group; C, ZV boosted group; D, HZ/su boosted group. The rectangles identify 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 an individual participant. A, ZV primary group; B, HZ/su primary group; C, ZV boosted group; D, HZ/su boosted group. The rectangles identify T cell clusters.
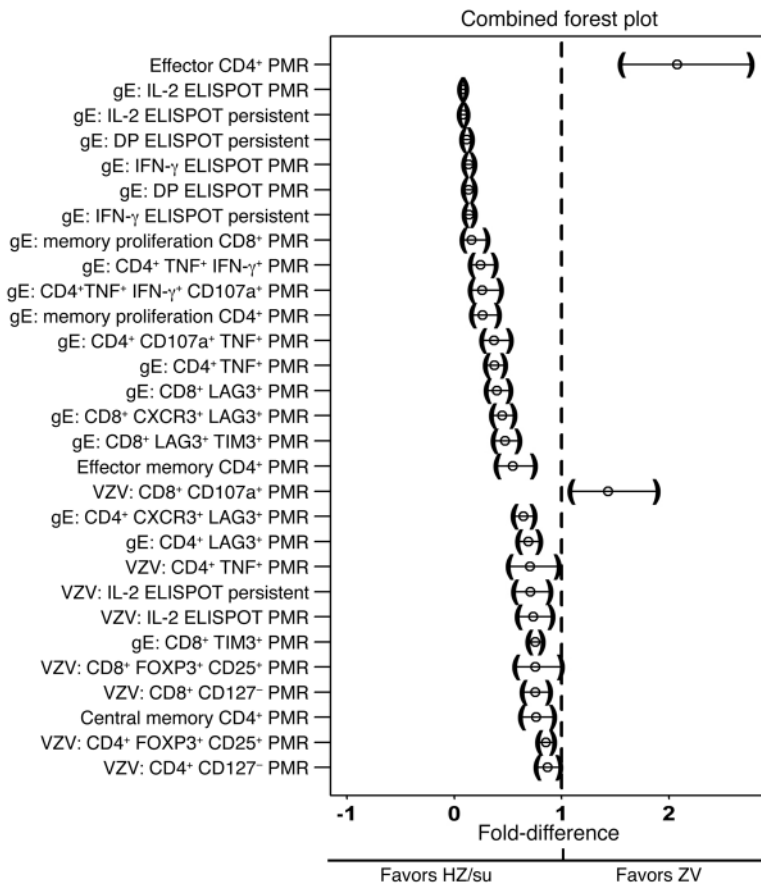
Figure 5. Hierarchical presentation of T cell responses that significantly differentiate the 2 vaccines. Data were derived from 158 participants for ELISpot, 94 for proliferation, and 60 for T cell differentiation and functional PMR. The plot shows means estimated for the fold differences of ZV/HZ/su results and 95% CI for significantly different parameters (95% CI does not overlap the null effect, i.e., equivalence, indicated by the dotted vertical line). All other parameters are shown in Supplemental Figure 7. The stimulant and T cell responses are indicated on the coordinate. Means of less than 1 indicate higher responses in the HZ/su group, and those greater than 1 indicate higher responses in the ZV group.

tion represented the immunologic mechanism necessary and sufficient for VZV-Th1 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 the persistence of Th1 responses attributed to their effects on IL-2 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 IL-2 PMR pathway (Supplemental Figure 6). The data showed nonsignificant ADE ($P > 0.1$) and highly significant ACME ($P \leq 0.01$) for both gE-Th1- and VZV-Th1 one-year responses, indicating that IL-2 PMR mediated the effect of vaccines on the 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 2 vaccines were the higher gE- and VZV-specific memory Th1 responses generated by HZ/su, including peak CD4$^+$ Tcm and Tem percentages, gE–IL-2 and VZV–IL-2 SFCs, 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% or more up to 4 years after HZ/su administration compared with approximately 40% protection by ZV after a similar interval (12, 16, 17, 29). Higher VZV-Treg and gE-Tcheck percentages at PMR in HZ/su compared with ZV recipients are also probably related to the higher memory responses in HZ/su. Tregs and Tcheck may play a role in Th1 differentiation by directing the immune response from effector to memory (30–32). Alternatively, they may signal that Teff cells are being quenched. In contrast with HZ/su, the immune response to ZV was characterized by higher VZV-CD4$^+$ and VZV-CD8$^+$ effectors at PMR. This may be due to the nature of this live virus vaccine, which includes an agent capable of multiple cycles of replications that sustain the Teff cells 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 cells in the circulation and with delayed increase of Th1 memory responses after ZV (33).

The very low or absent gE-Th1 responses before HZ/su administration, even in those who had received ZV 5 or more 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 first 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 first dose of HZ/su (34). Sei et al. (35) 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 2 important points: (a) the second dose of HZ/su is essential for immunogenicity and efficacy of this vaccine (this difference is not explained by ZV being administered as a single dose, since providing 2 doses of ZV does not significantly alter the immune response) (36, 37); and (b) biologically significant gE-Th1 responder T cells may arise from naive cells. Whether drawing responses from the naive T cell pool may be advantageous for the host because these cells have undergone fewer cycles of replication than memory cells and/or are less exhausted and, therefore, may generate longer lasting memory or more efficient killing is not known. Akondy et al. showed that ZV also draws Th1 responders from the naive 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–IL-2 and VZV–IL-2 PMR to HZ/su and ZV not only independently contributed to the persistence of Th1 responses after vaccination, but also mediated the effect of the vaccines on persistent Th1, indicating that IL-2 PMR was necessary for the persistence of Th1 responses after vaccination. Our findings contrast with a previous study in which persistence of IFN-γ responses to ZV was not predicted by the magnitude of the VZV-IFN-γ PMR (39). The difference underscores the importance of IL-2 as a predictor of immunogenicity. Currently, there is no mechanistic immune correlate of protection conferred by ZV or HZ/su. gE–IL-2 and/or VZV–IL-2 PMR is a strong candidate to fill this gap, which we are planning to verify in studies in which HZ is an end point, such as in immune-compromised hosts.

CD8+ T cells have a prominent role in protection against herpesviruses (40). Increased VZV-CD8+ Teff cells have been described during convalescence from chicken pox and HZ and after exogenous exposure to VZV or reactivation of VZV (41–43). gE-specific CD8+ Teff cell responses to HZ/su have not been previously demonstrated in humans, although the QS21 component of AS01$_B$ is known to promote antigen crosspresentation by dendritic cells (44, 45). CD8+ T cell responses to antigens coformulated with AS01$_B$ were 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 CD4+ T cell IL-2 production. Furthermore, sorted proliferating CD8+ T cells from HZ/su recipients respond with IFN-γ and/or IL-2 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 fewer Th1 cytokines and cytotoxicity markers compared with CD8+ T cells generated by ZV. In contrast, HZ/su generated higher CD8+ Tcheck and Treg PMR. The upregulation of Tcheck may quench the Teff cell function of CD8+ T cells, which explains the difficulty in demonstrating gE-specific CD8+ Teff cell 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.

Our study was the first, to our knowledge, 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 cell 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 cell and Tcheck studies were part of an exploratory analysis and need to be confirmed.

The immunologic responses to HZ/su may also provide insight into the immunologic mechanism or mechanisms 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 (a) VZV T cells that synapse with latently infected neurons to provide signals required to maintain latency or (b) VZV CMI, which limits replication of reactivated virus and therefore prevents symptomatic disease, for which there is growing evidence (51–54). 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 end points.

The high efficacy of HZ/su is exceptional among vaccines given to older adults and among investigational vaccines against herpesviruses. Compared with ZV, HZ/su is distinguished by robust and persistent memory responses. The AS01$_B$ adjuvant is critical for the magnitude of the Th response to HZ/su, as previously shown (23, 34, 57), and probably plays a role in its persistence. AS01$_B$ 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 had resided in the US at least 30 years; none had prior HZ. Exclusions from the study were immune suppression and recent administration of blood products or other vaccines. Arms A and B (Supplemental Figure 1 and Supplemental Table 1), 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 yoa, *n* = 22; or 70–85 yoa, *n* = 23). Arms C and D contained an additional 70 participants who were 70–85 yoa and had received ZV 5 or more years previously. These were randomly assigned to receive either an additional dose of ZV followed by placebo (arm C) or 2 doses of HZ/su (arm D). Participants were blinded to all vaccinations. 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 for arm B on days 7 and 67. PBMCs, plasma, and serum were cryopreserved within 4 hours of acquisition (58, 59).

Flow cytometric enumeration of VZV- and gE-specific T cell subsets. Thawed PBMCs 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) mAbs were added at 1 μg/ml. Brefeldin A (MilliporeSigma, 5 μg/ml), Monensin (MilliporeSigma, 5 μg/ml), and anti-CD107a (clone H4A3; BD 328609) were added for the last 16 hours. gE-stimulated and mock-stimulated cells were incubated for 18 hours, while wells with infectious VZV were incubated for 42 hours. At the end of the incubation, PBMCs were washed and incubated with Zombie Yellow Viability Stain (BioLegend). PBMCs were then washed in 1% BSA (MilliporeSigma) in PBS (Mediatech) (stain buffer), divided into 3 panels, and incubated with Abs 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-

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CF594; clone HIL-7R-M21; BD 562397; panel 2), CD25 (APC-Cy7; clone M-A251; BD 557753; 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 Abs against IL-10 (PE-Cy7; clone JES3-9D7; BioLegend 501419; panel 2), TGF-β (APC; clone TW4-2F8; BioLegend 349607; panel 2), TNF-α (APC-Cy7; clone MAb11; BioLegend 502943; panel 1), IFN-γ (BV421; clone B27; BD 502531; panel 1), and FoxP3 (PE; clone 259D/C7; BD 560082; panel 2) as appropriate. Unbound Abs were removed by washing with staining buffer and were fixed in 2% paraformaldehyde (Electron Microscopy Sciences) in PBS, and 200,000 or more events were acquired with the Gallios (Beckman Coulter) instrument and analyzed using FlowJo (Tree Star) software. The gating strategies are shown in Supplemental Figure 8. Supplemental Figure 9 shows the Treg specificity of the FOXP3+CD25+ marker combination as verified during staining optimization assays.

T cell proliferation measured by flow cytometry. Thawed PBMCs stained with CellTrace Violet (BioLegend) were cultured in the presence of infectious VZV, gE peptide pools as above, or mock stimulation for 5 days at 10^6 PBMCs/ml as above. On day 3, 3.3 IU/ml rhIL-2 (R&D Systems) was added to mock and VZV wells. At the end of the incubation, PBMCs were washed with PBS and stained with Zombie Yellow Viability Stain. PBMCs 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^dim populations.

Primary and secondary outcomes. The primary outcome was determined by FN-γ/IL-2 FluoroSpot results at 30 days after the last dose of vaccine in each group. The effects of vaccine, age, and prior ZV administration on the primary outcome were prespecified objectives. Secondary outcomes were flow cytometric enumeration of Tem cells, Teff cells, Tregs, and exhausted CD4+ and CD8+ T cells and identifying responses that clearly differentiated between the 2 vaccines. Description of adverse events was also a secondary objective.

Statistics. Frequencies (%) or means and SD 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, sex, 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 effects of vaccine on persistence of Th1 responses at 1 year were mediated through PMR responses. For each mediation analysis, 2 regression models were estimated per the methods outlined by Tingley et. al (60). The ADE was estimated directly from the regression coefficient describing the PMR-adjusted relationship between vaccine and persistence of Th1. The 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 set at 0.005 for CD4 and 0.01 for CD8, reflecting their detection thresholds. Linear regression models similar to FluoroSpot 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-stimulated 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 when 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 0s, sensitivity analyses were conducted to evaluate the impact of imputation and compared with the imputed results. Flow parameters with a median VZV- or gE-stimulated–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 the vaccine group, linear regression models were constructed for each parameter of interest, adjusting for baseline value. Age, sex, and booster status were evaluated as covariates and excluded from the models if not found significant ($P < 0.05$). To account for multiple comparisons, 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 noninferiority tests (arm A vs. B and arm C vs. D) regarding VZV- and gE-specific ELISpot responses between the 2 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 postvaccine 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 greater than 0.5 and would lead to the conclusion that the response to live ZV is noninferior to the response to recombinant gE vaccine.

With 45 subjects enrolled in each arm, A and B, and an assumed 10% dropout rate, 40 subjects in each arm would be available for analysis and could achieve over 90% power to detect noninferiority (arm A vs. B) using a 1-sided 2-sample *t* test with a significance level (α) of 0.025. With 35 subjects enrolled in each arm, C and D, and an assumed 10% dropout rate, 31 subjects in each arm would be available for analysis and could achieve over 80% power to detect noninferiority (arm C vs. D) using a 1-sided 2-sample *t* test with a significance level (α) of 0.025. The assumptions used in the power calculation are as follows: (a) 10% dropout rate; (b) common SD of 0.41 (assumed an extra variability) on the log-transformed scale in each group based on previously published data (4) administering the live herpes ZV to subjects 60–70 years of age; (c) noninferiority margin of 2-fold with respect to fold-rise ratio; and (d) true ratio (vaccine 1/vaccine 2) for fold-rise ratio of 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. Subjects provided informed consent prior to participation in the study.

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