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CELLULAR RESPONSE TO INFECTION

Breadth and Functionality of Varicella-Zoster Virus Glycoprotein-Specific Antibodies Identified after Zostavax Vaccination in Humans

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ABSTRACT Herpes zoster (HZ) (shingles) is the clinical manifestation of varicella-zoster virus (VZV) reactivation. HZ typically develops as people age, due to decreased cell-mediated immunity. However, the importance of antibodies for immunity against HZ prevention remains to be understood. The goal of this study was to examine the breadth and functionality of VZV-specific antibodies after vaccination with a live attenuated HZ vaccine (Zostavax). Direct enumeration of VZV-specific antibody-secreting cells (ASCs) via enzyme-linked immunosorbent spot assay (ELISPOT assay) showed that Zostavax can induce both IgG and IgA ASCs 7 days after vaccination but not IgM ASCs. The VZV-specific ASCs range from 33 to 55% of the total IgG ASCs. Twenty-five human VZV-specific monoclonal antibodies (MAbs) were cloned and characterized from single-cell-sorted ASCs of five subjects (>60 years old) who received Zostavax. These MAbs had an average of ~20 somatic hypermutations per VH gene, similar to those seen after seasonal influenza vaccination. Fifteen of the 25 MAbs were gE specific, whereas the remaining MAbs were gB, gH, or gI specific. The most potent neutralizing antibodies were gH specific and were also able to inhibit cell-to-cell spread of the virus *in vitro*. Most gE-specific MAbs were able to neutralize VZV, but they required the presence of complement and were unable to block cell-to-cell spread. These data indicate that Zostavax induces a memory B cell recall response characterized by anti-gE > anti-gI > anti-gB > anti-gH antibodies. While antibodies to gH could be involved in limiting the spread of VZV upon reactivation, the contribution of anti-gE antibodies toward protective immunity after Zostavax needs further evaluation.

IMPORTANCE Varicella-zoster virus (VZV) is the causative agent of chickenpox and shingles. Following infection with VZV, the virus becomes latent and resides in nerve cells. Age-related declines in immunity/immunosuppression can result in reactivation of this latent virus, causing shingles. It has been shown that waning T cell immunity correlates with an increased incidence of VZV reactivation. Interestingly, serum with high levels of VZV-specific antibodies (VariZIG; IV immunoglobulin) has been administered to high-risk populations, e.g., immunocompromised children, newborns, and pregnant women, after exposure to VZV and has shown some protection against chickenpox. However, the relative contribution of antibodies against individual surface glycoproteins toward protection from shingles in elderly/immunocompromised individuals has not been established. Here, we examined the breadth and function-

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ality of VZV-specific antibodies after vaccination with the live attenuated VZV vaccine Zostavax in humans. This study will add to our understanding of the role of antibodies in protection against shingles.

KEYWORDS antibody, B cell, cloning, herpes zoster, immunology, shingles, VZV, VZV glycoprotein, Zostavax

Chickenpox (varicella) and herpes zoster (shingles) are both caused by varicella-zoster virus (VZV). VZV is a member of the *Herpesviridae* family of DNA viruses, which cause both lytic and latent infections. After natural infection with VZV, the virus becomes latent in sensory ganglia. Previous work has shown that immunity induced by natural infection can protect against VZV reactivation (1–3). However, as the immune response to VZV wanes, either in the immunocompromised (i.e., after transplantation, chemotherapy, cancer, HIV/AIDS, or stress) or with age, clinically relevant reactivation of VZV may occur. This can result in the development of shingles, which may cause significant morbidity due to pain associated with postherpetic neuralgia (PHN). Zostavax is a live attenuated viral vaccine licensed for people over the age of 50 and has been shown to significantly decrease the incidence of shingles, by 63.9% in 60- to 69-year-olds and by 37.6% in people >70 years of age. Those who did develop shingles after receiving Zostavax saw a significant decrease in the amount of PHN (66.5% overall) (4). Studies of both Zostavax and the now-FDA-approved VZV vaccine Shingrix (gE protein in $AS01_B$) have demonstrated that boosting the immune response to VZV and/or the gE protein of VZV alone can decrease both the incidence and severity of shingles (4–13).

Earlier studies have shown that the incidence of shingles increases with age, primarily due to a decrease in T cell immunity (9, 14, 15). However, the relative importance of humoral immunity in protection against viral reactivation remains unclear. On one hand, VZV-specific antibodies do not decrease with age, suggesting that antibodies alone are not sufficient for protection against VZV reactivation (9). However, no study to date has evaluated whether neutralizing antibodies to VZV decrease with age; the previous studies measured only total VZV- or glycoprotein E-specific serum antibody levels. On the other hand, antibodies have been shown to be a correlate of vaccine-mediated protection in children receiving the live attenuated varicella vaccine Varivax (16). Moreover, there is evidence that sera from individuals with high titers of antibodies against VZV (VariZIG, VZIG, or ZIG) can protect some immunocompromised children and pregnant women from both subclinical and clinical varicella (17–20).

Four major glycoprotein complexes are located on the surface of VZV viral particles (glycoprotein B [gB], gC, gE/gI, and gH/gL (21)). Glycoprotein E is the most predominant and immunogenic glycoprotein on infected cells and virions (21–24). Glycoproteins E and I form heterodimers and are required for virus replication *in vivo* (25–27). Inhibition of gE/gI heterodimerization results in decreased gE maturation and surface expression, inhibiting gI incorporation into virions and thus blocking infection of skin and T cells *in vivo* (27). Glycoprotein B and the gH/gL heterodimer are the second and third most abundant and immunodominant VZV glycoproteins (28). Previous work has shown that gB and gH/gL are necessary and minimally sufficient for virion fusion to the cell, and they have been hypothesized to mediate VZV-induced fusion and virion entry into the cell (27, 29–32). A model of VZV fusion suggests that gH/gL can activate gB to trigger its fusogenic function (27).

Many studies have evaluated the antibody response to VZV in humans. During primary natural varicella infection and after primary varicella vaccination, gE and gB antibodies predominate, followed by gH-specific antibody responses (23, 24). Studies utilizing mouse and mouse-human chimeric hybridomas demonstrated that gE monoclonal antibodies (MAbs) neutralize VZV *in vitro* in a complement-dependent manner (33, 34). VZV gB-specific antibodies have been shown to neutralize the virus independently of complement and with lower 50% inhibitory concentrations (IC_{50}) than for gE

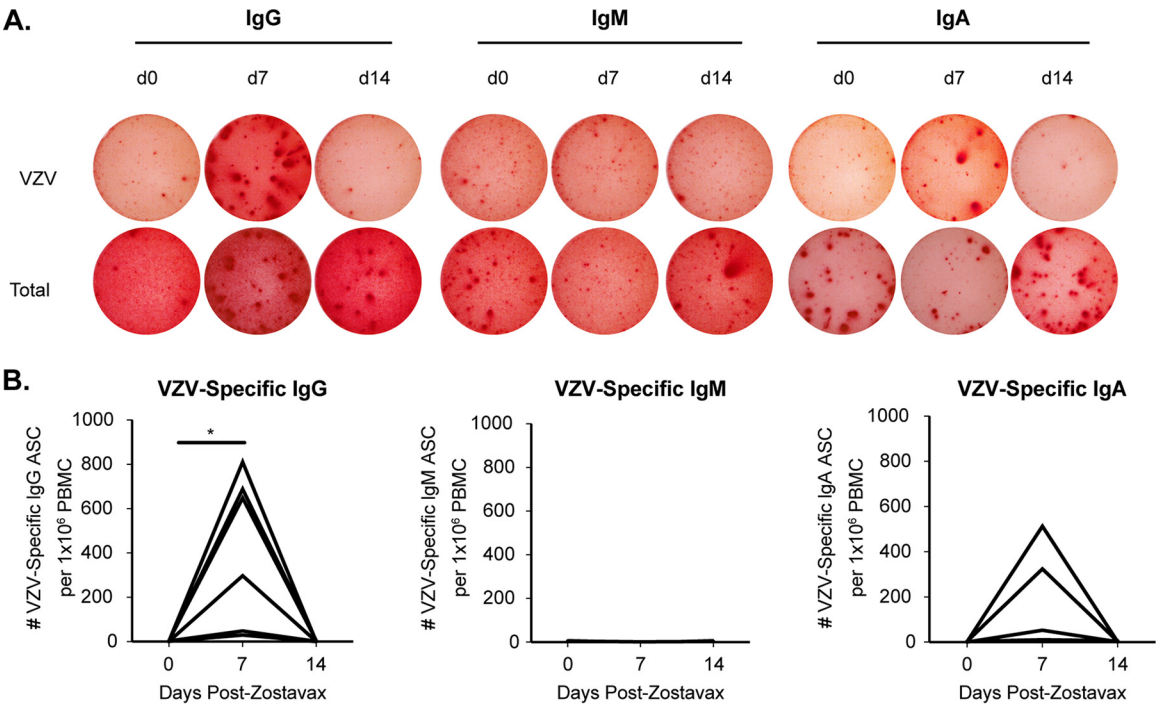

FIG 1 Kinetics of VZV-specific antibody-secreting cells (ASCs) after Zostavax vaccination in older adults. (A) Representative VZV-specific (top row) and total (bottom row) IgG (left), IgM (center), and IgA (right) ASCs were measured on days 0, 7, and 14 after Zostavax vaccination via ELISPOT assay. (B) Cumulative results for VZV-specific IgG, IgM, and IgA ASCs after Zostavax vaccination. *, $P < 0.05$ by the Student *t* test.

antibodies with complement (33). Interestingly, VZV-specific gH (gH/gL) antibodies have been shown by several groups to neutralize with and without complement as well as to inhibit cell-to-cell spread both *in vitro* and in a humanized SCID xenograft model (28, 35–44). Structural analysis of two VZV gH/gL monoclonal antibodies isolated from VZV-immune donors via phage display or single-cell antibody-secreting cell cloning IgG-94 (43) and IgG-RC (36) showed that both antibodies target the same site, which is composed of residues in both gH and gL. This epitope is in proximity to the site on gH/gL that activates gB, and thus targeting of this site may represent a potential vulnerability for VZV entry (45).

Using an unbiased approach, we examined the breadth and functionality of VZV-specific antibodies after vaccination with Zostavax in humans. We demonstrated that Zostavax induces VZV-specific IgG and IgA antibody-secreting cells (ASCs) but not IgM ASCs. Analysis of monoclonal antibodies cloned and characterized from single-sorted ASCs revealed that over half of the MAbs generated were gE specific, whereas the other MAbs were gI, gB, and gH specific. Functional analysis of these MAbs demonstrated that antibodies specific for gH displayed the most potent complement-independent neutralization and inhibition of cell-to-cell spread. gE-specific MAbs, as well as a one gB-specific MAb, could neutralize VZV only in the presence of complement and did not inhibit cell-to-cell spread. Overall, these results indicate that gH antibodies may be important *in vivo* for preventing virus spread, thus potentially limiting the tissue damage/postherpetic neuralgia after reactivation.

RESULTS

Zostavax induces IgG and IgA, but not IgM, ASCs 7 days after vaccination. In order to examine the breadth and functionality of the antibody response to vaccination by using an unbiased approach, we vaccinated seven subjects (60 to 71 years old) with Zostavax. Serial blood draws were obtained on days 0, 7, and 14 postvaccination. VZV-specific antibody-secreting cells (ASCs) peaked at day 7 postvaccination and returned to baseline by day 14 (Fig. 1A and B). This is consistent with what we have

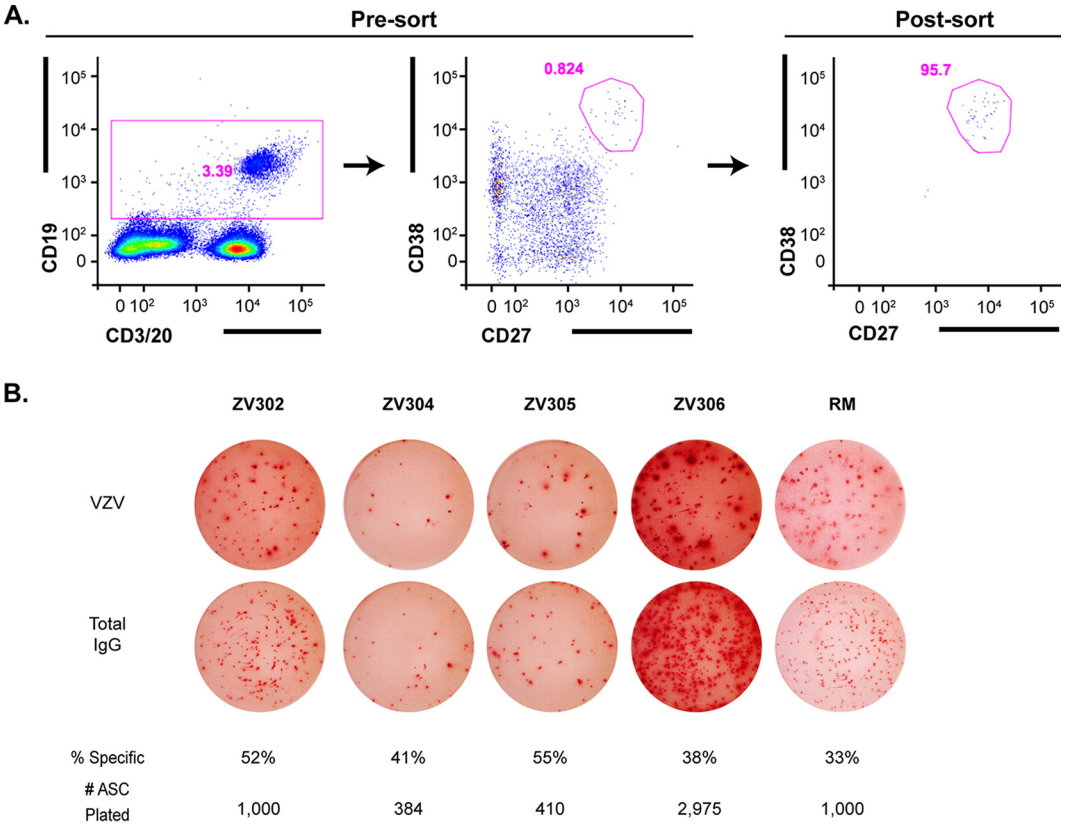

FIG 2 ASC sorting and frequency of VZV-specific ASCs. Five older adults were vaccinated with Zostavax, and PBMCs were collected on day 7. (A) ASCs were single-cell sorted into PCR plates using a FACSAria II (left and middle panels, presort; right panel, postsort to assess purity). (B) The frequency of VZV-specific ASCs was calculated via IgG ELISPOT assay. Top row, VZV-specific IgG ASCs; bottom row, total IgG ASCs. The percentages of specific ASCs were measured by taking the number of VZV-specific IgG ASCs divided by the total number of IgG ASCs detected. The number of ASCs plated per well is listed.

published previously for VZV-specific IgG ASCs following vaccination with Zostavax (46). IgG-producing ASCs dominated among VZV-specific ASCs, followed by IgA ASCs in a few subjects, and no VZV-specific IgM ASCs were detected. The lack of antigen-specific IgM ASCs and the appearance of IgG and IgA ASCs at day 7 are indicative of a true memory B cell recall response. To determine the specificities of the antibodies generated after Zostavax vaccination, we performed single-cell reverse transcription-PCR (RT-PCR) of sorted ASCs 7 days after vaccination to generate monoclonal antibodies as described previously (47–49). $CD19^{+}$ $CD27^{hi}$ $CD38^{hi}$ ASCs were first sorted in bulk 7 days after vaccination (Fig. 2A). Figure 2B shows that approximately 33 to 55% of sorted bulk IgG-producing ASCs were VZV specific via enzyme-linked immunosorbent spot assay (ELISPOT assay). Next, the ASCs were single-cell sorted into 96-well PCR plates and cloned as described previously (47). Paired heavy- and light-chain plasmids were transiently transfected, and supernatant was collected and screened on a VZV-infected cell lysate via enzyme-linked immunosorbent assay (ELISA). Supernatants that scored positive had antibodies purified and the specificity and functionality tested. Overall, we generated 25 VZV-specific monoclonal antibodies from five subjects. The last two subjects' ASCs were not cloned but were saved for future use. Due to a variable percent frequency of VZV-specific plasmablasts as well as potential primer bias, we cloned variable numbers of antibodies from the 5 subjects (RM, $n = 5$; ZV301, $n = 1$; ZV302, $n = 4$; ZV303, $n = 14$; and ZV304, $n = 1$).

Zostavax induces primarily a memory B cell recall response. Sequence analysis of VZV-specific monoclonal antibodies (Table 1) showed that the median level of somatic hypermutations (SHM) per VH gene was 20, with a range of 7 to 34 (Fig. 3). In contrast, antibody sequences derived from naive B cells averaged approximately zero

TABLE 1 Sequence analysis of VZV-specific monoclonal antibodies

			V region							CDR3 region	
Antibody	Specificity	Isotype	V gene	V % identity	No. of mutations	No. of silent mutations	No. of nonsilent mutations	J gene	D gene	CDR lengths[a]	Amino acid junction
RM-1A2	gE	IgG2	IGHV1-2*02	90	30	9	21	IGHJ4*02	IGHD3-9*01	8.8.14	CARIMYFEYDSWSDYW
		Kappa	IGKV1-39*01	96	12	5	7	IGKJ1*01		6.3.9	CQQSYSLPWTF
RM-1D1	gI	IgG1	IGHV3-11*01	97	8	2	6	IGHJ4*02	IGHD2-2*02	8.8.12	CAREPPYTSSIDYW
		Kappa	IGKV1-39*01	98	7	2	5	IGKJ1*01		6.3.9	CQQSYSTPWTF
RM-2D6	gH	IgG1	IGHV3-30*03	95	16	4	12	IGHJ4*02	IGHD3-3*01	8.8.15	CAKTSWARFLEWTFDYW
		Kappa	IGKV1-39*01	96	11	6	5	IGKJ5*01		6.3.9	CQQSHSTPTF
RM-5A2	gE	IgG1	IGHV4-59*01	94	17	5	12	IGHJ4*02	IGHD3-10*02	8.7.15	CARIGGVSFGERPIDYW
		Kappa	IGKV1-39*01	96	13	5	8	IGKJ4*01		6.3.9	CQQSYSIPLTF
RM-5B3	gE	?	IGHV5-51*01	89	34	13	21	IGHJ4*02	IGHD6-19*01	8.8.12	CARQRYSSGSFGYW
		Kappa	IGKV4-1*01	94	18	8	10	IGKJ4*01		12.3.9	CQQYETYPFTF
301-1G5	gE	IgG1	IGHV1-2*02	88	25	8	17	IGHJ4*02	IGHD7-27*01	8.8.15	CATLTRETGAPSTFDYW
		Kappa	IGKV4-1*01	91	16	5	11	IGHJ4*02		12.3.9	CQHYYNTPLTF
302-1B12	gI	IgG1	IGHV1-69*04	83	29	7	22	IGHJ6*02	IGHD1-26*01	8.8.15	CARGGSFSLGANGLDVW
		Kappa	IGKV3-20*01	92	6	1	5	IGKJ1*01		7.3.9	CQQHGSLPWTF
302-1C12	gI	IgA2	IGHV1-69*04	88	17	7	10	IGHJ3*01	IGHD4-11*01	8.8.16	CAIRGGMTTLTPEDLDVW
		Kappa	IGKV2D-29*01	93	16	7	9	IGKJ4*01		11.3.9	CMQSIHLPLTF
302-1D11	gE	IgA1	IGHV3-9*01	89	16	5	11	IGHJ6*02	IGHD2-21*02	8.8.22	CAKDQYCGGDCHSKSYYYYGMDVW
		Lambda	IGLV6-57*02	94	7	6	1	IGLJ2/3*01		8.3.10	CQSYDSQTAVIF
302-1G9	gH	IgA1	IGHV1-18*01	85	23	5	18	IGHJ4*02	IGHD4-23*01	8.8.16	CARDRGGSLAMVVGLDHW
		Kappa	IGKV1(D)-39*01	91	18	8	10	IGKJ4*01		6.3.9	CQQCYSAELTF
303-1A8	gE	IgG1	IGHV3-30-3*01	86	23	7	16	IGHJ4*02	IGHD4-23*01	8.8.10	CARDGSFGLDYW
		Kappa	IGKV1-6*02	94	8	2	6	IGKJ2*01		6.3.9	CLQDYNDPYTF
303-1A12	gB	IgA1	IGHV3-23*04	85	21	3	18	IGHJ4*02	IGHD2-21*02	8.8.11	CARDRDWNYFDKW
		Lambda	IGLV1-44*01	83	14	5	9	IGLJ1*01		8.3.11	CAAWDDRLNGYVF
303-1B2	gI	IgG1	IGHV4-30-4*01	91	10	2	8	IGHJ5*02	IGHD3-3*01	10.7.15	CARESIGDDFWSGLGPW
		Kappa	IGKV2-28*01	98	2	0	2	IGKJ2*01		11.3.9	CMQALQTPYTF
303-1C1	gI	IgG1	IGHV3-53*01	91	12	2	10	IGHJ4*02	IGHD6-25*01	8.7.14	CAREAYNSGTYYFDYW
		Kappa	IGKV1-39*01	90	12	3	9	IGKJ2*01		6.3.9	CQQSHTIPYTF
303-1C2	gB	IgA1	IGHV3-30*03	92	9	3	6	IGHJ4*02	IGHD2-21*02	8.8.14	CARDFSGVTTISLDSW
		Lambda	IGLV1-36*01	93	5	0	5	IGLJ2*01		8.3.11	CAAWDDSLNRGVF
303-1C6	gB	IgA1	IGHV3-23*01	85	26	8	18	IGHJ4*02	IGHD2-21*02	8.8.11	CARDRDWNYFDNW
		Lambda	IGLV1-44*01	89	16	5	11	IGLJ1*01		8.3.11	CAAWDDRLNGYVF
303-1C9	gE	IgG1	IGHV3-23*01	88	27	9	18	IGHJ5*02	IGHD5-24*01	8.8.15	CANCPGDSDNCYWFDPW
		Kappa	IGKV1-12*01	88	11	4	7	IGKJ4*01		6.3.9	CQQAKNFPLTF
303-1D7	gE	IgG1	IGHV3-30-3*01	87	23	10	13	IGHJ4*02	IGHD4-23*01	8.8.10	CARDGSFGLDYW
		Kappa	IGKV1-6*02	91	8	2	6	IGKJ2*01		6.3.9	CLQDYNDPYTF
303-1E3	gE	IgG1	IGHV4-30-4*01	89	20	7	13	IGHJ4*02	IGHD2-2*01	10.7.15	CARVRTSSTTSYYFDYW
		Kappa	IGKV1-39*01	93	13	5	8	IGKJ3*01		6.3.8	CQQSYRTLTF
303-1E8	gE	IgA2	IGHV4-61*02	89	18	4	14	IGHJ4*02	IGHD3-22*01	10.7.18	CARAPFYNDFSGYSYYFDYW
		Lambda	IGLV1-44*01	88	19	7	12	IGLJ1*01		8.3.12	CAAWDDSLNPLYVF
303-1E12	gE	IgG1	IGHV1-2*02	92	15	3	12	IGHJ4*02	IGHD2-21*02	8.8.13	CARWEGGDWFAFDFW
		Lambda	IGLV3-1*01	89	10	3	7	IGLJ1*01		6.3.11	CQAWDSSTASFVF
303-1F5	gE	IgA1	IGHV3-30*03	77	32	6	26	IGHJ4*02	IGHD3-9*01	8.8.15	CAKDILTGYYKGNFDYW
		Kappa	IGKV3-11*01	84	31	11	20	IGKJ4*01		6.3.9	CQLRNGWLFTF
303-1F7	gE	IgG1	IGHV4-39*07	88	23	8	15	IGHJ4*02	IGHD3-10*01	10.7.11	CATGALRRPFDSW
		Kappa	IGKV1-27*01	94	10	4	6	IGKJ1*01		6.3.9	CLKCNTAPWTF
303-1G1	gE	IgA1	IGHV4-31*03	94	10	3	7	IGHJ5*02	IGHD2-2*01	10.7.19	CARDSGYCSSTNCPQNWFDPW
		Kappa	IGKV1-39*01	80	8	0	8	IGKJ1*01		6.3.9	CQQSYTTPQTF
304-1A12	gE	IgA2	IGHV3-7*01	92	7	1	6	IGHJ6*02	IGHD3-3*01	8.8.18	CARSPRFLSQDYYYYVMDVW
		Kappa	IGKV1(D)-39*01	91	9	1	8	IGKJ1*01		6.3.10	CQQSFITRTWTF

[a]Amino acid lengths for CDR1:CDR2:CDR3.

mutations per VH gene. For reference, in Fig. 3 we also show previously reported data on the number of somatic mutations per VH gene in ASCs generated after seasonal influenza vaccination (50). Levels of SHM in ASC clones responding to Zostavax vaccination are similar to what is seen after influenza vaccination and indicate that Zostavax ASCs are derived from memory B cell precursors. We were able to identify two separate clones (same VDJ junction) with more than one member (different mutations) each for donor 303. The first clone includes antibodies 303-1A8 and 303-1D7 (Table 1). Both antibodies utilized the same variable-region genes (IGHV3-30-3*01 and IGKV1-6*02), had the same CDR3 sequences for both heavy and light chains, had the same number of total mutations in VH and VK, and had the same number of amino acid replacement mutations in VK. The number of replacement mutations in VH differed, as 303-1A8 had 16, compared with 13 for 303-1D7. These data indicate that 303-1A8 and 303-1D7 shared the same parental clone and that 303-1A8 had undergone further affinity maturation. The second clonal family consists of antibodies 303-1A12 and 303-1C6 (Table 1). Both antibodies utilized the same heavy- and light-chain variable region genes (IGHV3-23*04 and IGLV1-44*01) and had identical CDR3 sequences. These antibodies diverged slightly in that 303-1C6 had a higher total number of SHM than

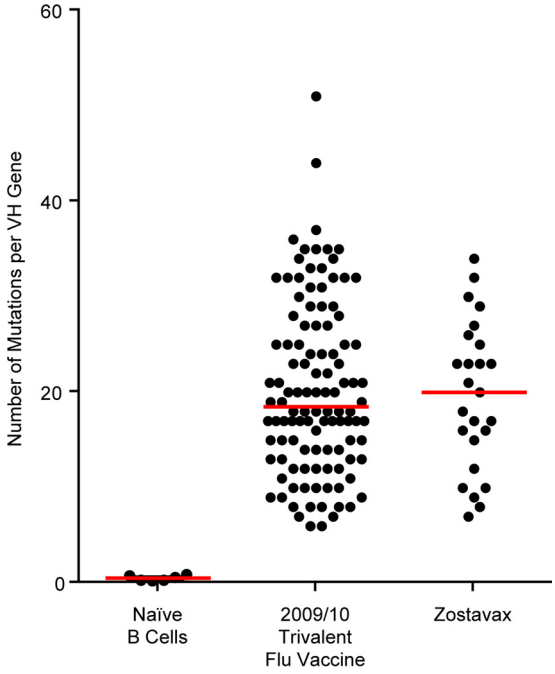

FIG 3 Comparison of the numbers of VH somatic hypermutations in naive B cells, ASCs sorted after vaccination with the 2009/10 influenza trivalent vaccine, and ASCs after Zostavax vaccination. Data from the 2009/10 influenza trivalent vaccine were published by Li et al. (50). IgBLAST was used to determine the number of somatic hypermutations (Fr1-CDR3 heavy chain) as described previously (49).

303-1A12 (silent plus replacement mutations). If we look at the number of replacement mutations resulting in an amino acid change, both antibodies had the same number of SHM in IgH ($n = 18$), but 303-1C6 had more IgL SHM than 303-1A12. These data indicate that 303-1C6 had undergone further affinity maturation.

VZV gE-specific antibodies are predominant in the recall response to Zostavax. Specificities of the 25 VZV-specific monoclonal antibodies were established as per the scheme depicted in Fig. 4. Briefly, 153 paired heavy- and light-chain genes were transiently transfected for antibody expression. Of the 153 supernatants tested on VZV-infected cell lysates, 54 were shown to be VZV-infected cell lysate positive via ELISA. This indicates that approximately 35% of all antibodies generated were VZV positive, which is similar to the percentage of VZV-specific IgG ASCs shown in Fig. 2B. Next, the supernatants were tested to see whether they bound to a lectin-purified VZV glycoprotein via ELISA. Twenty-five of 54 monoclonal antibodies bound to VZV glycoprotein (46%). Although it was not formally demonstrated, we believe that the remaining antibodies are specific to the other vaccine components, such as the lysate (MRC-5) in which VZV Oka is grown in to make Zostavax. We focused on surface glycoprotein-specific antibodies, as these are most likely to play a relevant role *in vivo*. Out of the 25 VZV glycoprotein-positive monoclonal antibodies, 15 were determined to bind VZV glycoprotein E via ELISA (60%). The remaining 40% of monoclonal antibodies that were glycoprotein E negative were tested via immunoprecipitation and liquid chromatography-tandem mass spectrometry (LC-MS/MS) to determine their specificity. Lectin-purified VZV glycoproteins from lysate or VZV-infected cell lysate (MAb 302-1G9) were used for immunoprecipitation, followed by protein identification by shotgun proteomics (Fig. 5). We identified 3 VZV-specific gB monoclonal antibodies (303-1A12, 303-1C2, and 303-1C6) (Fig. 5A). As discussed above, 303-1A12 and 303-1C6 are clonally related, and 303-1C6 has additional amino acid changes compared with 303-1A12 (Table 1). We also identified two VZV-specific gH-specific monoclonal antibodies (Fig. 5B). Based on the LC-MS/MS results, we concluded that RM-2D6 precipitates gH by itself, whereas 302-1G9 coprecipitates the gH/gL complex. This could be a result of the fact that an antibody to gH will pull

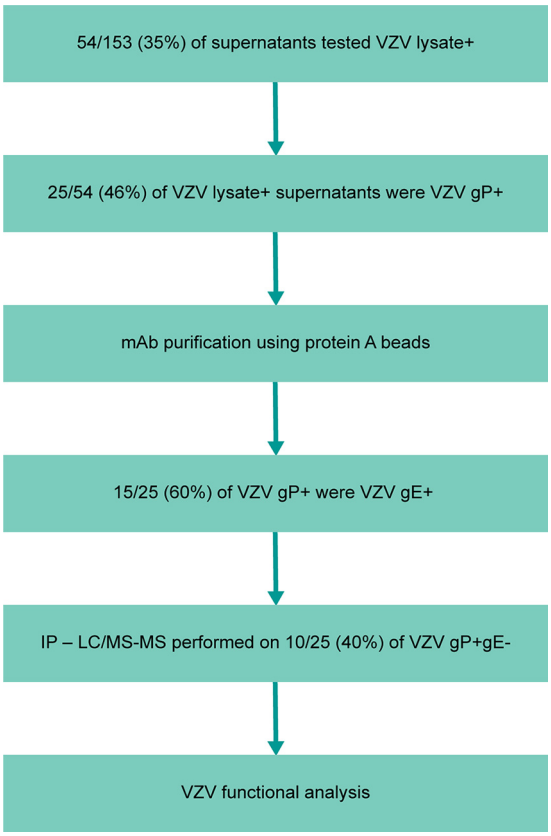

FIG 4 Work flow to identify glycoprotein-specific VZV monoclonal antibodies. Supernatants generated using ASCs single cell sorted, cloned, and expressed in Expi293 cells were tested for VZV lysate binding via ELISA. VZV glycoprotein-positive but non-gE antibodies (10/25) were tested via immunoprecipitation followed by LC/MS to determine the specificity. All glycoprotein-positive antibodies were then tested for neutralization and cell-to-cell spread inhibition *in vitro*.

down gL as gH/gL form a complex. Alternatively, this antibody could have an epitope between gH and gL. Epitope mapping was beyond the scope of this study and will be addressed in future studies. Finally, we identified 5 gI-specific monoclonal antibodies (Fig. 5C) (based on predicted molecular weight). Four of these gI-specific monoclonal antibodies, namely, RM-1D1, 302-1C12, 302-1B12, and 303-1C1, coprecipitate the gE/gI complex, as we can detect both VZV-specific glycoprotein E and glycoprotein I using LC-MS/MS. Similar to the rationale for gH and gL, gI-specific antibodies could pull down gE due to the gE/gI complex. Figure 5D demonstrates via Western blotting that these antibodies recognize epitopes on gI, as a glycoprotein-only lysate (including all possible glycoproteins) was run and shows only one band at the predicted gI molecular weight. Additionally, one gI-specific monoclonal antibody, 302-1B2, appears to predominantly precipitate only gI, as the MS signal intensity for gE is 2.5% (data not shown). In total, we were able to identify 15 gE-, 5 gI-, 3 gB-, and 2 gH-specific monoclonal antibodies (Fig. 5E). These results indicate gE is the predominant humoral target upon Zostavax vaccination. This is not surprising, as glycoprotein E is the most abundant glycoprotein present on the surfaces of VZV virions and infected cells (21).

VZV-specific antibodies can neutralize virus *in vitro*. After identifying the specificities of all 25 VZV-specific monoclonal antibodies, we tested the ability of these antibodies to neutralize VZV *in vitro* (Table 2 and Fig. 6). Green fluorescent protein (GFP)-tagged VZV strain Oka was grown in MRC-5 cells in order to make cell-free virus stock. As VZV is highly cell associated *in vitro* (51), it was critical to make a cell-free virus stock in order to have a reproducible number of input virions in the assay (52). VZV was preincubated with each monoclonal antibody or an influenza virus hemagglutinin

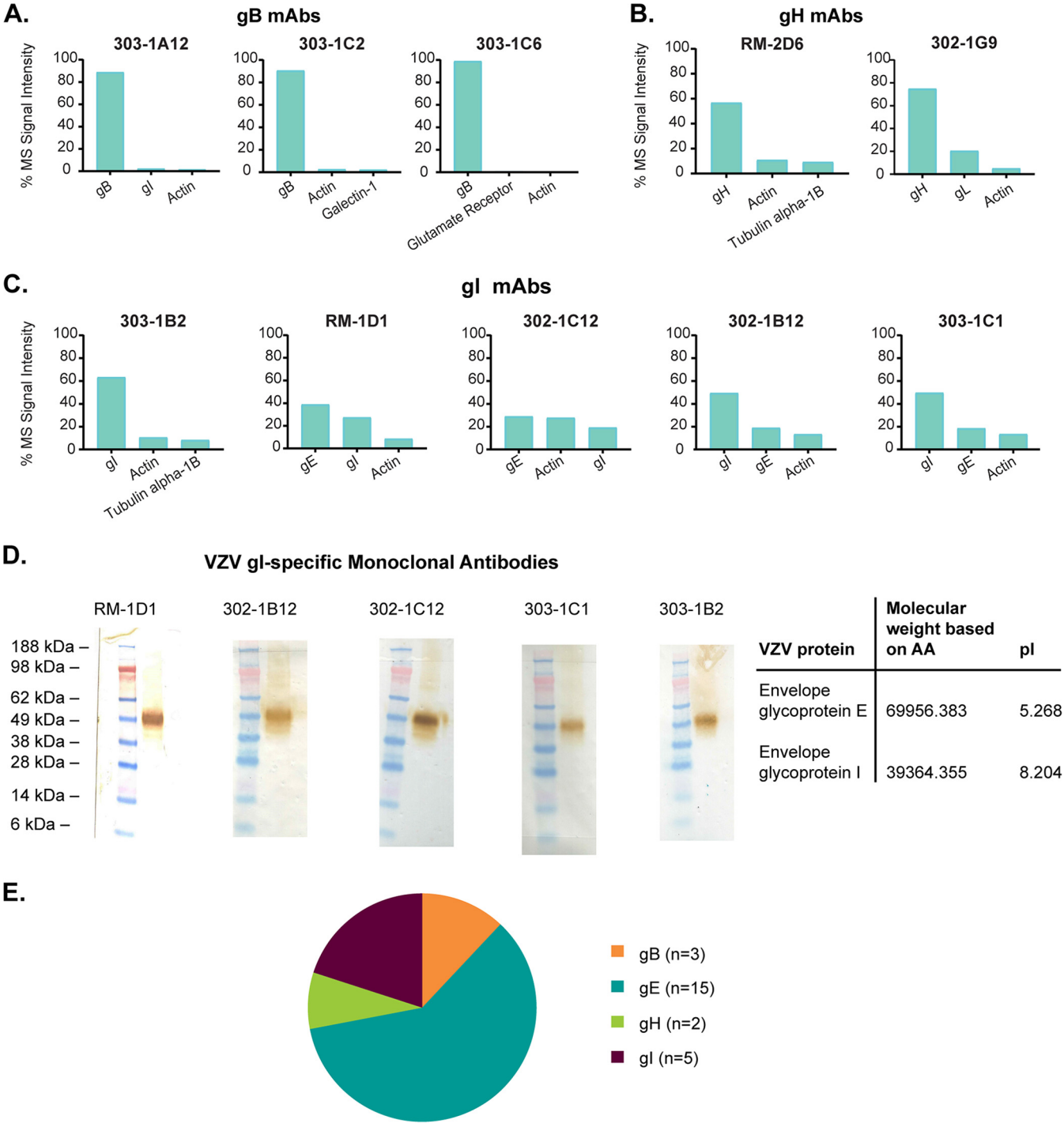

FIG 5 Identification of non-gE-specific monoclonal antibodies. Immunoprecipitation followed by nano-LC-MS/MS was done using the gP-specific monoclonal antibodies and the top three MS hits listed. (A) VZV gB-specific monoclonal antibodies. (B) VZV gH-specific monoclonal antibodies. (C) VZV gI-specific monoclonal antibodies. (D) Western blotting with RM-1D1, 302-1B12, 302-1C12, 303-1C1, and 303-1B2 antibodies was performed using a glycoprotein lysate (including all VZV glycoproteins). The table shows the predicted molecular weights and pIs. (E) Of the 25 VZV glycoprotein-positive antibodies, 15 were gE specific, 5 were gI specific, 3 were gB specific, and 2 were gH specific.

head-specific antibody as a control. After 1 h, 10 U/ml of guinea pig complement was added to half of the virus-plus-antibody wells and left for an additional 30 min. ARPE-19 cells were used for VZV infection, as previous groups have shown that they can produce high titers of VZV and show cytopathic effects (53), and the shape of these cells is more suitable for Acumen Cellista imaging. APRE-19 cells were infected for 5 to 7 days at

TABLE 2 *In vitro* IC_{50} values

Antibody	Specificity	IC_{50} (μg/ml)	
		With complement	Without complement
RM-1A2	gE	0.14	>10
RM-5A2	gE	0.87	>10
RM-5B3	gE	0.52	>10
301-1G5	gE	0.08	>10
302-1D11	gE	0.10	>10
303-1A8	gE	0.11	>10
303-1C9	gE	>10	>10
303-1D7	gE	0.11	>10
303-1E3	gE	0.14	>10
303-1E8	gE	0.10	>10
303-1E12	gE	0.14	>10
303-1F5	gE	>10	>10
303-1F7	gE	>10	>10
303-1G1	gE	0.15	>10
304-1A12	gE	0.17	>10
RM-1D1	gI	>10	>10
302-1B12	gI	>10	>10
302-1C12	gI	>10	>10
303-1B2	gI	>10	>10
303-1C1	gI	>10	>10
RM-2D6	gH	0.03	0.23
302-1G9	gH	0.06	0.09
303-1A12	gB	>10	>10
303-1C2	gB	3.04	5.27
303-1C6	gB	>10	>10
Irrelevant	Influenza virus hemagglutinin head	>10	>10

35°C, and a GFP readout was conducted to quantify infection. Overall, we identified 15 monoclonal antibodies that could neutralize VZV *in vitro* (Fig. 6A). Twelve of the 15 VZV gE-specific monoclonal antibodies could neutralize VZV only in the presence of complement, with an IC_{50} range of 0.08 to 0.87 μg/ml (Table 2 and Fig. 6B). Previous work from other groups has shown that gE-specific antibodies require complement to mediate neutralization *in vitro* (34, 54). None of the gI-specific MAbs could neutralize with or without complement (Fig. 6A). Both gH-specific monoclonal antibodies, RM-2D6 and 302-1G9, had the highest potencies for neutralizing VZV (range, 0.03 to 0.23 μg/ml with or without complement) and did not require the presence of complement. These data are in agreement with previous reports for gH antibodies (28, 35–44). These results confirm previous findings that antibodies directed against gH were the most potent neutralizers *in vitro*. One out of the three gB-specific monoclonal antibodies neutralized VZV weakly. 303-1C2 neutralized with (3.04 μg/ml) or without (5.27 μg/ml) complement with an IC_{50} ~100 times lower than that of the gH antibodies. Interestingly, all of the gE-specific antibodies required complement for *in vitro* neutralization but were less potent than the gH-specific antibodies (~3 times less). Representative neutralization curves for VZV preincubated with gH, gE, or gI antibodies are shown in Fig. 6C.

VZV anti-gH antibodies inhibit cell-to-cell spread *in vitro*. VZV has been shown to be highly cell associated, can induce cell fusion (i.e., formation of syncytia), and transmits through cell-to-cell spread without producing extracellular virions *in vitro* (55). In order to determine the effects of VZV monoclonal antibodies on cell-to-cell transmission, we performed an *in vitro* cell spread inhibition assay. Previous reports have shown that gH monoclonal antibodies can inhibit cell-to-cell spread *in vitro* (28, 35–43) and in a humanized SCID skin xenograft model (44). We tested the ability of all of our monoclonal antibodies to inhibit the cell-to-cell spread of VZV *in vitro*. Figure 7A shows representative wells with VZV alone or with an irrelevant MAb (against influenza virus hemagglutinin head), 303-1E8 (VZV gE specific), RM-2D6 (VZV gH specific), or 302-1G9 (VZV gH specific). There was significant inhibition of cell spread in a dose-dependent manner using the RM-2D6 (gH) and 302-1G9 (gH) monoclonal antibodies.

A.

mAb Specificity	Neutralizes With and Without Complement	Neutralizes Only With Complement	No Neutralization
gE	0	12	3
gH	2	0	0
gB	1	0	2
gI	0	0	5
Irrelevant	0	0	1

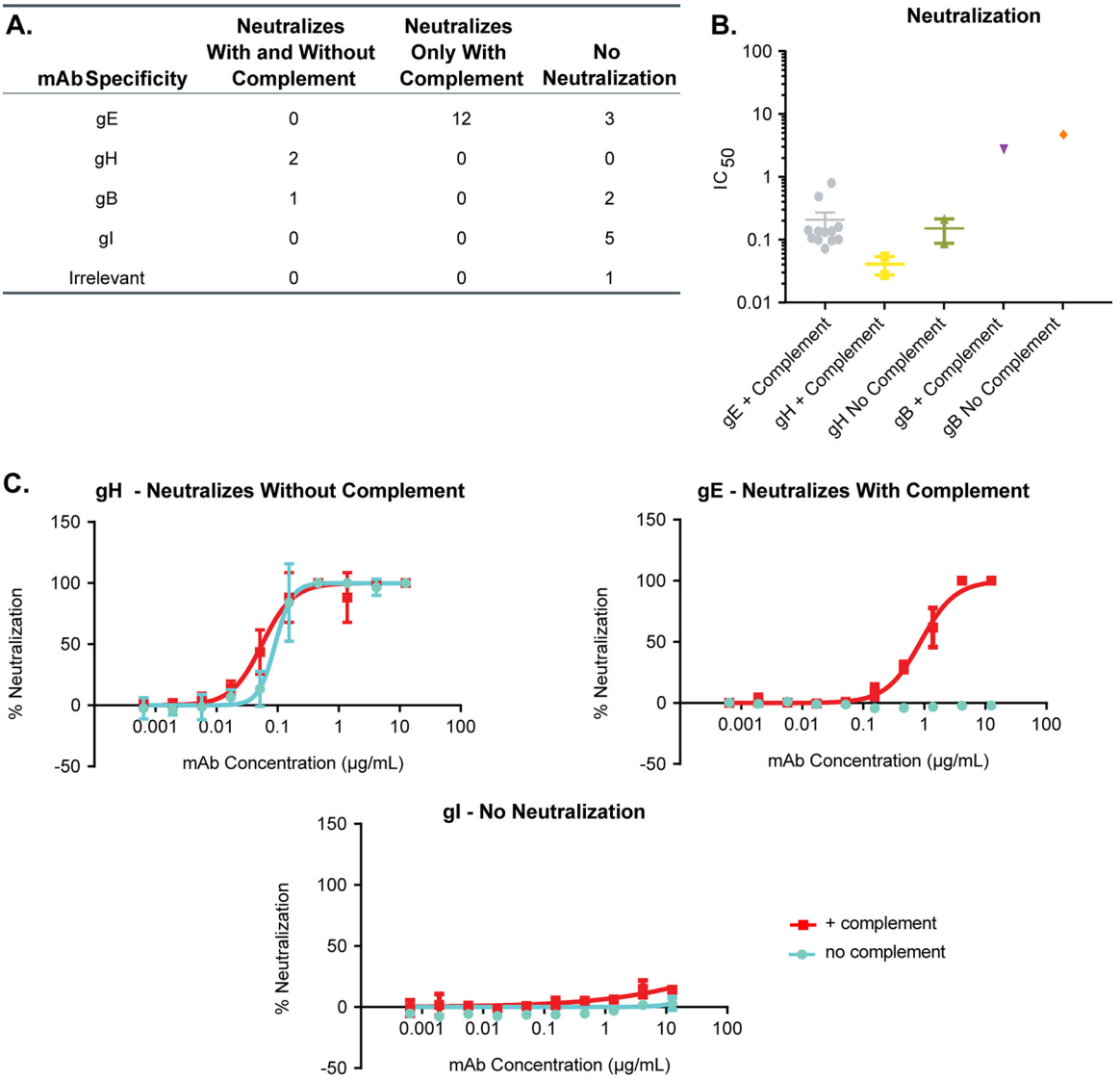

FIG 6 *In vitro* VZV neutralization. Purified VZV-specific monoclonal antibodies were tested for *in vitro* neutralization as described in Materials and Methods. Using an Acumen Cellista, the total number of cells expressing GFP per well was quantified. (A) Cumulative results with and without complement. (B) Cumulative IC_{50} results (in μg/ml) for all antibodies that showed neutralization with or without the addition of guinea pig complement. (C) Representative neutralization plots with gH-, gE-, and gI-specific monoclonal antibodies added. Red, guinea pig complement added; blue, without guinea pig complement.

The cumulative results are shown in Fig. 7B, with all of the gE antibodies shown in the top panel and the non-gE antibodies shown in the bottom panel. Only the gH-specific antibodies could inhibit cell-to-cell spread. Next, we tested whether complement was required for cell spread inhibition. Threefold dilutions of the gH and irrelevant control monoclonal antibodies were performed, starting at 30 μg/ml, with (Fig. 7C, left) or without (Fig. 7C, right) complement. Both 302-1G9 and RM-2D6 could inhibit cell-to-cell spread *in vitro* independent of complement and with similar IC_{50} results for each antibody (~1.4 to 6.2 μg/ml). Overall, our data are consistent with previously published results; only VZV gH-specific monoclonal antibodies have the ability to inhibit cell spread *in vitro*.

DISCUSSION

In this study, we used molecular techniques to examine the breadth and functionality of the VZV-specific response after Zostavax vaccination in humans. Although decreases in cell-mediated immunity have been shown to result in herpes zoster, we

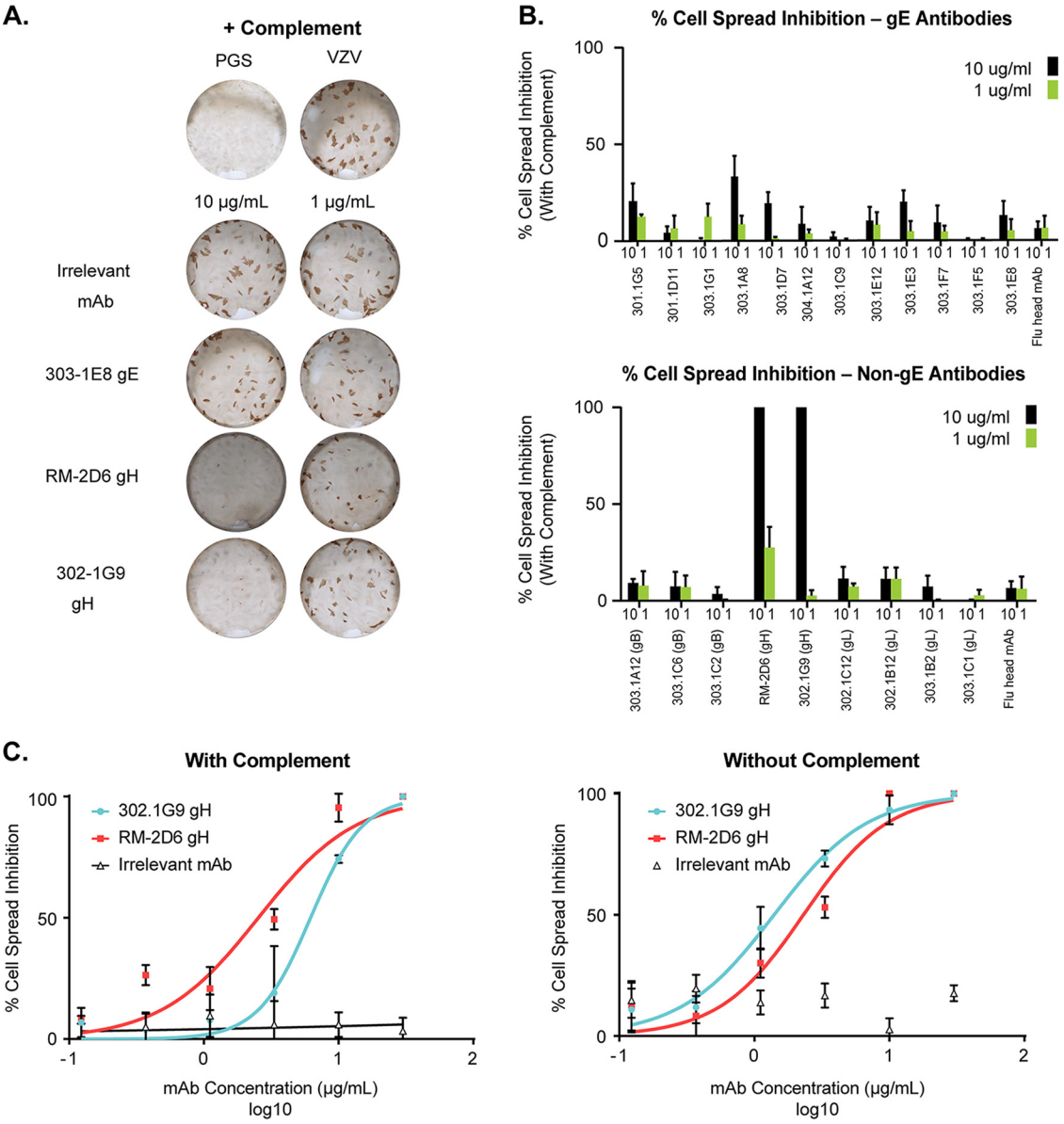

FIG 7 *In vitro* cell-to-cell spread inhibition. Cell-to-cell spread inhibition was performed as described in Materials and Methods. (A) Representative well images for samples treated with complement. PGS buffer was used as a negative control. (B) Cumulative data for anti-gE antibodies (top) and non-gE antibodies (bottom). Black bars represent antibodies added at 10 μg/ml, and green bars represent the same antibodies added at 1 μg/ml. (C) Cell spread inhibition curves with IC_{50} values (μg/ml) with anti-gH or irrelevant MAbs, with (left) or without (right) complement added.

evaluated whether VZV-specific antibodies play a role in *in vitro* protection. The humoral response induced by Zostavax is predominately a memory B cell recall response: it is composed of IgG and IgA ASCs, with no detectible IgM ASCs, and shows levels of somatic hypermutation similar to what is seen after seasonal influenza vaccination. The majority of antibodies were specific to VZV gE (15/25 = 60%), indicating that gE is the predominant antibody target after vaccination. We could detect 5 gI-specific monoclonal antibodies (5/25 = 20%). These MAbs recognized gI, as determined by SDS-PAGE and Western blotting, but some appeared to coprecipitate gE. Finally, we could detect three gB-specific antibodies (3/25 = 12%) and two gH antibodies (2/25 = 8%) (1 from RM and 1 from ZV303). Functional analyses of these antibodies demonstrated that the gH-specific MAbs were the most potent neutralizers (with or without complement) and could also inhibit cell-to-cell spread of VZV *in vitro*.

Neutralization IC_{50} for the gH antibodies generated in this study were 0.03 to 0.23 μg/ml (0.2 to 1.5 nM). These potencies are equal to if not better than those previously reported in the literature (range, 0.12 to 8,000 nM [35, 43, 56, 57]). One gB antibody (303-1C2) could also neutralize VZV *in vitro* with or without complement. However, 303-1C2 was much less potent than the gH MAbs (without complement, 16.6 times less potent; with complement, 73 times less potent). VZV gE-specific MAbs could neutralize *in vitro* only in the presence of complement but could not inhibit cell spread.

The data in this article are consistent with previous reports in the literature demonstrating that gH MAbs are the most potent neutralizers and can inhibit cell-to-cell spread *in vitro* (28, 35–44). VZV gB-specific antibodies have also been shown to neutralize *in vitro* (58). Previously described VZV-specific monoclonal antibodies were generated by (i) hybridoma generation after immunization of mice with VZV (34), (ii) fusion of Ig V genes from mouse hybridomas to human Ig constant regions (59), (iii) *in vitro* immunization of human lymphocytes with VZV antigens, followed by fusion of the lymphocytes to myeloma cells (42), or (iv) phage display panning of a library of human Ig genes derived from human splenocytes obtained from subjects with idiotypic thrombocytopenic purpura (33). Our approach to isolate ASCs producing anti-VZV antibodies is similar to that used in a previously published study (36). However, we were able to isolate our anti-VZV glycoprotein-specific monoclonal antibodies in an antigen-agnostic manner (from 5 separate subjects), giving more insight into the breadth of glycoprotein-specific ASCs during the response to Zostavax.

Antibodies can act through a variety of mechanisms to prevent virus entry, including blocking receptor engagement, preventing postbinding/prefusion events at the cell surface or inside endosomes, inhibiting release of progeny virus, opsonization, antibody-dependent cell-mediated cytotoxicity (ADCC), and activation of the complement cascade (60). As gH/gL and gB are important for virion fusion (27, 29–32), antibodies directed against these glycoproteins are likely to prevent viral attachment and thus interfere with cell-to-cell spread. VZV-specific gE antibodies have been shown to neutralize in a complement-dependent manner but do not inhibit cell-to-cell spread (34, 54). One possible mechanism for this is that binding of gE-specific antibodies to VZV particles leads to the activation of the complement cascade and inactivation of the virus, while infected cells are protected from complement-mediated lysis due to the presence of complement regulatory proteins on the cell surface and/or low cell surface expression of gE.

As the antibodies that were detected after Zostavax vaccination had a high degree of somatic hypermutations (median, 20; range, 7 to 34), it is unclear whether these mutations were a result of primary infection, exogenous exposure to varicella, or subclinical reactivation over time. There have been some reports that VZV DNA can be detected in the blood of some healthy individuals (61). These data suggest that subclinical reactivation and subsequent boosting of the immune response may occur as people age. However, the importance of endogenous versus exogenous boosting has yet to be fully determined. Since there are similar degrees of somatic hypermutation observed after vaccination with Zostavax and with seasonal influenza vaccine, endogenous/exogenous boosting of the VZV-specific memory B cell compartment could result in increased affinity maturation over time.

The biological importance of VZV-specific antibodies *in vivo* is unclear. Although patients with agammaglobulinemia are not more susceptible to severe varicella infection (62), treatment of some immunocompromised children and pregnant women with VariZIG (high-titer VZV polyclonal sera) has been shown to prevent severe varicella (17–20). Additionally, VZV-specific total antibody titers do not decrease with age (9). Of note, there are limited data evaluating whether VZV-specific neutralizing antibody titers or gH-specific antibody titers decrease with age. If the neutralizing antibody titers do not decrease with age, this would be further evidence that antibodies probably do not play a protective role against the development of shingles. It could be envisaged that antibodies may play a protective role in preventing disseminated VZV during primary infection when the virus titer in the blood is high. However, the role of antibodies in

preventing the development of shingles is probably limited due to the biological transport of the virus from the dorsal root ganglion to the skin through nerves and not through the blood. Evidence in this report demonstrates that gE antibodies can neutralize in the presence of complement *in vitro* but cannot inhibit cell-to-cell spread. The FDA-approved VZV vaccine Shingrix is a subunit vaccine containing VZV gE adjuvanted with $AS01_B$ (7, 8). This vaccine is >90% efficacious, but the correlative mechanism of protection is currently unknown (8). Our data suggest that gE-specific antibodies are not the most functionally potent VZV-specific antibodies (compared to gH-specific antibodies) in terms of neutralization and cell-to-cell spread. Thus, unless the $AS01_B$ adjuvant induces a gE antibody qualitatively different from what natural infection/Zostavax vaccination induces (i.e., can inhibit cell-to-cell spread) or acts differently *in vivo* where there is complement present, the most likely mechanism of protection for Shingrix could be attributed to T cells.

Overall, we identified 25 human VZV-specific antibodies via an unbiased approach from 5 separate subjects 7 days after receiving Zostavax. This is the first study that examined the breadth of the VZV-specific antibody response using molecular biology techniques. All 5 donors tested had gE-specific MAbs, and they were either the only antibody isolated (301 and 304) or the predominant antibody (RM, 302, and 303). Though we see skewing of the results to donor 303, we feel that this distribution as well as the previous literature shows that gE is the predominant antibody target.

Although the majority of antibodies were VZV gE specific, the most potent antibodies *in vitro* were directed against gH. If antibodies do play a protective role *in vivo* during chickenpox or shingles, then inclusion of gH (gH/gL) protein would be beneficial in designing a next-generation VZV vaccine. Additionally, gH-specific monoclonal antibody therapy could also be considered an alternative to VariZIG in VZV-negative immunocompromised patients or pregnant women.

MATERIALS AND METHODS

Study subjects. All studies were approved by the Emory University Institutional Review Board (IRB no. 00045821 [healthy blood subject protocol] and 00050285 [Zostavax-vaccinated subject protocol]). Study subjects provided written informed consent prior to participation. Seven subjects with prior natural immunity to VZV were recruited for collection of peripheral blood mononuclear cells (PBMC) and serum at days 0, 7, 14, and 28. Study demographics are listed in Table 1. Five subjects (RM and ZV301-304) were then used on day 7 for single-cell ASC sorting. The remaining two subjects' ASCs were sorted but not cloned due to limited resources.

PBMC isolation. PBMCs were isolated using BD Vacutainer CPT tubes, washed, and resuspended in complete FBS medium (RPMI 1640 plus 2% fetal bovine serum [FBS], 50 U/ml penicillin, 50 μg/ml streptomycin, 2 mM L-glutamine, and 50 μM 2-mercaptoethanol) for immediate use.

B cell ELISPOT assay. Millipore Multiscreen-HA 96-well plates (Millipore MAHA N4510, nonsterile) were coated with either 10 μg/ml of goat anti-human IgA+IgG+IgM(H+L) (Jackson, 109-055-064) to measure total IgA or IgM antibody-secreting cells (ASCs), 10 μg/ml of donkey anti-human IgG(H+L) (Jackson, 709-005-149) to measure total IgG ASCs, or 20 to 40 μg/ml of VZV-infected cell lysate (detergent free; Meridian Life Sciences, 7740) to measure VZV-specific ASCs. All were diluted in sterile phosphate-buffered saline (PBS), and 100 μl/well was added and incubated overnight at 4°C. Plates were washed with PBS plus 0.05% Tween 20 and with PBS and blocked with complete medium (RPMI 1640, 5% fetal bovine serum, 50 U/ml penicillin, 50 μg/ml streptomycin, 2 mM L-glutamine, and 50 μM 2-mercaptoethanol) for >2 h at 37°C. Blocking medium was discarded, and 100 μl/well of complete medium was added followed, by 100 μl/well of 1×10^7 total PBMCs. Twofold serial dilutions were carried out. In some instances, $CD19^+$ $CD20^{lo}$ $CD38^+$ $CD27^+$ cells were bulk sorted and added directly to the blocked ELISPOT assay plate. Figure 2 shows how many ASCs were added per well after sorting. Cells were then incubated for ~16 h at 37°C with 5% CO_2. Plates were washed with PBS followed by PBS plus 0.05% Tween 20, and 100 μl/well of donkey anti-human IgG biotin (Jackson, 709-066-098), goat anti-human IgA biotin (Jackson, 109-065-011), or goat anti-human IgM biotin (Caltag, H15015) diluted 1/1,000 in PBS–0.05% Tween 20–1% FBS was added and left for 2 h at room temperature. Plates were washed with PBS plus 0.05% Tween 20, and 100 μl/well of HRP-avidin D (Vector Laboratories, A-2004) diluted 1/1,000 in PBS–0.05% Tween 20–1% FBS was added and left for 1 h at room temperature. Plates were washed with PBS plus 0.05% Tween 20 followed by PBS, and 100 μl/well of 3-amino-9-ethylcarbazole (AEC) (Sigma, A-5754) in 0.1 M Na-acetate buffer at pH 5.0 (sodium acetate trihydrate [$C_2H_3O_2Na \cdot 3H_2O$] FW 136.1; Sigma, S-8625) was added, left for 5 to 15 min, and then washed thoroughly with distilled water. Plates were scanned on a CTL (Shaker Heights, OH) ImmunoSpot analyzer. Data are representative of the number of antibody-secreting cells (ASCs) per million PBMC.

ASC sorting. Freshly isolated PBMC from ~40 ml of blood were stained with anti-human CD19–fluorescein isothiocyanate (FITC) (BD 555412), anti-human CD38–phycoerythrin (PE) (BD 555460), anti-

human CD3–PE-Cy7 (BD 557851), anti-human CD20–PE-Cy7 (BD 335793), and anti-human CD27–allophycocyanin (APC) (Ebio, 17-0279) for 30 min on ice. Cells were washed and resuspended in PBS plus 2% FBS. Using a BD FACSAria II, antibody-secreting cells (ASCs) ($CD19^+$ $CD38^+$ $CD27^+$) were either single-cell sorted into a 96-well PCR plate containing 10 mM Tris-HCl with 40 U/μl of RNase inhibitor (Promega) and frozen at −80°C or bulk sorted into complete medium.

Generation of MAbs. The generation of monoclonal antibodies (MAbs) from single-cell-sorted ASCs was performed as described previously (47, 49, 63). Briefly, IgG and IgA heavy, kappa, and lambda variable regions were amplified by reverse transcription and PCR. cDNA was synthesized from random hexamers and used for a first-round IgGH, IgAH/IgMH, Igκ, and Igλ PCR. The first-round PCR cocktail of primers covered all families of variable (V) and joining (J) genes; this PCR was followed by a nested PCR to identify the sequences of the V and J genes of the IgGH, IgAH/IgMH, Igκ, and Igλ regions. Once the IgGH, IgAH/IgMH, Igκ, and Igλ gene families were known, highly specific primers were used to amplify these regions and put restriction enzyme sites for cloning into IgG1H, Igκ, and Igλ backbones for antibody expression. These antibodies were expressed in HEK293 (donor RM) or Expi293 (Invitrogen) (donors 301, 302, 303, and 304) cells. The antibodies were affinity purified using protein A-agarose beads (GenScript).

V family breadth and somatic hypermutation analysis. The heavy-chain variable (V) region sequences were analyzed to determine repertoire breadth as described previously (63). Briefly, the International ImmunoGeneTics (IMGT) database information system (www.imgt.org) was used to identify the V and J gene families. To analyze the number of somatic hypermutations in the VH gene, IgBLAST was used to assess the frequency of VH mutations as a combination of both silent and replacement (resulting in a change at the amino acid level) mutations from the framework region 1 to the complementarity-determining region 3 (CDR3). The frequencies of VH mutations in naive B cells and after seasonal trivalent influenza vaccination have been published previously (49, 50).

VZV lysate and gE-specific ELISAs. The binding specificity of isolated monoclonal antibodies was determined by ELISA. An initial VZV-infected cell lysate ELISA was performed. If the VZV lysate was positive, a VZV total glycoprotein ELISA was used to identify VZV gP-specific MAbs. A follow-up VZV gE ELISA was utilized for those MAbs showing specificity for VZV glycoproteins.

For the VZV-infected cell lysate and gP ELISA, Nunc C96 MaxiSorp Immunoplates (Thermo Scientific) were coated overnight at 4°C with either VZV-infected cell lysate (Meridian Life Sciences, 7740) or lectin-purified VZV glycoproteins (Virusys or in-house reagent) diluted in PBS at a concentration of 1 μg/ml. For the VZV gE ELISA, Pierce nickel-coated plates (Thermo Scientific) were coated overnight at 4°C with His-tagged recombinant VZV gE protein (Oka strain, in-house reagent) diluted in PBS at a concentration of 1 μg/ml. The remainder of the protocol was identical for the VZV lysate, VZV total glycoprotein, and VZV gE ELISAs. Protein-coated plates were washed six times with PBS–0.05% Tween 20 and blocked with blocking buffer (PBS–0.05% Tween 20 with 3% nonfat dry milk) for 1 h at room temperature on a plate rocker. Either supernatant from transiently transfected Expi293 cells diluted 1:2 in blocking buffer (VZV lysate ELISA) or VZV-specific monoclonal antibodies were 5-fold serially diluted in blocking buffer, starting at a concentration of 3 μg/ml, for a total of 4 to 6 dilutions. Blocking buffer was removed from the coated plates, and diluted antibodies were bound to the plate for 1.5 h at room temperature on a plate rocker. Antibody-bound plates were washed six times with PBS–0.05% Tween 20. Goat anti-human IgG–HRP (Southern Biotech) was diluted 1:2,000 in blocking buffer, added to the washed plates, and left for 1 h at room temperature on a plate rocker. Plates were washed six times with PBS–0.05% Tween 20 and developed for 5 min with SuperBlu-Turbo tetramethylbenzidine (TMB) solution (Virolabs) followed by ELISA stop solution for TMB (Virolabs). Absorbance was read at 450 nm on a Victor multilabel counter (Wallac/PerkinElmer), and antibody binding curves were visualized using nonlinear-fit four-parameter variable slope analysis in GraphPad Prism 7 software.

Immunocapture. Immunocapture experiments were performed with magnetic M-270 Epoxy Dynabeads (Thermo Fisher, 14302D) following the manufacturer's instructions. In brief, 30 μg of MAb was covalently coupled to 1 mg of magnetic beads. The reaction was performed overnight in an incubator shaker at 37°C. Unbound MAb was removed by washing first with PBS and then with PBS with 0.02% bovine serum albumin (BSA) (Pierce, 23209). Twenty-five micrograms of VZV glycoprotein lysate (Virusys) was added to the magnetic beads. Binding of the antigen was allowed by end-over-end rotation of the bead-antigen mixture for 14 h at 4°C. Unbound VZV glycoprotein was removed by three washes with 500 μl PBS. Antigen was released with 25 μl of 8 M urea (ultrapure; Riedel de Haen, 15604) in 100 mM ammonium bicarbonate (Thermo Fisher, BP2413). Control experiments were performed by coupling an irrelevant monoclonal IgG.

MS analysis. Half the volume (12.5 μl) of the released antigen solution was used for antigen identification. Reduction was performed in an incubator shaker for 20 min at 60°C with 1 μl of 500 mM Tris(2-carboxyethyl)phosphine (TCEP). The sample was then alkylated for 30 min at room temperature in the dark by adding a 1.5-fold molar excess of iodoacetamide (Pierce, 90034). Protein was then digested in an incubator shaker for 4 h at 37°C with 0.1 μg LysC (Roche Diagnostics, 11047825001). A second digest was performed overnight at 37°C with 0.1 μg trypsin (Promega, V5111) after the sample was diluted to 1.5 M urea using 100 mM ammonium bicarbonate. The digestion was stopped by adding 2 μl of formic acid. Samples were analyzed by nano-LC-MS/MS immediately after the digestion or stored at −80°C. Nano-LC-MS/MS analysis was performed on a splitless Ultimate 3000 ultraperformance liquid chromatography (UPLC) system (Dionex) that was directly coupled to an Orbitrap XL mass spectrometer (Thermo Fisher). For the analysis, 50 μl of the protein digest was loaded from the autosampler onto a C_{18} trapping column (300-μm inner diameter by 5 mm, C_{18} PepMap100, 5-μm particle size, 100-Å pore size; Thermo Scientific, catalog no. 160454) and then back eluted onto a 75-μm-inner-diameter analytical

column (15-cm Acclaim PepMap RSLC; particle size, 2 μm; Thermo Scientific, catalog no. 164534) by gradient elution, increasing the acetonitrile percentage within 50 min from 2% to 35% in 65 min. Peptides were directly eluted into the Orbitrap XL mass spectrometer, and high-mass-accuracy MS spectra were acquired in the Orbitrap mass analyzer at a resolution of 100,000. The top 5 MS/MS spectra were acquired in the ion trap mass analyzer with automated gain control and dynamic exclusion enabled. Sequence identification and label-free quantitation was performed using the software package Protein Discoverer 1.4 (Thermo Scientific), allowing only peptides with less than a 1% false-discovery rate as calculated by the percolator node as positive identifications. Protein sequences of the human as well as the VZV proteome were obtained from the UniProt server (www.uniprot.org).

Western blotting. VZV glycoprotein preparation (including all glycoproteins, at 2.5 μg per lane) were separated by Bis-Tris 4 to 12% SDS-PAGE (NuPAGE NP0336; Invitrogen) using MES (morpholineethanesulfonic acid) running buffer. Proteins were transferred onto a nitrocellulose membrane following the manufacturer's instructions using the iBlot system from Thermo Fisher (iB301002). Primary anti-VZV antibody and secondary detection antibody were applied using the Snap ID system from Millipore. In brief, the primary antibody was diluted 1:1,000. PBS with 0.1% Tween 20 and 0.5% BSA was used as a blocking buffer. HRP-labeled goat anti-human Fc antibodies (PerkinElmer, NEF802) were used as the detection antibody. The Western blot was developed using chloronaphthol-diaminobenzidine (CN/DAB) substrate (Thermo Scientific, 34000).

Virus. GFP-VZV (pOKA ORF11-GFP) was kindly provided by Marvin Sommer and Ann Arvin, Stanford University. GFP-tagged VZV Oka strain stocks were grown in MRC-5 cells. MRC-5 cells were cultured 35°C with 5% CO_2 in 2% complete EMEM (Eagle minimum essential medium [EMEM] [Corning] supplemented with 2% heat-inactivated fetal bovine serum [HyClone], 1% L-glutamine [Gibco], and 0.5% neomycin [Sigma]). Cell-associated virus was harvested into PGS buffer (in-house buffer) at 40 h postinfection, sonicated to disassociate virus from cells, and centrifuged at 1,500 rpm for 10 min to remove cell debris. Cell-free virus was flash frozen in liquid nitrogen and stored at −70°C.

Neutralization assay. Purified VZV-specific monoclonal antibodies were 3-fold serially diluted, starting at a concentration of 25 μg/ml, for a total of 10 dilutions. Antibodies were diluted into 2% complete DMEM/F-12 (Dulbecco's modification of Eagle's medium/Ham's F-12 50/50 mix [DMEM/F-12] [Corning] supplemented with 2% heat-inactivated fetal bovine serum [HyClone] and 1% penicillin-streptomycin [Corning]). GFP-tagged VZV Oka strain stocks were quick-thawed in a water bath, diluted to a concentration of 300 PFU/25 μl in PGS buffer, and kept on wet ice. Twenty-five microliters of diluted antibody was thoroughly mixed with 25 μl of diluted VZV-GFP (giving a final starting antibody concentration of 12.5 μg/ml) in a flat-bottom, clear-walled 96-well plate (Costar, 3598) and incubated at 35°C with 5% CO_2 for 1 h. Controls included VZV-GFP alone (no antibody) and blank wells (no antibody and no VZV-GFP). All conditions were performed in replicates of 8. After 1 h of incubation, 10 units/well of reconstituted guinea pig complement (Sigma) was added to half of the virus-antibody wells (totaling 4 replicates per condition) and incubated at 35°C with 5% CO_2 for an additional 30 min. ARPE-19 target cells were diluted in 2% complete DMEM/F-12, and 20,000 cells, in 50 μl, were added to each well, mixed thoroughly, and incubated at 35°C with 5% CO_2 for 1 h with shaking every 15 min. One hundred microliters of 2% complete DMEM/F-12 was then added to each well, and the plate was centrifuged at 1,200 rpm for 10 min to encourage virus-cell interaction and cell settling. The plate was incubated at 35°C with 5% CO_2 for 5 to 7 days, and the total number of cells expressing GFP per well was quantified using an Acumen Cellista (TTP LabTech). The percentage of viral neutralization for each well was defined as [(average of 4 replicates of VZV-GFP-only control − condition of interest)/average of 4 replicates of VZV-GFP-only control] × 100. IC_{50} values were calculated from dilution curves using GraphPad Prism 7 software.

***In vitro* cell-to-cell spread inhibition assay.** One hundred thousand MRC-5 cells (ATCC) per well were plated in a 12-well plates (Corning 3513) in 2 ml of complete Eagle's minimal essential medium (EMEM) (Sigma) with 10% fetal bovine serum, 1% of L-glutamine, and 0.5% neomycin for 2 to 3 days at 37°C with 5% CO_2 until >90% confluence was reached. GFP-labeled cell-free VZV was generated as discussed above (at 7.03×10^4 PFU/ml). Confluent MRC-5 wells were washed once with warm complete EMEM plus 10% FBS, and 100 μl of 1:125-diluted VZV in PGS buffer (Merck in-house) was added per well (~55 PFU/well). PGS buffer alone was added as a mock control. Plates were rocked back and forth 8 times and incubated for 15 min at 35°C with 5% CO_2. This was repeated 3 times for a total infection time of 1 h. After 1 h, 2 ml of EMEM plus 2% FBS plus 1% L-glutamine plus 0.5% neomycin was added and plates placed back at 35°C with 5% CO_2 for 16 to 18 h. The next day, wells were washed once with 2 ml of warm EMEM plus 2% FBS plus 1% L-glutamine plus 0.5% neomycin, and antibodies were diluted in EMEM plus 2% FBS plus 1% L-glutamine plus 0.5% neomycin starting at 30 μg/ml. Diluted antibodies (0.5 ml per well) were added and left for 60 min at 35°C with 5% CO_2. For the complement-treated wells, 65 μl of guinea pig complement (Sigma, S1639) was added and incubated for 30 min at 35°C with 5% CO_2. A 1.5-ml portion of EMEM plus 2% FBS plus 1% L-glutamine plus 0.5% neomycin was added, and plates were placed back at 35°C with 5% CO_2 for 4 more days.

To fix and immunostain the cells, wells were washed with PBS, and 2 ml of 90% acetone (Sigma, 534064) diluted in distilled water was added and left for 10 min at room temperature. Wells were washed once with PBS, and 0.5 ml of 1:2,000-diluted VZV glycoprotein E antibody (Abcam, Ab52549) in PBS plus 0.05% Tween 20 added and left for 30 min at 37°C. Wells were gently washed 2 times with PBS, and rabbit anti-mouse IgG peroxidase secondary antibody (Sigma, A9044) diluted 1:1,000 in PBS plus 0.05% Tween 20 was added at 0.5 ml per well and left for 30 min at 37°C. Wells were washed 2 times with PBS, and 150 μl of 1× DAB/Metal in peroxide buffer (Thermo Scientific, 1856090) was added, rocked back and forth 8 times, and incubated at room temperature for 15 min. Plates were then flipped over onto paper

towels and dried overnight at room temperature. The plates were scanned on a CTL Immunospot S6 reader (Shaker Heights, OH). The percentage of cell-to-cell spread inhibition for each condition was defined as [(average of 3 replicates of VZV-GFP-only control − condition of interest)/average of 3 replicates of VZV-GFP-only control] × 100. IC_{50} values were calculated from dilution curves using GraphPad Prism 7 software.

Accession number(s). Sequences were deposited in GenBank under the following accession numbers (heavy/light chains): 301-1G5, MH259708/MH259733; 302-1B12, MH259709/MH259734; 302-1C12, MH259710/MH259735; 302-1D11, MH259711/MH259736; 302-1G9, MH259712/MH259737; 303-1A8, MH259713/MH259738; 303-1A12, MH259714/MH259739; 303-1B2, MH259715/MH259740; 303-1C1, MH259716/MH259741; 303-1C2, MH259717/MH259742; 303-1C6, MH259718/MH259743; 303-1C9, MH259719/MH259744; 303-1D7, MH259720/MH259745; 303-1E3, MH259721/MH259746; 303-1E8, MH259722/MH259747; 303-1E12, MH259723/MH259748; 303-1F5, MH259724/MH259749; 303-1F7, MH259725/MH259750; 303-1G1, MH259726/MH259751; 304-1A12, MH259727/MH259752; RM-1A2, MH259728/MH259753; RM-1D1, MH259729/MH259754; RM-2D6, MH259730/MH259755; RM-5A2, MH259731/MH259756; and RM-5B3, MH259732/MH259757.

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