Pentameric complex of viral glycoprotein H is the primary target for potent neutralization by a human cytomegalovirus vaccine

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Human cytomegalovirus (HCMV) can cause serious morbidity/mortality in transplant patients, and congenital HCMV infection can lead to birth defects. Developing an effective HCMV vaccine is a high medical priority. One of the challenges to the efforts has been our limited understanding of the viral antigens important for protective antibodies. Receptor-mediated viral entry to endothelial/epithelial cells requires a glycoprotein H (gH) complex comprising five viral proteins (gH, gL, UL128, UL130, and UL131). This gH complex is notably missing from HCMV laboratory strains as well as HCMV vaccines previously evaluated in the clinic. To support a unique vaccine concept based on the pentameric gH complex, we established a panel of 45 monoclonal antibodies (mAbs) from a rabbit immunized with an experimental vaccine virus in which the expression of the pentameric gH complex was restored. Over one-half (25 of 45) of the mAbs have neutralizing activity. Interestingly, affinity for an antibody to bind virions was not correlated with its ability to neutralize the virus. Genetic analysis of the 45 mAbs based on their heavy- and light-chain sequences identified at least 26 B-cell linage groups characterized by distinct binding or neutralizing properties. Moreover, neutralizing antibodies possessed longer complementarity-determining region 3 for both heavy and light chains than those with no neutralizing activity. Importantly, potent neutralizing mAbs reacted to the pentameric gH complex but not to gB. Thus, the pentameric gH complex is the primary target for antiviral antibodies by vaccination.

monoclonal antibody | antiviral antibody

Human cytomegalovirus (HCMV) is an important pathogen in transplant patients (1–5), and its infection can lead to invasive end-organ diseases, such as pneumonitis and hepatitis, as well as vascular pathology contributing to graft failure (4, 6, 7). HCMV is also the most common cause of in utero viral infections in North America and Europe, affecting 0.5–2% of newborns annually (8–10). Congenital HCMV infection can lead to symptomatic diseases at birth and also cause developmental disabilities in children (10, 11). Maternal seropositivity before conception protects against congenital transmission (12, 13), and both maternal humoral and cellular immunity are likely to contribute to the protection (14–16). Antibodies in particular are important for preventing congenital infection, serving as the first line of defense against maternal infection. It may also play a role in preventing transmission to the fetus, supported by the results of a small, nonrandomized study in pregnant women with primary HCMV infection, in which the passive immunity of monthly infusions of HCMV hyperimmune human IgG (HCMV-HIG) (200 mg/kg maternal weight) was ~60% effective in protecting against congenital HCMV infection (17, 18). These studies suggest that it is feasible to develop a vaccine for preventing congenital HCMV infection and its sequelae. However, despite the fact that the Institute of Medicine has identified development of an effective vaccine for prevention of congenital HCMV as a top priority since 1999 (19), progress toward this goal has only been incremental (8, 20, 21). One of the hurdles to the efforts is our limited understanding of component of natural immunity associated with protection against HCMV infection.

HCMV is a large, complex virus, with a genome capable of encoding >150 proteins (22–26). Because of the strict species specificity, options of animal models for HCMV research are limited (27). Thus, the functions of most HCMV antigens in viral infection in vivo and their roles as targets for host immunity are poorly understood. Furthermore, culture systems of single cell types have limitations for studying HCMV pathogenesis. Immunohistochemistry studies showed that HCMV can infect varieties of cells in vivo, including endothelial, epithelial cells, fibroblasts, and leukocytes (28–36). Many HCMV end-organ diseases, such as pneumonitis and gastroenteritis, are due to infection of the epithelial/endothelial cells in the affected organ (35–39). However, common laboratory strains, such as AD169 and Towne, were culture-adapted in fibroblast cells, with genomic

Significance

Congenital human cytomegalovirus (HCMV) infection is an important cause of newborn disability, and developing a vaccine against congenital HCMV is a top priority. However, despite decades of efforts, a vaccine remains elusive. Previous vaccines lacked an antigen called pentameric glycoprotein H (gH) complex, essential for the virus to infect epithelial/endothelial cells, and these vaccines induced poor neutralizing antibodies. To support a unique vaccine concept featuring the pentameric gH complex, we established 45 mAbs from a rabbit immunized with an experimental vaccine. Over 50% of the mAbs have antiviral activity, and potent clones target the pentameric gH complex, thus establishing this antigen as the key for potent antiviral antibodies by vaccination. Our result contributes to the understanding of immune attributes of an effective vaccine against HCMV.

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mutations (22, 24, 40) and, more importantly, have lost their tropism to endothelial and epithelial cells, in contrast to pathogenic clinical isolates (32, 33, 41, 42).

Loss of viral tropism to endothelial and epithelial cells was mapped to various mutations in the viral UL131-128 locus, and these mutations abrogated the expression of the pentameric glycoprotein H (gH) complex, composed of gH, gL, UL128, UL130, and UL131 proteins, a determinant for viral tropism to endothelial and epithelial cells (42–44). Because the pentameric gH complex is missing in common laboratory strains (42, 43), its importance in viral tropism, viral pathogenesis, and vaccine design was not fully appreciated until recently (42, 45). With this understanding, it is not surprising that Towne virus and recombinant glycoprotein B (gB) vaccines, although with ∼50% efficacy against primary infection in the clinic (46–49), induced poor neutralizing titers against viral infection of epithelial cells, in contrast to immune sera from HCMV-seropositive donors (50, 51). Thus, missing the pentameric gH complex is likely a deficiency in antigen composition for both vaccines (50). Studies of monoclonal antibodies (mAbs) isolated from HCMV-seropositive donors or polyclonal IgG enriched for antigen specificity supported the hypothesis that the pentameric gH complex, not gB, appears to be important for neutralizing activity in human subjects with natural infection (52).

We recently described an experimental vaccine virus in which expression of the pentameric gH complex was restored (53). Unlike the parental AD169 virus and the recombinant gB vaccine, this virus can elicit high levels of neutralizing antibodies in rabbits and rhesus macaques (53). To support clinical development of this vaccine centered its concept on the pentameric gH complex, we established a comprehensive panel of 45 mAbs from a single rabbit that received vaccination. Of the 45 mAbs, 25 had neutralizing activity against viral entry in epithelial cells, including 11 elite neutralizers with ≥10-fold greater potency than HCMV-HIG. Biochemical analysis demonstrated that all elite neutralizers preferentially bound to the virus expressing the pentameric gH complex, and the majority of elite neutralizers (8 of 11) specifically recognized a recombinant form of the pentameric gH complex. Interestingly, binding affinity for intact virions was not correlated with neutralizing activity. Moreover, genetic analysis of the 45 mAbs based on their heavy- and light-chain sequences identified at least 26 B-cell linage groups characterized by distinct binding or neutralizing properties. In addition, neutralizing antibodies had longer complementarity-determining region 3 (CDR3) for both heavy and light chains than those of antibodies with no neutralizing activity. These data establish the importance of the pentameric gH complex as the primary target for potent neutralizing antibodies by vaccination, and support development of an experimental HCMV vaccine featuring the pentameric gH complex.

Results

A Panel of 45 mAbs from a Rabbit Vaccinated with HCMV Expressing the Pentameric gH Complex. Recent studies of the pentameric gH complex have indicated its importance in human immunity to natural HCMV infection and highlighted the antigenic deficiency of HCMV vaccines previously evaluated in the clinic (50–52, 54). We have built an experimental vaccine virus with the expression of its pentameric gH complex restored (53, 55). Immunization of rabbits with 10, 30, or 100 μg/dose of vaccine elicited neutralizing antibodies, with geometric mean titers at 670, 960, and 1,930, respectively, well within the range of titers commonly seen in HCMV-seropositive subjects (Fig. 1).

Biochemical and proteomics compositions of the vaccine and its parental AD169 virus were similar for all major viral structural proteins, except the pentameric gH complex, and UL116, UL103, and UL41A (Fig. S1). Thus, the newly added pentameric gH complex is likely the key target for potent neutralizing antibodies by vaccination. To test this hypothesis, we established a panel of 45 mAbs from a single rabbit immunized with the vaccine virus. All clones were confirmed as unique based on their genetic identity.

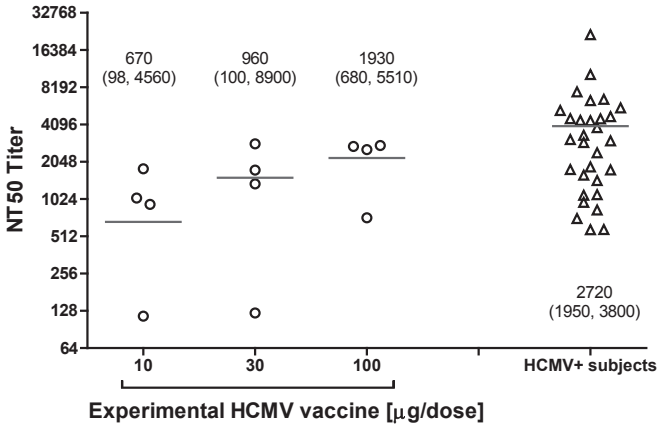


Fig. 1. A vaccine virus with the pentameric gH complex restored can elicit high neutralizing titers in rabbits. Groups of rabbits (n = 4) immunized with the vaccine virus at indicated dose at week 0, 3, and 8. The immune sera were collected at week 11 and evaluated along with prevaccination sera in viral neutralization assay. Prevaccination sera showed no neutralizing activity. A serum panel from HCMV-seropositive donor (n = 30) was included in the experiments as a comparison. NT50 titers represent the reciprocal serum dilutions to block 50% viral entry. The geometric means with 95% confidence intervals are marked in the plot for each group.

Binding Affinity to Virions Is Not Correlated with Neutralization Activity of Rabbit Anti-HCMV mAbs. To compare the functional attributes of all 45 mAbs, we quantified the ability of each mAb to neutralize and to bind the vaccine virus. An analysis of HCMV-HIG (e.g., CytoGam) is shown as an example and as a reference for comparison (Fig. 2 *A* and *B*). To quantify neutralization capacity and binding affinity, the mAb concentrations required to block viral entry by 50% (EC_{50} neutralizing) and to achieve 50% maximal binding (EC_{50} binding), respectively, were calculated through four-parameter curve fittings. If there was no reliable curve fitting (*Materials and Methods*), an arbitrary value of 100 μg/mL was assigned for the EC_{50}, indicative of the poor neutralization activity or weak binding affinity of that mAb. All EC_{50} values are included in Table S1.

We plotted EC_{50} values for neutralizing (y axis) versus binding (x axis) for each of the 45 mAbs (Fig. 2*C*). The EC_{50} neutralizing for HCMV-HIG (∼1 μg/mL; Fig. 2*A*), shown as a horizontal dotted line, was used to segregate mAbs based on their neutralizing potency: 25 mAbs with EC_{50} neutralizing of ≤1 μg/mL were considered neutralizing mAbs (triangles above the line), and 20 mAbs with EC_{50} neutralizing of >1 μg/mL nonneutralizing mAbs (circles below the line). The EC_{50} binding for all neutralizing mAbs ranged from 0.2 to 5 μg/mL, comparable to HCMV-HIG (∼2 μg/mL; Fig. 2*B*). In contrast, the majority of nonneutralizing mAbs (14 of 20) had higher binding affinity for the vaccine virus than HCMV-HIG (Fig. 2*C*, lower left quadrant). Thus, high binding affinity for a mAb appeared not associated with improved antiviral function.

To focus our characterization, we designated the mAbs with ≥10-fold neutralizing capacity or binding affinity than HCMV-HIG as elite neutralizers or elite binders, thus, with EC_{50} neutralization values of ≤0.1 μg/mL (filled triangles in Fig. 2*C*) and EC_{50} binding values of ≤0.2 μg/mL (filled circles in Fig. 2*C*), respectively. Interestingly, only mAb 57.4 can be classified as both an elite neutralizer and an elite binder.

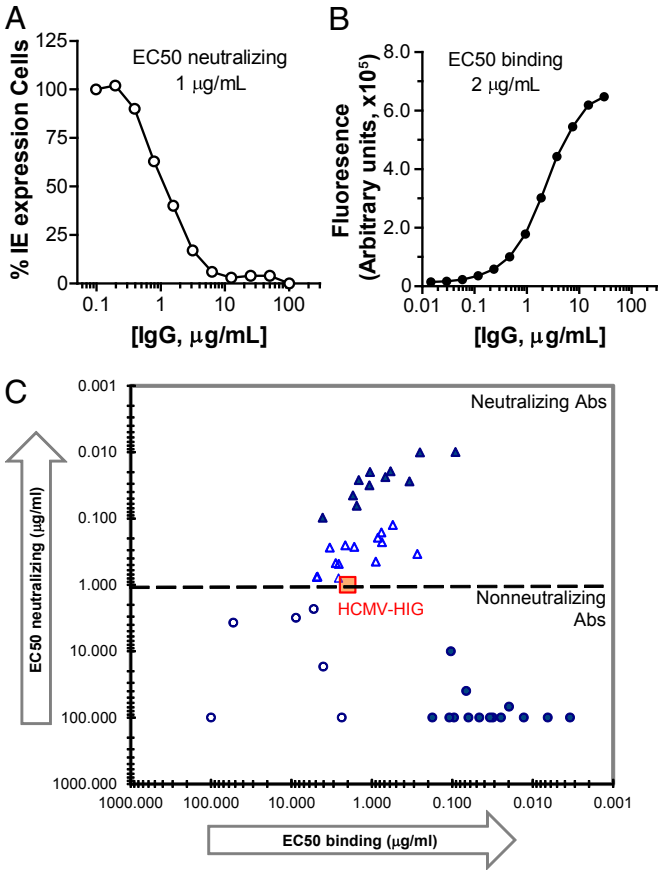


Fig. 2. Correlation analysis of neutralizing and binding properties of rabbit mAbs. The neutralizing and binding functions of each antibody were analyzed in viral neutralization and binding assays, respectively. Human CMV hyperimmune IgG (HCMV-HIG) (e.g., CytoGam) is provided as an example and also as a reference for comparison. HCMV-HIG is analyzed for neutralization in ARPE-19 cells (*A*) and binding to virions (*B*). EC_{50} neutralizing and EC_{50} binding, defined as the IgG concentration required to block 50% of viral infection (*A*) or reach 50% maximal binding signal (*B*), respectively, were calculated by four-parameter curve fitting. For mAbs with weak neutralizing or binding capacity, the EC_{50} value was arbitrarily assigned as 100 μg/mL (*Materials and Methods*). Each mAb was plotted for its EC_{50} neutralizing (*y* axis) and EC_{50} binding (*x* axis) (*C*). The solid square symbol in the center represents HCMV-HIG (e.g., CytoGam); the dashed horizontal line represents the EC_{50} neutralizing of HCMV-HIG, which is used as the threshold to differentiate neutralizing mAbs (triangles above the line) and nonneutralizing mAbs (circles below the line). The 11 elite neutralizing antibodies are identified by solid triangles. mAbs 57.4 and 276.10 are marked with the clone identification. The 11 elite binders are identified by filled circles.

Neutralizing Capacity of a mAb in Epithelial Cells Does Not Predict Its Neutralizing Ability in Fibroblasts. HCMV uses different entry mechanisms for infection of epithelial cells versus fibroblasts (56). Thus, we measured the EC_{50} neutralizing for the panel against the vaccine virus in a fibroblast cell line, MRC-5 cells (Table S1). By plotting EC_{50} neutralizing values in MRC-5 cells (*y* axis) versus ARPE-19 cells (*x* axis), we observed three distinct groups of mAbs (Fig. 3). Group C mAbs did not prevent viral infection in either cell line. Group B mAbs (17 of 45) neutralized virus in both cell types. Interestingly, only 5 of 11 elite neutralizers blocked viral infection to fibroblasts and epithelial cells. The remaining six elite neutralizers, including mAbs 57.4 and 276.10, fell into group A, which only neutralized virus in ARPE-19 cells. The discrepancy between neutralization capacity for the elite neutralizers in ARPE-19 versus MRC-5 cells is similar to the observations of human mAbs with potent neutralizing activity (54). These results suggest that the elite neutralizers recognize the pentameric gH complex, which is required for viral entry to epithelial cells, but not fibroblast cells (56).

Elite Neutralizers Show Preferential Binding to the Pentameric gH Complex-Restored Vaccine Virus over Parental AD169 Virus. To test the hypothesis that the elite neutralizers were specific to the pentameric gH complex, we first used virion-titration ELISA to compare the binding profiles of elite mAbs to parental AD169 virus versus the vaccine virus. The vaccine and AD169 virus had nearly identical composition including gB and gO (UL74), except the pentameric gH complex (53) (Fig. S1). Thus, any difference in the binding affinity of a mAb for parental AD169 versus the vaccine virus could be attributed to the pentameric gH complex. Three binding patterns were observed, as illustrated in Fig. 4: binding to the vaccine only (e.g., mAb 57.4; Fig. 4*A*), binding to both viruses, but with higher affinity for the vaccine (e.g., mAb 58.5; Fig. 4*B*), and comparable binding to both viruses (e.g., mAb 295.5; Fig. 4*C*). Of the 11 elite binders, 9 showed comparable binding to both viruses as exemplified by mAb 295.5 (Fig. 4*D*). In contrast, none of the elite neutralizers showed this binding pattern; mAbs 57.4 and 276.10 only reacted to the vaccine virus, whereas the remaining 9 of the elite neutralizers showed preferential binding to the vaccine virus over AD169 virus. Thus, the neutralizing mAbs possibly target the pentameric gH complex preferentially, consistent with our hypothesis that the pentameric gH complex is a key target for potent neutralizing antibodies.

A Majority of Elite Neutralizing mAbs Recognize a Recombinant Form of the Pentameric gH Complex. To determine whether the mAbs bind to gB or to the pentameric gH complex, we developed an ELISA using recombinant forms of both proteins (Fig. S2). Reactivity of the mAbs to the pentameric gH versus gB antigens at a single concentration of ~1 μg/mL is shown in Fig. 5. None of the elite neutralizers reacted to gB. Three elite binders (mAbs 272.7, 350.1, and 210.4) reacted strongly to gB, and none of these mAbs had any neutralizing activity in epithelial or fibroblast cells. These results are consistent with previous observations that gB is not effective in eliciting neutralizing mAbs against epi-

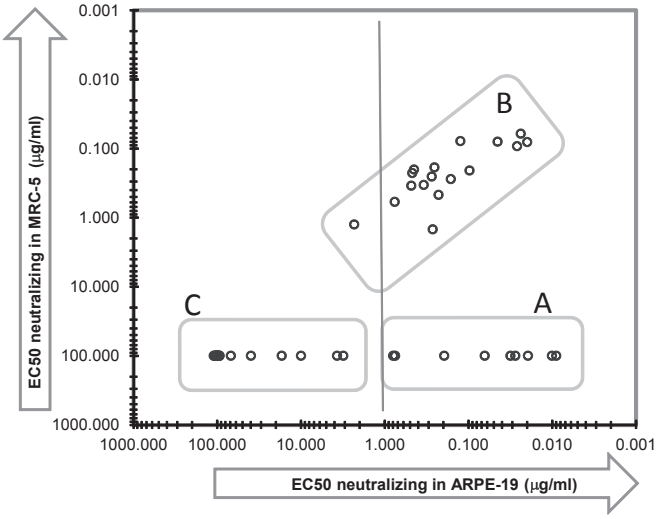


Fig. 3. Neutralizing properties of mAbs in ARPE-19 cells do not predict their activity in MRC-5 cells. The EC_{50} neutralizing values were calculated for each antibody in ARPE-19 epithelial cells and MRC-5 fibroblasts (Table S1). Three distinct groups are marked by circles for the mAbs only neutralizing in ARPE-19 cells (group *A*), the mAbs neutralizing in both ARPE-19 and MRC-5 cells (group *B*), and the mAbs nonneutralizing in either cell line (group *C*), respectively.

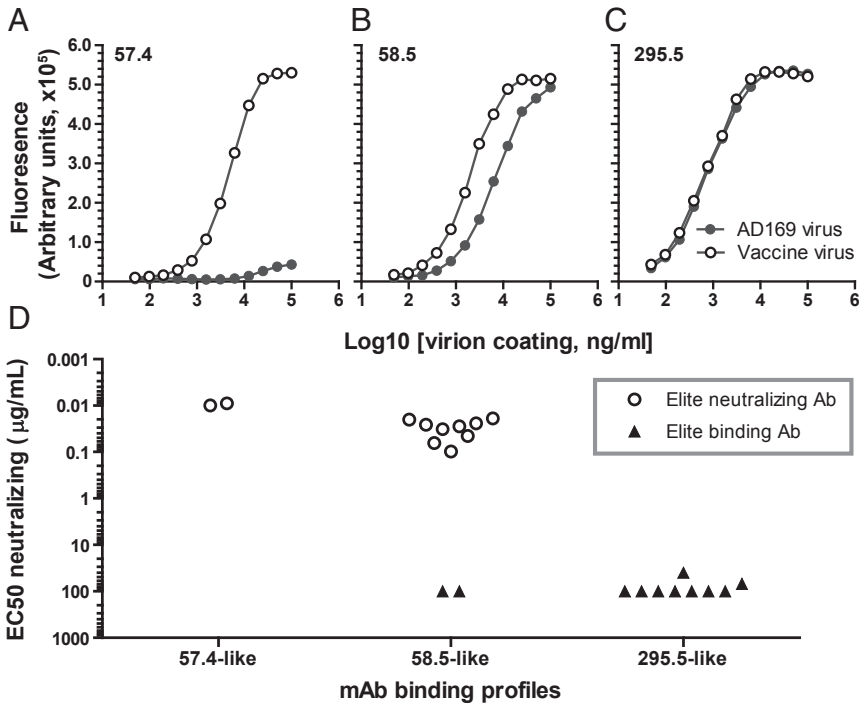


Fig. 4. Preferential binding of mAbs to the vaccine virus is associated with their neutralizing activity. AD169 virus and the vaccine virus were immobilized in titration on ELISA plates. Reactivity of rabbit mAb to AD169 versus the vaccine virions was tested at a fixed concentration of 2 μg/mL. The representative curves illustrate three patterns of binding to the antigen pair in *A* (mAb 57.4), *B* (mAb 58.5), and *C* (mAb 295.5), respectively. In *D*, the elite neutralizing mAbs (open circles) and elite binding mAbs (closed triangles) are plotted based on their binding patterns (*x* axis) and EC_{50} neutralizing value in ARPE-19 cells (*y* axis).

thelial cell entry (50, 53). In contrast, of the 11 elite neutralizers, 8 reacted to the pentameric gH complex, whereas only 2 of 11 elite binders (292.1 and 269.6) reacted to the pentameric gH complex (Fig. 5), with relatively weak signals for binding compared with the elite neutralizers. Thus, the majority of the elite neutralizers target the pentameric gH complex.

Phylogenetic Analysis Revealed at Least 26 Distinct Groups Reflecting Diverse B-Cell Lineages. To gain insight into the antibody response at the genetic level, we sequenced the variable regions of the light (V_K or V_L) and heavy (V_H) chains from the 45 mAbs. Phylogenetic trees were constructed based on the amino acid sequences of entire V_H regions (Fig. 6) and V_L regions (Fig. S3). Because an antibody heavy-chain CDR3 (HCDR3) best represents its junction-diversity and clonal specificity (57), we grouped the mAbs based on their HCDR3 sequence homologies into 26 lineage groups, 10 of which contained two or more mAbs.

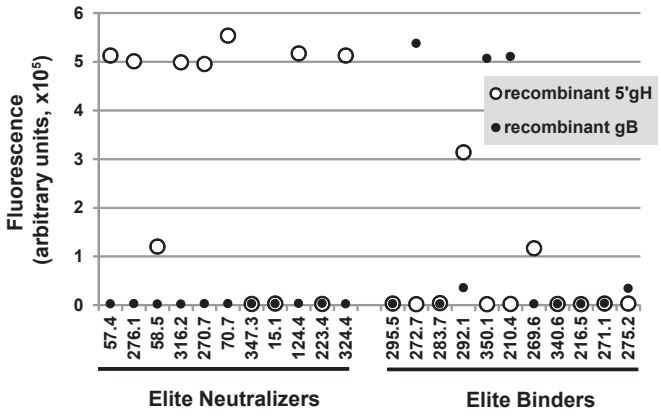
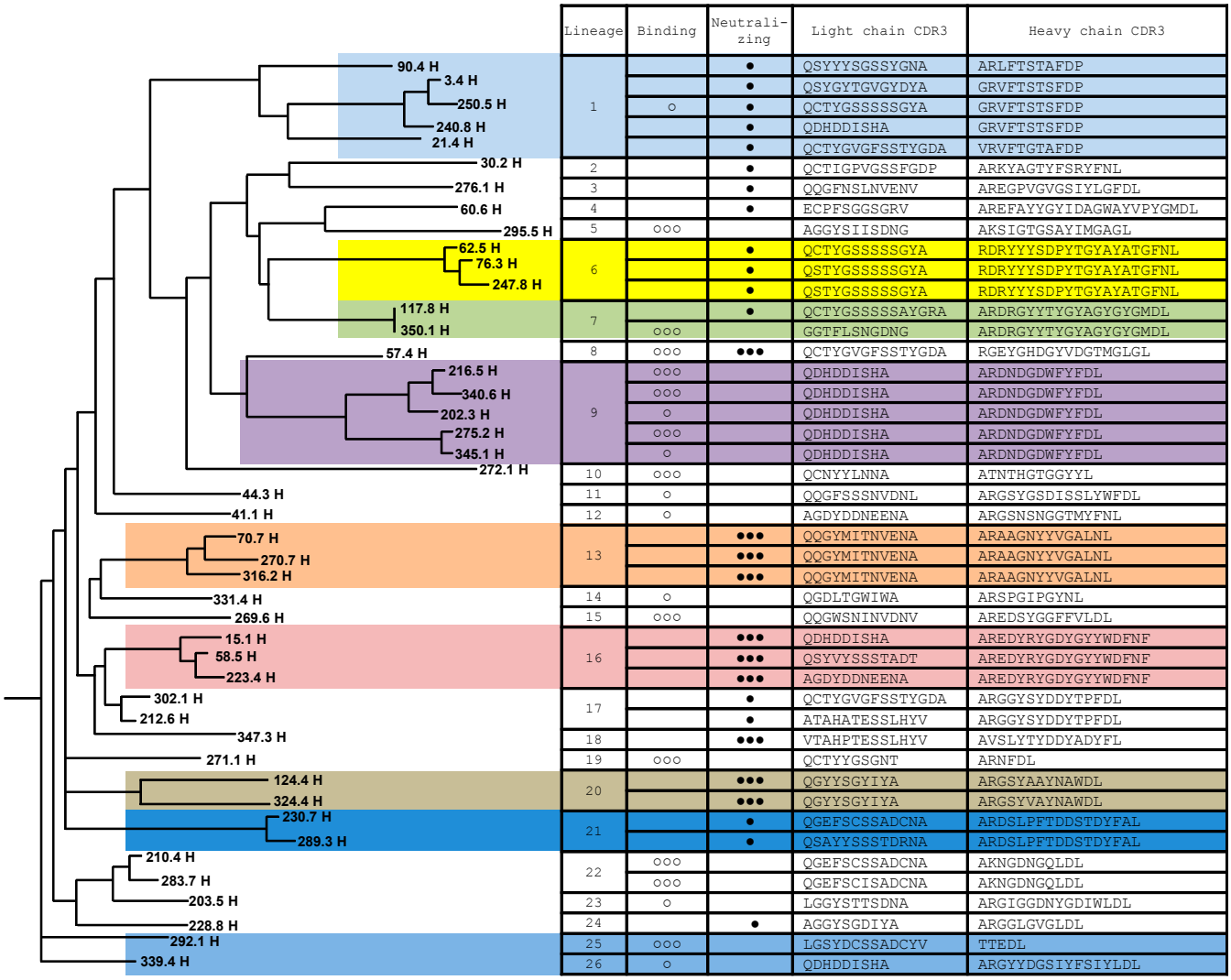


Fig. 5. Eight of the 11 elite neutralizing mAbs recognize pentameric gH complex in ELISA. Recombinant gB antigen or pentameric gH complex antigen was immobilized at 2 μg/mL on ELISA plates and tested for reactivity with elite neutralizing mAbs and elite binding mAbs in titration (Fig. S1). Fluorescent signals to either recombinant pentameric gH complex (recombinant 5′gH, open circles) or recombinant gB (solid dots) are plotted for each mAb at a concentration of ∼1 μg/mL.

Based on the HCDR3 sequence of the clusters in Fig. 6, we hypothesize that each of the 26 groups originated from a single B-cell lineage to a distinct epitope. The neutralizing and binding mAbs were largely segregated into distinct lineage groups. Eight of the 11 elite neutralizing mAbs were clustered in three lineage groups (13, 16, 20). The elite neutralizing mAb 347.3, the only member of lineage group 18, was closely related to the elite neutralizing lineage group 16. The elite neutralizing mAb 276.10 was grouped with the weakly neutralizing mAb 30.2. Like the elite neutralizers, the weakly neutralizing mAbs also tended to cluster in common lineage groups; five mAbs in lineage group 1, three in group 6, two in group 17, and two in group 21 were all weak neutralizers. Overall, the seven lineage groups accounted for 20 of the 25 neutralizing mAbs. In contrast to the neutralizing mAbs, nonneutralizing mAbs were more dispersed throughout the lineage groups, with the exception of lineage groups 9 and 22. All five mAbs in lineage group 9 were elite or intermediate binders, and two mAbs in lineage group 22 were elite binders. Ten nonneutralizing mAbs fell in lineage groups of a single antibody. The lack of relatedness among these nonneutralizing mAbs comparing to the neutralizing mAbs suggests that the antigens recognized by these nonneutralizing mAbs are more diverse than those by neutralizing mAbs.

For a given mAb, the length of its HCDR3 or light-chain CDR3 (LCDR3) may correlate with its functional attributes, such as viral neutralization, or physical interaction, such as binding. For example, longer HCDR3 loops were found in gp140-binding mAbs from HIV-infected patients compared with



Lineage	Binding	Neutralizing	Light chain CDR3	Heavy chain CDR3
1		●	QSYYYSGSSYGNA	ARLFTSTAFDP
		●	QSYGYTGVGYDYA	GRVFTSTSFDP
	○	●	QCTYGSSSSSGYA	GRVFTSTSFDP
		●	QDHDDISHA	GRVFTSTSFDP
		●	QCTYGVGFSSTYGDA	VRVFTGTAFDP
2		●	QCTIGPVGSSFGDP	ARKYAGTYFSRYFNL
3		●	QQGFNSLNVENV	AREGPVGVGSIYLGFDL
4		●	ECPFSGGSGRV	AREFAYYGYIDAGWAYVPYGMDL
5	○○○		AGGYSIISDNG	AKSIGTGSAYIMGAGL
6		●	QCTYGSSSSSGYA	RDRYYYSDPYTGYAYATGFNL
		●	QSTYGSSSSSGYA	RDRYYYSDPYTGYAYATGFNL
		●	QSTYGSSSSSGYA	RDRYYYSDPYTGYAYATGFNL
7		●	QCTYGSSSSSAYGRA	ARDRGYYTYGYAGYGYGMDL
	○○○		GGTFLSNGDNG	ARDRGYYTYGYAGYGYGMDL
8	○○○	●●●	QCTYGVGFSSTYGDA	RGEYGHDGYVDGTMGLGL
9	○○○		QDHDDISHA	ARDNDGDWFYFDL
	○○○		QDHDDISHA	ARDNDGDWFYFDL
	○		QDHDDISHA	ARDNDGDWFYFDL
	○○○		QDHDDISHA	ARDNDGDWFYFDL
	○		QDHDDISHA	ARDNDGDWFYFDL
10	○○○		QCNYYLNNA	ATNTHGTGGYYL
11	○		QQGFSSSNVDNL	ARGSYGSDISSLYWFDL
12	○		AGDYDDNEENA	ARGSNSNGGTMYFNL
13		●●●	QQGYMITNVENA	ARAAGNYYVGALNL
		●●●	QQGYMITNVENA	ARAAGNYYVGALNL
		●●●	QQGYMITNVENA	ARAAGNYYVGALNL
14	○		QGDLTGWIWA	ARSPGIPGYNL
15	○○○		QQGWSNINVDNV	AREDSYGGFFVLDL
16		●●●	QDHDDISHA	AREDYRYGDYGYYWDFNF
		●●●	QSYVYSSSTADT	AREDYRYGDYGYYWDFNF
		●●●	AGDYDDNEENA	AREDYRYGDYGYYWDFNF
17		●	QCTYGVGFSSTYGDA	ARGGYSYDDYTPFDL
		●	ATAHATESSLHYV	ARGGYSYDDYTPFDL
18		●●●	VTAHPTESSLHYV	AVSLYTYDDYADYFL
19	○○○		QCTYYGSGNT	ARNFDL
20		●●●	QGYYSGYIYA	ARGSYAAYNAWDL
		●●●	QGYYSGYIYA	ARGSYVAYNAWDL
21		●	QGEFSCSSADCNA	ARDSLPFTDDSTDYFAL
		●	QSAYYSSSTDRNA	ARDSLPFTDDSTDYFAL
22	○○○		QGEFSCSSADCNA	AKNGDNGQLDL
	○○○		QGEFSCISADCNA	AKNGDNGQLDL
23	○		LGGYSTTSDNA	ARGIGGDNYGDIWLDL
24		●	AGGYSGDIYA	ARGGLGVGLDL
25	○○○		LGSYDCSSADCYV	TTEDL
26	○		QDHDDISHA	ARGYYDGSIYFSIYLDL

Fig. 6. Phylogenetic analysis of 45 rabbit mAbs and their lineages in correlation to their binding and neutralizing properties. Phylogenetic trees were constructed based on the entire heavy-chain variable-region amino acid sequence, and lineage groups were classified based on similarities in the heavy-chain CDR3. Lineage groups containing two or more mAbs are highlighted with different background colors. The solid dots identify neutralizing mAbs, with those of three dots indicating the elite neutralizing mAbs, whereas the open circles identify nonneutralizing mAbs, with those of three circles indicating the elite binding mAbs.

IgG antibodies derived from non–gp140-binding B-cells (58). Thus, we analyzed the relationship between antibodies' HCDR3 or LCDR3 with their functions (Fig. 7). The 11 elite neutralizers had a longer average HCDR3 than that of the elite binders (15.6 aa versus 12.2; $P = 0.024$), whereas the average lengths of their LCDR3 were about the same (11.6 aa versus 10.8; $P = 0.266$). The comparison was also conducted for all neutralizing mAbs ($n = 25$) versus those with no such activity ($n = 20$), and the average sizes of HCDR3 and LCDR3 for the neutralizing antibodies, 15.9 and 12.3 aa, respectively, were significantly longer than those antibodies with no neutralizing activity, 13.0 and 10.9 aa, respectively ($P = 0.009$ in both comparisons). This result indicates that targets important for neutralization may be preferentially recognized by progenitor B-cell receptors with long HCDR3 or LCDR3. Interestingly, the average number of somatic mutations found in the neutralizing antibodies is not significantly different from that of the nonneutralizing antibodies for either V_H or V_L (Table S2). These observations indicate that targets important for viral neutralization favored those with long HCDR3 and/or LCDR3. In the vaccination model, antibody affinity maturation by somatic mutations played a secondary role for developing such neutralizing antibodies.

Discussion

Developing an HCMV vaccine is feasible, supported by observations that adoptive transfer of HCMV-HIG in pregnant women can prevent both viral transmission to fetus and congenital HCMV disease (17, 18). These studies also indicate that induction of neutralizing antibodies is an important goal for vaccination. However, the polyclonal nature of human HIG makes it poorly suited for the identification of antigens that are important for neutralizing antibodies, especially for a complex pathogen such as HCMV. Therefore, we characterized the humoral responses to HCMV using a comprehensive panel of 45 unique mAbs from a single vaccinated host. By reconstructing the host antibody responses at clonal levels, we found that (*i*) antibodies with neutralizing activity were not those with high binding affinity to the virus; (*ii*) neutralizing antibodies had longer HCDR3 or LCDR3 than those with no neutralizing activity; and

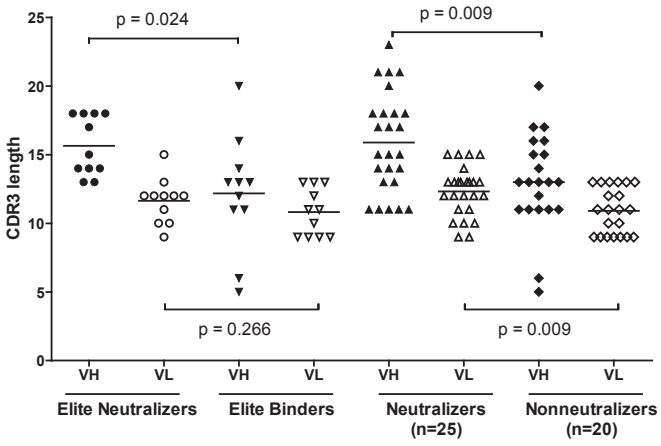


Fig. 7. Neutralizing function for an antibody is associated with long CDR3 for heavy or light chain. Heavy- and light-chain CDR3 lengths are plotted for the 11 elite neutralizing antibodies (circles), the 11 elite binding antibodies without mAb 57.4 (inverted triangles), all neutralizing antibodies (n = 25; triangles), and all nonneutralizing antibodies (n = 20; diamonds). The solid and open symbols represent heavy and light chains, respectively. Average CDR3 length is indicated by the horizontal line. Unpaired two-tailed t test was performed for statistical comparisons of indicated groups with P values marked.

(*iii*) a majority of the elite members among neutralizing mAbs recognized the pentameric gH complex.

The antibodies described here may be different from those isolated from humans (54), as the latter could have been continuously shaped by recurrent infection in the donors. Because HCMV is not expected to replicate in rabbits (53), the antibodies induced by vaccination were likely determined by the immune hierarchy of antigens as presented in their native forms on virions. Thus, screening for virus neutralization during clonal isolation was crucial, because the antigens ranked high in the immune hierarchy may not be important for viral neutralization. Indeed, the functions of binding to virions and viral neutralization were largely segregated, such that the elite binders had poor neutralizing activity and the elite neutralizers only showed average binding affinity. In addition, the neutralizing and nonneutralizing mAbs were largely segregated into distinct groups by homology analysis of their HCDR3. Genetically, neutralizing antibodies had significantly longer CDR3 for both heavy and light chains than those of nonneutralizing antibodies, whereas the numbers of somatic mutations were comparable for both groups. Numerically, antibodies with long HCDR3 or LCDR3 are relatively rare in B-cell repertoire and have been reported to have unique functions such as those antibodies with broad neutralizing capacities to HIV-1 or autoimmune potentials (59). Our results indicate that neutralization of HCMV was associated with selection of those antibodies of long HCDR3 and/or LCDR3, primarily determined by VDJ or VJ recombination and N/P addition (60). Different from the situation of HIV-1 infection where persistent infection may lead to increased somatic mutations (61), neutralizing functions of antibodies induced by the defined vaccination regimen in our study were minimally influenced by somatic mutations. One notable exception is mAb 57.4, which had a high mutation rate of 31% for its V_H, and its unique quality as the only antibody qualified both as elite neutralizer and elite binder was likely a combination of long CDR3 and efficient affinity maturation responding to vaccination. Furthermore, phylogenetic analysis indicated that antibodies with neutralizing activity were highly related. These results indicate that neutralizing antibodies were a minor population in the B-cell repertoire and they were likely targeting immunorecessive epitopes, or rare antigens, apparently more important in antiviral immunity than those immunodominant antigens such as gB.

The antiviral functions of an antibody typically occur through binding to its cognate antigen. It is intuitive that an antibody with higher binding affinity would have stronger neutralizing activity. Thus, the segregation of elite binders and elite neutralizers, with the exception of mAb 57.4, was unexpected. There are at least three possible explanations. First, it is possible that all of the described neutralizing mAbs intrinsically have lower affinity for their antigens. However, our results do not support this possibility, as seven elite neutralizers demonstrated high affinity for the recombinant pentameric gH complex, with binding signals plateauing at an IgG concentration as low as ∼100 ng/mL (Fig. S2). A second, more likely explanation is that the antigens for neutralizing mAbs are presented on the vaccine virus at lower density than the well-known immunodominant antigens such as gB. The weak binding signals of elite neutralizers to virions could be explained with the possibility that the pentameric gH complex was less abundant than gB on viral particles, thus in suboptimal concentrations as antigen, affecting the detection signals in ELISA. Proteomics analysis of the vaccine virions has shown similar compositions for major viral glycoproteins, including gM, gB, and gH/gL/gO complex (Fig. S1), similar to previously described for AD169 (62), and the pentameric gH complex is estimated about 1% of total virus mass. Last, it is a possibility, although difficult to control experimentally, that the antigens or epitopes recognized by the neutralizing antibodies were more susceptible to denaturation than those by the binding antibodies, and those antigens were compromised more extensively when the vaccine virions were immobilized on plates, hence affecting the detection signals of the neutralizing antibodies in ELISA.

With the results of our ELISA experiments, we can tentatively assign the targets for elite neutralizers. Two top neutralizing mAbs, 57.4 and 276.10, recognized epitopes unique to the pentameric gH complex. The remaining nine elite neutralizers showed stronger binding to the vaccine virus than to parental AD169 virus; these mAbs likely targeted epitopes involving the gH/gL, present in the pentameric gH complex as well as in the gH/gL/gO complex. Although the pentameric gH complex is unique to the vaccine virus, the gH/gL/gO or gH/gL complex is present in both AD169 virus and the vaccine (63, 64). Consistent with this possibility, six of these nine elite neutralizers reacted to recombinant pentameric gH complex; three remaining elite neutralizers, mAbs 347.3, 15.1, and 223.4, demonstrated binding preference to the vaccine virus over AD169 and they could recognize the epitope(s) of the pentameric gH complex but their epitope(s) may not be well presented on the recombinant form of the complex. Finally, most elite binders displayed no preference for the vaccine over AD169 virus. Thus, even though their precise epitopes are yet to be defined, the elite neutralizers had specificity to the pentameric gH complex. Taken together, the results support the hypothesis that vaccines such as AD169 have an antigenic deficiency of missing the pentameric gH complex and are thus unable to induce antibodies that efficiently block viral epithelial entry (53).

The pentameric gH complex is an attractive target in vaccine design in part because it is essential for viral infection of cell types directly linked to viral pathogenesis, including endothelial/epithelial cells and leukocytes (45). Antibodies targeting the pentameric gH complex could be more effective in preventing infection than antibodies to other antigens, as they act as sentinel at the portal entry for primary infection by blocking HCMV transmission at the mucosal contact surface (37, 65). Also, HCMV is disseminated via leukocytes within host and transmitted from leukocytes to vascular endothelial cells; antibodies against the pentameric gH complex can effectively block viral transmission from leukocytes to endothelial cells in culture (14, 66). In addition, the HCMV pathogenicity has been linked to its ability to infect endothelial cells (32, 33, 67), and infected vas-

cular endothelial cells have been postulated as the site for persistent infections (68). More importantly, histopathology studies of clinical tissue specimens have identified HCMV in organ-specific epithelial cells, such as retinal pigment epithelial cells and pulmonary alveolar epithelial cells (69, 70), and viral lesions in the epithelial cells have been implicated as the pathologic basis for HCMV retinitis and pneumonitis. These observations suggest that antibodies targeting the pentameric gH complex are not only important for prevention of viral transmission and dissemination, but also likely play a role in limiting viral infection of the cells central to HCMV persistent infection and end-organ disease (45).

Polyvalent antibodies to HCMV, such as HCMV-HIG, showed promise in two small trials for protection against congenital HCMV infection and disease (17, 18). However, infusion of pregnant women with 200 mg/kg HCMV-HIG was only ~60% effective against congenital infection (17). HCMV-HIG in transplantation settings is marginally beneficial (71). Previous HCMV vaccines, including Towne and recombinant gB vaccines, are not designed to induce antibodies to the pentameric gH complex, and they are limited in their efficacy against HCMV infection (46–48). The results of this study demonstrated that the pentameric gH complex is the primary target for functional antibodies by vaccination. Because the viral genes encoding the pentameric gH complex are conserved among clinical isolates (72), this complex is an ideal vaccination target. Our study demonstrates that an experimental HCMV vaccine with this complex can elicit neutralizing antibodies in rabbits, in quality and quantity, similar to those in human subjects with natural infection (51–55), and thus merits further evaluations in the clinic.

Materials and Methods

Cells, Reagents, and Viruses. Recombinant gB protein (Sino Biological) was based on the sequence of Towne strain with its furin-cleavage site mutated and the transmembrane region deleted (73). Recombinant pentameric gH complex was obtained from Redbiotec. The complex was constructed using the sequence of Towne strain with gH truncated of its cytoplasmic and transmembrane domains. The secreted complex was expressed in a baculovirus culturing system and assembled using the Redbiotec's rePAX technology. HCMV-HIG (CytoGam) was commercially manufactured and distributed by CSL. Human sera were obtained from local blood banks with donor's consent for research. All clinical samples were provided without any personal identifier. Parental AD169 virus from ATCC was propagated in MRC-5 cells (55). Vaccine virus was described previously (53).

Animals and Rabbit Hybridoma Culture. New Zealand White female rabbits of 3–4 mo of age were purchased from a specific pathogen-free colony (Covance). Animals were housed individually in a Merck animal facility, in accordance with the *Guide for the Care and Use of Laboratory Animals* (74), and the facility is credentialed by Association for Assessment and Accreditation of Laboratory Animal Care (aaalac.org). The study (APS ID 08089972060453) was approved by Merck's Institutional Animal Care and Use Committee. Rabbits were immunized with intramuscular injections of 10, 30, or 100 μg of vaccine virus in 0.5-mL saline at weeks 0, 3, and 8, and the immune sera were collected at week 11. One rabbit was boosted at week 14 i.v. with 500 μg of the vaccine virus, and the spleen was harvested 4 d later for hybridoma cultures. Rabbit hybridoma lines were generated at Epitomics (75). Approximately 500 hybridoma cultures were screened for the production of IgG, and then screened for neutralization of the vaccine virus and for binding to the vaccine virus (55). Seventy-five cultures were selected and cloned through two rounds of limiting dilution. After confirming their activities, 45 unique lines were established and expanded for mAb production.

Viral Neutralization Assay. A neutralization assay, based on the enumeration of cells expressing viral immediate-early (IE) antigen 24 h postinfection, was described previously (55). EC_{50} values, defined as IgG concentration required to block 50% viral entry, were calculated from four-parameter curve fitting using Prism 5 (GraphPad Software). A mAb would be considered non-neutralizing if the percentage of cells with IE expression as a function of antibody concentration did not follow a typical sigmoid curve, or if the fitting produced a value of $R^2 < 0.9$. Under these circumstances, an arbitrary value of 100 μg/mL was assigned as the EC_{50} neutralizing for the antibody.

ELISAs. Two types of ELISA were used. Antibody-titration ELISA was used to determine the binding affinity of each mAb to the antigen, recombinant proteins, or purified vaccine virions, with the antigen immobilized at at 2 μg/mL in PBS, on 96-well FluoroNunc MaxiSorp plates at 4 °C overnight. Plates were blocked with 3% (vol/vol) nonfat milk in PBS/0.05% Tween-20 and incubated with the mAb in a titration from 0.2 to 30 μg/mL Virion-titration ELISA was designed to quantitatively compare two or more antigens for their epitope specificity to a given antibody. In this case, for selected mAbs, we compared antibody reactivity to the parental AD169 virus versus the vaccine virus. The virions were immobilized from 0.1 to 100 μg/mL in twofold dilutions on microtiter plates at 4 °C overnight. Plates were blocked as above and then incubated with a fixed antibody concentration of 2 μg/mL. For both assays, plates were washed after antibody incubation and then HRP-conjugated goat anti-rabbit IgG was added (Southern Biotech). A fluorogenic HRP substrate, 10-acetyl-3,7-dihroxyphenoxazine (ADHP) (Virolabs) was added at 100 μL per well to generate resorufin at a concentration proportional to the HRP concentration (76, 77). Fluorescent signals with excitation at 531 nm were measured with emission at 595 nm in a plate reader (Victor III; Perkin-Elmer). EC_{50} binding values were calculated from four-parameter curve fitting using Prism 5. An antibody was considered a poor binder if the fluorescent signals as a function of antibody concentration did not follow a typical sigmoid curve, or if the fitting produced a value of $R^2 < 0.9$. Under these circumstances, an arbitrary value of 100 μg/mL was assigned as the EC_{50} binding for the antibody.

Cloning of mAb-Encoding Genes and Phylogenetic Analysis of Antibody Sequences. Coding sequences for mAbs were cloned from hybridoma cells as described previously, with minor modifications (75). Briefly, mRNA was isolated from rabbit cells using TRIzol extraction and reverse-transcribed to cDNA using SuperScript II kits (Invitrogen). Variable (V_H and V_L) regions were amplified by PCR using L chain and H chain primers (78). PCR products were gel purified using nucleospin gel extraction kits (Macherey-Nagel), ligated into pCR2.1 TA-clone vectors (Invitrogen), and plated onto S-Gal Amp^R plates for the selection of white colonies. The plasmids were independently extracted from multiple colonies using miniprep kits, and each clone was sequenced from both directions using M13R and M13F sequencing primers. Final sequences were confirmed by at least three identical sequencing results. A phylogenetic tree was built by alignment of entire V_H or V_L amino acid sequences of 45 rabbit mAbs. Sequence alignment and phylogenetic analysis were performed using the ClustalX software (79). Lineage groups were assigned based on HCDR3 sequence homology.

Statistical Analysis. Unpaired two-tailed *t* tests were performed where indicated, using Prism 5 software developed by GraphPad Software.

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