HCMV glycoprotein B subunit vaccine efficacy mediated by nonneutralizing antibody effector functions

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Human cytomegalovirus (HCMV) is the most common congenital infection worldwide, frequently causing hearing loss and brain damage in afflicted infants. A vaccine to prevent maternal acquisition of HCMV during pregnancy is necessary to reduce the incidence of infant disease. The glycoprotein B (gB) + MF59 adjuvant subunit vaccine platform is the most successful HCMV vaccine tested to date, demonstrating ∼50% efficacy in preventing HCMV acquisition in multiple phase 2 trials. However, the mechanism of vaccine protection remains unknown. Plasma from 33 postpartum women gB/MF59 vaccinees at peak immunogenicity was tested for gB epitope specificity as well as neutralizing and nonneutralizing anti-HCMV effector functions and compared with an HCMV-seropositive cohort. gB/MF59 vaccination elicited IgG responses with gB-binding magnitude and avidity comparable to natural infection. Additionally, IgG subclass distribution was similar with predominant IgG1 and IgG3 responses induced by gB vaccination and HCMV infection. However, vaccine-elicited antibodies exhibited limited neutralization of the autologous virus, negligible neutralization of multiple heterologous strains, and limited binding responses against gB structural motifs targeted by neutralizing antibodies including AD-1, AD-2, and domain I. Vaccinees had high-magnitude IgG responses against AD-3 linear epitopes, demonstrating immunodominance against this nonneutralizing, cytosolic region. Finally, vaccine-elicited IgG robustly bound membrane-associated gB on the surface of transfected or HCMV-infected cells and mediated virion phagocytosis, although were poor mediators of NK cell activation. Altogether, these data suggest that nonneutralizing antibody functions, including virion phagocytosis, likely played a role in the observed 50% vaccine-mediated protection against HCMV acquisition.

cytomegalovirus | vaccines | glycoprotein B | pediatrics

Human cytomegalovirus (HCMV) affects 1 out of every 150 live-born infants worldwide (1). In the United States alone, this equates to 40,000 children infected annually, of whom 8,000 develop long-term disabilities including microcephaly, intrauterine growth restriction, hearing/vision loss, or neurodevelopmental delay (2, 3)—more congenital disease than all 29 newborn conditions currently screened for in the United States combined (4). It is clear that preexisting maternal immunity affects the incidence of congenital infection because 30–40% of HCMV-seronegative women that acquire the virus during pregnancy transmit the infection to the fetus in utero in contrast to 1–2% following superinfection of HCMV-seroimmune women (2). Therefore, it is hypothesized that a maternal vaccine that prevents maternal HCMV acquisition, protects against viral transmission to the infant, or reduces the severity of congenital infection is an achievable goal (5).

A variety of HCMV vaccine candidates have been tested, including live-attenuated virus, glycoprotein subunit formulations, and single/bivalent DNA plasmids (reviewed in ref. 6). The HCMV glycoprotein B (gB) subunit vaccine administered with MF59 squalene adjuvant demonstrated moderate (∼50%) efficacy in preventing primary HCMV infection in cohorts of both postpartum (7) and adolescent women (8). Furthermore, this vaccine demonstrated a protective benefit against HCMV viremia and reduced clinical need for antiviral treatment in transplant recipients (9). As the primary viral fusion protein, HCMV gB is essential for entry into all cell types and is a known target of neutralizing antibodies (10, 11). However, previous investigations have reported that gB/MF59-elicited antibodies were poorly neutralizing (12–14), which raises questions about the mechanism underlying the partial gB vaccine efficacy observed in multiple clinical trials. An understanding of the gB/MF59-mediated protection is needed to rationally design immunogens that will improve upon the partial vaccine efficacy that was achieved clinically.

Glycoprotein B is a 907-amino acid, homotrimeric glycoprotein consisting of four distinct structural regions: an ectodomain, a membrane-proximal region (MPER), a transmembrane domain, and a cytoplasmic domain (*SI Appendix*, Fig. S1) (11, 15). Additionally, there are five distinct antigenic sites known to be targeted by gB-specific antibodies, identified as antigenic domains (AD) 1–5 (*SI Appendix*, Fig. S1) (10, 11, 15). Antibodies

Significance

The CDC estimates that every hour, a child is born in the United States with permanent neurologic disability resulting from human cytomegalovirus (HCMV) infection—more than is caused by Down syndrome, fetal alcohol syndrome, and neural tube defects combined. A maternal vaccine to block transmission of HCMV to the developing fetus is a necessary intervention to prevent these adverse outcomes. The gB/MF59 vaccine is the most successful tested clinically to date, achieving 50% reduction in HCMV acquisition. This manuscript establishes the function and epitope specificity of the humoral response stimulated by this vaccine that may explain the partial vaccine efficacy. Understanding the mechanism of gB/MF59-elicited protective immune responses will guide rational design and evaluation of the next generation of HCMV vaccines.

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See Commentary on page 6110.

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against AD-1, an uninterrupted ~80-amino acid epitope, are present in virtually all infected individuals (10) and can be either neutralizing or nonneutralizing (16). In contrast, AD-2–specific antibodies are present in only a subset of seropositive people (10), and this region consists of two unique linear epitopes: site 1 is perfectly conserved in all viral strains and a target of potently neutralizing antibodies, whereas site 2 is highly variable and targeted by only nonneutralizing antibodies (17). AD-3 is located within the cytosolic domain and is a known target of exclusively nonneutralizing antibodies (18, 19). Finally, AD-4 (domain II) and AD-5 (domain I) are conformational, globular protein domains that were recently identified and characterized as the targets of neutralizing antibodies (10). The antigen used for the gB/MF59 vaccine clinical trials (7–9) consisted of the full protein with two modifications to facilitate manufacture: (*i*) deletion of the transmembrane domain (75 amino acids) and (*ii*) mutation of the furin protease cleavage site (20).

To identify possible mechanisms accounting for the partial protection observed in clinical trials, we undertook an in-depth investigation into the characteristics and functionality of antibody responses elicited by gB/MF59 vaccination. Here we report on our observations that gB/MF59 vaccination resulted in an antibody profile quite distinct from that observed in the setting of natural HCMV infection. Furthermore, our data indicate that nonneutralizing antibody functions likely played a role in the observed 50% vaccine efficacy against HCMV acquisition.

Results

HCMV Neutralization and IgG Binding to gB-Neutralizing Domains. We obtained plasma samples from 33 gB/MF59 vaccinee participants in the phase 2 clinical trial conducted in a population of seronegative postpartum women (7). All subsequent studies used samples collected at peak immunogenicity (6.5 mo) or the next available time point (not exceeding 12 mo). gB/MF59 vaccination has been previously reported to elicit robust titers of gB immunogen-specific IgG (20, 21). We observed similar results for this subset of vaccinees, with high-magnitude plasma gB IgG binding exceeding that elicited in chronically infected, seropositive (SP) individuals (*SI Appendix*, Fig. S2*A*) ($\log_{10}$AUC: gB/MF59 = 6.32, SP = 5.64; $P = 0.03$, Wilcoxon rank sum test).

We first investigated the ability of vaccine-elicited antibodies to neutralize a panel of HCMV strains, including the autologous Towne strain (Fig. 1*A*), AD169 (Fig. 1*B*), and TB40/E [in fibroblasts (Fig. 1*C*) and epithelial cells (Fig. 1*D*)]. Assays were conducted in both the presence and absence of rabbit complement to assess the possibility of enhanced neutralization titers following complement fixation. A low level of vaccine-elicited neutralization was observed against the autologous Towne virus, although significantly reduced compared with the seropositive group (Towne median $\log_{10}ID_{50}$: gB/MF59 = 1.70, SP = 2.96; $P < 0.001$, pooled t test). Very few vaccinee samples had detectable neutralization against heterologous viruses, although neutralization of these strains was robust in the seropositive group (TB40/E epithelial cell median $\log_{10}ID_{50}$: gB/MF59 < 1; SP = 3.80; $P < 0.001$, Fisher's exact test). We observed minimal enhancement of vaccine-mediated neutralization activity in the presence of complement (Towne median vaccinee $\log_{10}ID_{50}$: no comp = 1.70; comp = 1.93), not as robust as previous reports (22). Additionally, we used a GFP reporter-based neutralization assay to confirm the lack of plasma neutralization observed. The GFP-based assay detected higher-level plasma neutralization in seropositive individuals than the IE-1 immunofluorescence-based assay (*SI Appendix*, Fig. S3) consistent with previous reports (12), yet still detected only low-level, infrequent neutralization in vaccinee plasma. Thus, the assay methodology did not explain the lack of neutralization observed against heterologous virus strains in gB/MF59 vaccinees.

Subsequently, we examined whether vaccine-elicited antibodies bound to previously identified gB-neutralizing epitopes (*SI Appendix*, Fig. S1) (10, 11), including AD-1 (Fig. 1*F*), AD-2 site 1 (Fig. 1*G*), domain I (AD-5) (Fig. 1*H*), domain II (AD-4) (Fig. 1*I*), and domain I + II combined (Fig. 1*J*). All gB antigens utilized for this study are described in *SI Appendix*, Table S1, with quality control data presented in *SI Appendix*, Fig. S4. Although vaccination elicited robust gB-binding responses, there was very poor targeting of these gB neutralizing epitopes. Compared with chronically HCMV-infected, seropositive women, there was significantly reduced vaccine-elicited binding against AD-1 (median $\log_{10}$MFI: gB/MF59 = 1.75, SP = 2.46; $P = 0.001$, pooled t test), AD-2 site 1 (median $\log_{10}$MFI: gB/MF59 = 0.65, SP = 1.70; $P = 0.002$, Satterthwaite t test), domain I (median $\log_{10}$MFI: gB/MF59 = 1.70, SP = 3.20; $P < 0.001$, pooled t test), and a domain I + II fused construct (median $\log_{10}$MFI: gB/MF59 = 2.60, SP = 3.14; $P < 0.001$, pooled t test).

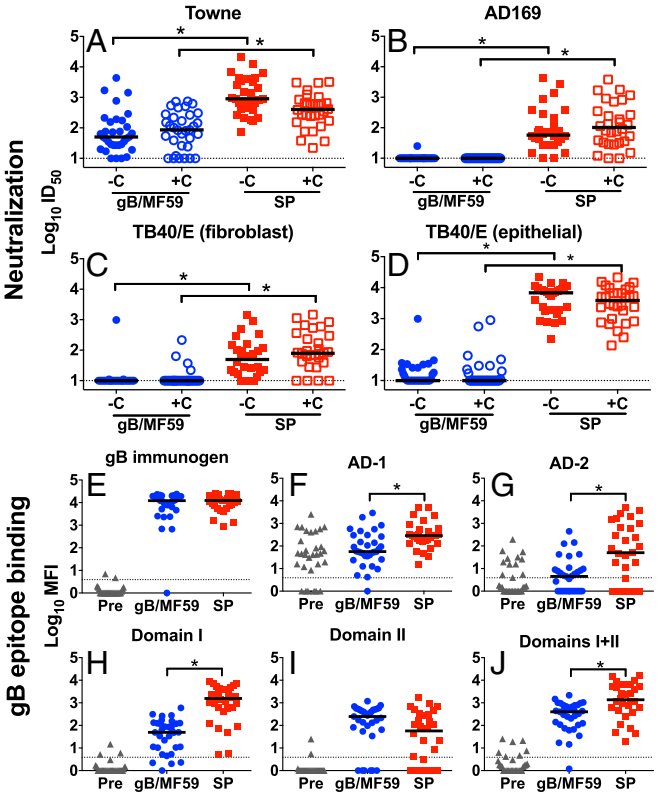

Fig. 1. Limited vaccine-elicited neutralization responses and poor gB neutralizing epitope binding following gB/MF59 immunization. (*A–D*) Neutralizing antibody responses and (*E–J*) gB neutralizing epitope binding were assessed for 33 gB/MF59 vaccinees (blue circles) and 30 seropositive, chronically HCMV-infected individuals (red squares). Neutralization was measured by IE-1 staining against Towne strain HCMV (*A*) and AD169 strain HCMV (*B*) in fibroblasts and TB40/E strain HCMV in both fibroblasts (*C*) and epithelial cells (*D*). Assays were conducted in both the presence (+C; open symbols) and absence (–C; solid symbols) of purified rabbit complement. Binding responses against (*E*) the gB immunogen and known gB neutralizing epitopes (*F*) AD-1, (*G*) AD-2, (*H*) domain I, (*I*) domain II, and (*J*) domain I + II combined were measured. Each data point represents the mean of two experimental replicates. Horizontal dotted lines for neutralization assays indicate the starting dilution, whereas dotted lines for neutralizing epitope binding indicate the threshold for positivity (preimmune control mean + 2 SDs). Black horizontal bars indicate the median values for each group. *$P < 0.05$, Fisher's exact test (neutralization), pooled t test (epitope binding).

Comparison of Neutralization Activity of Postpartum and Phase 1 gB/MF59 Vaccinee Plasma. Because our observed lack of heterologous virus neutralization and modest complement enhancement in gB/MF59 vaccinees was inconsistent with previous reports of phase 1 gB/MF59 vaccine immunogenicity (22), we obtained phase 1 gB/MF59 vaccinee sera (14) and complement-dependent neutralizing monoclonal antibodies (210.4, 272.7, and 350.1) reported by other investigators (22). We first noted that the level of gB binding was

comparable between the phase 1 and the postpartum phase 2 cohorts (*SI Appendix*, Fig. S5). In our neutralization assay, gB mAbs 210.4, 272.7, and 350.1 had neutralization enhanced in a complement-dependent manner in both fibroblasts (Fig. 2*A*) and epithelial cells (Fig. 2*B*). Our neutralization assays also confirmed previous findings of low-level, gB/MF59-induced heterologous virus neutralization in plasma of healthy adult phase 1 vaccinees that is robustly boosted with the addition of complement. Even without complement, phase 1 gB/MF59 sera had increased neutralization activity compared with postpartum vaccinees (median fibroblast $\log_{10}ID_{50}$, without complement: phase 1 = 1.32, postpartum <1; $P < 0.001$, Fisher's exact test). In the presence of complement, the median plasma neutralization titer of phase 1 gB/MF59 vaccinees was boosted more than 1 log in magnitude (median fibroblast phase 1 $\log_{10}ID_{50}$: −C = 1.32, +C = 2.57; $P = 0.018$, Wilcoxon signed rank test). However, no complement enhancement of plasma neutralization was observed for postpartum gB/MF59 vaccinees (Fig. 2).

Linear gB Epitope Binding. To map the epitopes targeted by vaccine-induced antibodies a peptide microarray library was created, consisting of 15-mers overlapping each subsequent peptide by 10 residues and spanning the entire gB ORF (Towne strain). We observed that vaccine-elicited linear epitope binding was quite distinct from that observed in the setting of chronic HCMV infection (Fig. 3 and *SI Appendix*, Fig. S6). Most notably, there was a negligible AD-2 site 1 response elicited by vaccination, which is known to be a target of potent gB-specific neutralizing antibodies (23) (AD-2 site 1 median $\log_{10}$MFI: gB/MF59 = 2.68, SP = 3.69; $P < 0.001$, Satterthwaite *t* test). Furthermore, vaccination resulted in dominant IgG response against the nonneutralizing AD-3 epitope located in the gB protein cytodomain (AD-3 median $\log_{10}$MFI: gB/MF59 = 5.23, SP = 4.14; $P < 0.001$, pooled *t* test), composing 76% of the linear gB IgG response in vaccinees compared with 32% in chronically HCMV-infected individuals (*SI Appendix*, Fig. S6 *A*, *B*, and *F*)

IgG Subclass and Binding to Membrane-Associated gB. Given the poor neutralizing antibody responses observed, we sought to investigate whether vaccine-elicited IgG responses had properties suggestive of the ability to mediate nonneutralizing antibody effector functions. First, we examined the IgG subclass of gB-directed responses and identified that both vaccinees and chronically HCMV-infected individuals had a similar response profile dominated by IgG1 and IgG3, with very little detectable IgG2 or IgG4 subclasses (Fig. 4 *A*–*D*). Furthermore, we examined whether vaccine-elicited antibodies could bind to membrane-associated gB expressed on the surface of both gB-transfected (Fig. 4 *E* and *F*) and TB40/E-infected cells (Fig. 4 *H* and *I*). Vaccine-elicited IgG bound to both transfected autologous Towne strain gB (Fig. 4*E*) and a heterologous strain gB (Fig. 4*F*) (most

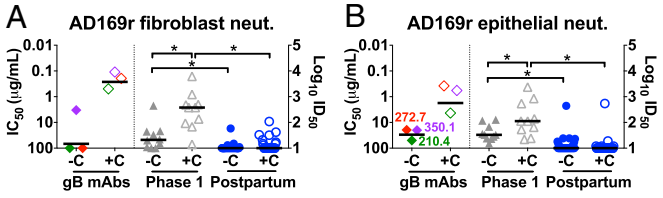

Fig. 2. Phase 1 gB/MF59 vaccinees exhibit enhanced plasma HCMV neutralization potency compared with postpartum gB/MF59 vaccinees. Neutralization for 10 phase 1 plasma samples was assessed by GFP-reporter expression against repaired AD169 strain HCMV in (*A*) fibroblasts and (*B*) epithelial cells. Assays were conducted in both the presence (+C; solid symbols) and absence (−C; open symbols) of purified rabbit complement. Controls are complement-dependent neutralizing mAbs 210.4 (green), 272.7 (red), and 350.1 (purple). *$P < 0.05$, Fisher's exact test (phase 1 vs. postpartum), Wilcoxon signed rank test (−C vs. +C).

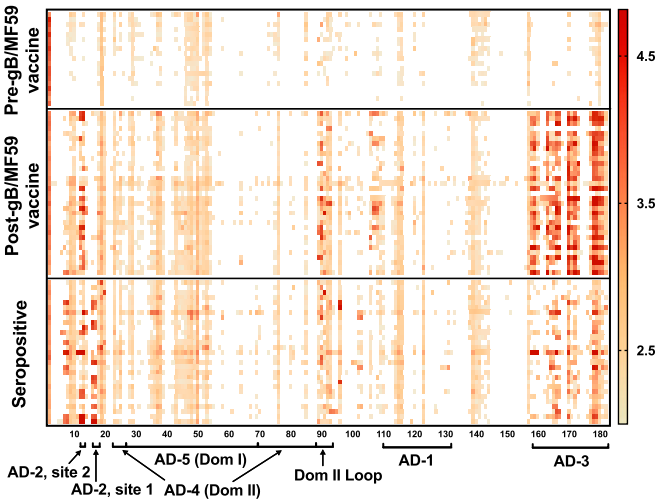

Fig. 3. Dominant linear epitope-binding response against cytosolic antigenic domain 3 following gB/MF59 immunization. The binding magnitude of antibody responses of Cytogam, 19 gB/MF59 vaccinees preimmunization, 32 gB/MF59 vaccinees postimmunization, and 30 chronically infected seropositive controls were assessed against a 15-mer peptide library spanning the entire Towne gB ORF (180 unique peptides). Each row indicates a single patient. Assay was completed in triplicate, and the binding magnitude is indicated as the log-scaled, median fluorescent intensity. White indicates median fluorescent intensity <100. Peptides corresponding to distinct gB antigenic domains are indicated along *x* axis.

frequently detected strain in infected vaccinees) more robustly than antibodies elicited by chronic HCMV infection (heterologous median % IgG binding: gB/MF59 = 13.2%, SP = 5.9%; $P < 0.001$, Satterthwaite *t* test). However, vaccine-elicited IgG bound TB40/E-infected cells less well than antibodies elicited by chronic infection (Fig. 4*H*) (median % infected cell binding: gB/MF59 = 6.7%, SP = 36.7%; $P < 0.001$, Satterthwaite *t* test), likely due to antibody binding to other glycoprotein epitopes in the seropositive group. Thus, we purified gB-specific IgG from both gB/MF59 vaccine and SP plasma and assessed the magnitude of the infected cell-associated gB binding. The purified gB-specific IgG revealed higher-magnitude infected cell-associated gB binding in the vaccinee group (Fig. 4*I*) (median % infected cell gB binding: gB/MF59 = 4.4%, SP = 1.7%; $P = 0.01$, Satterthwaite *t* test), consistent with the gB-transfected cell IgG-binding magnitude. Finally, we examined NK cell degranulation in the presence of plasma antibodies from gB vaccinees because this process is a prerequisite of both antibody-dependent cellular cytotoxicity (ADCC) and cytokine release by activated NK cells. Despite the vaccine eliciting robust gB transfected and infected cell IgG binding, minimal NK cell degranulation responses were detected in vaccinees using both gB-transfected (Fig. 4*G*) and TB40/E-infected target cells (Fig. 4*J*). In contrast, the majority of chronically HCMV-infected individuals had antibodies that mediated measurable, although low-magnitude, NK degranulation (TB40/E-infected targets, % CD107a+ NK cells: gB/MF59 = 4.9%, SP = 6.6%; $P < 0.001$, Wilcoxon rank sum test).

Antibody-Mediated Phagocytosis and Monocyte Infection. We next investigated the ability of vaccine-elicited antibodies to mediate phagocytosis. We developed highly specific, flow-based assays for measuring the phagocytosis of both gB-conjugated beads (*SI Appendix*, Fig. S7 *A* and *B*) and fluorescently conjugated HCMV virions (*SI Appendix*, Fig. S7 *C* and *D*) using a THP-1 monocyte cell line. Cellular uptake of HCMV was confirmed by confocal microscopy, demonstrating fluorescent virus either at the cell surface (*SI Appendix*, Fig. S7*E*) or internalized (*SI Appendix*, Fig. S7*F*). The small number of distinct viral foci observed following phagocytosis differs from the multiple, dispersed foci following

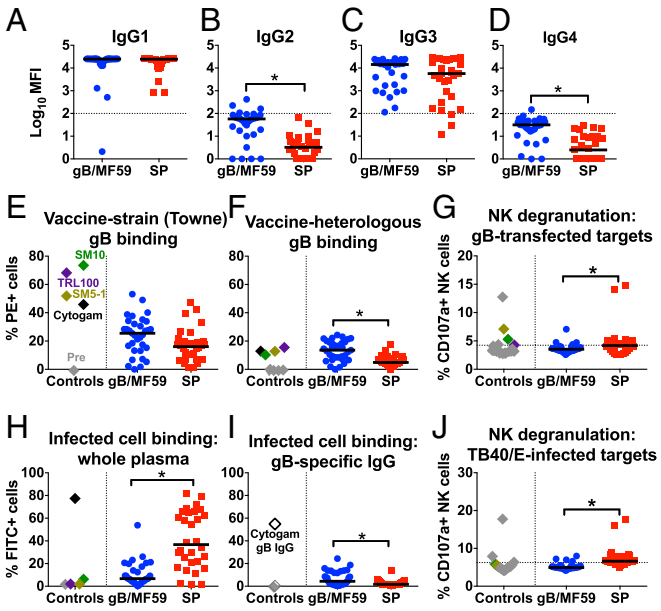

Fig. 4. gB/MF59 vaccination elicited high-magnitude IgG3 responses and robust membrane-associated gB IgG binding. The magnitude of gB-specific (*A*) IgG1, (*B*) IgG2, (*C*) IgG3, and (*D*) IgG4 subclass responses was assessed for 33 gB/MF59 vaccinees (blue circles) and 30 seropositive, chronically HCMV-infected individuals (red squares). Furthermore, the ability of plasma antibodies to bind to gB-transfected cells was assessed, including (*E*) autologous (Towne) and (*F*) heterologous gB. Likewise, binding to TB40/E-infected cells was quantified using both (*H*) whole plasma and (*I*) purified gB-specific IgG. Last, the ability of plasma antibodies to activate NK cells in the presence of either (*G*) gB mRNA-transfected ARPE target cells or (*J*) TB40/E-infected ARPE target cells was assessed by the percentage of NK cells expressing CD107a. Black horizontal bars indicate the median values for each group. Horizontal dotted lines in subclass plots (*A*–*D*) indicate the threshold for positivity, defined here as 100 MFI. The dotted line in the NK cell degranulation plots (*G* and *J*) represents the threshold for positivity (mean of preimmune samples +2 SDs). Each data point represents the mean value of two experimental replicates. For *E*–*J*, control values include Cytogam (black), AD-2 mAb TRL345 (purple), Dom I mAb SM10 (green), and Dom II mAb SM5-1 (brown). **P* < 0.05, Satterthwaite *t* test.

monocyte infection (*SI Appendix*, Fig. S7*H*). Additionally, we confirmed that phagocytosis did not lead to productive infection, because cells that phagocytosed TB40/E-mCherry virus did not exhibit mCherry expression at 48 h postphagocytosis (*SI Appendix*, Fig. S8). Robust vaccine-elicited phagocytosis of gB immunogen-coupled beads was observed, exceeding that in SP individuals (median % phagocytosing cells: gB/MF59 = 52.1, SP = 29.1; $P = 0.04$, pooled *t* test) (Fig. 5*A*). To determine whether the dominant AD-3 antibody response (Fig. 3) contributed to this vaccine-elicited gB phagocytosis observed, we investigated phagocytosis of gB ectodomain-coupled beads (Fig. 5*B*). There was a reduced magnitude of phagocytosis activity directed against the gB ectodomain in comparison with the full gB protein. Furthermore, there was no observable difference in gB ectodomain-directed phagocytosis activity between vaccinees and SP individuals (median % phagocytosing cells: gB/MF59 = 24.8, SP = 18.6; *P* = ns, pooled *t* test), suggesting that a proportion of the phagocytosis-mediating antibodies measured in vaccinees target cytodomain epitopes likely not exposed on the surface of an HCMV virion or infected cell. Additionally, we examined phagocytosis of whole HCMV virions and noted more robust virion phagocytosis mediated by plasma antibodies from chronically HCMV-infected individuals compared with vaccinees (Fig. 5*C*) (median % phagocytosing cells: gB/MF59 = 9.6%, SP = 17.6%; $P < 0.001$, pooled *t* test). To determine if antibodies targeting other glycoprotein epitopes in the SP group contribute to this difference, we evaluated virion phagocytosis mediated by purified gB-specific IgG from vaccinees and HCMV-infected individuals and observed similar levels of gB-specific phagocytosis (Fig. 5*D*) (median % positive cells: V = 11.3%, SP = 10.1%; *P* = ns, pooled *t* test). Additionally, we confirmed that although gB vaccine-elicited antibodies could mediate robust virion phagocytosis, they minimally blocked infection of THP-1 monocytes (Fig. 5 *E* and *F*) (normalized % neutralization at 1:100 dilution: gB/MF59 = 18.4%, SP = 82.8%; $P < 0.001$, Satterthwaite *t* test). Finally, we examined whether vaccine-elicited plasma antibodies could mediate whole virion phagocytosis in monocyte-derived macrophages. However, we observed a high level of nonspecific virus uptake by macrophages regardless of the addition of HCMV-specific antibodies (*SI Appendix*, Fig. S9).

Infected/Uninfected Vaccinee Immune Response Comparison and Antibody Response Correlation Matrix. Although this study was underpowered to detect a difference in immune responses between vaccinees who became infected during the course of the trial ($n = 11$) and those who did not ($n = 22$), we assessed the comparative magnitude of responses between these groups by uncorrected Mann–Whitney U or Fisher's exact tests (*SI Appendix*, Table S2). Although a majority of responses were nominally higher in protected vs. unprotected vaccinees, the only vaccine-elicited response that was potentially distinct between infected and uninfected vaccinees (raw $P < 0.05$) was IgG binding to AD-2, site 2 in the linear peptide microarray ($P = 0.01$, exact Wilcoxon rank sum test), which is a nonneutralizing epitope (11).

Last, we sought to investigate the relationship between measured antibody responses by creating a correlation matrix (*SI Appendix*, Fig. S10). We observed two distinct clusters of responses that appeared related to one another. The first cluster is composed of gB-specific phagocytosis activity (of both gB protein and whole HCMV virions), binding responses against free gB protein and membrane-associated gB, and gB-specific IgG1

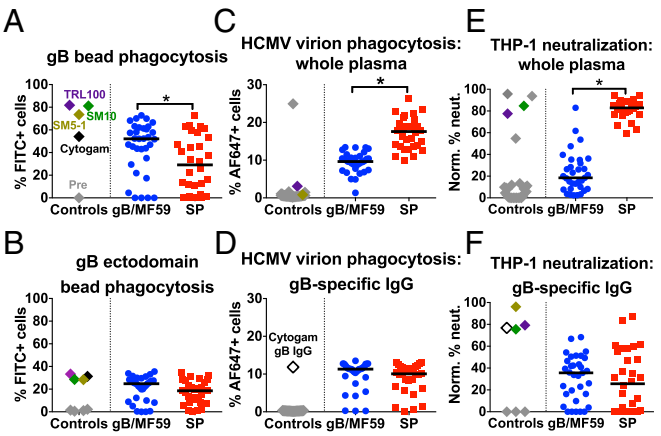

Fig. 5. gB vaccine elicits antibodies that mediate robust HCMV virion phagocytosis, although do not block monocyte infection. Flow-based assays were used to test the phagocytosis-mediating ability of plasma IgG from 33 gB/MF59 vaccinees (blue circles) and 30 chronically HCMV-infected individuals (red squares) of (*A*) gB immunogen-coupled fluorescent beads, (*B*) gB ectodomain-coupled fluorescent beads, and (*C* and *D*) fluorophore-conjugated whole HCMV virions. Last, the ability of vaccine-elicited antibodies to block infection of THP-1 cells was assessed at a single dilution (1:100), using both (*E*) whole plasma and (*F*) purified gB-specific IgG. Black horizontal bars indicate the median values for each group. The dotted line in the whole virion phagocytosis plots represents the threshold for positivity (mean of preimmune samples +2 SDs), whereas the dotted line for the TB40/E infection of THP-1 monocyte plots is the threshold for true neutralization activity (50%). Each data point represents the mean value of two experimental replicates. Control antibody values include Cytogam (black), AD-2 mAb TRL345 (purple), Dom I mAb SM10 (green), and Dom II mAb SM5-1 (brown). **P* < 0.05, pooled *t* test.

and IgG3 subclass responses. Neutralization activity, by contrast, was associated with a distinct alternate cluster, with robust correlations observed between the different viral strains and cell lines tested. NK cell activation was inversely correlated with several parameters in cluster 1 (phagocytosis, gB binding). Finally, some epitope-specific responses correlated with antibody function, including gB domain II-specific IgG responses with phagocytosis activity and AD-2 and domain I-specific IgG responses with neutralization activity. To evaluate whether these clustered correlations were driven by vaccinated vs. seropositive individuals, we performed an identical cluster analysis for vaccinee (*SI Appendix*, Fig. S11*A*) and seropositive antibody responses (*SI Appendix*, Fig. S11*B*) independently. Of note, the formation of cluster 1 was primarily driven by gB vaccine-elicited immune responses, whereas cluster 2 was primarily driven by HCMV infection, likely by IgG binding and neutralization against non-gB HCMV glycoprotein epitopes.

Discussion

Over the past decade, the HCMV vaccine field has largely shifted its focus away from the elicitation of gB-specific antibody responses and toward targeting of the gH/gL/UL128/UL130/UL131A pentameric complex (PC) because this protein construct was newly identified as a primary target of potent HCMV neutralizing antibodies (24). However, it is important to recognize that the gB/MF59 vaccine platform, which did not include the PC, achieved ∼50% vaccine efficacy in preventing primary HCMV infection in two phase 2 trials (7, 8) and demonstrated a protective benefit for transplant recipients (9) without the elicitation of potent neutralizing antibody responses (9, 12–14). Increasingly, the limitations of neutralizing antibodies in controlling HCMV cell-to-cell spread (23) and the protective capacity of nonneutralizing HCMV-specific antibodies are becoming recognized (25), indicating that the role of nonneutralizing antibodies needs to be further investigated as a potentially important end point for HCMV vaccine immunogenicity trials.

Using a cohort of postpartum, phase 2 gB/MF59 vaccinees, we demonstrated negligible neutralizing responses against heterologous strains and minimal complement-mediated enhancement of neutralization activity. However, in a cohort of young, healthy phase 1 gB/MF59 vaccinees we observed a different result, confirming reports of heterologous neutralization and robust complement-mediated enhancement of virus-neutralizing activity (22). Because the vaccine immunogen and adjuvant were identical in both trials, we hypothesize there is a physiologic and immunologic difference between the two cohorts. Pregnant women have long been known to have increased severity and/or susceptibility to specific infectious pathogens (26), suggesting a modified immunologic state during pregnancy. Reproductive hormones have been shown to modulate activity of the human immune system (26). For example, progesterone, which surges at the end of pregnancy and into the postpartum period, is immunosuppressive and can alter the Th1/Th2 balance (27), which could have affected antibody maturation. Regardless, it is evident that the partial vaccine efficacy achieved following gB/MF59 vaccination of postpartum women was not dependent on the induction of robust neutralizing antibody responses.

Analysis of the linear gB epitope IgG-binding profile revealed high-magnitude vaccine-elicited antibody responses against the AD-3 region located within the cytodomain of gB (11, 15). Indeed, an average of 76% of the total vaccine-elicited linear gB IgG binding was directed against this single region, in contrast to 32% in seropositive individuals. Because the AD-3 region is intracellular when gB is expressed on a cell membrane, it presumably does not give rise to antibodies that can bind to or neutralize infectious virus (28). It is unclear whether AD-3–directed antibody responses contributed to vaccine-mediated protection through mechanisms that remain to be defined or, alternatively, whether this response was merely a diversion away from more functional epitopes. Decoy immune responses away from functional epitopes have been described for other pathogens, most notably for HIV-1 (29). The restricted antibody response against other gB epitopes, including neutralizing epitopes such as AD-1, AD-2, domain I, and domain II, may be a consequence of a diversionary AD-3 immune response. It is therefore possible that a next-generation HCMV gB vaccine construct without the cytodomain AD-3 epitope would elicit a greater magnitude and breadth of epitope-specific IgG responses and possibly elicit more potently neutralizing antibodies.

gB/MF59 vaccination elicited a robust titer of gB-specific IgG3 subclass antibodies, which is unusual for a protein subunit vaccine (30, 31). The identified IgG1/IgG3 subclass profile is consistent with previous reports (32), although the relative abundance of gB-specific IgG subclasses could not be defined by our assay because of its semiquantitative nature. Previous studies have suggested that the subclass distribution of gB-specific IgG responses may depend on the adjuvant used for vaccination (33) or the structure of the gB immunogen (34). Specifically, adjuvant MF59 has been tied to enhanced IgG3 responses for other vaccines (30). Antigen-specific IgG3 has been implicated in virologic control of other pathogens such as HIV-1 (35), and it is anticipated that IgG3 mediates protective antiviral effects by binding to effector cell Fc receptors and facilitating nonneutralizing functions such as antibody-dependent cellular cytotoxicity (ADCC) (36) or antibody-dependent cellular phagocytosis (ADCP) (37). Nonneutralizing antibody effector functions have not been evaluated extensively for HCMV, although NK cells have been strongly implicated in control of HCMV replication (38). Moreover, there is some precedent for ADCC-mediated control of HCMV replication in vitro (39). A critical prerequisite for any vaccine-elicited nonneutralizing effector functions is antibody binding to membrane-associated glycoproteins. Our results demonstrate robust, strain-independent binding of vaccine-elicited antibodies to gB expressed on the surface of both gB-transfected and HCMV-infected cells, suggesting the possibility that these antibodies facilitate antiviral functions such as ADCC or ADCP.

Despite the high-magnitude vaccine-elicited IgG3 response and membrane-associated gB binding, no substantial evidence of ADCC was observed among vaccinees. However, we demonstrated that vaccine-elicited plasma antibodies could mediate a robust level of ADCP of both the gB immunogen alone and gB expressed on the surface of whole virions. Antibody-mediated uptake of whole virions has not to our knowledge been explored as a protective immune mechanism for HCMV, although it has been shown to play an important role in clearing other viral pathogens including influenza, West Nile virus, adenovirus, SARS, foot-and-mouth disease virus, and perhaps HIV-1 (37). As we observed robust vaccine-elicited IgG3 antibody responses, it should be noted that IgG3 has high affinity for the Fc receptors expressed on monocytes/macrophages (40) and that this IgG subclass has been associated with more robust uptake of opsonized virus (37, 41). Monocytes are widely recognized as an important target for HCMV latent infection and dissemination throughout the body (42). Our data suggest that phagocytosed virions are destroyed and do not initiate HCMV replication, yet subsequent studies should investigate whether antibody-mediated uptake of HCMV can facilitate initiation of latent/lytic HCMV infection.

One limitation of this study is that we did not assess the role of CD4+ or CD8+ T cells in gB/MF59 vaccine-elicited functional immunity, because stored mononuclear cells are not available. However, protein vaccines, and MF59-adjuvanted protein vaccines in particular, are quite poor at stimulating antigen-specific T cells (43). Nevertheless, this topic remains controversial because robust CD4+/CD8+ T cell immunity has been observed in a human cohort following three doses of an MF59-adjuvanted protein vaccine (44), and gB/MF59 vaccination of HCMV-seropositive subjects resulted in boosted gB-specific CD4+ T cells (13). Another shortcoming is that our study was underpowered to compare the magnitude of immune responses between vaccinees who acquired HCMV during the course of the vaccine trial and those who did not. Thus, we cannot say with certainty that nonneutralizing

antibody functions such as ADCP were associated with protection against HCMV infection.

This in-depth investigation of the immune responses elicited by the most efficacious HCMV vaccine candidate to date has revealed important biology of antibody functions that are potentially protective against HCMV infection. Although neutralizing antibodies are likely important in the control of cell-free HCMV dissemination, cellular responses and/or nonneutralizing antibody effector functions may be essential to eliminate the infected cell reservoir and contain direct cell-to-cell spread. Our data demonstrate that heterologous neutralizing antibody responses were not responsible for the partial efficacy observed in this HCMV vaccine clinical trial (7). Furthermore, gB/MF59-elicited antibodies can mediate robust, nonneutralizing antiviral functions, and such responses in the absence of potent neutralizing antibodies are the likely mechanism behind the clinically demonstrated 50% vaccine efficacy. Thus, the elicitation of nonneutralizing antibody responses against HCMV should be a consideration in the rational design of the next generation of HCMV vaccines for the elimination of congenital and transplant-associated HCMV infections.

Methods

The study cohort consisted of 33 postpartum women administered a glycoprotein B + MF59 adjuvant subunit vaccine during a phase 2, randomized, placebo-controlled clinical trial (7). Plasma antibody responses were assessed at the peak immunogenicity time point (6.5 mo). Neutralizing antibody responses were evaluated using both IE-1 immunofluorescence and GFP-reporter assays, in the presence and absence of purified rabbit complement (Cedarlane Laboratories). Antibody binding to gB epitopes was assessed via binding antibody multiplex assay as well as linear peptide microarray (JPT peptides). Finally, the ability of vaccine-elicited antibodies to bind membrane-associated gB and to mediate NK cell degranulation or monocyte phagocytosis was investigated by flow cytometry. Vaccine-elicited antibody response magnitude was compared with a cohort of 30 chronically HCMV-infected postpartum women. See *SI Appendix, Supplemental Materials and Methods*, for full details.

Institutional review board (IRB) approval was obtained from the University of Alabama at Birmingham (postpartum gB/MF59), Virginia Commonwealth University (phase 1 gB/MF59), and Harvard University (seropositive cohort). All subjects signed an approved consent form. The Duke IRB determined that the analysis of deidentified sera samples did not meet the definition of human subjects research.

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