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Full Title: HCMV glycoprotein B subunit vaccine efficacy was mediated by non-neutralizing antibody effector functions

Short Title: Mechanism of HCMV gB vaccine-mediated protection

Subject area: Immunology

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Abstract:

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 approximately 50% efficacy in preventing HCMV acquisition in phase II trials. However, the mechanism of vaccine protection remains unknown. Plasma from 33 gB/MF59 vaccinees at peak immunogenicity was tested for gB epitope specificity as well as neutralizing and non-neutralizing anti-HCMV effector functions, and compared to 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. Interestingly, vaccinees had high-magnitude IgG responses against AD-3 linear epitopes, demonstrating immunodominance against this non-neutralizing, cytosolic region. Finally, vaccine-elicited IgG robustly bound trimeric, membrane-associated gB on the surface of transfected or HCMV-infected cells and mediated virion phagocytosis, though were poor mediators of NK cell activation. Altogether, these data suggest that non-neutralizing antibody functions, including virion phagocytosis, likely played a role in the observed 50% vaccine-mediated protection against HCMV acquisition.

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.

Introduction:

Human cytomegalovirus (HCMV) is the most common congenital viral infection (1), impacting 1 out of every 150 live born infants worldwide (2). 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 (3, 4) – more congenital disease than all 29 newborn conditions currently screened for in the US combined (5). It is clear that preexisting maternal immunity impacts the incidence of congenital infection, as 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 (3). Furthermore, infants born to HCMV-seropositive women are also less likely to exhibit symptoms of congenital infection at birth and can have milder neurologic sequelae (6, 7). Therefore, many hypothesize 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 (8-10). Given the global burden of disease of congenital HCMV, such a vaccine remains a Tier 1 priority of the National Academy of Medicine (11).

A variety of candidate HCMV vaccine approaches have been attempted, including live attenuated virus (12, 13), glycoprotein subunit formulations (14, 15), viral vectors (16, 17), and single/bivalent DNA plasmids (18, 19). Importantly, 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 (14) and adolescent women (15). Furthermore, this vaccine demonstrated a protective benefit against HCMV viremia and reduced clinical need for antiviral treatment in transplant recipients (20). gB is the viral fusogen which facilitates the fusion of viral envelope and host membrane during viral entry (21). This glycoprotein is essential for entry into all cell types, including trophoblast progenitor cells (22), and is a known target of neutralizing antibodies (23, 24). However, previous investigations have

reported that gB/MF59-elicited antibodies were poorly neutralizing (25-27), raising 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 4 distinct structural regions: an ectodomain, a membrane-proximal region (MPER), a transmembrane domain, and a cytoplasmic domain (Fig. S1) (28, 29). Additionally, there are 5 distinct antigenic sites known to be targeted by gB-specific antibodies, identified as antigenic domain (AD) 1-5 (Fig. S1) (23, 28, 29). Antibodies against AD-1, an uninterrupted ~80 amino acid epitope, are present in virtually all infected individuals (23) and can be either neutralizing or non-neutralizing (30, 31). In contrast, AD-2 specific antibodies are present in only subset of seropositive people (23, 32), and this region consists of 2 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 non-neutralizing antibodies (32). AD-3 is located within the cytosolic domain and is a known target of exclusively non-neutralizing antibodies (33-35). 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 (23, 36-38). The antigen used for the gB/MF59 vaccine clinical trials (14, 15, 20) consisted of the full protein with two modifications to facilitate manufacture: (1) deletion of the transmembrane domain (75 amino acids) and (2) mutation of the furin protease cleavage site (39).

To identify possible mechanisms accounting for the partial protection against HCMV infection following gB/MF59 vaccination, we undertook an in-depth investigation into the characteristics and functionality of the antibody responses elicited by this gB subunit vaccine. As other groups have reported, we observed that gB/MF59 vaccine-elicited antibodies mediated limited neutralization. Here we report on our observations that gB/MF59 vaccination results in an antibody profile quite distinct from that observed in the setting of natural HCMV infection.

112 Furthermore, we have observed that the poorly-neutralizing, gB/MF59-elicited antibodies can
113 mediate robust non-neutralizing antiviral responses, such as phagocytosis, leading us to
114 propose that non-neutralizing antibody functions played a role in the observed 50% vaccine
115 efficacy against HCMV acquisition.

Results:

Immunogen and HCMV virion binding IgG responses/avidity.

We obtained plasma samples from 33 gB/MF59 vaccinee participants in the phase 2 clinical trial conducted in a population of seronegative postpartum women (14). All subsequent studies used samples collected at peak immunogenicity (6.5 months), or the next available (not exceeding 12 months). The gB/MF59 vaccine platform has been previously reported to elicit robust titers of gB immunogen-specific IgG (39, 40). We observed similar results for this subset of vaccinees (gB/MF59), with high-magnitude plasma gB IgG binding exceeding that elicited in chronically-infected, seropositive (SP) individuals (Fig. 1A) (log_{10}AUC: gB/MF59=6.32, SP=5.64; p=0.03, Wilcoxon rank sum test). In contrast, much lower IgG binding of vaccinee sera was observed against whole HCMV virions compared to seropositive individuals (Fig. 1B) (log_{10}AUC: gB/MF59=0.48, SP=2.91; p<0.001, Saittertherwaite t test), likely due to naturally HCMV-elicited antibodies targeting a variety of other CMV glycoproteins and/or multiple episodes of reactivation or reinfection boosting the response in seropositives. Additionally, we assessed the avidity of the interaction between vaccine-elicited antibodies and the gB immunogen (Fig. 1C) or whole virions (Fig. 1D) as a marker for the strength of the antibody–antigen interaction generated by B cell somatic hypermutation. We identified that the relative avidity index (RAI) of vaccine-elicited antibodies against the gB immunogen was similar to that observed in chronic infection, though somewhat reduced when assessed against whole virions (median RAI: gB/MF59=0.81, SP=0.99; p<0.001, pooled t test).

HCMV neutralization and IgG binding to gB-neutralizing domains.

We next investigated the ability of vaccine-elicited antibodies to neutralize a panel of HCMV strains, including the autologous Towne strain (Fig. 2A), AD169 (Fig. 2B), and TB40/E (in fibroblasts, Fig. 2C, and epithelial cells, Fig. 2D). 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, though significantly reduced compared to 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, though 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 $log_{10}ID_{50}$: comp=1.70; no comp=1.93), not quite as robust as previous reports (41). Subsequently, we examined whether vaccine-elicited antibodies bound to previously-identified gB neutralizing epitopes (23, 28), including AD-1 (Fig. 2F), AD-2 site 1 (Fig. 2G), Domain I (AD-5) (Fig. 2H), Domain II (AD-4) (Fig. 2I), and Domain I+II combined (Fig. 2J). Intriguingly, though vaccination elicited robust gB-binding responses, there was very poor targeting of these gB neutralizing epitopes. Compared to 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, Saittertherwaite 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). Thus, the poor targeting of known gB neutralizing epitopes by vaccination likely explains the lack of neutralization responses observed.

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 open reading frame (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, Fig. S2). 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 (42) (AD-2 site 1 median log_{10}MFI: gB/MF59=2.68, SP=3.69; p<0.001, Saittertherwaite t test). Furthermore, vaccination resulted in dominant IgG response against the non-neutralizing 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), comprising 76% of the linear gB IgG response in vaccinees compared to 32% in chronically HCMV-infected individuals (Fig. S2A,B,F)

IgG subclass distribution 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 non-neutralizing 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. 4A-D). Furthermore, we examined whether vaccine-elicited immune responses could bind to membrane-associated gB expressed on the surface of both gB-transfected (Fig. 4E,F) and TB40/E-infected cells (Fig. 4 H,I). Vaccine-elicited IgG bound to both transfected autologous Towne strain gB (Fig. 4E) and a heterologous strain gB (Fig. 4F) (most 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, Saittertherwaite t test). Yet, vaccine-elicited IgG bound TB40/E-infected cells less well than antibodies elicited by chronic infection (Fig. 4H) (median % infected cell binding: gB/MF59=6.7%, SP=36.7% ; p<0.001, Saittertherwaite 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. 4I) (median % infected cell gB binding: gB/MF59=4.4%, SP=1.7%; p=0.01, Saittertherwaite 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 as this process is a prerequisite of both antibody-dependent cellular cytotoxicity (ADCC) and cytokine release by activated NK cells. Interestingly, 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. 4G) and TB40/E-infected target cells (Fig. 4J). In contrast, the majority of chronically HCMV-infected individuals had antibodies that mediated measurable, though 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 monocyte phagocytosis. We developed highly-specific, flow-based assays for measuring the phagocytosis of both gB-conjugated beads (Fig. 5A,B) and fluorescently-conjugated HCMV virions (Fig. 5C,D). Cellular uptake of HCMV was confirmed by confocal microscopy, demonstrating fluorescent virus either at the cell surface (Fig. 5E) or internalized (Fig. 5F). The small number of distinct viral foci observed following phagocytosis differs from the multiple, dispersed foci following monocyte infection (Fig. 5H). Additionally, we confirmed that phagocytosis did not lead to productive infection, as cells that phagocytosed TB40/E-mCherry virus did not exhibit mCherry expression at 48 hours post phagocytosis (Fig. S3). 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. 5I). 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. 5J). There was a reduced magnitude of phagocytosis activity directed against the gB ectodomain in comparison to 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 to vaccinees (Fig. 5K) (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. 5L) (median % positive cells: V=11.3%, SP=10.1%; p=ns, pooled t test). Finally, we confirmed that, though gB vaccine-elicited antibodies could mediate robust virion phagocytosis, they minimally blocked infection of THP-1 monocytes (Fig. 5M,N) (normalized % neutralization at 1:100 dilution: gB/MF59=18.4%, SP=82.8%; p<0.001, Satterthwaite t test).

Assay correlation matrix.

Lastly, we sought to investigate the relationship between measured antibody responses by creating a correlation matrix (Fig. 6). We observed two distinct clusters of responses that appeared related to one another. The first cluster is comprised 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 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. Intriguingly, 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. Of note, the formation of cluster 1 was primarily driven by gB-elicited immune responses following vaccination, while cluster 2 was primarily driven by binding and neutralization against non-gB CMV glycoprotein epitopes (Fig. S4A,B).

252 **Discussion:**

253 Over the past decade, the HCMV vaccine field has largely shifted its focus away from
254 the elicitation of gB-specific antibody responses and towards the targeting of the
255 gH/gL/UL128/UL130/UL131A pentameric complex (PC) because this protein construct was
256 newly identified as a primary target of potent HCMV neutralizing antibodies (24, 43). Yet, it is
257 important to recognize that the gB/MF59 vaccine platform, which did not include the PC,
258 achieved approximately 50% vaccine efficacy in preventing primary HCMV infection in two
259 phase II trials (14, 15) and demonstrated a protective benefit for transplant recipients (20)
260 without the elicitation of potent neutralizing antibody responses (20, 25, 26). Increasingly, both
261 the limitations of neutralizing antibodies in controlling HCMV cell-to-cell spread (42) and the
262 protective capacity of non-neutralizing HCMV-specific antibodies are becoming recognized (44),
263 indicating that the role of non-neutralizing antibodies needs to be further investigated as a
264 potentially-important endpoint for HCMV vaccine immunogenicity trials.

265 In this study, we confirmed previous reports of negligible gB/MF59-elicited heterologous
266 and autologous neutralization responses using a panel of viral strains representing diverse gB
267 genotypes: AD169 (gB2), TB40/E (gB4), and Towne (gB1), the autologous virus that is the basis
268 of the vaccine gB antigen (45). While we observed complement-mediated enhancement of
269 HCMV-seropositive neutralization titers in fibroblast cells (2-4 fold), but not in epithelial cells,
270 consistent with previous reports (46). Furthermore, we observed minimal complement-mediated
271 enhancement of gB/MF59 vaccine recipient neutralizing responses, though not as robust as
272 reported previously (41). The limited complement-mediated enhancement is in spite of high
273 levels of gB-specific IgG3 antibodies in the plasma of both vaccinees and seropositive
274 individuals, which is frequently associated with robust complement fixation (47, 48). Congruent
275 with the poor vaccine-elicited neutralizing antibody response, we observed that gB vaccination
276 failed to elicit responses against well-characterized conformational and linear gB neutralizing
277 epitopes such as AD-1, AD-2, Domain I, and Domain II. This finding suggests that these

important neutralizing epitopes were either: 1) inadequately exposed to the immune system on the protein immunogen, or 2) that their post-fusion conformation presented via the protein immunogen did not accurately mimic the pre-fusion conformation on the viral envelope, as has been described for HSV-1 gB (49).

Intriguingly, 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 (Fig. 3, Fig. S1) (28, 29). 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 (50). 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, where a vaccine construct containing both gp120 and gp41 elicited a memory B cell response in which 93% targeted the gp41 region (51). This gp41-dominant response is hypothesized to have occurred due to preexisting memory B cells directed against a gp41-crossreactive epitope within intestinal microbiota, thus biasing the antibody response in a form of molecular mimicry. The cause of the immune dominance of the AD-3 region in gB/MF59 vaccinees is unclear, though this epitope may be more accessible on soluble protein compared to its typical intraluminal location on whole virions or HCMV-infected cells. The restricted antibody response against other gB epitopes, including neutralizing epitopes such as AD-1, AD-2, Domain I, and Domain II (Fig. S2) may be a consequence of the high-magnitude linear antibody responses against the AD-3 region. It is therefore possible that a gB vaccine construct without the cytodomain AD-3 epitope would target a greater breadth of epitopes 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 (52, 53), and possibly due to use of squalene adjuvant MF59 (52). Antigen-specific IgG3 has been implicated in virologic control of other pathogens such as HIV-1 (54), and is anticipated that IgG3 mediates protective antiviral effects by binding to effector cell Fc receptors and facilitating non-neutralizing functions such as antibody-dependent cellular cytotoxicity (ADCC) (55) or antibody-dependent cellular phagocytosis (ADCP) (56). Non-neutralizing antibody effector functions have not been evaluated extensively for HCMV, though NK cells have been strongly implicated in control of HCMV replication (57-60). Moreover, there is some precedent for ADCC-mediated control of HCMV replication *in vitro* in patients with severe AIDS and concomitant HCMV retinitis (61). Furthermore, purified HCMV hyperimmune gamma globulin (Cytogam) has been observed to dramatically enhance the antiviral function of macrophages or NK cells in culture (61). A critical prerequisite for any vaccine-elicited non-neutralizing effector functions is antibody binding to membrane-associated glycoproteins. Importantly, 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. Yet, 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, though it has been shown to play an important role in clearing other viral pathogens including influenza (62), West Nile virus (63), adenovirus (64), SARS coronavirus (65), foot-and-mouth disease virus (66), and perhaps HIV-1 (56). 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 (48), and that this IgG subclass has been associated with more robust uptake of opsonized virus (56, 67). Monocytes are widely recognized as an important target for HCMV latent infection and dissemination throughout the body (68). Our data suggests that phagocytosed virions are destroyed and do not initiate HCMV replication, yet the fate of a phagocytosed virion is relatively unexplored and 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, as stored mononuclear cells are not available. Yet protein vaccines, and MF59-adjuvanted protein vaccines in particular, are generally poor at stimulating antigen-specific T cells (69), and previous studies have indicated that MF59 induces a Th2 polarized immune response (70). Nevertheless, this topic remains controversial, as one group has reported robust CD4+/CD8+ T cell immunity in a human cohort following 3 doses of an MF59-adjuvanted protein vaccine (71). Another shortcoming is that we did not have access to a sufficient number of vaccinee samples to compare humoral 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 non-neutralizing antibody functions such as ADCP were associated with protection against HCMV infection. Nevertheless, this investigation has expanded the repertoire of HCMV-specific antibody functional assays and informs which vaccine-elicited antibody functions were potentially-responsible for the partial vaccine efficacy, guiding subsequent investigation in HCMV vaccine trials.

Some researchers have argued that conventional immune metrics currently used as a surrogate marker of effective anti-HCMV immunity, such as neutralization, may not tell the whole story (72). 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. Though neutralizing antibody

356 responses are likely important in the control of cell-free HCMV dissemination, cellular responses
357 and/or non-neutralizing antibody effector functions may be essential to eliminate the infected
358 cell reservoir and contain direct cell-to-cell spread. Our data suggest for the first time that
359 gB/MF59-elicited antibodies can mediate robust, non-neutralizing antiviral functions, and that
360 such responses in the absence of potent neutralizing antibodies are the likely mechanism
361 behind the clinically-demonstrated 50% vaccine efficacy. Thus, the elicitation of non-neutralizing
362 antibody responses against HCMV should be a consideration in the rational design of the next
363 generation of HCMV vaccines for the elimination of congenital and transplant-associated HCMV
364 infections.
365

366 **Methods:**

367 Full methods are available in the supplemental material.

Author contributions:

C.S.N. and S.R.P. designed research; C.S.N., T.H., and E.C. performed research; C.S.N., T.H., E.C., G.X., and N.V. analyzed data; R.F.P contributed samples and expertise; and C.S.N., J.P., and S.R.P. wrote the paper.

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References:

1. Swanson EC & Schleiss MR (2013) Congenital cytomegalovirus infection: new prospects for prevention and therapy. *Pediatr Clin North Am* 60(2):335-349.
2. Manicklal S, Emery VC, Lazzarotto T, Boppana SB, & Gupta RK (2013) The "Silent" Global Burden of Congenital Cytomegalovirus. *Clinical Microbiology Reviews* 26(1):86-102.
3. Kenneson A & Cannon MJ (2007) Review and meta-analysis of the epidemiology of congenital cytomegalovirus (CMV) infection. *Rev Med Virol* 17(4):253-276.
4. Ross SA & Boppana SB (2005) Congenital cytomegalovirus infection: outcome and diagnosis. *Semin Pediatr Infect Dis* 16(1):44-49.
5. Centers for Disease C & Prevention (2008) Impact of expanded newborn screening--United States, 2006. *MMWR Morb Mortal Wkly Rep* 57(37):1012-1015.
6. Boppana SB, Rivera LB, Fowler KB, Mach M, & Britt WJ (2001) Intrauterine transmission of cytomegalovirus to infants of women with preconceptional immunity. *The New England journal of medicine* 344(18):1366-1371.
7. Fowler KB*, et al.* (1992) The outcome of congenital cytomegalovirus infection in relation to maternal antibody status. *The New England journal of medicine* 326(10):663-667.
8. Plotkin S (2015) The history of vaccination against cytomegalovirus. *Med Microbiol Immunol* 204(3):247-254.
9. McVoy MA (2013) Cytomegalovirus vaccines. *Clin Infect Dis* 57 Suppl 4:S196-199.
10. Adler SP (2013) Immunization to prevent congenital cytomegalovirus infection. *Br Med Bull* 107:57-68.
11. Anonymous (2000) *Vaccines for the 21st Century: A Tool for Decisionmaking,* The National Academies Collection: Reports funded by National Institutes of Health, eds Stratton KR, Durch JS, & Lawrence RSWashington (DC)).
12. Elek SD & Stern H (1974) Development of a vaccine against mental retardation caused by cytomegalovirus infection in utero. *Lancet* 1(7845):1-5.
13. Plotkin SA, Farquhar J, & Horberger E (1976) Clinical trials of immunization with the Towne 125 strain of human cytomegalovirus. *The Journal of infectious diseases* 134(5):470-475.
14. Pass RF*, et al.* (2009) Vaccine prevention of maternal cytomegalovirus infection. *The New England journal of medicine* 360(12):1191-1199.
15. Bernstein DI*, et al.* (2016) Safety and efficacy of a cytomegalovirus glycoprotein B (gB) vaccine in adolescent girls: A randomized clinical trial. *Vaccine* 34(3):313-319.
16. Adler SP*, et al.* (1999) A canarypox vector expressing cytomegalovirus (CMV) glycoprotein B primes for antibody responses to a live attenuated CMV vaccine (Towne). *The Journal of infectious diseases* 180(3):843-846.
17. Wussow F*, et al.* (2014) Human cytomegalovirus vaccine based on the envelope gH/gL pentamer complex. *PLoS pathogens* 10(11):e1004524.
18. Wloch MK*, et al.* (2008) Safety and immunogenicity of a bivalent cytomegalovirus DNA vaccine in healthy adult subjects. *The Journal of infectious diseases* 197(12):1634-1642.
19. Jacobson MA*, et al.* (2009) A CMV DNA vaccine primes for memory immune responses to live-attenuated CMV (Towne strain). *Vaccine* 27(10):1540-1548.
20. Griffiths PD*, et al.* (2011) Cytomegalovirus glycoprotein-B vaccine with MF59 adjuvant in transplant recipients: a phase 2 randomised placebo-controlled trial. *Lancet* 377(9773):1256-1263.
21. Vanarsdall AL & Johnson DC (2012) Human cytomegalovirus entry into cells. *Curr Opin Virol* 2(1):37-42.
22. Zydek M*, et al.* (2014) HCMV infection of human trophoblast progenitor cells of the placenta is neutralized by a human monoclonal antibody to glycoprotein B and not by antibodies to the pentamer complex. *Viruses* 6(3):1346-1364.

23. Potzsch S*, et al.* (2011) B cell repertoire analysis identifies new antigenic domains on glycoprotein B of human cytomegalovirus which are target of neutralizing antibodies. *PLoS pathogens* 7(8):e1002172.
24. Fouts AE, Chan P, Stephan JP, Vandlen R, & Feierbach B (2012) Antibodies against the gH/gL/UL128/UL130/UL131 complex comprise the majority of the anti-cytomegalovirus (anti-CMV) neutralizing antibody response in CMV hyperimmune globulin. *Journal of virology* 86(13):7444-7447.
25. Cui X, Meza BP, Adler SP, & McVoy MA (2008) Cytomegalovirus vaccines fail to induce epithelial entry neutralizing antibodies comparable to natural infection. *Vaccine* 26(45):5760-5766.
26. Sabbaj S, Pass RF, Goepfert PA, & Pichon S (2011) Glycoprotein B vaccine is capable of boosting both antibody and CD4 T-cell responses to cytomegalovirus in chronically infected women. *The Journal of infectious diseases* 203(11):1534-1541.
27. Frey SE*, et al.* (1999) Effects of antigen dose and immunization regimens on antibody responses to a cytomegalovirus glycoprotein B subunit vaccine. *The Journal of infectious diseases* 180(5):1700-1703.
28. Burke HG & Heldwein EE (2015) Correction: Crystal Structure of the Human Cytomegalovirus Glycoprotein B. *PLoS pathogens* 11(11):e1005300.
29. Chandramouli S*, et al.* (2015) Structure of HCMV glycoprotein B in the postfusion conformation bound to a neutralizing human antibody. *Nature communications* 6:8176.
30. Schoppel K*, et al.* (1996) Antibodies specific for the antigenic domain 1 of glycoprotein B (gpUL55) of human cytomegalovirus bind to different substructures. *Virology* 216(1):133-145.
31. Wagner B*, et al.* (1992) A continuous sequence of more than 70 amino acids is essential for antibody binding to the dominant antigenic site of glycoprotein gp58 of human cytomegalovirus. *Journal of virology* 66(9):5290-5297.
32. Meyer H, Sundqvist VA, Pereira L, & Mach M (1992) Glycoprotein gp116 of human cytomegalovirus contains epitopes for strain-common and strain-specific antibodies. *J Gen Virol* 73 (Pt 9):2375-2383.
33. Kniess N, Mach M, Fay J, & Britt WJ (1991) Distribution of linear antigenic sites on glycoprotein gp55 of human cytomegalovirus. *Journal of virology* 65(1):138-146.
34. Silvestri M, Sundqvist VA, Ruden U, & Wahren B (1991) Characterization of a major antigenic region on gp55 of human cytomegalovirus. *J Gen Virol* 72 (Pt 12):3017-3023.
35. Mach M (2005) Antibody-mediated neutralization of infectivity Cytomegaloviruses: Molecular Biology and Immunology. *Caister Academic Press*:265-283.
36. Spindler N*, et al.* (2013) Characterization of a discontinuous neutralizing epitope on glycoprotein B of human cytomegalovirus. *Journal of virology* 87(16):8927-8939.
37. Spindler N*, et al.* (2014) Structural basis for the recognition of human cytomegalovirus glycoprotein B by a neutralizing human antibody. *PLoS pathogens* 10(10):e1004377.
38. Wiegers AK, Sticht H, Winkler TH, Britt WJ, & Mach M (2015) Identification of a neutralizing epitope within antigenic domain 5 of glycoprotein B of human cytomegalovirus. *Journal of virology* 89(1):361-372.
39. Pass RF*, et al.* (1999) A subunit cytomegalovirus vaccine based on recombinant envelope glycoprotein B and a new adjuvant. *The Journal of infectious diseases* 180(4):970-975.
40. Mitchell DK, Holmes SJ, Burke RL, Duliege AM, & Adler SP (2002) Immunogenicity of a recombinant human cytomegalovirus gB vaccine in seronegative toddlers. *Pediatr Infect Dis J* 21(2):133-138.
41. Li F FD, Tang A, Rustandi RR, Troutman MC, Espeseth AS, Zhang N, An Z, McVoy M, Zhu H, Ha S, Wang D, Adler SP, Fu TM (2017) Complement enhances in vitro neutralizing potency of antibodies to human cytomegalovirus glycoprotein B (gB) and immune sera induced by gB/MF59 vaccination. *Nature vaccines* 2.

42. Jacob CL, *et al.* (2013) Neutralizing antibodies are unable to inhibit direct viral cell-to-cell spread of human cytomegalovirus. *Virology* 444(1-2):140-147.
43. Chandramouli S, *et al.* (2017) Structural basis for potent antibody-mediated neutralization of human cytomegalovirus. *Sci Immunol* 2(12).
44. Bootz A, *et al.* (2017) Protective capacity of neutralizing and non-neutralizing antibodies against glycoprotein B of cytomegalovirus. *PLoS pathogens* 13(8):e1006601.
45. Chou SW & Dennison KM (1991) Analysis of interstrain variation in cytomegalovirus glycoprotein B sequences encoding neutralization-related epitopes. *The Journal of infectious diseases* 163(6):1229-1234.
46. Spiller OB, Hanna SM, Devine DV, & Tufaro F (1997) Neutralization of cytomegalovirus virions: the role of complement. *The Journal of infectious diseases* 176(2):339-347.
47. Bindon CI, Hale G, Bruggemann M, & Waldmann H (1988) Human monoclonal IgG isotypes differ in complement activating function at the level of C4 as well as C1q. *The Journal of experimental medicine* 168(1):127-142.
48. Vidarsson G, Dekkers G, & Rispens T (2014) IgG subclasses and allotypes: from structure to effector functions. *Frontiers in immunology* 5:520.
49. Zeev-Ben-Mordehai T, *et al.* (2016) Two distinct trimeric conformations of natively membrane-anchored full-length herpes simplex virus 1 glycoprotein B. *Proceedings of the National Academy of Sciences of the United States of America* 113(15):4176-4181.
50. Mach M (2006) antibody mediated neutralization of infectivity. *Cytomegaloviruses molecular biology and immunology*, ed Reddehase MJ (Caister Academic Press, Norfolk, UK), pp 265-283.
51. Williams WB, *et al.* (2015) HIV-1 VACCINES. Diversion of HIV-1 vaccine-induced immunity by gp41-microbiota cross-reactive antibodies. *Science* 349(6249):aab1253.
52. Fouda GG, *et al.* (2015) Infant HIV type 1 gp120 vaccination elicits robust and durable anti-V1V2 immunoglobulin G responses and only rare envelope-specific immunoglobulin A responses. *The Journal of infectious diseases* 211(4):508-517.
53. Chung AW, *et al.* (2014) Polyfunctional Fc-effector profiles mediated by IgG subclass selection distinguish RV144 and VAX003 vaccines. *Science translational medicine* 6(228):228ra238.
54. Yates NL, *et al.* (2014) Vaccine-induced Env V1-V2 IgG3 correlates with lower HIV-1 infection risk and declines soon after vaccination. *Science translational medicine* 6(228):228ra239.
55. Chung AW, *et al.* (2015) Dissecting Polyclonal Vaccine-Induced Humoral Immunity against HIV Using Systems Serology. *Cell* 163(4):988-998.
56. Tay MZ, *et al.* (2016) Antibody-Mediated Internalization of Infectious HIV-1 Virions Differs among Antibody Isotypes and Subclasses. *PLoS pathogens* 12(8):e1005817.
57. Kuijpers TW, *et al.* (2008) Human NK cells can control CMV infection in the absence of T cells. *Blood* 112(3):914-915.
58. Cosman D, *et al.* (2001) ULBPs, novel MHC class I-related molecules, bind to CMV glycoprotein UL16 and stimulate NK cytotoxicity through the NKG2D receptor. *Immunity* 14(2):123-133.
59. Biron CA & Brossay L (2001) NK cells and NKT cells in innate defense against viral infections. *Curr Opin Immunol* 13(4):458-464.
60. Arase H, Mocarski ES, Campbell AE, Hill AB, & Lanier LL (2002) Direct recognition of cytomegalovirus by activating and inhibitory NK cell receptors. *Science* 296(5571):1323-1326.
61. Forthal DN, Phan T, & Landucci G (2001) Antibody inhibition of cytomegalovirus: the role of natural killer and macrophage effector cells. *Transpl Infect Dis* 3 Suppl 2:31-34.
62. Fujimoto I, Pan J, Takizawa T, & Nakanishi Y (2000) Virus clearance through apoptosis-dependent phagocytosis of influenza A virus-infected cells by macrophages. *Journal of virology* 74(7):3399-3403.

535 63. Chung KM, Thompson BS, Fremont DH, & Diamond MS (2007) Antibody recognition of
536 cell surface-associated NS1 triggers Fc-gamma receptor-mediated phagocytosis and clearance
537 of West Nile Virus-infected cells. *Journal of virology* 81(17):9551-9555.
538 64. Zsengeller Z, Otake K, Hossain SA, Berclaz PY, & Trapnell BC (2000) Internalization of
539 adenovirus by alveolar macrophages initiates early proinflammatory signaling during acute
540 respiratory tract infection. *Journal of virology* 74(20):9655-9667.
541 65. Yasui F*, et al.* (2014) Phagocytic cells contribute to the antibody-mediated elimination of
542 pulmonary-infected SARS coronavirus. *Virology* 454-455:157-168.
543 66. Quattrocchi V*, et al.* (2011) Role of macrophages in early protective immune responses
544 induced by two vaccines against foot and mouth disease. *Antiviral Res* 92(2):262-270.
545 67. Rozsnyay Z*, et al.* (1989) Distinctive role of IgG1 and IgG3 isotypes in Fc gamma R-
546 mediated functions. *Immunology* 66(4):491-498.
547 68. Wills MR, Poole E, Lau B, Krishna B, & Sinclair JH (2015) The immunology of human
548 cytomegalovirus latency: could latent infection be cleared by novel immunotherapeutic
549 strategies? *Cell Mol Immunol* 12(2):128-138.
550 69. Foged C, Hansen J, & Agger EM (2012) License to kill: Formulation requirements for
551 optimal priming of CD8(+) CTL responses with particulate vaccine delivery systems. *Eur J*
552 *Pharm Sci* 45(4):482-491.
553 70. Singh M*, et al.* (1998) A comparison of biodegradable microparticles and MF59 as
554 systemic adjuvants for recombinant gD from HSV-2. *Vaccine* 16(19):1822-1827.
555 71. Galli G*, et al.* (2009) Adjuvanted H5N1 vaccine induces early CD4+ T cell response that
556 predicts long-term persistence of protective antibody levels. *Proceedings of the National*
557 *Academy of Sciences of the United States of America* 106(10):3877-3882.
558 72. Britt WJ (2017) Congenital Human Cytomegalovirus Infection and the Enigma of
559 Maternal Immunity. *Journal of virology* 91(15).
560 73. Wang D*, et al.* (2011) Quantitative analysis of neutralizing antibody response to human
561 cytomegalovirus in natural infection. *Vaccine* 29(48):9075-9080.
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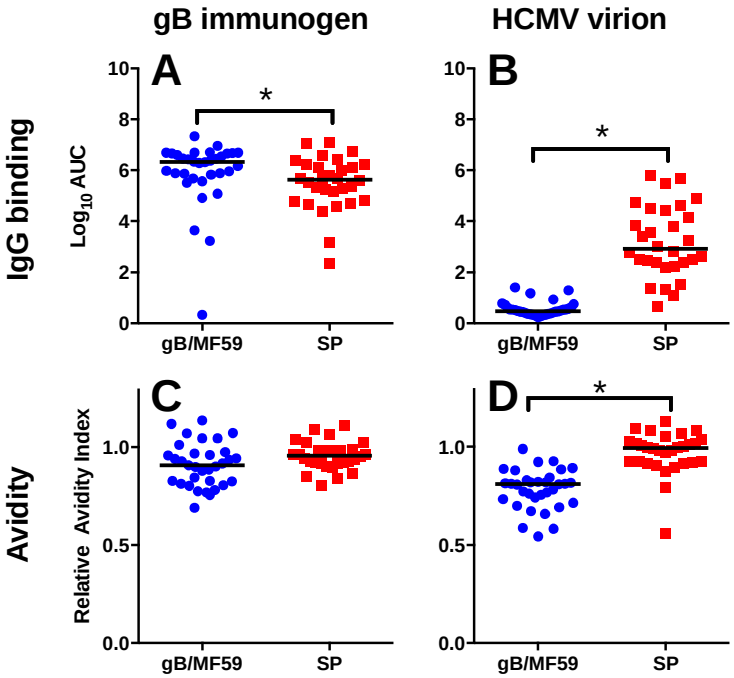
Figures:



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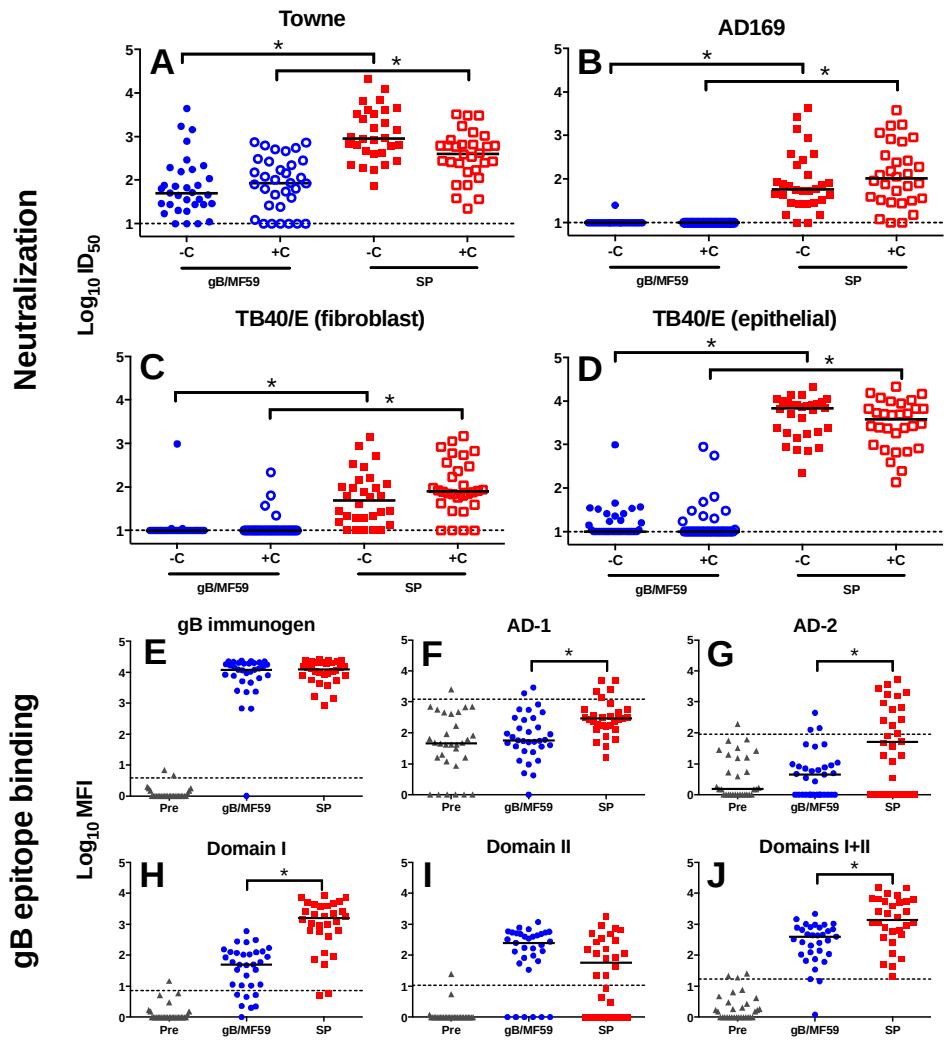


Figure 2. Limited vaccine-elicited neutralization responses and poor gB neutralizing epitope-binding following gB/MF59 immunization. Neutralizing antibody responses (A-D) and gB neutralizing epitope binding (E-J) were assessed for 33 gB/MF59 vaccinees (blue circle) and 30 seropositive, chronically-HCMV infected individuals (red square). Neutralization was measured 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", solid symbols) and absence ("-C", open symbols) of purified rabbit complement. In comparison to seropositive, chronically-infected individuals, gB/MF59 vaccine-elicited neutralization titers were reduced against each viral strain (A-D). Binding responses against the gB immunogen (E) and known gB neutralizing epitopes AD-1 (F), AD-2 (G), Domain I (H), Domain II (I), and Domain I+II combined (J) were measured. In comparison to seropositive women, gB/MF59 vaccination elicited reduced binding against AD-1, AD-2, Domain I, and Domain I+II combined. Each data point represents the mean of 2 experimental replicates. Horizontal dotted lines for neutralization assays indicates the starting dilution, whereas dotted lines for neutralizing-epitope binding indicate the threshold for positivity (preimmune control mean + 2 standard deviations). Black horizontal bars indicate the median values for each group. *=p<0.05, Fisher's exact test (neutralization), pooled t test (epitope binding).

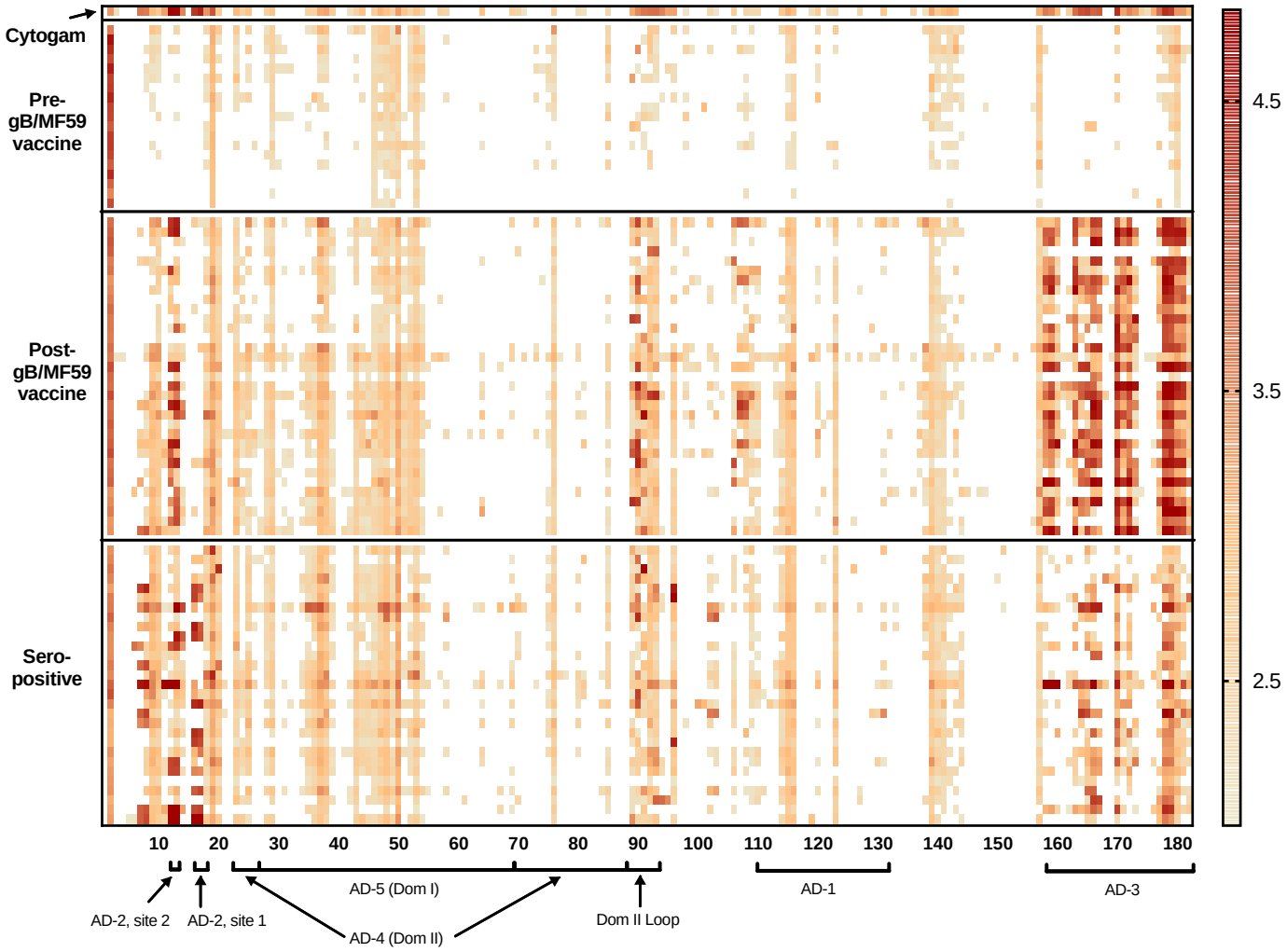
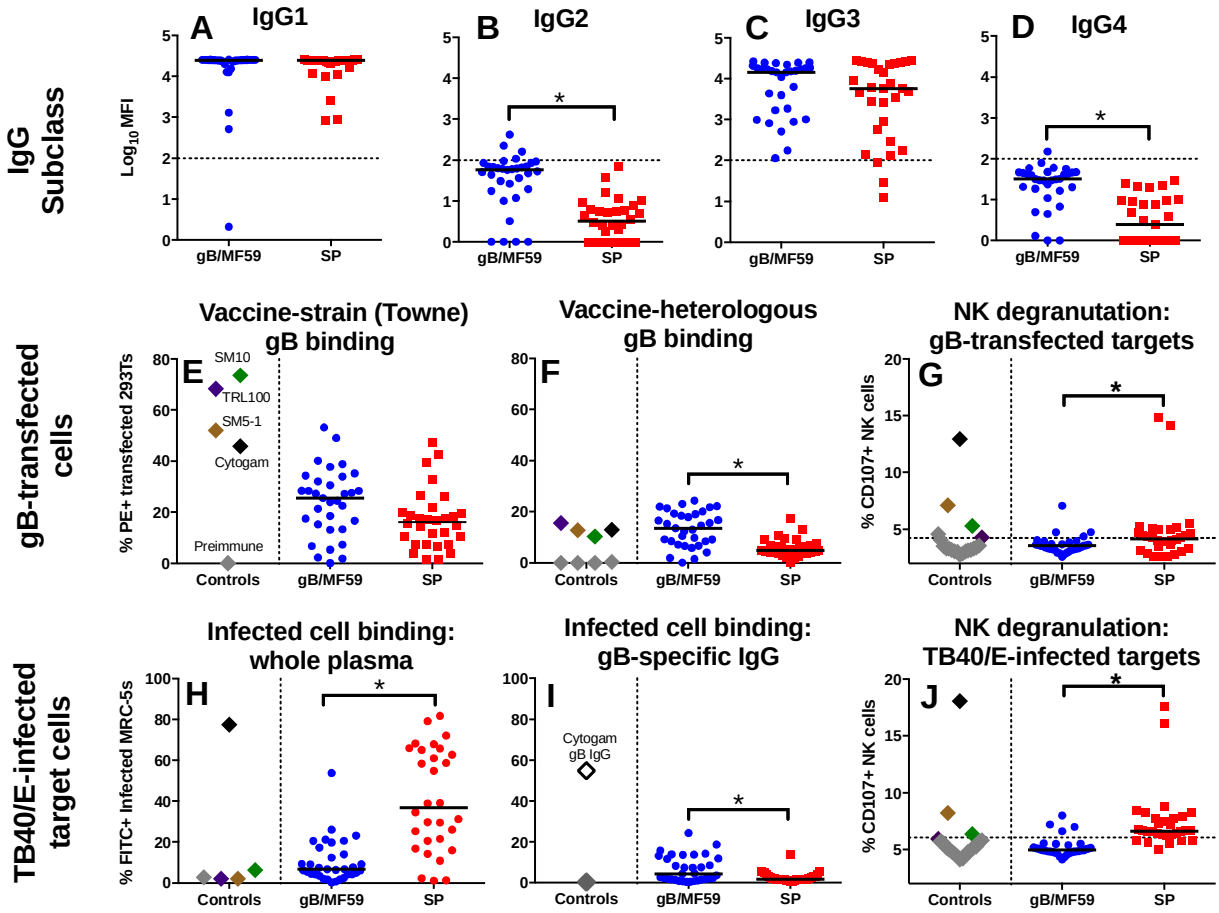


Figure 3. Dominant linear epitope binding response against cytosolic antigenic domain 3 following gB/MF59 immunization. The binding magnitude of antibody responses of Cytogam, 19 gB/MF59 vaccinees pre-immunization, 32 gB/MF59 vaccinees post-immunization, and 30 chronically-infected seropositive controls were assessed against a 15-mer peptide library spanning the entire Towne gB open reading frame (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.



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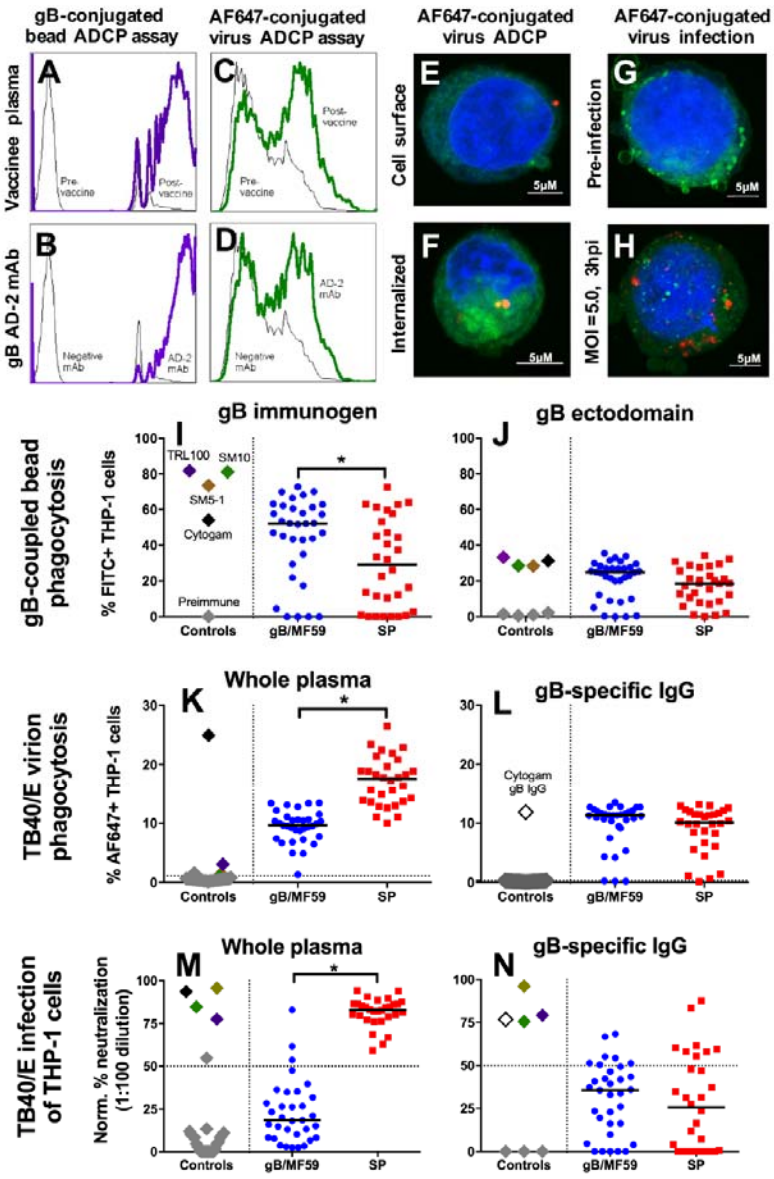

Figure 5. gB vaccine elicits antibodies that mediate robust HCMV virion phagocytosis, though do not block monocyte infection. A flow cytometry-based assay was created to assess antibody-mediated phagocytosis of both gB-coupled fluorescent beads (A,B) and fluorophore-conjugated virus (C,D). Histogram plots of fluorescent intensity indicate the sensitivity of the assay for sera (A,C) and a gB AD-2 specific mAb (B,D). The assay was validated by confocal microscopy (E-G) of THP-1 cells that have either phagocytosed (E,F) or been infected with (G,H) fluorescently-labeled virus. Nuclear material is shown in blue, plasma membrane in green, and AF647-tagged virus in red. Images indicate that phagocytosing cells can either have virus bound to the cell surface (E) or internalized (F). These 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 gB immunogen-coupled fluorescent beads (I), gB ectodomain-coupled fluorescent beads (J), and fluorophore-conjugated whole HCMV virions (K,L). In comparison to seropositive, chronically HCMV-infected women, more robust phagocytosis of the gB immunogen and whole HCMV virions (gB-specific activity) was observed among gB/MF59 vaccinees. Lastly, the ability of vaccine-elicited antibodies to block infection of THP-1 cells was assessed at a single dilution (1:100), using both

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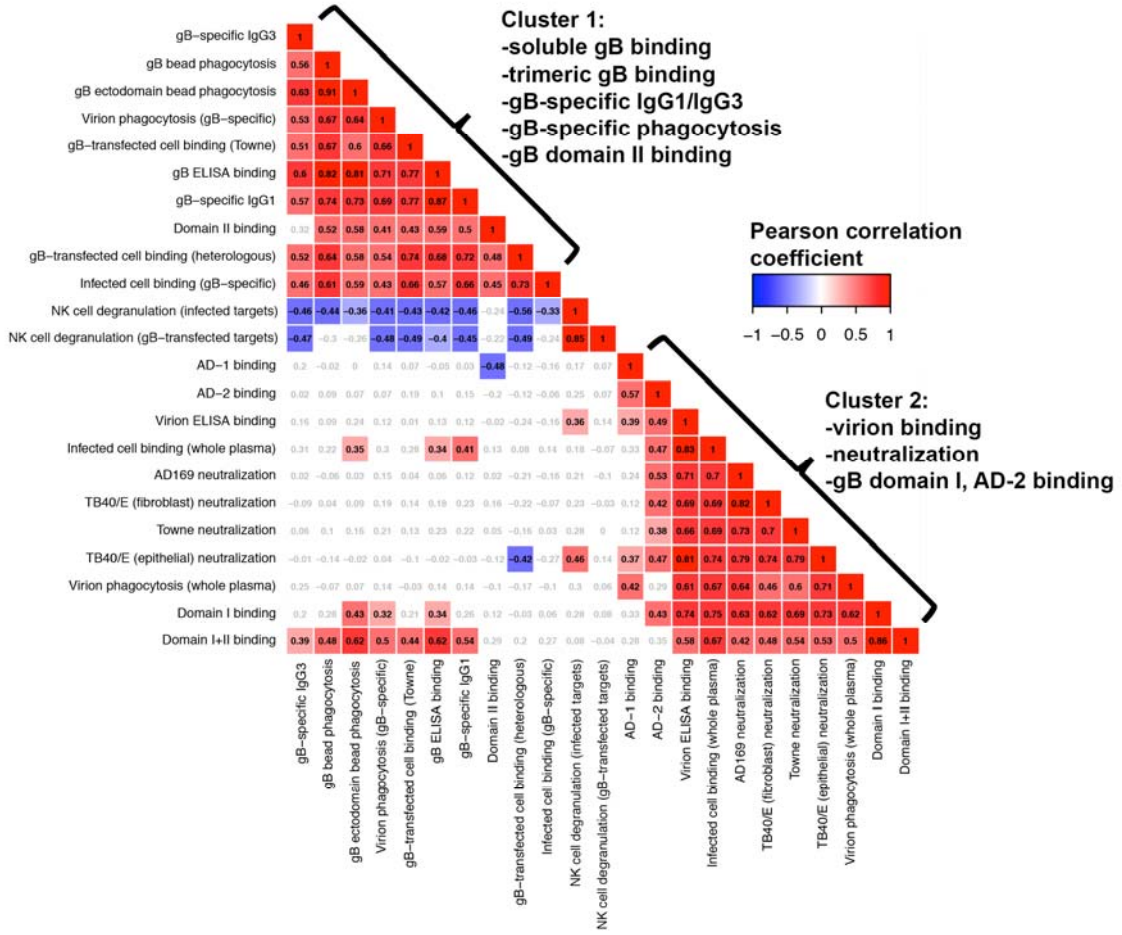

Figure 6. Phagocytosis activity is highly correlated with gB-binding IgG and IgG3 magnitude. A correlation matrix was constructed using data from all 63 tested samples (33 gB/MF59 vaccinee + 30 seropositive) to identify whether assay results correlate with one another. Assays are clustered based on similarity. The Pearson coefficient for each correlation is displayed in the matrix, ranging from -1.0 (blue) to +1.0 (red). Non-significant correlations (p>0.01) are displayed in gray text. Two distinct clusters were identified. The first cluster consists of gB-binding (protein and membrane-associated), IgG1/IgG3 subclass, and ADCP. The second cluster consists of whole virus binding and neutralization activity.

Figure Legends:

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