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1 **Specificity and effector functions of non-neutralizing gB-specific monoclonal**
2 **antibodies isolated from healthy individuals with human cytomegalovirus infection**
3
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24 **Abstract:**

25 Human cytomegalovirus (HCMV) is the most common congenital infection, and the
26 leading nongenetic cause of sensorineural hearing loss (SNHL) in newborns globally. A
27 gB subunit vaccine administered with adjuvent MF59 (gB/MF59) is the most efficacious
28 tested to-date, achieving 50% efficacy in preventing infection of HCMV-seronegative
29 mothers. We recently discovered that gB/MF59 vaccination elicited primarily non-
30 neutralizing antibody responses, that HCMV strains acquired by vaccinees more often
31 included strains with gB genotypes that are distinct from the vaccine antigen, and that
32 protection against HCMV acquisition correlated with ability of vaccine-elicited antibodies
33 to bind to membrane associated gB. Thus, we hypothesized that gB-specific non-
34 neutralizing antibody binding breadth and function are dependent on their epitope and
35 genotype specificity as well as their ability to interact with membrane-associated gB.
36 Twenty-four gB-specific monoclonal antibodies (mAbs) isolated from naturally HCMV-
37 infected individuals were mapped for gB domain specificity by binding antibody multiplex
38 assay (BAMA) and for genotype preference binding to membrane-associated gB
39 presented on transfected cells. We defined their non-neutralizing functions including
40 antibody dependent cellular phagocytosis (ADCP) and antibody dependent cellular
41 cytotoxicity (ADCC). The isolated gB-specific non-neutralizing mAbs were primarily
42 specific for Domain II and linear antigenic domain 2 site 2 (AD2). We observed variability
43 in mAb gB genotype binding preference, with increased binding to gB genotypes 2 and
44 4. Functional studies identified two gB-specific mAbs that facilitate ADCP and have
45 binding specificities of AD2 and Domain II. This investigation provides novel

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46 understanding on the impact of gB domain specificity and antigenic variability on gB-
47 specific non-neutralizing antibody responses.
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69 **Importance:**

70 HCMV is the most common congenital infection worldwide, but development of a
71 successful vaccine remains elusive. gB-specific non-neutralizing mAbs, represent a
72 distinct anti-HCMV Ab subset implicated in the protection against primary infection during
73 numerous phase-II gB/MF59 vaccine trials. By studying non-neutralizing gB-specific
74 mAbs from naturally infected individuals, this study provides novel characterization of
75 binding site specificity, genotypic preference, and effector cell functions mediated by
76 mAbs elicited in natural infection. We found that a panel of twenty-four gB-specific non-
77 neutralizing mAbs bind across multiple regions of the gB protein, traditionally through to
78 be targeted by neutralizing mAbs only, and bind differently to gB depending if the protein
79 is soluble versus embedded in a membrane. This investigation provides novel insight into
80 the gB-specific binding characteristics and effector cell functions mediated by non-
81 neutralizing gB-specific mAbs elicited through natural infection, providing new endpoints
82 for future vaccine development.
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92 **Introduction:**

93 Each year, an estimated 40,000 children in the U.S. are born with congenital
94 human cytomegalovirus (HCMV) infection (cCMV), with roughly 8,000 afflicted children
95 developing long term sequelae of disease such as sensorineural hearing loss,
96 neurodevelopmental delay, visual impairment, and psychomotor disability [1, 2]. While
97 mothers with primary HCMV infection during pregnancy have rates of vertical
98 transmission up to 40%, mothers with HCMV reactivation or reinfection can also transmit
99 the virus to their developing infant but at a rate in the range of <1-4% [3-5]. Additionally,
100 HCMV infection post-transplantation and consequently graft rejection, remain significant
101 complications for solid-organ transplant patients, especially for those HCMV naïve
102 recipients with transplants from HCMV seropositive donors [6, 7]. Although the protective
103 correlates of immunity against HCMV infection have not been fully elucidated, there is
104 ample evidence that immune factors elicited by natural infection can confer some degree
105 of protection for mothers, infants, and immunosuppressed individuals against HCMV
106 reinfection or reactivation. Thus, understanding how natural immunity against HCMV
107 mediates protection may offer the basis for development of an effective HCMV vaccine.

108 Numerous HCMV vaccine candidates have been tested clinically, including live-
109 attenuated virus, viral glycoprotein subunit formulations, viral vectors, and single/bivalent
110 DNA plasmids, yet few have demonstrated sufficient protection as compared to natural
111 immunity to warrant late stage clinical development [8]. Interestingly, the glycoprotein B
112 (gB/MF59) subunit vaccine achieved nearly 50% efficacy in preventing HCMV primary
113 infection in distinct cohorts of HCMV seronegative postpartum women and adolescent
114 girls [9, 10]. Moreover, in HCMV seronegative patients receiving organs from HCMV

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115 seropositive donors, the gB/MF59 vaccine was shown to reduce the magnitude and
116 duration of posttransplant HCMV viremia [11]. gB is a viral fusion protein, essential for
117 viral infection of host cells, and is a target of both neutralizing and non-neutralizing
118 antibodies [12]. Counterintuitively, the gB/MF59 vaccination elicited limited neutralizing
119 responses when compared with HCMV seropositive individuals [13, 14]; yet, gB/MF59
120 vaccination elicited effector-cell mediated antibody responses, including antibody
121 dependent cellular phagocytosis (ADCP). This observation prompted a hypothesis that
122 non-neutralizing antibodies against gB may contribute to protection against HCMV
123 acquisition [13, 14]. Moreover, effector antibody functions mediated by NK cells may be
124 crucial for targeting HCMV-infected cells, further underlying the significance of non-
125 neutralizing antibodies in anti-HCMV immunity [15, 16].

126 gB is a homotrimeric class III fusion protein that contains 5 defined antigenic
127 domains, namely AD1 - AD5, with AD2 site 1, AD4 (Domain II), and AD5 (Domain I) as
128 the target of neutralizing antibodies, and AD2 site 2, AD1, and the furin cleavage site as
129 reported primary targets of non-neutralizing antibodies [12, 17]. The transmembrane and
130 intravirion domains of gB (AD3), are thought to be obscured from antibody recognition in
131 the membrane-associated portion. Yet, the epitope-specific antibody responses
132 stimulated by natural HCMV infection and gB/MF59 vaccination demonstrate AD3
133 immunodominance, with 76% of total linear gB-specific antibody responses directed at
134 this region in naturally HCMV infected vs 32% in gB/MF59 vaccinated individuals [12].
135 Variation in epitope-specific antibody binding may also stem from gB genotype-specific
136 differences amongst clinical HCMV strains [17]. In fact, differences amongst HCMV gB
137 genotypes correlate with cell tropism during HCMV infection [18]. Interestingly, it was

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138 recently reported that the ability of gB/MF59 vaccine sera to bind to membrane associated
139 gB, via a transfected cell binding assay, predicts risk of HCMV acquisition [19].
140 Furthermore, like HSV gB and VSV protein G, HCMV gB is undergoes significant
141 conformational transition from prefusion to postfusion states to mediate viral and host-cell
142 membrane fusion [20, 21]. Thus, the CMV vaccine field needs characterization of
143 antibodies that bind distinctly to soluble postfusion gB constructs and cell-associated gB,
144 with the latter possibility representing a distinct prefusion-like structure.

145 Because there are stark differences in the gB epitope binding profiles of naturally-
146 infected individuals and gB/MF59 vaccinees [13, 22], closing the gap in our understanding
147 of immune-protection offered by prior exposure to natural HCMV infection and vaccination
148 will require investigation into how gB as an antigen induces potentially protective effector
149 antibody responses. The aim of this study is to characterize the non-neutralizing gB-
150 specific antibody responses in natural HCMV infection for a panel of gB-specific mAbs
151 isolated by memory B cell cultures from three naturally infected individuals [23]. Towards
152 this goal, we analyzed a panel of 24 mAbs by defining their target specificity for gB
153 domains as well as genotype specificity, and analyzed effector functions for these mAbs
154 in ADCC and ADCP assays. Understanding gB diversity, and how gB-specific antibodies
155 bind and mediate antiviral functions across genotypes to different antigenic domains, will
156 be critical if we hope to optimize anti-gB antibody responses in future iterations of a HCMV
157 vaccine.
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161 **Methods:**

162 **gB-specific monoclonal antibodies (mAbs)**. gB-specific monoclonal antibodies
163 (mAbs) were isolated from healthy adult volunteers with previous natural HCMV
164 infection. Subjects were consented for blood sampling in accordance with NIH
165 guidelines [23]. B cell isolation and recombinant monoclonal antibody isolation and
166 production using a human IgG1 backbone was performed as previously described [23].
167 Briefly, total RNA from isolated memory B cells, which screened positive for binding
168 activity against gB, was converted to cDNA using a reverse transcription kit (Invitrogen),
169 and IgG genes were identified by PCR primers. After cloning the variable regions of gB-
170 specific mAbs from B cells from three donors the VH and VL sequences were cloned
171 into a human IgG1 vector and recombinantly expressed in HEK293 cells in Zhiqiang
172 An's laboratory at the University of Texas Health Science Center at Houston.
173

174 **Postfusion gB ectodomain trimer and gB domain protein expression and**
175 **purification.** A pαH expression plasmid encoding an artificial signal sequence, residues
176 32-692 of HCMV gB from strain AD-169 with solubilized fusion loops, as described
177 previously [12, 24], and C-terminal 6xHis and TwinStrep tags was used to transiently
178 transfect 293F cells. Secreted postfusion gB trimers were purified using Strep-Tactin
179 resin (IBA Life Sciences) before being run over a Superose 6 size-exclusion column
180 (GE Healthcare) in 2 mM Tris pH 8.0, 200 mM NaCl and 0.02% NaN_3. Sequences
181 encoding HCMV glycoprotein B (gB) Domain II (AA 112-133 + 143-438) from the Merlin
182 strain was tagged at the 5' end with the UL132 signal peptide sequence
183 MPAPRGLLRATFLVLVAFGLLLHMDFS and hemagglutinin (HA) tag, and at the 3' end

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184 with an avidin and polyhistidine tag. The discontinuous sequence encoding gB Domain
185 II (AA 112-133 + 343-438) was joined with the flexible linker Ile-Ala-Gly-Ser-Gly. For
186 Merlin strain gB Domain I (AA133-343), the 5' HA and 3' avidin/polyhistidine tags
187 omitted due to hypothesized steric hinderance. Nucleotides were codon optimized for
188 mammalian cells, synthesized *de novo* (Genscript), then cloned into pcDNA3.1(+)
189 mammalian expression vector (Invitrogen) via *Bam*HI at the 5′ end and *Eco*RI site at the
190 3′ end. Plasmids were transiently transfected into 293F suspension cells as previously
191 described using polyethyleneimine transfection reagent (Sigma-Aldrich) [25].
192 Supernatant was harvested 5 days later, and purified using Nickel-NTA resin for gB
193 Domain II (Thermo Fisher Scientific), and lectin resin (VWR) for gB Domain I. Purity and
194 identity were confirmed by Western blot using polyclonal CMV IgG (Cytogam – CSL
195 Behring) or monoclonal antibodies SM10 (Domain I) and SM5-1 (Domain II).
196
197 **Mapping gB-specific mAb domain specificity by BAMA.** Antibody responses against
198 full length gB protein (only missing the transmembrane domain), gB ectodomain, and gB
199 domains were measured as previously described [26]. Carboxylated fluorescent beads
200 (Luminex) were covalently coupled to purified HCMV antigens and incubated with
201 monoclonal antibodies in assay diluent (phosphate-buffered saline, 5% normal goat
202 serum, 0.05% Tween 20, and 1% Blotto milk). The gB domain antigen panel, included full
203 length gB (generously provided by Sanofi), gB ectodomain, gB domain I, gB domain II,
204 gB AD-1 (myBiosource), and biotinylated linear gB AD-2 site 2 (biotin-
205 AHSRSGSVQRVTSS), and biotinylated linear gB AD-2 site 1 (biotin-NETIYNTTLKYGD)
206 are previously described [13]. All gB proteins are based on the Towne strain gB (genotype

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207 1). CMV glycoprotein–specific antibody binding was detected with phycoerythrin-
208 conjugated goat anti-human IgG (2 μg/mL, Southern Biotech). Beads were washed and
209 acquired on a Bio-Plex 200 instrument (Bio-Rad), and results were expressed as mean
210 fluorescence intensity. A panel CMV seronegative plasma samples (n=30) were included
211 to determine nonspecific baseline levels of binding. Minimal background activity was
212 observed, so the threshold for positivity for each antigen was set at the mean value (100
213 MFI) of negative control sera to each antigen + two standard deviations. Blank beads
214 were used in all assays to account for nonspecific binding. All assays included tracking
215 of CMV immunoglobulin (Cytogam, generously gifted by CSL Behring) standard by Levy-
216 Jennings charts. The preset assay criteria for sample reporting were coefficient of
217 variation per duplicate values of ≤20% for each sample and ≥100 beads counted per
218 sample. All mAbs were analyzed at a concentration of 30 μg/mL for each antigen: full-
219 length gB (gift of Sanofi), gB ectodomain, gB Domain I, gB Domain II, gB AD-1 and gB
220 AD-2 sites 1 and 2. This concentration was predetermined to be within the linear range
221 of binding based on testing serial dilutions of a small subset of gB specific mAbs (1-155,
222 1-235, 2-43, 1-189, 3-54, and 3-74).
223
224 **gB-specific mAb binding strength measured via ELISA.** gB-specific monoclonal
225 antibody binding responses were measured against full length gB protein (Sanofi), gB
226 regional epitopes (Ectodomain, Domain I), or gB peptides (AD2 site 1, AD2 site 2). All
227 gB constructs were solubilized in 0.1 M $NaHCO_3$, with proteins plated at a concentration
228 of 3 μg/mL and peptides at 10 μg/mL respectively. Plates were washed then blocked
229 before adding gB-specific mAbs at either 5 μg/mL for conformational protein ELISAs or

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230 100 µg/mL for peptide ELISAs. After incubation with mAbs, plates were washed and
231 incubated with a 1:5000 goat-anti human HRP conjugated IgG secondary (Jackson
232 ImmunoResearch). ELISAs were developed with SeraCare ELISA kit and read at 450
233 nm.
234

235 **Assessment of gB-specific mAb avidity via surface plasmon resonance (SPR).**
236 Postfusion trimeric gB ectodomain was captured on an NTA sensor chip to ~500
237 response units (RUs) per cycle using a Biacore X100 (GE Healthcare). The chip was
238 doubly regenerated using 0.35 M EDTA and 0.1 M NaOH followed by 0.5 mM $NiCl_2$.
239 Three samples containing only buffer were injected over both ligand and reference flow
240 cells, followed by single injections of each mAb at a concentration of 25 nM. Samples
241 that did not initially result in interpretable sensorgrams were repeated using a
242 concentration of 250 nM. The resulting data were double-reference subtracted and fit to
243 a 1:1 binding model using the Biacore X100 Evaluation software.
244

245 **gB genotype-specific transfected cell binding.** HEK293T cells were cultured
246 overnight to ~50% confluency in a T25 flask, and then co-transfected using TransIT-
247 mRNA Transfection Kit (Mirus Bio) with a GFP-expressing mRNA plasmid (Miltenyi
248 Biotec) and a second plasmid encoding either full length gB from genotypes 1, 2, 3, 4,
249 or 5 (UPenn, Drew Weissman). Transfected cells were incubated at 37℃ and 5% CO_2
250 for 24 hours, washed with PBS once, and detached using 0.05% trypsin + EDTA
251 (Thermo Fisher Scientific). Cells were re-suspended in DMEM complete medium and
252 counted using Countess Automated Cell Counter (Invitrogen). 100,000 cells were

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253 placed in tubes and stained with LIVE/DEAD Aqua Dead Cell Staining Kit (Thermo
254 Fisher Scientific) diluted 1:1000 at room temperature for 20 minutes. 50,000 live cells
255 were placed in each well of a 96-well V-bottom plate (Corning). The plates were
256 centrifuged at 1,200 x g for 5 minutes and the supernatants were aspirated. Cells were
257 incubated with monoclonal antibodies, which were diluted to 5 μg/ml in duplicate in
258 DMEM complete medium, at 37℃ and 5% CO_2 for 2 hours. After washing with wash
259 buffer (PBS + 1% FBS) twice, cells were incubated with PE-conjugated mouse anti-
260 human IgG Fc (Southern Biotech) diluted 1:200 at 4℃ for 30 minutes. Following two
261 additional wash steps, cells in tubes and plates were resuspended and fixed in PBS +
262 10% formalin for 10 minutes at room temperature. Fixed cells were washed once and
263 resuspended in wash buffer for flow cytometry. Events were acquired on LSR II
264 machine (BD biosciences) using high-throughput sampler (HTS). Data were analyzed
265 with Flowjo software (Tree Star, Inc.), and the PE+ population was identified from the
266 live GFP+ cell population for each sample. Non-specific binding of PE-conjugated
267 mouse anti-human IgG Fc was corrected in the analysis.
268

269 **Natural killer (NK) cell CD107a degranulation assay:** Cell-surface expression of
270 CD107a was used as a marker for NK cell degranulation (7, 8). ARPE cells were plated
271 at $3x10^4$ cells/well in a 96-well flat-bottom tissue culture plate and allowed to incubate
272 for 24 hours at 37°C. Cells were infected with AD169r-GFP at an MOI of 1.0 or
273 transfected with gB-encoding mRNA using the TransIT mRNA transfection kit (Mirus
274 Biosciencies), then incubated a further 48 hours at 37°C. Following incubation,
275 supernatant was removed and the transfected or infected cell monolayers were washed

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276 once with RPMI 1640 containing 10% FBS, HEPES, Pen-Strep-L-Glut, Gentamicin (R10
277 media) before addition of NK cells. Primary human NK cells were isolated from
278 peripheral blood mononuclear cells (PBMC) after overnight rest in R10 media with
279 10ng/mL IL-15 (Miltenyi Biotech) by depletion of magnetically labeled cells (Human NK
280 cell isolation kit, Miltenyi Biotech). $5x10_4$ live NK cells were added to each well
281 containing gB transfected or HCMV-infected ARPE19 cell monolayers. mAbs were
282 diluted in R10 and added to the cells at a final dilution of 25μg/mL in duplicate. Brefeldin
283 A (GolgiPlug, 1 µl/ml, BD Biosciences), monensin (GolgiStop, 4µl/6mL, BD
284 Biosciences), and CD107a-FITC (BD Biosciences, clone H4A3) were added to each
285 well and the plates were incubated for 6 hours at 37ºC in a humidified 5% CO_2
286 incubator. NK cells were then gently resuspended, taking care not to disturb the ARPE-
287 19 cell monolayer, and the NK containing supernatant was collected and transferred to
288 96-well V-bottom plates. The recovered NK cells were washed with PBS, and stained
289 with LIVE/DEAD Aqua Dead Cell Stain at a 1:1000 dilution for 20 minutes at room
290 temperature. The cells were then washed with 1%FBS PBS and stained for 20 minutes
291 at room temperature with the following panel of fluorescently conjugated antibodies
292 diluted in 1%FBS PBS: CD56-PECy7 (BD Biosciences, clone NCAM16.2), CD16-
293 PacBlue (BD Biosciences, clone 3G8), and CD69-BV785 (BioLegend, Clone FN50).
294 The cells were then washed with 1%FBS PBS, fixed and permeabilized for 20 minutes
295 at 4ºC (BD Fixation/Permeabilization solution), washed with BD Perm/Wash, and
296 stained for intracellular interferon (IFN)-γ-BV711 (BioLegend, clone 4S.B3) and tumor
297 necrosis factor-alpha (TNF)-α- BV650 (BD Biosciences, clone Mab11) for 30 minutes at
298 4ºC. The cells were then washed twice and re-suspended in 1% paraformaldehyde

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299 fixative for flow cytometric analysis. Data analysis was performed using FlowJo software
300 (v9.9.6). Data is reported as the % of CD107a+ live NK cells (singlets, lymphocytes,
301 aqua blue-, CD56+ and/or CD16+, CD107a+). The threshold for positivity is the mean
302 response of preimmune samples plus two standard deviations. CD69, TNFα, and IFNγ
303 were not included in the final analysis due to the low frequency of CD107a+ responses.
304

305 **Antibody dependent cellular phagocytosis (ADCP):** Approximately 3.5x10$_6$ PFU of
306 concentrated, sucrose gradient-purified AD169r-GFP virus was transferred to a 100,000
307 kDa Amicon filter (Millipore), then buffer exchanged with 1x PBS, concentrated down to
308 approximately 100 µL, and transferred to a microcentrifuge tube. Next, 10 µg of AF647
309 NHS ester (Invitrogen) reconstituted in DMSO was added to the concentrated, purified
310 virus for direct fluorescent conjugation, then this reaction mixture was incubated at room
311 temperature for 1 hour with constant agitation. The reaction was quenched with 80 µL of
312 1 M Tris-HCl, pH 8.0, then the fluorophore-labelled virus was diluted 25x in wash buffer
313 (PBS + 0.1% FBS). mAbs were diluted to 0.1 mg/mL in wash buffer, then 10 µL of each
314 diluted mAb was combined with 10 µL of diluted, fluorophore-conjugated virus in a
315 round-bottom, 96-well plate and allowed to incubate at 37°C for 2 hours. Following this
316 incubation step, 50,000 THP-1 cells were added to each well, suspended in 200 µL
317 primary growth media. Plates were centrifuged at 1200x g and 4°C for 1 hour in a
318 spinoculation step, then incubated at 37°C for an additional hour. Cells were re-
319 suspended and transferred to a 96-well V-bottom plate, then washed twice prior to
320 fixation in 100 µL DPBS + 1% formalin. Events were acquired on LSR II machine (BD
321 biosciences) using the HTS. The % AF647+ cells was calculated from the full THP-1 cell

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322 population and reported for each sample. A cutoff for a sample mediating ADCP was
323 defined as > 99% AF647+ signal from THP1 cells incubated with fluorophore
324 conjugated virus and an HCMV seronegative control.
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345 **Results**

346 *Mapping the domain-specificity of non-neutralizing gB-specific mAbs from HCMV*
347 *seropositive individuals*

348 We first mapped the binding specificity of the panel of gB-specific mAbs for defined
349 neutralizing and non-neutralizing antigenic domains of gB by BAMA. The most frequent
350 binding specificity was against Domain II (37.5%), as well as AD2 site 2 (12.5%) and
351 AD3/MPER (12.5%) (Figure 1A). While 4 mAbs demonstrated detectable binding to
352 both linear AD2 Site 1 and Site 2, these mAbs are likely AD2 Site 2 specific, given that
353 previously described AD2 Site 1 mAbs have neutralizing activity [27, 28]. Interestingly,
354 there were three mAbs, 1-155, 1-237, and 3-18, which bound full length gB, (Sanofi),
355 but did not bind a gB ectodomain that excludes the membrane proximal region (MPER)
356 and the cytodomain containing AD3, suggesting specificity for one of these regions.
357 There are two groups of clonal populations of mAbs found in Individual 1, including
358 mAbs 1-189, 1-190, 1-191, 1-192 which bind full length gB and gB ectodomain
359 conformationally, but not to a defined epitope (termed “conformational” binding), and
360 mAbs 1-223 and 1-224. Antigenic domain-specificity of gB-specific mAbs was
361 heterogenous in Individual 1, but was dominated by Domain II in individuals 2 (57.1%)
362 and 3 (66.7%) (Figure 1B). The binding profile for the combined panel of 24 gB-specific
363 mAbs notably excludes any AD1 or AD5 (Domain I) binding mAbs, despite the
364 polyclonal CMV-IgG preparation Cytogam binding to all antigens (data not shown).
365

366 *mAb binding kinetics to full length gB and trimeric post-fusion gB ectodomain*

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367 Next, we sought to describe the binding kinetics of each gB-specific mAb to the full
368 length gB protein vs a trimeric post-fusion gB ectodomain construct. By understanding
369 discrepancies in binding between full length gB and the post-fusion gB ectodomain, we
370 aimed to highlight novel HCMV AD3 or MPER-specific mAbs and their binding kinetics.
371 Multiple mAbs (1-155, 1-189, 1-191, 1-192, 1-237) exhibited poor binding strength to
372 post-fusion gB (ectodomain), but retained robust binding to full length gB. MAbs 3-18
373 and 3-58 exhibited poor gB binding to both full length gB and post fusion gB ectodomain
374 (Table 1).

375 Three isolated gB-specific mAbs with binding to both the linear AD2 Site 1 and
376 Site 2 regions, regardless of individual donor, demonstrated both high binding strength
377 and avidity for full length gB (EC_{50} 0.001 – 0.009 μg/mL) and gB ectodomain (0.002 –
378 0.013 μg/mL) (Table 1). gB mAbs with AD2 site 2 only specificity also had robust
379 binding to full length gB (EC_{50} 0.003 – 0.006 μg/mL) as well as gB ectodomain (0.004 –
380 0.008 μg/mL) (Table 1). Of the mAbs which bound Domain II, all except three, 1-228, 3-
381 58 and 3-74, showed strong binding to both full length gB and gB ectodomain (EC_{50} <
382 0.020 μg/mL) (Table 1). Specifically, mAb 3-74, which binds Domain II predominantly,
383 demonstrated poor binding and avidity to both full length gB and gB ectodomain by
384 ELISA and SPR. Two Domain II-specific mAbs bound with an interesting pattern to gB
385 ectodomain. Domain II-specific mAb 1-228 had strong binding to full length gB (EC_{50}
386 0.001 μg/mL) but very poor binding to gB ectodomain by ELISA (EC_{50} 8.014 μg/mL) and
387 no detectable (N.D.) avidity by SPR (Table 1). Further, Domain II-specific mAb 3-58 had
388 poor binding strength to both full length gB (EC_{50} 5.73 μg/mL) and gB ectodomain (EC_{50}
389 20.56 μg/mL) (Table 1).

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390 MAbs which bound to the full length gB and not the ectodomain in the gB domain
391 mapping, and therefore potentially bind AD3/MPER, 1-155 and 1-237, followed
392 predictable patterns of binding with strong binding to full length gB ($EC_{50} < 0.004$ μg/mL)
393 and negligible binding strength and avidity to gB ectodomain (Figure 1A, Table 1). mAb
394 3-18, also determined to be AD3/MPER specific by BAMA, demonstrated poor binding
395 measured by both ELISA ($EC_{50} > 100$ μg/mL) and SPR (K_D 22.5 nM) to the ectodomain
396 as well Finally, those mAbs which didn't have a definable epitope specificity by gB
397 domain mapping, and termed "conformational", had highly variable binding strength to
398 gB ectodomain, but fairly strong binding to full length gB. Interestingly, mAb 2-32, which
399 bound both full length gB (EC_{50} 0.003 μg/mL) and gB ectodomain (EC_{50} 0.006 μg/mL)
400 well via ELISA, as well as has binding detected to both the full length gB and gB
401 ectodomain antigens via BAMA (Figure 1A), demonstrated undetectable binding to gB
402 ectodomain by SPR (N.D., not detectable).
403
404 *Cell associated gB DNA transfected-cell binding*
405 We have recently discovered that the magnitude of binding to gB DNA transfected cells
406 is a correlate of protection against primary HCMV acquisition in postpartum and
407 adolescent women vaccinated with gB/MF59 in phase II clinical trials [19]. Next, we
408 investigated whether binding to cell associated gB is dependent upon gB-specific mAb
409 antigenic site specificity. gB-specific mAbs with AD-2 specificity demonstrated high
410 magnitude binding to cell associated gB more consistently than mAbs from any other
411 specificity represented in our panel (Figure 2A). AD-3 specific mAbs bind poorly to cell
412 associated gB, consistent with epitope location in the transmembrane and cytosolic

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413 compartment [12]. Notably, binding to cell associated gB was not correlated to strength
414 of binding to soluble gB measured by ELISA (Figure 2B). These findings demonstrate
415 that gB-mAbs of various specificities, except for AD3, are capable of binding cell
416 associated gB with high magnitude.
417
418 *Cell associated gB genotype-specific mAb binding*
419 We previously reported that gB/MF59 vaccinees may have had reduced acquisition of
420 HCMV strains with gB1 genotype, the genotype matched to the vaccine construct,
421 suggesting strain-specific protection [29]. As such, we next explored how non-
422 neutralizing gB-specific mAbs differentially recognize gB genotypes 1-5, as expressed
423 on the surface of a cell. Comparison of total, and genotype specific, gB transfected cell
424 bound populations of each gB-specific mAb (Figure 3A) highlights significant variability
425 for cell-associated gB binding across epitope specificities. While a majority of gB-
426 specific mAbs bound all 5 genotypes, there was unequal binding magnitude across
427 genotypes for the same mAb. To compare genotype preference amongst gB-specific
428 mAbs (Figure 3B), we next assessed the percent representation of each genotype in the
429 sum total gB genotype binding for each mAb. Here, there emerged a clear preference
430 for gB-specific mAbs isolated from these naturally-infected donors for gB genotypes 2
431 and 4, representing the cumulative genotype preference for 11 of 14 gB-specific mAbs
432 with gB genotype specific binding (Figure 3C). Genotype-dominant binding was
433 determined by mAbs which bound a single gB genotype with a % PE positive population
434 greater than 5x the % PE positive population of the lowest bound gB genotype. These
435 findings are consistent across epitope specificities. Interestingly, mAbs which were

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436 unable to bind gB ectodomain measured by ELISA or BAMA, but bound full length gB
437 and classified as AD3 specific, demonstrated equivalent cell associated gB binding
438 across all tested gB genotypes (Figure 3A). These data implicate differences in epitope
439 presentation of soluble gB and cell-associated gB.

440

441 *Antibody dependent cellular cytotoxicity (ADCC)*

442 Antibody-mediated NK cell cytotoxicity has been demonstrated as a crucial mechanism
443 for controlling HCMV infection [30, 31]. To distinguish the domain specificity of gB-
444 specific mAbs which mediate ADCC, we next screened our panel of mAbs for ability to
445 mediate two well defined NK phenotypes of cytotoxic killing, CD107a upregulation,
446 indicative of degranulation, (Figure 4A) and CD16 downregulation, indicative of NK cell
447 activation (Figure 4B) in the presence of HCMV AD169r infected cell targets. While a
448 number of mAbs across multiple domains demonstrated measurable CD107a
449 degranulation, they were comparable to the negative control Synagis (mAb against
450 respiratory syncytial virus). Next, a more specific marker of NK cell activation, CD16
451 downregulation calculated into a downregulation index (DRI), was measured for each
452 gB-specific mAb. Notably, no gB-specific mAb demonstrated significant CD16
453 downregulation whereas HCMV hyperimmunoglobulin (Cytogam) had demonstrable
454 activity.

455

456 *gB AD2 Site 1/Site 2 and Domain II-specific mAbs 1-235 and 3-74 mediate ADCP*

457 In a study of functional antibody responses to gB/MF59 vaccination, vaccinees were
458 found to demonstrate limited neutralization responses, but robust ADCP [13]. To identify

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459 the domain specificity of gB-specific mAbs which can mediate ADCP, we screened our
460 panel of gB-specific mAbs for ability to mediate whole virion phagocytosis (Figure 5A).
461 Two gB-specific mAbs, 1-235 and 3-74, mediated phagocytosis of AD169r-GFP virions,
462 which exceeded the baseline ADCP level of non-specific mAbs and seronegative
463 control plasma by 19.4% and 14.7% respectively. To better assess their ADCP potency
464 in both THP-1 cells and primary monocytes isolated from healthy donors (Figure 5B),
465 mAbs 1-235 and 3-74 were titrated from a concentration of 100 μg/mL. ADCP increased
466 in a dose-dependent manner for 1-235 and 3-74 in both THP1 cells and primary
467 monocytes. Importantly, the gB epitope specificity of ADCP mediating mAb 1-235 was
468 AD2 Site 1 and Site 2, while 3-74 specificity was Domain II (Figure 1A).
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482 **Discussion**

483 Identification of the dominant non-neutralizing antibody responses elicited by the
484 partially-protective gB/MF59 vaccine has suggested a shift in the paradigm of
485 immunogenicity endpoints for consideration when designing the next generation of
486 HCMV vaccines [13]. While protective immunity through virus neutralization to prevent
487 primary infection is considered the "gold standard" of HCMV vaccine development, next
488 generation vaccine regimens will need to consider these non-neutralizing anti-viral
489 antibodies. Indeed, strategies such as cell-to-cell spread enable immunologically-covert
490 spread of infection even in the presence of neutralizing antibodies [32]. In fact,
491 prophylactic passive immunization with both neutralizing and non-neutralizing mAbs
492 were considered equally protective in a murine cytomegalovirus challenge model [33].
493 Accordingly, non-neutralizing antibodies which mediate effector functions like ADCP,
494 like those described in this study, may have potential importance as immunological
495 endpoints of a protective HCMV vaccine.

496 A majority (> 90%) of gB-specific antibodies from B cell clones have no
497 neutralizing activity [34]. Indeed, the partially-protective gB/MF59 vaccine elicited limited
498 heterologous neutralization [13]. While the specificity and characteristics of neutralizing
499 gB-specific mAbs have been well described, the types of naturally-elicited gB-specific
500 mAbs that mediate non-neutralizing effector functions which may be critical for
501 protection against HCMV acquisition remain to be fully characterized.

502 In this study, 24 non-neutralizing gB-specific mAbs isolated from naturally
503 HCMV-infected individuals were assessed for epitope binding specificity and affinity, gB
504 genotype preference, and Fc-mediated effector functions. These non-neutralizing mAbs

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505 bound predominantly to Domain II, or to one or both of the binding sites of AD2. Domain
506 II is largely the target of neutralizing antibodies, with one study identifying Domain II-
507 specific binding by neutralizing antibodies in greater than 90% of HCMV seropositive
508 subjects tested. Interestingly, three mAbs from the panel demonstrated robust binding
509 to the full length gB protein but failed to bind a gB ectodomain, suggesting specificity
510 within the AD3 or MPER region, a portion of the gB construct located in the cytodomain
511 of membrane associated gB [12]. This potentially hidden region on an intact virion or
512 infected cell could certainly be exposed through protein shedding from cell lysis or
513 disruption of the HCMV virion [35], similarly to other viral structural antigens such as
514 pp150 and pp28. However, it also raises questions regarding the structure of this
515 membrane-associated region of the protein. Interestingly, none of the non-neutralizing
516 gB-specific mAbs in this panel had appreciable binding to AD1 or Domain I. AD1 is
517 targeted by both neutralizing and non-neutralizing mAbs [34, 36]. As such, the lack of
518 Domain I and AD1 binding from this representative panel could be the result of sampling
519 bias from only three individuals [23], by selection bias of excluding mAbs which bind to
520 traditionally neutralizing domains, or non-optimal conformation of the protein used for B
521 cell sorting to isolate these mAbs.

522 Antibodies mediating neutralization after gB/MF59 vaccination and natural
523 infection can be strain specific [13, 37] and may not provide equivalent degrees of
524 protection against all strains. It has been reported that breakthrough HCMV infections in
525 the vaccine subjects receiving gB/MF59, which is a gB genotype 1 based vaccine, were
526 more likely to be infected with HCMV strains expressing gB genotypes 3 or 5 than
527 strains 1, 2, or 4 [29]; evidence suggesting gB/MF59 induced antibodies were limited

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528 due to its strain-specific neutralization or strain-specific antibody effector functions. Our
529 study offers the first evidence that critical gB-specific non-neutralizing antibodies have
530 different strain specific gB recognition when displayed on the surface of a cell. This
531 panel of non-neutralizing gB-specific mAbs generally bound with greater preference to
532 gB genotypes 2 and 4. These findings are in concordance with phylogenetic clustering
533 of the 5 clinically significant HCMV gB genotypes 1/2/4 and 3/5 into two supergroups
534 [29]. The lack of knowledge regarding which HCMV gB genotype or genotypes infected
535 the individuals from which the mAbs were isolated limits the assertions we can make
536 about how gB genotype specific antibodies may be raised by natural infection. Clinically,
537 prevalence of HCMV gB variants may be influenced by geography, immune status, and
538 prior infection with HCMV of other gB variants [38-40]. Future efforts might address the
539 question of gB variant specific non-neutralizing recognition and antibody function by
540 utilizing mAbs isolated from individuals that have been tested for endogenous viral
541 strains or have known exposure to a specific gB genotype variant.

542 A notable finding from studying full-length gB-binding mAbs that may be AD3- or
543 MPER- specific, is their breadth of membrane associated gB binding. These mAbs, 1-
544 155, 1-237, and 3-18 demonstrate negligible gB ectodomain and postfusion gB trimer
545 binding by both ELISA and SPR yet yield comparable signals for transfected cell binding
546 to other gB ectodomain binding mAbs. With AD3 thought to be buried in the cytodomain
547 [12], these findings raise the question: how do presentation of gB epitopes differ as a
548 soluble protein versus a membrane-associated protein? As a viral fusogen, gB is
549 thought to undergo transformation from a prefusion conformation to a postfusion
550 conformation to facilitate entry into a host cell [21, 41]. While this study does not define

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551 a specific prefusion structure, it does highlight how a distinct prefusion-like gB on a cell
552 surface may expose different epitopes than soluble postfusion gB, potentially
553 accounting for improved membrane associated gB binding of AD3-specific mAbs.
554 Notably, it was recently reported that the ability of gB/MF59 vaccine sera to bind to gB
555 transfected cells predicts risk of HCMV acquisition [19]. Taken together, these findings
556 warrant further investigation of gB conformation-specific antibody binding, to parse out
557 the epitope binding specificity and effector functions of gB vaccine-elicited Abs.

558 This study demonstrates the first effort to identify gB domain specificity and
559 function of antibodies that mediate Fc receptor functions like ADCP and ADCC elicited
560 by natural HCMV infection. While little is known about gB-specific non-neutralizing
561 functions in naturally-infected individuals, investigations of gB/MF59 vaccinees has
562 shown this class of antibody may indeed be a desirable target of vaccines that aim to
563 protect against primary HCMV acquisition [13]. It is now evident that non-neutralizing
564 Abs are not limited to traditional “non-neutralizing” epitopes [12, 17]. Even more, these
565 mAbs, while preferentially binding to certain gB genotypes, retain the ability to bind
566 across a spectrum of gB genotype variants. Ultimately this work contributes to the field
567 of HCMV vaccinology by emphasizing the impact of epitope binding specificity, binding
568 strength, and genotype breadth amongst functional non-neutralizing gB-specific mAbs.
569 By improving our knowledge of gB immunogenicity elicited by natural infection, and the
570 specificity of antibody responses that may mediate key functions other than traditional
571 neutralization, this work informs rational design of new HCMV vaccines that aim to
572 reduce the devastating burden of HCMV disease in both congenital infection and
573 transplant settings.

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574 **Acknowledgements:**

575 The authors recognize Justin Pollara and Whitney Edwards for their assistance with the
576 antibody dependent cellular cytotoxicity assays. The gB-specific monoclonal antibodies
577 were kindly provided by Dai Wang at Merck through a collaboration with Zhiqiang An's
578 laboratory at the University of Texas Health Science Center at Houston. This work was
579 supported by grants from Merck Research Labs and the Welch Foundation (AU-0042-
580 20030616 to Z.A), NIH/ National Institute of Allergy and Infectious Disease R21
581 (R21AI136556 to S.R.P). Dr Permar consults for Merck, Pfizer, Sanofi, and Moderna
582 vaccine programs and has sponsored programs from Merck and Moderna around CMV
583 vaccine immunity. A patent application covering the mabs described in the article has
584 been submitted by Merck &Co., Inc. and University of Texas. D.W. is a current
585 employee and stockholders of Merck & Co., Inc.

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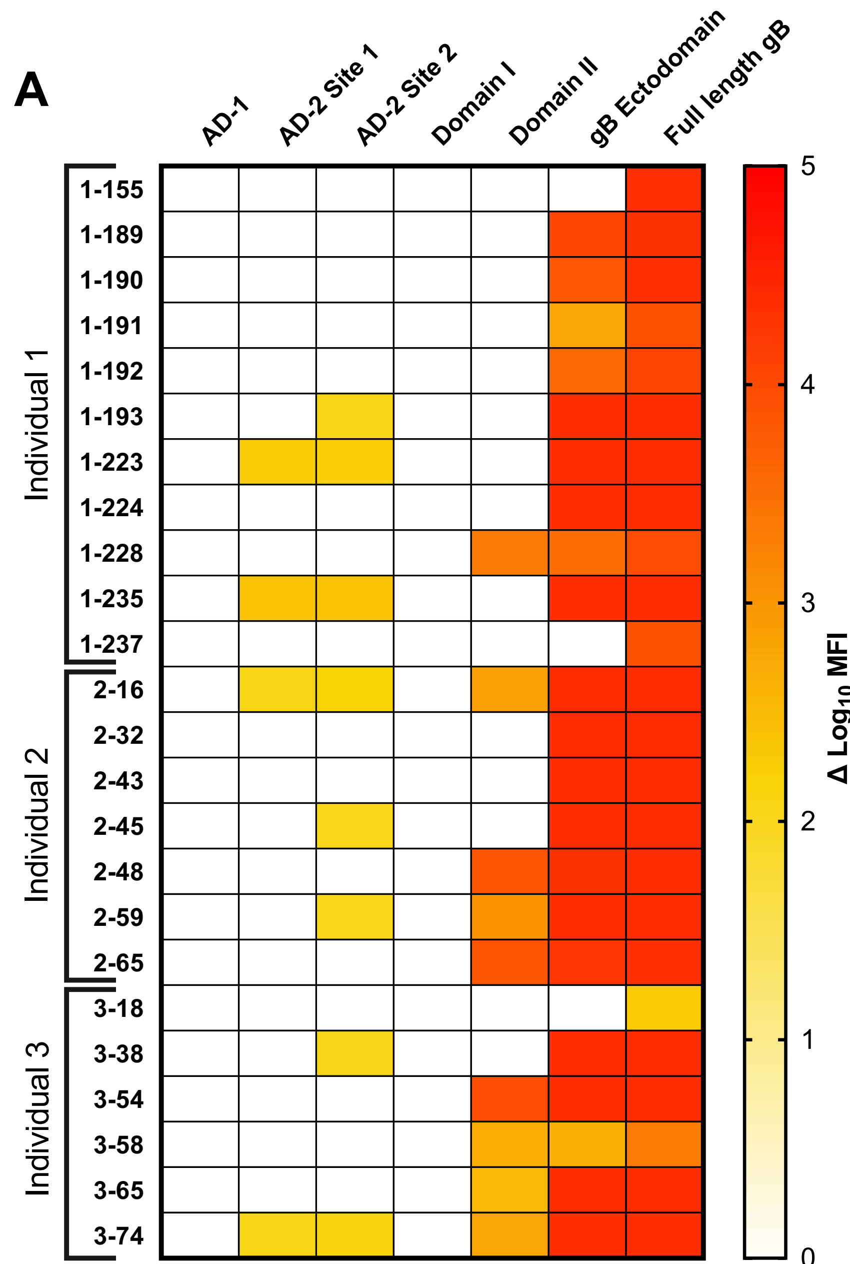


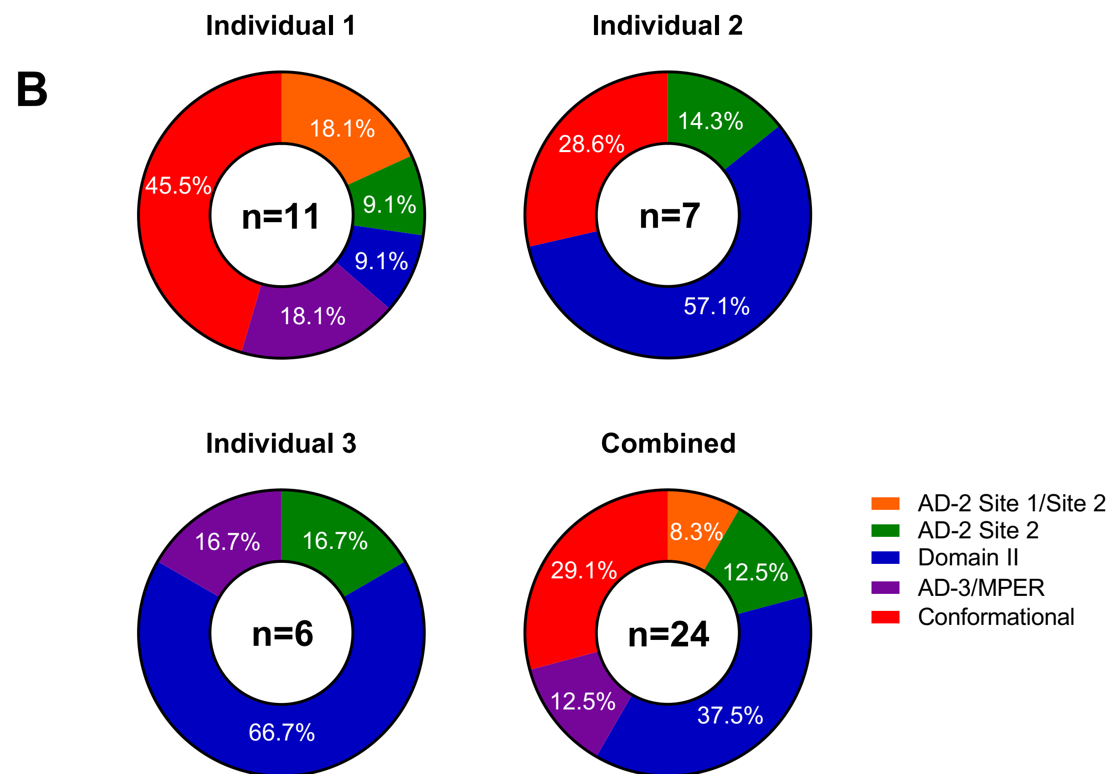
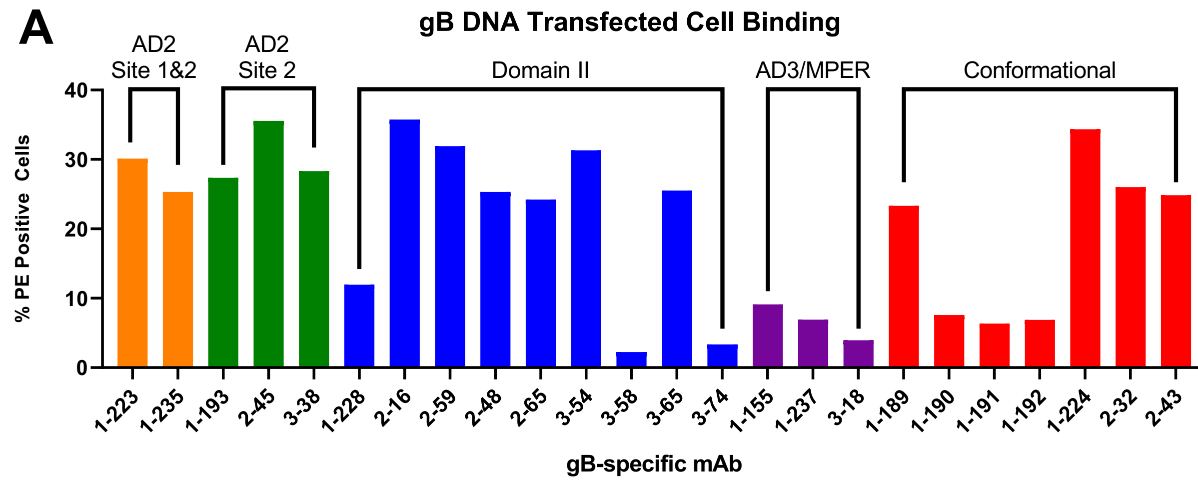


Figure 1. gB domain-specific mAb binding determined by BAMA. (A) Heat map of gB domain-specific binding strength for gB-specific mAbs represented as $\log_{10}$ mean fluorescent intensity (MFI). (B) Pie charts representing the percentage of total gB-specific mAbs binding to each domain specificity assessed by BAMA per each naturally HCMV infected individual and all individuals combined. AD-2 Site 1/Site 2: Binding to both the AD-2 Site 1 and Site 2 peptide, AD-2 site 2: binding to the site 2 peptide only, Domain II: binding to Domain II only, AD3/MPER: binding to full length gB, but not gB ectodomain, Conformational: binding to full length gB and gB ectodomain, but no other domain.

Monoclonal Antibody	Heavy Chain [a]	Light Chain [a]	CDRH3 Length [a]	Neutralization IC50 (µg/mL) [a]	Epitope Specificity	Full length gB binding EC_{50} (µg/mL) [b]	gB ectodomain binding EC_{50} (µg/mL) [b]	gB ectodomain apparent avdity KD (nM) [c]
1-155	IGHV1-69*01	IGLV2-11*01	14	0	AD3/MPER	0.002	6.903	6.2
1-189	IGHV1-69*01	IGKV4-1*01	11	0	Conformational	0.007	2.562	702
1-190	IGHV1-69*01	IGKV4-1*01	11	0	Conformational	0.019	3.716	1.88
1-191	IGHV1-69*01	IGKV4-1*01	11	0	Conformational	0.009	6.163	10.8
1-192	IGHV1-69*06	IGKV4-1*01	11	0	Conformational	0.010	> 100	116
1-193	IGHV1-18*01	IGKV2-30*01	22	1	AD2 Site 2	0.003	0.004	0.0003
1-223	IGHV1-69*03	IGLV2-23*01	22	8.3	AD2 Site 1/Site 2	0.009	0.013	0.301
1-224	IGHV1-69*03	IGKV3-20*01	22	6.4	Conformational	0.006	0.011	0.116
1-228	IGHV3-13*01	IGKV1-33*01	10	0	Domain II	0.001	8.014	N.D.
1-235	IGHV4-31*03	IGKV4-1*01	11	0	AD2 Site 1/Site 2	0.001	0.002	0.019
1-237	IGHV1-69*01	IGKV4-1*01	12	0	AD3/MPER	0.003	2.656	5.27
2-16	IGHV1-69*01	IGKV3-15*01	15	11	Domain II	0.002	0.004	0.0004
2-32	IGHV4-59*01	IGKV4-1*01	12	0	Conformational	0.003	0.006	N.D.
2-43	IGHV6-1*01	IGKV4-1*01	14	15	Conformational	0.003	0.004	0.0034
2-45	IGHV4-34*02	IGKV3-15*01	24	9.8	AD2 Site 2	0.003	0.006	0.0004
2-48	IGHV1-2*02	IGKV3-20*01	14	14.6	Domain II	0.003	0.006	0.113
2-59	IGHV4-34*02	IGLV1-47*01	18	2.3	Domain II	0.006	0.010	0.0016
2-65	IGHV1-2*02	IGKV3-20*01	14	6.4	Domain II	0.007	0.018	0.081
3-18	IGHV3-21*01	IGKV1D-8*01	26	0	AD3/MPER	16.370	> 100	22.5
3-38	IGHV1-18*01	IGKV4-1*01	14	0	AD2 Site 2	0.006	0.008	0.0003
3-54	IGHV1-46*01	IGKV1-5*03	22	1	Domain II	0.006	0.011	0.474
3-58	IGHV3-30*03	IGLV3-10*01	34	0	Domain II	5.733	20.560	1.82
3-65	IGHV3-33*01	IGKV4-1*01	16	0	Domain II	0.003	0.003	0.036
3-74	IGHV3-30-3*01	IGLV3-25*03	28	0	Domain II	0.601	0.723	1.52

Table 1. **gB-specific mAb immunogenetics, binding strength, and avidity to full length gB and gB ectodomain**

a. Heavy chain and light chain genes, neutralization IC50 (µg/mL), as well as CDRH3 length listed as described in (Xia et al 2017).
b. binding to full length gB and post fusion gB ectodomain EC_{50} (µg/mL) measured by ELISA.
c. binding kinetics to post fusion gB ectodomain measured by SPR



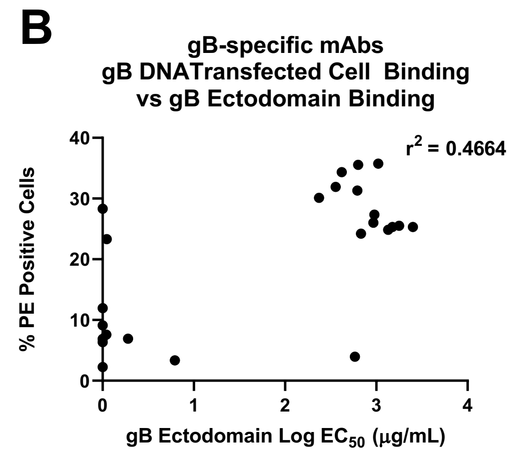


Figure 2. gB DNA transfected cell binding. (A) gB-specific mAb binding to cell-associated gB on the surface of gB DNA transfected cells. (B) gB-specific mAbs generally bind with high magnitude to cell-associated gB, but this binding strength is not highly correlated to strength of binding (EC_{50}) to soluble gB ectodomain measured by ELISA.

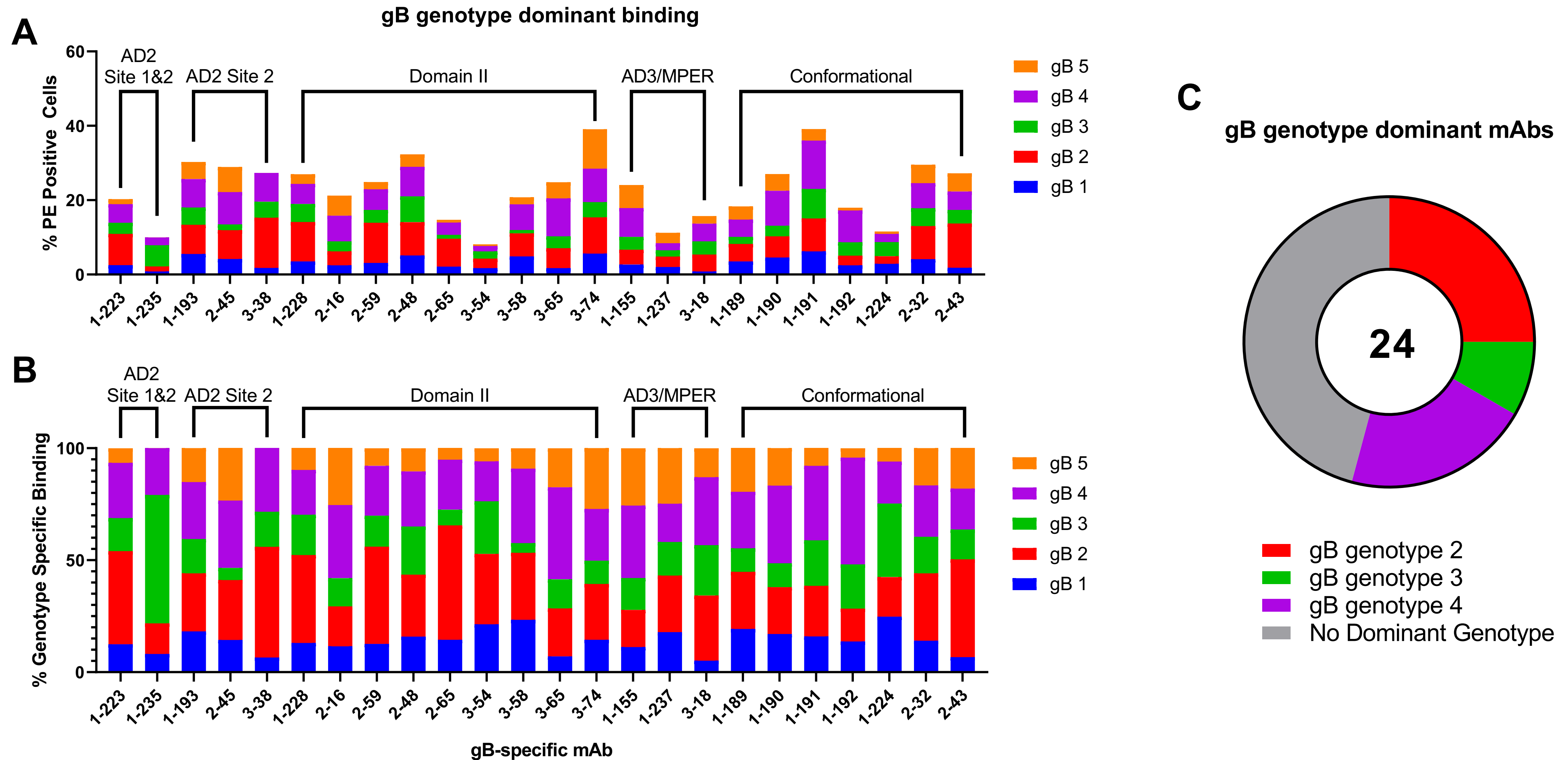


Figure 3. Cell associated gB genotype-specific mAb binding. (A) Percentage of gB genotype-specific transfected cells bound by gB-specific mAbs, grouped by domain specificity. (B) Percentage of gB genotype-specific transfected cells bound by the total population of gB transfected cells, demonstrating genotype preference for each gB-specific mAbs, listed by domain specificity. (C) gB-genotype preference for gB-specific mAbs defined if % PE positive population of a single transfected gB genotype was greater than 5 times the % PE positive population of the lowest bound cell-associated gB genotype by that mAb.

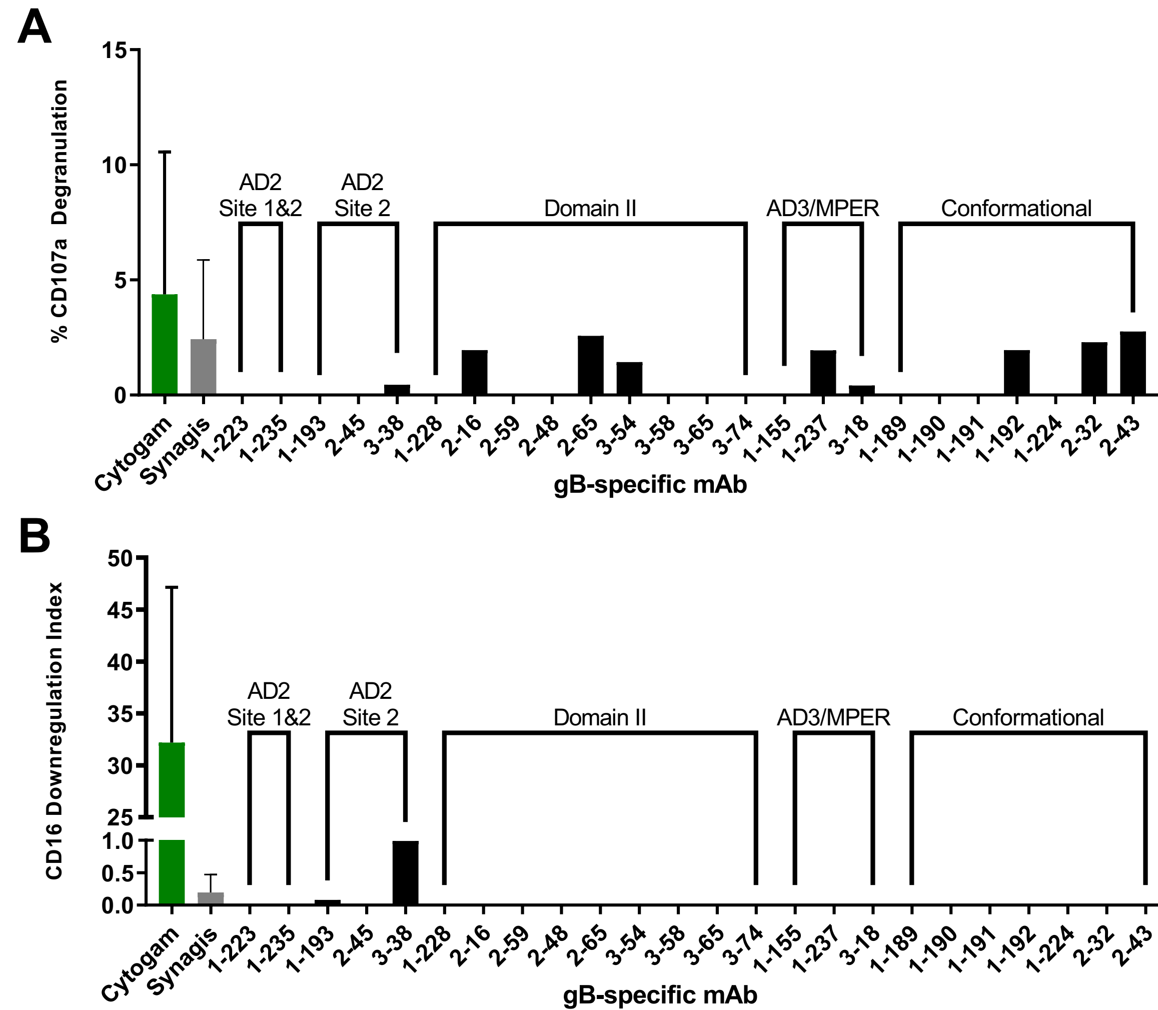


Figure 4. **gB-specific mAbs mediate negligible NK activation ADCC responses when compared to polyclonal CMV-specific antibody preparation (Cytogam).** gB-specific mAbs were screened for mediation of NK cell (A) CD107a degranulation and (B) CD16 downregulation (DRI) as measures of antibody-mediated NK cell activation. Data are presented as mAb specific response against AD169r-GFP infected ARPE19 cells minus mock infected negative control cells.

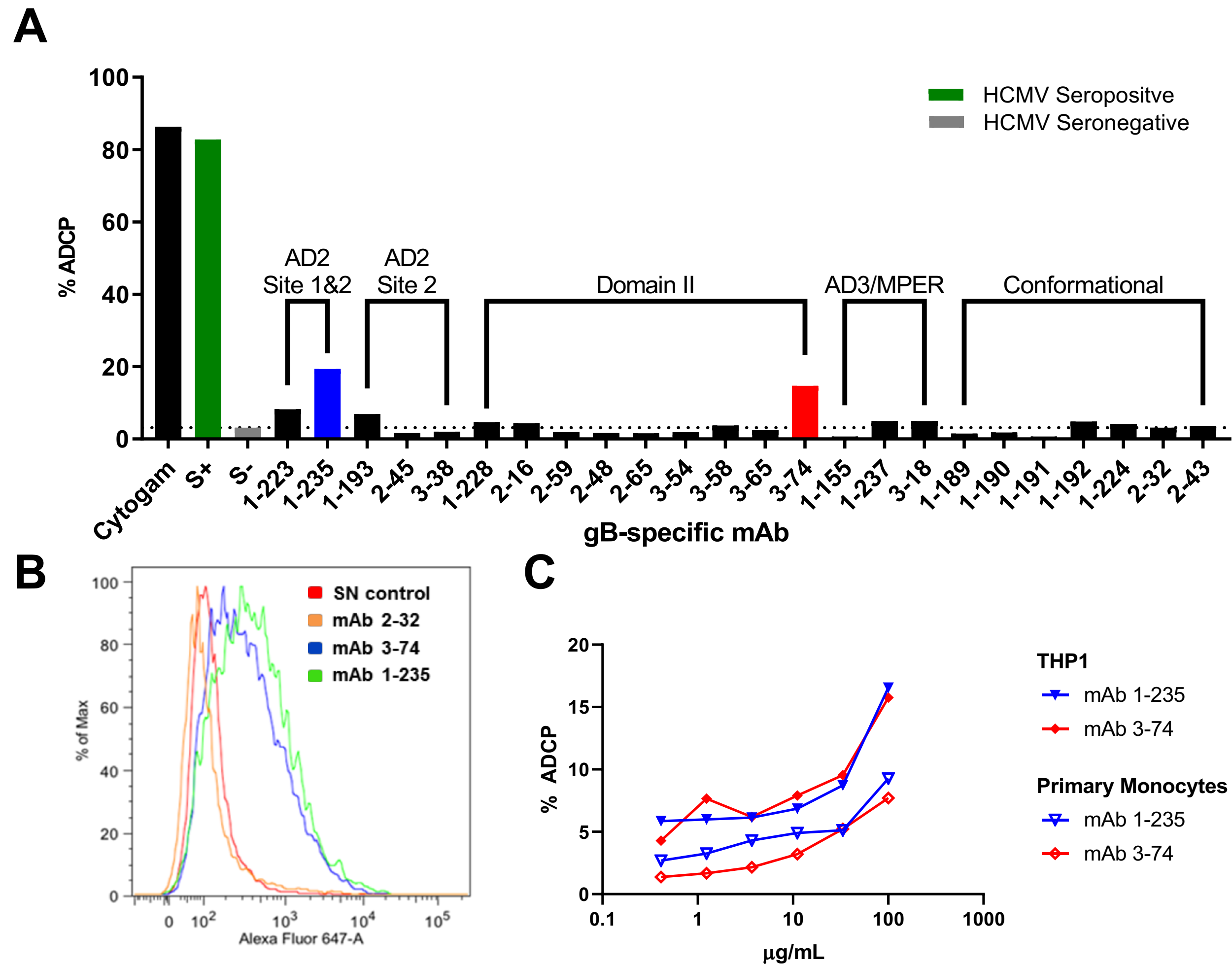


Figure 5. **gB AD2 and Domain-1 specific mAbs 1-235 and 3-74 mediate antibody dependent cellular phagocytosis (ADCP).** (A) gB-specific mAbs were screened for ADCP activity. (B) A representative histogram for ADCP mediating mAbs as compared to a HCMV seronegative control. The magnitude of ADCP was titrated over a dilution series for the two ADCP mediating mAbs using (C) THP1 cells (solid symbols) and primary monocytes (open symbols).