

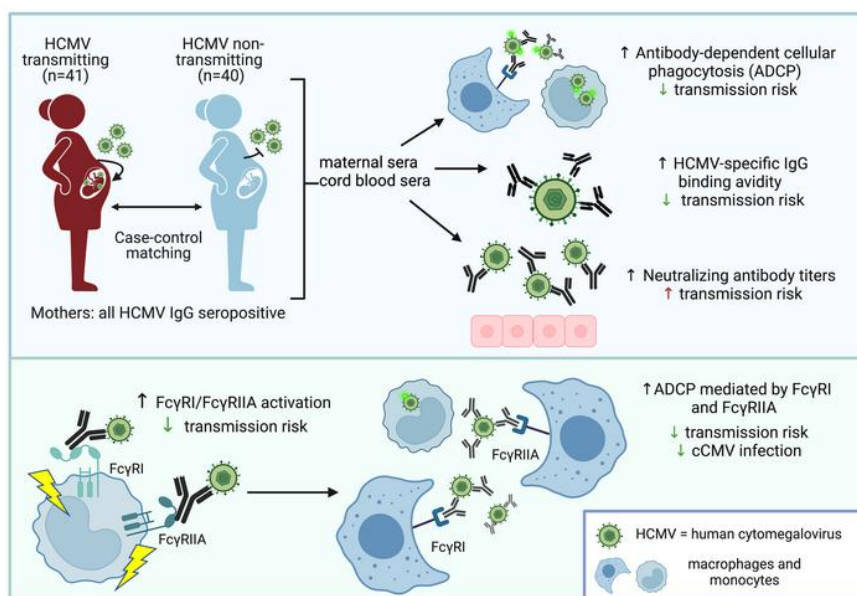
# Maternal Fc-mediated non-neutralizing antibody responses correlate with protection against congenital human cytomegalovirus infection

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## Graphical abstract



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2 congenital human cytomegalovirus infection

3 Running title: Fc antibody responses in congenital HCMV

4

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21

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25 Moderna and Merck. She also serves on the board of the National CMV Foundation and as an educator  
26 on CMV for Medscape, KMW has a sponsored research project from Moderna on immune correlates of  
27 congenital CMV infection. The other authors have declared that no other conflict of interest exists.

28 **Abstract**

29 Human cytomegalovirus (HCMV) is the most common congenital infection and a leading cause of  
30 stillbirth, neurodevelopmental impairment, and pediatric hearing loss worldwide. Development of a  
31 maternal vaccine or therapeutic to prevent congenital HCMV has been hindered by limited knowledge of  
32 the immune responses that protect against HCMV transmission in utero. To identify protective antibody  
33 responses, we measured HCMV-specific IgG binding and anti-viral functions in paired maternal and cord  
34 blood sera from HCMV seropositive transmitting (n=41) and non-transmitting (n=40) mother-infant dyads  
35 identified via a large U.S.-based public cord blood bank. We found that high avidity IgG binding to HCMV  
36 and antibody-dependent cellular phagocytosis (ADCP) were associated with reduced risk of congenital  
37 HCMV infection. We also determined that HCMV-specific IgG activation of FcγRI and FcγRII was  
38 enhanced in non-transmitting dyads and that increased ADCP responses were mediated through both  
39 FcγRI and FcγRIIA expressed on human monocytes. These findings suggest that engagement of  
40 FcγRI/FcγRIIA and Fc effector functions including ADCP may protect against congenital HCMV infection.  
41 Taken together, these data can guide future prospective studies on immune correlates against cCMV  
42 transmission and inform HCMV vaccine and immunotherapeutic development.

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## 55 **Introduction**

56 Human cytomegalovirus (HCMV) is the most common congenital infection worldwide, affecting 1 out of  
57 200 births or nearly 1 million newborns annually (1, 2). Most congenital HCMV (cCMV) infections are  
58 asymptomatic, yet serious disease outcomes can occur including stillbirth, intrauterine growth restriction,  
59 neonatal multi-organ disease, neurodevelopmental impairment, and sensorineural hearing loss. (3, 4)  
60 Moreover, cCMV infection has recently been linked to an elevated risk of acute lymphoblastic leukemia  
61 (5-7). Newborn screening for and public awareness of cCMV remains limited, leaving most cases  
62 undiagnosed and the true burden of disease underestimated (5, 8, 9). There are no licensed vaccines or  
63 therapeutics to prevent cCMV and an improved understanding of protective immunity against congenital  
64 HCMV transmission is urgently needed to guide novel interventions.

65

66 HCMV is a ubiquitous, host-restricted  $\beta$ -herpesvirus with multiple envelope glycoproteins and complexes  
67 including glycoprotein B (gB) and gHgL “dimer,” which can associate with gO to form the gHgLgO “trimer”  
68 or pUL128/130/131 to form the “pentamer” complex (10). HCMV envelope glycoproteins mediate viral  
69 entry, and following primary infection, the host remains latently infected for life (10-12). Over 80% of  
70 reproductive-aged women worldwide are latently infected with HCMV and congenital transmission occurs  
71 in maternal primary and nonprimary infection (i.e., reactivation from latency or reinfection with new  
72 strains) (2, 12-16). Mothers with primary infection have a 30% risk of fetal transmission whereas those  
73 with nonprimary infection have a 1-4% risk (2, 17-19), suggesting that preexisting maternal immunity  
74 partially protects against cCMV. In maternal primary infection, high avidity IgG binding to HCMV and anti-  
75 pentamer IgG levels are correlated with decreased congenital transmission risk (20-23). However, in  
76 maternal nonprimary infection, protective immunity remains unclear, as HCMV-specific IgG levels and  
77 neutralizing antibody titers do not always correlate with reduced congenital transmission (24-26).

78

79 Identifying protective immune responses in maternal primary and nonprimary infection is necessary to  
80 develop effective interventions to prevent cCMV. High avidity HCMV-specific IgG binding and neutralizing  
81 antibodies have been the main targets of vaccines and therapeutics (10, 27). Yet, maternal treatment

82 with HCMV hyperimmunoglobulin, a pooled preparation of high avidity, neutralizing antibodies, to prevent  
83 fetal transmission following primary infection has had limited efficacy (28-33).

84

85 Emerging evidence indicates that non-neutralizing antibody functions also protect against HCMV  
86 infection, but these have not been targeted in HCMV vaccines or immunotherapeutics to-date (34-37).  
87 Moreover, whether Fc-mediated non-neutralizing antibody functions (e.g., antibody-dependent cellular  
88 phagocytosis (ADCP) and antibody-dependent cellular cytotoxicity (ADCC)) protect against cCMV has  
89 not been explored. In this study, we focused on ADCP since vaccine trials suggest that Fc effector  
90 functions independent of ADCC mediate protection against HCMV (34, 38). We hypothesized that ADCP  
91 may protect against cCMV infection since ADCP can eliminate virus:IgG immune complexes and virally-  
92 infected cells, which could prevent systemic maternal viral replication, dissemination, and transmission  
93 across the maternal-fetal interface (39).

94

95 To identify protective immune responses against cCMV transmission, we compared antibody profiles in  
96 HCMV seropositive transmitting and non-transmitting mother-infant dyads identified as donors to a U.S.-  
97 based public cord blood bank. In our primary analysis, we compared 13 predefined IgG binding,  
98 neutralizing, and non-neutralizing antibody responses in transmitting versus non-transmitting women. In  
99 an exploratory analysis, we used systems serology to define differences between transmitting and non-  
100 transmitting dyads and examined the role of Fc-mediated immunity in congenital HCMV transmission.  
101 Insights from this study can inform vaccine and therapeutic development to prevent cCMV infection, a  
102 major cause of perinatal and pediatric morbidity worldwide.

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108 **Results**

109 **Baseline characteristics of HCMV transmitting and non-transmitting mother-infant dyads**

110 Our study included sera from 81 mother-infant dyads identified retrospectively as donors to the Carolinas  
111 Cord Blood Bank (CCBB), a large U.S.-based public cord blood bank (**Supplementary Figure 1**).  
112 Congenital HCMV infection (cCMV) was defined based on the presence of HCMV viremia in the donated  
113 cord blood plasma. Forty-one dyads with cCMV infection (“HCMV transmitting”) were matched to forty  
114 dyads with HCMV IgG seropositive mothers that gave birth to cCMV uninfected infants (“HCMV non-  
115 transmitting”). Matching criteria included infant sex, infant race, maternal age, and delivery year. Only  
116 women with healthy, uncomplicated pregnancies that gave birth at term were included in our study and  
117 cord blood donors were screened for signs of 1) neonatal sepsis, 2) congenital infection (petechial rash,  
118 thrombocytopenia, hepatosplenomegaly), and 3) congenital abnormalities. Demographic and clinical  
119 characteristics were comparable between transmitting and non-transmitting dyads, though cCMV cases  
120 had a non-significantly higher rate of Cesarean section (56% vs. 40%, Fisher’s exact  $p = 0.22$ , **Table 1**).

121  
122 To assess whether mothers may have had primary or nonprimary HCMV infection during pregnancy, we  
123 measured HCMV-specific IgG avidity and IgM in maternal sera collected at delivery (40). HCMV IgG  
124 avidity indexes were similar between transmitting and non-transmitting mothers (**Table 1**). Yet, 11/41  
125 (26.8%) of transmitting mothers had detectable HCMV-specific IgM compared to only 2/40 (5%) of non-  
126 transmitting mothers (**Table 1**). These data suggest that transmitting women likely had a higher rate of  
127 primary infection or reinfection during pregnancy, which are known risk factors for congenital transmission  
128 (40, 41). To further assess comparability between groups, we quantified HCMV viral loads in maternal  
129 sera and found that a similar proportion of transmitting (26.8%) and non-transmitting (37.5%) women had  
130 low-level HCMV DNAemia (**Table 1**), similar to observations in healthy HCMV seropositive women (42).

131  
132 **Maternal and cord blood sera from HCMV transmitting dyads have high HCMV-specific IgG levels**

133 We first quantified IgG binding against 3 distinct HCMV strains including TB40E (an endotheliotropic  
134 strain expressing pentamer), AD169r (a lab-adapted strain with repaired pentamer expression), and

135 Toledo (a low-passage clinical isolate lacking pentamer) (**Supplementary Figure 2A-D**). Median IgG  
136 binding against each HCMV strain was similar between transmitting and non-transmitting groups except  
137 for cord blood IgG binding to AD169r, which was lower in infected infants (**Figure 1A**). Within transmitting  
138 dyads, whole virus IgG binding was lower in cord blood versus maternal sera across all strains (**Figure**  
139 **1B**). However, IgG binding against envelope glycoproteins including gB, pentamer, gH/gL/gO, and gH/gL  
140 was significantly elevated (2.5-10 fold) in transmitting versus non-transmitting dyads, and glycoprotein-  
141 specific IgG was efficiently transferred into cord blood of infected infants (**Figure 1C-E**). These data  
142 reveal that infants with cCMV infection received high levels of maternal HCMV-specific IgG via placental  
143 antibody transfer. To explore if other HCMV-specific antibodies were associated with protection, we  
144 measured IgG binding against HCMV tegument proteins pp28, pp150, and the viral replication factor  
145 UL44, which are known to elicit potent IgG responses. IgG binding to pp150 and UL44 was also higher  
146 in transmitting versus non-transmitting dyads with some differences within dyads (**Figure 1F-G**).  
147 Altogether, these findings indicate that higher quantity of HCMV-specific IgG at the delivery timepoint is  
148 not correlated with lower congenital HCMV transmission risk.

149

### 150 **HCMV non-transmitting women have higher relative IgG binding to whole virus antigen and cell-** 151 **associated gB**

152 In our recent study on placental IgG transfer in cCMV infection (43), we observed that HCMV transmitting  
153 women had elevated total IgG levels (i.e., hypergammaglobulinemia). In this larger cohort, we also found  
154 that total IgG levels were higher in transmitting versus non-transmitting women (**Supplementary Figure**  
155 **3A**). When adjusting for total IgG levels, IgG binding against pentamer, gH/gL/gO, gH/gL, and UL44  
156 remained higher in transmitters, but non-transmitters had higher relative IgG binding to whole virus  
157 antigens and cell-associated gB (**Supplementary Figure 3B-C**). These data indicate that IgG binding to  
158 gB expressed in the native conformation on a virion or infected cell surface and other HCMV antigens not  
159 captured in our study may be associated with reduced cCMV transmission risk.

160

161 **HCMV-specific IgG binding avidity is increased in non-transmitting versus transmitting dyads**

162 We next assessed the quality of HCMV-specific IgG in transmitters versus non-transmitters by measuring  
163 IgG binding avidity. Maternal sera from non-transmitters had higher avidity IgG binding to AD169r and  
164 Toledo, but not TB40E, and cord blood IgG binding avidity was increased across all strains in uninfected  
165 versus infected infants (**Figure 2A**). Within dyads, whole virus IgG binding avidity was lower in paired  
166 cord blood versus maternal sera in transmitting but not non-transmitting dyads (**Figure 2B**). HCMV  
167 glycoprotein-specific IgG binding avidity was also lower in the cord blood of infected versus uninfected  
168 infants, though no significant differences were observed within dyads (**Figure 2C-D**). In a sensitivity  
169 analysis excluding mothers with detectable HCMV-specific IgM as a surrogate biomarker for recent  
170 primary infection or reinfection, many of these avidity differences persisted. Non-transmitting dyads still  
171 had higher avidity IgG binding to HCMV, and low avidity HCMV-specific IgG was enriched in the cord  
172 blood of infected infants (**Supplementary Figure 4A-C**). Altogether, these findings suggest that high  
173 avidity HCMV-specific IgG in the maternal and fetal circulation is associated with protection against cCMV  
174 infection, even when excluding mothers with recent primary infection or reinfection.

175

176 **Neutralizing and non-neutralizing antibody functions in transmitting and non-transmitting dyads**

177 Next, we compared neutralizing and non-neutralizing antibody functions in transmitting and non-  
178 transmitting dyads (**Supplementary Figure 5A-D**). Neutralizing antibody titers were 1.5-4 fold higher in  
179 transmitting versus non-transmitting dyads across strains and cell types (**Figure 3A-C**). Within dyads,  
180 neutralizing titers were mostly similar in paired cord blood and maternal sera (**Figure 3D-F**). These data  
181 indicate that HCMV neutralizing antibodies are effectively transferred across the placenta regardless of  
182 transmission status. In contrast, HCMV-specific ADCP, a non-neutralizing antibody response, was higher  
183 in non-transmitting versus transmitting women (**Figure 3G**). This difference was significant for Toledo ( $p$   
184 = 0.0057, FDR-corrected  $p$  = 0.011) with a trend towards increased ADCP of TB40E ( $p$  = 0.053) and  
185 AD169r ( $p$  = 0.068), which may not have reached statistical significance due to lower overall ADCP  
186 measured against these strains (**Supplementary Figure 5E-F**). Within dyads, ADCP was highly enriched  
187 in paired cord blood versus maternal sera (**Figure 3H**). Higher ADCP in fetal versus maternal circulation

188 may be due to fewer inhibitory factors, such as IgA, or could indicate enhanced placental transfer of  
189 ADCP-mediated IgG, a phenomenon that has been observed for ADCC-eliciting antibodies (44). Overall,  
190 these data suggest that non-neutralizing antibody responses may be important for preventing cCMV.

191

### 192 **ADCP and high avidity IgG binding to HCMV correlate with decreased risk of cCMV infection**

193 For our primary analysis, we hypothesized that 13 maternal antibody responses would be correlated with  
194 reduced risk of cCMV infection (**Table 2**). Using univariate logistic regression, 12 of the 13 variables were  
195 significantly associated with cCMV transmission risk. However, high magnitude IgG binding to HCMV  
196 envelope glycoproteins and neutralization were associated with increased risk, whereas high avidity IgG  
197 binding and ADCP were associated with decreased risk (**Table 2**). After adjusting for maternal total IgG  
198 and HCMV-specific IgM, HCMV glycoprotein-specific IgG binding and neutralization were still associated  
199 with increased risk, but IgG binding avidity was no longer significantly associated with reduced risk  
200 (**Supplementary Table 1**). ADCP against Toledo remained significantly associated with protection  
201 against cCMV transmission in both adjusted univariate regression models (**Supplementary Table 1**).

202

203 Since many immune variables in our predefined primary analysis were strongly correlated, we used least  
204 absolute shrinkage and selection operator (LASSO) for feature selection prior to multivariable analysis.  
205 LASSO is an approach to minimize overfitting a regression model that shrinks the coefficients of poorly  
206 predictive variables to zero, thereby removing them from the model. First, the cohort was randomly split  
207 into a training and test dataset and a 5-fold nested cross-validation with 5 repeats was used to train the  
208 LASSO model. LASSO-selected features included magnitude of pentamer IgG binding, avidity of gB IgG  
209 binding, avidity of gHgLgO IgG binding, and ADCP against Toledo strain (**Table 2**). Higher pentamer IgG  
210 binding was associated with increased risk whereas higher ADCP and IgG binding avidity were  
211 associated with decreased risk of cCMV infection in this multivariable model using the LASSO-selected  
212 features. In the out-of-sample test data, this 4-parameter LASSO model had a 0.75 accuracy (95% CI:  
213 0.48-0.93) in predicting cCMV transmission risk with 1.00 equaling perfect prediction and 0.45 equaling  
214 the random prediction rate after class label permutation (**Supplementary Figure 6**).

215

216 Next, we used a systems serology approach leveraging principal components analysis (PCA) to explore  
217 differences in HCMV-specific antibody responses in transmitting versus non-transmitting dyads. PC1  
218 accounted for 57 and 59% of the variance, respectively; however, PC2, which accounted for 16 and 17%  
219 of the variance, was superior at delineating between transmitting and non-transmitting groups (**Figure**  
220 **4A-B**). The top contributors to PC2 included ADCP against Toledo, TB40E, and AD169r, IgG binding  
221 avidity to HCMV glycoproteins, and IgG binding magnitude to Toledo, TB40E, and AD169r strains (**Figure**  
222 **4**). These PCA results further establish that IgG binding to HCMV antigens distinct from the major  
223 envelope glycoproteins, high avidity HCMV-specific IgG binding, and ADCP responses were enriched in  
224 the maternal and cord blood sera of non-transmitting compared to transmitting dyads.

225

#### 226 **HCMV-specific IgG binding to FcγRI and FcγRII differs in transmitting and non-transmitting dyads**

227 After identifying ADCP as a potential correlate of protection, we sought to understand why ADCP was  
228 enhanced in non-transmitting dyads. We hypothesized that HCMV-specific IgG from non-transmitting  
229 dyads may better engage the host Fcγ receptors (FcγRs) on innate immune cells that mediate ADCP. To  
230 explore this hypothesis, we measured HCMV-specific IgG binding to FcγRI and FcγRII including the  
231 activating FcγRIIA and inhibitory FcγRIIB (45-47) (**Supplementary Figure 7**). To compare FcγR binding  
232 between groups, we normalized FcγR-specific IgG binding to total IgG binding to antigen-coated beads  
233 at baseline. Normalized HCMV-specific IgG binding to FcγRI was significantly higher in non-transmitting  
234 versus transmitting dyads (**Figure 5A-B**) and highly enriched in the cord blood of uninfected infants  
235 (**Supplementary Figure 8A**). HCMV-specific IgG binding to FcγRI was also negatively correlated ( $p <$   
236  $0.05$ ) with HCMV viral loads in the cord blood of infected infants, suggesting that FcγRI engagement may  
237 help control viremia. Normalized gB-, pentamer-, gH/gL/gO-, and gH/gL-specific IgG binding to FcγRIIA  
238 was higher in transmitters whereas pp28-, pp150- and UL44-specific IgG binding to FcγRIIA was higher  
239 in non-transmitters (**Figure 5C-D, Supplementary Figure 8B**). Only gHgLgO- and pp150-specific IgG  
240 binding to FcγRIIB differed between groups, with the former higher in transmitters and the latter higher  
241 in non-transmitters (**Figure 5E-F**). These findings suggest that engagement of FcγRI, and to a lesser

242 extent FcγRIIA, may mediate protection against cCMV transmission, which we sought to explore further  
243 using a functional signaling assay.

244

#### 245 **FcγRI and FcγRII activation is enhanced in non-transmitting dyads and correlated with ADCP**

246 To quantify HCMV-specific IgG activation of FcγRI and FcγRII, we used mouse BW thymoma cell lines  
247 expressing chimeric human FcγRs that secrete mouse IL-2 upon IgG engagement as a quantitative read-  
248 out for FcγR activation (48, 49). We first confirmed that each BW cell line was expressing the FcγR of  
249 interest (**Figure 6A-C**). HCMV-specific FcγRI activation was 3-4 fold higher in non-transmitting dyads  
250 (**Figure 6D**). There was also a trend towards higher FcγRIIA activation and significantly higher FcγRIIB  
251 activation in non-transmitting dyads (**Figure 6E-F**). It has been hypothesized that higher IgG binding to  
252 activating versus inhibitory FcγRs improves anti-viral phagocytosis since preferential IgG binding to  
253 FcγRIIA over FcγRIIB has been correlated with enhanced ADCP in HIV infection (50, 51). Yet, we found  
254 that the ratio of FcγRIIA to FcγRIIB IgG engagement did not correlate with ADCP or decreased cCMV  
255 transmission risk in our cohort (**Supplementary Figure 9**). Instead, higher FcγRI, FcγRIIA, and FcγRIIB  
256 activation all correlated with higher ADCP ( $p < 0.0001$ ) (**Figure 6G-I, Supplementary Figure 10**). These  
257 data imply that engagement of host FcγRI and FcγRII may both help mediate effective ADCP of HCMV.

258

#### 259 **ADCP of HCMV is mediated by FcγRI and FcγRIIA on human monocytes**

260 To test our hypothesis that FcγRI and FcγRII engagement mediates enhanced ADCP in non-transmitting  
261 dyads, we measured ADCP in the presence and absence of FcγR blocking antibodies. We found that the  
262 human monocyte cell line we used to measure ADCP, THP1s, had high expression of FcγRI and FcγRIIA  
263 but not FcγRIIB (**Figure 7A, Supplementary Figure 11A**). Blocking FcγRII with a pan anti-FcγRII and  
264 FcγRIIA-specific but not FcγRIIB-specific antibody inhibited ADCP (**Figure 7B-C, Supplementary**  
265 **Figure 11B**). Notably, blocking FcγRII at high Cytogam concentrations lead to a much larger decrease  
266 in ADCP than at intermediate or low Cytogam levels (**Figure 7B-C, Supplementary Figure 11B**). These  
267 data indicate that FcγRI mediates ADCP most effectively at intermediate antibody levels and less  
268 efficiently at elevated antibody levels. Blocking FcγRI inhibited ADCP to a similar degree across Cytogam

269 concentrations, suggesting that FcγRII-mediated ADCP is directly correlated with HCMV-specific  
270 antibody levels (**Figure 7C**). In maternal sera samples, blocking FcγRI only (median decrease = 40%),  
271 FcγRII only (median decrease = 70%) and FcγRI plus FcγRII (median decrease = 90%) inhibited ADCP  
272 to varying degrees (**Figure 7D-G**). Non-transmitting dyads had significantly higher ADCP responses  
273 compared to transmitting dyads across all FcγR blocking conditions (**Figure 7E, Supplementary Figure**  
274 **12**) and FcγRI-mediated ADCP was particularly enhanced in non-transmitters (**Figure 7G**). Altogether,  
275 these findings suggest that ADCP mediated by FcγRI and to a lesser extent FcγRIIA is associated with  
276 protection against cCMV transmission.

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## 296 **Discussion**

297 Despite decades of research, protective immune responses against congenital HCMV transmission have  
298 remained elusive and there are no licensed vaccines or therapeutics to prevent cCMV (12). Using a case-  
299 control cohort of cord blood donor HCMV transmitting (n=41) and non-transmitting (n=40) mother-infant  
300 dyads, we identified Fc-mediated immunity and ADCP as novel potential correlates of protection against  
301 cCMV infection. These findings can guide future prospective studies on immune correlates against cCMV  
302 transmission and inform HCMV vaccine and immunotherapeutic development.

303

304 In our recent complementary study, we discovered that placental IgG transfer was modestly decreased  
305 in cCMV infection (43), prompting us to investigate HCMV-specific IgG transfer in HCMV transmitting  
306 pregnancies. Our finding that cCMV-infected infants had high levels of neutralizing and non-neutralizing  
307 HCMV-specific IgG suggests that reduced IgG transfer into the fetal circulation is not a risk factor for  
308 congenital infection. Low avidity HCMV-specific IgG was enriched in the cord blood of infected infants,  
309 which has been proposed as a mechanism of antibody-dependent enhancement (52, 53). However, we  
310 speculate that this phenomenon is likely an indication of recent maternal primary infection or reinfection  
311 leading to an abundance of low avidity antibodies present early in pregnancy that were then transferred  
312 across the placenta. Altogether, our results further demonstrate that high-avidity HCMV-specific IgG in  
313 the maternal and fetal circulation is associated with protection against cCMV infection (20, 23, 43, 54).

314

315 HCMV antigen-specific IgG levels and neutralizing antibody titers have been correlated with protection  
316 against cCMV transmission in some studies (21, 24), yet these antibody responses were not associated  
317 with decreased transmission risk in our cohort. Our findings are consistent with a recent study by Dorfman  
318 et al. that also observed higher maternal gB- and pentamer-specific IgG levels at delivery were associated  
319 with cCMV infection (25). Though higher HCMV-specific IgG levels were associated with increased  
320 transmission, antibody-dependent enhancement of infection is unlikely given compelling epidemiological  
321 and experimental evidence that maternal antibodies help protect against congenital transmission (2, 55-  
322 57). Since both studies measured maternal HCMV-specific antibody responses at the delivery timepoint,

323 we infer instead that elevated IgG levels and neutralizing antibody titers in transmitters are likely due to  
324 “boosting” from active HCMV infection or reactivation during pregnancy (25). This interpretation is  
325 bolstered by existing cCMV immune correlates literature measuring antibody responses at different  
326 timepoints in pregnancy. In maternal primary infection, Boppana et al. also identified higher gB-specific  
327 IgG titers in transmitting versus non-transmitting women at delivery (20). In contrast, Vanarsdall et al.  
328 found that seropositive transmitting and non-transmitting women had similar gB-, pentamer-, and  
329 gH/gL/gO-specific IgG levels and neutralization titers when measured in the first trimester (26). Moreover,  
330 Huang et al. recently demonstrated that higher pp150-specific IgG levels were associated with reduced  
331 cCMV transmission risk in HCMV seropositive women when measured early in gestation (58). Altogether,  
332 these data suggest that elevated HCMV-specific IgG levels and neutralization titers at delivery are not  
333 causally associated with cCMV infection but correlated with transmission risk because transmitting  
334 women are more likely to experience sustained viral replication during pregnancy.

335

336 Our study is the first to examine Fc-mediated antibody responses in cCMV transmission, so our finding  
337 that ADCP was associated with reduced cCMV infection represents an important step forward for the  
338 field. ADCP can defend against both cell-associated and cell-free virus through phagocytosis of virally-  
339 infected cells or virus:IgG immune complexes (59, 60). Since HCMV infection primarily spreads cell-to-  
340 cell in vivo, it is logical that ADCP would be associated with protection against placental transmission  
341 (61). FcγRI and FcγRIIA, which we demonstrate mediate this ADCP, are highly expressed on maternal-  
342 and fetal-derived monocytes and macrophages at the maternal-fetal interface (62-64), so it is intriguing  
343 to consider whether these innate immune cells employ Fc-mediated functions against HCMV (39). Future  
344 studies should investigate if ADCP or other Fc-mediated antibody functions protect against cCMV  
345 transmission systemically and/or at the maternal-fetal interface. Notably, different biophysical and  
346 biochemical properties of IgG including subclass and glycosylation also modify FcγR binding affinity and  
347 downstream effector functions, so whether these Fc characteristics modulate cCMV transmission risk  
348 should also be explored (65-67). Altogether, our data suggest that enhanced phagocytosis, anti-viral  
349 signaling, and/or antigen presentation following IgG binding to FcγRI and FcγRIIA may help protect

350 against cCMV infection, though the role of the inhibitory FcγRIIB remains unclear as this receptor was  
351 not expressed on the monocytes we used to measure ADCP (47, 59, 68).

352

353 These findings can inform the development of vaccines and immunotherapeutics to prevent cCMV  
354 infection. Maternal treatment with HCMV hyperimmune globulin during primary infection to prevent  
355 congenital transmission has not been efficacious in two randomized clinical trials despite showing some  
356 efficacy in smaller observational studies (28-33). In our study, we observed that FcγRI-mediated ADCP  
357 was reduced at elevated Cytogam levels. Whether poor engagement of Fc-mediated immunity partially  
358 explains the limited efficacy of HCMV hyperimmune globulin is unknown but targeting certain Fc effector  
359 functions may improve polyclonal or monoclonal antibodies to prevent cCMV transmission.

360

361 Our results have important implications for HCMV vaccine development beyond the context of congenital  
362 infection. The most successful HCMV vaccine to-date was a gB subunit vaccine with an MF59 adjuvant  
363 that achieved ~50% efficacy, yet the antibody responses mediating this protection have remained elusive  
364 (35, 37, 38, 69). Neutralizing antibodies were poorly elicited by the gB/MF59 vaccine, leading researchers  
365 to hypothesize that non-neutralizing antibody responses mediated vaccine efficacy (34, 36, 38). Though  
366 ADCC responses were poorly stimulated in gB/MF59 vaccinees, ADCP responses were robustly induced  
367 (34, 36, 38). Moreover, there was a trend ( $\beta = -0.420$ ,  $p = 0.120$ ) towards ADCP being associated with  
368 protection against virus acquisition in our recent study of adolescent and postpartum gB/MF59 vaccinees  
369 (36). Notably, IgG binding to cell-associated gB, which we identified as an immune correlate of protection  
370 for these gB/MF59 vaccinees, was correlated with ADCP and FcγRI/FcγRIIA activation in our mother-  
371 infant cohort study (36). Taken together, these data suggest that Fc-mediated IgG responses against gB  
372 expressed on the surface of a virion or cell may protect against HCMV infection. Non-neutralizing  
373 antibody responses against other targets should also be explored as non-structural HCMV antigens were  
374 recently identified as potent targets of ADCC (70). Cumulatively, these findings indicate that Fc-mediated  
375 and polyfunctional antibody responses against diverse HCMV antigens should be investigated as  
376 potential correlates of protection in HCMV infection and vaccination (51, 65, 66, 71).

377

378 Our study has several limitations. Due to the cross-sectional and retrospective nature of this cord blood  
379 bank donor cohort, we could not definitively identify maternal primary versus nonprimary HCMV infections  
380 and the timing of maternal infection. Because of this caveat, caution is warranted in interpreting our  
381 results since differences between transmitting and non-transmitting groups may be biased by a higher  
382 rate of primary infection and/or reinfection in transmitting cases. This limitation highlights the need for  
383 future prospective studies with longitudinal sampling throughout pregnancy to define protective antibody  
384 responses against cCMV infection in both maternal primary and nonprimary infection. We also did not  
385 have information on the HCMV strains infecting transmitting versus non-transmitting women, so could  
386 not assess if maternal exposure to different HCMV strains were contributing to differences in antibody  
387 responses between groups. Placental biospecimens and maternal PBMCs were not collected, so we  
388 could not investigate placental infection nor maternal cellular immune correlates of protection, even  
389 though CMV-specific T cell responses have been associated with reduced cCMV transmission (23, 27,  
390 72). Since long-term clinical outcomes were unavailable, we could not assess whether antibody  
391 responses correlated with protection against long-term disease sequelae, though all infants were born  
392 without signs of cCMV infection. Statistical power was limited by small sample size (n = 41 HCMV  
393 transmitting dyads), yet this represents one of the largest U.S.-based cohorts to assess cCMV immune  
394 correlates to-date and one of the few studies without the confounder of maternal HIV co-infection (21,  
395 23-26, 73). Our study focused on ADCP and did not measure other Fc effector functions including ADCC,  
396 antibody-dependent neutrophil phagocytosis (ADNP), and antibody-dependent complement deposition  
397 (ADCD) due to limited sera sample volumes, but these Fc functions should be explored in future studies.

398

399 Development of an efficacious HCMV vaccine has been considered a “top tier priority” by the U.S.  
400 National Academy of Medicine for over 20 years but identifying immune correlates of protection to inform  
401 vaccine development has proved challenging (74, 75). Our study suggests that eliciting HCMV-specific  
402 IgG that engages FcγRI/FcγRIIA and mediates non-neutralizing Fc effector functions such as ADCP may  
403 be an important immune response in the prevention of cCMV transmission and a promising new approach

404 for HCMV vaccinology. These findings will guide future studies on correlates of protection against HCMV  
405 and may inform the development of novel vaccines and therapeutics to prevent cCMV infection.

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431 **Methods**

432

433 *Study population.* We analyzed maternal and cord blood sera from 81 mother-infant dyads recruited from  
434 2008-2017 as donors to the Carolinas Cord Blood Bank (CCBB). The CCBB collects maternal and cord  
435 blood biospecimens at delivery and mother-infant dyads were identified from over 29,000 CCBB donor  
436 records (**Supplementary Figure 1**). Maternal donors underwent infectious diseases screening for  
437 HCMV, hepatitis B virus, syphilis, hepatitis C virus, HIV-1/2, HTLV I and II, Chagas Disease, and West  
438 Nile virus. All mothers in our study were HCMV IgG seropositive and negative for other infectious  
439 diseases. Cord blood plasma was screened by the CCBB for HCMV infection with a Real-Time PCR  
440 COBAS AmpliPrep/TaqMan nucleic acid test (WHO HCMV reference standard, limit of quantification 147  
441 IU/mL (76)), and all cord blood units positive for HCMV underwent a second confirmatory PCR test.

442

443 Cases of congenital HCMV infection (cCMV) were defined as mother-infant dyads with cord blood that  
444 screened positive for HCMV viremia at birth per PCR testing. "HCMV transmitting" cases with cCMV  
445 infection (n=41) were matched to a target of 1 "HCMV non-transmitting" seropositive mother (n=40) with  
446 no HCMV viremia detected in the cord blood (**Supplementary Figure 1**). Maternal HCMV IgG  
447 seropositivity and avidity were confirmed by a whole virion HCMV ELISA and HCMV IgM seropositivity  
448 was determined using a clinical diagnostic ELISA (Bio-Rad). Maternal sera was screened for HCMV  
449 DNAemia via quantitative PCR (qPCR). For qPCR, 150-200ul of maternal sera was ultracentrifuged then  
450 DNA was extracted using the DNA QIAamp Kit (Qiagen) and tested in duplicate using SybrSelect and  
451 300nM of forward and reverse primers to amplify the HCMV immediate early-1 (IE1) gene (24). HCMV  
452 viral loads were interpolated from an IE1 plasmid standard curve. Maternal sera was also screened for  
453 hypergammaglobulinemia (total IgG >15,000 mg/dL) via ELISA as previously described (43).

454

455 *Cell culture and HCMV virus growth.* Human retinal pigment epithelial cells (ARPEs), human foreskin  
456 fibroblasts (HFFs), human embryonic kidney (HEK)-293T cells, and human monocytes (THP-1s) were  
457 acquired from ATCC and cultured according to ATCC protocols. For differentiation, THP-1 cells were

458 cultured in R10 media with 100nM PMA and incubated for 48 hours. HCMV strains TB40/E, AD169r, and  
459 Toledo were propagated as previously described (36, 77). HFF cells (Toledo) or ARPE cells (TB40E and  
460 AD169r) were plated in T175 flasks, rested overnight and infected (MOI = 0.01) the next day once cells  
461 reaching ~80% confluency. Infected HFFs or ARPEs were incubated for ~14 days until 90-95% of cells  
462 showed cytopathic effect. Harvested cells were either sonicated or subjected to multiple freeze/thaws  
463 and then centrifuged to pellet cells before the cell-associated “supernatant” was combined with collected  
464 cell-free supernatant. This combined cell-associated and cell-free virus stock was filtered (0.45- $\mu$ m) then  
465 concentrated on a 20% sucrose cushion at 20,000 rpm in a Beckman SW28 rotor for 1.5 hours. The  
466 concentrated virus pellet was resuspended in HFF or ARPE cell growth medium (0.2 M sucrose) and the  
467 titer of concentrated virus stocks was determined with HFFs (Toledo) or ARPEs (TB40E and AD169r) by  
468 the limiting dilution technique in 96-well plates.

469

470 *Whole virion HCMV IgG binding and avidity.* 384-well ELISA plates were coated with 33 PFU/well of  
471 TB40/E, 2700 PFU/well of AD169r, or 1000 PFU/well of Toledo virus (optimized PFU based on Cytogam  
472 binding in **Supplementary Figure 2**) diluted in 0.1 M sodium bicarbonate buffer then incubated overnight  
473 before blocking. Maternal and cord blood sera serial dilutions were tested starting at 1:30, plated in  
474 duplicate, then incubated before adding horseradish peroxidase (HRP)-conjugated goat anti-human IgG  
475 Fc (Jackson ImmunoResearch; 109-035-008). Plates were developed with tetramethylbenzidine (TMB)  
476 and peroxidase substrate (KPL) then optical density (OD) at 450nm was measured via SpectroMax.  
477 HCMV-specific IgG concentrations were interpolated from the linear range of a 5-parameter HCMV-  
478 hyperimmune globulin (Cytogam) standard curve. HCMV binding to monoclonal IgG antibodies against  
479 gB (TRL-345, in-house), pentamer (TRL-310, in-house), pp65 (1-L-11, Thermo), and UL44 (M612460,  
480 Genway Bio) were also included for comparisons across strains. For IgG avidity, duplicate wells were  
481 treated with 7M urea or 1X PBS between the primary and secondary incubation steps and relative avidity  
482 index (RAI) was calculated as (OD with urea)/(OD with PBS)x100%. Duplicates with coefficients of  
483 variance (CVs) >20% were repeated.

484

485 *HCMV glycoprotein-specific IgG binding and avidity.* A binding antibody multiplex assay (BAMA) was  
486 used to quantify HCMV glycoprotein-specific IgG binding and avidity (77, 78). HCMV gB ectodomain,  
487 pentamer complex, gH/gL/gO, gH/gL, pp28, pp150 and UL44 antigens were covalently coupled to  
488 intrinsically fluorescent beads (Bio-Rad). Maternal and cord blood sera were diluted at 1:500 (for gB  
489 ectodomain, pentamer complex, gH/gL/gO, gH/gL) and at 1:25 (for pp28, pp150, UL44) in assay diluent,  
490 plated in duplicate, then co-incubated with antigen-coupled beads. Antigen-specific IgG binding was  
491 detected with mouse anti-human IgG-PE (Southern Biotech) and mean fluorescent intensity (MFI) was  
492 acquired on a Bio-Plex 200 (Luminex). For avidity, duplicate wells were incubated with sodium citric acid  
493 (pH = 4.0) or 1X PBS (pH = 7.4) between the primary and secondary incubations and RAI was calculated  
494 as  $(\text{MFI with sodium citric acid})/(\text{MFI with PBS}) \times 100\%$ . A serial dilution of HCMV-hyperimmunoglobulin  
495 (Cytogam) was included as a positive control and the cut-off for positivity was determined by calculating  
496 the mean MFI of seronegative sera binding plus 3 standard deviations. Blank beads and wells were  
497 included to account for background signal. Duplicates with CVs >25% were repeated.

498

499 *Fc receptor (FcR) binding by HCMV glycoprotein-specific IgG.* FcR binding by HCMV glycoprotein-  
500 specific IgG was measured using a modified BAMA (36, 79). Purified human FcR1a, FcR2a (clone H131),  
501 and FcR2b were produced by the DHVI Protein Production Facility and biotinylated in-house. Maternal  
502 and cord blood sera were diluted at 1:500 (for gB ectodomain, pentamer complex, gH/gL/gO, gH/gL) and  
503 at 1:25 (for pp28, pp150, UL44) in assay diluent before incubation with HCMV antigen-coated beads, as  
504 above. Next, biotinylated human FcRs were complexed with streptavidin-PE (BD Biosciences) then co-  
505 incubated with antibody-bound beads after washing. MFI was acquired on a Bio-Plex 200 and duplicates  
506 with CVs >25% were repeated.

507

508 *Cell-associated HCMV glycoprotein B (gB) IgG binding.* A gB-transfected cell binding assay was used to  
509 measure IgG binding to cell-associated gB (36). HEK-293T cells were co-transfected with DNA plasmids  
510 expressing green fluorescent protein (GFP) and full-length gB (Sino Biological) using the Effectene  
511 Transfection Kit (Qiagen). After incubation at 37°C for 48 hours, 200,000 live cells/well were plated into

512 96-well U-bottom plates then centrifuged and washed before a 5 minute incubation in Human TruStain  
513 Fc Block (1:1000 dilution; BioLegend). In duplicate, cells were then co-incubated with maternal and cord  
514 blood sera diluted 1:500 or with controls for 2 hours at 37°C. A serial dilution of HCMV-  
515 hyperimmunoglobulin (Cytogam) and a gB-specific monoclonal antibody (TRL-345, in-house) were  
516 included as positive controls and seronegative sera samples were included as negative controls.  
517 Following incubation, cells were stained with Live/Dead Near-IR (1:1000; Invitrogen) then washed and  
518 stained with PE-conjugated goat anti-human IgG-Fc (1:200; Southern Biotech). Lastly, cells were washed  
519 and fixed with 10% Formalin. Events were acquired on an LSRII flow cytometer, and the percentage of  
520 PE-positive cells was calculated from the live, GFP-positive cell parent population using FlowJo (36).  
521 Unstained cells and single color-stained cells were included for setting gates and compensation. The cut-  
522 off for positivity was the mean signal of HCMV seronegative samples plus 3 standard deviations.  
523 Duplicates with CVs >50% were repeated.

524

525 *Neutralization.* HCMV neutralization was measured by high-throughput fluorescence bioimaging (36).  
526 Epithelial cells (ARPEs), fibroblasts (HFFs) or differentiated monocytes/macrophages (THP-1s) were  
527 plated in 384-well clear, flat-bottom plates and then incubated at 37°C overnight. To quantify  
528 neutralization, maternal and cord blood sera samples were diluted 1:10 followed by an 8-point serial  
529 dilution and then co-incubated with HCMV strains AD169r (MOI = 2) or Toledo (MOI = 1) for 2 hours at  
530 37°C to allow immune complex formation. Virus-only wells and an 8-point HCMV-hyperimmunoglobulin  
531 (Cytogam) serial dilution were included as positive controls while seronegative samples and no virus  
532 wells were included as negative controls. After addition of the virus:sera mixture, cells were incubated at  
533 37°C for 24-48 hours (depending on optimized conditions for each virus strain and cell type) then fixed  
534 in 10% Formalin. To quantify HCMV infection, plates were stained with mouse anti-HCMV immediate-  
535 early 1 (IE1) gene (1:500; MAB810 Millipore) followed by goat anti-mouse IgG-AF488 (1:500; Millipore)  
536 and cell nuclei were stained with DAPI (1:10,000; Thermo Fisher). After staining, plates were visualized  
537 with a Cellomics fluorescent plate reader (**Supplementary Figure 5**) to enumerate total cell count,  
538 infected cell count, and percent infected cells in each well. Following image acquisition, neutralization

539 titers corresponding to the dilution that resulting in a 50% reduction in percent infected cells (ID50) were  
540 calculated using interpolation in GraphPad Prism (**Supplementary Figure 5**). Samples containing wells  
541 with low cell counts and with duplicates with CVs >50% were repeated.

542

543 *Whole virion HCMV antibody-dependent cellular phagocytosis (ADCP)*. HCMV strains TB40/E, AD169r,  
544 and Toledo were conjugated to fluorochrome AF647 (Invitrogen) to measure ADCP (36). In brief,  $2.0 \times 10^6$   
545 PFU of TB40/E,  $1.0 \times 10^7$  PFU of AD169r, or  $1.0 \times 10^7$  PFU of Toledo virions were buffer-exchanged with  
546 1X PBS using a 100,000-kDa Amicon filter (Millipore) and conjugated to 10 $\mu$ g of AF647 N-  
547 hydroxysuccinimide ester reconstituted in DMSO during a 1-hour incubation with constant agitation. The  
548 conjugation reaction was quenched with 80ul of 1M tris-HCl (pH = 8.0) and fluorescently-labeled virus  
549 was diluted 25X in wash buffer. A serial dilution of HCMV-hyperimmunoglobulin (Cytogam) was included  
550 as a positive control while seronegative sera samples and an anti-RSV monoclonal antibody (Synagis)  
551 were included as negative controls. In a 96-well plate, fluorescently-labeled virus was co-incubated with  
552 maternal sera, cord blood sera (1:10) or controls at 37°C for 2 hours to allow immune complex formation  
553 before adding 50,000 THP-1 cells per well. Plates were then centrifuged (1200g) at 4°C for 1 hour in a  
554 spinoculation step before a 1-hour incubation at 37°C to allow for phagocytosis. Next, cells were  
555 transferred to a 96-well U-bottom plate, washed and fixed with 1% Formalin. Events were acquired on an  
556 LSRII flow cytometer, and the percentage of AF647-positive cells was calculated from the live THP-1  
557 monocyte cell parent population using FlowJo (gating strategy in **Supplementary Figure 5**). Unstained  
558 cells and single color-stained cells were included as controls for setting gates and compensation. The  
559 cut-off for positivity was the mean signal of HCMV seronegative samples plus 3 standard deviations and  
560 duplicates with CVs >50% were repeated.

561

562 To measure Fc $\gamma$ R expression, THP1s were stained with anti-human CD64-PE (clone 10.1, eBioscience),  
563 CD32-PE (clone 6C4, eBioscience), CD32A-FITC (clone IV.3, StemCell Technologies), CD32B-APC  
564 (clone S18005H, Biolegend), CD16-PE (clone CB16, eBioscience), and Ig-PE isotype control (clone  
565 P3.6.2.8.1, eBioscience). Cells were fixed with 2% paraformaldehyde and acquired on a LSRII flow

566 cytometer and analyzed using FlowJo v10.7.2. For FcγR blocking, THP1 cells were co-incubated with  
567 purified anti-human CD64 (clone 10.1 Biolegend), CD32 (clone AT10, Bio-Rad), CD32A (clone IV.3,  
568 StemCell Technologies) or CD32B (clone S18005H, Biolegend) for 1.5 hours at 37°C prior to co-  
569 incubation with virus:sera immune complexes. The remainder of the ADCP assay was performed as  
570 described above. An ADCP phagocytosis score to account for non-specific background ADCP signal was  
571 calculated as follows: (%AF647 positive cells \* AF647 MFI in sera sample)/(%AF647 positive cells \*  
572 AF647 MFI in PBS control wells)\*100%.

573

574 *Fcγ receptor signaling assay.* HCMV-specific IgG signaling through FcγRs was measured using a  
575 previously published approach (48). Briefly, we used mouse BW thymoma cells stably expressing  
576 chimeric FcR-CD3ζ, which contains an extracellular human FcR and intracellular CD3ζ signaling domain,  
577 to quantify anti-viral IgG activation of host FcγRs. To confirm FcγR expression, 0.5×10<sup>6</sup> BW cells were  
578 added to each well in a 96-well plate. The non-transfected parental BW cell line and BW cell lines  
579 expressing human CD64 (FcR1a), CD32a (FcR2a), or CD32b (FcR2b) were stained for surface  
580 expression of human FcγRs with 5μl anti-human CD64-PE (clone 10.1, eBioscience), 5μl anti-human  
581 CD32-PE (clone 6C4, eBioscience), 5μl CD16-PE (clone CB16, eBioscience), and 5μl anti-human Ig-PE  
582 isotype control (clone P3.6.2.8.1, eBioscience) then cells were fixed with 2% paraformaldehyde. Events  
583 were acquired on a LSRII flow cytometer and analyzed using FlowJo v10.7.2.

584

585 To quantify FcγR activation, 96-well plates were coated with 20,000 PFU/well of HCMV strain AD169r  
586 and incubated at 4°C overnight. After coating, plates were washed with assay buffer (1X PBS + 1% FBS)  
587 then blocked with buffer at room temperature for 1 hour. After blocking, HCMV-coated plates were co-  
588 incubated with maternal or cord blood sera diluted 1:10 in BW cell media at 37°C for 1 hour. HCMV-  
589 hyperimmunoglobulin (Cytogam), seronegative and no antibody conditions were included as controls.  
590 Following immune complex formation, plates were washed with BW media before adding 100,000 FcR1a,  
591 FcR2a, or FcR2b-expressing BW cells per well. In separate wells, the parental (non-transfected) BW  
592 cells were added as a negative control. Cells were incubated at 37°C with immune complex coated plates

593 for 20 hours before being transferred to V-bottom plates and pelleted (1200 rpm). Cell supernatants were  
594 harvested and mouse IL-2 (mIL-2) levels in culture supernatants were measured using ELISA. For the  
595 mIL-2 ELISA, 384-well plates were coated with 3µg/mL of purified rat anti-mouse IL-2 (BD biosciences)  
596 and incubated at 4°C overnight before blocking. Purified mIL-2 and BW cell culture supernatants were  
597 added in duplicate and incubated for 1 hour at room temperature. After primary incubation, plates were  
598 incubated with rat anti-mIL2 conjugated to biotin (BD biosciences; 1:2000) followed by streptavidin-HRP  
599 (1:8000) for 1 hour and 30 minutes respectively. Plates were developed with TMB/KPL then OD at 450nm  
600 was measured via SpectroMax and mIL-2 concentrations were interpolated from a 5-parameter mIL-2  
601 standard curve using GraphPad Prism. Duplicates with CVs >30% were repeated.

602

603 *Statistics.* All primary raw and analyzed data underwent independent data quality control (QC) by a  
604 second lab member prior to statistical analysis and inclusion in the study. For all analyses, interpolated  
605 antibody or cytokine concentrations, MFI values and neutralization titers were log-transformed to  
606 normalize the data distribution. Antibody responses below the limit of detection were set equal to the limit  
607 of detection for statistical analyses. To assess differences between HCMV transmitting and non-  
608 transmitting mother-infant dyads, immune variables were compared between groups using Mann-  
609 Whitney U/Wilcoxon rank-sum and within dyads using Wilcoxon signed rank tests. Statistical significance  
610 was defined a priori as  $p < 0.05$  with a two-tailed test and a Benjamini-Hochberg correction for multiple  
611 comparisons. For the primary immune correlate analysis, 13 predefined maternal humoral immune  
612 variables were included for univariate logistic regression analysis. Using the 13 predefined immune  
613 variables from our primary immune correlate analysis, LASSO regression analysis was performed using  
614 the caret R package and 5-fold cross-validation with 5 repeats was performed. For the LASSO regression,  
615 the cohort was randomly split into two independent datasets, which were used for training the LASSO  
616 model and testing the predictive performance of the model respectively. To test the random prediction  
617 rate of the trained LASSO model, class lab permutation was performed on the dataset and model  
618 accuracy characteristics were assessed. All statistical analyses were completed in R v4.1.1 and  
619 GraphPad Prism v9.1. The principal components analysis (PCA) plots were rendered using ggplot2 and

620 correlation matrices were plotted using the corrplot package in R. All other figures were generated using  
621 GraphPad Prism.

622

623 *Study Approval.* Approval was obtained from Duke's Institutional Review Board (Pro00089256) to use  
624 de-identified clinical data and biospecimens provided by the CCBB. No patients were prospectively  
625 recruited for this study and all samples were acquired retrospectively from the CCBB biorepository from  
626 donors who had previously provided written consent for banked biospecimens to be used for research.

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647 **Author contributions**

648 ECS, JAJ, JHH, KMW, and SRP designed the research study. ECS, IGM, CP, MJH, SJB and HW  
649 conducted the experiments and acquired the data. ECS, IGM and SJB completed the primary data  
650 analysis. CEW and ECS completed the statistical analyses with oversight from KMW. SRP and KMW  
651 acquired funding for the study. JK provided the biospecimens and clinical data for the study. ECS wrote  
652 the primary draft of the manuscript. ECS, IGM, CP, JAJ, CEW, SJB, MJH, HW, JHH, JK, GGF, KMW and  
653 SRP all contributed to writing and editing the manuscript.

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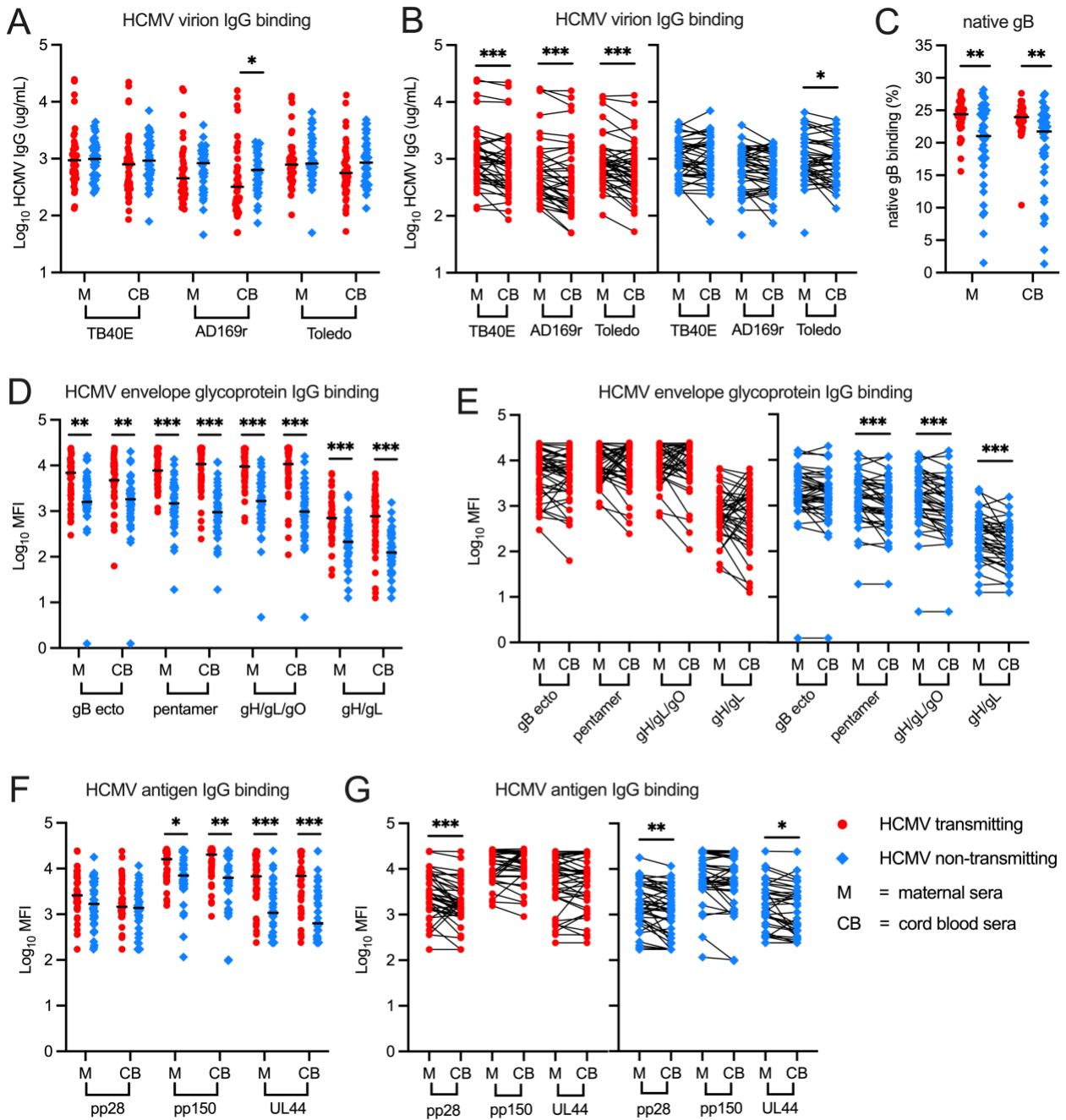
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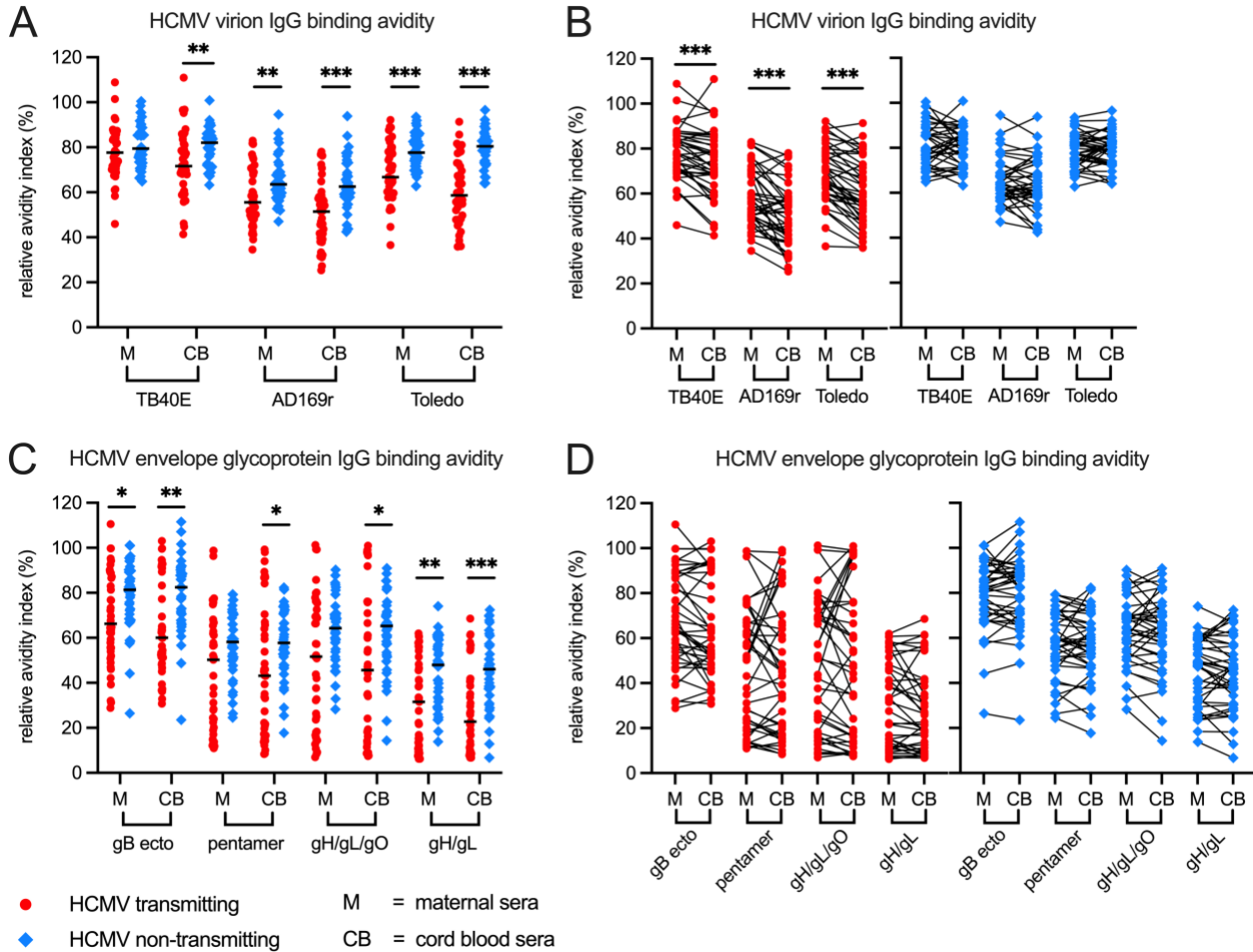
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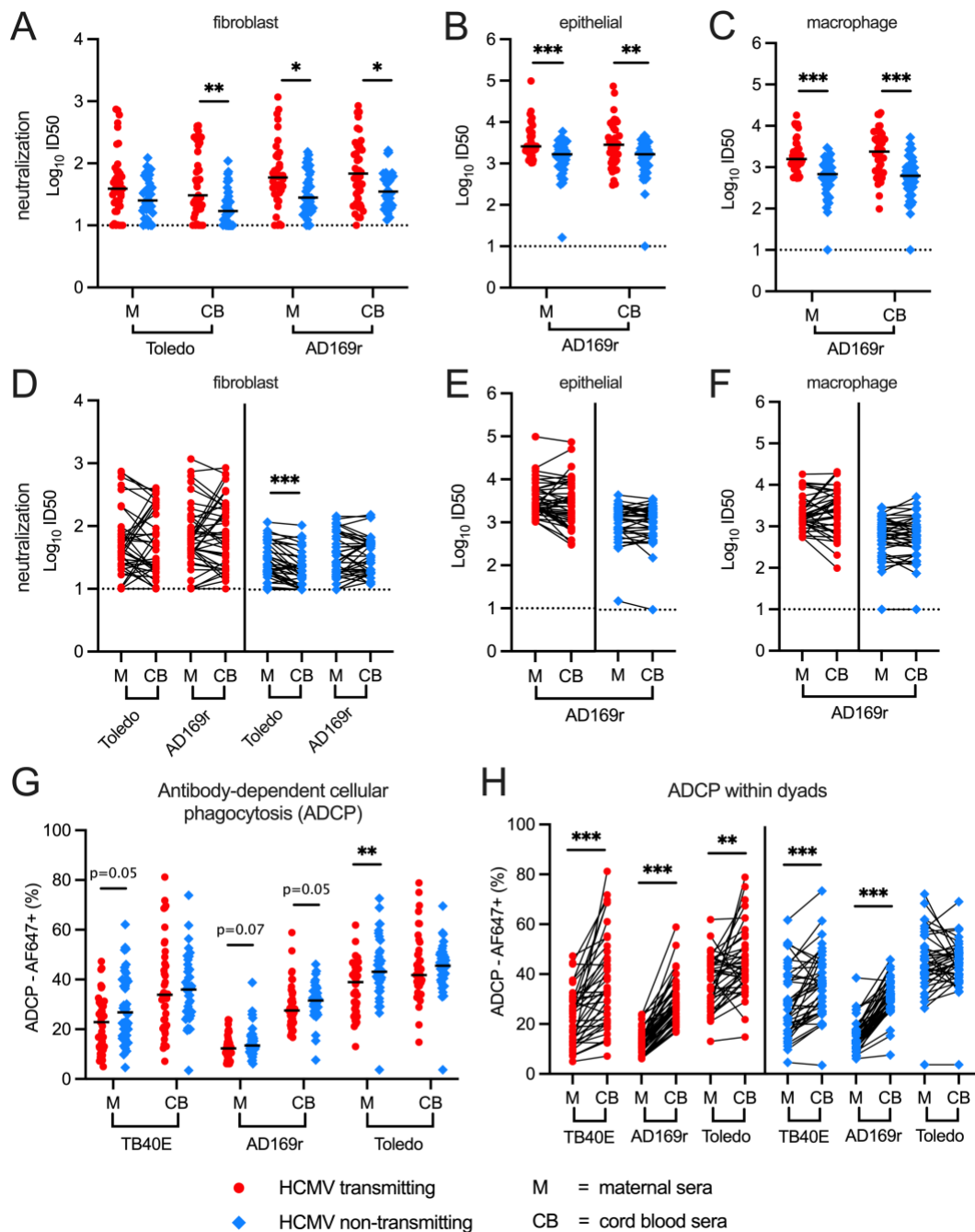
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954 **Figure 1. HCMV-specific IgG binding and transplacental IgG transfer in HCMV transmitting and**  
 955 **non-transmitting mother-infant dyads.** HCMV-specific IgG levels against HCMV strains TB40E,  
 956 AD169r, and Toledo were measured using enzyme-linked immunosorbent assay (ELISA). IgG binding to  
 957 cell-associated gB was quantified using a flow-based assay with HEK293T cells transfected with full-  
 958 length gB. HCMV antigen IgG binding was measured using a Luminex-based binding antibody multiplex  
 959 assay and reported as mean fluorescent intensity (MFI). IgG binding responses in maternal (M) and cord  
 960 blood (CB) sera were compared between and within HCMV transmitting (red circles, n = 41) and non-  
 961 transmitting (blue squares, n = 40) mother-infant dyads. (A-B) IgG binding to HCMV virus antigens (A)  
 962 in transmitting versus non-transmitting dyads and (B) within paired maternal and cord blood sera. (C) IgG  
 963 binding to cell-associated gB in transmitting versus non-transmitting dyads. (D-E) IgG binding to HCMV

964 envelope glycoproteins (D) in transmitting versus non-transmitting dyads and (E) within paired maternal  
 965 and cord blood sera. (F-G) IgG binding to HCMV antigens (F) in transmitting versus non-transmitting  
 966 dyads and (G) within paired maternal and cord blood sera. gB ecto = gB ectodomain. Black bars denote  
 967 median. FDR-corrected P values reported for Mann-Whitney U test (1A, 1C, 1D, 1F) or Wilcoxon signed-  
 968 rank test (1B, 1E, 1G). \* P < 0.05, \*\* P < 0.01, \*\*\* P < 0.001.  
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 972 **Figure 2. HCMV-specific IgG binding avidity is increased in non-transmitting versus transmitting**  
 973 **mother-infant dyads.** HCMV-specific IgG binding avidity against HCMV strains TB40E, AD169r, and  
 974 Toledo were measured using whole virion enzyme-linked immunosorbent assay (ELISA) with an  
 975 additional dissociation step using urea and relative avidity index (RAI) was calculated as (OD with  
 976 urea/OD without urea)x100%. HCMV glycoprotein-specific IgG binding avidity was measured using a  
 977 Luminex-based binding antibody multiplex assay with an additional dissociation step with sodium citrate  
 978 and RAI was calculated as (MFI with sodium citrate/MFI with 1X PBS)x100%. IgG binding avidity in  
 979 maternal (M) and cord blood (CB) sera were compared between and within HCMV transmitting (red  
 980 circles, n = 41) and non-transmitting (blue squares, n = 40) mother-infant dyads. (A-B) Whole virus  
 981 HCMV-specific IgG binding avidities (A) in transmitting versus non-transmitting dyads and (B) within  
 982 paired maternal and cord blood sera. (C-D) HCMV glycoprotein-specific IgG binding avidities (C) in  
 983 transmitting versus non-transmitting dyads and (D) within paired maternal and cord blood sera. gB ecto  
 984 = gB ectodomain. Black bars denote median. FDR-corrected P values reported for Mann-Whitney U test  
 985 (2A, 2C) or Wilcoxon signed-rank test (2B, 2D). \* P < 0.05, \*\* P < 0.01, \*\*\* P < 0.001.

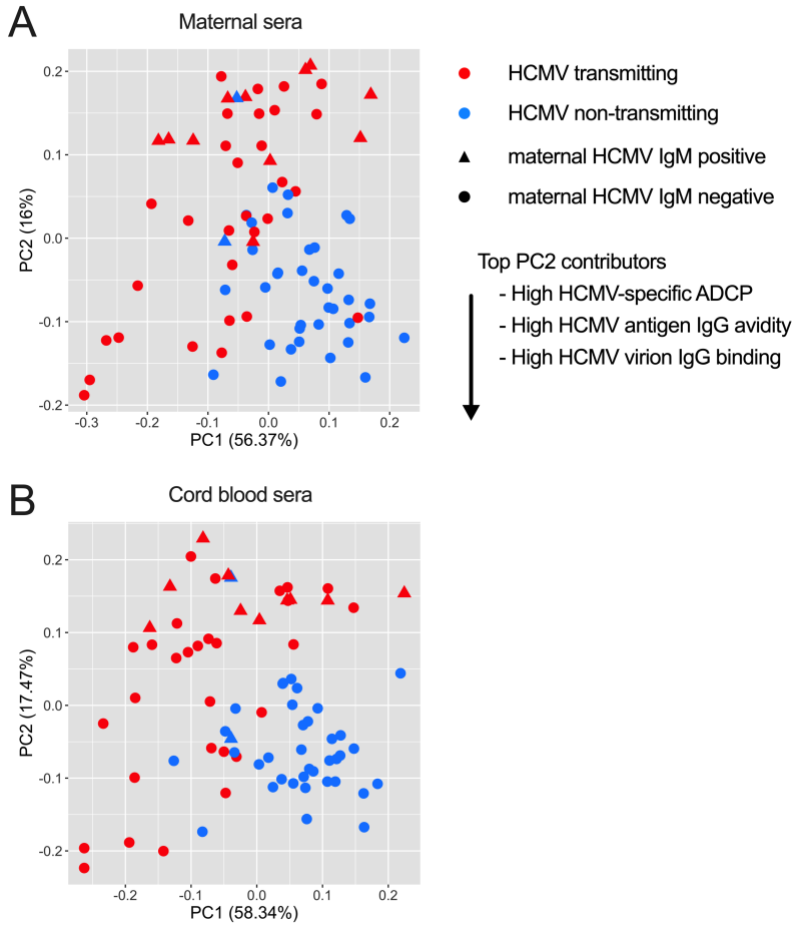


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**Figure 3. Neutralizing and non-neutralizing antibody responses differ in HCMV transmitting compared to non-transmitting mother-infant dyads.** Functional anti-viral antibody responses in maternal (M) and cord blood (CB) sera were compared between and within HCMV transmitting (red circles, n = 41) and non-transmitting (blue squares, n = 40) mother-infant dyads. Neutralization was measured by HCMV IE1 staining and titers were calculated as the inhibitory dilution 50 (ID50), equivalent to the sera dilution that inhibited 50% of the max infection in virus only wells. (A-C) Neutralization titers against HCMV strains Toledo and/or AD169r in (A) fibroblasts (HFFs), (B) epithelial cells (ARPEs), and (C) macrophages (differentiated THP1s) in transmitting versus non-transmitting dyads and (D-F) within paired maternal and cord blood sera. Antibody-dependent cellular phagocytosis (ADCP) of AF647 fluorophore-conjugated HCMV virions by THP1 monocytes was quantified using a flow-based assay and calculated as percentage AF647 positive cells. (G-H) HCMV-specific ADCP (G) in transmitting versus non-transmitting dyads and (H) within paired maternal and cord blood sera. Black bars denote median.

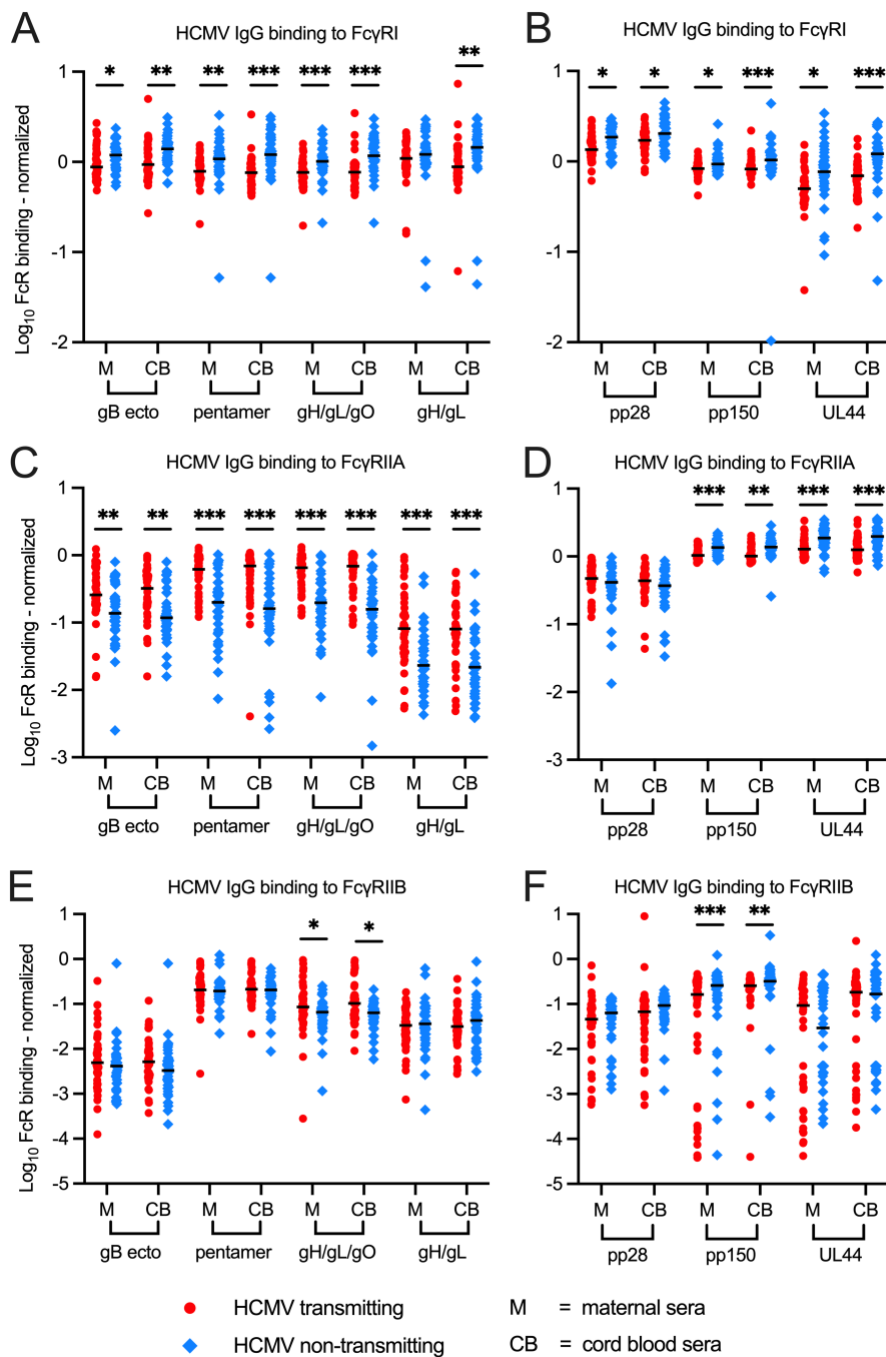
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Dotted lines indicate the lower limit of detection (ID50 = 10). FDR-corrected P values reported for Mann-Whitney *U* test (3A-C, 3G) or Wilcoxon signed-rank test (3D-F, 3H). \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$



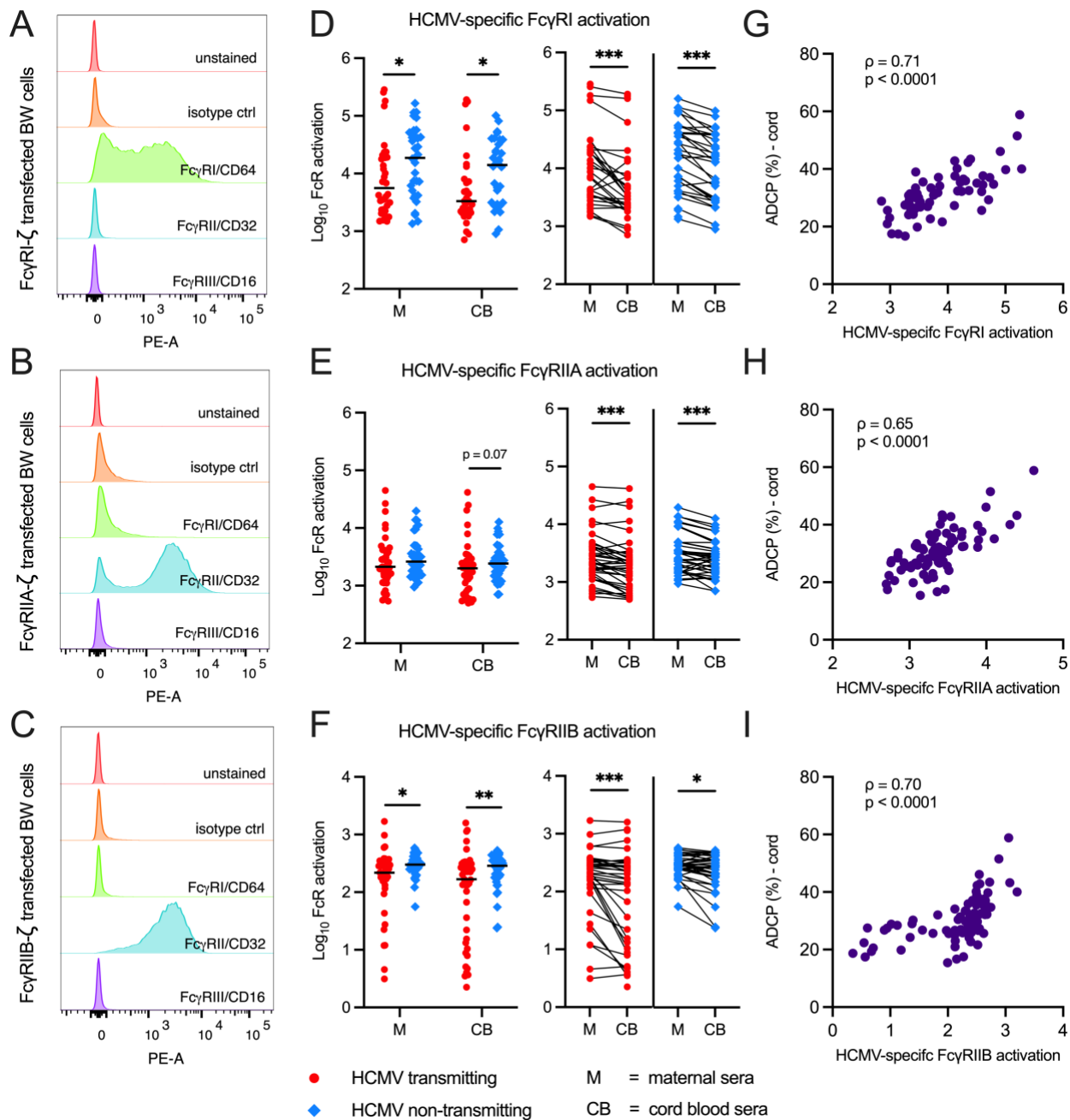
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**Figure 4. Principal components analysis (PCA) highlights distinct HCMV-specific antibody responses in HCMV transmitting versus non-transmitting dyads.** Principal components analysis (PCA) across antibody responses in HCMV transmitting (red,  $n = 41$ ) and non-transmitting (blue,  $n = 40$ ) mother-infant dyads. Triangles ( $n=14$ ) indicate dyads where mothers screened positive for HCMV-specific IgM responses and circles ( $n=67$ ) indicate dyads where mothers had no detectable HCMV-specific IgM responses. Scatterplot of PC1 and PC2 for (A) maternal and (B) cord blood sera.

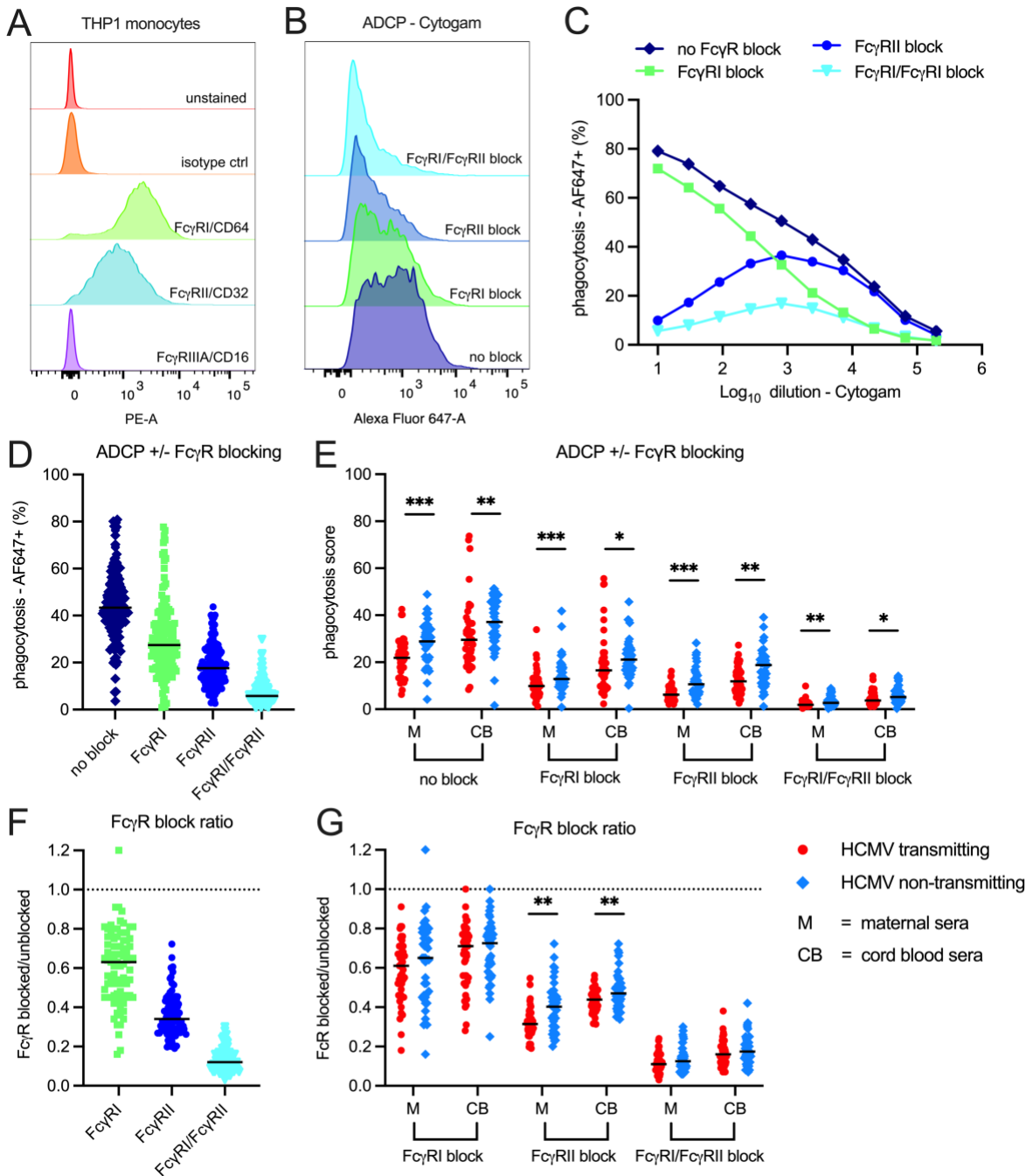


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**Figure 5. HCMV-specific IgG binding to Fc $\gamma$ RI, Fc $\gamma$ RIIA and Fc $\gamma$ RIIB differs in transmitting and non-transmitting dyads.** HCMV antigen-specific IgG binding to Fc $\gamma$  receptors (Fc $\gamma$ R) was measured using a Luminex-based binding antibody multiplex assay with a biotinylated Fc $\gamma$ R and streptavidin-PE detection antibody. HCMV antigen-specific IgG binding to host Fc $\gamma$ R was normalized as a ratio of total antigen-specific IgG binding and was compared between transmitting (red circles, n = 41) and non-transmitting (blue squares, n = 40) mother-infant dyads. (A-B) HCMV-specific IgG binding to activating Fc $\gamma$ RI, (C-D) activating Fc $\gamma$ RIIA (high affinity H131 variant), and (E-F) inhibitory Fc $\gamma$ RIIB. gB ecto = gB ectodomain. Black bars denote median. FDR-corrected P values reported for Mann-Whitney U test (5A-F). \* P < 0.05, \*\* P < 0.01, \*\*\* P < 0.001.



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 1021 **Figure 6. HCMV-specific IgG activation of FcγRI, FcγRIIA and FcγRIIB is increased in non-transmitting**  
 1022 **compared to transmitting mother-infant dyads.** HCMV-specific IgG activation of FcγRs was measured  
 1023 using maternal (M) and cord blood (CB) sera from HCMV transmitting (red circles, n = 41) and  
 1024 non-transmitting (blue squares, n = 40) mother-infant dyads. To quantify HCMV-specific IgG activation of  
 1025 FcγRs, mouse BW cell lines stably expressing chimeric human FcγRs fused to a mouse CD3ζ signaling  
 1026 domain were co-cultivated with virus:sera immune complexes for 20 hours. Activation of FcγRs by  
 1027 immune complexes triggered CD3ζ signaling and mouse IL-2 secretion, which was measured by ELISA  
 1028 as a quantitative read-out of HCMV-specific IgG signaling through host FcγRs. (A-C) Flow cytometry of  
 1029 BW cell lines including unstained cells (red), isotype control (orange), anti-FcγRI/CD64 (light green), anti-  
 1030 FcγRII/CD32 (blue), and anti-FcγRIII/CD16 (purple) PE-conjugated antibodies. (D-F) HCMV-specific IgG  
 1031 activation of (D) FcγRI, (E) FcγRIIA, (F) FcγRIIB in transmitting versus non-transmitting dyads  
 1032 and within paired maternal and cord blood sera. (G-I) Spearman correlations between HCMV-specific IgG  
 1033 FcγR activation and ADCP. Black bars denote median. P values for Mann-Whitney U test (6D-F between  
 1034 groups) or Wilcoxon signed-rank test (6D-F within dyads). \* P < 0.05, \*\* P < 0.01, \*\*\* P < 0.001.



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**Figure 7. Antibody-dependent cellular phagocytosis (ADCP) of HCMV is mediated by Fc $\gamma$ RI and Fc $\gamma$ RIIA expressed on human monocytes.** ADCP of AF647 fluorophore-conjugated HCMV virions (Toledo strain) by THP1 monocytes was quantified using a flow-based assay. In blocking experiments, THP1 monocytes were pre-incubated with Fc $\gamma$ R blocking antibodies for 90 minutes prior to co-incubating virus:sera immune complexes with THP1 monocytes. (A) Flow cytometry of THP1 monocytes including unstained THP1s (red), isotype control (orange), anti-Fc $\gamma$ RI/CD64 (light green), anti-Fc $\gamma$ RII/CD32 (blue), and anti-Fc $\gamma$ RIIIA/CD16 (purple) PE-conjugated antibodies. (B-C) ADCP facilitated by CytoGam under no blocking and Fc $\gamma$ R blocking conditions. (D) ADCP across unblocked and Fc $\gamma$ R blocking conditions in all sera samples (n=162). (E) ADCP across unblocked and Fc $\gamma$ R blocking conditions in maternal (M) and cord blood (CB) sera from HCMV transmitting (red circles, n = 41) and non-transmitting (blue squares, n = 40) mother-infant dyads. (F) Ratio of Fc $\gamma$ R blocked/unblocked ADCP responses, calculated as (% AF647+ with FcR block)/(% AF647+ with no FcR block) in each sera sample (n=162). (G) Ratio of Fc $\gamma$ R

1048 blocked/unblocked ADCP responses in transmitting versus non-transmitting dyads. Black bars denote  
1049 median. P values for Mann-Whitney U test (7E, 7G). \* P < 0.05, \*\* P < 0.01, \*\*\* P < 0.001.

Table 1. Baseline characteristics of HCMV transmitting and non-transmitting cord blood bank donor mother-infant dyads

Mother-infant dyad characteristics	HCMV transmitting (n=41)	HCMV non-transmitting (n=40)
<b>Infant sex, n (%)</b>		
Female	17 (41.5%)	16 (40.0%)
Male	24 (58.5%)	24 (60.0%)
<b>Infant race/ethnicity, n (%)</b>		
White	26 (63.4%)	24 (60.0%)
Black or African American	8 (19.5%)	8 (20.0%)
Hispanic or Latino	2 (4.9%)	2 (5.0%)
Other	5 (12.2%)	6 (15.0%)
<b>Maternal age (years), median [IQR]</b>	27 [23, 31]	28 [24, 33]
<b>Gestational age (months), median [IQR]</b>	39.0 [39.0, 40.0]	39.0 [38.0, 40.0]
<b>Delivery year, median [range]</b>	2013 [2010, 2015]	2012 [2008, 2017]
<b>Delivery type, n (%)</b>		
Vaginal	18 (43.9%)	24 (60.0%)
Cesarean section	23 (56.1%)	16 (40.0%)
<b>Maternal HCMV IgG avidity index, median [IQR]</b>	77.6 [70.4-84.5]	79.5 [73.3-86.3]
<b>Maternal HCMV IgM seropositivity, n (%)</b>		
Seropositive	11 (26.8%)	2 (5.0%)
Seronegative	30 (73.2%)	38 (95.0%)
<b>Maternal sera HCMV DNAemia</b>		
Positive	11 (26.8%)	15 (37.5%)
Negative	30 (73.2%)	25 (62.5%)
<b>Maternal sera HCMV viral copies, median [range]<sup>a</sup></b>	346 [256-1052]	365 [260-719]
<b>Cord blood sera HCMV viral copies, median [range]<sup>c</sup></b>	727 [137-18,100]	ND

ND = not detected

<sup>a</sup> Maternal HCMV viral copies listed in viral copies/mL, lower limit of detection = 250 copies/mL<sup>b</sup> Cord blood HCMV viral copies detected listed in IU/mL, lower limit of detection = 137 copies/mL

HCMV transmitting and non-transmitting dyads were matched on maternal age (+/- 3 years), infant race, sex, and delivery year (+/- 3 years)

**Table 2. Univariate and LASSO regression analysis of maternal humoral immune correlates of congenital HCMV transmission.** Logistic regression analysis on 13 primary variables of maternal sera anti-HCMV antibody responses comparing HCMV transmitting and HCMV IgG seropositive non-transmitting mothers.

Antibody response	Univariate logistic regression		LASSO regression <sup>c</sup>
	Beta coefficient (SE) <sup>b</sup>	P value	Beta coefficient <sup>b</sup>
IgG binding to cell-associated gB (%)	0.206 (0.068)	<b>0.003</b>	-
gB ectodomain IgG binding (Log MFI)	0.738 (0.229)	<b>0.001</b>	-
pentamer IgG binding (Log MFI)	1.749 (0.384)	<b>&lt;0.0001</b>	1.53
gHgLgO IgG binding (Log MFI)	1.469 (0.323)	<b>&lt;0.0001</b>	-
gB IgG avidity (%)	-0.030 (0.013)	<b>0.021</b>	-0.07
pentamer IgG avidity (%)	-0.023 (0.011)	<b>0.033</b>	-
gHgLgO IgG avidity (%)	-0.027 (0.011)	<b>0.011</b>	-0.58
Fibroblast neutralization (Log ID <sub>50</sub> ) <sup>a</sup>	0.688 (0.258)	<b>0.008</b>	-
Epithelial neutralization (Log ID <sub>50</sub> ) <sup>a</sup>	1.543 (0.424)	<b>&lt;0.0001</b>	-
Macrophage neutralization (Log ID <sub>50</sub> ) <sup>a</sup>	1.544 (0.397)	<b>&lt;0.0001</b>	-
Whole virion Toledo ADCP (%)	-0.058 (0.022)	<b>0.008</b>	-0.09
Whole virion TB40E ADCP (%)	-0.040 (0.019)	<b>0.032</b>	-
Whole virion AD169r ADCP (%)	-0.077 (0.043)	0.073	-

<sup>a</sup> Neutralization measured against HCMV strain AD169r

<sup>b</sup> Positive beta coefficients are associated with increased risk and negative beta coefficients are associated with decreased risk of congenital HCMV transmission

<sup>c</sup> Beta coefficients shown for significant variables included in model after LASSO feature selection

"-" Symbol indicates that the variable was not selected in the LASSO regression model following feature selection

ADCP = antibody-dependent cellular phagocytosis

SE = standard error

Bold indicates statistical significance (P value < 0.05)