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

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Running title: Fc antibody responses in congenital HCMV

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Conflict of interest statement: We have read the journal’s policy and the authors of this manuscript have the following financial conflict of interest to disclose: SRP is a consultant for Moderna, Merck, Pfizer, GSK, Dynavax, and Hoopika CMV vaccine programs and leads sponsored research programs with Moderna and Merck. She also serves on the board of the National CMV Foundation and as an educator on CMV for Medscape, KMW has a sponsored research project from Moderna on immune correlates of congenital CMV infection. The other authors have declared that no other conflict of interest exists.
Abstract

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

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

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

Identifying protective immune responses in maternal primary and nonprimary infection is necessary to develop effective interventions to prevent cCMV. High avidity HCMV-specific IgG binding and neutralizing antibodies have been the main targets of vaccines and therapeutics (10, 27). Yet, maternal treatment
with HCMV hyperimmunoglobulin, a pooled preparation of high avidity, neutralizing antibodies, to prevent fetal transmission following primary infection has had limited efficacy (28-33).

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

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

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

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

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

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

We first quantified IgG binding against 3 distinct HCMV strains including TB40E (an endotheliotropic strain expressing pentamer), AD169r (a lab-adapted strain with repaired pentamer expression), and
Toledo (a low-passage clinical isolate lacking pentamer) (Supplementary Figure 2A-D). Median IgG binding against each HCMV strain was similar between transmitting and non-transmitting groups except for cord blood IgG binding to AD169r, which was lower in infected infants (Figure 1A). Within transmitting dyads, whole virus IgG binding was lower in cord blood versus maternal sera across all strains (Figure 1B). However, IgG binding against envelope glycoproteins including gB, pentamer, gH/gL/gO, and gH/gL was significantly elevated (2.5-10 fold) in transmitting versus non-transmitting dyads, and glycoprotein-specific IgG was efficiently transferred into cord blood of infected infants (Figure 1C-E). These data reveal that infants with cCMV infection received high levels of maternal HCMV-specific IgG via placental antibody transfer. To explore if other HCMV-specific antibodies were associated with protection, we measured IgG binding against HCMV tegument proteins pp28, pp150, and the viral replication factor UL44, which are known to elicit potent IgG responses. IgG binding to pp150 and UL44 was also higher in transmitting versus non-transmitting dyads with some differences within dyads (Figure 1F-G). Altogether, these findings indicate that higher quantity of HCMV-specific IgG at the delivery timepoint is not correlated with lower congenital HCMV transmission risk.

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

In our recent study on placental IgG transfer in cCMV infection (43), we observed that HCMV transmitting women had elevated total IgG levels (i.e., hypergammaglobulinemia). In this larger cohort, we also found that total IgG levels were higher in transmitting versus non-transmitting women (Supplementary Figure 3A). When adjusting for total IgG levels, IgG binding against pentamer, gHgLgO, gHgL, and UL44 remained higher in transmitters, but non-transmitters had higher relative IgG binding to whole virus antigens and cell-associated gB (Supplementary Figure 3B-C). These data indicate that IgG binding to gB expressed in the native confirmation on a virion or infected cell surface and other HCMV antigens not captured in our study may be associated with reduced cCMV transmission risk.
HCMV-specific IgG binding avidity is increased in non-transmitting versus transmitting dyads

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

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

Next, we compared neutralizing and non-neutralizing antibody functions in transmitting and non-transmitting dyads (Supplementary Figure 5A-D). Neutralizing antibody titers were 1.5-4 fold higher in transmitting versus non-transmitting dyads across strains and cell types (Figure 3A-C). Within dyads, neutralizing titers were mostly similar in paired cord blood and maternal sera (Figure 3D-F). These data indicate that HCMV neutralizing antibodies are effectively transferred across the placenta regardless of transmission status. In contrast, HCMV-specific ADCP, a non-neutralizing antibody response, was higher in non-transmitting versus transmitting women (Figure 3G). This difference was significant for Toledo (p = 0.0057, FDR-corrected p = 0.011) with a trend towards increased ADCP of TB40E (p = 0.053) and AD169r (p = 0.068), which may not have reached statistical significance due to lower overall ADCP measured against these strains (Supplementary Figure 5E-F). Within dyads, ADCP was highly enriched in paired cord blood versus maternal sera (Figure 3H). Higher ADCP in fetal versus maternal circulation
may be due to fewer inhibitory factors, such as IgA, or could indicate enhanced placental transfer of ADCP-mediating IgG, a phenomenon that has been observed for ADCC-eliciting antibodies (44). Overall, these data suggest that non-neutralizing antibody responses may be important for preventing cCMV.

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

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

Since many immune variables in our predefined primary analysis were strongly correlated, we used least absolute shrinkage and selection operator (LASSO) for feature selection prior to multivariable analysis. LASSO is an approach to minimize overfitting a regression model that shrinks the coefficients of poorly predictive variables to zero, thereby removing them from the model. First, the cohort was randomly split into a training and test dataset and a 5-fold nested cross-validation with 5 repeats was used to train the LASSO model. LASSO-selected features included magnitude of pentamer IgG binding, avidity of gB IgG binding, avidity of gHgLgO IgG binding, and ADCP against Toledo strain (Table 2). Higher pentamer IgG binding was associated with increased risk whereas higher ADCP and IgG binding avidity were associated with decreased risk of cCMV infection in this multivariable model using the LASSO-selected features. In the out-of-sample test data, this 4-parameter LASSO model had a 0.75 accuracy (95% CI: 0.48-0.93) in predicting cCMV transmission risk with 1.00 equaling perfect prediction and 0.45 equaling the random prediction rate after class label permutation (Supplementary Figure 6).
Next, we used a systems serology approach leveraging principal components analysis (PCA) to explore differences in HCMV-specific antibody responses in transmitting versus non-transmitting dyads. PC1 accounted for 57 and 59% of the variance, respectively; however, PC2, which accounted for 16 and 17% of the variance, was superior at delineating between transmitting and non-transmitting groups (Figure 4A-B). The top contributors to PC2 included ADCP against Toledo, TB40E, and AD169r, IgG binding avidity to HCMV glycoproteins, and IgG binding magnitude to Toledo, TB40E, and AD169r strains (Figure 4). These PCA results further establish that IgG binding to HCMV antigens distinct from the major envelope glycoproteins, high avidity HCMV-specific IgG binding, and ADCP responses were enriched in the maternal and cord blood sera of non-transmitting compared to transmitting dyads.

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

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

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

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

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

To test our hypothesis that FcγRI and FcγRII engagement mediates enhanced ADCP in non-transmitting dyads, we measured ADCP in the presence and absence of FcγR blocking antibodies. We found that the human monocyte cell line we used to measure ADCP, THP1s, had high expression of FcγRI and FcγRIIA but not FcγRIIB (Figure 7A, Supplementary Figure 11A). Blocking FcγRII with a pan anti-FcγRII and FcγRIIA-specific but not FcγRIIB-specific antibody inhibited ADCP (Figure 7B-C, Supplementary Figure 11B). Notably, blocking FcγRII at high Cytogam concentrations lead to a much larger decrease in ADCP than at intermediate or low Cytogam levels (Figure 7B-C, Supplementary Figure 11B). These data indicate that FcγRI mediates ADCP most effectively at intermediate antibody levels and less efficiently at elevated antibody levels. Blocking FcγRI inhibited ADCP to a similar degree across Cytogam...
concentrations, suggesting that FcγRII-mediated ADCP is directly correlated with HCMV-specific antibody levels (Figure 7C). In maternal sera samples, blocking FcγRI only (median decrease = 40%), FcγRII only (median decrease = 70%) and FcγRI plus FcγRII (median decrease = 90%) inhibited ADCP to varying degrees (Figure 7D-G). Non-transmitting dyads had significantly higher ADCP responses compared to transmitting dyads across all FcγR blocking conditions (Figure 7E, Supplementary Figure 12) and FcγRI-mediated ADCP was particularly enhanced in non-transmitters (Figure 7G). Altogether, these findings suggest that ADCP mediated by FcγRI and to a lesser extent FcγRIIA is associated with protection against cCMV transmission.
Discussion

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

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

HCMV antigen-specific IgG levels and neutralizing antibody titers have been correlated with protection against cCMV transmission in some studies (21, 24), yet these antibody responses were not associated with decreased transmission risk in our cohort. Our findings are consistent with a recent study by Dorfman et al. that also observed higher maternal gB- and pentamer-specific IgG levels at delivery were associated with cCMV infection (25). Though higher HCMV-specific IgG levels were associated with increased transmission, antibody-dependent enhancement of infection is unlikely given compelling epidemiological and experimental evidence that maternal antibodies help protect against congenital transmission (2, 55-57). Since both studies measured maternal HCMV-specific antibody responses at the delivery timepoint,
we infer instead that elevated IgG levels and neutralizing antibody titers in transmitters are likely due to “boosting” from active HCMV infection or reactivation during pregnancy (25). This interpretation is bolstered by existing cCMV immune correlates literature measuring antibody responses at different timepoints in pregnancy. In maternal primary infection, Boppana et al. also identified higher gB-specific IgG titers in transmitting versus non-transmitting women at delivery (20). In contrast, Vanarsdall et al. found that seropositive transmitting and non-transmitting women had similar gB-, pentamer-, and gH/gL/gO-specific IgG levels and neutralization titers when measured in the first trimester (26). Moreover, Huang et al. recently demonstrated that higher pp150-specific IgG levels were associated with reduced cCMV transmission risk in HCMV seropositive women when measured early in gestation (58). Altogether, these data suggest that elevated HCMV-specific IgG levels and neutralization titers at delivery are not causally associated with cCMV infection but correlated with transmission risk because transmitting women are more likely to experience sustained viral replication during pregnancy.

Our study is the first to examine Fc-mediated antibody responses in cCMV transmission, so our finding that ADCP was associated with reduced cCMV infection represents an important step forward for the field. ADCP can defend against both cell-associated and cell-free virus through phagocytosis of virally-infected cells or virus:IgG immune complexes (59, 60). Since HCMV infection primarily spreads cell-to-cell in vivo, it is logical that ADCP would be associated with protection against placental transmission (61). FcγRI and FcγRIIA, which we demonstrate mediate this ADCP, are highly expressed on maternal- and fetal-derived monocytes and macrophages at the maternal-fetal interface (62-64), so it is intriguing to consider whether these innate immune cells employ Fc-mediated functions against HCMV (39). Future studies should investigate if ADCP or other Fc-mediated antibody functions protect against cCMV transmission systemically and/or at the maternal-fetal interface. Notably, different biophysical and biochemical properties of IgG including subclass and glycosylation also modify FcγR binding affinity and downstream effector functions, so whether these Fc characteristics modulate cCMV transmission risk should also be explored (65-67). Altogether, our data suggest that enhanced phagocytosis, anti-viral signaling, and/or antigen presentation following IgG binding to FcγRI and FcγRIIA may help protect
against cCMV infection, though the role of the inhibitory FcγRIIB remains unclear as this receptor was not expressed on the monocytes we used to measure ADCP (47, 59, 68).

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

Our results have important implications for HCMV vaccine development beyond the context of congenital infection. The most successful HCMV vaccine to-date was a gB subunit vaccine with an MF59 adjuvant that achieved ~50% efficacy, yet the antibody responses mediating this protection have remained elusive (35, 37, 38, 69). Neutralizing antibodies were poorly elicited by the gB/MF59 vaccine, leading researchers to hypothesize that non-neutralizing antibody responses mediated vaccine efficacy (34, 36, 38). Though ADCC responses were poorly stimulated in gB/MF59 vaccinees, ADCP responses were robustly induced (34, 36, 38). Moreover, there was a trend (β = -0.420, p = 0.120) towards ADCP being associated with protection against virus acquisition in our recent study of adolescent and postpartum gB/MF59 vaccinees (36). Notably, IgG binding to cell-associated gB, which we identified as an immune correlate of protection for these gB/MF59 vaccinees, was correlated with ADCP and FcγRI/FcγRIIA activation in our mother-infant cohort study (36). Taken together, these data suggest that Fc-mediated IgG responses against gB expressed on the surface of a virion or cell may protect against HCMV infection. Non-neutralizing antibody responses against other targets should also be explored as non-structural HCMV antigens were recently identified as potent targets of ADCC (70). Cumulatively, these findings indicate that Fc-mediated and polyfunctional antibody responses against diverse HCMV antigens should be investigated as potential correlates of protection in HCMV infection and vaccination (51, 65, 66, 71).
Our study has several limitations. Due to the cross-sectional and retrospective nature of this cord blood bank donor cohort, we could not definitively identify maternal primary versus nonprimary HCMV infections and the timing of maternal infection. Because of this caveat, caution is warranted in interpreting our results since differences between transmitting and non-transmitting groups may be biased by a higher rate of primary infection and/or reinfection in transmitting cases. This limitation highlights the need for future prospective studies with longitudinal sampling throughout pregnancy to define protective antibody responses against cCMV infection in both maternal primary and nonprimary infection. We also did not have information on the HCMV strains infecting transmitting versus non-transmitting women, so could not assess if maternal exposure to different HCMV strains were contributing to differences in antibody responses between groups. Placental biospecimens and maternal PBMCs were not collected, so we could not investigate placental infection nor maternal cellular immune correlates of protection, even though CMV-specific T cell responses have been associated with reduced cCMV transmission (23, 27, 72). Since long-term clinical outcomes were unavailable, we could not assess whether antibody responses correlated with protection against long-term disease sequelae, though all infants were born without signs of cCMV infection. Statistical power was limited by small sample size (n = 41 HCMV transmitting dyads), yet this represents one of the largest U.S.-based cohorts to assess cCMV immune correlates to-date and one of the few studies without the confounder of maternal HIV co-infection (21, 23-26, 73). Our study focused on ADCP and did not measure other Fc effector functions including ADCC, antibody-dependent neutrophil phagocytosis (ADNP), and antibody-dependent complement deposition (ADCD) due to limited sera sample volumes, but these Fc functions should be explored in future studies.

Development of an efficacious HCMV vaccine has been considered a “top tier priority” by the U.S. National Academy of Medicine for over 20 years but identifying immune correlates of protection to inform vaccine development has proved challenging (74, 75). Our study suggests that eliciting HCMV-specific IgG that engages FcγRI/FcγRIIA and mediates non-neutralizing Fc effector functions such as ADCP may be an important immune response in the prevention of cCMV transmission and a promising new approach.
for HCMV vaccinology. These findings will guide future studies on correlates of protection against HCMV and may inform the development of novel vaccines and therapeutics to prevent cCMV infection.
Methods

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

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

Cell culture and HCMV virus growth. Human retinal pigment epithelial cells (ARPEs), human foreskin fibroblasts (HFFs), human embryonic kidney (HEK)-293T cells, and human monocytes (THP-1s) were acquired from ATCC and cultured according to ATCC protocols. For differentiation, THP-1 cells were
cultured in R10 media with 100nM PMA and incubated for 48 hours. HCMV strains TB40/E, AD169r, and Toledo were propagated as previously described (36, 77). HFF cells (Toledo) or ARPE cells (TB40E and AD169r) were plated in T175 flasks, rested overnight and infected (MOI = 0.01) the next day once cells reaching ~80% confluency. Infected HFFs or ARPEs were incubated for ~14 days until 90-95% of cells showed cytopathic effect. Harvested cells were either sonicated or subjected to multiple freeze/thaws and then centrifuged to pellet cells before the cell-associated “supernatant” was combined with collected cell-free supernatant. This combined cell-associated and cell-free virus stock was filtered (0.45-μm) then concentrated on a 20% sucrose cushion at 20,000 rpm in a Beckman SW28 rotor for 1.5 hours. The concentrated virus pellet was resuspended in HFF or ARPE cell growth medium (0.2 M sucrose) and the titer of concentrated virus stocks was determined with HFFs (Toledo) or ARPEs (TB40E and AD169r) by the limiting dilution technique in 96-well plates.

Whole virion HCMV IgG binding and avidity. 384-well ELISA plates were coated with 33 PFU/well of TB40/E, 2700 PFU/well of AD169r, or 1000 PFU/well of Toledo virus (optimized PFU based on Cytogam binding in Supplementary Figure 2) diluted in 0.1 M sodium bicarbonate buffer then incubated overnight before blocking. Maternal and cord blood sera serial dilutions were tested starting at 1:30, plated in duplicate, then incubated before adding horseradish peroxidase (HRP)-conjugated goat anti-human IgG Fc (Jackson ImmunoResearch; 109-035-008). Plates were developed with tetramethylbenzidine (TMB) and peroxidase substrate (KPL) then optical density (OD) at 450nm was measured via SpectroMax. HCMV-specific IgG concentrations were interpolated from the linear range of a 5-parameter HCMV-hyperimmune globulin (Cytogam) standard curve. HCMV binding to monoclonal IgG antibodies against gB (TRL-345, in-house), pentamer (TRL-310, in-house), pp65 (1-L-11, Thermo), and UL44 (M612460, Genway Bio) were also included for comparisons across strains. For IgG avidity, duplicate wells were treated with 7M urea or 1X PBS between the primary and secondary incubation steps and relative avidity index (RAI) was calculated as (OD with urea)/(OD with PBS)x100%. Duplicates with coefficients of variance (CVs) >20% were repeated.
**HCMV glycoprotein-specific IgG binding and avidity.** A binding antibody multiplex assay (BAMA) was used to quantify HCMV glycoprotein-specific IgG binding and avidity (77, 78). HCMV gB ectodomain, pentamer complex, gH/gL/gO, gH/gL, pp28, pp150 and UL44 antigens were covalently coupled to intrinsically fluorescent beads (Bio-Rad). Maternal and cord blood sera were diluted at 1:500 (for gB ectodomain, pentamer complex, gH/gL/gO, gH/gL) and at 1:25 (for pp28, pp150, UL44) in assay diluent, plated in duplicate, then co-incubated with antigen-coupled beads. Antigen-specific IgG binding was detected with mouse anti-human IgG-PE (Southern Biotech) and mean fluorescent intensity (MFI) was acquired on a Bio-Plex 200 (Luminex). For avidity, duplicate wells were incubated with sodium citric acid (pH = 4.0) or 1X PBS (pH = 7.4) between the primary and secondary incubations and RAI was calculated as (MFI with sodium citric acid)/(MFI with PBS)x100%. A serial dilution of HCMV-hyperimmunoglobulin (Cytogam) was included as a positive control and the cut-off for positivity was determined by calculating the mean MFI of seronegative sera binding plus 3 standard deviations. Blank beads and wells were included to account for background signal. Duplicates with CVs >25% were repeated.

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

**Cell-associated HCMV glycoprotein B (gB) IgG binding.** A gB-transfected cell binding assay was used to measure IgG binding to cell-associated gB (36). HEK-293T cells were co-transfected with DNA plasmids expressing green fluorescent protein (GFP) and full-length gB (Sino Biological) using the Effectene Transfection Kit (Qiagen). After incubation at 37°C for 48 hours, 200,000 live cells/well were plated into
96-well U-bottom plates then centrifuged and washed before a 5 minute incubation in Human TruStain Fc Block (1:1000 dilution; BioLegend). In duplicate, cells were then co-incubated with maternal and cord blood sera diluted 1:500 or with controls for 2 hours at 37°C. A serial dilution of HCMV-hyperimmunoglobulin (Cytogam) and a gB-specific monoclonal antibody (TRL-345, in-house) were included as positive controls and seronegative sera samples were included as negative controls. Following incubation, cells were stained with Live/Dead Near-IR (1:1000; Invitrogen) then washed and stained with PE-conjugated goat anti-human IgG-Fc (1:200; Southern Biotech). Lastly, cells were washed and fixed with 10% Formalin. Events were acquired on an LSRII flow cytometer, and the percentage of PE-positive cells was calculated from the live, GFP-positive cell parent population using FlowJo (36).

Duplicates with CVs >50% were repeated.

**Neutralization.** HCMV neutralization was measured by high-throughput fluorescence bioimaging (36).

Epithelial cells (ARPEs), fibroblasts (HFFs) or differentiated monocytes/macrophages (THP-1s) were plated in 384-well clear, flat-bottom plates and then incubated at 37°C overnight. To quantify neutralization, maternal and cord blood sera samples were diluted 1:10 followed by an 8-point serial dilution and then co-incubated with HCMV strains AD169r (MOI = 2) or Toledo (MOI = 1) for 2 hours at 37°C to allow immune complex formation. Virus-only wells and an 8-point HCMV-hyperimmunoglobulin (Cytogam) serial dilution were included as positive controls while seronegative samples and no virus wells were included as negative controls. After addition of the virus:sera mixture, cells were incubated at 37°C for 24-48 hours (depending on optimized conditions for each virus strain and cell type) then fixed in 10% Formalin. To quantify HCMV infection, plates were stained with mouse anti-HCMV immediate-early 1 (IE1) gene (1:500; MAB810 Millipore) followed by goat anti-mouse IgG-AF488 (1:500; Millipore) and cell nuclei were stained with DAPI (1:10,000; Thermo Fisher). After staining, plates were visualized with a Cellomics fluorescent plate reader (**Supplementary Figure 5**) to enumerate total cell count, infected cell count, and percent infected cells in each well. Following image acquisition, neutralization
titers corresponding to the dilution that resulting in a 50% reduction in percent infected cells (ID50) were calculated using interpolation in GraphPad Prism (Supplementary Figure 5). Samples containing wells with low cell counts and with duplicates with CVs >50% were repeated.

Whole virion HCMV antibody-dependent cellular phagocytosis (ADCP). HCMV strains TB40/E, AD169r, and Toledo were conjugated to fluorochrome AF647 (Invitrogen) to measure ADCP (36). In brief, 2.0x10^6 PFU of TB40/E, 1.0x10^7 PFU of AD169r, or 1.0x10^7 PFU of Toledo virions were buffer-exchanged with 1X PBS using a 100,000-kDa Amicon filter (Millipore) and conjugated to 10µg of AF647 N-hydroxysuccinimide ester reconstituted in DMSO during a 1-hour incubation with constant agitation. The conjugation reaction was quenched with 80ul of 1M tris-HCl (pH = 8.0) and fluorescently-labeled virus was diluted 25X in wash buffer. A serial dilution of HCMV-hyperimmunglobulin (Cytogam) was included as a positive control while seronegative sera samples and an anti-RSV monoclonal antibody (Synagis) were included as negative controls. In a 96-well plate, fluorescently-labeled virus was co-incubated with maternal sera, cord blood sera (1:10) or controls at 37°C for 2 hours to allow immune complex formation before adding 50,000 THP-1 cells per well. Plates were then centrifuged (1200g) at 4°C for 1 hour in a spinoculation step before a 1-hour incubation at 37°C to allow for phagocytosis. Next, cells were transferred to a 96-well U-bottom plate, washed and fixed with 1% Formalin. Events were acquired on an LSRII flow cytometer, and the percentage of AF647-positive cells was calculated from the live THP-1 monocyte cell parent population using FlowJo (gating strategy in Supplementary Figure 5). Unstained cells and single color-stained cells were included as controls for setting gates and compensation. The cut-off for positivity was the mean signal of HCMV seronegative samples plus 3 standard deviations and duplicates with CVs >50% were repeated.

To measure FcγR expression, THP1s were stained with anti-human CD64-PE (clone 10.1, eBioscience), CD32-PE (clone 6C4, eBioscience), CD32A-FITC (clone IV.3, StemCell Technologies), CD32B-APC (clone S18005H, Biolegend), CD16-PE (clone CB16, eBioscience), and Ig-PE isotype control (clone P3.6.2.8.1, eBioscience). Cells were fixed with 2% paraformaldehyde and acquired on a LSRII flow
cytometer and analyzed using FlowJo v10.7.2. For FcγR blocking, THP1 cells were co-incubated with purified anti-human CD64 (clone 10.1 Biolegend), CD32 (clone AT10, Bio-Rad), CD32A (clone IV.3, StemCell Technologies) or CD32B (clone S18005H, Biolegend) for 1.5 hours at 37°C prior to co-incubation with virus:sera immune complexes. The remainder of the ADCP assay was performed as described above. An ADCP phagocytosis score to account for non-specific background ADCP signal was calculated as follows: (%AF647 positive cells * AF647 MFI in sera sample)/(%AF647 positive cells * AF647 MFI in PBS control wells)*100%.

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

To quantify FcγR activation, 96-well plates were coated with 20,000 PFU/well of HCMV strain AD169r and incubated at 4°C overnight. After coating, plates were washed with assay buffer (1X PBS + 1% FBS) then blocked with buffer at room temperature for 1 hour. After blocking, HCMV-coated plates were co-incubated with maternal or cord blood sera diluted 1:10 in BW cell media at 37°C for 1 hour. HCMV-hyperimmunoglobulin (Cytogam), seronegative and no antibody conditions were included as controls. Following immune complex formation, plates were washed with BW media before adding 100,000 FcR1a, FcR2a, or FcR2b-expressing BW cells per well. In separate wells, the parental (non-transfected) BW cells were added as a negative control. Cells were incubated at 37°C with immune complex coated plates
for 20 hours before being transferred to V-bottom plates and pelleted (1200 rpm). Cell supernatants were harvested and mouse IL-2 (mIL-2) levels in culture supernatants were measured using ELISA. For the mIL-2 ELISA, 384-well plates were coated with 3μg/mL of purified rat anti-mouse IL-2 (BD biosciences) and incubated at 4°C overnight before blocking. Purified mIL-2 and BW cell culture supernatants were added in duplicate and incubated for 1 hour at room temperature. After primary incubation, plates were incubated with rat anti-mIL2 conjugated to biotin (BD biosciences; 1:2000) followed by streptavidin-HRP (1:8000) for 1 hour and 30 minutes respectively. Plates were developed with TMB/KPL then OD at 450nm was measured via SpectroMax and mIL-2 concentrations were interpolated from a 5-parameter mIL-2 standard curve using GraphPad Prism. Duplicates with CVs >30% were repeated.

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

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

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


Figure 1. HCMV-specific IgG binding and transplacental IgG transfer in HCMV transmitting and non-transmitting mother-infant dyads. HCMV-specific IgG levels against HCMV strains TB40E, AD169r, and Toledo were measured using enzyme-linked immunosorbent assay (ELISA). IgG binding to cell-associated gB was quantified using a flow-based assay with HEK293T cells transfected with full-length gB. HCMV antigen IgG binding was measured using a Luminex-based binding antibody multiplex assay and reported as mean fluorescent intensity (MFI). IgG binding 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. (A-B) IgG binding to HCMV virus antigens (A) in transmitting versus non-transmitting dyads and (B) within paired maternal and cord blood sera. (C) IgG binding to cell-associated gB in transmitting versus non-transmitting dyads. (D-E) IgG binding to HCMV
envelope glycoproteins (D) in transmitting versus non-transmitting dyads and (E) within paired maternal and cord blood sera. (F-G) IgG binding to HCMV antigens (F) in transmitting versus non-transmitting dyads and (G) within paired maternal and cord blood sera. gB ecto = gB ectodomain. Black bars denote median. FDR-corrected P values reported for Mann-Whitney U test (1A, 1C, 1D, 1F) or Wilcoxon signed-rank test (1B, 1E, 1G). * P < 0.05, ** P < 0.01, *** P < 0.001.

Figure 2. HCMV-specific IgG binding avidity is increased in non-transmitting versus transmitting mother-infant dyads. HCMV-specific IgG binding avidity against HCMV strains TB40E, AD169r, and Toledo were measured using whole virion enzyme-linked immunosorbent assay (ELISA) with an additional dissociation step using urea and relative avidity index (RAI) was calculated as (OD with urea/OD without urea)x100%. HCMV glycoprotein-specific IgG binding avidity was measured using a Luminex-based binding antibody multiplex assay with an additional dissociation step with sodium citrate and RAI was calculated as (MFI with sodium citrate/MFI with 1X PBS)x100%. IgG binding avidity 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. (A-B) Whole virus HCMV-specific IgG binding avidities (A) in transmitting versus non-transmitting dyads and (B) within paired maternal and cord blood sera. (C-D) HCMV glycoprotein-specific IgG binding avidities (C) in transmitting versus non-transmitting dyads and (D) within paired maternal and cord blood sera. gB ecto = gB ectodomain. Black bars denote median. FDR-corrected P values reported for Mann-Whitney U test (2A, 2C) or Wilcoxon signed-rank test (2B, 2D). * P < 0.05, ** P < 0.01, *** P < 0.001.
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.
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

**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.
Figure 5. HCMV-specific IgG binding to FcγRI, FcγRIIA and FcγRIIB differs in transmitting and non-transmitting dyads. HCMV antigen-specific IgG binding to Fcγ receptors (FcγRs) was measured using a Luminex-based binding antibody multiplex assay with a biotinylated FcγR and streptavidin-PE detection antibody. HCMV antigen-specific IgG binding to host FcγRs 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 FcγRI, (C-D) activating FcγRIIA (high affinity H131 variant), and (E-F) inhibitory FcγRIIB. 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.
Figure 6. HCMV-specific IgG activation of FcγRI, FcγRIIA and FcγRIIB is increased in non-transmitting compared to transmitting mother-infant dyads. HCMV-specific IgG activation of FcγRs was measured using maternal (M) and cord blood (CB) sera from HCMV transmitting (red circles, n = 41) and non-transmitting (blue squares, n = 40) mother-infant dyads. To quantify HCMV-specific IgG activation of FcγRs, mouse BW cell lines stably expressing chimeric human FcγRs fused to a mouse CD3ζ signaling domain were co-cultivated with virus:sera immune complexes for 20 hours. Activation of FcγRs by immune complexes triggered CD3ζ signaling and mouse IL-2 secretion, which was measured by ELISA as a quantitative read-out of HCMV-specific IgG signaling through host FcγRs. (A-C) Flow cytometry of BW cell lines including unstained cells (red), isotype control (orange), anti-FcγRI/CD64 (light green), anti-FcγRI/CD32 (blue), and anti-FcγRII/CD16 (purple) PE-conjugated antibodies. (D-F) HCMV-specific IgG activation of (D) FcγRI, (E) FcγRIIA, (F) FcγRIIB in transmitting versus non-transmitting dyads and within paired maternal and cord blood sera. (G-I) Spearman correlations between HCMV-specific IgG FcγR activation and ADCP. Black bars denote median. P values for Mann-Whitney U test (6D-F between groups) or Wilcoxon signed-rank test (6D-F within dyads). * P < 0.05, ** P < 0.01, *** P < 0.001.
Figure 7. Antibody-dependent cellular phagocytosis (ADCP) of HCMV is mediated by FcγRI and FcγRIIA 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 FcγR 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-FcγRI/CD64 (light green), anti-FcγRII/CD32 (blue), and anti-FcγRIII/CD16 (purple) PE-conjugated antibodies. (B-C) ADCP facilitated by Cytogam under no blocking and FcγR blocking conditions. (D) ADCP across unblocked and FcγR blocking conditions in all sera samples (n=162). (E) ADCP across unblocked and FcγR 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 FcγR blocked/unblocked ADCP responses, calculated as (% AF647+ with FcR block)/(% AF647+ with no FcR block) in each sera sample (n=162). (G) Ratio of FcγR...
blocked/unblocked ADCP responses in transmitting versus non-transmitting dyads. Black bars denote 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

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

ND = not detected

a Maternal HCMV viral copies listed in viral copies/mL, lower limit of detection = 250 copies/mL

b 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.

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

<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)