Plasmablast Response to Primary Rhesus Cytomegalovirus Infection in a Monkey Model of Congenital CMV Transmission

Running Title: Plasmablast Response to Primary RhCMV Infection

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ABSTRACT

Human cytomegalovirus (HCMV) is the most common congenital infection worldwide, and the leading infectious cause of neurologic deficits and hearing loss in newborns. Development of a maternal HCMV vaccine to prevent vertical virus transmission is a high priority, yet protective maternal immune responses following acute infection are poorly understood. To characterize the maternal humoral immune response to primary CMV infection, we investigated the plasmablast and early antibody repertoire using a nonhuman primate model with two acutely rhesus CMV (RhCMV) infected animals – a CD4+ T cell-depleted dam that experienced fetal loss shortly after vertical RhCMV transmission and an immunocompetent dam that did not transmit RhCMV to her infant. Compared to the CD4+ T cell-depleted dam that experienced fetal loss, the immunocompetent, nontransmitting dam had a more rapid and robust plasmablast response that produced a high proportion of RhCMV-reactive antibodies, including the first identified monoclonal antibody specific for soluble and membrane-associated RhCMV envelope glycoprotein B (gB). Additionally, we noted that plasmablast RhCMV-specific antibodies had similar variable gene usage and maturation to those observed in a monkey chronically co-infected with simian immunodeficiency virus (SIV) and RhCMV. This study reveals characteristics of the early maternal RhCMV-specific humoral immune responses to primary RhCMV infection in rhesus monkeys, and could contribute to a future understanding of what antibody responses should be targeted by a vaccine to eliminate congenital HCMV transmission. Furthermore, the identification of a RhCMV gB-specific monoclonal antibody underscores the possibility of modeling future HCMV vaccine strategies in this nonhuman primate model.
INTRODUCTION

Human cytomegalovirus (HCMV) is a ubiquitous herpesvirus with seroprevalence rates ranging from 40-90% of the world's adult population (1). HCMV is the most common cause of congenital infection worldwide, affecting 0.64% of all live-born infants, totaling 40,000 newborns per year in the United States alone (2). Of these 40,000 annual congenital HCMV infections, it is estimated that 5,400 cases result in long-term neurological sequelae, and an additional 400 cases result in fetal death (3). One of the most common neurological manifestations of congenital HCMV infection is microcephaly, a condition that has gained global attention following the recent outbreak of Zika virus in the Americas (4). HCMV is also the leading cause of sensorineural hearing loss in newborns, accounting for up to 25% of all childhood deafness (5-7). In recognition of the major impact of congenital HCMV infection on pediatric health, the development of a protective maternal HCMV vaccine has remained a “top priority” of the National Academy of Medicine for the past fifteen years (8-10).

The rate of congenital HCMV transmission is higher among women who acquire primary infection during pregnancy (30-35%) than among HCMV-seropositive women (0.2-2%) (2, 11-13). Furthermore, in utero HCMV transmission following primary maternal CMV infection more frequently results in permanent and severe neurological deficits, especially when transmitted during the earliest stages of fetal development (14). Collectively, these findings suggest that preexisting maternal immunity can provide partial protection against congenital transmission and plays a role in the prevention of adverse pregnancy outcomes such as neurological impairment, and, in some cases, fetal loss. However, the maternal immune correlates of protection, which will frame the immunologic targets of a HCMV vaccine, are not yet clearly defined.

Previous human cohort studies have suggested that antibodies likely play an important role in controlling both vertical transmission of HCMV and reducing the severity of clinical outcomes following infant infection. Pregnant women with early avidity maturation of HCMV-
specific IgG antibodies appear to be at a lower risk of vertical transmission (15). Additionally, in comparison to HCMV-transmitting mothers, non-transmitting mothers exhibited earlier antibody responses to surface glycoprotein pentameric complex (gH/gL/UL128/UL130/UL131A), and antibodies with this specificity were found to inhibit HCMV cell-to-cell spread and infection of cells by free virus (16, 17). Furthermore, infants with an increased number of B cells present at birth had a reduced incidence of long-term impairment resulting from congenital HCMV infection, suggesting that antibodies may be critical for effective anti-HCMV immunity during fetal development (18). Despite these findings, attempts to prevent transplacental HCMV transmission by passive infusion of HCMV-specific hyperimmune globulin to pregnant women following primary infection did not result in a significant reduction in rates of congenital infection (19). However, perhaps the elicitation or delivery of more potent functional antibodies may be an optimal strategy to prevent placental HCMV transmission (16).

The role of maternal humoral immunity in preventing severe fetal outcomes is further supported by our recently described rhesus monkey model of congenital CMV transmission (20). In our model, two groups of RhCMV-seronegative pregnant females (immunocompetent and CD4+ T cell-depleted dams) were inoculated intravenously with a swarm of 3 RhCMV strains (180.92, UCD52, and UCD59) at 8 weeks of gestation. In 2 of 3 immunocompetent females, intrauterine RhCMV transmission occurred between 2 and 4 weeks post infection. Of interest, while all fetuses of the immunocompetent dams were carried to term and there were no signs of congenital CMV pathogenesis, a single RhCMV-seronegative, immunocompetent dam did not transmit RhCMV to her infant despite the absence of pre-existing immunity. In contrast, intrauterine transmission occurred within 1 to 3 weeks of infection in 4 of 4 CD4+ T cell-depleted pregnant dams, of which 3 experienced fetal loss at week 3 post infection while the fourth animal delivered a full-term infant with symptomatic CMV disease. Most notably, CD4+ T cell-depletion caused a delay in the appearance of RhCMV-neutralizing antibodies until 3 weeks post infection, which may have contributed to protection against the severe fetal outcome (20).
Given the rapidity of intrauterine transmission, dampened maternal anti-RhCMV antibody function, and fetal loss and/or disease after inoculation of CD4+ T cell-depleted dams, we sought to characterize the acute maternal B cell responses in both a CD4+ T cell-depleted, RhCMV-transmitting dam as well as an immunocompetent non-transmitting dam in order to define the early humoral immune response to RhCMV infection during pregnancy.

Here, we applied a newly developed rhesus plasmablast phenotyping and sorting methodology (21) to define the kinetics and monoclonal antibody repertoire of the plasmablast response to acute RhCMV infection in both a RhCMV-transmitting and non-transmitting dam. We also compared the early antibody responses to that of the memory B cell repertoire of a chronically RhCMV and SIV co-infected rhesus monkey. The recovered monoclonal antibodies were further assessed for RhCMV antigen-specific binding and genetic/maturation characteristics in comparison to those isolated from the memory B cell population of a chronically RhCMV/SIV co-infected rhesus monkey. Our characterization of the plasmablast response in rhesus monkeys following primary RhCMV infection will guide future studies evaluating immunologic responses to acute viral infection in rhesus monkey models.

Furthermore, our dissection of the potentially-protective role of the early maternal humoral immune response against congenital RhCMV transmission could inform our understanding of the characteristics of maternal antibodies that prevent congenital HCMV transmission in humans.
MATERIALS AND METHODS

Characterization and isolation of plasmablasts from acutely RhCMV-infected rhesus dams. The plasmablast population in peripheral blood mononuclear cells (PBMCs) from RhCMV-transmitting and non-transmitting dams during primary RhCMV infection were phenotypically characterized and isolated using a panel of fluorescently-conjugated monoclonal antibodies and flow cytometry as previously describe (21). Plasmablasts were defined by the following flow cytometry phenotype: CD14-/CD16-/CD3-/CD20-/HLAD R+/CD11c-/CD123-/CD80+ (Fig. S1). Gating controls were determined using fluorescence minus one (FMO) controls for the full panel (CD3, CD20, CD14, CD16, CD11c, CD123, HLA-DR, CD80, surface IgG, AquaVD) (Table S1). Plasmablasts were isolated using the following flow cytometry phenotype: CD14-/CD16-/CD3-/CD20-/HLAD R+/CD11c-/CD123-/CD80+/ surface IgG+; surface IgG expression was added to the sorting criteria to potentially enhance the yield of RhCMV-specific antibody-producing plasmablasts. Though well-defined in humans, plasmablasts are not as well-defined phenotypically in rhesus monkeys. Thus, plasma blast responses were first characterized in a rhesus monkey who received a DTaP booster vaccination (Fig. S2). This technique was then applied to PBMCs isolated from the immunocompetent RhCMV non-transmitting dam (251-05) in addition to a CD4+ T cell-depleted, RhCMV-transmitting monkey (369-09) for comparison. Complete blood counts were performed on peripheral blood at each time point to be able to calculate absolute numbers of plasmablasts.

Isolation of memory B cells from a chronically RhCMV/SIV co-infected monkey. Memory B cells, defined as CD20+/CD3-/surface IgD- lymphocytes, were identified and sorted from PBMCs collected from an adult rhesus monkey naturally infected with RhCMV and intravenously inoculated with simian immunodeficiency virus (SIV) one year prior to evaluation. Memory B cells were sorted as single cells into 96-well plates containing an RNA stabilizing
mixture (22). Of note, the use of an SIV-infected monkey was a matter of convenience and was not intended to model HCMV/HIV co-infection.

**PCR amplification, sequencing, and data analysis of immunoglobulin heavy (V\text{H}) and light (V\text{L}) variable genes isolated from plasmablasts and memory B cells.** The immunoglobulin (Ig) V\text{H}, D\text{H},J\text{H}, and V\text{L},J\text{L} genes of the sorted plasma cell and memory B cells were amplified by RT and nested PCR using the method and primer sets as reported (22). Nested V\text{H} and V\text{L} gene PCR methods were completed as described (23). Briefly, PCR products of Ig V\text{H} and V\text{L} genes were purified using a PCR purification kit and sequenced in forward and reverse directions using an ABI 3700 instrument and BigDye sequencing kit. The isotype of Ig heavy chain and light chain were determined by comparing the constant region sequences of the isolated Ig V\text{H} and V\text{L} PCR products with the constant and variable region sequences of defined rhesus Ig genes. Functional V\text{H} and V\text{L} gene pairing and inferred unmutated ancestors were determined using Cloanalyst, as well as gene segment usage, somatic mutations, and complimentary determining regions 3 (CDR3) length.

**Expression of V\text{H} and V\text{L} as full-length IgG\text{1}, recombinant monoclonal antibodies.** Using the methods described (23), the isolated Ig V\text{H} and V\text{L} gene pairs were assembled by PCR into the linear full-length Ig heavy and light chain gene expression cassettes for production of recombinant monoclonal antibodies by transfection in the human embryonic kidney cell line, 293T (22). Three days following transfection with purified PCR products of the paired Ig heavy and light chain gene expression cassettes, cell culture supernatants containing recombinant monoclonal antibodies were harvested and quantified for expressed IgG levels and screened for antibody reactivity (23).
Evaluation of plasma and monoclonal antibody reactivity to RhCMV. To test transiently-expressed recombinant monoclonal antibodies and plasma for RhCMV-specific reactivity, transfection supernatants were assessed for binding to whole free virus and viral lysates prepared from fibroblast-adapted 180.92 (grown in primary rhesus fibroblasts and passaged in telomerized rhesus fibroblasts) and epithelial cell-tropic UCD59/UCD52 (grown/passaged in monkey kidney epithelial cells) (24, 25), by ELISA as previously described (20). The positivity cut-off was defined as an OD>0.1 which was twice that of antibody negative control wells. Antibodies were also screened for polyreactivity against an SIV envelope (Env) antigen, SIVmac251gp120 (26). Antibodies with reactivity against SIV gp120, similarly defined by an OD>0.1 and twice that of irrelevant transfected antibody negative control wells, were considered to demonstrate SIV Env reactivity. Rhesus anti-influenza HA IgG monoclonal antibody (CH65) was included as a negative control.

Assessment of plasmablast mAb specificity by RhCMV glycoprotein-expressing Modified Vaccinia Ankara (MVA) vectors. Flow cytometry was used to investigate rhesus monkey antibody binding specificity as described (27), with the following modifications. Briefly, BHK-21 cells were infected with MVA-RhUL128C (expressing all 5 pentameric complex proteins gH/gL/UL128/UL130/UL131A), MVA-RhgB or MVA-RhgB/pp65 at a multiplicity of infection (MOI) of 5. At 4h post infection, the cells were fixed and permeabilized using Cytofix/Cytoperm solution (BD Biosciences). Rhesus monoclonal antibodies were diluted in Perm/Wash buffer (BD Biosciences) to obtain a concentration of 500ng/ml and added to the cells for 1h at 4°C. After washing with Perm/Wash buffer, Alexa Fluor 647 Goat Anti-Rhesus IgG (H+L) (SouthernBiotech) was added at a dilution of 1:2,000 for 1h at 4°C. After an additional wash, the cells were resuspended in PBS-0.1% BSA and acquired using a Gallios flow cytometer (Beckman Coulter). Analysis was performed using FlowJo software (Tree Star). Uninfected BHK-21 cells were used as a control. GFP expression from infected cells was used to confirm
MVA infection since all the constructs contained a GFP expression cassette (28, 29).

Immunoblotting was performed to confirm antibody binding using lysates from BHK-21 cells infected with MVA-RhUL128C or MVA-RhgB at a MOI of 5 for 24h. Infected and uninfected cells (2.5x10^5) were resuspended in 250µl reducing Laemmli buffer (DTT 0.1 M). Proteins were boiled, electrophoretically separated by SDS-PAGE, and blotted to a PVDF membrane. Purified rhesus monoclonal antibody was incubated with the membrane at a concentration of 10ng/ml. Polyclonal anti-RhgB mouse antiserum (30) was used at a dilution of 1:3,000 and hybridoma supernatant of anti-BR-5 was used at a dilution of 1:1,500. Proteins were visualized with secondary antibodies; anti-rhesus (KPL) or anti-mouse IgG (Thermo Scientific) Ab coupled to horseradish peroxidase (HRP), followed by chemiluminescence detection using Pierce ECL Western blot substrate (Thermo Fisher Scientific).

Assessment of plasmablast mAb binding to soluble and membrane-associated RhCMV glycoproteins. First, to isolate membrane-associated RhCMV proteins, confluent telomerase rhesus fibroblast (teloRF) cells were infected with RhCMV (either UCD52 or 180.92) at an MOI of 1. At 72hpi, cells were harvested by manual cell scraping, washed in PBS, then the cytosolic and membrane protein fractions isolated using the Mem-PER Plus Membrane Protein Extraction Kit (Thermo Fisher Scientific). Next, soluble RhCMV glycoprotein complexes (gB and gH/gL/UL128/UL131A) and prepared RhCMV membrane-associated proteins from virally-infected cells were electrophoretically separated by SDS-PAGE (2µg/well) on a NuPAGE Novex 4-12% Bis-Tris Protein Gel (Thermo Fisher Scientific) under non-reducing conditions, then blotted to a PVDF membrane (Thermo Fisher Scientific). The membrane was blocked with casein blocking buffer, then purified monoclonal antibodies were incubated with the membrane at a dilution of 1:100 for rhesus mAbs, 1:500 for IE-1 and pp65b mAbs (kindly provided by Daniel Cawley, Oregon Health Sciences University), and 1:10,000 for RhCMVIg (whole IgG purified from RhCMV-seropositive monkeys). Proteins were detected using alkaline...
phosphatase-conjugated secondary antibodies (anti-monkey for rhesus mAbs; anti-mouse for IE-1 and pp65b; Thermo Fisher Scientific), then visualized with stabilized alkaline phosphatase substrate (Promega).

Neutralization assays. These methods were completed as described (20). Briefly, telomerized rhesus fibroblasts (TeloRF) and monkey kidney epithelial (MKE) cells were seeded into 96-well plates and incubated for 2 days at 37°C and 5% CO₂ to achieve 100% confluency. After 2 days, serial dilutions (1:10 to 1:30,000) of heat-inactivated rhesus plasma were incubated with RhCMV 180.92 or RhCMV UCD52 in a 50µl volume for 45min at 37°C. The virus/plasma dilutions were then added in duplicate to wells containing telo-RF or MKE cells, respectively, and incubated at 37°C for 2h. After washing, cells were incubated at 37°C for an additional 3h. Infected cells were then fixed for 20min at -20°C with 1:1 methanol/acetone, rehydrated in PBS with calcium (3x5min), and processed for immunofluorescence with 0.6mg/mL mouse anti-RhCMV IE-1 monoclonal antibody (Daniel Cawley, Oregon Health Sciences University) followed by a 1:500 dilution of goat anti-mouse IgG-Alexa Fluor 488 antibody (Millipore). Nuclei were stained with DAPI for 5min (Pierce) and imaged at 10x magnification. ImageJ software was used to automatically count cells from a single field of view.

Statistical analyses. Comparison of the \(V_\text{H}\) mutation frequency and \(V_\text{H}\) CDR3 length for 251-05 (NT/CD4+), 369-09 (T/CD4-), and the chronically RhCMV/SIV-infected monkey was performed using the nonparametric Kruskal-Wallis test. A p-value of <0.05 was considered as significant and was not adjusted for pair-wise comparisons. All comparisons were performed using GraphPad Prism version 6.04 and were two-tailed.
RESULTS

Kinetics of the early antibody and plasmablast responses in acutely RhCMV-infected pregnant rhesus monkeys. Two RhCMV-seronegative, pregnant rhesus macaques (251-05 and 369-09) at 8 weeks gestation were intravenously inoculated with a swarm of RhCMV strains. Animal 369-09, which had been treated with a CD4+ T cell depleting antibody one week prior to inoculation, had a high maternal plasma viral load and evidence of intrauterine transmission (virus present in amniotic fluid by PCR) within one week of RhCMV infection. Fetal loss occurred in this animal at 3 weeks post infection. By contrast, animal 251-05 did not receive the CD4+ T cell depleting antibody prior to RhCMV inoculation, had a peak maternal plasma viral load approximately 2 logs lower than that of the CD4+ T cell-depleted dam, and did not transmit the virus to her fetus (Fig. 1A and B). In the setting of acute primary RhCMV infection, both animals had peak plasma anti-RhCMV binding IgM and IgG responses by 2 weeks post infection (Fig. 1C and D), but the magnitude of the IgM and IgG responses of 369-09 (transmitting, CD4+ T cell-depleted [T/CD4-]) was 0.5-1 log lower than that of 251-05 (non-transmitting, immunocompetent [NT/CD4+]) (Fig. 1C and D). Importantly, RhCMV antigen-specific (RhgB, RhgH/gL-PC) IgG antibodies were detectable by 2 weeks post infection in the NT/CD4+ dam, but were delayed in the T/CD4- female until 5 weeks post infection (Fig. 1E and F). The higher titer of gH/gL-PC-specific antibodies in the T/CD4- dam from weeks 6-12 post infection was unexpected, but could be due to greater antigen exposure in this CD4+ T cell-depleted animal with increased plasma viral load (Fig. 1F). Furthermore, antibodies capable of neutralizing RhCMV infection of fibroblasts and epithelial cells were produced by the NT/CD4+ dam by 2 and 3 weeks post infection, respectively, yet were delayed until over 3 weeks post infection in the T/CD4- dam (Fig. 1G and H). The viral load and antibody kinetics data contained in Fig. 1 was adapted from a previous publication (20) which established this rhesus macaque model of congenital cytomegalovirus transmission.
To complement our assessment of the early antibody kinetics in each of the two monkeys, we measured the kinetics of the plasmablast response over the first 4 to 5 weeks of primary RhCMV infection. Plasmablasts, defined as CD14-/CD16-/CD3-/CD20-/CD11c-/CD123-/CD80+/HLADR+ lymphocytes (Fig. S1, Fig. S2) (21), were detectable in peripheral blood as early as 1 week after infection in the NT/CD4+ dam, continued their expansion through week 2 post infection, and diminished by week 4 post infection (Fig. 2A). In contrast, no clear peripheral plasmablast population expansion was observed in the T/CD4- dam through week 5. Notably, the population of plasmablasts detected in peripheral blood at week 0 in the T/CD4- dam may be a response to the CD4+ T cell-depleting antibody that was administered intravenously the week prior. The absolute number of plasmablasts in the periphery of the T/CD4- dam, calculated using the circulating absolute lymphocyte count as determined by the complete blood count, was lower than that in the NT/CD4+ monkey with absolute peak plasmablast counts of 1.8×10^3/µl and 5.5x10^3/µl (3 weeks post infection), respectively. Thus, CD4+ T cell depletion delayed the peak plasmablast response following RhCMV infection and may have resulted in incomplete development of the overall plasmablast response.

**Isolation and characterization of the acute plasmablast antibody repertoire during primary RhCMV infection.** Plasmablasts were single-cell sorted from the NT/CD4+ dam at week 2 post infection and the T/CD4- dam at week 3 post infection using the following flow cytometry phenotype: CD14-/CD16-/CD3-/CD20-/HLADR+/CD11c-/CD123-/CD80+/IgG+. Of 80 single-sorted plasmablasts isolated from both the NT/CD4+ and T/CD4- monkeys, immunoglobulin variable gene PCR amplification, purification, and sequencing yielded 14 heavy and light chain pairs without nonsense mutations (17.5% efficiency) and 9 functional heavy and light chain pairs (11.3% efficiency) for the NT/CD4+ and T/CD4- monkey, respectively.

To examine the specificity of the plasmablast antibodies at the peak plasmablast response during acute primary RhCMV infection, we screened the antibodies that were...
generated from the isolated plasmablasts for reactivity against RhCMV virions and virus lysates.

Antibody polyreactivity was assessed by binding to control antigen SIVmac gp120. Of the 14
antibodies generated by plasmablasts at week 2 post infection in the NT/CD4+ female, 3
(21.4%) were RhCMV-reactive, 3 (21.4%) were polyreactive and bound to both RhCMV
antigens and SIVmac gp120, and 8 (57.1%) were not reactive to either RhCMV or SIV antigens
(Fig. 3A). Among the 9 antibodies generated from the plasmablasts of the T/CD4- female, only
1 (11.1%) was solely RhCMV-specific, 4 (44.4%) were polyreactive to RhCMV virions and
SIVmac gp120, and 4 (44.4%) were nonreactive (Fig. 3B, Dataset S1).

We also compared the acutely-elicited monoclonal antibodies isolated from plasmablasts
of the two acutely RhCMV-infected monkeys to that of circulating memory B cells harvested
from an adult female rhesus monkey (206-96) chronically co-infected with RhCMV and SIV
(Dataset S2). In rhesus monkey breeding colonies, RhCMV seroconversion typically occurs by
one year of age (31), and therefore it is expected that memory B cell response to RhCMV in this
monkey may have undergone affinity maturation over several years leading to a higher degree
of antigen affinity. In addition, RhCMV/SIV co-infected monkeys often sustain RhCMV disease
due to an inability to contain viral replication (32), yet also experience B cell dysregulation.
Remarkably, of the 51 antibodies isolated from total circulating memory B cells of the
RhCMV/SIV co-infected monkey, 27 bound to either RhCMV lysate and/or intact virions (52.9%,
Fig. 3C). Relatively few bound only to whole RhCMV virions (n=2, 3.9%), which may reflect
binding to conformation-dependent surface glycoprotein complexes, whereas 15.7% bound to
only viral lysate (n=8). The majority of the remaining RhCMV-specific antibodies bound to both
whole virions and viral lysate (n=17, 33.3%) (Fig. 3C, Dataset S2). In this monkey, a relatively
small percentage of RhCMV-reactive antibodies (n=5, 9.8%) were polyreactive with SIV gp120
(Fig. 3C, Dataset S2). Additionally, only 4 of 52 isolated antibodies were reactive to SIV gp120
alone (7.8%), despite coinfection with SIV for more than 1 year. The remaining 20 of 52
antibodies from this chronically RhCMV/SIV co-infected monkey were neither RhCMV nor SIV gp120 reactive (Fig. 3C, Dataset S2).

Classification of plasmablast antibody isotype and gene usage in acute primary RhCMV infection vs. memory B cell antibodies during chronic RhCMV/SIV co-infection. Antibodies isolated from the plasmablast response following acute RhCMV infection of the NT/CD4+ dam were equally distributed between IgM and IgG isotypes (28.6% each), whereas isolated plasmablasts from the T/CD4- female and chronically RhCMV-infected memory B cell population were predominantly IgG isotype antibodies (77.8% and 84.3%, Fig. 4A). The predominant variable heavy chain family used by all plasmablasts isolated during acute RhCMV infection were V_H3 and V_H4, consistent with those used by antibodies isolated from memory B cells of the chronically RhCMV/SIV co-infected animal (Fig. 4B). When comparing total antibodies isolated from each of the 3 animals, the specific V_H gene usage had similar distributions (Fig. 4C, upper pie charts). In acute infection, 4-F was the predominant V_H gene used by the NT/CD4+ dam (21.4%), whereas the T/CD4- female showed no V_H gene preference. When comparing specific V_H gene usage of antibodies reactive with RhCMV antigens, V_H 4-D was predominant in the NT/CD4+ (33.3%) monkey and second-most predominant in the RhCMV/SIV (14.8%) monkey, yet the T/CD4- female showed no clear preference for any particular V_H gene (Fig. 4D). V_H gene 4-F was most predominant in the RhCMV/SIV co-infected monkey (22.2%), and also appeared in the antibody repertoire in acute RhCMV infection in both the NT/CD4+ dam and the T/CD4- dam.

Mutation frequency and heavy chain CDR3 length of plasmablast antibodies in primary maternal RhCMV infection vs. circulating memory B cell antibodies during chronic RhCMV/SIV co-infection. To examine the genetic characteristics and degree of affinity maturation within the plasmablast response of the NT/CD4+ and T/CD4- monkeys, we analyzed
the heavy chain somatic hypermutation frequency as well as HCDR3 of antibodies at the peak of the plasmablast response following acute RhCMV infection (Fig. 4D and E). For comparison, we included antibodies isolated from the chronically RhCMV/SIV co-infected monkey. The median variable heavy chain (V\(_H\)) somatic hypermutation rates for nonreactive antibodies from the NT/CD4+, T/CD4- and chronically RhCMV/SIV co-infected monkeys were 2.6%, 7.3%, and 6.1%, respectively which were not statistically different between any of the animals (p-value NS, Kruskal Wallis). The median HCDR3 length of nonreactive antibodies were also found to be similar among all three monkeys (p-value NS, Kruskal Wallis). No differences were observed in the median V\(_H\) mutation frequency in RhCMV-reactive antibodies in the three monkeys: 3%, 2.4%, and 5.4% in the NT/CD4+, T/CD4-, and RhCMV/SIV monkeys, respectively (p-value NS, Kruskal Wallis). Additionally, no significant differences were found in the HCDR3 length of RhCMV-reactive antibodies produced by the acutely infected NT/CD4+ and T/CD4- dams or the chronically RhCMV/SIV infected animal, which had median lengths of 17, 18, and 14 amino acids, respectively (p-value NS, Kruskal Wallis).

**Evaluation of RhCMV-reactive plasmablast mAb specificities.** We investigated the specificity of isolated plasmablast mAbs that bound strongly to RhCMV whole virions. The process of antibody screening is detailed in Fig. 5A. A total of 7 mAbs both exhibited binding against RhCMV whole virions and were efficiently produced in transient transfection (concentration of at least 0.75 µg/mL) – 4 from 251-05 (NT/CD4+) and 3 from 369-09 (T/CD4-) (Fig. 5A, Dataset S1). These mAbs were purified and screened for RhCMV glycoprotein recognition. Antibody specificity was first assessed by intracellular flow cytometry staining of permeabilized BHK-21 cells infected with modified vaccinia Ankara (MVA) expressing RhCMV pentameric complex gH/gL/UL128/UL130/UL131A (termed MVA-UL128C), RhCMV gB, or a combination of RhCMV gB and tegument protein, RhCMV pp65. Of the antibodies screened, only a single antibody (DH669) isolated from the NT/CD4+ dam exhibited specificity for RhgB.
(Fig. 5B and Fig. S3), an essential surface glycoprotein required for entry into all cell types. Recognition of Rgb by DH669 was confirmed by Western blot against both MVA-expressed (Fig. 5C) and soluble Rgb protein (Fig. 5D). Furthermore, this mAb was not cross-reactive with soluble HCMV gB protein by ELISA. DH669 is IgG1 subclass with variable heavy chain gene 1~F*02 (3% mutated, CDR3 length of 7 amino acids), and kappa variable light chain gene 4~3*01. The neutralization activity of DH669 was assessed against 180.92 in telomerized rhesus fibroblasts and UCD52 in monkey kidney epithelial cells (starting concentration 100µg/mL), but no neutralizing activity was detected in either cell line. No tested mAbs demonstrated binding to either MVA-expressed or soluble Rgb complex (Fig. S4).

We further investigated binding of these 7 mAbs against cytosolic and membrane-associated proteins isolated from RhCMV-infected fibroblasts. We first confirmed that the cytosolic protein preparations isolated from RhCMV-infected cells contained a variety of RhCMV antigens including RhCMV IE-1 and RhCMV pp65b (Fig. S4). Moreover, we noted that DH669 bound to membrane-associated Rgb from both 180.92 (fibroblast-tropic, F) and UCD52 (epithelial-tropic, E) in addition to the soluble Rgb protein (Fig. 5D, Fig. S4). Intriguingly, 4 of the remaining 6 mAbs also exhibited binding to membrane-associated Rgb (Fig. 5D, Fig. S4). Furthermore, these mAbs were reactive against Rgb expressed on the surface of both UCD52-infected and 180.92-infected cells, and are therefore not specific to Rgb from a single viral strain (Fig. 5D, Fig. S4). These membrane-associated Rgb-binding mAbs were isolated from plasmablasts of both the 251-05 (NT/CD4+) and 369-09 (T/CD4-), suggesting that they are frequently elicited in both settings.
DISCUSSION

There remains a gap in our understanding of the immune responses to primary HCMV infection during pregnancy, and in particular those that prevent viral transmission to the fetus (1). Using a rhesus monkey model of congenital RhCMV transmission, we previously observed that CD4+ T cell-depleted monkeys had delayed development of maternal RhCMV-neutralizing antibodies, which was associated with high rates of congenital transmission and fetal loss (20). Given this observation, this study sought to characterize the early maternal humoral response following primary RhCMV infection by dissecting the antibody repertoire of plasmablasts. Compared to the immunocompetent RhCMV non-transmitting dam, the CD4+ T cell-depleted dam had slower kinetics and reduced magnitude of a peripheral plasmablast response, as well as distinct antibody specificity and V_{H} gene expression.

To our knowledge, this is the first study to evaluate the plasmablast response to acute viral infection in rhesus monkeys and to characterize the plasmablast antibody repertoire in the setting of primary maternal CMV infection. Plasmablast responses can be triggered by a T cell-independent (34) or T cell-dependent pathway (33, 34). In the former, plasmablasts are activated to produce polyspecific antibodies with poor affinity maturation (35). In the T cell-dependent pathway, CD4+ follicular helper T cells secrete IL-21 which facilitates germinal center B cell selection events and differentiation of activated B cells into plasmablasts (36). By studying the antibody repertoire in acutely infected monkeys with differential CD4+ T cell status, we have gained insight into the importance of each of these pathways in the kinetics and specificity of an early antibody response in acute RhCMV infection. We realize the limitations of our study due to the antibody repertoire of a small number of animals being represented. However, our data suggests that a rapid plasmablast response against RhCMV potentially contributes to protection against congenital transmission and is dependent upon CD4+ T cell help. Therefore, the role of CD4+ T cells in the development of RhCMV-specific antibody responses to primary infection should be investigated further.
In addition to distinct kinetics of the plasmablast response in the dams with disparate immune competency and fetal outcome, we also observed possible differences in the plasmablast antibody repertoire. RhCMV-reactive plasmablasts from 251-05 (NT/CD4+) showed a preference for V_H4 family, including genes 4~D and 4~F, which resembles the V_H gene expression profile from the monkey chronically co-infected with RhCMV/SIV. In contrast, RhCMV-reactive antibodies from 369-09 (T/CD4-) showed no clear preference for V_H family or gene expression. Comparison of the genetic characteristics of these antibodies to those isolated from memory B cells from two acutely infected animals with a chronic RhCMV/SIV co-infection allowed us to assess the relationship between RhCMV-binding antibodies produced early after infection to the highly evolved antibody repertoire present during chronic RhCMV infection. The similarity of V_H gene usage between 251-05 (NT/CD4+) and the chronically RhCMV/SIV co-infected monkey suggests that this immunocompetent dam had the potential to develop a high-affinity RhCMV-binding clonal lineage. There was no discernable difference between either the percentages of plasmablast RhCMV-specific antibodies or mutation frequency between the NT/CD4+ dam and T/CD4-. However, there were a higher percentage of polyreactive antibodies in the T/CD4- dam (3/14, 21%) compared to the NT/CD4+ dam (4/9, 44%) perhaps suggesting decreased affinity maturation in the absence of CD4+ T cell help.

One surprising observation was that remarkable 53% of the memory B cell pool that was RhCMV-specific in the chronically RhCMV/SIV co-infected monkey, compared to just 17.6% of the antibodies that were SIV-reactive. This trend correlates well with the highly-prevalent HCMV-specific memory T cell responses in HCMV-seropositive individuals, comprising approximately 10% of both the CD4+ and CD8+ memory T cell compartments in blood (37). Additionally, it has been shown that a higher proportion of HCMV-specific memory CD4+ T cells compared to HIV-1-specific CD4+ T cells exist in individuals with chronic HCMV/HIV co-infections (38).
Of the 7 antibodies tested for RhCMV glycoprotein specificity, we were able to identify that only 1 (DH669) recognized MVA-expressed or soluble RhgB. Intriguingly, 4 additional mAbs bound to membrane-associated RhgB, though not to MVA-expressed or soluble RhgB. We hypothesize that this binding variability could indicate protein conformational differences between the MVA-expressed/soluble RhgB protein and RhgB expressed on the surface of RhCMV-infected cells, raising the possibility that these mAbs may be directed against pre-fusion or cell-associated RhgB protein epitopes. gB is the primary protein that mediates viral fusion with the host cell and has been a topic of vaccine research for many decades (39), culminating in multiple phase II clinical trials in which a gB subunit vaccine demonstrated moderate (~50%) efficacy (40, 41). This is the first reported isolation of an anti-RhCMV monoclonal antibody, suggesting the future possibility of modeling antibody-based prophylaxis/treatment of congenital HCMV transmission in the rhesus monkeys with RhCMV glycoprotein-specific mAbs. It is noteworthy that DH669 was non-neutralizing. Though the majority of gB-specific mAbs are non-neutralizing in humans (42), gB is recognized as an important target of neutralizing activity (43). Furthermore, it is unknown whether neutralization, and not other antibody functions such as antibody-dependent cellular cytotoxicity (ADCC), are protective against congenital CMV transmission.

Of note, none of the isolated plasmablast mAbs bound to MVA-expressed or soluble RhCMV gH/gL/UL128/UL130/UL131A pentameric complex (RhPC). One potential explanation is that our mAb isolation and glycoprotein-specificity analysis was completed at 1-2 weeks post infection. RhgB, similar to HCMV gB, is an immunodominant target and elicits high titers of antibodies early during the course of infection (44). Indeed, in humans, the development of HCMV PC-specific antibodies is delayed several weeks following the appearance of RhCMV gB reactive antibodies (45). The timing of the development of RhPC-reactive mAbs in this model is important since the pentameric complex is known to be a target of neutralizing antibodies for both HCMV (46, 47) and RhCMV (48), and therefore is perhaps an important vaccine
It remains unknown whether the kinetics of rhesus monkey anti-RhPC antibody response following natural infection are analogous to the human humoral response to HCMV PC, and more extensive mapping of the plasmablast and B cell repertoire following primary RhCMV infection is needed.

Our study of the plasmablast antibody repertoire in rhesus monkeys is limited by a small number of experimental subjects. However, we were able to investigate early maternal humoral response to acute RhCMV infection, which is important for modeling prevention of primary congenital CMV transmission and disease. Our analysis suggests that the delayed plasmablast response in the CD4+ T cell-depleted dam may have contributed to congenital infection and/or disparate fetal outcomes. This work will enable future studies to identify the characteristics, specificity, and function of antibodies that ought to be elicited by maternal vaccination to bring an end to the leading infectious cause of brain damage and sensorineural hearing loss in infants.
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**FIGURE LEGENDS**

**FIG 1** Differences in the early RhCMV-specific antibody response between a RhCMV non-transmitting (NT/CD4+) and RhCMV-transmitting (T/CD4-) pregnant monkey following acute maternal RhCMV challenge. (A-B) Maternal plasma (●) and amniotic fluid ( ) RhCMV DNA loads in the immunocompetent, RhCMV non-transmitting dam (NT/CD4+, 251-05, in blue throughout) and CD4+ T cell-depleted RhCMV-transmitting dam (T/CD4-, 369-09, in red throughout). Arrow indicates time of fetal loss due to spontaneous abortion. (C-D) IgM and IgG responses measured against whole RhCMV virions. (E-F) RhCMV gB and gH/gL/-PC glycoprotein specific responses. The dotted lines indicate the limit of detection at week 0+2 s.d. (G-H) Neutralizing titers assessed against RhCMV variants in fibroblast and epithelial cells.

**FIG 2** Kinetics of plasmablast response in a RhCMV-nontransmitting, immunocompetent (NT/CD4+) dam (251-05) and RhCMV-transmitting (T/CD4-) dam following acute maternal RhCMV challenge. (A) The plasmablast population (defined as CD80+/HLADR+ of negatively-selected lineage), outlined in black, following primary RhCMV infection in 251-05 (NT/CD4+, above) and in 369-09 (T/CD4-, below). Percentages shown denote the percent of CD80+/HLADR+ out of negatively-selected lineage. (B) Percent plasmablasts (calculated as CD80+/HLADR+ of viable lymphocytes [AqVi-/CD14-]) (left) and absolute plasmablast counts (right) are shown for 251-05 (NT/CD4+, in blue) and 369-09 (T/CD4-, in red) acutely infected with RhCMV.

**FIG 3** RhCMV reactivity of antibodies isolated from plasmablasts of dams acutely infected with RhCMV and from total memory B cells of a monkey chronically co-infected with RhCMV/SIV. Plasmablasts at peak response were sorted from 251-05 (NT/CD4+) at 2 weeks post infection (wkpi), 369-09 (T/CD4-) at 3 wkpi and screened for RhCMV reactivity and specificity. Total
memory B cells were sorted from peripheral blood of 206-96, a rhesus monkey chronically co-infected with RhCMV and SIV for 1 year. Proportions of isolated monoclonal antibodies with RhCMV or SIV reactivity, and antibodies that were nonreactive to RhCMV or SIV are shown.

**FIG 4** Isotype, subclass, and genetic analysis of antibodies in acute and chronic RhCMV infection. Distribution of immunoglobulin isotype (A), \( V_H \) family (B), and \( V_H \) gene usage (C), from total monoclonal antibodies (upper pie charts) and RhCMV-reactive antibodies (lower pie charts) isolated from NT/CD4+ (251-05, in blue), T/CD4- (369-09, in red) during acute infection as well as from a chronically RhCMV/SIV co-infected monkey (206-96, in gray). \( V_H \) mutation frequency (D) and HCDR3 lengths (E) of isolated RhCMV-reactive (●) and nonreactive (■) monoclonal antibodies were similar between each monkey.

**FIG 5** Characterization of plasmablast mAb glycoprotein binding specificities. (A) Flowchart depicting the sorting/screening of monoclonal antibodies (mAbs) from plasmablasts of 2 monkeys (B) BHK-21 cells infected with MVA recombinants were analyzed by flow cytometry for intracellular staining of RhCMV UL128C (expressing gH/gL/UL128/UL130/UL131A), gB, or gB/pp65. (Left panel) Staining of MVA infected cells was performed with DH669 as primary antibody and Alexa Fluor 647-coupled secondary anti-rhesus IgG Ab. (Right panel) GFP expression was analyzed for confirming MVA infection of BHK-21 cells since all the constructs contain a GFP expression cassette. Uninfected cells were used as a control. (C) Recognition of linear RhgB by DH669 (left panel) and anti-RhgB polyclonal serum (center panel). Immunoblots were performed by using a lysate from MVA-RhUL128C (PC) or MVA-RhgB (gB) infected BHK-21. Uninfected cells (U) were used as a control. For loading control, samples were analyzed with anti-MVA BR-5 Ab (right panel). (D) (top row) The binding of RhCMV Ig (whole IgG from RhCMV-seropositive monkeys) and DH669, (middle row) plasmablast mAbs from 251-05 (NT/CD4+), and (bottom row) plasmablast mAbs from 369-09 (T/CD4-) was assessed to soluble
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