Maternal CD4$^+$ T cells protect against severe congenital cytomegalovirus disease in a novel nonhuman primate model of placental cytomegalovirus transmission

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Elucidation of maternal immune correlates of protection against congenital cytomegalovirus (CMV) is necessary to inform future vaccine design. Here, we present a novel rhesus macaque model of placental rhesus CMV (rhCMV) transmission and use it to dissect determinants of protection against congenital transmission following primary maternal rhCMV infection. In this model, asymptomatic intrauterine infection was observed following i.v. rhCMV inoculation during the early second trimester in two of three rhCMV-seronegative pregnant females. In contrast, fetal loss or infant CMV-associated sequelae occurred in four rhCMV-seronegative pregnant macaques that were CD4$^+$ T-cell depleted at the time of inoculation. Animals that received the CD4$^+$ T-cell–depleting antibody also exhibited higher plasma and amniotic fluid viral loads, dampened virus-specific CD8$^+$ T-cell responses, and delayed production of autologous neutralizing antibodies compared with immunocompetent monkeys. Thus, maternal CD4$^+$ T-cell immunity during primary rhCMV infection is important for controlling maternal viremia and inducing protective immune responses that prevent severe CMV-associated fetal disease.

congenital cytomegalovirus | rhesus model | T-cell immunity | neutralizing antibody | rhesus CMV

Human congenital cytomegalovirus (CMV) infection occurs in 0.7% of all pregnancies (1) and is a major cause of permanent sensorineural and neurocognitive disabilities in infants worldwide. The rate of congenital CMV transmission is as high as 50% among women who acquire primary CMV infection during pregnancy, compared with less than 2% in women with chronic infection (2). Furthermore, congenital CMV transmission following primary maternal infection causes the most severe fetal outcomes including microcephaly, intracranial cyst formation, seizures, and intrauterine growth restriction (3).

Although evident, the protective immune correlates of preconceptual immunity have not been determined. Currently, the guinea pig animal model is used to study protective immune responses against congenital CMV infection, as it is the only other species known to be susceptible to placental transmission of its species-specific CMV (4). Despite having limited sequence homology to human CMV (HCMV) (5), guinea pig CMV (GPCMV) crosses the placental barrier at a similar rate following acute maternal infection and establishes fetal infection with comparable CMV-associated sequelae (6). Several vaccine strategies including live-attenuated virus (7, 8), passive immunization of antibodies specific for glycoprotein B (gB) and the gH/gL complex (9, 10), and recombinant gB subunit immunization (11) have proven to be effective at reducing the rate of congenital GPCMV infection and preventing fetal demise. In human clinical trials, gB immunization was only 50% effective in reducing postpartum maternal virus acquisition (12) and passive immunization with CMV hyperimmune globulin of women with primary CMV infection within 6 wk of presumed acquisition did not achieve a significant reduction in the rate of fetal infection compared with placebo controls (13). These findings address the need for a relevant large-animal model with a more closely related CMV genome and wider availability of tools to assess the maternal immune system.

Nonhuman primate models are widely used in the preclinical evaluation of vaccine candidates, as they are anatomically, physiologically, and immunologically similar to humans. Furthermore, their widespread use has led to the development of an extensive set of immunological tools that allow rigorous probing and characterization of vaccine-elicited immune responses. Rhesus macaques, a common nonhuman primate animal model, have previously been used to study CMV pathogenesis in the setting of primary and secondary infection (14–16). Rhesus CMV (rhCMV), which has greater sequence and structural homology to HCMV than GPCMV (17–19), results in asymptomatic infection and establishment of a persistent and lifelong

Significance

Congenital cytomegalovirus (CMV) is the leading infectious cause of childhood hearing loss and brain damage worldwide. Yet, despite its high prevalence and ranking as a top priority for vaccine development, the immune correlates of protection that could guide vaccine development remain undefined. Using a novel nonhuman primate model of congenital CMV transmission, we demonstrate a critical role for maternal CD4$^+$ T cells in the induction of protective maternal immune responses that prevent fetal demise. In addition to establishing placental CMV transmission for the first time (to our knowledge) in nonhuman primates, this study reveals an association between delayed maternal virus-specific neutralizing antibody responses and severe fetal outcome, providing insight into the mechanism by which maternal CD4$^+$ T cells impact congenital CMV disease.

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infection, similar to that in humans.. Importantly, previous studies have shown that rhCMV inoculated intraperitoneally or intracranially into the developing fetus can induce neurological defects similar to those observed in congenitally infected human infants (20, 21). However, because of the high rate of rhCMV seroprevalence among animals of reproductive age (22) and repeated exposure to rhCMV within breeding colonies where rhCMV is endemic (23), there have been no previous reports of fetal or neonatal macaques exhibiting sequelae consistent with congenital rhCMV infection.

Here, to our knowledge, we report the first nonhuman primate model of congenital CMV transmission using rhCMV-seronegative rhesus monkeys. In this model, $CD4^+$ T-cell–depleted or immunocompetent rhCMV-seronegative monkeys were inoculated with a defined mixture of rhCMV strains in the early second trimester of pregnancy. Following detection of placental rhCMV transmission, fetuses were observed for signs of rhCMV-associated sequelae, and the maternal immune responses between mothers with severely affected fetuses and asymptomatic or noninfected infants were evaluated to identify potential immune correlates of protection against congenital CMV disease in nonhuman primates.

Results

Rhesus Macaque Model of Congenital rhCMV Transmission. Two groups of rhCMV-seronegative (rhCMVsn) pregnant rhesus macaques ($n = 3$ each) were i.v. inoculated with a high-titer mixture of rhCMV strains, including the fibroblast-passaged 180.92 (24) and limited fibroblast-passaged, epithelial cell-tropic UCD52 and UCD59 (25) viruses in a 2:1:1 ratio during week 8 of a 24-wk gestation (Fig. S1). To maximize the likelihood of successful in utero rhCMV transmission, the first group of rhCMVsn dams (group 1) was treated i.v. with a recombinant rhesus anti-$CD4^+$ T-cell–depleting antibody (CD4R1) 1 wk before rhCMV infection, as reduced $CD4^+$ T-cell counts are associated with increased susceptibility to CMV infection in humans and monkeys (26–28). The second group of rhCMVsn dams (group 2) remained immunocompetent. To control for the effect of maternal $CD4^+$ T-cell depletion alone on fetal outcome, three rhCMV-seropositive (rhCMVsp) pregnant females (group 3) were treated with the $CD4^+$ T-cell–depleting antibody at the same gestational time point as group 1 but were not challenged with rhCMV.

The inoculum chosen for this study included multiple rhCMV virus stocks with differential cell tropism to enhance the probability of transplacental transmission. However, a fourth rhCMVsn animal (274-98) included in group 1 was inoculated with rhCMV 180.92 only, which itself is a mixture of rhCMV variants that either encode a full-length genome or a partially deleted ULb′ region, resulting in elimination of several immune-modulatory proteins but retaining an intact *UL128-UL131* region encoding surface proteins important for in vivo replication (29). The dosage of the inoculum [$1–2 \times 10^6$ 50% tissue culture infective dose ($TCID_{50}$)/strain], consistent with previous rhCMV challenge experiments (2×10^5 to 2×10^6 $TCID_{50}$) (29, 30), was administered i.v. based on studies conducted by Barry and colleagues (25) that have shown variable kinetics and magnitude of viremia following s.c. inoculation. Intravenous inoculation was expected to achieve localization of rhCMV progeny at the maternal–fetal interface with consistent kinetics. In addition, it was important to establish a proof-of-principle that rhCMV was indeed capable of transplacental transmission. Subsequent studies will refine routes of maternal inoculation to better recapitulate maternal HCMV exposure and subsequent fetal infection.

Efficiency of the maternal $CD4^+$ T-cell antibody depletion was determined by flow cytometry of peripheral blood $CD4^+$ T cells (Fig. S2). Within 1 wk of $CD4^+$ T-cell–depleting antibody treatment, we observed a complete loss of circulating $CD4^+$ T cells in group 1 and group 3 dams (Fig. S3*A*). Peripheral blood $CD4^+$ T cells were detected again after 1–2 wks but persisted at low levels throughout the duration of pregnancy. In contrast, the number of $CD4^+$ T cells in group 2 animals remained unchanged during acute and chronic rhCMV infection. As expected, there was a reciprocal increase in $CD8^+$ T cells in group 1 and 3 macaques (Fig. S3*B*), and an expansion in peripheral effector memory $CD4^+$ and $CD8^+$ T cells in all rhCMV-infected animals following acute infection (Fig. S3 *C* and *D*).

Quantitative real-time PCR (QPCR) was used to assess maternal plasma viremia in rhCMVsn $CD4^+$ T-cell–depleted and immunocompetent females following rhCMV inoculation. In all dams, peak viremia occurred between 3 and 5 wks postinfection, reaching a mean peak titer of 2.87×10^6 DNA copies per mL (range: 1.76×10^5 to 7.5×10^6) in group 1 females (Fig. 1 *A–D*). This was approximately one-half log higher than that of group 2 females (mean, 6.85×10^5 DNA copies per mL; range, 6.79×10^4 to 9.31×10^5) (Fig. 1 *E–G*).

Diagnosis and Outcome of Congenital rhCMV Infection. Detection of CMV DNA in amniotic fluid (AF) by PCR is the gold standard for diagnosing congenital CMV transmission in humans. Using amniocentesis and AF QPCR, we observed a 100% (four of four) transmission rate in $CD4^+$ T-cell–depleted group 1 animals (Fig. 1 *A–D*) and a 66% (two of three) transmission rate in immunocompetent group 2 animals (Fig. 1 *E–G*), defined by having at least two of six AF PCR replicate wells positive for detectable rhCMV DNA following inoculation. The mean virus load in the AF of group 1 dams ranged from 24 to 580 rhCMV DNA copies per mL and appeared as early as 1 or 2 wks postinfection, yet appeared slightly later (2 or 4 wks postinfection) and was lower magnitude (16–71 copies per mL) in group 2 dams, potentially attributable in part to the higher viral loads in the plasma of dams lacking peripheral $CD4^+$ T cells. In contrast, animals in group 3 chronically infected with rhCMV that were depleted of $CD4^+$ T cells, had little to no detectable plasma viremia during pregnancy, and AF rhCMV DNA was only detected in a single PCR replicate from animal 234-07 at week 9 of gestation. This rare and inconsistent virus detection was not considered to be confirmatory of congenital

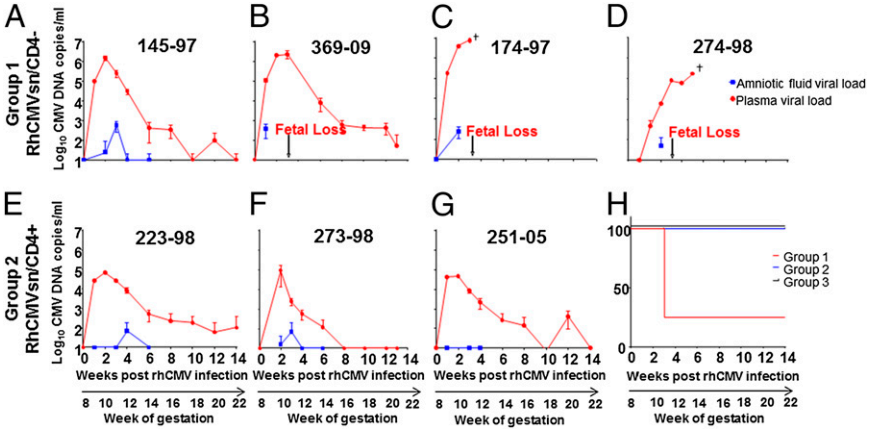

Fig. 1. Congenital rhCMV transmission and pregnancy outcome in rhCMV-seronegative, $CD4^+$ T-cell–depleted and nondepleted females following peak maternal viremia. (*A–G*) rhCMV copy number in maternal plasma and AF was assessed by QPCR in rhCMV-seronegative, $CD4^+$ T-cell–depleted group 1 and immunocompetent group 2 females. Data are shown as the mean ± SD from three or more replicates. Identification numbers for the pregnant females are displayed above each graph. Crosses signify maternal euthanasia. (*H*) Percent survival of fetuses following maternal rhCMV inoculation or $CD4^+$ T-cell depletion.

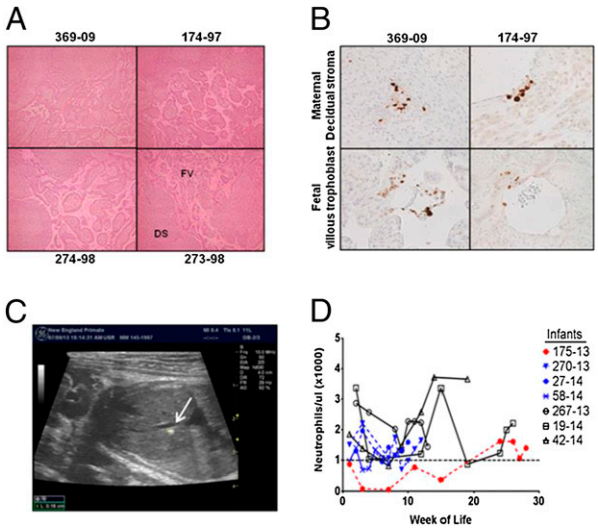

Fig. 2. rhCMV detection in placental tissue and potential CMV-associated sequelae in congenitally rhCMV-infected live-born infants. (*A*) Hematoxylin–eosin staining of placenta tissue from all CD4$^+$ T-cell–depleted group 1 females that experienced fetal loss (369-09, 174-97, and 274-98) and from 273-98, an immunocompetent group 2 dam with an asymptomatic, congenitally infected infant. DS, decidual stroma; FV, fetal villi. (*B*) Immunohistochemical staining of rhCMV-IE1 protein in placental tissue from two of the group 1 females. (*C*) Fetal sonogram of the live-born infant from group 1 revealed a liver calcification at week 20 of gestation, indicated by the white arrow. (*D*) Absolute neutrophil counts from the infant(s) born to group 1, group 2, and group 3 dams are displayed as red, blue, and black lines, respectively. Dashed lines signify infants with intrauterine rhCMV infection. The lower limit of normal neutrophil counts for rhesus infants, aged 7–30 d, is marked by the black dotted line.

rhCMV transmission. Importantly, three of the four group 1 females experienced spontaneous abortion at week 3 postinfection, whereas all group 2 dams carried fetuses to term (Fig. 1*H*). This high rate of fetal loss was not caused by CD4$^+$ T-cell depletion alone, as the control group 3 females all delivered full-term infants. Comparison of placental tissue from CD4$^+$ T-cell–depleted females that underwent spontaneous abortion to that of immunocompetent animals with asymptomatic infants showed no evidence of pathology that would indicate poor placental health as a cause of fetal loss (Fig. 2*A*).

Of note, euthanasia was required for two of the four group 1 dams around the time of peak maternal viremia (Fig. 1 *C* and *D*). Necropsy of dam 174-97 demonstrated a massive small bowel intussusception but lacked evidence of rhCMV enteritis or other obvious causal condition, making it unclear whether loss of the fetus, which occurred days before maternal death, was a result of poor maternal health or congenital rhCMV infection. Necropsy of dam 274-98 revealed widespread rhCMV dissemination, viral myocarditis, and congestive heart failure. Although the timing of fetal loss in this instance also preceded maternal death, we cannot exclude the possibility that the spontaneous abortion was a consequence of poor maternal health related to rhCMV dissemination. Still, the fetal loss, combined with detectable rhCMV in AF, suggest an important role for maternal CD4$^+$ T cells in protection against both severe maternal disease and fetal outcome during primary maternal rhCMV infection.

To investigate the extent of intrauterine rhCMV infection, we performed immunohistochemical staining for the rhCMV immediate early protein 1 (*IE1*) on available placental and aborted fetal tissue of group 1 dams (Fig. 2*B*). rhCMV-*IE1* was visible in the decidual stroma and villous trophoblasts in two of the three placentas. Additionally, sonography of the rhCMV-exposed fetuses was conducted in utero to identify early signs of congenital rhCMV infection characteristic of infected human fetuses, including microcephaly, intracranial or intrahepatic calcifications, and intrauterine growth restriction. Fetal sonography of the single live-born infant from group 1 (175-13) revealed an intrahepatic calcification at week 20 of gestation (Fig. 2*C*). No additional signs of rhCMV infection were present in this or other rhCMV-exposed fetuses (Fig. S4 *A* and *B*).

Another method used to diagnose congenital CMV infection in human infants is the detection of CMV DNA in urine or saliva within the first weeks of life (31). We performed QPCR on available infant urine and saliva samples collected between 1 and 3 wks of life, and also on aborted fetal tissue and placenta from group 1 females (Table S1). rhCMV DNA was present in the placenta of all three spontaneous abortions and in all aborted fetal tissue. The three live-born infants born to rhCMVsn females with rhCMV DNA in the AF also had detectable rhCMV DNA in saliva between weeks 1 and 3 of life. Interestingly, the saliva viral load in the single live-born infant of the group 1 dams (175-13) was ~1.5 orders of magnitude higher than other asymptomatic, rhCMV-infected infants, suggesting a higher virus burden in this infant. rhCMV was not detected in the urine or saliva of infants 267-13 and 19-14, born to the rhCMVsp group 3 dams, one of whom had detectable rhCMV DNA in the AF. However, saliva from another infant in this group (42-14) had low-level virus detectable in the first week of life, in addition to 1 of 12 PCR-positive replicates in the urine at week 3 of life and very low rhCMV DNA copies in the placenta tissue in 4 of 12 PCR replicates. These results suggest the possibility that congenital rhCMV infection can occur in naturally seropositive animals in the setting of CD4$^+$ T-cell depletion, an important finding for the assessment of maternal immune responses and congenital transmission during virus reactivation or reinfection.

Following birth, rhesus infants were also assessed for hematologic abnormalities, such as thrombocytopenia and neutropenia, which are characteristic of congenital CMV infection in humans (32). Infant 175-13, with the high saliva virus load and identified liver lesion, also displayed neutrophil counts below the normal level for juvenile monkeys at the time of birth and throughout 28 wks of life (Fig. 2*D*). One uninfected infant (58-14) born to a group 2 female displayed low levels of all hematopoietic cell types, yet no other major hematologic abnormalities were observed in the rhCMV-exposed infants at birth (Fig. S4*C*).

Identification of the Congenitally Transmitted/Founder Virus. Recent deep-sequence analysis revealed that, despite high CMV intrahost genetic variability in adults, human congenital CMV infection appears to result from a limited number of transmitted/founder variants (33). We sought to identify which of the three rhCMV strains included in our inoculum replicated most efficiently in maternal plasma of group 1 animals and which strain(s) crossed the placental barrier to result in congenital rhCMV infection. We

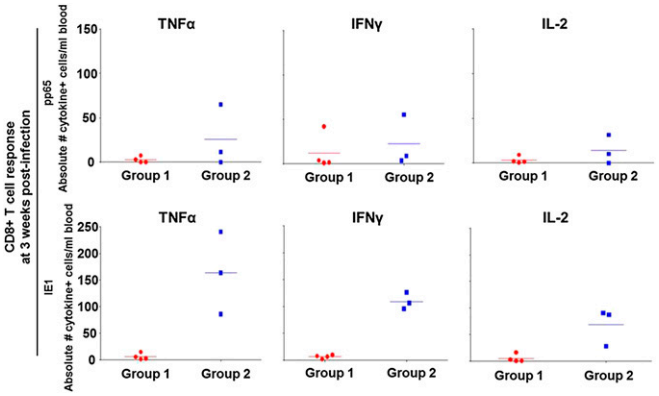

Fig. 3. Intracellular cytokine production by CD8$^+$ T cells in CD4$^+$ T-cell–depleted and immunocompetent, acutely rhCMV-infected pregnant monkeys. Flow cytometry analysis of maternal rhCMV-*pp65* (*Top*) or *IE1*-specific (*Bottom*) intracellular cytokine CD8$^+$ T-cell responses in group 1 and group 2 females at 3 wks postinfection. Data are expressed as absolute number of cytokine-producing CD8$^+$ T cells per microliter of whole blood after subtraction of background of unstimulated cells.

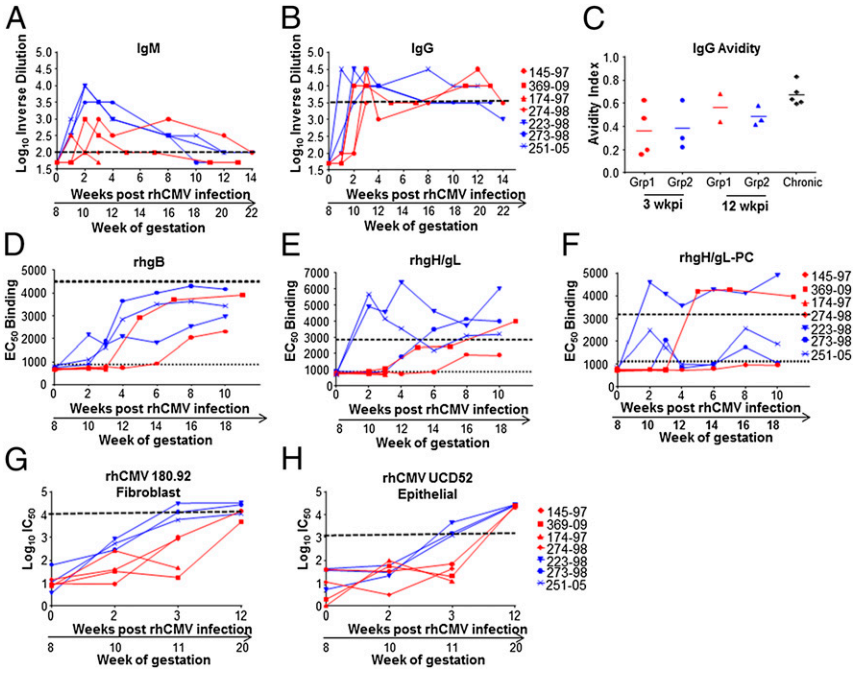

Fig. 4. The appearance of rhCMV-neutralizing antibodies is delayed in acutely rhCMV-infected, $CD4^+$ T-cell–depleted females. (*A* and *B*) rhCMV-specific IgM and IgG antibody endpoint titers in plasma from group 1 (red) and group 2 (blue) females were determined by ELISA. Dotted lines represent the mean IgM and IgG endpoint titer of three chronically infected rhesus macaques. (*C*) rhCMV IgG antibody avidity was measured in group 1 (red) and group 2 (blue) females. The IgG avidity index of five chronically infected rhesus monkeys (♦) is shown as a reference for high avidity. (*D*–*F*) EC_{50} titers were measured for IgG antibodies specific to rhCMV gB (*D*), gH/gL (*E*), and the pentameric complex (*F*) in plasma from group 1 (red) and group 2 (blue) females. The limit of detection as indicated by the lower dotted lines is the average of week 0 time points plus 2 SD. The mean IgG antibody EC_{50} titer from six chronically infected animals is represented by the upper dotted line. (*G* and *H*) Neutralizing activity of maternal group 1 (red) and group 2 (blue) plasma against rhCMV 180.92 in rhesus fibroblasts and rhCMV UCD52 in monkey epithelium. Dotted lines indicate the mean neutralizing antibody titers of three chronically infected rhesus monkeys.

performed single-genome amplification (SGA) and sequence analysis of the rhCMV *UL128* exon 1 region, which had sufficient nucleotide variability to distinguish between the three viral stocks (Fig. S5*A*). Between 90% and 100% of plasma variants had sequence homology with rhCMV UCD52, whereas the remaining plasma variants were identified as rhCMV UCD59 (Fig. S5*B*). To confirm our findings, we performed deep-sequencing analysis on two of the three maternal plasma samples. In our analysis, we identified 20 regions across the genome, 100–280 bp in length, for which there were five or more reads and that included at least two single nucleotide polymorphisms (SNPs) that could be used to distinguish between rhCMV 180.92, UCD52, and UCD59 (Fig. S5*E*). Similar to SGA, deep sequencing revealed UCD52 as the dominant strain in the plasma of animal 369-09 in all 20 genes examined. However, data from the second animal, 174-97, indicate plasma variants to be a mixed population of UCD52 and UCD59 variants as exactly one-half of the gene loci examined (10 of 20) were either UCD52 or UCD59 dominant. This discrepancy is likely a result of limited depth of genome coverage obtained by nonspecific deep-sequence analysis as many of the genes were almost equally represented by both rhCMV UCD52 and UCD59 variants, or alternatively may be due to the increased sensitivity of this assay. The absence of rhCMV 180.92 by SGA, and limited detection by deep sequencing in each of these monkeys is not surprising as only 5% of the variants within this fibroblast-adapted stock encode a full-length genome whereas the remaining 95% lack a portion of the ULb′ region encoding numerous immune-modulatory genes that are important for in vivo replication (29). Accordingly, the group 1 dam (274-98) that was infected with rhCMV 180.92, but not UCD52 or UCD59, had only rhCMV 180.92 sequences detected in maternal plasma, of which 86% of the circulating virus encoded the full-length genome (Fig. S5 *C* and *D*). We also performed SGA on DNA extracted from the AF of all three group 1 dams that received a swarm of rhCMV strains which revealed the presence of only rhCMV UCD52 (Fig. S5*B*). These findings suggest that congenital rhCMV infection in rhesus monkeys may result from a restricted number of transmitted viruses, or alternatively that epithelial-tropic UCD52 may have a fitness advantage for systemic and intrauterine virus replication that should be further explored.

Maternal rhCMV-Specific Cellular Immunity. One mechanism by which depletion of $CD4^+$ T cells could have impacted fetal outcome following acute rhCMV infection, is through its effect on both the cellular and humoral arms of adaptive immunity. $CD4^+$ T cells are important for generation of the pathogen-specific memory $CD8^+$ T cells, yet the role of maternal $CD8^+$ T-cell function in congenital CMV transmission is unknown. To determine whether maternal $CD8^+$ T-cell function is correlated with protection against severe congenital rhCMV disease in rhesus monkeys, we compared the $CD8^+$ T-cell TNFα, IFNγ, and IL-2 intracellular cytokine response to a pool of overlapping rhCMV-phosphoprotein 65 (*pp65*) and *IE1* peptides in group 1 and group 2 dams (Fig. S6). At 3 wk postinfection, concurrent with the timing of fetal loss, only low levels of rhCMV *pp65*-specific $CD8^+$ T-cell responses were observed in either group (Fig. 3). In contrast, *IE1*-specific responses were present and generally higher magnitude in group 2 compared with group 1 dams, with TNFα-secreting cells being the most abundant (Fig. 3). The observed differences in $CD8^+$ T-cell cytokine production raise the possibility that the absence of maternal $CD4^+$ T cells in group 1 females may have caused a blunting effect, albeit modest, on the early rhCMV-specific $CD8^+$ T-cell response, which could contribute to the adverse pregnancy outcomes following primary rhCMV infection. Unlike the rhCMV-specific $CD8^+$ T-cell responses, rhCMV-specific $CD4^+$ T-cell responses of both $CD4^+$ T-cell–depleted and nondepleted seronegative dams were mostly undetectable during acute infection (Fig. S7).

Maternal rhCMV-Specific Humoral Immunity. Previous studies have indicated that, although virus-specific antibody production in pregnant women with primary CMV infection does not differ between transmitting and nontransmitting females, the presence of high-avidity CMV-specific IgG responses is associated with reduced risk of congenital HCMV transmission (34). Thus, we measured both the kinetics of maternal rhCMV-specific IgM and IgG antibody responses (Fig. 4 *A* and *B*) and IgG avidity (Fig. 4*C*) in the group 1 and group 2 dams. Peak rhCMV-specific IgM antibody responses appeared by week 2 postinfection in most animals, but were 0.5–1 log lower in group 1 females (Fig. 4*A*). The kinetics of the acute maternal rhCMV-specific IgG responses were more variable between and within study groups than the IgM responses; however, similar magnitude peak titers were reached by week 3 postinfection in all dams (Fig. 4*B*). The IgG avidity index was also similar in both $CD4^+$ T-cell–depleted and nondepleted dams at weeks 3 ($P = 0.857$) and 12 postinfection ($P = 0.8$), yet

were lower than that of chronically rhCMV-infected monkeys at both time points ($P = 0.03$, $P = 0.056$; Fig. 4*C*).

Preexisting CMV-neutralizing antibodies, particularly those directed against the *gH/gL/UL128-131* pentameric glycoprotein complex (gH/gL-PC), in the sera of naturally seropositive women are hypothesized to contribute to the partial protection against congenital HCMV transmission (35). In our study, all group 2 dams had detectable *gB* and gH/gL-PC specific antibody responses by week 3 postinfection, and two of the three group 2 dams, including the single nontransmitter, also had antibodies specific for the *gH/gL* complex as early as 2 wks postinfection (Fig. 4 *D*–F). In contrast, these responses were not present in group 1 dams at 2 or 3 wks postinfection. Of the two surviving $CD4^+$ T-cell–depleted group 1 females, *gB* binding antibodies did not appear until 5–8 wks postinfection (Fig. 4*D*), and only one group 1 dam developed antibodies to the gH/gL-PC over the course of pregnancy, emerging at week 5 postinfection (Fig. 4*F*). We next determined the neutralizing capacity of antibodies in maternal plasma at early time points after rhCMV inoculation in both fibroblast and epithelial cell lines using the autologous challenge virus strains rhCMV 180.92 and UCD52, respectively. Neutralizing responses detected in both fibroblasts and epithelial cells developed more rapidly and were higher magnitude in group 2 dams than group 1 dams at week 3 postinfection (Fig. 4 *G* and *H*). The results from these studies suggest that maternal production of rhCMV-neutralizing antibodies against *gB* and gH/gL-PC within the first 3 wks following rhCMV inoculation is impacted by the presence or absence of maternal $CD4^+$ T-cell immunity and could play a significant role in fetal outcome following rhCMV infection independent of maternal health.

Discussion

To our knowledge, our study is the first to report intrauterine CMV transmission in a nonhuman primate animal model. In this model, congenital rhCMV transmission was observed in $CD4^+$ T-cell–depleted and immunocompetent, rhCMVsn dams following i.v. rhCMV inoculation during the early second trimester of pregnancy. As in humans, congenital rhCMV infection was diagnosed via the detection of viral DNA in AF, and confirmed by detection of virus in aborted tissue or infant saliva and urine collected within the first few weeks of life.

In our study, the rate of congenital rhCMV transmission was 66% (two of three) among immunocompetent dams. Although the two infected infants in this group showed no congenital rhCMV-associated sequelae, only a small percentage (10–15%) of congenitally infected human newborns display clinical signs of HCMV infection at birth (1). The rhCMV transmission rate in rhCMVsn, $CD4^+$ T-cell–depleted dams was 100% and resulted in a high rate (three of four) of spontaneous abortion and a single live-born infant with detectable sequelae of infection. Although congenital CMV is a known cause of fetal loss in humans (36) and is frequently found in the tissues of stillborn fetuses (37), two of the four $CD4^+$ T-cell–depleted dams required euthanasia shortly after the discovery of fetal loss introducing the confounding factor of poor maternal health contributing to adverse fetal outcome. Poor $CD4^+$ T-cell immunity in many clinical settings is strongly associated with enhanced susceptibility to CMV infection (26–28), which was true for both pregnant dams and their fetuses in this study. However, it is evident from our study that congenital rhCMV transmission can occur in immunocompetent dams following primary maternal rhCMV infection. To avoid the confounding effect of maternal health in this model, future experiments could minimize the viral titer used or exclude $CD4^+$ T-cell depletion. Also included in our model was a group of $CD4^+$ T-cell–depleted, naturally rhCMVsp dams. Among this group, a single infant had detectable virus in saliva during the first week of life yet had undetectable viral DNA in AF. Further investigation into whether congenital rhCMV transmission can occur in rhCMVsp dams will be critical, as approximately one-half of all congenital HCMV infections in developed regions are a result of secondary maternal infection.

It is evident from our study that $CD4^+$ T cells modulate maternal immune responses during acute infection and may be important determinants of fetal outcome. During acute infection, $CD4^+$ T-cell cytokine production and the interaction between $CD4^+$ T cells and antigen-presenting cells stimulate proliferation of the cytotoxic $CD8^+$ T-cell population, which in turn eliminates virus-infected cells. Impaired $CD4^+$ T-cell functionality in primary human and rhesus CMV infection are associated with higher viral loads and prolonged virus excretion (15, 38, 39). Thus, as expected, $CD4^+$ T-cell–depleted dams exhibited higher plasma virus loads than immunocompetent dams, which could have directly contributed to increased rates of placental transmission. Despite having more replication, the $CD8^+$ T-cell response to rhCMV-specific proteins was dampened in $CD4^+$ T-cell–depleted dams at 3 wks postinfection when fetal loss was observed, most notably TNFα production. In humans, pregnancy outcome is largely dependent on the balance of cytokines, particularly TNFα, which is expressed in almost every cell type found at the fetal–maternal interface (40). High levels of TNFα have been directly linked to fetal loss (41), but this cytokine is also required for the recruitment of macrophages that participate in maintaining pregnancy (40). Thus, it may be of interest to investigate the role of virus-specific $CD8^+$ T-cell cytokine production and function in rhCMV placental transmission, or to more closely examine these responses during severe and asymptomatic congenital rhCMV infection.

Antibody production and maturation following acute rhCMV infection are also dependent on $CD4^+$ T-cell help. We did not, however, observe notable differences in the levels of rhCMV-reactive IgM and IgG antibodies or in IgG antibody avidity between $CD4^+$ T-cell–depleted and immunocompetent dams. Instead, we observed reduced rhCMV glycoprotein-specific antibody responses to both *gB* and the pentameric complex, and a delay in antibody neutralization function in fibroblast and epithelial cells at 2 and 3 wks postinfection in animals that received the $CD4^+$ T-cell–depleting antibody. Although our findings are supportive of previous studies that implicate a correlation between weakly neutralizing antibodies and enhanced rates of congenital HCMV transmission (42), it remains to be determined whether delivery of strongly neutralizing rhCMV antibodies are sufficient for protection against rhCMV transmission and disease, and which glycoprotein complex(es) should be targeted. However, recent analysis of HCMV transmitting and nontransmitting women identified an association between the presence of neutralizing antibodies against the pentamer complex and reduced congenital infection (35). This, together with our findings that viruses encoding the full-length ULb′ region, which includes *UL128–UL131* genes required for epithelial cell entry, replicated most efficiently in vivo and were the only viruses detected in AF stresses the importance of exploring passive immunization as a strategy for maternal vaccination and highlights the need to determine whether viruses lacking the pentameric complex can establish fetal infection.

The current guinea pig model of congenital CMV infection is a mainstay in the field, with the distinct advantage that these small animals are more high-throughput and less resource-heavy than large-animal models. Furthermore, the structural resemblance of the guinea pig placenta to that of humans and longer gestational period compared with other rodent models contribute to its value as a suitable model to test novel vaccine and therapeutic strategies (43). However, preclinical vaccine studies that have shown significant reduction in the rate of congenital infection and pup mortality in guinea pigs have not often predicted the outcome of human trials, a likely reflection of the evolutionary differences of both the species and the species-specific CMV (7–10). Our development of a rhesus monkey model of congenital CMV transmission that is more physiologically similar to humans in reproductive and placental biology (44) and uses a more genetically related CMV to that of humans than small-animal models (17, 18) provides the field with a highly relevant tool that may better predict preclinical efficacy of vaccine candidates. In addition, given the availability of vast resources to evaluate the rhesus immune response, the rhesus monkey congenital CMV model may allow more rigorous analysis of the maternal immune correlates of protection against congenital CMV infection, including the role of cell-mediated immunity that could inform a protective vaccine.

Materials and Methods

Animals and Procedures. The animal protocol was approved by the Harvard Medical School Area Standing Committee on Animals and the Duke University Medical Center Institutional Animal Care and Use Committee. Harvard Medical School has an approved Animal Welfare Assurance on file with the Office for Laboratory Animal Welfare (Assurance number A3431-01). Indian rhesus macaques were housed at the New England Primate Research Center and maintained in accordance with institutional and federal guidelines for the care and use of laboratory animals (45). Paired mating, pregnancy screening, sample collection, experimental $CD4^+$ T-cell depletion, and rhCMV inoculation were performed as described in *SI Materials and Methods*.

Diagnosis of Congenital rhCMV Infection and Genomic Analysis. Congenital rhCMV infection was diagnosed following the detection of rhCMV DNA in maternal AF by QPCR and was further confirmed by immunohistochemistry staining of placental tissue using an anti-rhCMV IE1 polyclonal rabbit sera and assessment of virus load in fetal tissue or infant saliva and urine specimens by QPCR. Details for each diagnostic assay are provided in *SI Materials and Methods*. SGA and deep-sequence analysis were used to determine the dominant rhCMV strain replicating in maternal plasma and AF samples. Primer sets, conditions, and the method of analysis are outlined in *SI Materials and Methods*.

Humoral and Cellular Immune Analysis. Maternal antibody kinetics, avidity, and specificity were measured using whole virion or MVA ELISAs and are further described in *SI Materials and Methods*. Neutralization EC_{50} titers obtained from telo-RFs and MKEs were calculated using the method of Reed and Muench following manual counting of rhCMV-infected cells stained with a fluorescently labeled rhCMV IE1 monoclonal antibody. Cell maintenance and assay conditions are reported in *SI Materials and Methods*. Characterization of maternal peripheral $CD4^+$ and $CD8^+$ T cells was performed by FLOW cytometry using the fluorescently labeled monoclonal antibodies listed in Table S2. Gating strategies for each population are outlined in Fig. S2. Additional gates used to analyze $CD4^+$ and $CD8^+$ intracellular cytokine T-cell responses are reported in Fig. S7. Detailed protocols are available in *SI Materials and Methods*.

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1. Dollard SC, Grosse SD, Ross DS (2007) New estimates of the prevalence of neurological and sensory sequelae and mortality associated with congenital cytomegalovirus infection. *Rev Med Virol* 17(5):355–363.
2. Kenneson A, Cannon MJ (2007) Review and meta-analysis of the epidemiology of congenital cytomegalovirus (CMV) infection. *Rev Med Virol* 17(4):253–276.
3. Cheeran MCJ, Lokensgard JR, Schleiss MR (2009) Neuropathogenesis of congenital cytomegalovirus infection: Disease mechanisms and prospects for intervention. *Clin Microbiol Rev* 22(1):99–126.
4. Choi YC, Hsiung GD (1978) Cytomegalovirus infection in guinea pigs. II. Transplacental and horizontal transmission. *J Infect Dis* 138(2):197–202.
5. Kanai K, et al. (2011) Re-evaluation of the genome sequence of guinea pig cytomegalovirus. *J Gen Virol* 92(Pt 5):1005–1020.
6. Griffith BP, Lucia HL, Hsiung GD (1982) Brain and visceral involvement during congenital cytomegalovirus infection of guinea pigs. *Pediatr Res* 16(6):455–459.
7. Bia FJ, et al. (1984) Vaccination against transplacental cytomegalovirus transmission: Vaccine reactivation and efficacy in guinea pigs. *J Infect Dis* 149(3):355–362.
8. Leviton MP, et al. (2013) An attenuated cytomegalovirus vaccine with a deletion of a viral chemokine gene is protective against congenital CMV transmission in a guinea pig model. *Clin Dev Immunol* 2013:906948.
9. Chatterjee A, Harrison CJ, Britt WJ, Bewtra C (2001) Modification of maternal and congenital cytomegalovirus infection by anti-glycoprotein b antibody transfer in guinea pigs. *J Infect Dis* 183(11):1547–1553.
10. Auerbach MR, et al. (2014) A neutralizing anti-gH/gL monoclonal antibody is protective in the guinea pig model of congenital CMV infection. *PLoS Pathog* 10(4):e1004060.
11. Schleiss MR, et al. (2014) Glycoprotein B (gB) vaccines adjuvanted with AS01 or AS02 protect female guinea pigs against cytomegalovirus (CMV) viremia and offspring mortality in a CMV-challenge model. *Vaccine* 32(23):2756–2762.
12. Pass RF, et al. (2009) Vaccine prevention of maternal cytomegalovirus infection. *N Engl J Med* 360(12):1191–1199.
13. Revello MG, et al.; CHIP Study Group (2014) A randomized trial of hyperimmune globulin to prevent congenital cytomegalovirus. *N Engl J Med* 370(14):1316–1326.
14. Lockridge KM, et al. (1999) Pathogenesis of experimental rhesus cytomegalovirus infection. *J Virol* 73(11):9576–9583.
15. Antoine P, et al. (2012) Functional exhaustion of $CD4^+$ T lymphocytes during primary cytomegalovirus infection. *J Immunol* 189(5):2665–2672.
16. Yue Y, Zhou SS, Barry PA (2003) Antibody responses to rhesus cytomegalovirus glycoprotein B in naturally infected rhesus macaques. *J Gen Virol* 84(Pt 12):3371–3379.
17. Hansen SG, Strelow LI, Franchi DC, Anders DG, Wong SW (2003) Complete sequence and genomic analysis of rhesus cytomegalovirus. *J Virol* 77(12):6620–6636.
18. Rivailler P, Kaur A, Johnson RP, Wang F (2006) Genomic sequence of rhesus cytomegalovirus 180.92: Insights into the coding potential of rhesus cytomegalovirus. *J Virol* 80(8):4179–4182.
19. Yue Y, Barry PA (2008) Rhesus cytomegalovirus a nonhuman primate model for the study of human cytomegalovirus. *Adv Virus Res* 72:207–226.
20. London WT, et al. (1986) Experimental congenital disease with simian cytomegalovirus in rhesus monkeys. *Teratology* 33(3):323–331.
21. Tarantal AF, et al. (1998) Neuropathogenesis induced by rhesus cytomegalovirus in fetal rhesus monkeys (*Macaca mulatta*). *J Infect Dis* 177(2):446–450.
22. Vogel P, Weigler BJ, Kerr H, Hendrickx AG, Barry PA (1994) Seroepidemiologic studies of cytomegalovirus infection in a breeding population of rhesus macaques. *Lab Anim Sci* 44(1):25–30.
23. Asher DM, Gibbs CJ, Jr, Lang DJ, Gajdusek DC, Chanock RM (1974) Persistent shedding of cytomegalovirus in the urine of healthy Rhesus monkeys. *Proc Soc Exp Biol Med* 145(3):794–801.
24. Kaur A, et al. (1996) Cytotoxic T-lymphocyte responses to cytomegalovirus in normal and simian immunodeficiency virus-infected rhesus macaques. *J Virol* 70(11):7725–7733.
25. Oxford KL, et al. (2011) Open reading frames carried on UL/b′ are implicated in shedding and horizontal transmission of rhesus cytomegalovirus in rhesus monkeys. *J Virol* 85(10):5105–5114.
26. Kaur A, et al. (2002) Decreased frequency of cytomegalovirus (CMV)-specific $CD4^+$ T lymphocytes in simian immunodeficiency virus-infected rhesus macaques: Inverse relationship with CMV viremia. *J Virol* 76(8):3646–3658.
27. Widmann T, et al. (2008) Levels of CMV specific CD4 T cells are dynamic and correlate with CMV viremia after allogeneic stem cell transplantation. *PLoS One* 3(11):e3634.
28. Bronke C, et al. (2005) Dynamics of cytomegalovirus (CMV)-specific T cells in HIV-1-infected individuals progressing to AIDS with CMV end-organ disease. *J Infect Dis* 191(6):873–880.
29. Assaf BT, et al. (2014) Limited dissemination and shedding of the UL128 complex-intact, UL/b′-defective rhesus cytomegalovirus strain 180.92. *J Virol* 88(16):9310–9320.
30. Yue Y, et al. (2008) Evaluation of recombinant modified vaccinia Ankara virus-based rhesus cytomegalovirus vaccines in rhesus macaques. *Med Microbiol Immunol (Berl)* 197(2):117–123.
31. Boppana SB, et al.; National Institute on Deafness and Other Communication Disorders CHIMES Study (2011) Saliva polymerase-chain-reaction assay for cytomegalovirus screening in newborns. *N Engl J Med* 364(22):2111–2118.
32. Boppana SB, Ross SA, Fowler KB (2013) Congenital cytomegalovirus infection: Clinical outcome. *Clin Infect Dis* 57(Suppl 4):S178–S181.
33. Renzette N, Bhattacharjee B, Jensen JD, Gibson L, Kowalik TF (2011) Extensive genome-wide variability of human cytomegalovirus in congenitally infected infants. *PLoS Pathog* 7(5):e1001344.
34. Boppana SB, Britt WJ (1995) Antiviral antibody responses and intrauterine transmission after primary maternal cytomegalovirus infection. *J Infect Dis* 171(5):1115–1121.
35. Lilleri D, et al. (2013) Fetal human cytomegalovirus transmission correlates with delayed maternal antibodies to gH/gL/pUL128-130-131 complex during primary infection. *PLoS One* 8(3):e59863.
36. Boppana SB, Pass RF, Britt WJ, Stagno S, Alford CA (1992) Symptomatic congenital cytomegalovirus infection: Neonatal morbidity and mortality. *Pediatr Infect Dis J* 11(2):93–99.
37. Iwasenko JM, et al. (2011) Human cytomegalovirus infection is detected frequently in stillbirths and is associated with fetal thrombotic vasculopathy. *J Infect Dis* 203(11):1526–1533.
38. Antoine P, et al. (2014) Postnatal acquisition of primary rhesus cytomegalovirus infection is associated with prolonged virus shedding and impaired $CD4^+$ T lymphocyte function. *J Infect Dis* 210(7):1090–1099.
39. Tu W, et al. (2004) Persistent and selective deficiency of $CD4^+$ T cell immunity to cytomegalovirus in immunocompetent young children. *J Immunol* 172(5):3260–3267.
40. Calleja-Aguius J, Muttukrishna S, Jauniaux E (2012) The role of tumor necrosis factor-receptors in pregnancy with normal and adverse outcome. *IJICMR* 2012(4):1–15.
41. Toder V, Fein A, Carp H, Torchinsky A (2003) TNF-α in pregnancy loss and embryo maldevelopment: A mediator of detrimental stimuli or a protector of the fetoplacental unit? *J Assist Reprod Genet* 20(2):73–81.
42. Maidji E, McDonagh S, Genbacev O, Tabata T, Pereira L (2006) Maternal antibodies enhance or prevent cytomegalovirus infection in the placenta by neonatal Fc receptor-mediated transcytosis. *Am J Pathol* 168(4):1210–1226.
43. Schleiss MR, McVoy MA (2010) Guinea pig cytomegalovirus (GPCMV): A model for the study of the prevention and treatment of maternal-fetal transmission. *Future Virol* 5(2):207–217.
44. De Ruick EPCT, Van Esch E (2008) The macaque placenta: A mini-review. *Toxicol Pathol* 36(7):1085–1185.
45. National Research Council (2011) *Guide for the Care and Use of Laboratory Animals* (National Academies Press, Washington, DC), 8th Ed.
46. Keele BF, et al. (2008) Identification and characterization of transmitted and early founder virus envelopes in primary HIV-1 infection. *Proc Natl Acad Sci USA* 105(21):7552–7557.
47. Wussow F, et al. (2013) A vaccine based on the rhesus cytomegalovirus UL128 complex induces broadly neutralizing antibodies in rhesus macaques. *J Virol* 87(3):1322–1332.
48. Abel K, et al. (2011) Vaccine-induced control of viral shedding following rhesus cytomegalovirus challenge in rhesus macaques. *J Virol* 85(6):2878–2890.
49. Tarantal AF (2005) Ultrasound imaging in rhesus (Macaca mulatta) and long-tailed (Macaca fascicularis) macaques: Reproductive and research applications. *The Laboratory Primate*, ed Wolfe-Coote S (Elsevier Academic, San Diego), pp 331–334.
50. Tarantal AF, Hendrickx AG (1988) Use of ultrasound for early pregnancy detection in the rhesus and cynomolgus macaque (*Macaca mulatta* and *Macaca fascicularis*). *J Med Primatol* 17(2):105–112.