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Use of specific-pathogen-free (SPF) rhesus macaques to better model oral pediatric cytomegalovirus infection

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Abstract

Congenital human cytomegalovirus (HCMV) infection can result in lifelong neurological deficits. Seronegative pregnant woman often acquire primary HCMV from clinically asymptomatic, but HCMV-shedding children. Potential age-related differences in viral and immune parameters of primary RhCMV infection were examined in an oral rhesus CMV infection model in specific pathogen free macaques.

Note/Short Paper

Worldwide, human cytomegalovirus (HCMV) infection is the most common congenital infection, affecting ≈ 0.7% of all fetuses. Congenitally infected infants can suffer lifelong neurological sequelae (7, 9, 18), and clinically healthy babies at birth can develop neurological complications in the first years of life. In the US, 0.5–2% of all infants acquire HCMV in utero. Seronegative young infants can acquire HCMV through breast-milk (16) or in day care settings, e.g. through HCMV-contaminated saliva on toys (11, 13). Similar to HCMV-infected adults, infants generally do not develop clinical symptoms upon HCMV acquisition, but, in contrast to adults, shed virus for prolonged periods of time (3). As their infection goes largely unnoticed, HCMV-infected kids can transmit the virus to seronegative pregnant women who either have or do not have preconceptional immunity to HCMV. It is now well-established that HCMV can re-infect HCMV-immune women. In the absence of an HCMV vaccine, interventions aimed at stopping or reducing HCMV shedding in infants could provide an effective means of preventing congenital HCMV infection.

The host factors responsible for prolonged viral shedding in infants are only poorly understood. To overcome sample limitations from young children, we sought to develop an infant rhesus CMV infection model because RhCMV infection in adult macaques is highly similar to human HCMV infection (5, 10). Previously, a direct comparison of RhCMV-specific immune responses between infant and adult macaques in correlation to virological

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outcome was not possible, because RhCMV is ubiquitous in macaque colonies and >90% of animals have seroconverted by 6 months of age. The generation of Specific Pathogen Free (SPF) macaque colonies in which animals are bred to be free of multiple viruses, including RhCMV (4), now enables controlled RhCMV pathogenesis studies in different age groups.

To mimic oral HCMV infection in children, 4-week old infant (n=5) and 5-year old young adult SPF macaques were orally (via the buccal pouch) infected with a natural RhCMV isolate (1x 10^6 PFU/ml) using a blunted syringe, and followed for 1 year. All infants, but only 1 of 3 adult animals, seroconverted within 2–4 weeks (10, 19, 20) (Figure 1A). The 2 seronegative adults received a second RhCMV dose at week 16. One animal seroconverted within 4 weeks, the other animal remained seronegative for 8 weeks, but had RhCMV antibodies by week 12 after the 2nd infection (Figure 1A). The data suggest that infants compared to adults have enhanced susceptibility to *oral* RhCMV infection. The MHC status of the infants at study entry was unknown, and therefore we cannot make any conclusion about possible genetic factors influencing susceptibility to RhCMV infection or pathogenesis outcome. In addition to binding antibodies, all animals developed RhCMV-specific neutralizing antibodies (1, 2) (Figure 1B). Although two of the adults showed the highest 50% neutralization titers (NT50), no statistically significant differences in NT50 values were detected between infant and adult animals. As all infant and 2 adult animals seroconverted within 4 weeks of RhCMV infection, animal #A3 likely became naturally infected, because the adult animals were co-housed. Animals #A1 and #A2 had qPCR-detectable RhCMV DNA in saliva at a single time point (8, 15) (Table 1), but it could not be conclusively determined whether these animals were actively shedding and transmitted RhCMV to #A3, because the RhCMV copy number was below the cut-off value (100 copies/ml). Consistent with observations in human infant HCMV infection, infant macaques showed pronounced high titer RhCMV shedding in saliva and urine (3, 14, 17, 21) (Table 1).

Better control of virus shedding in adults could not be explained by antibody responses because binding and neutralizing titers did not differ between infant and adult animals and persisted in both groups throughout the study period. Around the time of seroconversion, all animals showed an increase in Ki67 positive cells within the total CD4 and CD8 T cell populations, with the frequencies of Ki67 positive CD8 T cells being significantly higher in adults than in infants (data not shown). Analysis of antigen-specific T cells in longitudinally collected blood samples by intracellular cytokine staining (IL-2, IFN-γ, TNF-α and CD107) showed that all animals developed CD4 and CD8 T cell responses to whole RhCMV lysate, RhCMV pp65 and RhCMV IE1 (Figure 1C) (1, 2, 12). There were no differences, however, in the magnitude, persistence, or quality of infant and adult T cell responses. Generally, RhCMV-specific T cells produced only a single cytokine, IFN-γ or TNF-α. Dual-positive cytokine responses were detected in 4 of 5 infant and 2 of 3 adult animals within the CD4, and in 4 of 5 infants and 1 of 3 adult animals within the CD8 T cell population (data not shown). These results are in contrast to the age-dependent increase in HCMV-specific T cells (6, 17), and to a similar age-dependency of RhCMV-specific T cell responses in *non*-SPF macaques (own unpublished data). As 5 year-old macaques are comparable to 15 to 19 year old human teenagers, the age difference between the two animal groups in the current study might have been too small. Alternatively, the SPF status could have affected immune responsiveness.

In this proof-of-concept study, we established an oral RhCMV infection model in infant SPF rhesus macaques. The findings can be summarized as follows: (i) The susceptibility to oral RhCMV infection appears to decline with age, as 2 of 3 older animals required multiple oral RhCMV exposures to become infected, although this route proved to be 100% effective in infants. Future studies should determine host factors enhancing susceptibility to RhCMV infection within the oral microenvironment. (ii) Similar to HCMV infection in humans,

infant macaques shed RhCMV more persistently and at higher titers compared to adult macaques. Viral shedding in saliva and urine might represent the most reliable marker to assess control of CMV infection, and efficacy of drug treatments and vaccines. (iii) Larger animal studies are needed to define immune parameters associated with better control of RhCMV in adult compared to young animals. In the limited study here, differences in the magnitude or quality of RhCMV-specific T and B cell responses could not be detected. However, this was the first time SPF animals were available, and as the SPF colony ages, more detailed virological and immunological studies, including tissue analysis, could be performed with larger animals groups.

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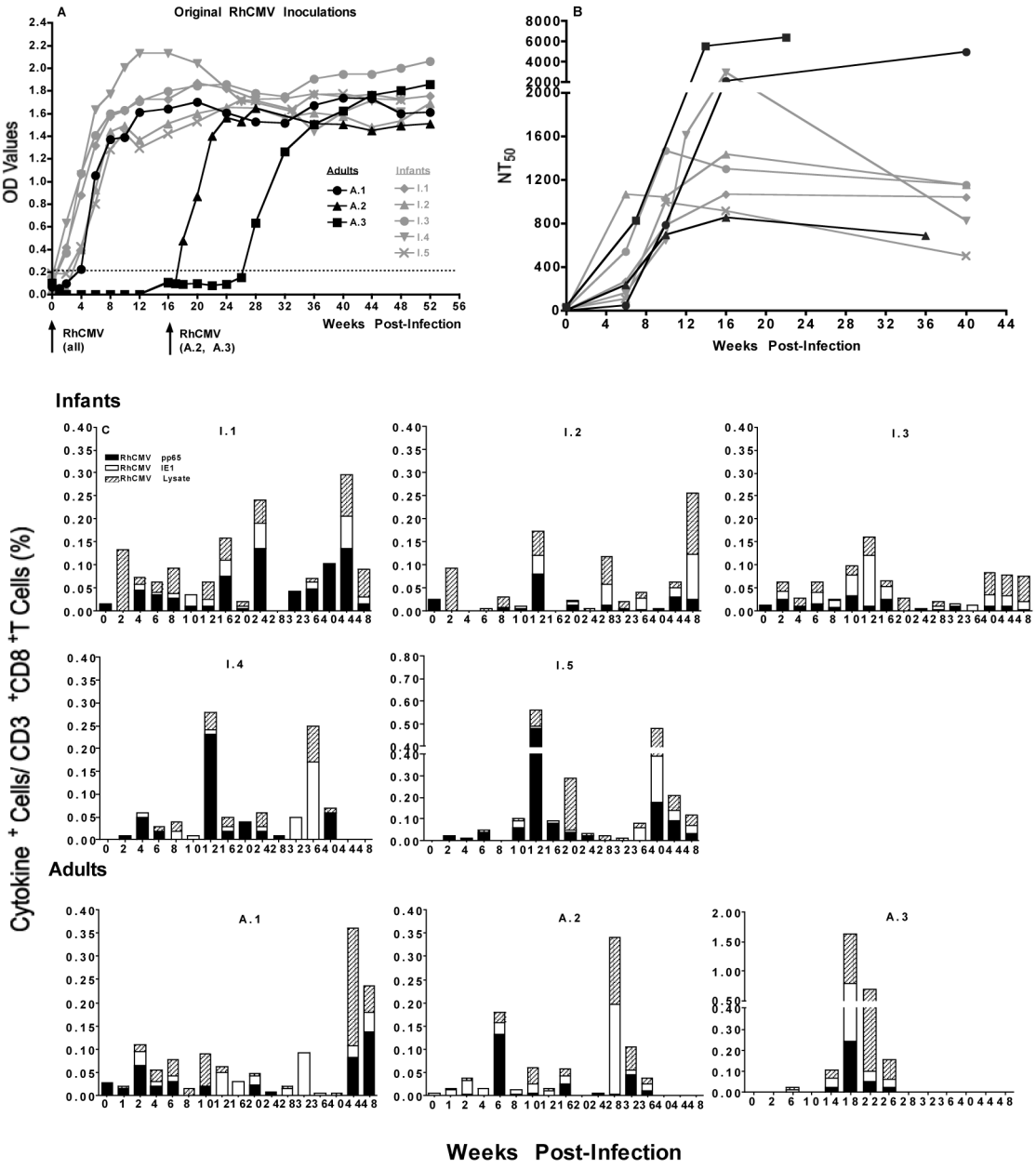

Figure 1. Immune responses to oral RhCMV infection

Panel A: RhCMV-specific antibody development. OD values for RhCMV-specific binding antibodies in longitudinally collected plasma samples (1:100 dilution) were determined by ELISA using RhCMV lysate as antigen. Infant animals are depicted in grey symbols and lines and adult animals in black symbols and lines. Arrows below the x-axis indicate the times of oral RhCMV exposure. The weeks post infection for all animals are based on the first oral RhCMV exposure at week 0. The dashed line shows the threshold for a sample to be considered RhCMV antibody positive based on the analysis of a pool of plasma collected from RhCMV-negative animals.

Panel B: Neutralizing antibody titers. Neutralizing antibodies for RhCMV were determined at weeks 0, 6, 10, 16 and 40 post RhCMV infection. For animal A.3 only samples from weeks 0, 7, 14 and 22 were available, assuming that infection occurred

between weeks 24–26 or weeks 10–12 after the first or second RhCMV inoculation, respectively. Note that the time points for the adult animals in Panel B were adjusted to reflect the actual time of RhCMV infection (e.g. for animal A.3 week 7 post infection in Panel B corresponds to week 33 in Panel A). Shown are the NT_{50} titers for individual infant and adult macaques.

Panel C: RhCMV-specific T cell responses in peripheral blood. Although dual cytokine-positive cells were detected, the graph here only shows the sum of single cytokine positive cells (TNF-α+IL-2+IFN-γ) specific for RhCMV pp65 (black bars), RhCMV IE1 (white bars) and/or RhCMV lysate (striped bars) at individual time points post RhCMV infection. The total frequencies of single cytokine positive cells determined by Boolean gating analysis within the $CD8^+$T cell populations in individual animals. Animal numbers are indicated on top of each graph.

Table 1

RhCMV Detection

Weeks Post Infection	0	2	4	6	7	8	10	12	14	16	18	20	22	24	26	28	32	36	40	44	48	52	Compartment
Infants **I.1**	-	-	-	-		-	-	-		-		-		-		-	-	-	-	-	-	-	**Plasma**
	NS	-	-	-		-	-	-		-		-		-		-	-	-	-	-	-	+++	**Saliva**
	NS	NS	NS	NS		NS	NS	NS		NS		++		+++		NS	+	+	NS	NS	NS	NS	**Urine**
I.2	-	-	-	-		-	-	-		-		-		-		-	-	-	-	-	-	-	**Plasma**
	NS	-	-	+/−		-	-	-		-		-		-		+/−	-	+/−	+/−	-	+/−	-	**Saliva**
	NS	NS	NS	NS		NS	NS	NS		NS		-		-		NS	+++	NS	NS	NS	NS	NS	**Urine**
I.3	-	-	+/−			+/−	+/−	-		-		-		-		-	-	-	-	-	-	-	**Plasma**
	-	-	-	-		-	-	-		+++		+/−		++		+++	++	++	+/−	++	+	+/−	**Saliva**
	NS	NS	NS	NS		NS	NS	NS		NS		NS		NS		NS	-	+++	NS	NS	NS	NS	**Urine**
I.4	-	-	-	-		-	-	-		NT		NT		NT		NT	NT	NT	NT	NT	NT	NT	**Plasma**
	-	-	-	-		-	-	+/−		-		+		NS		++	+	+/−	+	+	+/−	NS	**Saliva**
	NS	NS	NS	NS		+/−	NS	+		+		++		++		+	+++	NS	+	NS	+++	NS	**Urine**
I.5	-	-	-	-		-	-	+/−		NT		NT		NT		NT	NT	NT	NT	NT	NT	NT	**Plasma**
	-	+	+/−	++		+	++	+/−		+/−		+/−		+		-	+/−	+/−	-	+/−	NS	NS	**Saliva**
	NS	NS	NS	NS		NS	NS	+/−		-		NS		+		+	NS	NS	NS	NS	-	NS	**Urine**
Adults **A.1**	-	-	-	-		-	-	-		-		-		-		-	-	-	-	-	-	-	**Plasma**
	-	-	-	+/−		-	-	-		-		-		-		-	-	-	-	-	-	-	**Saliva**
	-	NS	NS	NS		NS	NS	NS		NS		-		-		NS	NS	NS	-	-	NS	NS	**Urine**
A.2	-	-	-	-		-	-	-		-		-		-		-	-	-					**Plasma**
	-	-	-	-		-	-	-		+/−		-		-		-	-	-					**Saliva**
	NS	-	-	-		-	NS	-		NS		-		-		NS	NS	NS					**Urine**
A.3	-	-		-	NT		-		-		-		-		-								**Plasma**
	-	-		-	NT		-		-		-		-		-								**Saliva**
	NS	-		-	NT		NS		NS		NS		NS		NS								**Urine**

NS = no sample available

NT = not tested

+/− less<10 copies (LOD)

* >10, but less <10^4 copies

** >10^4, but less <10^5 copies

*** >10^5 copies