A Novel Non-Replication Competent Cytomegalovirus Capsid Mutant Vaccine Strategy Is Effective in Reducing Congenital Infection

Running title: Guinea Pig Cytomegalovirus DISC vaccine

K. Yeon Choi,[1] Matthew Root,[1] Alistair McGregor[1]#

[1]Department of Microbial Pathogenesis & Immunology, Texas A&M University, Health Science Center, College of Medicine, College Station, Texas, United States of America.

#Address correspondence to Alistair McGregor, mcgregor@medicine.tamhsc.edu

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Abstract

Congenital cytomegalovirus (CMV) is a leading cause of mental retardation and deafness in newborns. The guinea pig is the only small animal model for congenital CMV. A novel CMV vaccine was investigated as an intervention strategy against congenital guinea pig cytomegalovirus (GPCMV). In this disabled infectious single cycle (DISC) vaccine strategy, a GPCMV mutant lacked the ability to express an essential capsid gene (*UL85* homolog, *GP85*), except when grown on a complementing cell line. In vaccinated animals, the GP85 mutant virus (GP85 DISC) induced an antibody response to important glycoprotein complexes considered neutralizing target antigens (gB, gH/gL/gO and gM/gN). The vaccine also generated a T cell response to the pp65 homolog (GP83), determined via a newly established guinea pig interferon gamma ELISPOT assay. In a congenital protection study, GP85 DISC vaccinated animals and non-vaccinated control group were challenged during pregnancy with wild type GPCMV (10^5 pfu). Animals went to term and viral load in target organs of pups analyzed. Based on live pup births in the vaccinated and control groups (94.1% vs 63.6%) the vaccine was successful in reducing mortality (P=0.0002). Additionally, pups from the vaccinated group had reduced CMV transmission with 23.5% infected target organs compared to 75.9% in the control group .Overall, these preliminary studies indicate that a DISC CMV vaccine strategy has the ability to induce an immune response similar to that of natural virus infection but has the increased safety of a non-replication competent virus, which makes this approach attractive as a CMV vaccine strategy.

Importance

Congenital CMV is a leading cause of mental retardation and deafness in newborns. An effective vaccine against CMV still remains an elusive goal despite over fifty years of CMV research. The guinea pig, with a placenta structure similar to humans, is the only small animal model for congenital CMV and recapitulates disease symptoms (eg. deafness) in newborn pups. In this report, a novel vaccine strategy against congenital guinea pig cytomegalovirus (GPCMV) was developed, characterized and tested for efficacy. This disabled infectious single cycle (DISC) vaccine strategy induced a neutralizing antibody, or T cell response to important target antigens. In a congenital protection study, animals were protected against CMV in comparison to the non-vaccinated group (52% reduction of transmission). This novel vaccine was more effective than previously tested gB based vaccines and most other strategies live virus vaccines. Overall, the DISC vaccine is a safe and promising approach against congenital CMV.

53 **Introduction**

54 Human cytomegalovirus (HCMV), a betaherpesvirus, has very closely evolved with their human
55 host. Virus infection in a healthy host is normally asymptomatic but leads to a lifelong infection.
56 In contrast, infection of an immune compromised host (AIDS and transplant patients) or virus
57 reactivation because of an impaired immune system can have severe consequences of morbidity
58 and mortality but established antiviral therapy can potentially reduce the impact of the disease in
59 these patients (1). Another important aspect of cytomegalovirus disease is congenital infection,
60 where the virus crosses the placenta and infects the fetus in utero. This occurs in approximately
61 2% of live births in the US and can lead to serious symptomatic disease including impaired
62 vision, mental retardation and sensorineural hearing loss (SNHL) in newborns (2-6). Established
63 antiviral therapy cannot be used because of possible teratogen and toxic side effects associated
64 with the drugs to the fetus in utero (1). However, long term (6 month) valganciclovir antiviral
65 therapy is now recommended for infants with CNS involvement to improve SNHL and
66 development outcome (7). Importantly, the greatest risk of congenital infection is to mothers
67 who acquire a primary infection during pregnancy and prior immunity can reduce the risk by up
68 to 69% (8). Hence, the impact of a vaccine is potentially substantial, especially in the US, EU
69 and Japan, where up to 50% of women of child bearing age are negative for HCMV (9-11) and
70 therefore at a greater risk of primary infection during pregnancy.
71
72 Any proposed intervention for the prevention or treatment of HCMV infection should ideally be
73 evaluated in a pre-clinical model. Unfortunately, HCMV is extremely species-specific.
74 Consequently, animal model pathogenicity, vaccine and antiviral studies are carried out using
75 animal-specific CMVs (12-16). The guinea pig is unique insofar as it is the only small animal

model to allow the study of congenital CMV infection, unlike the mouse or rat model (17). Both human and guinea pig placentas are hemomonochorial containing a homogenous layer of trophoblast cells separating maternal and fetal circulation (18-20). Additionally, as with human pregnancy, the guinea pig gestation period (approximately 65 days) can be divided into trimesters. Importantly, GPCMV congenital infection causes disease in the fetus and in newborn pups similar to those found in humans including SNHL (21-23). Consequently, the guinea pig model is well suited for testing of intervention strategies aimed at preventing congenital CMV infection (1, 24, 25).

A major drawback in GPCMV research has largely been overcome by the recent sequencing of the viral genome and the development of infectious BAC clones of GPCMV (15, 26-29). Manipulation of an infectious GPCMV BAC has allowed the preliminary study of some viral genes (1, 30-36). Analysis of the viral genome (15, 29) indicated that GPCMV encodes homologs to the HCMV glycoproteins (gB, gH, gL, gM, gN, gO) in genes co-linear with the HCMV genome (designated *GP55*, *GP75*, *GP115*, *GP100*, *GP73* and *GP74* respectively). In HCMV, these six glycoproteins (gB, gH, gL, gM, gN, gO) are required for fibroblast cell entry and they form the glycoprotein complexes, gCI (gB), gCII (gM/gN), gcIII (gH/gL/gO) on the viral membrane (37-39). In HCMV, these complexes are important neutralizing antibody targets and vaccine candidates (40-44). We recently demonstrated that GPCMV forms functionally similar glycoprotein complexes and these complexes are essential for infection of fibroblast cells as well as important target antigens (36). Additionally, GPCMV forms a homolog pentameric complex (gH/gL/UL128-131) that is necessary for epithelial tropism in HCMV (45) and GPCMV (Coleman et al., paper submitted) as well as other cell types (46).

99

100 In both HCMV and GPCMV, the viral glycoprotein gB is the immunodominant neutralizing viral
101 antigen (47-51). A recombinant HCMV gB has been investigated as a candidate subunit vaccine
102 in phase 2 clinical trials but this provides at best approximately 50% efficacy (41). This is
103 despite high antibody titers which are effective in neutralizing virus on fibroblasts (41, 52).
104 Importantly, separate studies of sera from gB vaccinated individuals is less effective at
105 neutralizing virus infection on endothelial and epithelial cells in comparison to convalescent sera
106 from HCMV infected individuals (53-55). This demonstrated the importance of other viral
107 neutralizing target antigens for infection on these cell types. Consequently, other target antigens
108 should be considered important in the development of a vaccine against congenital CMV. The
109 importance of other target antigens can be demonstrated in the guinea pig model where a gB
110 vaccine, despite high antibody titers, fails to fully protect against congenital CMV in the guinea
111 pig model (51, 56, 57). The gH/gL complex has been identified as a potentially important target
112 antigen and in a congenital GPCMV model. In this study, a novel antibody therapy strategy has
113 been shown to be effective in reducing the incidence of congenital CMV (36, 58). Consequently,
114 an effective immune response to both gB and gH/gL are likely important factors for a successful
115 vaccine against congenital CMV.

116

117 Although patients convalescent for HCMV have an antibody response to various important
118 neutralizing antigens, they also have a heightened immune response to the major tegument
119 protein pp65, which is considered to be the major T cell target antigen (59). The pp65 antigen
120 has been explored as a T cell target vaccine strategy in animal models and in clinical trials (60).
121 GPCMV encodes a homolog to pp65 (30) and a pp65 homolog (GP83) vaccine strategy based on

a defective alphavirus delivery approach resulted in partial protection against congenital GPCMV infection, which indicated the potential importance of a T cell mediated immune response against congenital infection (61). However, HCMV also encodes other T cell target antigens (IE1, IE2, pp150 and gB) which are also likely important for the generation of a protective T cell immune response against HCMV and most likely congenital CMV (62). Importantly, GPCMV encodes homologs to these proteins. Undoubtedly, development of a successful vaccine strategy against congenital CMV infection may require an approach that induces an immune response to multiple target antigens, which include both antibody and T cell response and mimic a natural CMV infection to induce long term protection.

Potentially, one the most effective vaccine strategies would be to use an attenuated virus as it would mimic a natural infection. However, a live attenuated virus may have the potential to establish latency in the host and therefore could be considered a high risk vaccine strategy. An alternative is the use of a non-replication competent virus, which infects cells in a similar manner to a wild type virus and expresses an array of viral antigens but is incapable of producing progeny virus because of a specific essential gene knockout. The GPCMV vaccine strategy described in this study was based on a targeted knockout of a capsid gene which was essential for virus assembly but relatively unimportant as a vaccine target. Importantly, CMV capsid genes are highly conserved between HCMV and animal CMV and the process of capsid assembly in HCMV is well studied and defined (63). The defective infectious single cycle (DISC) vaccine strategy was based on a *UL85* homolog (*GP85*) mutant that encodes the minor capsid protein which dimerizes with itself and the minor capsid binding protein (UL46) to form a triplex as part of a fundamental building block for capsid assembly (63). The GP85 capsid gene

145 was placed under the control of the tet-off advanced transactivation system (64) in a GPCMV
146 BAC and mutant virus propagated on a newly developed tet-off advanced guinea pig fibroblast
147 cell line. Animals vaccinated with the *GP85* DISC GPCMV induced an immune response to
148 important antibody target antigens (gB, gH/gL and gM/gN). Additionally, DISC vaccinated
149 animals induced a T cell response to the pp65 homolog (GP83) which was evaluated in a novel
150 interferon gamma ELISPOT assay. Finally, in a congenital infection vaccine protection study the
151 GP85 DISC vaccinated animals induced a highly protective immune response against congenital
152 infection compared to a non-vaccinated control group. Overall, the GPCMV DISC vaccine
153 strategy is a highly promising intervention strategy against congenital infection.
154

155 ## Materials & Methods

156 **Cells, viruses and oligonucleotides**

157 GPCMV (strain 22122, ATCC VR682), first generation GPCMV BAC (26, 27) derived viruses
158 were propagated on guinea pig fibroblast lung cells (GPL; ATCC CCL 158) or tet-off GPL cells
159 (see below) in F-12 medium supplemented with 10% fetal calf serum (FCS, Life Technologies),
160 10,000 IU of penicillin/liter, 10 mg of streptomycin/liter (Life Technologies), and 7.5% $NaHCO_3$
161 (Life Technologies) at 37°C/5% CO_2. Virus titrations were carried out on six-well plates.
162 Plaques were stained with 10% Giemsa stain or visualized by fluorescence microscopy. High
163 titer stock viruses were generated as previously described (36). Pathogenic wild type GPCMV
164 used in congenital CMV challenge studies were serially maintained as salivary gland stocks from
165 infected guinea pigs. All oligonucleotides were synthesized by Sigma-Genosys (The Woodlands,
166 TX) and are listed in Table 1.
167

Tet-Off Advanced GPL cell line.

In order to generate a Tet-Off advanced GPL cell line, GPL cells in 6 well plates were transfected with pTet-Off-Advanced plasmid (Clontech Laboratories) (4µg/ well) and cells maintained under neomycin, G418 (Life Technologies), antibiotic selection (400-600µg/ ml) in complete F-12 media as described in the previous section. Selection media was changed every third day. Individual colonies of cells were identified and clonally isolated by cloning rings and seeded into separate T-25 flasks and expanded under G418 selection (400µg/ml) as separate cell lines. Thirty individual cell lines were generated and frozen low pass stocks were maintained in liquid nitrogen following a standard protocol. G418 resistant GPL cell lines were screened for expression of the tTA2 transactivator (Clontech Laboratories) by the ability of the cell line to enable expression of a luciferase reporter gene placed under pTREtight promoter control in a recombinant expression plasmid pTREtightLUC (Clontech Laboratories). A transactivation/ expression assay for luciferase expression was analyzed by bioluminescence imaging of the plasmid transfected wells. GPL or presumptive GPL-tet-off (tTA positive) cells in separate 6 well dishes were transfected with a pTRELuc plasmid (Clontech Laboratories) (2µg/well) following a standard transfection protocol (36). At 24 hours post expression, the media in the wells was replaced with fresh F-12 complete media (without G418). D-luciferin substrate (100 µg/ml)(Promega)) was also added to the transfected well and incubated at 37°C. At 15 min post incubation, the plates were imaged (IVIS 50 Xenogen)(35) for 5 min. Control GPL 6 well plates transfected with pTREtightLuc were also imaged. As a positive control for bioluminescence imaging separate GPL cells in six well dishes were transfected with a second luciferase expression plasmid pcDNA3-Luc (McGregor, unpublished construct). This plasmid encodes the luciferase reporter cassette under HCMVIE promoter control on the backbone of a pcDNA3.0

plasmid (Life Technologies). The pcDNA3-Luc construct provided the maximal level of luciferase expression/ bioluminescence on both GPL and Tet-off GPL cell lines and was not dependent upon tTa2 transactivation for expression. On pTREtightLuc transfected cells bioluminescent signal was only detected on cells expressing tTA2 (tet-off advanced transactivator).

Cloning of GPCMV genes and generation of GP85 locus shuttle vectors

The GPCMV sequence was based on the complete 22122 viral genome sequence (Genbank accession #AB592928.1). Generation of individual shuttle vectors for specific gene knockout and construction of transient expression vectors are described in more detail below. For generation of GP85 5'UTR mutants an initial construct was generated with flanking sequences (GP85 and GP86 coding sequences) for recombination to enable deletion of the original GP85 5'UTR sequence and substitution of specific sequences. The targeted deletion (Figure 1) removed all of the intergenic non-coding sequences between GP85 and GP86 ORF except for 3 bases prior to the GP85 ATG start and 4 bases after the stop coding of GP86 (deletion GPCMV co-ordinates: bases 135907-136179). The left arm GP85 flanking sequence was PCR amplified with primers FEcGP85FLK and RBmGP85FLKand the 0.9kb sequence (GPCMV co-ordinates: bases 134992-135906) was cloned as a *Eco*R I /*Bam*H I fragment into pUC19 to generate pGP85FLK. The right arm GP86 flanking sequence was PCR amplified with primers FBmGP86FLK and RHdGP86FLK and the 0.5kb sequence (GPCMV co-ordinates: bases136180-136681) was cloned as a *Bam*H I/*Hind* III fragment into pUC19 to generate pGP86FLK. Both constructs were sequenced to verify GPCMV sequence (data not shown). Next the GP86FLK cassette was isolated from pGP86FLK as a *Bam*H I/*Hind* III fragment and cloned

214 into pGP85FLK cut with *Bam*H I/*Hind* III to generate a construct carrying both left and right
215 flanking recombination arms separated by a *Bam*H I site. The modified construct was designated
216 pGP85/GP86. Next, a kanamycin (Km) cassette from pACYC177 (NEB) (36) was PCR
217 amplified with primers FBgXhBmKm and RBgEcVSlKm. The 1.1kb PCR product was isolated
218 by agarose gel electrophoresis and digested with *Bgl*II and cloned into pGP85/GP86 cut with
219 *Bam*H I. The correct orientation of the construct carried the Km cassette running in the opposite
220 direction to GP85 and GP86 genes and was designated pGP8586Km+ (data not shown). Next,
221 the SV40 polyA sequence from peGFP-C1 (Clontech Laboratories) was PCR amplified with the
222 primers FSV40AEcSl and RSV40AEcSl. The 330 bp fragment had novel *Eco*R I and *Sal* I sites
223 introduced into both 5' and 3' ends and was cloned initially into pNEB193 (NEB) cut with
224 *Eco*RI to generate pNEBSV40polyA. The SV40polyA was next isolated as a *Sal* I fragment from
225 pNEBSV40polyA and cloned into pGP8586Km+ cut with *Sal* I which introduced the
226 SV40polyA sequence downstream of the GP86 stop codon in a plasmid designated
227 pGP8586SV40AKm. Next, the TREtight promoter was PCR amplified as a *Bam*H I fragment
228 from pTREtight (Clontech Laboratories) and cloned initially into pNEB193 (NEB) to generate
229 pNEBTRE and promoter sequence verified by sequencing (data not show). The TREtight
230 promoter as a *Bam*H I fragment was then isolated and cloned into pGP8586SV40AKm cut with
231 *Bam*H I and orientation determined by *Xho* I digestion. The correct construct placed the
232 pTREtight promoter directly upstream of the GP85coding sequence. This construct, which was
233 used to generate the DISC virus strain, was designated pGP8586TRESV40AKm. The cloning
234 strategy for the shuttle vectors is summarized in Figure 2. In order to further demonstrate the
235 essential nature of the GP85 protein, an additional GP85 knockout shuttle vector was generated
236 synthetically (DNA2.0) which deleted the majority of the GP85 coding sequence pSYDGP85

(codons 58-241 deleted). This shuttle vector was further modified by the insertion of a Km *Bam*H I cassette (36) to enable selection of the GPCMV GP85 knockout mutant BAC. The GP85 deletion knockout vector was designated as pSYDGP85Km.

Generation of gene mutant GPCMV BACmids and analysis of GPCMV BAC mutants.

An inducible ET recombination system (GeneBridges) was introduced into DH10B bacterial cells containing a first generation GPCMV BAC plasmid (26, 27) and mutagenesis of the GPCMV BAC was performed using linearized shuttle vectors encoding kanamycin marker as previously described (32). Isolated mutant GPCMV BAC colonies were characterized by separate *Eco*R I and *Hind* III restriction digestions of BAC DNA to verify the accuracy of the predicted genome configuration after mutation (26, 27). Insertion of the Km drug resistance cassette into the viral genome introduced a novel *Hind* III restriction enzyme site at the site of mutation to enable verification of locus modification. Specific gene modifications were confirmed by comparative PCR analysis between wild type and mutant GPCMV BACs using common flanking primers for each gene (Table 1). PCR reactions were carried out using conditions described in McGregor et al. (32) except the extension time at 72^{o}C was modified based on the size of each gene (based on 30 sec extension per 500 bases). The gene knockout for mutants was further verified by sequencing of the cloned PCR product.

Generation of mutant GPCMV

For generation of recombinant viruses, large-scale GPCMV BAC DNA was purified from *E. coli* DH10B strain using a maxi plasmid kit (Qiagen). BAC DNA was transfected onto GPL or GPL-tet-off cells in six well dishes using Lipofectamine 2000 (Invitrogen) as previously described

(33). GPCMV BAC transfections were carried out with two independent clones for each gene knockout/modification. Transfections were followed for at least 4 weeks for the production of viral plaques. GFP positive viral plaques were detected via microscopy (33). Non-infectious mutants produced only single GFP positive cells that did not progress to viral plaques. GPCMV mutant BAC transfections were carried out multiple times (minimum of 6 times) for each clone. Rescue of lethal knockout mutants was by co-transfection of the mutant GPCMV BAC DNA with a 1.7 kb PCR product of the wild type GP85/GP86 locus generated from wild type GPCMV BAC DNA with primers FEcGP85FLK and RHdGP86FLK (Table 1)

Ethics

Guinea pig (Hartley) animal studies were carried out under IACUC (Texas A&M University or University of Minnesota). All study procedures were carried out in strict accordance with the recommendations in the "Guide for the Care and Use of Laboratory Animals of the National Institutes of Health." Animals were observed daily by trained animal care staff, and animals that required care were referred to the attending veterinarian for immediate care or euthanasia. Terminal euthanasia was carried out by lethal CO_2 overdose followed by cervical dislocation in accordance with IACUC protocol and NIH guidelines. Animals purchased from Charles River Laboratories were verified as seronegative for GPCMV by toe nail clip bleed and anti-GPCMV ELISA of sera as previously described (36). Animal studies were carried out to determine: (1) the immune response (antibody) to the GPCMV DISC vaccine; (2) the T cell immune response to GPCMV pp65 homolog protein; (3) the level of protection against congenital GPCMV by the DISC vaccine. Initial DISC vaccine regime studies were carried as described in the results section. Congenital GPCMV protection studies were carried out as described in the section

below and results section. Additionally, animals hyperimmune to wild type GPCMV were evaluated for antibody response to GPCMV and specific glycoprotein complexes via ELISA as well as neutralizing antibody titers. Guinea pigs (n=5) were made hyperimmune to GPCMV by three injections of wild type GPCMV via subcutaneous route (10^5 pfu per injection). Each injection was separated by an interval of 4 weeks. Animals were evaluated after 1st and last injection for anti-GPCMV titer. Animals were euthanized by approximately 4 weeks after the last injection and sera pooled for evaluation of antibody titers (anti-GPCMV, anti-gB, anti gH/gL, and anti- gM/gN). Additionally, splenocytes from individual animals were used to evaluate the T cell response to GP83 via interferon gamma ELISPOT assay as described in materials and methods.

Congenital GPCMV vaccine protection studies

Seronegative female guinea pigs were randomly assigned to two different groups. Group 1 (n=14) were vaccinated subcutaneously with the GPCMV DISC vaccine ($1x10^3$ pfu) and boosted with a second inoculation 1month later with a repeat dose ($1x\ 10^3$pfu). Animals were confirmed as seroconverted for GPCMV and paired with seronegative males for mating. Dams were confirmed as pregnant by palpitation at approximately day 20-25 of gestation. A second control group of non-vaccinated seronegative females (Group 2, n=15) were also paired for mating. At late second trimester/early third trimester, pregnant animals in both groups were challenged with the salivary gland stock of wild type GPCMV (10^5 pfu) via subcutaneous inoculation and animals were allowed to go to term. The viral load in target organs (liver, lung, spleen, brain) of live or still born pups was evaluated by real time PCR.

306 **Real time PCR**

307 Tissues were collected from euthanized guinea pigs to determine the viral load. For pups from
308 congenital infection studies, tissues (lung, liver, spleen, brain) were collected within 3 days post
309 birth. Pup specific placenta was collected and preserved for DNA extraction when applicable.
310 For tissue DNA extraction, FastPrep 24 (MP Biomedical) was used to homogenize tissues as a
311 20% weight/volume homogenate in Lysing Matrix D (MP Biomedicals). DNA was extracted
312 using the QIAxtractor (Qiagen) according to manufacturer's tissue protocol instructions. Viral
313 load was determined by real time PCR on a Lightcycler 480 (Roche Applied Science). Primers
314 and hydrolysis probe were designed using the Lightcycler Probe Design2 program to amplify a
315 product from the GPCMV GP44 gene: Forward primer 5'TCTCCACGGTGAAAGAGTTGT;
316 Reverse primer 5'GTGCTGTCGGACCACGATA; hydrolysis probe 5'FAM-
317 TCTTGCTCTGCAGGTGGACGA-BHQ1. PCR master mix contained Lightcycler Probes
318 Master (Roche Life Science), 0.4 µM primers and 0.1 µM probe, 0.4U uracil N-glycosylase
319 (UNG) in 25µl total reaction volume including 10 µl of DNA per reaction. Standard controls and
320 no template controls (NTC) were run with each assay for quantification. Lightcycler480
321 amplification parameters were: UNG step for 10 minutes at 40°C followed by activation at 95°C
322 for 10 minutes, then 45 cycles of denaturation at 95°C for 15s, annealing at 56°C for 15s,
323 elongation at 72°C for 10s. Data was collected by 'single' acquisition during the extension step.
324 Data was analyzed with the LightCycler Data Analysis Software (Version 1.5.1; Roche).
325 Standard curve was generated using serial dilutions of GPCMV GP44 plasmid (33) at known
326 concentrations for quantification and assay sensitivity. The sensitivity of the assay was
327 determined to be 5 copies/reaction. Viral load was expressed as copy number/mg tissue. Results
328 calculated were a mean value of triplicate PCR runs per sample.

329

330 **ELISAs, GPCMV neutralization assays and western analysis**

331 Anti-GPCMV ELISA and specific glycoprotein complex ELISAs (anti gB, anti gH/gL and anti-
332 gM/gN) were carried out as previously described (36). MaxiSorp ELISA plates (NUNC) were
333 coated with 0.25µg of either Ag+ or Ag- preparations diluted in carbonate coating buffer
334 overnight at 4°C, washed in PBST then blocked with 2% nonfat dry milk. Test sera were diluted
335 in blocking buffer from 1:80 to 1:5120 in doubling dilutions, incubated for 2 hours at 37°C and
336 then reacted with anti-Guinea Pig IgG peroxidase antibody (Sigma) diluted (1:1000) in blocking
337 buffer for an additional 1 hour at 37°C before reacting with TMB membrane peroxidase
338 substrate (KPL). Net OD (absorbance 450nm) was attained by subtracting OD of Ag- from OD
339 of Ag+. ELISA reactivity was considered positive if the net OD was greater than or equal to 0.2
340 as determined by GPCMV negative serum. GPCMV neutralization assays were performed on
341 GPL fibroblast cells with a GFP tagged GPCMV (vAM403, (26) and neutralization assays were
342 performed as previously described (36). Final neutralizing antibody titer is the highest dilution
343 producing a 50% or greater reduction in plaques compared to virus only control. Western blots
344 were carried out as previously described (36) using wild type virus infected cell lysate or sucrose
345 purified virions plus control mock infected GPL cell lysate. Sera from a vaccinated animals, R1
346 regime (1/2000 dilution), was used in conjunction with anti-guinea pig IgG-HRP conjugate,
347 1/500 (Sigma).

348

349 **Guinea pig interferon gamma enzyme-linked immunospot (ELISPOT) assay**

350 Anti-guinea pig interferon gamma (IFNγ) monoclonal antibodies used in the assay were based on
351 previously characterized monoclonal antibodies against guinea pig IFNγ (65, 66). Hybridoma

cell lines for the production of the monoclonal antibodies were a generous gift from Dr. Schäfer, Robert Koch-Institute, Germany. Large scale antibody production and purification was carried out by Genscript. IFN γ ELISPOT assays were performed in PVDF membrane 96 well plates. Membranes were pre-soaked with 70% EtOH and then washed with 1X PBS to thoroughly remove the alcohol prior to coating with 0.5µg guinea pig IFNγ capture antibody, V-E4, and incubated overnight at 4°C. Membranes were washed with 1X PBS then blocked with RPMI + 10% FCS for 2 hours at room temperature. Splenocytes were prepared as previously described (67). Briefly, isolated spleen harvested from euthanized animals were immediately homogenized in PBS then put through a 70µm cell strainer and RBC lysed. Splenocytes were washed with 1X PBS three times before being resuspended in RPMI media containing 10% FBS, antibiotic/antimycotic. Cells were counted with trypan blue and $1x10^5$ cells were used per well. Blocked membranes were washed once with PBS before splenocytes were added. GPCMV GP83 peptide pools (see below) were added to each well of cells at final concentration of 5µg/ml. Con A (10µg/ml) was used as positive control and other controls included cells only control, DMSO control (peptide background), and GFP (nonspecific peptide control) and media only control. Plates are covered with foil then incubated at 37°C in 5% CO_2 cell culture incubator for 18 hours. Membranes were washed with wash buffer (1X PBS + 0.1% tween 20) 5 times before detection antibody, N-G3 biotinylated was added and incubated at room temperature for 2 hours. Detection antibody was diluted to 1µg/ml with diluent (1X PBS, 1% BSA, 0.05% tween 20) and filtered through a 0.2µm filter before use. Membranes were washed with wash buffer 3 times before 100µl of streptavidin-AP (R&D Systems), diluted 1:3200 in diluent, were added, incubated for 1.5 hours at room temperature. 100 µl BCIP/NBT (Life Technologies) were added to membranes washed 3X in wash buffer before incubation for 30 mins at room temperature

375 covered from light. Membranes were washed 2X in distilled water, inverted to blot and dried
376 before counting the spots on ImmunoSpot S6 (CTL). Final counts were calculated based on spot
377 forming cells (SFC) per 10^6 cells after background spots (cells only without any stimulation)
378 were subtracted.
379 A total of 140 pepscreen 9 amino acid peptides overlapping by 5 amino acids were generated
380 (Sigma Aldrich, The Woodlands, TX) to create the GPCMV GP83 peptide library expanding the
381 full length gene. Nine amino acid peptides were used to target CD8+ T cell activation (68). Each
382 peptide was reconstituted to 10mg/ml with DMSO. Peptide pools were generated using similar
383 configuration matrix previously described (68, 69). The GP83 matrix consisted of 24 peptide
384 pools in a 12x12 grid with each pool containing 12 peptides. Pools IX, X, XI, XII contained 11
385 peptides and Pool XXIV had 8 peptides. Additional DMSO was added to keep the concentration
386 the same in all pools. All peptide pools were diluted to 10µg/ml working stock in RPMI for
387 stimulation. The matrix was designed for each peptide to be included in exactly 2 pools keeping
388 the number of pools at a minimum. The intersection of positive pools corresponds to the
389 stimulating peptides.
390
391 **Statistical analysis**
392 Mean antibody titers (ELISA and NA) in the vaccine study and hyperimmune GPCMV infected
393 animals were compared by Mann-Whitney test. In congenital studies, pup outcome and
394 transmission rates were compared by Fisher's exact test. GPCMV viral load in specific target
395 organs of pups were compared by Student t test or/and Mann Whitney test depending on sample
396 size. All comparisons were two-tailed.
397

Results

Generation of a GPCMV DISC Virus Strain

A lethal gene knockout was engineered into the GPCMV genome to render the virus incapable of productive infection unless the mutant virus was grown on a complementing cell line. This mutant virus was the basis of the GPCMV vaccine strategy and was referred to as a DISC virus (defective infectious single cycle virus). This terminology was originally developed for a herpes simplex vaccine strategy, which was based on a glycoprotein (gH) knockout virus (70). The GPCMV DISC vaccine strategy was based on a targeted knockout of a capsid gene, as the gH glycoprotein was considered potentially important for glycoprotein complex formation and consequently a basis for important target antigen(s). In contrast, the viral capsid genes are essential for virus assembly but in themselves are relatively unimportant vaccine targets. Additionally, a capsid mutant was considered unlikely to interfere with expression of other viral proteins or the assembly of viral glycoprotein complexes since capsid assembly occurs independently in the nucleus. Importantly, CMV capsid genes are highly conserved between HCMV and animal CMV and the process of capsid assembly in HCMV is well studied and defined (63). Additionally, capsid assembly is conserved between alphaherpesviruses (eg. HSV) and HCMV (71). GPCMV encodes homologs of the HCMV capsid genes and earlier electron microscopy studies demonstrated a similar icosahedral capsid assembly process in the nucleus as well as a similar virus maturation process (15, 72). The GPCMV DISC vaccine strategy was based on a *UL85* homolog (*GP85*) mutant that was predicted to encode the minor capsid protein (GP85). In HCMV, UL85 dimerizes with itself and the minor capsid binding protein (UL46) to form a triplex as part of a fundamental building block for capsid assembly (63). GPCMV encodes a UL46 homolog (GP46), which would enable triplex formation with GP85. A BLAST

alignment of the predicted GP85 and UL85 proteins demonstrated a high level of sequence conservation between open reading frames (56 % identity) (Figure 3). Transient plasmid expression of the GP85 protein in GPL cells demonstrated that the protein was located in both the nucleus and cytoplasm (data not shown). A targeted deletion knockout of the *GP85* gene (codons 58-241) in a GPCMV BAC resulted in a lethal knockout of the virus, which demonstrated the essential nature of *GP85* (data not shown).

The first stage in the generation of a DISC virus was the development of a complementing cell line for virus growth. In the case of the GPCMV *GP85* DISC strain, the classical approach of a cell line which expressed the missing gene *in trans* was substituted for another strategy that employed the tet-off advanced system where cells expressed the transactivator (64). Control of the *GP85* gene expression in a recombinant virus was achieved by placing the gene under the control of the tet-off transactivating protein tTA2 (64). Potentially, this approach has the advantage of placing multiple viral genes under the control of the same tet-off system/tet-off transactivating protein and therefore bypasses the requirement for a cell line expressing the essential target gene(s) *in trans*. Establishment of the tet-off advanced system required the development of a GPL cell line that expressed the tet-off transactivator, tTA2 (Clontech Laboratories). The procedure for generation of the GPL tet-off cell lines was described in the materials and methods. Overall, 30 potential tet-off advanced cell lines were isolated and screened for tTA2 transactivator by transfection of a luciferase reporter plasmid (Clontech Laboratories) under TREtight (tet-off) promoter control (pTREtightLUC). Expression of luciferase was assayed by bioluminescence imaging of transfected plates at 24 hr post transfection. Four cell lines (A4, A20, A21 and D7) were identified which continued to exhibit

consistent levels of tTA2 expression as determined by luciferase reporter gene transactivation bioluminescence assay. Figure 4 shows the transient expression assays used to screen candidate cell lines. Plate bioluminescence imaging was employed to ensure that the results demonstrated activity across the complete intact monolayer. Control plasmid transfections (pTREtightLUC and pTet-Off-Advanced) were performed on GPL cells to provide an idea of minimal acceptable level of activity of reporter gene transactivation. Additionally, pCDNALuc (Luciferase under HCMV MIE control) was used to provide a guide for efficient direct expression of the reporter gene (see Figure 1). Cell lines A20 and A21 (Figure 1) were subsequently used for the propagation of the GP85DISC virus strain as they consistently exhibited the highest levels of reporter gene transactivation.

In order to generate the *GP85* DISC GPCMV mutant, the first generation GPCMV BAC was modified at the *GP85/GP86* intergenic locus. The UTR region upstream of the GP85 ORF was deleted (Figure 1) and replaced by partial or complete sequences to generate a GP85 DISC mutant as described in materials and methods using specific *GP85/GP86* locus shuttle vectors (Figure 2). Three specific *GP85* GPCMV BAC mutants were generated (Figure 4). The first mutant replaced the GP85 UTR sequence (272 bp (135907-136179) with a Km cassette (designated GP85/GP86Km GPCMV BAC). The second mutant replaced the UTR with a Km cassette and SV40 polyA sequence downstream of the *GP86* coding sequence (designated GP85/GP86Km/polyA GPCMV BAC). The third mutant replaced the *GP85* UTR with a Km cassette and SV40 polyA sequence and also introduced a TREtight promoter directly upstream of the GP85 ORF. This third GPCMV BAC mutant was designated TREGP85 DISC GPCMV BAC. Verification of the *GP85* locus mutations was confirmed by Hind III restriction profile

analysis of wild type and mutant GPCMV BACs. Insertion of a Km cassette introduced a novel *Hind* III site into the *GP85/GP86* locus and disrupted the *Hind* III 'A' fragment (approximately 44 kb, GPCMV co-ordinates 102379-146446), which was modified to produce as expected two novel fragments in the TREGP85 DISC GPCMV BAC profile of approximately 34.5 kb and 10.8kb after inclusion of the extra cassette sequence and deletion sequence. Other modifications also produced a similar pattern of modified *Hind* III 'A' fragment (Figure 5). Specific modification to the *GP85/GP86* locus was also verified by PCR analysis of the modified locus based on primers used to create the shuttle vector (left arm co-ordinates 134992-135906/right arm co-ordinates 136180-136681). The *GP85/GP86* locus and deleted intergenic sequence is shown in Figure 1. Figure 5(iv) shows the 1.7 kb wild type locus (GPCMV co-ordinates 134992-136681) and the 2.5 kb PCR product of the locus with GP85 5'UTR deletion and insertion of a 1.1 kb Km cassette. PCR of the TREGP85 GPCMV BAC GP85/GP86 locus demonstrated a modified locus of 3.1 kb as a result of the insertion of Km/ SV40 polyA/ pTREtight cassette and deletion of the original *GP85* 5' UTR. Note that GP85/GP86/Km/polyA GPCMV BAC was correctly modified based on restriction profile and PCR analysis but data was not included to limit redundancy.

All three mutant GPCMV BAC clones were transfected onto both GPL and GPL tet off cell lines as described in materials and methods. Separate transfections of the three *GP85* GPCMV BAC mutants failed to generate infectious virus on GPL cells and individually transfected cells remained GFP positive but viral plaques and virus spread across the monolayer failed to occur (Figure 6). The *GP85* mutants could be rescued back to wild type virus by co-transfection of the BAC DNA with rescue PCR product of the wild type *GP85/GP86* locus as described in materials

and methods (Figure 5). Separate transfection of all three GP85 mutant GPCMV BACs onto the GPL tet-off cells resulted in viable virus production for the TREGP85 GPCMV BAC which produced GFP positive virus that spread across the monolayer. In contrast, the other *GP85* mutant transfected well remained as single GFP positive cells (data not shown). Monolayers were monitored daily for 3-4 weeks with frequent changes in media. Transfection of each mutant was carried out multiple times (>40) and additional mutants were also tested (data not shown) but the same result was obtained each time. It was concluded that the tTA2 transactivator in the tet-off cell line specifically enabled expression of the *GP85* gene when under TREtight promoter control in the TREGP85 GPCMV BAC derived virus. Virus stocks of the *GP85* DISC virus strain were generated on tet-off GPL cells and the growth kinetics of the mutant virus on the complementing tet-off GPL cell line was evaluated. Figure 7 demonstrated that the DISC virus grew with attenuated growth kinetics on the tet-off cell line compared to wild type virus but the normal growth kinetics of the wild type virus demonstrated that expression of the tet-off transactivator did not interfere with GPCMV growth. Potentially, the expression kinetics of GP85 protein (gene under tet-off control) compared to the other triplex component UL46 protein (gene under GPCMV promoter control) impaired the stoichiometry of protein expression to enable optimal triplex formation. However, an in depth evaluation of protein expression would require the development of custom antibodies to GP85 and GP46 for future western blot assays. The GP85 DISC virus was able to successfully infect normal GPL cells as verified by GFP reporter gene expression in infected GPL cells but did not produce any titratable progeny virus during a 7 day infection of GPL cells (data not shown). It was concluded that the GP85 DISC strain was restricted to the tet-off GPL cells for production of progeny virus but retained the ability to infect non-complementing cells. The GP85 DISC strain expressed an array of viral

antigens on non-supporting GPL cells which could be detected by western blot analysis using hyper immune sera from convalescent guinea pigs (data not shown). The immune response to GPCMV was also evaluated by western blot. Figure 8 shows a typical result for western blots of R1 DISC vaccinated animal sera. Evaluation was carried out by western blots of sucrose purified virus as well as GPCMV virus infected GPL cell lysate (Figure 8). These results demonstrated that antibodies were generated against both structural and non-structural proteins.

Antibody immune response against viral glycoprotein complexes

Vaccine regime I (2 shot vaccine) vs vaccine regime II (3 shot vaccine)

Two vaccination regimes were investigated to evaluate the antibody response to GPCMV GP85 DISC vaccine (Figure 9). In the first vaccine study, regime I (R1), GPCMV negative female guinea pigs (n=6) were vaccinated subcutaneously with 10^3 pfu DISC virus and subsequently boosted 4 weeks post initial infection with an equivalent dose. Sera collected 4 weeks post booster (8 weeks post initial infection) were tested for GPCMV seroconversion and the level of neutralizing antibodies. All animals seroconverted and showed relatively high anti-GPCMV ELISA titers (1:1280-5120, mean 1:2987) but this was lower than pooled hyperimmune animal sera convalescent for wild type GPCMV (mean titer 1:5000). The immune response to specific glycoproteins (gB, gH/gL and gM/gN) was also evaluated from DISC vaccinated animals using newly developed ELISAs (36). ELISA titers of the DISC vaccinated animals for anti-gB were not as robust when compared to wildtype GPCMV hyperimmune sera (1:640 vs 1:1600, $p < 0.05$). Similarly, the glycoprotein complex gH/gL titer was lower (1:216 vs 1:960, $p < 0.05$) (Figure 10A). However, the immune response of DISC vaccinated animals to the gM/gN complex was slightly stronger but not statistically significant compared to hyper-immune sera

(1:704 vs 1:480). The neutralizing antibody titer of DISC vaccinated animals on GPL cells was lower at 1:960 vs 1:4200 ($p < 0.05$) for hyper immune sera (Figure 10B).

In the second vaccine study, regime II (R2), Figure 9, GPCMV negative female guinea pigs (n=5) were vaccinated subcutaneously with 10^3 pfu DISC virus and subsequently received equivalent booster inoculations of 10^3 DISC virus at 4 and 8 weeks post initial inoculation. Sera collected 4 weeks post second boost (12 weeks post initial vaccination) were tested for anti-GPCMV titers and specific immune response to glycoprotein complexes described for R1 samples. The additional DISC vaccination in R2 animals appeared to enhance the immune response to GPCMV. The anti-GPCMV ELISA titer ranged from 1:2560-1:10240 serum dilution with the mean titer (1:5120) resembling hyper-immune GPCMV animals (Figure 10A). Specific glycoprotein complex ELISAs demonstrated an increase in the immune response to both gB (1:3000 vs hyperimmune titer 1:1600) and gH/gL (1:2700 vs hyperimmune 1:960, $p <0.05$). The antibody titer against gM/gN was also increased (1:4000 vs hyperimmune 1:480, $p < 0.05$). Although the second booster strategy (R2) increased the immune response to all specific complexes tested, the impact on the gM/gN immune response was relatively unusual as titer levels were above that of hyperimmune animals. The reason for this dramatic change in the gM/gN immune response is undetermined at this time and is worthy of further investigation. It should be noted that although our vaccine studies are compared to the immune response in hyperimmune animals, the actual immune response to the GPCMV glycoprotein complexes as well as the anti-GPCMV titers are more varied and lower in naturally infected animals based on results obtained from GPCMV positive animals purchased from various animal vendors (data not shown).

559

560 Overall, the DISC vaccine strategy in vaccinated animals was more successful with a two
561 booster approach (R2) compared to a single booster (R1). In the R2 study, the anti-GPCMV
562 titers were increased and importantly, the antibody response for the specific glycoprotein
563 complexes were also increased compared to R1. However, despite improved values in the R2
564 study with titers equivalent or better than the hyperimmune sera, the neutralizing titer of animals
565 in the R2 study were below that for hyperimmune sera (1:2000 vs 1:4200, $p < 0.05$) (Figure 10B)
566 and only slightly higher than the R1 study. Potentially, a missing component of the immune
567 response in DISC vaccinated animals compared to wild type virus infected animals was an
568 immune response to the homolog pentameric complex (58) as the DISC vaccine was based on a
569 lab adapted virus that lacked a full length *UL128-131* homolog locus (15, 26). The immune
570 response to the homolog penatemric complex is currently under investigation in our laboratory
571 via the use of attenuated GPCMV mutants as well as a second generation GPCMV DISC
572 vaccine.

573

574

575 **T cell immune response to the pp65 homolog protein**

576 The T cell response to the DISC virus vaccine was evaluated by a newly developed guinea pig
577 specific IFNγ ELISPOT assay as described in materials and methods. Pools of 9mer peptides
578 designed to target CD8+ T cell response were used to pulse isolated splenocytes from animals in
579 R1 and R2 study groups as well as wild type GPCMV infected animals. The peptide pool matrix
580 was designed to optimally utilize the largest number of peptides in the most efficient and cost
581 effective manner (68, 69). The GP83 protein has a predicted length of 565 amino acids which

582 generated 140 overlapping 9mer peptides. This enabled 24 pools of peptides in a 12x12 square
583 matrix with each peptide appearing in two pools. The intersection of positive pools identified the
584 stimulating peptides narrowing down the potential candidate peptides that can be tested
585 individually. The concentration of peptide pools used was determined by testing different
586 concentrations based on HCMV IFNγ ELISPOT results (73, 74). For the GP83 peptide pools a
587 final concentration of 5 µg/ml gave the highest stimulation of IFNγ producing cells. Con A
588 (positive control) produced an average of 5840 spot forming cells (SFC) per 10^6 stimulated cells
589 (±235). Unstimulated cells (634 SFC/10^6 cells ±5.8) and DMSO stimulated cells (548 SFC/10^6
590 cells ±16) produced similar results, which was used to establish the baseline background level.
591 Each animal response to GP83 peptides stimulation was slightly different but at least 50% of the
592 animals responded to peptide pools V, VIII, X, and XX (data not shown). An increase in IFNγ
593 secreting cells in response to peptide pools X and XX occurred in all animals. Spot forming cells
594 from group R1 responding to pool X (2621 SFC/10^6 cells ±508) or pool XX (2227 SFC/10^6 cells
595 ±590) were similar to those seen in wild type GPCMV infected cells, stimulated with pool X
596 (2900 SFC/10^6 cells ±468) and pool XX (3220 SFC/10^6 cells ±1362). Furthermore, there were no
597 significant difference in response to pool X (3220 SFC/106 cells ±1114) or pool XX (2918
598 SFC/106 cells ±990) in group R2 (Figure 11), despite a higher humoral response to GPCMV
599 antigens. Certain GP83 peptide pools (Pools II and IV) failed to routinely stimulate the
600 splenocytes above background in infected animals (Figure 11).
601
602 Overall, the DISC vaccine was confirmed to induce a T cell response against the pp65 homolog
603 protein which is considered a major T cell target in HCMV. Encouragingly, the DISC vaccine T
604 cell response was similar to animals challenged with wild type live virus. An analysis of the

605 immune response in naturally infected animals remains to be investigated to determine if there is
606 a difference in response compared to vaccinated or hyperimmune animals. The stimulation of
607 splenocytes from DISC vaccinated animals by GP83 peptide pools allowed the identification of a
608 "hotspot" region within the GP83 protein which will enable a narrower range of peptides to be
609 used in future studies and enable identification of the most reactive T cell epitope. However, the
610 use of outbred Harley guinea pigs in future studies may have to be substituted with inbred strain
611 2 animals to limit animal to animal variation in the GP83 T cell response.
612

613 **Congenital GPCMV protection study (2 shot vaccine regime I)**

614 Previous sections indicated that the DISC vaccine strategy induced an effective antibody and T
615 cell response against GPCMV in vaccinated animals. Consequently, the DISC virus vaccine
616 strategy was evaluated for an ability to protect against congenital GPCMV under vaccine regime
617 I (see Figure 9). Initially, 29 female guinea pigs seronegative for GPCMV were randomly
618 assigned into two groups: Group 1, vaccinated (DISM, n=14); Group 2, control unvaccinated
619 (NOD, n=15). Group 1 animals were vaccinated with 10^3 pfu subcutaneously with the GP85
620 DISC virus and 1 month post vaccination the animals received an equivalent booster dose of the
621 DISC strain. Evaluation of their sera status revealed that the animals had similar range of mixed
622 anti-GPCMV ELISA titers which ranged from 1:160 to 1:2560 and neutralizing antibody titers as
623 animals from R1 study (data not shown). Once animals were confirmed to have an immune
624 response to the DISC vaccine, they were paired with seronegative male guinea pigs for mating.
625 The control group 2 (non-vaccinated animals) were also paired for mating at this time with
626 seronegative males. Dams were confirmed as pregnant by palpation (20-25 days of gestation). At
627 approximately late 2^{nd} trimester, dams were challenged subcutaneously with 10^5 pfu of wild type

628 salivary gland stock virus and animals were allowed to go to term. The viral load in target organs
629 (liver, lung, spleen, brain) of live or still born pups was evaluated by real time PCR. Table 2
630 summarizes the pregnancy outcome in the two animal groups. Overall, more dams carried
631 pregnancy to term in the vaccinated group (100% vs 69.2%) which had a higher proportion of
632 live pups (94.1% vs 63.6%). Additionally, the DISM group had a greater overall number of live
633 pups (48 vs 28). Although there were 3 dead pups in the vaccinated animal group, all of these
634 pups were determined to be negative for GPCMV and death was attributed to a complication of
635 pregnancy. In contrast, all 16 still born pups in the control non-vaccinated group were positive
636 for GPCMV, with 80% of tissue (liver, lung, spleen or brain) analyzed positive for GPCMV.
637 Additionally, in the non-vaccinated group there were two litters re-absorbed by the dam. Based
638 on the outcome (live vs stillborn pups) between groups, the DISC vaccine was considered to be
639 effective in protecting against congenital CMV mortality (P = 0.0002, based on live births of
640 94.1% vs 63.6%). In this study, the CMV mortality rate in the control group was 36.4% based on
641 dead pups for term litters. This excluded 2 litters reabsorbed by the dams in the control group. In
642 previously published congenital GPCMV studies, with a challenge virus of 10^5 pfu, the mortality
643 rate ranges from 34-81% (51, 61, 75-79). Therefore the present study falls within this range.
644 However, in previous studies the higher mortality rates were obtained by including animals that
645 aborted within two weeks of virus challenge. This scenario did not occur in our studies but re-
646 absorbed litters were excluded from the present study which could have increased the mortality
647 rate.
648
649 Based on pup tissue positive for GPCMV, a transmission rate of 75.9% for the control group was
650 reduced to 23.5% in the vaccine group (P=0.0001). The transmission rate in the control group

651 was roughly similar to the congenital GPCMV transmission rate observed for control non-
652 vaccinated groups in other published studies using challenge virus at 10^5 pfu. In previous studies,
653 the GPCMV transmission rate ranged from 50-85% with the majority in the range 65-85%
654 (average rate 74.4% (51, 75-79). In one study, with virus challenge at 10^6 pfu, the transmission
655 rate was 70% (80) which indicated that a higher dose of challenge virus does not necessarily
656 result in a higher transmission rate. Analysis of viral load of pup tissue from both groups
657 demonstrated that the tissue from the control non-vaccinated group had a higher frequency of
658 GPCMV positive tissue (Table 3). A 52.4% reduction in GPCMV transmission in the vaccinated
659 group (23.5% transmission) compared to the control group (75.9% transmission) indicated that
660 the DISC vaccine strategy was more effective compared to studies with live GPCMV vaccine
661 strains (77, 79) and gB based vaccine strategies (75, 76, 78, 81). Outcome in comparison to
662 previous GPCMV vaccine studies is covered in more detail in the discussion section.
663
664 The majority of pup tissues in the vaccinated group were negative for virus but a small number
665 of pups were positive. However, while the viral load in target tissue of these individual positive
666 pups was lower than that found in comparable tissue of the non-vaccinated group, it was not
667 statistically significant, except for the brain (Table 4). An important benchmark for the vaccine
668 strategy was the prevention of CMV infection in pup brains. Consequently, although the vaccine
669 strategy was successful in reducing congenital infection, the approach failed to completely
670 prevent viral infection of pup brains with 5/51 positive for low level of virus. In the non-
671 vaccinated group, 13/29 pups had brain infection and the viral loads were higher than seen in the
672 DISM group (Table 4, $p = 0.05$).
673

674 Congenital transmission rate from dams with anti-GPCMV titer of 1:640 or less was 32.4%
675 compared to 5.9% ($p = 0.042$) from dams with anti-GPCMV titer of 1:1280 or greater (Table 5).
676 Interestingly, a comparative analysis of individual animal anti-GPCMV antibody titers with
677 congenital CMV transmission demonstrated that a high anti-GPCMV ELISA was not necessarily
678 a predictor for protection against congenital infection. However, the incidence of congenital
679 CMV in the high titer group was significantly less. If anti-GPCMV ELISA titers were grouped as
680 low (1:160), medium (1:320-1:640) and high (1:1280-1:2560), then congenital infection occurred
681 across all groups: low titer (2 pups); medium titer (9 pups); high titer (1 pup) (see Table 6).
682 Potentially, an additional booster vaccination could have induced a stronger immune response as
683 indicated in DISC vaccine R2 studies. However, the rationale for testing efficacy with the R1
684 vaccine strategy was to match previous recombinant vaccine studies that used live attenuated
685 GPCMV strains (77, 79, 80, 82). Outcome in comparison to previous GPCMV vaccine studies is
686 covered in more detail in the discussion section. Overall, the DISC vaccine strategy was effective
687 in reducing congenital infection compared to the control group. Additionally, the DISC vaccine
688 was more effective than previously published studies with most of the attenuated live GPCMV
689 vaccines.
690

691 **Discussion**

692 Vaccines strategies against congenital CMV that have gone forward to human clinical trials have
693 mainly focused on a single target antigen (eg. gB glycoprotein) and neutralizing antibodies.
694 However, these strategies have at best provided 50% efficacy (41). Potentially, a vaccine against
695 congenital CMV has to induce both an antibody to key neutralizing glycoprotein complexes that
696 are essential for virus entry and additionally target important T cell target antigens to provide a

comprehensive protective immune response against CMV. It is unlikely that a subunit vaccine or a recombinant vector delivery system (eg. MVA) could deliver such a complicated array of antigens and successfully evoke an immune response equivalent to CMV convalescent patients. An attenuated live recombinant CMV vaccine strategy is potentially an effective approach since this approach mimics a natural infection and in theory induces an immune response equivalent to convalescent CMV immunity. However, attenuation covers a wide spectrum of viral mutants and potentially some or all attenuated live vaccine strains have a safety risk associated with them, more especially if the virus is able to establish latency in the host and contribute to disease later in life. A potentially safer CMV vaccine strategy is the use of a non-replication competent virus. This approach was investigated in guinea pig CMV and the term DISC virus was adopted for the type of virus vaccine studied. DISC (defective infectious single cycle) described accurately an important feature of our vaccine strategy. The recombinant virus lacked the ability to express an essential gene (*GP85* encoding the small capsid protein) and as such the virus could infect cells but lacked the ability to make progeny virus because of a defect in capsid assembly. Consequently, the DISC virus stock could only be propagated on a complementing cell line. The term DISC virus was originally used to describe a HSV-1 vaccine strategy, where the gH glycoprotein had been knocked out and the gH protein was supplied *in trans* in a complementing cell line (70). The DISC GPCMV approach deviated from this original strategy for HSV as the gH glycoprotein in GPCMV was considered highly important for the generation of target antigen complexes (36). A capsid mutant was deemed more suitable as capsid proteins are essential for the generation of progeny virus but in themselves are relatively unimportant target antigens for CMV. However, a RhCMV gH knockout mutant virus was generated (83) and is currently under investigation as a CMV vaccine in rhesus macaques. In an additional further development to the

DISC vaccine strategy, the essential capsid gene was not expressed by a complementing cell line *in trans*. Instead, the tet-off system (tet-off advanced) was introduced into guinea pig fibroblast cells to enable generation of a complementing cell line. This also required modifying the *GP85* gene in GPCMV to place gene expression under the control of the tet-off transactivator (tTA2) to enable GP85 DISC virus mutant growth on the tet-off cell line. This approach potentially enabled the development of next generation DISC virus strains with additional mutations (eg. pp71 expression) under tet-off promoter control with a single controlling complementing cell line. In MCMV, a spread deficient virus vaccine strategy was investigated for a M94 mutant virus. In this study a reverse strategy was used of incorporating the tet-on transactivator into the recombinant virus which also carried a M94 knockout. The complementing cell line encoded the M94 gene which was induced by the transactivator expression under MCMV infection. A similar strategy could have been employed for the GPCMV GP85 mutant, more especially since a GP85 deletion mutant was also generated as part of the initial study. A potential downside of this approach would have been viral expression of the transactivator in the host animal. However, the M94 mutant vaccine strategy was safe even in innate immune knockout mice and highly protective against challenge with a pathogenic strain of MCMV because of an induced T cell response (CD4+ and CD8+) as well as effective neutralizing antibodies (84).

In the GPCMV study, a newly developed interferon gamma ELISPOT assay demonstrated that the *GP85* DISC vaccine evoked a T cell response to the homolog pp65 protein (GP83). Specific regions within the GP83 protein were identified as producing a heightened response. Additional studies are required to better define if the GP83 response is mainly CD4+ or CD8+ based. A previous GPCMV study demonstrated that the GP83 antigen was partially protective against

743 congenital CMV. This previous study employed a defective alphavirus system that encoded
744 GP83 and both CD4+ and CD8+ responses were induced and defined by flow cytometry (61).
745 Mouse monoclonal antibodies to guinea pig CD3, CD4 and CD8 markers are available (85) and
746 so further characterization of the T cell response to GP83 would be possible. However, an
747 evaluation of the T cell response to other homolog T cell target antigens (eg. IE1and IE2) is also
748 a high priority for future studies in GPCMV. Currently, splenocytes are necessary to perform this
749 new guinea pig ELISPOT assay. Therefore it was not possible to correlate the level of T cell
750 response to the pp65 antigen and protection against congenital infection since animals were
751 required to take their pregnancy to term at which point the challenge virus would have
752 complicated the results from any ELISPOT assay. Consequently, this aspect could not be
753 evaluated for the congenital vaccine study but remains an important aspect that in this study is
754 only partially defined.
755
756 Both mouse and rhesus macaque CMV animal models have demonstrated the importance of a T
757 cell response to the virus to prevent virus dissemination and vaccine studies suggest that other
758 target antigens in addition to pp65 are important targets (86, 87). However, in the context of
759 congenital CMV the lack of a MCMV congenital model prevents any evaluation. Technical
760 issues and additional expense have limited RhCMV studies but a recent congenital RhCMV
761 study suggested that a CD4 response does have a controlling role in protection against congenital
762 CMV infection but that this impacted on both CD8+ cells and antibody response (88).
763
764 It is likely that an important component of protection against congenital CMV is neutralizing
765 antibodies directed to critical viral complexes necessary for cell entry. Importantly, antibodies

766 can cross the placenta and therefore anti-CMV antibodies can function in both maternal and fetal
767 compartments (89). In an earlier study, we characterized the GPCMV gH/gL/gO and gM/gN
768 homolog glycoprotein complexes and demonstrated their essential nature and their importance as
769 immune targets with newly developed ELISAs (36). Overall, the DISC vaccine strategy
770 generated a high anti-GPCMV antibody titer and the vaccine regime with 2 booster shots
771 resulted in an antibody response similar to that of natural immunity in hyperimmunized animals.
772 The DISC vaccine induced antibody responses to gB and gH/gL with similar titers found in
773 animals convalescent for natural infection, whereas the immune response to the gM/gN complex
774 was weaker under natural infection. The neutralizing antibody titer was on average lower than
775 that seen in convalescent animals. Unfortunately, other GPCMV vaccine studies do not currently
776 perform advanced glycoprotein specific ELISAs and therefore it is difficult to evaluate previous
777 reports alongside this current research. Recent studies based on live attenuated GPCMV vaccines
778 have demonstrated a robust anti-GPCMV antibody titer and a specific immune response to gB
779 based on western blot analysis. In our present study, two vaccine regimes were explored for the
780 DISC virus in guinea pigs and it appeared that a two booster strategy dramatically increased the
781 immune response to the DISC virus compared to a single booster. A two booster DISC vaccine
782 strategy induced an antibody response comparable to that seen for live attenuated GPCMV (anti-
783 GPCMV and gB titers) and a single booster DISC resulted in lower titers. In contrast, GPCMV
784 gB specific vaccine strategies) produced antibody titers higher than natural convalescent
785 immunity (51, 76, 78, 90) but these gB vaccine strategies were less successful in protecting
786 against congenital infection in comparison to the DISC vaccine. The most effective gB based
787 vaccine strategies reduced transmission rates from: 82 to 45% (gB recombinant baculovirus/
788 GSK adjuvant); 77 to 41% (gB DNA vaccine); 79 to 59% (MVA gB); (75, 76, 78). Potentially, a

789 failing of these gB vaccine strategies was that the gB lacked a C-terminal domain and therefore
790 an inability of the recombinant gB to multimerize as seen in natural infection. Consequently, a
791 large amount of the gB immune response was potentially directed to the linear homolog AD-1
792 domain (91) rather than other gB antigen targets. However, a more recent study by Cardin et al
793 (81) with a rLCMV vector, which encoded gB with a C-terminal domain but lacking a
794 transmembrane domain, did not increase protection against transmission (reduced from 83% to
795 60%) despite generation of high titer antibody to gB.
796
797 Previous congenital vaccine studies based on live attenuated GPCMV include a PKR mutant
798 virus (77), a MHC class I down regulation mutant (79) and GP83 knockout strain (80). The
799 vaccine strategies were able to reduce transmission by varying levels: 83% to 57% (79); 65% to
800 33% (77); 70% to 17% (80). Surprisingly, the pp65 homolog (GP83) knockout mutant would
801 appear to be the most effective live vaccine strategy despite a previous vaccine strategy using a
802 defective alphavirus encoding GP83 demonstrating the importance of the T cell response to
803 GP83 in protecting against congenital infection (85% reduced to 47%) (61). Currently, only the
804 GP83 knockout mutant vaccine strategy is more effective than the DISC vaccine in reducing the
805 congenital transmission rate. However, the *GP83* mutant virus has near normal growth kinetics
806 in tissue culture as does a HCMV pp65 mutant (30, 92). Consequently, a live HCMV vaccine
807 based on a pp65 knockout might present an unacceptable risk compared to a DISC vaccine
808 strategy, which would have a greater safety factor due to its inability to replicate outside of the
809 supporting cell line.
810

811 Unfortunately, the more effective DISC vaccine regime was evaluated after the congenital
812 vaccine protection study had been initiated with a single vaccine booster approach. Nonetheless,
813 the DISC vaccine was highly effective in protecting against congenital CMV and importantly
814 high anti-GPCMV titers were not necessarily an indicator for effective protection against
815 congenital infection. As noted above, it is likely that the T cell response to various target
816 antigens was also important in the prevention of congenital infection in the setting of the current
817 vaccine and the immune response to the GP83 was an indicator of a positive T cell response to
818 the DISC vaccine. However, a key component missing from the current DISC vaccine was the
819 homolog pentameric complex. Clinical strains of HCMV, unlike lab adapted strains, encode a
820 pentameric glycoprotein complex (gH/gL/UL128/130/131) for virus entry into epithelial and
821 endothelial cells via an alternative endocytic pathway of cell entry rather than a gB plus gH/gL
822 mediated cell membrane fusion approach (93-98). The pentameric complex is considered an
823 important neutralizing target for HCMV on epithelial/endothelial cells and presumably for
824 congenital infection, given the epi/endothelial structure of the placenta. The importance of the
825 endocytic pathway in virus infection of cells is underscored by the fact that a gB subunit HCMV
826 vaccine despite a high titer neutralizing immune response on fibroblasts lacks an effective ability
827 to neutralize infection of endothelial and epithelial cells compared to convalescent sera (41, 52-
828 55). In RhCMV, the pentameric complex has been demonstrated to be an important
829 pathogenicity factor as well as a neutralizing target antigen (99, 100). In GPCMV, a homolog
830 *UL128-131* locus has been identified and the ability of a pentameric complex to form has been
831 investigated (28, 58). We have recently demonstrated that this complex is important for virus
832 tropism to epithelial cells, pathogenicity and congenital infection. Importantly, the homolog
833 pentameric complex is highly immunogenic (101). Virus serially passaged as salivary gland

834 stock in animals is stably maintained but virus on fibroblast cells rapidly undergoes mutations or
835 deletions in this region. Our current DISC vaccine strategy is based on the 1st generation
836 GPCMV BAC which carried a deletion in the homolog *UL128-131* locus (15, 26). Consequently,
837 the DISC vaccine was incapable of generating an immune response to the pentameric complex
838 which is potentially an important component of the immune response to GPCMV in
839 convalescent animals. Importantly, we have recently identified the pentameric complex as an
840 important target for neutralization of virus infection of epithelial cells (101). Therefore
841 introduction of the pentameric complex in a second generation DISC vaccine is likely to have a
842 significant impact on the efficacy of a future DISC vaccine congenital CMV protection study. It
843 is important to note that all of the live GPCMV attenuated vaccine strategies evaluated to date do
844 not encode a viable pentameric complex and so modification to these strains could potentially
845 enhance the efficacy of these vaccine strategies. Indeed, a new GP83 knockout mutant virus that
846 also encodes a pentameric complex was highly effective in inducing a robust immune response
847 and reducing congenital transmission (101).
848
849 In conclusion, the first generation DISC vaccine against congenital GPCMV is a safer strategy
850 than the use of a live attenuated virus as it lacks the ability to produce progeny virus and
851 disseminate in the host. Importantly, the virus evokes an immune response similar to
852 naturalinfection which includes an antibody response to viral glycoprotein complexes necessary
853 for cell entry and a T cell response to the pp65 homolog. The vaccine strategy was successful in
854 fully preventing pup deaths and highly reduced the level and incidence of pups with congenital
855 CMV compared to a non-vaccinated control group. This promising vaccine strategy has potential
856 room for improvement by the inclusion of the homolog pentameric complex which is considered

857 an important complex for virus infection of epithelial (Coleman et al., 2016 in review) and a
858 variety of other cell types. Potentially, additional neutralizing antibodies to this complex will
859 greatly reduce congenital transmission.
860
861

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864 assistance.
865
866

867 **Figure legends**

868 **Figure 1.** Nucleotide sequence of the GP85/GP86 locus. Sense genome strand shown. Both
869 GP85 and GP86 are encoded on the complementary strand. The sequence used for flanking arms
870 in shuttle vector (Figure S1B) recombination are highlighted. GP85 flanking sequence GPCMV
871 bases 134992-135906. GP86 flanking sequence 136180-136681. Deleted intergenic sequence
872 135907-136179.

873

874 **Figure 2**. Cloning strategy for generation of a shuttle vector encoding GP85 under Tet-off (TRE-
875 tight) promoter control. 1-2. Part of the GP85 (5') and GP86 (3') coding sequence were
876 separately cloned via PCR into pUC19 to generate pGP85FLK and pGP86FLK. 3. The GP86
877 coding sequence was then cloned into pGP85FLK as a BamH I/Hind III fragment to generate
878 pGP85GP86 which lacked the intergenic sequence between the GP85 and GP86. 4-5. A Bgl II
879 Km cassette was introduced to generate pGP8586Km+ which was digested with Sal I to enable
880 cloning of a SV40 polyA cassette to generate pGP8586SV40AKm(6). The TREtight promoter
881 from pTREtight (Clontech) was PCR cloned as a BamH I fragment immediately upsteam of the
882 GP85 coding sequence (7) to generate pGP8586TRESV40AKm. Shuttle vectors (5, 6 and 7)
883 linearized with Pme I were used to modify the GP85/GP86 intergenic locus in the GPCMV BAC
884 via homologous recombination in separate reactions to introduce the modified sequence as
885 described in materials and methods to generate the DISC GPCMV BAC and other BAC mutants.

886

887 **Figure 3**. BLAST alignment of the predicted minor capsid protein (mCP) from GPCMV (GP85)
888 and HCMV, Towne strain (UL85). (i) Co-linear location of GP85 and UL85 genes in GPCMV
889 and HCMV respectively. (ii) BLAST search of GP85 protein identifies it as a member of the

890 Herpes V23 (capsid protein) superfamily. (iii) BLAST (NCBI BLASTp) alignment of GP85 and
891 UL85 proteins. Score= 354 bits (908), Expect= 2e-120, Method: Compositional matrix adjust.
892 Identities= 172/305 (56%), Positives= 226/305 (74%), Gaps= 4/305 (1%).
893
894 **Figure 4. Characterization of GPL-tet-off cell lines via luciferase reporter gene**
895 **transactivation.** GPL cells (A-D) or candidate GP-tet-off cells (E-H) were transfected with a
896 luciferase reporter plasmid under TREtight promoter control (pTREtightLUC). Wells A and C
897 were additionally transfected with tet-off transactivator (tTA2) expression plasmid. Well B was
898 transfected with a luciferase reporter gene plasmid under HCMV MIE promoter control
899 (pcDNA3LUC). At 24 hours post transfection, luciferase substrate (D-Luciferin) was added and
900 plates imaged for bioluminescence for 5 min (IVIS 50, Xenogen). Shown are black and white
901 image of six well plates with superimposed photon emission intensity. Vertical color bar range
902 indicates highest (red) to lowest (purple) levels of bioluminescence (p/sec/cm^2/sr) for imaged
903 samples. Tet-off cell line assays show duplicate wells for A20 (E and G) and A21 (F and H).
904
905 **Figure 5. Modification of the GP85 5'UTR region in a GPCMV BAC and generation of a**
906 **DISC GP85 strain BAC.** (i) Schematic of the GPCMV genome with location of the BAC
907 insertion and Hind III sites indicated. The *GP85/GP86* locus is encoded in the Hind III 'A'
908 fragment. (ii) Modifications to the 5'UTR in the *GP85/GP86* loci in wild type (1) and mutants:
909 GP85/GP86KmR BAC (2); GP85/GP86 KmR polyA BAC; TRE GP85 (DISC) BAC (3). Also
910 shown are the approximate size of the wild type and mutant loci based on external primer used to
911 generate the original *GP85/GP86* shuttle vector. (iii*) Eco*R I restriction profile analysis of wild
912 type or mutant GPCMV BACs described in (ii): wild type GPCMV BAC (1); GP85/GP86KmR

913 BAC (2); TRE GP85 (DISC) BAC (3). Original Hind III "A' genomic fragment indicated in blue
914 and modified fragment indicated by red dots. (iv) PCR amplification of the *GP85/GP86* loci in
915 wild type and mutant GPCMV BACs: wild type (1); GP85/GP86 KmR (2); TRE GP85 DISC
916 (3). DNA ladder (kb) Invitrogen.
917
918 **Figure 6. Regeneration of a DISC GP85 GPCMV from GP85 BAC mutants requires a TRE**
919 **promoter and a cell line expressing a tet-off transactivator (tTA2).** GP85 mutant GPCMV
920 BACs were individually transfected onto GPL cells: GP85/GP86 KmR (A); GP85/86 KmR
921 polyA (B); TRE GP85, DISC (C). Mutants were also transfected onto GPL-tet-off cells. TRE
922 GP85, DISC (D) shown. Individual transfected cells expressed GFP encoded by the BAC. Virus
923 spread was detected by GFP spread across the cell monolayer. Images taken at 16 days post
924 transfection.
925
926
927 **Figure 7. Growth kinetics of DISC GP85 GPCMV vs wild type GPCMV on GPL tet-off**
928 **cells.** GPL tet-off cells were infected at a moi of 1 pfu/cell with respective virus in separate wells
929 of six well dishes. Sample were taken at different days post infection and titrated in duplicate as
930 previously described (33). Results plotted as virus titer against days post infection.
931
932 **Figure 8.** Western blot s of wild type GPCMV. GPCMV (purified virus particles or total cell
933 lysate) were seperated by SDS-PAGE and immunoblotted with anti-GPCMV sera (1/2000
934 dilution) from DISC vaccinated animal and anti-guinea pig IgG-HRP (1/500, Sigma) as
935 described in materials and methods. Blot A, sucrose purified GPCMV. Blot B, GPCMV infected

GPL cell lysate. Control lanes MI, GPL cells uninfected. Protein size indicated in kDa using ladder (BioRad).

Figure 9. Overview of DISC GP85 vaccination schedules and preconception vaccine study. (a) and (b). In the initial characterization of the immune response to the DISC vaccine two vaccine regimes were employed: R1 (a); R2 (b). DISC virus inoculations are indicated with an arrow and virus symbol. Animals bled for immune response at indicated weeks post initial vaccination. In (a) and (b) animals were euthanized at the end of the vaccination schedule (and after confirmation of seroconversion) to determine T cell response via ELISPOT assay as described in materials and methods. (c) Preconception vaccine study (group 1) following vaccine regime R1. During late 2nd trimester of pregnancy animals were challenged with wild type GPCMV (10^5pfu) and animals allowed to go to term. (d) Control non-vaccinated animals (group 2) for congenital vaccine study.

Figure 10. Antibody immune response to GPCMV and specific viral glycoprotein complexes (gB, gH/gL and gM/gN) for DISC GP85 vaccinated guinea pigs determined by ELISAs.

(A) Immune response of animals vaccinated with DISC GP85 under R1 (blue) or R2 (orange) regimes were compared to animals hyper-immunized wild type GPCMV (grey). ELISA assays: anti-GPCMV; anti-gB; anti-gH/gL ; anti-gM/gN. Mean values for each group with standard deviation represented with error bars. Statistically significant with $P < 0.05$ in *a* hyperimmune vs R1; *b* R1 vs R2; *c* hyperimmune vs R2 compared by Mann Whitney test. (B) Neutralizing antibody titer on fibroblast cells of hyperimmune sera compared to R1 and R2 regimes.

Statistically significant with $P < 0.05$ in *d* hyperimmune vs R1; *e* hyperimmune vs R2 compared by Mann Whitney test.

Figure 11. Evaluation of animal T cell response to GPCMV vaccine. Interferon gamma ELISPOT of splenocyte responses to GPCMV GP83 peptide pool in R1, R2 vaccinated and wt GPCMV infected animals. Assay was carried out as described in materials and methods with splenocytes isolated from DISC GP85 vaccinated animals (vaccine regimes R1 or R2) or wild type GPCMV infected animals. Overlapping peptides (9mers) spanning the complete GP83 protein sequence were assigned to 24 peptide pools (I-XXIV). Results shown for selected GP83 peptide pools: II (green); IV (black); X (orange); XX (blue). Con A (red) was used as positive control stimulation and negative controls included unstimulated (grey) and DMSO control (yellow). Final counts were calculated based on spot forming cells (SFC) per 10^6 cells after background spots (cells only without any stimulation) were subtracted. GP83 peptide pools X and XX highly stimulated cells whereas pools II and IV represent poorly stimulated cells.

Table 1. Oligonucleotides

Table 2. Congenital infection outcome for live vs dead pups.

Table 3. Impact of DISC vaccine in transmission based on number of GPCMV positive pups in each specific tissue group

Table 4. Congenital infection outcome determined by viral load in target tissue of pups.

Table 5. Congenital CMV outcome related to anti-GPCMV titer.

Table 6. Individual congenital CMV outcome related to anti-GPCMV titer

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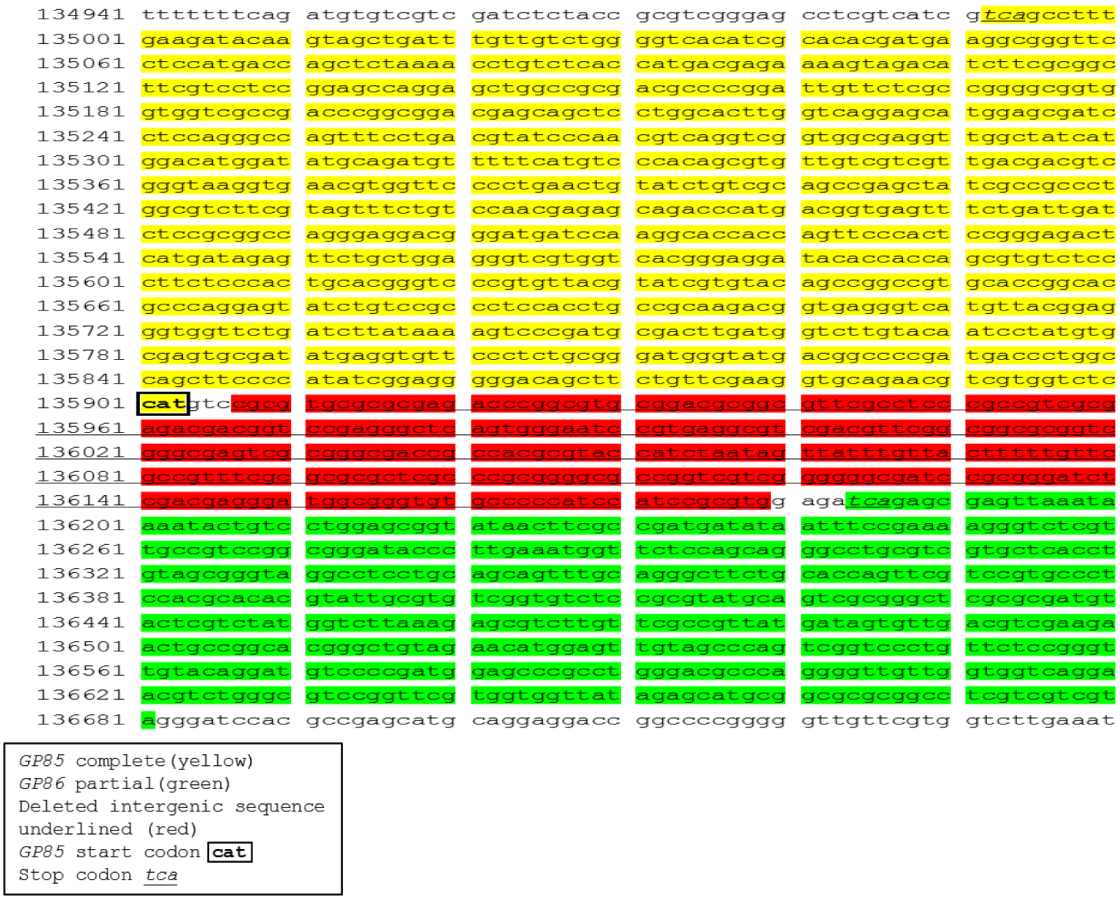
Figure 1



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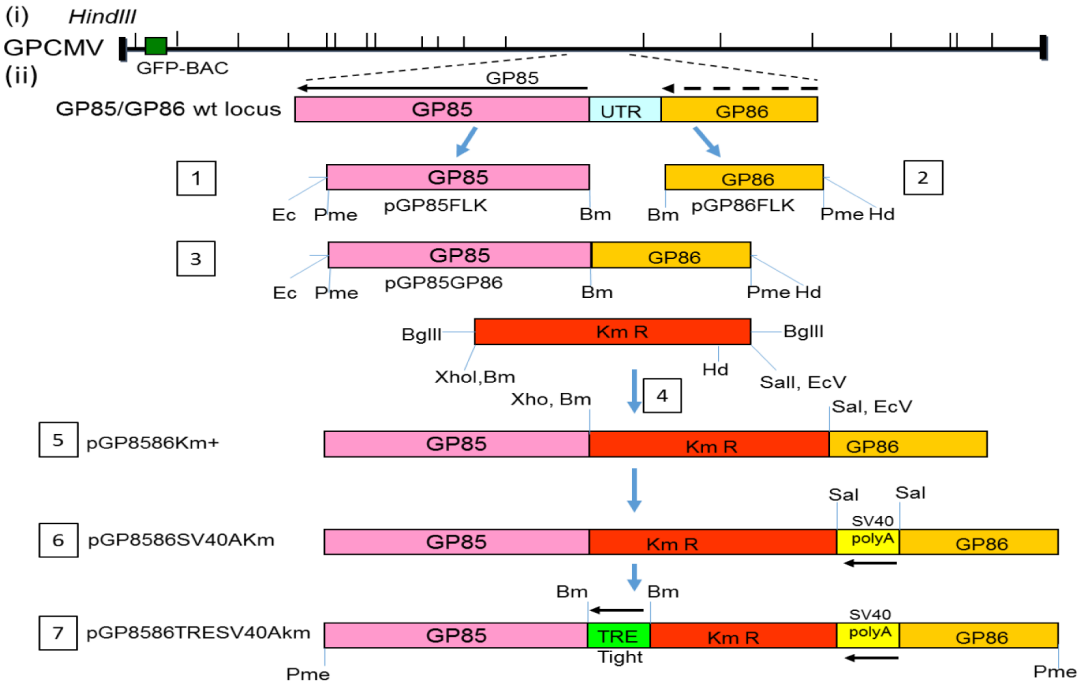

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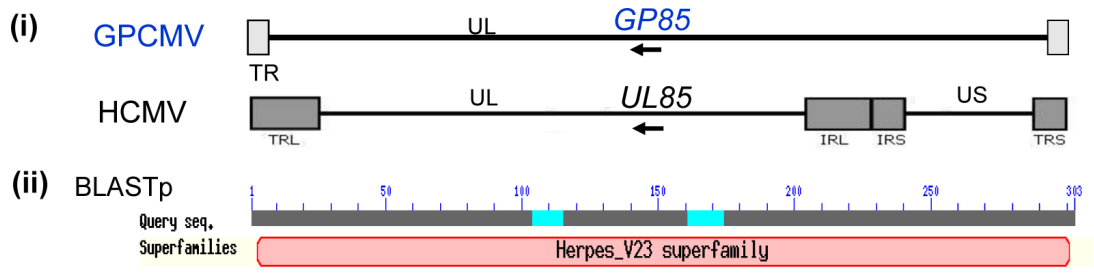

(iii)

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GP85   1    METTTFCTFEQKLSPSDMGKLARVIGAVIPIPQREHLISHSHIGLYKTIKSHRDFYKIRT  60
            ME   FCTF+ KLS +D+GKL +++ AV+PIPQR HLI H  +GL++ +   R + ++R
UL85   4    MEANIFCTFDHKLSIADVGKLTKLVAAVVPIPQRLHLIKHYQLGLHQFVDHTRGYVRLRG  63
GP85   61   TLRNMTLTVLRQVEGGQILLGVPVHGRLYTIRNTGPVQWEKGDTLVVYPPV-TTTLQQNS  119
             LRNMTLT++R+VEG QILL VP HG LYT+ NTGPV WEKGD L V PP+    L + +
UL85   64   LLRNMTLTLMRRVEGNQILLHVPTHGLLYTVLNTGPVTWEKGDALCVLPPLFHGPLAREN  123
GP85   120  IMSLPEWELVVPWIIPSSLAAEINQKLTVMGLLSLDRNYEDARAAIARLRQIQFRGTTFT  179
            +++L +WELV+PWI+P  LA EINQ+L +MGL SLDR+YE+ +AA+ +L+ I FR  TFT
UL85   124  LLTLGQWELVLPWIVPMPLALEINQRLLIMGLFSLDRSYEEVKAAVQQLQTITFRDATFT  183
GP85   180  LPDVVNDDNTLWDMKNICISMSMIANLATDLTLGYVRKLALEDRSMLLTKCQELLVRRVG  239
            +PD V D + L DMK  C+SMSM+ANLA++LT+ YVRKLALED SMLL KCQELL+R
UL85   184  IPDPVIDQHLLIDMKTACLSMSMVANLASELTMTYVRKLALEDSSMLLVKCQELLMRL--  241
GP85   240  DHHRP-GENNPGRRGQLLAPEDEAAKMSTFLVMVRQVLELVMEEPAFIVCDVTPDNKSAT  298
            D  R  GE     R Q ++P+DE A++S   VM+RQ+ +L+ E+  F VCDV+PDNKSAT
UL85   242  DRERSVGEPRTPARPQHVSPDDEIARLSALFVMLRQLDDLIREQVVFTVCDVSPDNKSAT  301
GP85   299  CIFKG  303
            CIFKG
UL85   302  CIFKG  306
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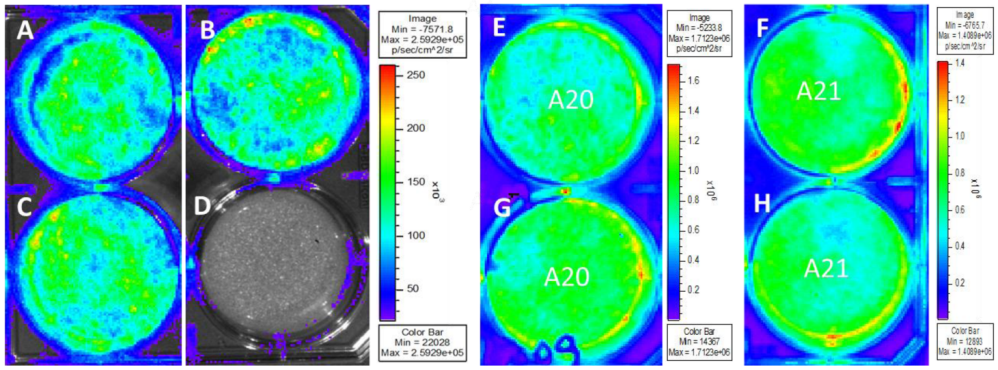
Figure 4



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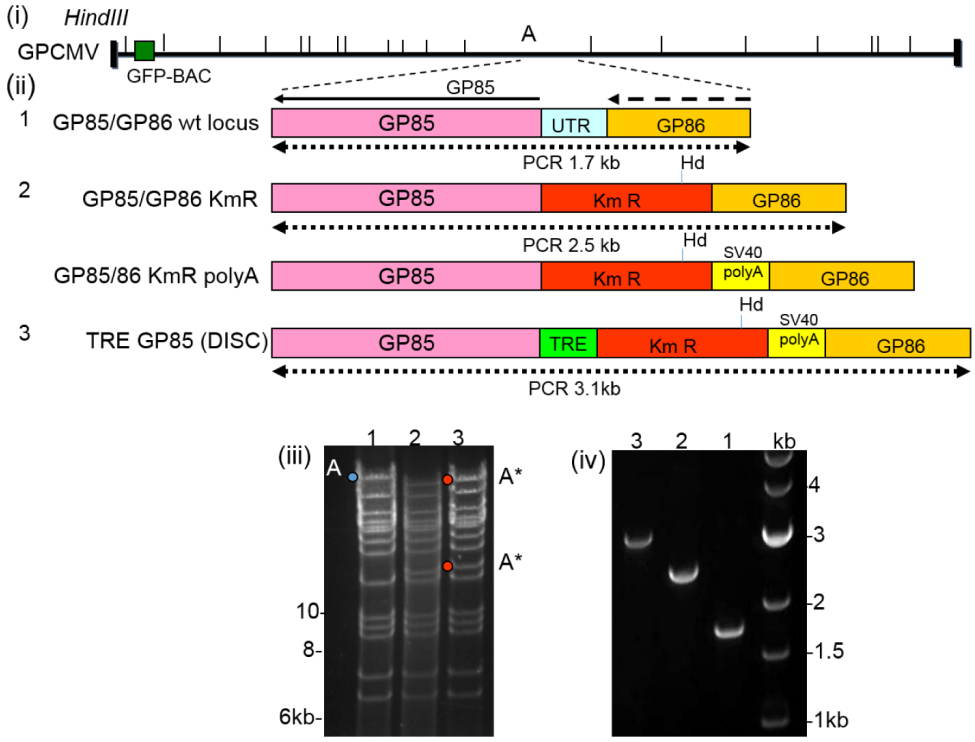

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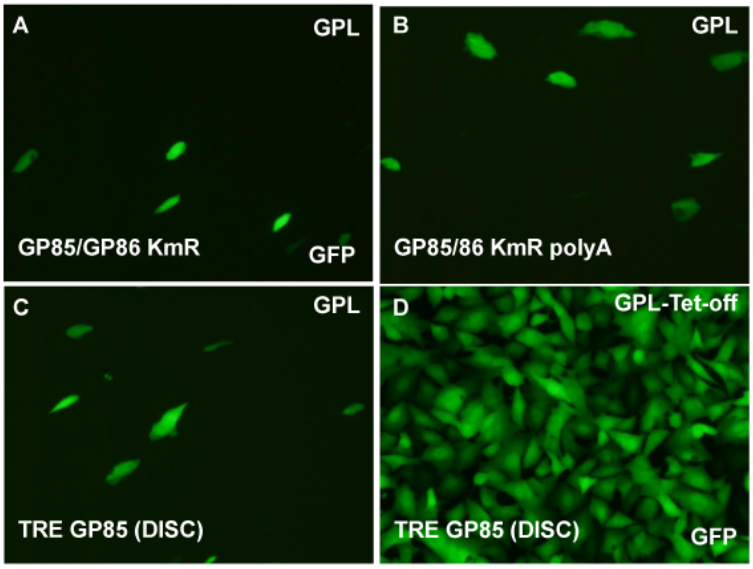

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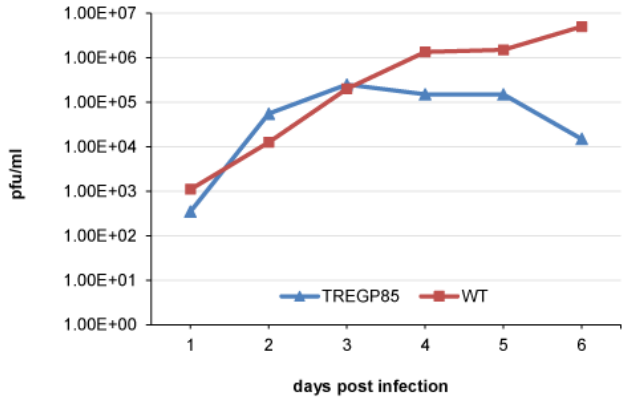

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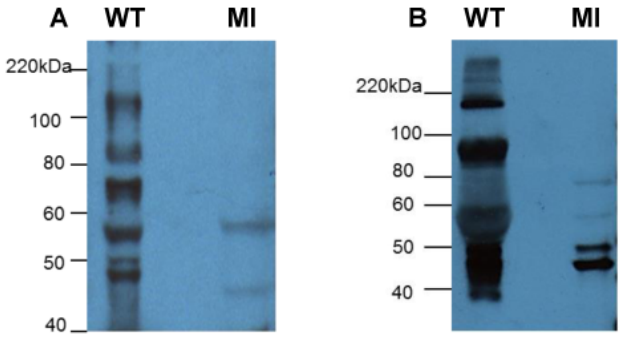

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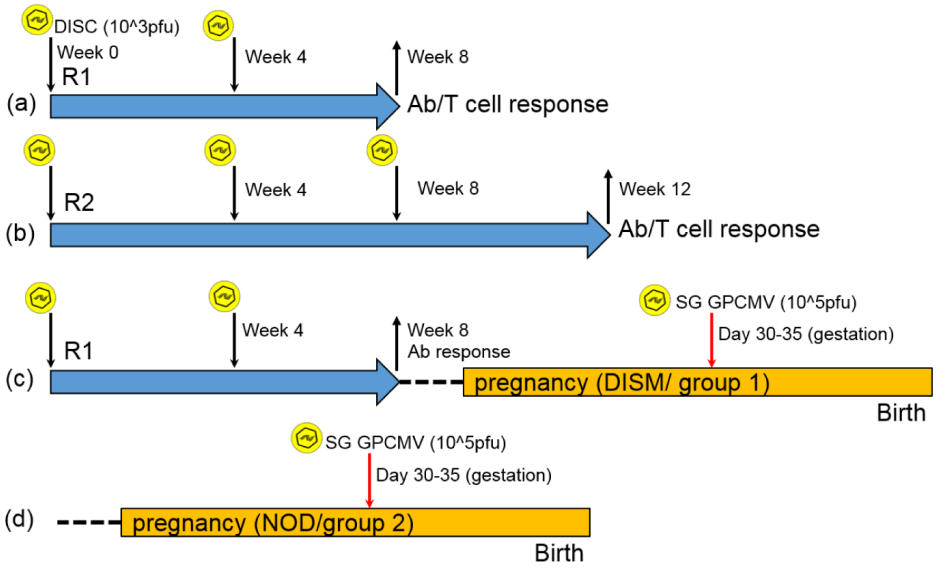
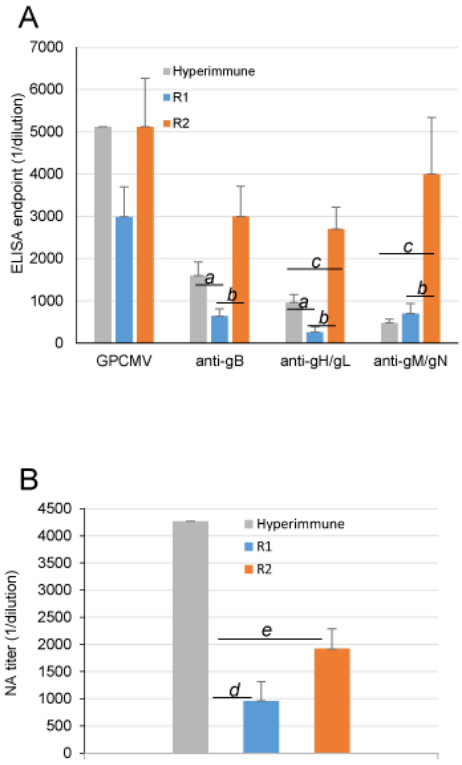


Figure 10



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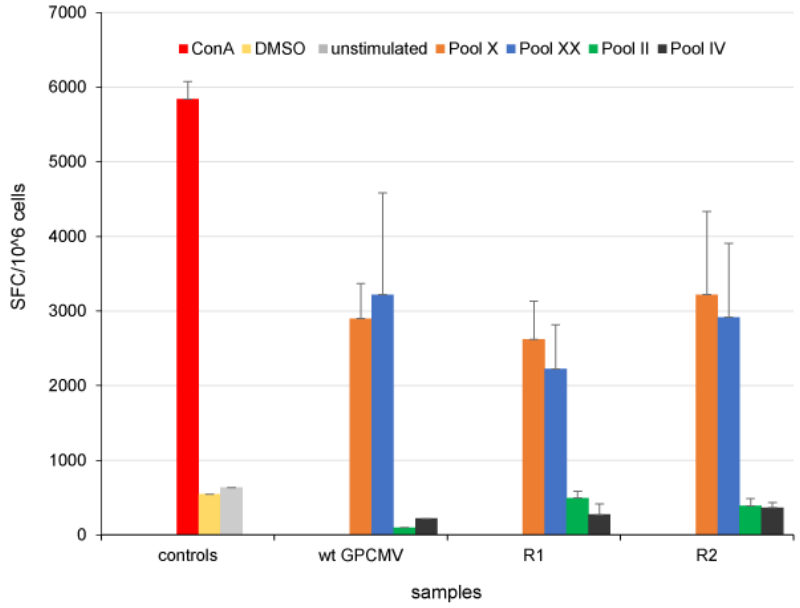

Table 1. Oligonucleotides

Oligo	Sequence
FEcGP85FLK	5'GATAGAGAATTCGTTAAACTCAGCCTTTGAAGATACAAGT AGCTGATTTGTTGTC
RBmGP85FLK	5'CAGACATGGATCCGACATGGAGACCACGACGTTCTGCACC TTCG
FBmGP86FLK	5'TAGAGTCGGATCCGAGATCAGAGCGAGTTAAATAAAATA CTGTCCTGGAG
RHdGP86FLK	5'GAGATAGCAAGCTTGTTTAAACTACGACGACGAGGCCGCG CGCCGCATGCTCTATAACCAC
FBgXhBmKm	5'AGATAGAAGATCTCTCGAGGATCCGATTTATTCAACAAAG CCACG
RBgEcVSlKm	5'AGCATGAAGATCTGATATCGTCGACGCCAGTGTTACAACC AATTAACC
FSV40AEcSl	5'ATAGATAGAATTCGTCGACTTAATTAATCTCTAGAGGATC ATAATCAGCCATACCACATTTGTAG
RSV40AEcSl	5'AGATAGAGAATTCGTCGACGATATCGCAGTGAAAAAAAT GCTTTATTTGTGAAATTTG
FTREBm	5'ATAGATAGGATCCTCGAGTTTACTCCCTATCAGTGATAG
RTREBm	5'AAGATAGGATCCAGGCGATCTGACGGTTCACTAAACGAGC

Table 2. Congenital infection outcome for live vs dead pups

Groups	Litters					Total pups	
	Total	Live only	Dead only	Mixed	Reabsorbed	Live born	Stillborn
DISM (N=14)	13	11	0	2	0	48* (94.1%)	3 (5.9%)
NOD (N=15)	13	8	4	1	2	28* (63.6%)	16 (36.4%)

*P = 0.0002 compared to control (Fisher's exact test)

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Table 3. Impact of DISC vaccine in transmission based on number of GPCMV positive pups in each specific tissue group

Vaccine	Lung	Liver	Spleen	Brain	CMV+ pups[a]
DISM	7/51 (13.7%)	2/51 (3.9%)	2/51 (3.9%)	5/51 (9.8%)	12/51 (23.5%)
NOD	17/29 (58.62%)	9/29 (31.03%)	19/29 (65.52%)	13/29 (44.83%)	22/29 (75.9%)
P value[b]	0.0001	0.0013	0.0001	0.0006	0.0001

[a]GPCMV detected in at least one organ

[b]No. of CMV+ specific organ *p* vs NOD (Fisher's exact test)

Table 4. Congenital infection outcome determined by viral load in target tissue of pups

Vaccine	**Lung**[a]	**Liver**[a]	**Spleen**[a]	**Brain**[a]
DISM	2.5 ± 2	2.1 ± 1.7	2.2 ± 1.5	2.9 ± 2.6
NOD	2.6 ± 2	2.6 ± 2.1	3.2 ± 2.9	3.3 ± 2.7
P value	NS [b, c]	NS [b]	NS [b]	0.05 [c]

[a]viral load expressed in $\log_{10}$ genome copies/mg tissue
[b]Student t test
[c]Mann-Whitney test
NS = not significant ($p > 0.05$)

Table 5. Congenital CMV outcome related to anti-GPCMV titer.

Anti-GPCMV titer	Pups (live/dead)	CMV+ pups
Low-mid range		
1:160-1:640	33/1	11/34* (32.4%)
High range		
1:1280-1:2560	15/2	1/17* (5.9%)

*p = 0.042 (Fisher's exact test)

Table 6. Individual congenital CMV outcome related to anti-GPCMV titer

DISC vaccinated mothers	Anti-GPCMV titer	Pups (live/dead)	#CMV+ pups
DISM11	1:160[a]	3/0	2
DISM12	1:160[a]	5/0	0
DISM6	1:320[b]	1/0	0
DISM14	1:320[b]	4/0	4
DISM1	1:640[b]	3/0	0
DISM3	1:640[b]	5/0	0
DISM5	1:640[b]	4/0	2
DISM7	1:640[b]	4/0	1
DISM10	1:640[b]	4/1	2
DISM2	1:1280[c]	3/0	0
DISM9	1:1280[c]	4/0	0
DISM13	1:1280[c]	3/2	1
DISM8	1:2560[c]	5/0	0

[a] anti-GPCMV low range titer ≤1:160
[b] anti-GPCMV mid range titer 1:320-1:640
[c] anti-GPCMV higj range titer ≥1:1280