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**Identification and functional characterization of a novel Fc gamma-binding glycoprotein in Rhesus Cytomegalovirus**

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26 **Abstract**

27 Receptors recognizing the Fc part of immunoglobulin G (FcγRs) are key determinants in  
28 antibody-mediated immune responses. Members of the *Herpesviridae* interfere with this  
29 immune regulatory network by expressing viral FcγRs (vFcγRs). Human cytomegalovirus  
30 (HCMV) encodes four distinct vFcγRs that differ with respect to their IgG-subtype specificity  
31 and their impact on antibody-mediated immune function *in vitro*. The impact of vFcγRs on  
32 HCMV pathogenesis and immunomodulation *in vivo* is not known. The evolutionary closest  
33 animal model of HCMV is rhesus CMV (RhCMV) infection of rhesus macaques. To enable  
34 the characterization of vFcγR function in this model, we studied IgG binding by RhCMV. We  
35 show that lysates of RhCMV-infected cells contain an IgG-binding protein of 30kDa encoded  
36 by the gene *Rh05* that is a predicted type I glycoprotein belonging to the *RL11* gene family.  
37 Upon deletion of *Rh05*, IgG-Fc binding by RhCMV strain 68-1 is lost whereas ectopic  
38 expression of Rh05 results in IgG binding to transfected cells consistent with Rh05 being a  
39 vFcγR. Using a set of reporter cell lines stably expressing human and rhesus FcγRs we further  
40 demonstrate that *Rh05* antagonizes host FcγR activation. Compared to *Rh05*-intact RhCMV,  
41 RhCMVΔ*Rh05* showed an increased activation of host FcγR upon exposure of infected cells  
42 to IgG from RhCMV-seropositive animals suggesting that Rh05 protects infected cells from  
43 opsonization and IgG-dependent activation of host FcγRs. However, antagonizing host FcγR  
44 activation by Rh05 was not required for the establishment and maintenance of infection of  
45 RhCMV, even in a seropositive host, as shown by the induction of T cell responses to  
46 heterologous antigens expressed by RhCMV lacking the gene region encoding Rh05. In  
47 contrast to viral evasion of NK cells or T cell recognition, the evasion of antibody-mediated  
48 effects does not seem to be absolutely required for infection or re-infection. The identification  
49 of the first vFcγR that efficiently antagonizes host FcγR activation in the RhCMV genome  
50 will thus permit more detailed studies of this immunomodulatory mechanism in promoting  
51 viral dissemination in the presence of natural or vaccine-induced humoral immunity.

52 **Importance**

53 Rhesus cytomegalovirus (RhCMV) offers a unique model for studying human  
54 cytomegalovirus (HCMV) pathogenesis and vaccine development. RhCMV infection of non-  
55 human primates greatly broadened the understanding of mechanisms by which CMVs evade  
56 or re-program T cell and NK cell responses in vivo. However, the role of humoral immunity  
57 and viral modulation of anti-CMV antibodies has not been studied in this model. There is  
58 evidence from in vitro studies that HCMVs can evade humoral immunity. By gene mapping  
59 and with the help of a novel cell-based reporter assay system we characterized the first  
60 RhCMV encoded IgG-Fcγ binding glycoprotein as a potent antagonist of rhesus FcγR  
61 activation. We further demonstrate that, unlike evasion of T cell immunity, this viral Fcγ  
62 receptor is not required to overcome anti-CMV immunity to establish secondary infections.  
63 These findings enable more detailed studies of the in vivo consequences of CMV evasion  
64 from IgG responses in non-human primate models.

65

66

## 67 Introduction

68 As prototypical members of the  $\beta$ -subgroup of the herpesvirus family, cytomegaloviruses  
69 (CMVs) establish lifelong infection characterized by viral latency and reactivation. Human  
70 and animal CMVs share sophisticated mechanisms to evade a multitude of antiviral host  
71 immune responses including both innate and adaptive arms of the immune system (1, 2). With  
72 respect to cell-mediated immunity, it has been shown that human cytomegalovirus (HCMV)  
73 can efficiently evade direct recognition of infected target cells by natural killer (NK) cells as  
74 well as T lymphocytes using a large repertoire of viral gene products that interfere with  
75 antigen presentation, surface receptor transport or innate receptor signaling (3, 4).  
76 Complementing viral evasion of cell-mediated immune responses are strategies for evasion of  
77 humoral immunity such as counteracting IgG-mediated antiviral immunity. Ribosomal  
78 profiling identified more than 750 translational products that include many potentially  
79 antigenic proteins during the sequential immediate-early (IE), early (E) and late (L) phases of  
80 gene expression (5). Despite exposure of these potential viral antigens to the host's immune  
81 system, human and animal CMVs maintain lifelong chronic infections with occasional  
82 reactivation. Moreover, CMVs are able to reinfect CMV-immune hosts despite the presence  
83 of CMV-specific humoral and cellular immune responses (6, 7). Potentially due to viral  
84 immune evasion capabilities, anti-HCMV IgG preparations such as intravenous hyperimmune  
85 immunoglobulin (IVIG) or monoclonal antibodies (mAbs) displayed only limited, if any,  
86 efficacy in various clinical settings (8-13). In non-human primate models, prevention of fetal  
87 transmission only occurred when IVIG was concentrated from plasma of donors that were  
88 pre-selected for high neutralization activity whereas IVIG from non-selected plasma was only  
89 partially protective suggesting that RhCMV is able to escape antibody control (14).  
90 Specific viral mechanisms that counteract antibody effector functions might be responsible for  
91 limiting the ability of antibodies to control viral infection and dissemination. HCMV evasion  
92 from IgG-Fc mediated effector functions can be attributed to a set of IgG-Fc binding

glycoproteins (vFcγRs) encoded by the HCMV genes *UL118/119* (gp68) and *RL11* (gp34) (15). These vFcγRs were shown to efficiently antagonize host IgG-Fc receptor (FcγR) activation in a cell-based *in vitro* reporter assay performed on IVIG-opsonized infected cells (16). In addition, *RL12* and *RL13* have been shown to have vFcγR activity (14). While HCMV is the only known human β-herpesvirus to encode such glycoproteins, it is not the only herpesvirus for which vFcγRs have been described. Mouse cytomegalovirus (MCMV) encodes the Ig-like glycoprotein fcr-1/m138 (17). Deletion of *m138* from the MCMV genome results in drastic attenuation of MCMV *in vivo* (18). However, since *m138* has both Fcγ-related and -unrelated immunoevasive functions (19-21) the role of Fcγ-modulation for viral pathogenesis has yet to be established. HSV-1 and VZV glycoproteins E and I (gE/gI) form an IgG-Fc binding heterodimer (22, 23). By clearing antigen/antibody complexes from the infected cell surface (24) the HSV-1 gE/gI complex promotes immune evasion *in vivo* (25). Interestingly, the VZV gE protein is the major component of the recently developed highly efficient subunit VZV vaccine (26).

Immune responses most prominently governed by host FcγRs include antibody dependent cell-mediated cytotoxicity (ADCC), antibody dependent cell-mediated phagocytosis (ADCP) and the induction of a pro-inflammatory cytokine profile by various immune cells including NK cells, macrophages, dendritic cells, B cells and neutrophils expressing FcγRs (27). FcγRs are further classified by their affinity to IgG-Fc and are highly conserved between humans and non-human primates showing strong cross-reactivity (28, 29). There are four known activating receptors comprising the high affinity receptor CD64/FcγRI, the medium affinity receptors CD32A/FcγRIIA and CD32C/FcγRIIC, and the low affinity receptor CD16A/FcγRIIIA. CD32B/FcγRIIB is the only known inhibitory receptor with a medium affinity to IgG-Fc and a single cytosolic ITIM motif (27). Although their affinity to IgG-Fc is also dependent on the IgG subclass, all FcγRs show their highest affinity towards IgG1 while optimal binding in general can only be observed to immune complexed IgG with an intact

glycan profile (30). In recent years FcγR-mediated immune responses have proven to be an essential factor in the antiviral effect of non-neutralizing but also neutralizing IgG specific for important pathogenic viruses like Influenza A (31, 32) and HIV (33, 34).

CMVs are highly species specific, which prevents studying HCMV directly in an animal model. While the closest relative of HCMV is chimpanzee CMV (CCMV), experimentation in these animals is no longer possible. In contrast, infection of rhesus macaques (RM) (*Macaca mulatta*) with rhesus cytomegalovirus (RhCMV) is a tractable model and the genomes of non-human primate (NHP) CMVs encode homologs of most of the HCMV gene families (35, 36). Therefore, RhCMV infection has emerged as a state of the art model allowing the study of primate CMV disease infection, immune responses and pathology *in vivo* (37), including important aspects of congenital infection (14, 38). While in this model RhCMV genes linked to evasion from CD8<sup>+</sup> T lymphocyte and NK cell responses have been extensively investigated (6, 39), little is known about the ability of RhCMV to evade antibody mediated immunity.

Here we demonstrate that the RhCMV *RL11* gene family member *Rh05* encodes an IgG-Fc binding glycoprotein. Similar to HCMV vFcγRs, this type 1 transmembrane protein is transported to the cell surface where it efficiently antagonizes FcγR activation triggered by immune IgG. In addition, Rh05 was able to antagonize human FcγRIIIa/CD16a activation by cells opsonized with a rhesusized monoclonal IgG antibody. Interestingly, Rh05 was not required for RhCMV super-infection, suggesting that evasion of pre-existing antibodies is not essential for the establishment of secondary infections. These results thus represent the first identification of a vFcγR in RhCMV and highlight the close evolutionary relationship of human and rhesus IgG and FcγRs consistent with the RM/RhCMV model being particularly relevant when studying viral evasion of IgG effector functions *in vivo*.

## Materials and Methods

145

146 **Cells.** All cells were cultured in a 5% CO<sub>2</sub> atmosphere at 37°C. Telomerized rhesus  
147 fibroblasts (TRF), HEK293T cells and Hela cells were maintained in Dulbecco's modified  
148 Eagle's medium (DMEM, Gibco) supplemented with 10% (vol/vol) fetal calf serum (FCS,  
149 Biochrom) and antibiotics (1x Pen/Strep, Gibco). TRF were generated from rhesus fibroblasts  
150 (RF) obtained from animals housed at Oregon National Primate Research Center (ONPRC)  
151 and life-extended as described previously (40). BW5147 mouse thymoma cells (BW, obtained  
152 from ATCC: TIB-47) were maintained at 3x10<sup>5</sup> to 9x10<sup>5</sup> cells/ml in Roswell Park Memorial  
153 Institute medium (RPMI GlutaMAX, Gibco) supplemented with 10% (vol/vol) FCS,  
154 antibiotics, sodium pyruvate (1x, Gibco) and β-mercaptoethanol (0.1 mM, Gibco).

155

156 **Generation of purified Fab and Fc fragments from whole serum.** IgG was isolated from  
157 pre-existing serum samples of healthy, RhCMV-naïve RM at the Oregon National Primate  
158 Research Center (ONPRC). Fab and Fc fragments were generated using the Pierce™ Fab  
159 Preparation Kit (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's  
160 instructions. Protein concentrations of the purified samples were determined using a  
161 NanoDrop® ND-1000 (Thermo Fisher Scientific, Waltham, MA, USA) and equal amounts of  
162 protein for each sample were separated on an SDS polyacrylamide gel. To visualize the  
163 purified fragments, the gel was fixed with methanol and silver stained using the  
164 SilverQuest™ Silver Staining Kit (Thermo Fisher Scientific, Waltham, MA, USA).

165

166 **Metabolic labelling of cells.** TRFs were grown in 60mm tissue culture dishes (1.5x10<sup>6</sup> cells  
167 per dish) and removed using a cell-scraper. Cells from two dishes were pooled and transferred  
168 into a 50ml conical tube. The cells were washed twice with PBS and incubated for 1 hour in  
169 starvation mix (DMEM complete without cysteine or methionine). Afterwards, the cells were  
170 pelleted and re-suspended in 1ml starvation mix, transferred into a 1.5ml Safe-Lock



171 Eppendorf centrifugation tube and 300 $\mu$ Ci of  $^{35}$ S were added per sample. The cells were  
172 rocked for 30 minutes at 37°C, pelleted and washed once with PBS. Finally the cells were  
173 lysed with NP40 lysis buffer containing protease inhibitors for 45 minutes at 4°C. Cell debris  
174 was removed by centrifugation at 16.100 x g for 20 minutes. The lysates were stored at -80°C.

175

176 **Immunoprecipitation of purified Fab, Fc and IgG from metabolically labeled cells.** Cell  
177 lysates were pre-cleared by adding protein A/G agarose beads, incubated for 1 hour at 4°C  
178 followed by pelleting the beads by centrifugation. The supernatant was transferred to a new  
179 tube, incubated again with protein A/G agarose beads at 4°C overnight followed by  
180 centrifugation. The pre-cleared lysates were transferred into a new Eppendorf tube and  
181 incubated with 10  $\mu$ g of either purified Fab, purified Fc or whole IgG with the addition of  
182 protease inhibitors overnight at 4°C. Protein A/G agarose beads were added to the mixture  
183 and the lysates were incubated for 1h while rocking at 4°C. The beads were pelleted, the  
184 supernatant was discarded and the beads were washed four times with NET buffer (50mM  
185 Tris pH 7.5, 5mM EDTA, 150mM NaCl, 0.5% NP-40) before resuspension in EndoH buffer.  
186 The samples were boiled for 10 minutes and split in equal parts with Endoglycosidase H  
187 being added to one part. All samples were incubated at 37°C overnight. 2x Laemmli  
188 Sample Buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromphenol blue,  
189 0.125M Tris HCl, pH 6.8) was added and the samples were boiled for 5 minutes and frozen at  
190 -80 °C.

191

192 **Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).** 10% SDS  
193 PAGE gels were generated using standard methods. Half of the immunoprecipitate described  
194 above was loaded onto the gel and electrophoresis was performed for 90 minutes at 100V.  
195 Gels were fixed and dried onto Whatman papers using a Slab Gel Dryer Model SGD5040  
196 (Savant). The dried gel was exposed to autoradiography film at -80°C for at least one week.

197 The film was developed using an SRX-101A film processor (Konica Minolta,  
198 Marunouchi, Chiyoda, Tokyo, Japan).

199

200 **Viruses and construction of recombinant mutants.** The primary RhCMV isolate UCD59  
201 was kindly provided by Dr. Peter Barry (UC Davis, CA) and has been isolated from RM at the  
202 CNPRC (41). The primary RhCMV isolates 19269 and 24514, as well as the CyCMV isolate  
203 31908, were isolated from animals at the ONPRC as described (42, 43). 68-1 RhCMV/gag  
204 and 68-1.2 RhCMV/gag were also previously described (44, 45). In both constructs, an  
205 expression cassette for the Simian Immune Deficiency Virus (SIV) *Gag* gene was inserted  
206 into the *Rh211* gene. The  $\Delta$ Rh14-Rh29 deletion mutant was generated on the basis of 68-1  
207 RhCMV/gag by homologous Red-mediated recombination (46) using primers with 50bp  
208 homology flanking the desired deletion. In the  $\Delta$ Rh01-Rh13.1 construct, SIVgag replaced the  
209 gene *Rh01* thus using the endogenous Rh01 promoter for SIVgag expression. Downstream of  
210 SIVgag an aminoglycoside 3'-phosphotransferase (KanR) cassette flanked by FRT sides was  
211 inserted which permits selection of recombinant clones and subsequent excision of the  
212 selection marker using a heat shock inducible flippase (FLP) (47). The constructs were  
213 analyzed by restriction digest with XmaI and Sanger sequencing across the introduced  
214 deletion. Recombinant viruses were reconstituted by electroporation of the BAC DNA into  
215 primary RF. Viral cultures were expanded to generate purified viral stocks for experiments.  
216 To generate single ORF deletions in RhCMV we utilized the *en passant* method that allows  
217 for "scarless" homologous recombination (48). Recombination primers with 100bp overhangs  
218 were designed so that the first 100bp of the sense-primer and the first 50bp of the antisense-  
219 primer at the 5'terminal end corresponded to DNA sequences either directly upstream or  
220 downstream of the intended deletion. The 50bp directly upstream of the intended deletion in  
221 the sense-primer were repeated in the antisense-primer to create a homologous sequence in  
222 the intermediate BAC construct. As a template to create the insertion cassette for homologous

223 recombination, we used a plasmid containing the aminoglycoside 3'-phosphotransferase  
224 (KanR) selectable marker with an upstream I-SceI unique restriction site. The primer binding  
225 sites for the recombination primers were designed to bind the 5'-end of the I-SceI restriction  
226 site and the 3'-end of the KanR selection marker. The KanR cassette was removed by  
227 arabinose induced expression of the I-SceI restriction enzyme in *E.coli* strain GS1783 and by  
228 simultaneous induction of the Red recombination genes by heat shock, leading to the  
229 homologous recombination of the introduced repeated 50bp sequences and the "scarless"  
230 removal of the targeted ORF. Deletion of the ORF was confirmed by restriction digest with  
231 XmaI and by Sanger sequencing across the deletion. Recombinant viruses were reconstituted  
232 and analyzed as described above.

233

234 **Analysis of RhCMVΔRh05 growth kinetics by multi-step growth curve.** Primary rhesus  
235 fibroblast were seeded out in 24 well plates ( $5 \times 10^4$  cells per well) and were infected with  
236 either RhCMV 68-1 or RhCMV 68-1 ΔRh05 at an MOI of 0.01. Supernatants from two wells  
237 per sample and time point were harvested every 3<sup>rd</sup> day starting at day 3 and the supernatants  
238 were cleared by centrifugation at  $16.100 \times g$  for 5 minutes before storing them at  $-80^\circ\text{C}$ . Viral  
239 titers of each sample were determined by 50% tissue culture infective dose (TCID<sub>50</sub>) assays  
240 on primary rhesus fibroblasts and the growth curves were graphed using the arithmetic mean  
241 of the two biological repeats per sample.

242

243 **Molecular cloning, transient transfection and lentiviral transduction.** Rh05 and rhesus-  
244 CD4 (ACC# D63347) were synthesized as gBlock fragments flanked by *NheI* and *BamHI*  
245 restriction sites (Integrated DNA Technologies, IDT) and cloned into the pIRES\_eGFP  
246 expression vector upstream of an internal ribosomal entry site (IRES) and the gene for green  
247 fluorescent protein (GFP). Transient expression of recombinant protein was achieved by  
248 transfection of Hela cells using Superfect transfection reagent (Qiagen). BW-reporter cells

249 stably expressing chimeric *Macaca mulatta* Fcγ-receptor-CD3ζ receptors were generated by  
250 lentiviral transduction using HEK293T cells as a packaging cell line. Fcγ-receptor-CD3ζ  
251 chimeric receptors were designed by fusion of the extracellular domain of the respective  
252 rhesus-Fcγ receptors (RhCD16: ACC# XP\_014968661; RhCD32a: ACC# XP\_014968622;  
253 RhCD32b: ACC# XP\_014968682; RhCD64: ACC# NP\_001244233) with the mouse CD3  
254 signaling module as described (49). The Rh-Fcγ receptors were synthesized as gBlock  
255 fragments flanked by *NheI* and *BamHI* restriction sites (IDT). gBlocks were then cloned into  
256 the puc2CL6IPwo lentiviral vector using the above mentioned restriction sites. For every  
257 construct one 10 cm dish of packaging cell line at roughly 70% density was transfected with  
258 the target construct and 2 supplementing vectors providing the VSV gagpol and VSV-G-env  
259 proteins (6μg of DNA each) using Polyethylenimine (22.5μg/ml) and Polybrene (4μg/ml,  
260 Merck Millipore) in a total volume of 7 ml (2 ml of 15 min pre-incubated transfection mix in  
261 serum-free DMEM added to 5 ml of fresh full DMEM. After a medium change, virus  
262 supernatant harvested from the packaging cell line two days after transfection was then  
263 incubated with target BW cells overnight (3.5ml supernatant on  $1 \times 10^6$  target cells) followed  
264 by expansion and pool selection using 2μg/ml of Puromycin.

265  
266 **Flow cytometry.**  $1 \times 10^6$  BW cells were washed in PBS, equilibrated in staining buffer (PBS,  
267 3% FCS) and sedimented at 1000g and 10°C for 3 minutes. Cells were resuspended in 100μl  
268 of either primary antibody solution followed by conjugate antibody solution or conjugate  
269 antibody solution alone (1/100 in staining buffer). Every incubation step was carried out at  
270 4°C for 1h and followed by 3 washing steps in staining buffer. Dead cells were stained using  
271 DAPI. After the final wash, cells were resuspended in 400μl staining buffer and analyzed on a  
272 FACS Fortessa instrument (BD Bioscience). Human IgG-Fc-TexasRed (Rockland) and anti-  
273 human-IgG-FITC (Miltenyi Biotec) were used as conjugates. PE-conjugation was performed  
274 using an ab102918 labelling kit by abcam as suggested by the supplier.

275

276 **Fcγ-receptor activation assay.** The assay was performed as described earlier (49). Briefly, in  
277 a standard assay, target cells were incubated with dilutions of *Macaca mulatta* sera (RhCMV-  
278 infected TRF) or mAbs (transfected Hela) in DMEM supplemented with 10% (vol/vol) FCS  
279 for 30min at 37°C. Cells were washed before co-cultivation with BW-reporter cells (ratio E:T  
280 20:1) for 16h at 37°C in a 5% CO<sub>2</sub> atmosphere. Cross-link activation of reporter cells was  
281 performed by direct coating of target antibody to an ELISA-plate (Nunc Maxisorp 96 well,  
282 flat transparent) followed by a blocking step and incubation with 2x10<sup>5</sup> reporter cells per well.  
283 For all activation assays, mouse IL-2 secretion was quantified by anti-IL-2 ELISA as  
284 described earlier (49). RhCMV-seropositive rhesus macaque serum was provided by the  
285 German Primate Center Göttingen from pre-existing samples.

286

287 **Statistical analysis.** Statistical analysis was performed using a two-way analysis of variance  
288 (ANOVA) together with Tukey's range. Analyses were performed using the Prism 6 software  
289 (GraphPad).

290

291 **Rhesus macaques.** Adult *Macaca mulatta* were used at the Oregon National Primate  
292 Research Center (ONPRC) which is accredited by the Association for Assessment and  
293 Accreditation of Laboratory Animal Care. The experiments were conducted in compliance  
294 with the Animal Welfare Act in accordance with the "Guide for the Care and Use of  
295 Laboratory Animals," Institute of Laboratory Animals Resources, National Research Council  
296 and approved by the Institutional Animal Care and Use Committees (IACUC) that adhere to  
297 national guidelines established in the Animal Welfare Act (7 U.S.C. Sections 2131–2159) and  
298 the Guide for the Care and Use of Laboratory Animals (8th Edition) as mandated by the U.S.  
299 Public Health Service Policy.

300 Three purpose-bred, pedigreed, male RM were used. At assignment, these RM were positive  
301 for RhCMV but free of Macacine herpesvirus 1, D-type simian retrovirus, simian T-  
302 lymphotropic virus type 1, simian immunodeficiency virus, and TB. The RM were sedated  
303 with ketamine HCl or Telazol® for subcutaneous administration of  $5 \times 10^6$  PFU of either 68-1  
304 RhCMV/gag, RhCMV $\Delta$ Rh01-13.1/gag or 68-1.2 RhCMVgag, respectively, on day 0.

305

306 **T cell assays.** SIVgag-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses were measured bi-weekly in  
307 PBMC by intracellular cytokine staining (ICS) (44, 45, 50, 51). Briefly, PBMC were  
308 incubated with consecutive 15mer peptide mixes (11 amino acid overlap) comprising SIVgag  
309 and the co-stimulatory molecules CD28 and CD49d (BD Biosciences) for 1h, followed by  
310 addition of Brefeldin A (Sigma-Aldrich) for an additional 8hrs. Co-stimulation without  
311 peptides served as background control. Alternatively, the MHC-E-restricted SIVgag supertope  
312 peptides (Gag69<sub>276-284</sub> RMYNPTNIL and Gag120<sub>482-490</sub> EKQRESREK) or MHC-II-restricted  
313 supertope peptides (Gag53<sub>211-222</sub> AADWDLQHPQP and Gag73<sub>290-301</sub> PKEPFQSYVDRF)  
314 were used in this assay.

315 Stimulated cells were fixed, permeabilized and stained (44, 45, 50, 51) using combinations of  
316 the following fluorochrome-conjugated mAbs: SP34-2 (CD3; Pacific Blue, Alexa700), L200  
317 (CD4; AmCyan, BV510), SK-1 (CD8 $\alpha$ ; PerCP-Cy5.5), MAB11 (TNF $\alpha$ ; FITC, PE), B27  
318 (IFN $\gamma$ ; APC), FN50 (CD69; PE, PE-TexasRed), B56 (Ki-67; FITC), and in polycytokine  
319 analyses, JES6-5H4 (IL2; PE, PE Cy-7). Data was collected on an LSR-II (BD Biosciences).  
320 Analysis was performed using FlowJo software (Tree Star). Lymphocytes were gated for  
321 CD3<sup>+</sup> and progressive gating on CD4<sup>+</sup> and CD8<sup>+</sup> T cell subsets. Antigen-responding cells in  
322 both CD4<sup>+</sup> and CD8<sup>+</sup> T cell populations were determined by their intracellular expression of  
323 CD69 and one or more cytokines. After subtracting background, the raw response frequencies  
324 were memory corrected (44, 45, 50, 51) using combinations of the following mAbs to define  
325 the memory vs. naïve subsets: SP34-2 (CD3; Alexa700, PerCP-Cy5.5), L200 (CD4;

326 AmCyan), SK-1 (CD8 $\alpha$ ; APC, PerCP-cy-5.5), MAB11 (TNF $\alpha$ ; FITC), B27 (IFN $\gamma$ ; APC),  
327 FN50 (CD69; PE), CD28.2 (CD28; PE-TexasRed), DX2 (CD95; PE), 15053 (CCR7; Pacific  
328 Blue), and B56 (Ki-67; FITC).  
329

## 330 Results

331

### 332 RhCMV glycoprotein binding to IgG

333 To determine whether RhCMV encodes viral proteins binding to IgG, purified rhesus IgG  
334 from RhCMV-seronegative RM was incubated with detergent lysates of [35S]methionine-  
335 labeled, RhCMV-infected telomerized rhesus fibroblasts (TRF). For control, we used Fab-  
336 fragments generated from rhesus IgG. In addition to the fibroblast-adapted laboratory strain  
337 68-1 which carries a number of gene deletions (36), we also used the primary RhCMV isolate  
338 UCD59 (42) and the recently characterized RhCMV isolates 19269 and 24514 as well as the  
339 cynomolgus CMV (CyCMV) isolate 31908 (43). Bound proteins were eluted from the  
340 protein A/G agarose beads and, where indicated, digested with Endoglycosidase H (EndoH)  
341 to monitor glycan processing during intracellular transport, followed by separation using  
342 SDS-PAGE. As shown in **Fig. 1**, RhCMV and CyCMV -infected, but not uninfected cell  
343 lysates, contained a single protein species of ~60kDa bound to IgG. This protein was  
344 observed in 68-1-infected cell lysates as well as in lysates from cells infected with primary  
345 NHP CMV isolates. Upon EndoH treatment the molecular weight of the protein was reduced  
346 to ~30kDa suggesting that the protein is highly glycosylated. Both EndoH-sensitive and  
347 EndoH-resistant bands were observed consistent with newly synthesized, EndoH-sensitive  
348 protein sub-populations in the endoplasmic reticulum (ER) that eventually egress to the cell  
349 surface.

350

### 351 *Rh05* encodes a viral FcγR

352 HCMV encodes four vFcγRs: *RL11* (gp34), *RL12*, *RL13* and *UL119/118* (gp68). *RL11*, *RL12*  
353 and *RL13* belong to the *RL11* gene family, encoding for a highly polymorphic glycoprotein  
354 family which is also found in RhCMV (36). HCMV gp68 is conserved in RhCMV, including  
355 the spliced gene structure, with the putative homologue encoded by *Rh152/151* (35).



356 However, the gp68 homologue is truncated in RhCMV 68-1 (36) rendering it possibly non-  
357 functional. Moreover, the molecular weight of the putative viral Fc receptor was considerably  
358 less than predicted for the gp68 homologue of RhCMV. Therefore, we hypothesized that the  
359 viral IgG-binding protein was likely a member of the *RL11* family. In RhCMV, the *RL11*  
360 family is encoded in the 5' end upstream of the open reading frame (ORF) *Rh29* (**Fig. 2A**). To  
361 determine whether the putative vFcγR is encoded in this gene region we generated two  
362 deletion mutants lacking *Rh01-Rh13.1* and *Rh14-Rh29* in RhCMV 68-1 by BAC  
363 recombineering (**Fig. 2A**). Replacement of the desired genomic regions by a FRT-flanked  
364 KanR cassette was confirmed by restriction digest. Upon electroporation of the BACs, virus  
365 was easily recovered, consistent with genes encoded in this genomic region being non-  
366 essential for growth *in vitro* as reported for RhCMV (52) and HCMV (53). To determine  
367 whether ΔRh01-13.1 and ΔRh14-29 contained or lacked the putative IgG binding protein we  
368 metabolically labeled infected RF as above and incubated detergent cell lysates with complete  
369 IgG, Fab-fragments, or Fc-fragments bound to Protein A/G agarose beads or control beads.  
370 Upon electrophoretic separation we observed that lysates of ΔRh14-29-infected cells  
371 contained the ~60kDa (or 30kDa upon deglycosylation) protein that was immunoprecipitated  
372 with both IgG and Fc, but not with F(ab)2 or beads alone (**Fig. 2B**). In contrast, the 60kDa  
373 protein was not observed in ΔRh01-13.1-infected cell lysates (**Fig. 2C**) consistent with the  
374 putative vFcγR being encoded in the 5'-terminal region of the genome.

375 To determine which gene(s) in the *Rh01-Rh13.1* region encoded the putative vFcγR we  
376 deleted individual genes in this region from the 68-1 BAC (**Fig. 3A**). Upon reconstitution of  
377 the single deletion constructs we evaluated IgG binding upon infection of RF. As shown in  
378 **Fig. 3B**, IgG was able to immunoprecipitate the putative vFcγR from all deletions mutants  
379 except ΔRh05. To ensure that lack of binding was not due to lack of infection and or gene  
380 expression, we also confirmed that ΔRh05 was not essential for infection and growth *in vitro*

381 (Fig. 3C). These results suggest that the *Rh01-Rh13.1* gene region contains a single vFcγR  
382 encoded by *Rh05*.

383 The gene *Rh05* encodes for an *RL11* family protein of 273 amino-acids (AA) with a predicted  
384 molecular weight of 30.19 kDa. The Rh05 protein displays a type I transmembrane topology  
385 with a predicted cleavable amino-terminal signal peptide (AA1-21), a predicted  
386 transmembrane domain (AA181-207) and a 65AA long cytoplasmic domain (Fig. 4).  
387 Homologous proteins are found in old-world NHP CMVs (Fig. 4). In contrast, none of the  
388 RL11-family proteins of human, great ape, or new world NHP CMV seem be direct homologs  
389 of Rh05. The ectodomain is predicted to belong to the immunoglobulin superfamily and  
390 contains nine putative N-linked glycosylation sites, several of which being highly conserved,  
391 consistent with the protein being highly glycosylated. Also conserved is the C-terminal AA  
392 sequence PATLWL[T/S][K/R] which might represent a subcellular sorting signal. The  
393 predicted characteristics of this protein are thus consistent with the observed MW and  
394 glycosylation pattern of the Fcγ-binding viral protein.

395  
396 **Recombinant Rh05 is an IgG-Fc binding cell surface protein which antagonizes human**  
397 **FcγRIIIA/CD16 activation**

398 To examine whether Rh05 has the capacity to counteract host Fcγ-receptor activation, as  
399 reported for the IgG-Fc binding HCMV proteins *RL11/gp34* and *UL119-118/gp68* (16), we  
400 introduced recombinant Rh05 into an established human Fcγ-receptor activation assay (49).  
401 As a target surface antigen we chose rhesus-CD4 (RhCD4) that can be detected with a  
402 recombinant rhesusized IgG1 monoclonal antibody (αRhCD4 mAb). To this end, we co-  
403 transfected Hela cells with RhCD4 (pCDNA3.1 vector) and a polycistronic pIRES\_eGFP  
404 vector encoding either recombinant HCMV gp68, RhCMV Rh05 or CD99 control protein  
405 together with GFP as an expression marker which allowed us to monitor transfection  
406 efficiency (Fig. 5A). As a first step, we wanted to determine whether Rh05 alone would be

407 sufficient to bind to the Fc portion of IgG on the cell surface. By staining the vFcγR and  
408 RhCD4 co-transfected Hela cells with a TexasRed-conjugated human IgG-Fc fragment and  
409 gating on the above mentioned GFP-positive population we observed that Rh05 is a potent  
410 IgG-Fc binding protein compared to HCMV gp68 which served as a positive control (**Fig. 5B,**  
411 **left**). A human IgG-Fc fragment was used as previous observations already showed high  
412 cross-reactivity between human and nonhuman primate IgG-Fc (28, 29). In these experiments,  
413 HCMV gp68 was expressed as a fusion protein to the transmembrane domain and cytosolic  
414 tail of human CD4 since this fusion protein reaches higher densities on the plasma membrane  
415 upon transient expression than wildtype gp68 (Kolb and Hengel, unpublished observation).  
416 Surface expression of co-transfected RhCD4 and binding of αRhCD4 to its antigen in co-  
417 transfected Hela cells was demonstrated by detection of RhCD4 using PE-conjugated  
418 αRhCD4 (**Fig. 5B, right**). Gating on GFP-positive cells allowed us to conclude that cells  
419 expressing Rh05, gp68 or CD99 uniformly expressed the target antigen RhCD4 and that  
420 surface levels of RhCD4 are not affected by co-transfected genes of interest (**Fig. 5B, right**).  
421 To address the antagonistic potential of Rh05, the co-transfected cells were then co-cultured  
422 with a reporter cell line expressing the human FcγRIIIA/CD16 ectodomain fused to the CD3-  
423 ζ-chain signaling module (BW5147-human-CD16-ζ) after adding graded amounts of  
424 αRhCD4. Reporter cell activation was quantified by measuring IL-2 production using a  
425 sandwich ELISA as described previously (49). As shown in **Fig. 5C**, compared to the  
426 expression of a non-Fcγ-binding control molecule (CD99) we observed a significant and  
427 antibody dose-dependent reduction of CD16-reporter cell activation by target cells expressing  
428 Rh05 that exceeded the inhibition mediated by gp68. Control BW cells lacking the CD16  
429 FcγR (parental cells) were not activated. Taken together, these data demonstrate that Rh05  
430 represents an IgG-Fc binding glycoprotein with the potential to antagonize the activation of  
431 host FcγRs.

432

433 **Rh05 protects RhCMV-infected cells from Fcγ-receptor activation by opsonizing IgG**

434 The potent inhibition of human CD16 activation by Rh05 supported our hypothesis that this  
435 vFcγR might protect infected cells from *Macaca mulatta* FcγR-dependent effector  
436 mechanisms. To this end we generated BW reporter cells encoding chimeric rhesus (Rh)  
437 CD16, RhCD32A, RhCD32B or RhCD64 consisting of the extracellular FcγR domain fused  
438 to the transmembrane and intracellular domains of the mouse CD3ζ chain. FcγR-activation  
439 can thus be monitored by production of interleukin-2 (IL-2). Surface expression and intact  
440 ligand binding of these chimeric Rh-FcγRs was demonstrated by flow cytometry using a  
441 TexasRed conjugated human IgG-Fc fragment (**Fig. 6A, left**). Next, the ability of these  
442 reporter cell lines to generate IL-2 upon FcγR activation was verified by receptor cross-  
443 linking by immobilized IgG of human and rhesus origin. All reporter cell lines responded to  
444 human IgG1 mAb Rituximab® or αRhCD4 (**Fig. 6A, middle**). Of note, BW-RhCD16ζ  
445 yielded lower signals compared to the other cell lines including BW cells expressing human-  
446 CD16ζ. This could be due to the fact that IgG from individual sources can have highly  
447 varying affinities to certain isoforms of Rh-FcγRs (29). Interestingly, the dose-response of  
448 BW-Rh64ζ cells in this context did not reach an activation plateau that was maintained at high  
449 antibody concentrations, but displayed a maximum response at lower antibody concentrations  
450 (**Fig. 6A, right**). In contrast, all other reporter cell lines (including reporter cells expressing  
451 hCD64) showed the typical sigmoidal dose-response with plateau activation to the  
452 immobilized antibodies above a given antibody concentration (data not shown). While we  
453 cannot fully explain this observation, it is possible that RhCD64 reaches suboptimal activation  
454 with high amounts of immobilized IgG due to its intrinsic molecular characteristics as a high-  
455 affinity FcγR which bind to but are not activated by monomeric IgG (29, 30).

456 With these reporter cell lines in hand, we then set out to assess the effect of Rh05 on Rh-FcγR  
457 activation. To this end, TRF infected with RhCMV 68-1 or RhCMVΔRh05 were incubated  
458 with polyclonal immune serum from RhCMV-positive or -negative animals and then co-

459 cultured with the respective reporter cell lines. As expected, surface antigen levels were  
460 similar between cells infected with either RhCMV 68-1 or RhCMV $\Delta$ Rh05, as demonstrated  
461 by flow cytometry detecting the bound anti-RhCMV serum via a FITC-conjugated polyclonal  
462 anti-human antibody (**Fig. 6B, left**). In contrast, IgG-Fc binding was only observed for TRF  
463 infected with RhCMV 68-1, but not RhCMV $\Delta$ Rh05 consistent with a complete loss of Fc-  
464 binding activity upon deletion of Rh05 (**Fig. 6B, right**). Applying the Fc $\gamma$ R reporter assay,  
465 serum from the RhCMV-seropositive animal elicited the typical dose-dependent response in  
466 the reporter cell lines, except for RhCD64 which again showed maximal stimulation at lower  
467 serum concentrations (**Fig. 6C**). Serum from the RhCMV-negative animal did not induce IL-2  
468 in any of the reporter cells (**Fig. 6C**). Importantly, compared to cells infected with RhCMV  
469 68-1, cells infected with RhCMV $\Delta$ Rh05 induced significantly higher reporter cell activation  
470 for all examined activating Rh-Fc $\gamma$ Rs at dilutions of RhCMV-immune serum that elicited  
471 maximal stimulation (**Fig. 6C**). Although there was a similar tendency for the inhibitory  
472 RhCD32B receptor, the differences between the RhCMV $\Delta$ Rh05 and 68-1 RhCMV did not  
473 reach statistical significance. Based on these results we conclude that Rh05 limits the ability  
474 of IgG antibodies bound to infected cells to activate host Fc $\gamma$ Rs thus counteracting  
475 opsonization and subsequent Fc $\gamma$ R mediated immune responses.

#### 477 **Re-infection by RL11-family-deleted RhCMV**

478 A unique aspect of both RhCMV and HCMV is their ability to establish secondary persistent  
479 infections in CMV-immune hosts. We previously demonstrated that viral evasion of CD8<sup>+</sup> T  
480 cells by US6-family viral inhibitors of MHC-I antigen presentation is necessary for RhCMV  
481 to re-infect RhCMV-seropositive animals (6). Furthermore, preventing the activation of NK  
482 cells by inhibiting the cell surface expression of ligands for activating NK-cell receptors  
483 proved to be essential for RhCMV infection in both RhCMV seropositive and seronegative  
484 hosts (39). Therefore, we were wondering whether the vFc $\gamma$ R Rh05 would be required for

485 RhCMV to overcome pre-existing humoral immunity. T cell responses to heterologous  
486 antigens expressed by RhCMV can be used as a surrogate measure for the ability of RhCMV  
487 to re-infect seropositive animals (6). Thus, we took advantage of the SIVgag gene inserted  
488 during the construction of  $\Delta$ Rh01-13.1 (see material and methods).  $5 \times 10^6$  PFU of  $\Delta$ Rh01-13.1  
489 was inoculated sub-cutaneously and the T cell response to SIVgag was measured biweekly in  
490 PBMC by intracellular cytokine staining (ICS) using overlapping peptides spanning the  
491 SIVgag sequence. As shown in **Fig. 7A**,  $\Delta$ Rh01-13.1 elicited robust SIVgag-specific  
492 responses for both CD4<sup>+</sup> and CD8<sup>+</sup> T-cells that were comparable to inoculation of 68-1  
493 RhCMV/gag into a different animal. While these results were only obtained in one animal,  
494 they clearly demonstrate that the gene region containing Rh05 is not essential for infection  
495 and re-infection.

496 We recently reported that recombinant viruses based on strain 68-1, but not the pentamer-  
497 intact derivative RhCMV 68-1.2, elicit CD8<sup>+</sup> T cells that recognize peptides exclusively in  
498 the context of MHC class II or the non-polymorphic MHC-E molecule instead of polymorphic  
499 MHC-Ia (44, 51). Moreover, some MHC-II and MHC-E-restricted SIVgag peptide epitopes,  
500 termed “supertopes”, are consistently recognized in every animal tested so far (>100 animals).  
501 To determine whether genes encoded in the Rh01-13 region affected this T cell programming  
502 we measured the CD8<sup>+</sup> T cell responses to two MHC-II and two MHC-E supertopes. Similar  
503 to total SIVgag responses, we observed that both 68-1 RhCMV/gag and  $\Delta$ Rh01-13.1 elicited  
504 supertope-specific CD8<sup>+</sup> T cells in contrast to 68-1.2 RhCMV/gag that failed to elicit CD8<sup>+</sup>  
505 T cells to these supertopes (**Fig. 7B**). These results suggest that deletion of Rh05, or any of  
506 the other genes encoded in the 5-terminal region of RhCMV, does not impact the ability of  
507 RhCMV 68-1 to elicit CD8<sup>+</sup> T cells to unconventional epitopes.

508 **Discussion**

509 Our results demonstrate that RhCMV Rh05 encodes an IgG-Fc binding glycoprotein that  
510 immobilizes antibodies at the cell surface. Using a cell-based assay to measure rhesus IgG  
511 mediated activation of rhesus FcγRs, we further show that Rh05 expressed on the surface of  
512 infected cells is a potent antagonist of host FcγR activation by anti-CMV antibodies. Based on  
513 these results we conclude that Rh05 is a vFcγR that counteracts the ability of CMV-specific  
514 antibodies to trigger activating host FcγRs thus supporting viral immune evasion.

515 Rh05 is the first vFcγR identified in RhCMV. Although Rh05 does not show direct homology  
516 to any of the previously identified vFcγRs in HCMV, the protein belongs to the same *RL11*  
517 gene family as three of the four HCMV vFcγRs: *RL11* (gp34), *RL12*, and *RL13* (16, 54).  
518 Similar to gp34 which is able to block all of the activating human FγRs - FcγRI (CD64),  
519 FcγRIIa (CD32a) and FcγRIIIa (CD16) – we observed that Rh05 reduced the activation of  
520 homologous rhesus FcγRs. The diverse RL11 glycoprotein family is characterized by the ~ 80  
521 AA RL11 domain containing a conserved tryptophan and two cysteine residues (55, 56). In  
522 addition to encoding vFcγRs, members of this gene family have been involved in various  
523 immunomodulatory functions (57-61) as well as viral modulation of angiogenesis, cell  
524 differentiation and reactivation (62, 63). Mutations in the RL13 glycoprotein are rapidly  
525 selected in both HCMV and NHP CMVs in tissue culture due to increased growth of RL13-  
526 defective variants (43, 64). Due to two frame-shift mutations, RhCMV strain 68-1 used in this  
527 study is also predicted to lack a functional RL13 homologue (Rh13.1) suggesting that the  
528 negative impact of this protein on viral growth *in vitro* is conserved (36). However, it is  
529 presently not known whether intact Rh13.1 also shares the ability to bind Fc with HCMV  
530 RL13. Similarly, it is not known whether the RhCMV homologue of HCMV *UL118/119*  
531 (gp68) is a functional vFcγR. However, given the significant homology including the spliced  
532 gene structure, this is highly likely. Interestingly, the *Rh151/152* gene encoding the gp68  
533 homolog is truncated and possibly non-functional in RhCMV 68-1 (36). Conceivably,



534 wildtype RhCMV could thus encode additional vFcγRs compared to RhCMV 68-1. However,  
535 we observed only a single viral protein band corresponding in size to Rh05  
536 immunoprecipitating with IgG in lysates from cells infected with low passage isolates of  
537 RhCMV and CyCMV (**Fig.1**). Thus, it is also conceivable that Rh05 is the only vFcγR in  
538 NHP CMVs. By studying the homologs of RL13 and gp68 in isolation we will be able to  
539 examine this possibility.

540 To determine the impact of vFcγR expression on host Fc receptor activation we introduced  
541 Rh-FcγRs into our previously developed FcγR activation assay (49). We showed that this  
542 assay delivered reproducible, quantifiable measurements of FcγR activation via immune IgG  
543 when applied to infected cells opsonized with polyclonal serum in the context of herpes  
544 simplex virus, HCMV and influenza virus (16, 31, 65). In a mouse influenza virus model,  
545 comparative FcγR assay results closely correlated with the protective capacity of antiviral  
546 IgGs *in vivo* (31). By generating Rh-FcγRs fused to mouse CD3ζ we were able to measure the  
547 antibody dose-dependent effect of FcγR-activation by antibody binding to RhCMV-infected  
548 cells. In doing so, we uncovered an unexpected IgG concentration-dependent optimum of  
549 rhesus CD64/FcγRI activation (**Fig. 6A,C**). In contrast, human FcγRI activation plateaued at  
550 high concentrations in this assay system (16). The finding that higher antibody concentrations  
551 result in lower FcγR activation could potentially reflect a unique feature, possibly a specific  
552 isoform, of the high affinity rhesus FcγRI.

553 It is thus possible that the rhesus FcγRI receptors are functionally different from human FcγRI  
554 receptors. However, the homology between RM and human FcγRs is approximately 95%,  
555 87% and 91% for FcγRI, RII and RIII, respectively (29). Some polymorphisms are observed  
556 in RM, particularly for FcγRIIA, some of which resulting in impaired antibody binding (29).  
557 However, the allotypic variants in this study (FcγRI-3, FcγRIIA-1, FcγRIIB-1, FcγRIIA-1)  
558 were previously shown to be fully functional but differed with respect to IgG subclass



559 specificity (29). Importantly, Rh05 was able to interfere with the activation of each activating  
560 RM FcγR by polyclonal RM serum, suggesting that Rh05 broadly binds IgG subclasses.

561 Unlike RhCMV lacking the gene region Rh182-189, encoding proteins that prevent MHC-I  
562 antigen presentation, or RhCMV lacking NKG2D-ligand-retaining Rh159, deletion of the  
563 gene region encompassing Rh05 did not affect the ability of RhCMV to overcome pre-  
564 existing immunity and establish a secondary infection. If Rh05 is indeed the only vFcγR  
565 encoded by RhCMV, this result would indicate that evasion of antibodies is not essential for  
566 super-infection. Alternatively, Rh05 is not the only vFcγRs and other, yet to be identified,  
567 vFcγRs support reinfection. In either case however, these results do not rule out that Rh05  
568 supports viral replication, dissemination and/or shedding. For instance, although strain 68-1  
569 RhCMV is clearly able to establish secondary persistent infections in RhCMV-seropositive  
570 RM, this highly passaged strain is clearly attenuated compared to low-passage isolates such as  
571 UCD59 resulting in decreased plasma viral titers and decreased shedding during acute  
572 infection (41). A more detailed study requiring a larger cohort size will thus be required to  
573 quantify the impact of Rh05 on RhCMV infection.

574 It will also be interesting to study the impact of Rh05 deletion, alone or together with  
575 additional putative vFcγRs discussed above, in settings of passive immunization with anti-  
576 RhCMV antibodies. The importance of IgG-Fc interaction with host FcγRs for protection by  
577 passive immunization against viruses has been illustrated in animal models of influenza and  
578 HIV (32, 33, 66). In the case of HIV, it has further been shown that viral Ab escape mutants  
579 arise in an Fc-dependent manner (33). However, large DNA viruses like CMV likely contain  
580 multiple epitopes targeted by antibodies, which renders it difficult for the virus to escape  
581 immune pressure by mutation. Conceivably, vFcγRs evolved to enable antibody escape by  
582 CMVs regardless of the epitope targeted thus limiting the ability of both neutralizing and non-  
583 neutralizing antibodies to prevent viral spread *in vivo*. This immune evasion mechanism might  
584 therefore limit the efficacy of passively administered immunoglobulins to prevent congenital

585 infection by CMV (9). The identification of a vFcγR in a highly relevant animal model of  
586 HCMV will help to develop a better understanding of the role of vFcγRs in counteracting  
587 immune responses elicited by vaccines and immunotherapies which might be improved by  
588 reagents that block vFcγR function.

589

590

591

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602

603

604 **Figure legends**

605 **Figure 1: RhCMV encodes an IgG binding protein**

606 To detect IgG binding proteins, lysates from metabolically labelled TRFs were incubated with  
607 serum from RhCMV-naïve RM and total IgG was immunoprecipitated using Protein A/G  
608 Agarose. Endoglycosidase H (EndoH) was added where indicated. A) Uninfected cell lysate.  
609 B) TRFs were infected with RhCMV 68-1 (MOI = 3) for 72 hrs prior to metabolic labelling.  
610 Infected cell lysates were either untreated, incubated with purified Fab fragments or whole  
611 serum. Immunoprecipitates were separated by SDS-PAGE and protein bands visualized by  
612 autoradiography. C) TRFs were infected with RhCMV 68-1 or the low passage isolate  
613 UCD59 (MOI = 3) for 72 hrs prior to metabolic labelling and immunoprecipitation. D) TRFs  
614 were infected with RhCMV 68-1 or the indicated RhCMV and CyCMV low passage isolates  
615 (MOI = 3) for 72 hrs prior to metabolic labeling. IgG immunoprecipitations after incubation  
616 with CMV naïve RM serum were performed using Protein A/G Agarose. Arrows indicate a  
617 single EndoH sensitive glycoprotein species. \*indicates a non-specific protein.

618  
619 **Figure 2: The IgG-binding protein is encoded in the 5' end of the RhCMV genome**

620 A) Schematic overview of the 5'-end genomic region of RhCMV encompassing the *RL11*  
621 gene family. All *RL11* gene family members are highlighted in dark grey. Two deletion  
622 mutants,  $\Delta$ Rh01-Rh13.1 and  $\Delta$ Rh14- $\Delta$ Rh29, that together span the entire *RL11* gene family,  
623 were constructed. The exact region that was deleted in each mutant is indicated by the boxed  
624 area. B) and C) TRFs were infected with the indicated deletion mutants or with RhCMV 68-1  
625 WT control at an MOI of 3 for 72 hrs prior to metabolic labelling. Lysates were either mock  
626 incubated or incubated with purified Fab fragments, purified Fc fragments or whole serum.  
627 IgG was immunoprecipitated and treated with EndoH where indicated. Arrows indicate the  
628 glycosylated and de-glycosylated forms of the RhCMV encoded protein that co-precipitates  
629 with RM IgG or RM IgG Fc fragments from RhCMV 68-1 and from RhCMV $\Delta$ Rh14-29, but

630 not from RhCMV $\Delta$ Rh01-13.1. \*indicates a non-specific protein. All other unmarked proteins  
631 species are also non-specific.

632

633 **Figure 3: Rh05 encodes a viral Fc binding protein**

634 A) Schematic overview of the RhCMV deletion mutants constructed by BAC recombineering.

635 The entire viral ORF was deleted in each case as indicated by the boxes. B)

636 Immunoprecipitations of IgG of RhCMV-naïve serum incubated with lysates from TRFs

637 infected with the single deletion mutants (MOI-3) for 72 hrs. Half of every sample was

638 EndoH treated as indicated. Arrows indicate the glycosylated and non-glycosylated form of

639 the IgG binding protein. \*indicates a non-specific protein. C) Multistep growth curve of

640 RhCMV 68-1 and RhCMV $\Delta$ Rh05 on primary rhesus fibroblasts. The cells were infected with

641 an MOI of 0.01, samples were harvested every third day and viral titers were determined by

642 TCID<sub>50</sub>.

643

644 **Figure 4: RhCMV Rh05 is conserved in old world monkey CMV species**

645 An alignment of the predicted amino acid sequence of Rh05 with putative homologues of

646 Cynomolgus CMV 31908 (CyCMV), Simian CMV Colburn (SCMV), Baboon CMV

647 OCOM4-37 (BaCMV) and Drill monkey CMV OCOM6-2 (DrCMV) was generated using the

648 CLUSTAL O (1.2.4) multiple sequence alignment tool. Highlighted are the predicted signal

649 sequence (green, predicted using the SignalP 4.1 Server), transmembrane region (blue,

650 predicted using the Phobius Server) and potential glycosylation sites (red, using the NetNGlyc

651 1.0 Server). Additionally, amino acids that have been defined as conserved across the

652 RL11 family of proteins were circled in black.

653

654 **Figure 5: Rh05 binds IgG-Fc and antagonizes antibody-dependent Fc $\gamma$ R activation**

655 HeLa cells were co-transfected with the target antigen rhesus-CD4 (RhCD4; pcDNA3.1) and  
656 either of the indicated genes of interest (*CD99*, HCMV *UL119-118* and RhCMV *Rh05*;  
657 p\_IRES-eGFP). A) GFP positive cells, gated on live cells using DAPI, were plotted against  
658 side scatter. The GFP-positive population, indicated by a gate, demonstrates similar  
659 transfection rates for each of the genes of interest. B) Left Panel: GFP-positive cells from A  
660 were analyzed for Fc $\gamma$ -binding by flow cytometry using Texas Red conjugated human-Fc $\gamma$   
661 fragment. RhCMV Rh05 and HCMV gp68 bound to IgG-Fc whereas CD99 was negative.  
662 Right Panel: Surface expression levels of RhCD4 are not affected by co-expressed genes of  
663 interest. RhCD4 was detected in the GFP-positive population from A using a PE-conjugated  
664 rhesusized anti-RhCD4 mAb. C) Rh05 antagonizes antibody-dependent Fc $\gamma$ -receptor  
665 activation. HeLa cells co-transfected with RhCD4 and the indicated genes of interest were  
666 incubated with rhesusized anti-RhCD4 mAb and subsequently co-cultured with BW reporter  
667 cells expressing the chimeric human receptor CD16 $\zeta$  (left) or parental BW5147 cells (right).  
668 IL-2 levels corresponding to reporter activation were quantified using ELISA. Error bars =  
669 SD. Two-way ANOVA (Tukey); gp68 vs. CD99 (black), Rh05 vs. CD99 (orange).

670

671 **Figure 6: Rh05 antagonizes Fc $\gamma$ R-stimulation by infected cells**

672 A) Left: Surface expression of chimeric rhesus Fc $\gamma$ -receptors RhCD16 $\zeta$ , RhCD32A $\zeta$ ,  
673 RhCD32B $\zeta$  and RhCD64 $\zeta$  on stably transduced BW cells was detected using Texas Red-  
674 conjugated human Fc $\gamma$  fragment. Parental BW cells were used as a control. Middle: Chimeric  
675 rhesus Fc $\gamma$ Rs are activated upon IgG-Fc binding. Indicated BW reporter cells were assessed  
676 for activation by immobilized antibodies (Rtx = Rituximab;  $\alpha$ RhCD4 = recombinant  
677 rhesusized anti-rhesus-CD4 mAb). All values are means of technical duplicates and represent  
678 plateau activation determined by incubation on titrated amounts of antibody (not shown).  
679 Right: dose-response upon RhCD64 reporter cell activation by titrated amounts of Rtx. B)  
680 TRF cells were infected with RhCMV 68-1 or RhCMV $\Delta$ Rh05 using centrifugal enhancement

681 at an MOI=2 for 72hr. Left: Infected cells were incubated with serum from a RhCMV sero-  
682 positive monkey and overall surface antigen expression was detected via a FITC-conjugated  
683 rabbit anti-human-IgG polyclonal antibody. Right: Infected cells were probed with a  
684 TexasRed-conjugated human IgG-Fc fragment. C) Rh05 antagonizes rhesus-FcγR activation  
685 by antibody bound to infected cells. Infected cells were incubated with serum dilutions of  
686 RhCMV-positive or RhCMV-negative monkeys and subsequently co-cultured with the  
687 indicated BW reporter cells. IL-2 levels corresponding to reporter activation were quantified  
688 using ELISA. Error bars = SEM; CMV-positive sera = averages of 2 independent  
689 experiments; CMV-negative sera = averages of 1 experiment. Two-way ANOVA (Tukey).  
690 Asterisks show statistical comparison of reporter responses to infected cells opsonized by  
691 RhCMV-positive serum.

692

693 **Figure 7: Rh05 is not required for superinfection.**

694 At day 0, a RhCMV-positive RM was infected subcutaneously with  $5 \times 10^6$  PFU of the  
695 indicated recombinant virus and the SIVgag-specific T cell responses in PBMC were  
696 monitored by ICS for CD69, TNFα and IFNγ either using overlapping SIVgag 15mer peptide  
697 mixes to measure total responses (A) or the indicated MHC-E and MHC-II supertopes to  
698 measure epitope-specific responses (B). Results are shown as a percentage of total memory  
699 CD4<sup>+</sup> or CD8<sup>+</sup> T cells.

700

701

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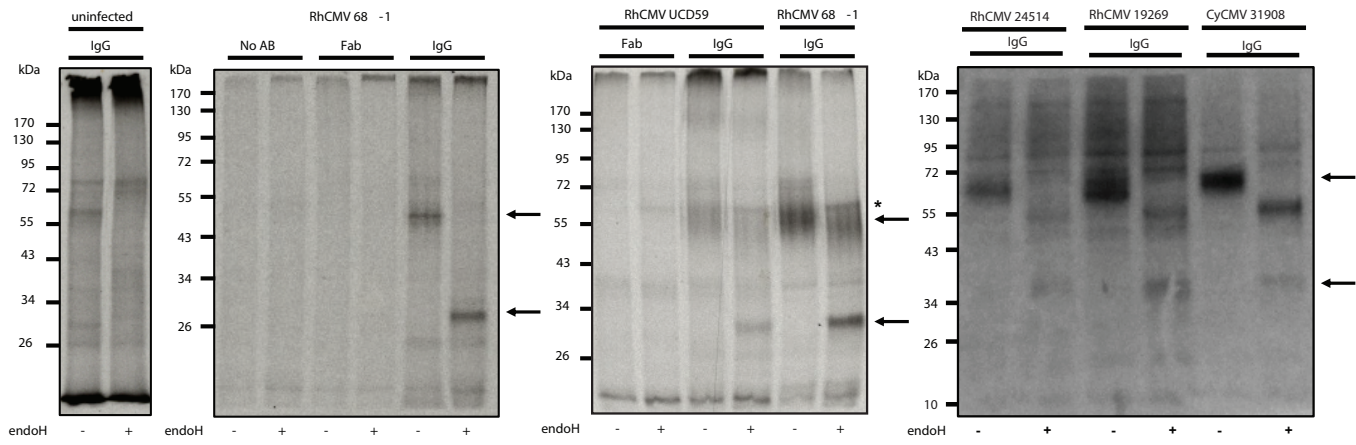


Figure 1

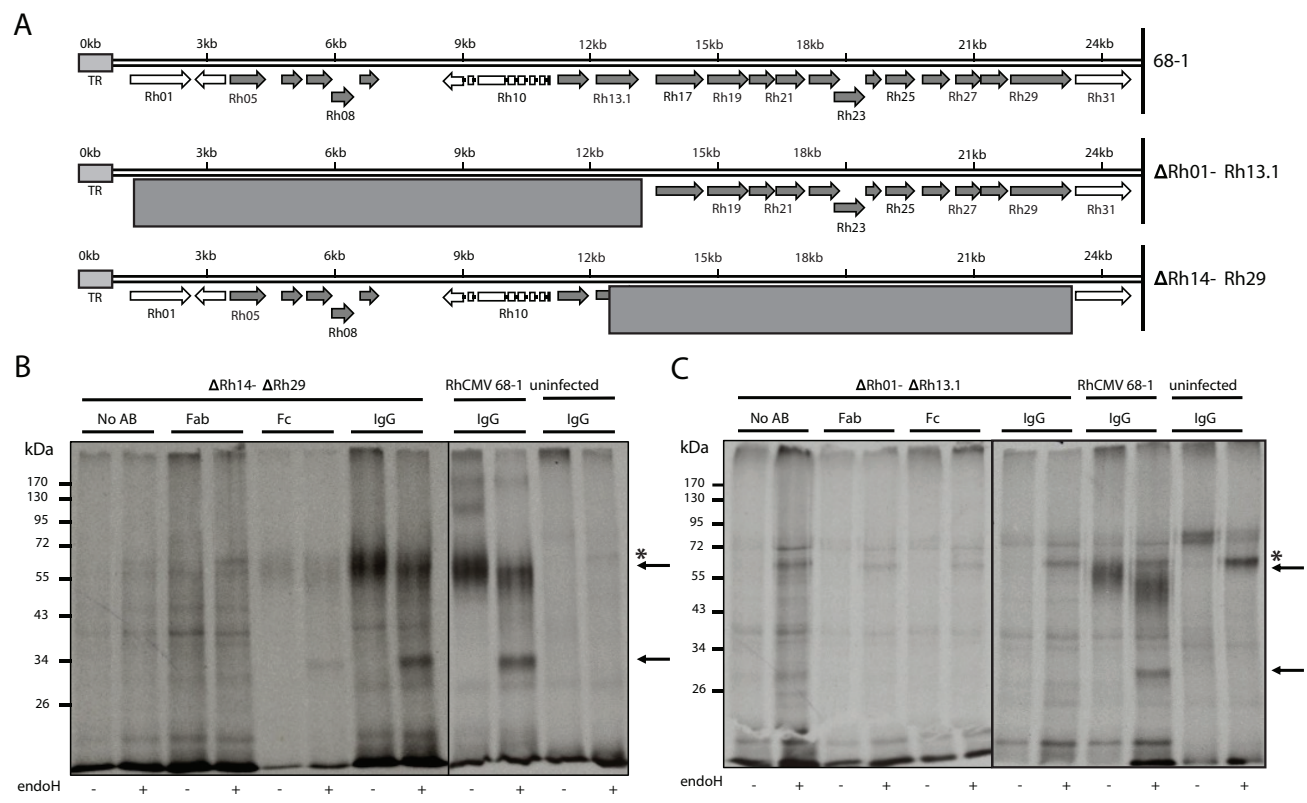
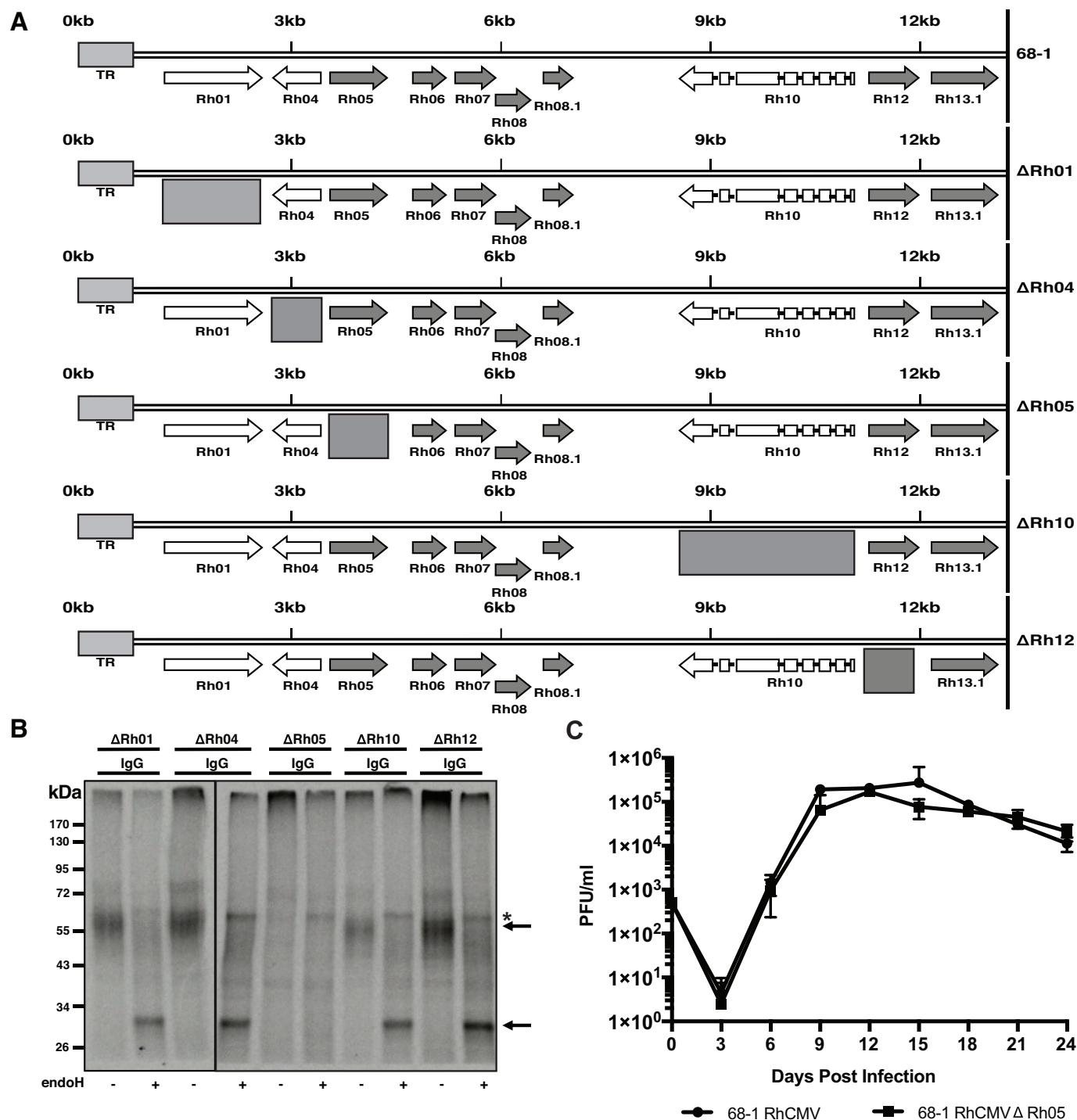


Figure 2



**Figure 3**

RhCMV	<u>MCPGLFTYIT-LTGMVMHTVSGN</u> NPRQLLCNVTFRFPGNNVSQVRLSTGDNVTFLYNVSQGH	59
CyCMV	<u>MCPGLFTYIT-LTGMVMHAVSGN</u> NPRQLLCNVTFRFPGTNVSQVRLSAGDNVTFLYNVSQGH	59
SCMV	<u>MCSGVFHYLTVFTGIVLTAVSGNSGK</u> -----NNNVTLVEVGIGQNVTLNYTRPSSH	51
BaCMV	<u>MCPGLFLFLE-ITGIAMTAASGSATGST</u> -----RTQPSMTQVALCPGGNVTFNYSRPQGH	54
DrCMV	<u>MCPGLFLFLE-ITGMVMTA</u> ISGGEG-----SRPLNVTQVQLCPGSNVTFNYTRPQGH	51
	** *: * :: : ** : : : * *	
RhCMV	SLSWLYS <u>N</u> LTA---N SSRHLRKYTLCSVTSNYRMTETRNNMCLHCNRSSLTLC SARPQDS	116
CyCMV	SLTWLYS <u>N</u> LTA---N SSRHLRKYTLCSVTSSYRMTETRNNMCLHCNRSSLTLC SARPQDS	116
SCMV	DVSWIYT <u>N</u> RTI---GNNHHFKRYSVCSFTSGYKRMENRNLNCIN <u>N</u> CTN <u>N</u> HSLTLCNIRPQDA	108
BaCMV	SVFWKYT <u>N</u> LTK---PAHKHLHQYVICLTGSIYILKETRNSMNMKCN <u>N</u> RS LQLYNVRPQDA	111
DrCMV	SMSWLYS <u>N</u> YSKMSEKRYKHLRHYLICLTSSYTMSETRNSMCMRCD <u>N</u> KSLTLCNMRPQDA	111
	.. : * *: * : : : * : : * : * : * : * : * : * : * : * : * : * : *	
RhCMV	GLYVLRDDT <u>N</u> NTDVMRCNVTVTGNGQLPVTHRPHSR---PTVTRIS--SAHLSGITLGNQ	171
CyCMV	GLYVLRDET <u>N</u> NTDVMRCNVTVTGNGQLSVTHRPHSR---PTVTRIS--SAHLSGITLGHE	171
SCMV	GLYVLRDYT <u>N</u> NHSDLFMY <u>N</u> VTV <u>N</u> CTIPHTQSTTKKTTTVSALVSRIQ--TASMSHVQ---P	163
BaCMV	GLYELHDHTNNSVLMVF <u>N</u> VTVRTV VAPQVTGMI----I-YTVSRVYHTSTHENGVT---K	163
DrCMV	GLYELRDHTNNSAVMVY <u>N</u> VTVRTLSAPTVRGTT----V-FRVVYQTHASTPHRGIV---K	163
	*** *: * *: * : : : * * * *	
RhCMV	KHSPTTWNT <u>WMVHISFATMALACFGVAVVLSGCVCL</u> RSVRAWTQKYRPLNEDPAPQKIDF	231
CyCMV	KHPNTTWNT <u>WMVHISFATMALACFGVAVVLSGCVCL</u> RSVRAWTQKYRPLNEDPAPQKIDF	231
SCMV	KPVKGNWET <u>WLIHISFASAALTCFAMAVILSGCVCARSLRAWAN</u> <u>N</u> YSQLKEPNEKEE---	220
BaCMV	HRIGNGWD <u>WMVHLSFATVAMTCFALAVILSGCVCA</u> RSIRAWSNNYRQLKD---QPD---	217
DrCMV	QKL R NGWDS <u>WMVHLSFATVAMTCFALAVILSGCVCARSLRAW</u> SNNYRQLKTTVDKEE---	220
	: * : * : * : * : * : * : * : * : * : * : * : * : * : * : *	
RhCMV	PDGTMKEHPHVTVIEPTKSADGTVVGLSAVSDDKPATLWLSR	273
CyCMV	PDGTMKEHPHVTVIEPTKSADGTVVGLSAVSDDKPATLWLSR	273
SCMV	-----YCDVIKVTEEEKVPIDMLESSVVDKQPATLWLT	255
BaCMV	-----SCDVIKLPEEEKVPIDVLTAVTDDKQPATLWLT	251
DrCMV	-----HCDVIRVTEDKKIPIDMLESSVVDKAPATLWLT	255
	. * : * : : * : * : * : * : * : * : * : * : * : *	

Figure 4

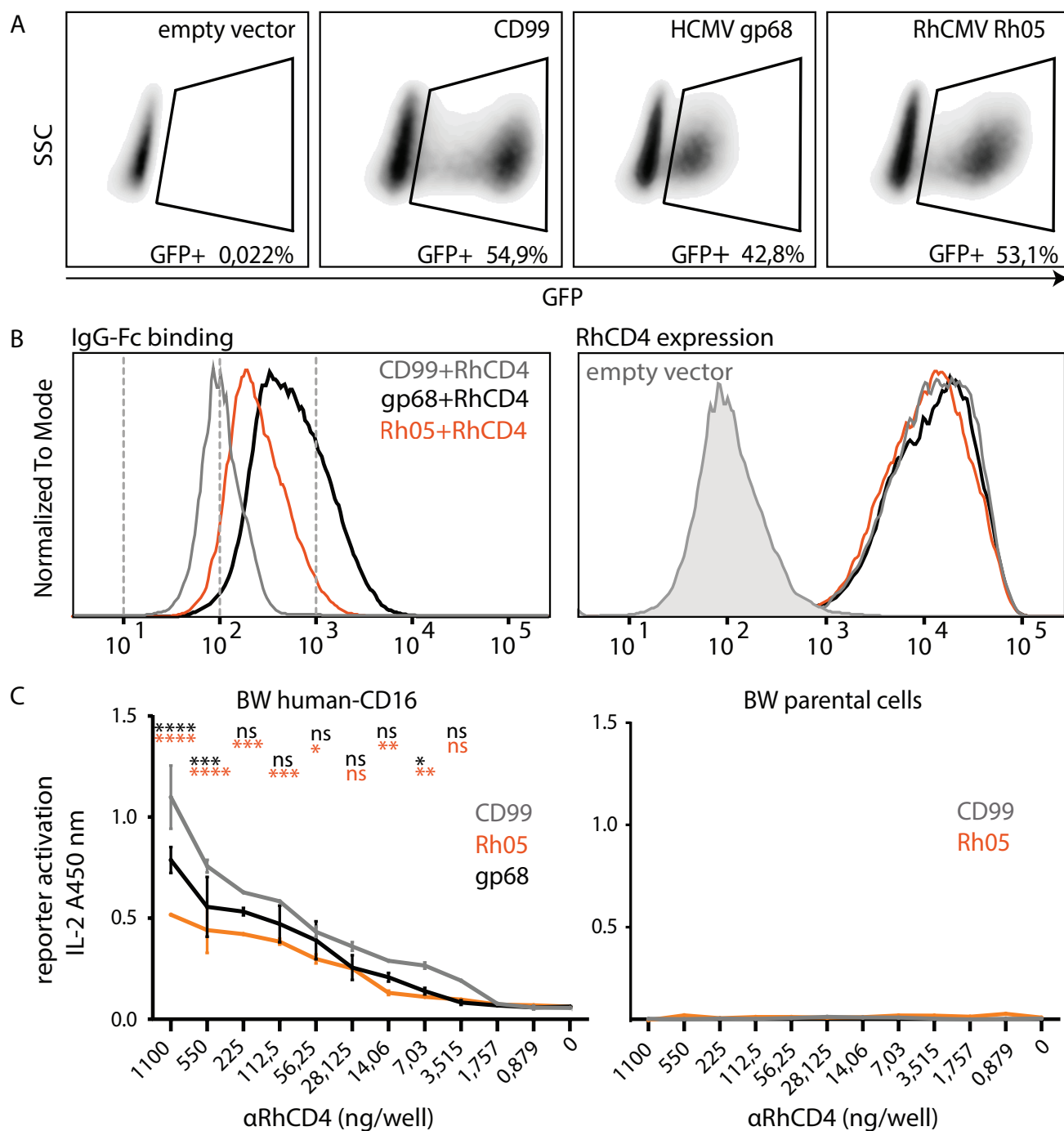


Figure 5

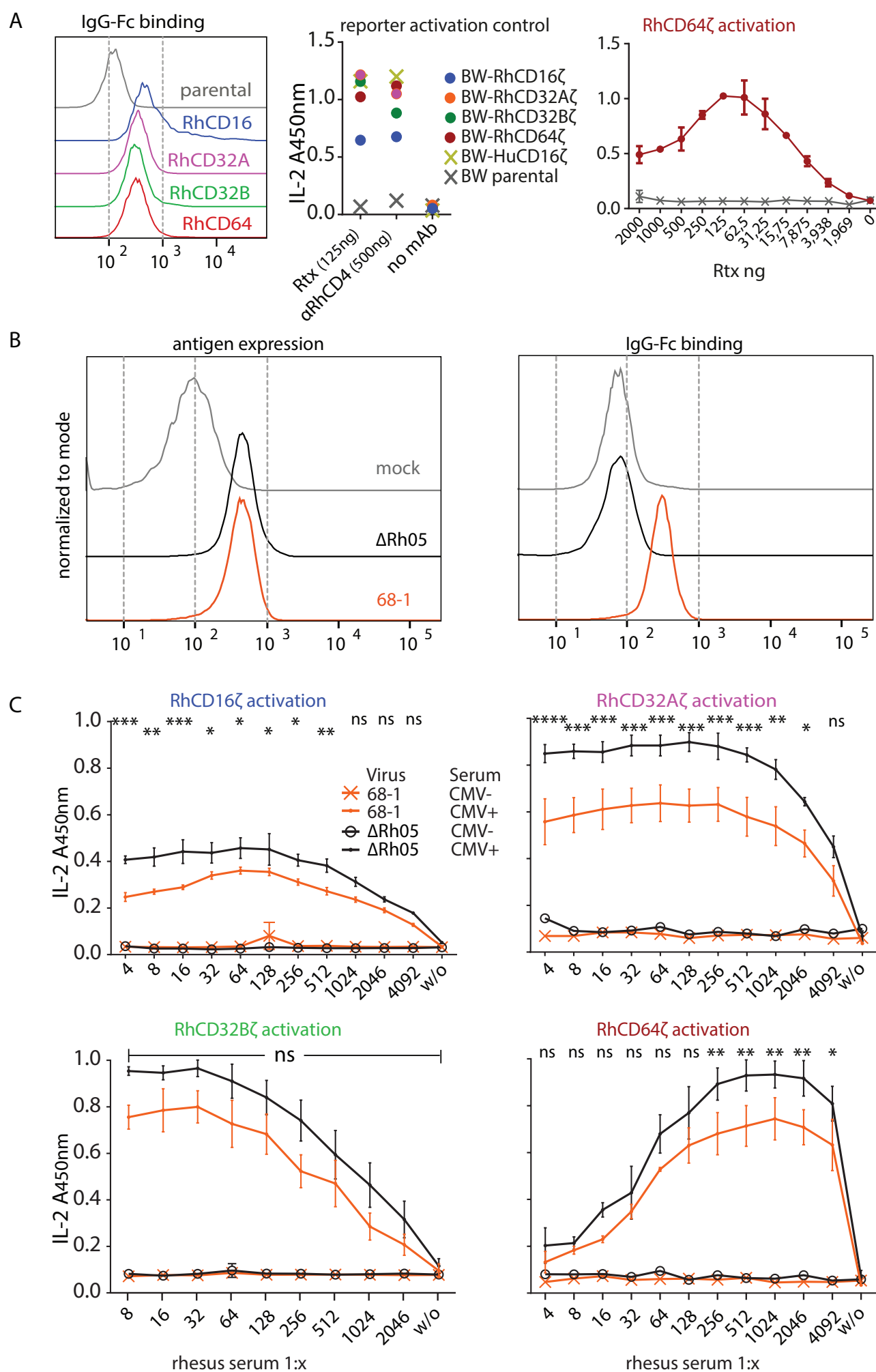


Figure 6

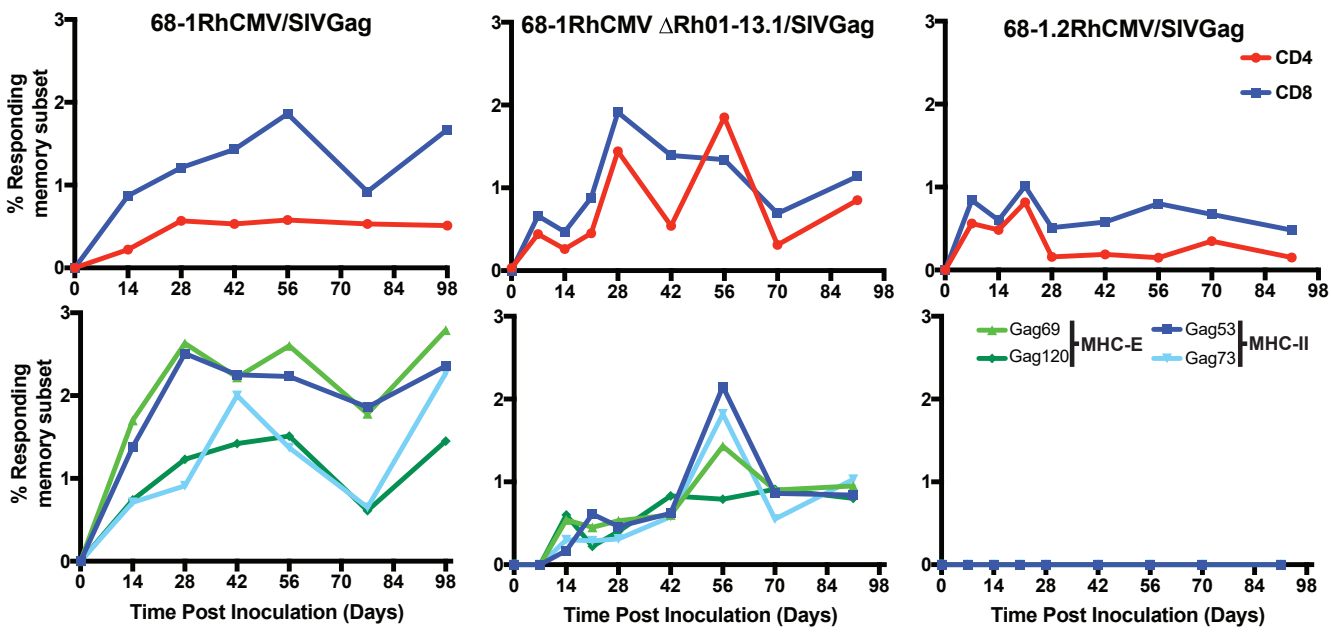


Figure 7