1   **Identification and functional characterization of a novel Fc gamma-binding**

2   **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 RhCMVΔRh05 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 β-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

93 glycoproteins (vFcγRs) encoded by the HCMV genes *UL118/119* (gp68) and *RL11* (gp34)

94 (15). These vFcγRs were shown to efficiently antagonize host IgG-Fc receptor (FcγR)

95 activation in a cell-based *in vitro* reporter assay performed on IVIG-opsonized infected cells

96 (16). In addition, *RL12* and *RL13* have been shown to have vFcγR activity (14). While

97 HCMV is the only known human β-herpesvirus to encode such glycoproteins, it is not the

98 only herpesvirus for which vFcγRs have been described. Mouse cytomegalovirus (MCMV)

99 encodes the Ig-like glycoprotein fcr-1/m138 (17). Deletion of *m138* from the MCMV genome

100 results in drastic attenuation of MCMV *in vivo* (18). However, since *m138* has both Fcγ-

101 related and -unrelated immunoevasive functions (19-21) the role of Fcγ-modulation for viral

102 pathogenesis has yet to be established. HSV-1 and VZV glycoproteins E and I (gE/gI) form

103 an IgG-Fc binding heterodimer (22, 23). By clearing antigen/antibody complexes from the

104 infected cell surface (24) the HSV-1 gE/gI complex promotes immune evasion *in vivo* (25).

105 Interestingly, the VZV gE protein is the major component of the recently developed highly

106 efficient subunit VZV vaccine (26).

107 Immune responses most prominently governed by host FcγRs include antibody dependent

108 cell-mediated cytotoxicity (ADCC), antibody dependent cell-mediated phagocytosis (ADCP)

109 and the induction of a pro-inflammatory cytokine profile by various immune cells including

110 NK cells, macrophages, dendritic cells, B cells and neutrophils expressing FcγRs (27). FcγRs

111 are further classified by their affinity to IgG-Fc and are highly conserved between humans

112 and non-human primates showing strong cross-reactivity (28, 29). There are four known

113 activating receptors comprising the high affinity receptor CD64/FcγRI, the medium affinity

114 receptors CD32A/FcγRIIA and CD32C/FcγRIIC, and the low affinity receptor

115 CD16A/FcγRIIIA. CD32B/FcγRIIB is the only known inhibitory receptor with a medium

116 affinity to IgG-Fc and a single cytosolic ITIM motif (27). Although their affinity to IgG-Fc is

117 also dependent on the IgG subclass, all FcγRs show their highest affinity towards IgG1 while

118 optimal binding in general can only be observed to immune complexed IgG with an intact

119   glycan profile (30). In recent years FcγR-mediated immune responses have proven to be an

120   essential factor in the antiviral effect of non-neutralizing but also neutralizing IgG specific for

121   important pathogenic viruses like Influenza A (31, 32) and HIV (33, 34).

122   CMVs are highly species specific, which prevents studying HCMV directly in an animal

123   model. While the closest relative of HCMV is chimpanzee CMV (CCMV), experimentation

124   in these animals is no longer possible. In contrast, infection of rhesus macaques (RM)

125   (*Macaca mulatta*) with rhesus cytomegalovirus (RhCMV) is a tractable model and the

126   genomes of non-human primate (NHP) CMVs encode homologs of most of the HCMV gene

127   families (35, 36). Therefore, RhCMV infection has emerged as a state of the art model

128   allowing the study of primate CMV disease infection, immune responses and pathology *in*

129   *vivo* (37), including important aspects of congenital infection (14, 38). While in this model

130   RhCMV genes linked to evasion from CD8+ T lymphocyte and NK cell responses have been

131   extensively investigated (6, 39), little is known about the ability of RhCMV to evade antibody

132   mediated immunity.

133   Here we demonstrate that the RhCMV *RL11* gene family member *Rh05* encodes an IgG-Fc

134   binding glycoprotein. Similar to HCMV vFcγRs, this type 1 transmembrane protein is

135   transported to the cell surface where it efficiently antagonizes FcγR activation triggered by

136   immune IgG. In addition, Rh05 was able to antagonize human FcγRIIIa/CD16a activation by

137   cells opsonized with a rhesusized monoclonal IgG antibody. Interestingly, Rh05 was not

138   required for RhCMV super-infection, suggesting that evasion of pre-existing antibodies is not

139   essential for the establishment of secondary infections. These results thus represent the first

140   identification of a vFcγR in RhCMV and highlight the close evolutionary relationship of

141   human and rhesus IgG and FcγRs consistent with the RM/RhCMV model being particularly

142   relevant when studying viral evasion of IgG effector functions *in vivo*.

143

144   **Materials and Methods**

145

146　**Cells.** All cells were cultured in a 5% CO_2 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 $3x10^5$ to $9x10^5$ 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 ($1.5x10^6$ 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 300μCi 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 μg 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 ΔRh14-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 ΔRh01-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 ($5x10^4$ 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 3rd day starting at day 3 and the supernatants

238   were cleared by centrifugation at 16.100 x g for 5 minutes before storing them at -80°C. Viral

239   titers of each sample were determined by 50% tissue culture infective dose ($TCID_{50}$) 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 *Nhe1* and *BamH1*

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 *Nhe1* and *BamH1* 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 $1x10^6$ target cells) followed

264   by expansion and pool selection using 2μg/ml of Puromycin.

265

266   **Flow cytometry.** $1x10^6$ 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_2 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 $2x10^5$ 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   lymphotrophic virus type 1, simian immunodeficiency virus, and TB. The RM were sedated

303   with ketamine HCl or Telazol® for subcutaneous administration of $5x10^6$ PFU of either 68-1

304   RhCMV/gag, RhCMVΔRh01-13.1/gag or 68-1.2 RhCMVgag, respectively, on day 0.

305

306   **T cell assays.** SIVgag-specific CD4+ and CD8+ 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$_{276-284}$ RMYNPTNIL and Gag120$_{482-490}$ EKQRESREK) or MHC-II-restricted

313   supertope peptides (Gag53$_{211-222}$ AADWDLQHPQP and Gag73$_{290-301}$ 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 (CD8α; PerCP-Cy5.5), MAB11 (TNFα; FITC, PE), B27

318   (IFNγ; 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+ and progressive gating on CD4+ and CD8+ T cell subsets. Antigen-responding cells in

322   both CD4+ and CD8+ 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 (CD8α; APC, PerCP-cy-5.5), MAB11 (TNFα; FITC), B27 (IFNγ; 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 RhCMVΔRh05, 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 RhCMVΔRh05 consistent with a complete loss of Fc-

464   binding activity upon deletion of Rh05 (**Fig. 6B, right**). Applying the FcγR 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 RhCMVΔRh05 induced significantly higher reporter cell activation

470   for all examined activating Rh-FcγRs 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 RhCMVΔRh05 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 FcγRs thus counteracting

475   opsonization and subsequent FcγR mediated immune responses.

476

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+ 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 vFcγR 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 ΔRh01-13.1 (see material and methods). $5x10^6$ PFU of ΔRh01-13.1

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**, ΔRh01-13.1 elicited robust SIVgag-specific

492   responses for both CD4+ and CD8+ 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+ 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+ 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 ΔRh01-13.1 elicited

504   supertope-specific CD8+ T cells in contrast to 68-1.2 RhCMV/gag that failed to elicit CD8+

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+ 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, FcγRIIIA-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 the 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

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, ΔRh01-Rh13.1 and ΔRh14-ΔRh29, 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 RhCMVΔRh14-29, but

630   not from RhCMVΔRh01-13.1. *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 RhCMVΔRh05 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     $TCID_{50.}$

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 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 FcγR 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 Fcγ-binding by flow cytometry using Texas Red conjugated human-Fcγ

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 Fcγ-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 CD16ζ (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 FcγR-stimulation by infected cells**

672   A) Left: Surface expression of chimeric rhesus Fcγ-receptors RhCD16ζ, RhCD32Aζ,

673   RhCD32Bζ and RhCD64ζ on stably transduced BW cells was detected using Texas Red-

674   conjugated human Fcγ fragment. Parental BW cells were used as a control. Middle: Chimeric

675   rhesus FcγRs are activated upon IgG-Fc binding. Indicated BW reporter cells were assessed

676   for activation by immobilized antibodies (Rtx = Rituximab; αRhCD4 = 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 RhCMVΔRh05 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 5×10^6 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     $CD4^+$ or $CD8^+$ T cells.

700

701

REFERENCES

1. **Powers, C., V. DeFilippis, D. Malouli, and K. Früh.** 2008. Cytomegalovirus immune evasion. Curr Top Microbiol Immunol **325:**333-59.

2. **Hengel, H., U. H. Koszinowski, and K. K. Conzelmann.** 2005. Viruses know it all: new insights into IFN networks. Trends Immunol **26:**396-401.

3. **Halenius, A., C. Gerke, and H. Hengel.** 2015. Classical and non-classical MHC I molecule manipulation by human cytomegalovirus: so many targets-but how many arrows in the quiver? Cell Mol Immunol **12:**139-53.

4. **Wilkinson, G. W., P. Tomasec, R. J. Stanton, M. Armstrong, V. Prod'homme, R. Aicheler, B. P. McSharry, C. R. Rickards, D. Cochrane, S. Llewellyn-Lacey, E. C. Wang, C. A. Griffin, and A. J. Davison.** 2008. Modulation of natural killer cells by human cytomegalovirus. J Clin Virol **41:**206-12.

5. **Stern-Ginossar, N., B. Weisburd, A. Michalski, V. T. Le, M. Y. Hein, S. X. Huang, M. Ma, B. Shen, S. B. Qian, H. Hengel, M. Mann, N. T. Ingolia, and J. S. Weissman.** 2012. Decoding human cytomegalovirus. Science **338:**1088-93.

6. **Hansen, S. G., C. J. Powers, R. Richards, A. B. Ventura, J. C. Ford, D. Siess, M. K. Axthelm, J. A. Nelson, M. A. Jarvis, L. J. Picker, and K. Früh.** 2010. Evasion of CD8+ T cells is critical for superinfection by cytomegalovirus. Science **328:**102-6.

7. **Ross, S. A., N. Arora, Z. Novak, K. B. Fowler, W. J. Britt, and S. B. Boppana.** 2010. Cytomegalovirus reinfections in healthy seroimmune women. J Infect Dis **201:**386-9.

8. **Ishida, J. H., A. Patel, A. K. Mehta, P. Gatault, J. M. McBride, T. Burgess, M. A. Derby, D. R. Snydman, B. Emu, B. Feierbach, A. E. Fouts, M. Maia, R. Deng, C. M. Rosenberger, L. A. Gennaro, N. S. Striano, X. C. Liao, and J. A. Tavel.** 2017. Phase 2 Randomized, Double-Blind, Placebo-Controlled Trial of RG7667, a Combination Monoclonal Antibody, for Prevention of Cytomegalovirus Infection in High-Risk Kidney Transplant Recipients. Antimicrob Agents Chemother **61:**e01794-16.

729   9.   **Revello, M. G., T. Lazzarotto, B. Guerra, A. Spinillo, E. Ferrazzi, A. Kustermann, S.**
730   **Guaschino, P. Vergani, T. Todros, T. Frusca, A. Arossa, M. Furione, V. Rognoni, N.**
731   **Rizzo, L. Gabrielli, C. Klersy, and G. Gerna.** 2014. A randomized trial of hyperimmune
732   globulin to prevent congenital cytomegalovirus. N Engl J Med **370:**1316-26.

733 10.   **Nigro, G., S. P. Adler, R. La Torre, and A. M. Best.** 2005. Passive immunization during
734   pregnancy for congenital cytomegalovirus infection. N Engl J Med **353:**1350-62.

735 11.   **Boeckh, M., R. A. Bowden, B. Storer, N. J. Chao, R. Spielberger, D. K. Tierney, G.**
736   **Gallez-Hawkins, T. Cunningham, K. G. Blume, D. Levitt, and J. A. Zaia.** 2001.
737   Randomized, placebo-controlled, double-blind study of a cytomegalovirus-specific
738   monoclonal antibody (MSL-109) for prevention of cytomegalovirus infection after allogeneic
739   hematopoietic stem cell transplantation. Biol Blood Marrow Transplant **7:**343-51.

740 12.   **Hodson, E. M., C. A. Jones, G. F. Strippoli, A. C. Webster, and J. C. Craig.** 2007.
741   Immunoglobulins, vaccines or interferon for preventing cytomegalovirus disease in solid
742   organ transplant recipients. Cochrane Database Syst Rev**:**CD005129.

743 13.   **Raanani, P., A. Gafter-Gvili, M. Paul, I. Ben-Bassat, L. Leibovici, and O. Shpilberg.**
744   2009. Immunoglobulin prophylaxis in hematopoietic stem cell transplantation: systematic
745   review and meta-analysis. J Clin Oncol **27:**770-81.

746 14.   **Nelson, C. S., D. V. Cruz, D. Tran, K. M. Bialas, L. Stamper, H. Wu, M. Gilbert, R.**
747   **Blair, X. Alvarez, H. Itell, M. Chen, A. Deshpande, F. Chiuppesi, F. Wussow, D. J.**
748   **Diamond, N. Vandergrift, M. R. Walter, P. A. Barry, M. Cohen-Wolkowiez, K. Koelle,**
749   **A. Kaur, and S. R. Permar.** 2017. Preexisting antibodies can protect against congenital
750   cytomegalovirus infection in monkeys. JCI Insight **2:**e94002.

751 15.   **Atalay, R., A. Zimmermann, M. Wagner, E. Borst, C. Benz, M. Messerle, and H. Hengel.**
752   2002. Identification and expression of human cytomegalovirus transcription units coding for
753   two distinct Fcgamma receptor homologs. J Virol **76:**8596-608.

754 16.   **Corrales-Aguilar, E., M. Trilling, K. Hunold, M. Fiedler, V. T. Le, H. Reinhard, K.**
755   **Ehrhardt, E. Merce-Maldonado, E. Aliyev, A. Zimmermann, D. C. Johnson, and H.**

756 **Hengel.** 2014. Human cytomegalovirus Fcgamma binding proteins gp34 and gp68 antagonize

757 Fcgamma receptors I, II and III. PLoS Pathog **10:**e1004131.

758 17. **Thale, R., P. Lucin, K. Schneider, M. Eggers, and U. H. Koszinowski.** 1994. Identification

759 and expression of a murine cytomegalovirus early gene coding for an Fc receptor. J Virol

760 **68:**7757-65.

761 18. **Crnkovic-Mertens, I., M. Messerle, I. Milotic, U. Szepan, N. Kucic, A. Krmpotic, S.**

762 **Jonjic, and U. H. Koszinowski.** 1998. Virus attenuation after deletion of the cytomegalovirus

763 Fc receptor gene is not due to antibody control. J Virol **72:**1377-82.

764 19. **Arapovic, J., T. Lenac Rovis, A. B. Reddy, A. Krmpotic, and S. Jonjic.** 2009. Promiscuity

765 of MCMV immunoevasin of NKG2D: m138/fcr-1 down-modulates RAE-1epsilon in addition

766 to MULT-1 and H60. Mol. Immunol. **47:**114-22.

767 20. **Lenac, T., M. Budt, J. Arapovic, M. Hasan, A. Zimmermann, H. Simic, A. Krmpotic, M.**

768 **Messerle, Z. Ruzsics, U. H. Koszinowski, H. Hengel, and S. Jonjic.** 2006. The herpesviral

769 Fc receptor fcr-1 down-regulates the NKG2D ligands MULT-1 and H60. J Exp Med

770 **203:**1843-50.

771 21. **Mintern, J. D., E. J. Klemm, M. Wagner, M. E. Paquet, M. D. Napier, Y. M. Kim, U. H.**

772 **Koszinowski, and H. L. Ploegh.** 2006. Viral interference with B7-1 costimulation: a new role

773 for murine cytomegalovirus fc receptor-1. J Immunol **177:**8422-31.

774 22. **Johnson, D. C., and V. Feenstra.** 1987. Identification of a novel herpes simplex virus type 1-

775 induced glycoprotein which complexes with gE and binds immunoglobulin. J Virol **61:**2208-

776 16.

777 23. **Olson, J. K., and C. Grose.** 1998. Complex formation facilitates endocytosis of the varicella-

778 zoster virus gE:gI Fc receptor. J Virol **72:**1542-51.

779 24. **Ndjamen, B., A. H. Farley, T. Lee, S. E. Fraser, and P. J. Bjorkman.** 2014. The herpes

780 virus Fc receptor gE-gI mediates antibody bipolar bridging to clear viral antigens from the cell

781 surface. PLoS Pathog **10:**e1003961.

782   25.   **Nagashunmugam, T., J. Lubinski, L. Wang, L. T. Goldstein, B. S. Weeks, P. Sundaresan,**

783   **E. H. Kang, G. Dubin, and H. M. Friedman.** 1998. In vivo immune evasion mediated by the

784   herpes simplex virus type 1 immunoglobulin G Fc receptor. J Virol **72:**5351-9.

785   26.   **Cunningham, A. L.** 2016. The herpes zoster subunit vaccine. Expert Opin Biol Ther **16:**265-

786   71.

787   27.   **Nimmerjahn, F., and J. V. Ravetch.** 2007. Fc-receptors as regulators of immunity. Adv

788   Immunol **96:**179-204.

789   28.   **Rogers, K. A., F. Scinicariello, and R. Attanasio.** 2006. IgG Fc receptor III homologues in

790   nonhuman primate species: genetic characterization and ligand interactions. J Immunol

791   **177:**3848-56.

792   29.   **Chan, Y. N., A. W. Boesch, N. Y. Osei-Owusu, A. Emileh, A. R. Crowley, S. L. Cocklin,**

793   **S. L. Finstad, C. H. Linde, R. A. Howell, I. Zentner, S. Cocklin, A. R. Miles, J. W.**

794   **Eckman, G. Alter, J. E. Schmitz, and M. E. Ackerman.** 2016. IgG Binding Characteristics

795   of Rhesus Macaque FcgammaR. J Immunol **197:**2936-47.

796   30.   **Bruhns, P., B. Iannascoli, P. England, D. A. Mancardi, N. Fernandez, S. Jorieux, and M.**

797   **Daeron.** 2009. Specificity and affinity of human Fcgamma receptors and their polymorphic

798   variants for human IgG subclasses. Blood **113:**3716-25.

799   31.   **Van den Hoecke, S., K. Ehrhardt, A. Kolpe, K. El Bakkouri, L. Deng, H. Grootaert, S.**

800   **Schoonooghe, A. Smet, M. Bentahir, K. Roose, M. Schotsaert, B. Schepens, N.**

801   **Callewaert, F. Nimmerjahn, P. Staeheli, H. Hengel, and X. Saelens.** 2017. Hierarchical and

802   Redundant Roles of Activating FcgammaRs in Protection against Influenza Disease by M2e-

803   Specific IgG1 and IgG2a Antibodies. J Virol **91:**e02500-16.

804   32.   **DiLillo, D. J., G. S. Tan, P. Palese, and J. V. Ravetch.** 2014. Broadly neutralizing

805   hemagglutinin stalk-specific antibodies require FcgammaR interactions for protection against

806   influenza virus in vivo. Nat Med **20:**143-51.

807   33.   **Horwitz, J. A., Y. Bar-On, C. L. Lu, D. Fera, A. A. K. Lockhart, J. C. C. Lorenzi, L.**

808   **Nogueira, J. Golijanin, J. F. Scheid, M. S. Seaman, A. Gazumyan, S. Zolla-Pazner, and**

809   **M. C. Nussenzweig.** 2017. Non-neutralizing Antibodies Alter the Course of HIV-1 Infection

810   In Vivo. Cell **170:**637-648 e10.

811   34.   **Chung, A. W., M. P. Kumar, K. B. Arnold, W. H. Yu, M. K. Schoen, L. J. Dunphy, T. J.**

812   **Suscovich, N. Frahm, C. Linde, A. E. Mahan, M. Hoffner, H. Streeck, M. E. Ackerman,**

813   **M. J. McElrath, H. Schuitemaker, M. G. Pau, L. R. Baden, J. H. Kim, N. L. Michael, D.**

814   **H. Barouch, D. A. Lauffenburger, and G. Alter.** 2015. Dissecting Polyclonal Vaccine-

815   Induced Humoral Immunity against HIV Using Systems Serology. Cell **163:**988-98.

816   35.   **Hansen, S. G., L. I. Strelow, D. C. Franchi, D. G. Anders, and S. W. Wong.** 2003.

817   Complete sequence and genomic analysis of rhesus cytomegalovirus. J Virol **77:**6620-36.

818   36.   **Malouli, D., E. S. Nakayasu, K. Viswanathan, D. G. Camp, 2nd, W. L. Chang, P. A.**

819   **Barry, R. D. Smith, and K. Früh.** 2012. Reevaluation of the Coding Potential and Proteomic

820   Analysis of the BAC-Derived Rhesus Cytomegalovirus Strain 68-1. J Virol **86:**8959-73.

821   37.   **Powers, C., and K. Früh.** 2008. Rhesus CMV: an emerging animal model for human CMV.

822   Med Microbiol Immunol **197:**109-15.

823   38.   **Bialas, K. M., T. Tanaka, D. Tran, V. Varner, E. Cisneros De La Rosa, F. Chiuppesi, F.**

824   **Wussow, L. Kattenhorn, S. Macri, E. L. Kunz, J. A. Estroff, J. Kirchherr, Y. Yue, Q.**

825   **Fan, M. Lauck, D. H. O'Connor, A. H. Hall, A. Xavier, D. J. Diamond, P. A. Barry, A.**

826   **Kaur, and S. R. Permar.** 2015. Maternal CD4+ T cells protect against severe congenital

827   cytomegalovirus disease in a novel nonhuman primate model of placental cytomegalovirus

828   transmission. Proc Natl Acad Sci U S A **112:**13645-50.

829   39.   **Sturgill, E. R., D. Malouli, S. G. Hansen, B. J. Burwitz, S. Seo, C. L. Schneider, J. L.**

830   **Womack, M. C. Verweij, A. B. Ventura, A. Bhusari, K. M. Jeffries, A. W. Legasse, M. K.**

831   **Axthelm, A. W. Hudson, J. B. Sacha, L. J. Picker, and K. Früh.** 2016. Natural Killer Cell

832   Evasion Is Essential for Infection by Rhesus Cytomegalovirus. PLoS Pathog **12:**e1005868.

833   40.   **Chang, W. L., V. Kirchoff, G. S. Pari, and P. A. Barry.** 2002. Replication of rhesus

834   cytomegalovirus in life-expanded rhesus fibroblasts expressing human telomerase. J Virol

835   Methods **104:**135-46.

836   41.   **Oxford, K. L., L. Strelow, Y. Yue, W. L. Chang, K. A. Schmidt, D. J. Diamond, and P. A.**

837   **Barry.** 2011. Open reading frames carried on UL/b' are implicated in shedding and horizontal

838   transmission of rhesus cytomegalovirus in rhesus monkeys. J Virol **85:**5105-14.

839   42.   **Yue, Y., A. Kaur, A. Lilja, D. J. Diamond, M. R. Walter, and P. A. Barry.** 2016. The

840   susceptibility of primary cultured rhesus macaque kidney epithelial cells to rhesus

841   cytomegalovirus strains. J Gen Virol **97:**1426-38.

842   43.   **Burwitz, B. J., D. Malouli, B. N. Bimber, J. S. Reed, A. B. Ventura, M. H. Hancock, L. S.**

843   **Uebelhoer, A. Bhusari, K. B. Hammond, R. G. Espinosa Trethewy, A. Klug, A. W.**

844   **Legasse, M. K. Axthelm, J. A. Nelson, B. S. Park, D. N. Streblow, S. G. Hansen, L. J.**

845   **Picker, K. Früh, and J. B. Sacha.** 2016. Cross-Species Rhesus Cytomegalovirus Infection of

846   Cynomolgus Macaques. PLoS Pathog **12:**e1006014.

847   44.   **Hansen, S. G., J. B. Sacha, C. M. Hughes, J. C. Ford, B. J. Burwitz, I. Scholz, R. M.**

848   **Gilbride, M. S. Lewis, A. N. Gilliam, A. B. Ventura, D. Malouli, G. Xu, R. Richards, N.**

849   **Whizin, J. S. Reed, K. B. Hammond, M. Fischer, J. M. Turner, A. W. Legasse, M. K.**

850   **Axthelm, P. T. Edlefsen, J. A. Nelson, J. D. Lifson, K. Früh, and L. J. Picker.** 2013.

851   Cytomegalovirus vectors violate CD8+ T cell epitope recognition paradigms. Science

852   **340:**1237874.

853   45.   **Hansen, S. G., C. Vieville, N. Whizin, L. Coyne-Johnson, D. C. Siess, D. D. Drummond,**

854   **A. W. Legasse, M. K. Axthelm, K. Oswald, C. M. Trubey, M. Piatak, Jr., J. D. Lifson, J.**

855   **A. Nelson, M. A. Jarvis, and L. J. Picker.** 2009. Effector memory T cell responses are

856   associated with protection of rhesus monkeys from mucosal simian immunodeficiency virus

857   challenge. Nat Med **15:**293-9.

858   46.   **Muyrers, J. P., Y. Zhang, V. Benes, G. Testa, J. M. Rientjes, and A. F. Stewart.** 2004. ET

859   recombination: DNA engineering using homologous recombination in E. coli. Methods Mol

860   Biol **256:**107-21.

861   47.   **Cherepanov, P. P., and W. Wackernagel.** 1995. Gene disruption in Escherichia coli: TcR

862   and KmR cassettes with the option of Flp-catalyzed excision of the antibiotic-resistance

863   determinant. Gene **158:**9-14.

864   48.   **Tischer, B. K., G. A. Smith, and N. Osterrieder.** 2010. En passant mutagenesis: a two step
865   markerless red recombination system. Methods Mol Biol **634:**421-30.

866   49.   **Corrales-Aguilar, E., M. Trilling, H. Reinhard, E. Merce-Maldonado, M. Widera, H.**
867   **Schaal, A. Zimmermann, O. Mandelboim, and H. Hengel.** 2013. A novel assay for
868   detecting virus-specific antibodies triggering activation of Fcgamma receptors. J Immunol
869   Methods **387:**21-35.

870   50.   **Hansen, S. G., J. C. Ford, M. S. Lewis, A. B. Ventura, C. M. Hughes, L. Coyne-Johnson,**
871   **N. Whizin, K. Oswald, R. Shoemaker, T. Swanson, A. W. Legasse, M. J. Chiuchiolo, C.**
872   **L. Parks, M. K. Axthelm, J. A. Nelson, M. A. Jarvis, M. Piatak, Jr., J. D. Lifson, and L.**
873   **J. Picker.** 2011. Profound early control of highly pathogenic SIV by an effector memory T-
874   cell vaccine. Nature **473:**523-7.

875   51.   **Hansen, S. G., H. L. Wu, B. J. Burwitz, C. M. Hughes, K. B. Hammond, A. B. Ventura,**
876   **J. S. Reed, R. M. Gilbride, E. Ainslie, D. W. Morrow, J. C. Ford, A. N. Selseth, R.**
877   **Pathak, D. Malouli, A. W. Legasse, M. K. Axthelm, J. A. Nelson, G. M. Gillespie, L. C.**
878   **Walters, S. Brackenridge, H. R. Sharpe, C. A. Lopez, K. Früh, B. T. Korber, A. J.**
879   **McMichael, S. Gnanakaran, J. B. Sacha, and L. J. Picker.** 2016. Broadly targeted CD8(+)
880   T cell responses restricted by major histocompatibility complex E. Science **351:**714-20.

881   52.   **Lilja, A. E., W. L. Chang, P. A. Barry, S. P. Becerra, and T. E. Shenk.** 2008. Functional
882   genetic analysis of rhesus cytomegalovirus: Rh01 is an epithelial cell tropism factor. J Virol
883   **82:**2170-81.

884   53.   **Murphy, E., I. Rigoutsos, T. Shibuya, and T. E. Shenk.** 2003. Reevaluation of human
885   cytomegalovirus coding potential. Proc Natl Acad Sci U S A **100:**13585-90.

886   54.   **Cortese, M., S. Calo, R. D'Aurizio, A. Lilja, N. Pacchiani, and M. Merola.** 2012.
887   Recombinant human cytomegalovirus (HCMV) RL13 binds human immunoglobulin G Fc.
888   PLoS One **7:**e50166.

889   55.   **Davison, A. J., P. Akter, C. Cunningham, A. Dolan, C. Addison, D. J. Dargan, A. F.**
890   **Hassan-Walker, V. C. Emery, P. D. Griffiths, and G. W. Wilkinson.** 2003. Homology

891 between the human cytomegalovirus RL11 gene family and human adenovirus E3 genes. J

892 Gen Virol **84:**657-63.

893 56. **Sekulin, K., I. Gorzer, D. Heiss-Czedik, and E. Puchhammer-Stockl.** 2007. Analysis of the

894 variability of CMV strains in the RL11D domain of the RL11 multigene family. Virus Genes

895 **35:**577-83.

896 57. **Perez-Carmona, N., P. Martinez-Vicente, D. Farre, I. Gabaev, M. Messerle, P. Engel,**

897 **and A. Angulo.** 2018. A prominent role of the human cytomegalovirus UL8 glycoprotein

898 restraining pro-inflammatory cytokine production by myeloid cells at late times during

899 infection. J Virol **92:**e02229-17.

900 58. **Bruno, L., M. Cortese, G. Monda, M. Gentile, S. Calo, F. Schiavetti, L. Zedda, E.**

901 **Cattaneo, D. Piccioli, M. Schaefer, E. Notomista, D. Maione, A. Carfi, M. Merola, and Y.**

902 **Uematsu.** 2016. Human cytomegalovirus pUL10 interacts with leukocytes and impairs TCR-

903 mediated T-cell activation. Immunol Cell Biol **94:**849-860.

904 59. **Gabaev, I., L. Steinbruck, C. Pokoyski, A. Pich, R. J. Stanton, R. Schwinzer, T. F.**

905 **Schulz, R. Jacobs, M. Messerle, and P. C. Kay-Fedorov.** 2011. The human

906 cytomegalovirus UL11 protein interacts with the receptor tyrosine phosphatase CD45,

907 resulting in functional paralysis of T cells. PLoS Pathog **7:**e1002432.

908 60. **Engel, P., N. Perez-Carmona, M. M. Alba, K. Robertson, P. Ghazal, and A. Angulo.**

909 2011. Human cytomegalovirus UL7, a homologue of the SLAM-family receptor CD229,

910 impairs cytokine production. Immunol Cell Biol **89:**753-66.

911 61. **Gabaev, I., E. Elbasani, S. Ameres, L. Steinbruck, R. Stanton, M. Doring, T. Lenac**

912 **Rovis, U. Kalinke, S. Jonjic, A. Moosmann, and M. Messerle.** 2014. Expression of the

913 human cytomegalovirus UL11 glycoprotein in viral infection and evaluation of its effect on

914 virus-specific CD8 T cells. J Virol **88:**14326-39.

915 62. **Crawford, L. B., J. H. Kim, D. Collins-McMillen, B. J. Lee, I. Landais, C. Held, J. A.**

916 **Nelson, A. D. Yurochko, and P. Caposio.** 2018. Human Cytomegalovirus Encodes a Novel

917 FLT3 Receptor Ligand Necessary for Hematopoietic Cell Differentiation and Viral

918 Reactivation. MBio **9:**e00682-18.

919   63.   **MacManiman, J. D., A. Meuser, S. Botto, P. P. Smith, F. Liu, M. A. Jarvis, J. A. Nelson,**

920   **and P. Caposio.** 2014. Human cytomegalovirus-encoded pUL7 is a novel CEACAM1-like

921   molecule responsible for promotion of angiogenesis. MBio **5:**e02035.

922   64.   **Stanton, R. J., K. Baluchova, D. J. Dargan, C. Cunningham, O. Sheehy, S. Seirafian, B.**

923   **P. McSharry, M. L. Neale, J. A. Davies, P. Tomasec, A. J. Davison, and G. W. Wilkinson.**

924   2010. Reconstruction of the complete human cytomegalovirus genome in a BAC reveals RL13

925   to be a potent inhibitor of replication. J Clin Invest **120:**3191-208.

926   65.   **Corrales-Aguilar, E., M. Trilling, H. Reinhard, V. Falcone, A. Zimmermann, O. Adams,**

927   **S. Santibanez, and H. Hengel.** 2016. Highly individual patterns of virus-immune IgG

928   effector responses in humans. Med Microbiol Immunol **205:**409-24.

929   66.   **Forthal, D., T. J. Hope, and G. Alter.** 2013. New paradigms for functional HIV-specific

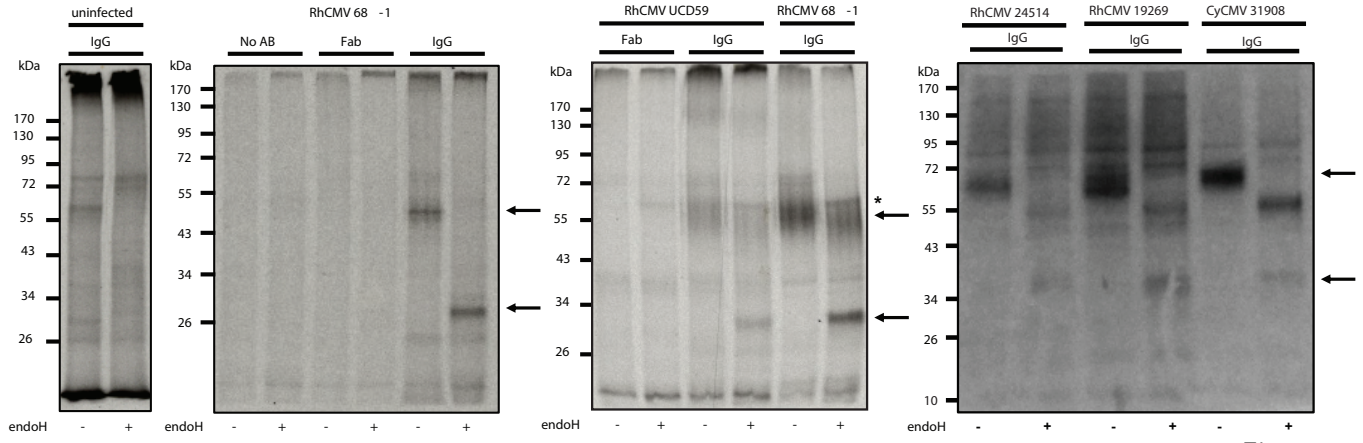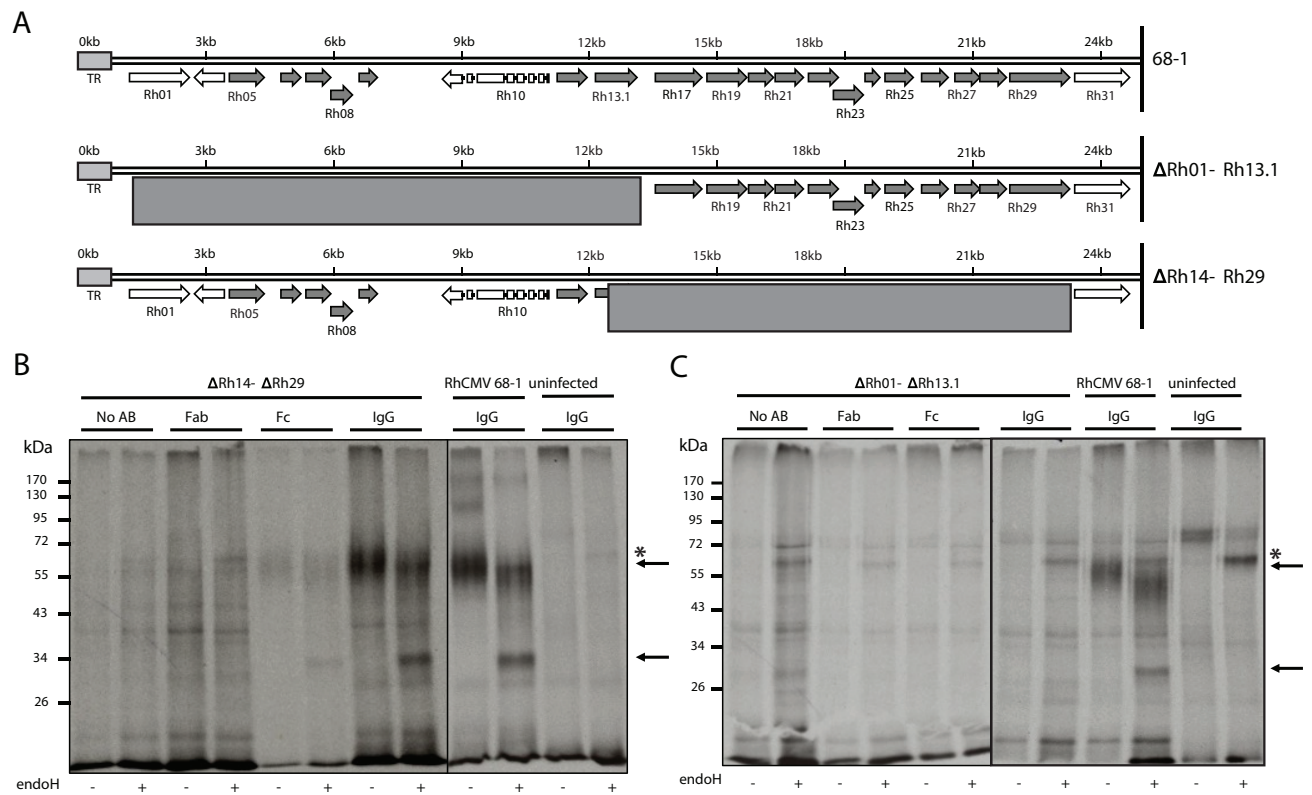930   nonneutralizing antibodies. Curr Opin HIV AIDS **8:**393-401.
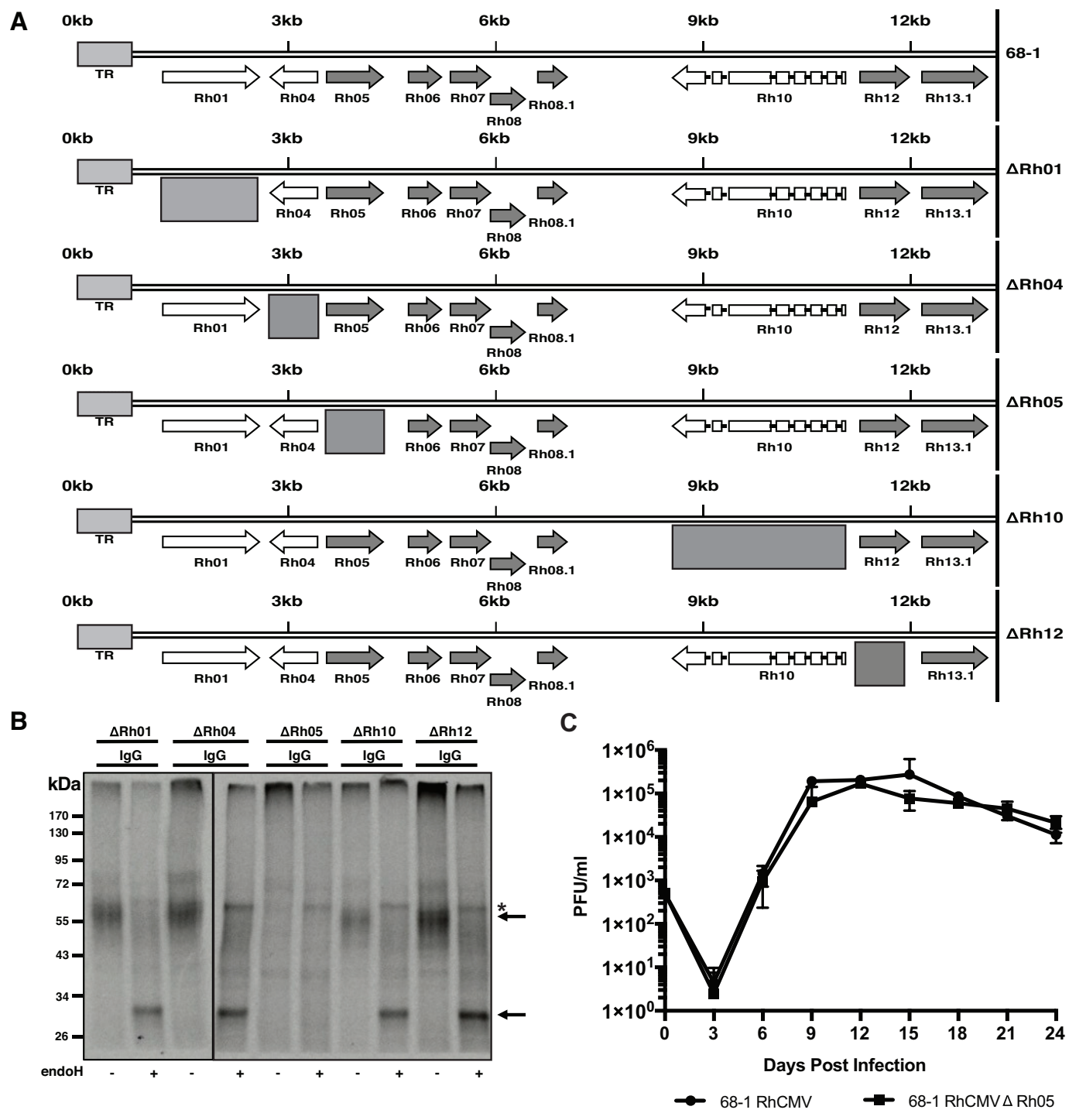
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Figure 1

Figure 2

Figure 3

```
RhCMV    MCPGLFTYIT-LTGMVMHTVSGNPRQLLCNVTRFPGNNVSQVRLSTGDNVTFLYNVSQGH    59
CyCMV    MCPGLFTYIT-LTGMVMHAVSGNPRQLLCNVTRFPGTNVSQVRLSAGDNVTFLYNVSQGH    59
SCMV     MCSGVFHYLTVFTGIVLTAVSGNSGK---------NNNVTLVEVGIGQNVTLNYTRPSSH    51
BaCMV    MCPGLFLFLE-ITGIAMTAASGSATGST-----RTQPSMTQVALCPGGNVTFNYSRPQGH    54
DrCMV    MCPGLFLFLE-ITGMVMTAISGGEG--------SRPLNVTQVQLCPGSNVTFNYTRPQGH    51
         ** *:* ::   :**:.: : **.          .:: * :  * ***: *.  ..*

RhCMV    SLSWLYSNLTA---NSSRHLRKYTLCSVTSNYRMTETRNNMCLHCNRSSLTLCSARPQDS   116
CyCMV    SLTWLYSNLTA---NSSRHLRKYTLCSVTSSYRMTETRNNMCLHCNRSSLTLCSARPQDS   116
SCMV     DVSWIYTNRTI---GNNHHFKRYSVCSFTSGYKRMENRNLMCINCTNHSLTLCNIRPQDA   108
BaCMV    SVFWKYTNLTK---PAHKHLHQYVICTLTGSYILKETRNSMNMKCNNRSLQLYNVRPQDA   111
DrCMV    SMSWLYSNYSKMSEKRYKHLRHYLICTLTSSYTMSETRNSMCMRCDNKSLTLCNMRPQDA   111
         .: |*| *:* :       :*:::* :*:.*..*   *.** * :.|*| . ** * . ****:

RhCMV    GLYVLRDDTNNTDVMRCNVTVTGNGQLPVTHRPHSR---PTVTRIS--SAHLSGITLGNQ   171
CyCMV    GLYVLRDETNNTDVMRCNVTVTGNGQLSVTHRPHSR---PTVTRIS--SAHLSGITLGHE   171
SCMV     GLYVLRDYTNHSDLFMYNVTVNCTIPHTQSTTKKTTTVSALVSRIQ--TASMSHVQ---P   163
BaCMV    GLYELHDHTNNSVLMVFNVTVRTVVAPQVTGMI----I-YTVSRVYHTSTHENGVT---K   163
DrCMV    GLYELRDHTNNSAVMVYNVTVRTLSAPTVRGTT----V-FRVVYQTHASTPHRGIV---K   163
         *** *:* **:: ::   ****          *          :: :

RhCMV    KHSPTTWNTWMVHISFATMALACFGVAVVLSGCVCLRSVRAWTQKYRPLNEDPAPQKIDF   231
CyCMV    KHPPNTWNTWMVHISFATMALACFGVAVVLSGCVCLRSVRAWTQKYRPLNEDPAPQKLDF   231
SCMV     KPVKGNWETWLIHISFASAALTCFAMAVILSGCVCARSLRAWANNYSQLKEPNEKEE---   220
BaCMV    HRIGNGWDTWMVHLSFATVAMTCFALAVILSGCVCARSIRAWSNNYRQLKD---QPD---   217
DrCMV    QKLRNGWDSWMVHLSFATVAMTCFALAVILSGCVCARSLRAWSNNYRQLKTTVDKEE---   220
         :       *::*::*:***: *:.**.:**.****** **.***::*  *:        .

RhCMV    PDGTMKEHPHVTVIEPTKSADGTVVGLSAVSDDKPATLWLSR   273
CyCMV    PDGTMKEHPHVTVIEPTKSADGTVVGLSAVSDDKPATLWLSR   273
SCMV     -------YCDVIKVTEEKKVPIDMLESSVVDAKQPATLWLTK   255
BaCMV    -------SCDVIKLPEEKKVPIDVLTA-VTDDKQPATLWLTK   251
DrCMV    -------HCDVIRVTEDKKIPIDMLESSVVDAKAPATLWLTK   255
                .* :   *.   ::   ... . ******::
```
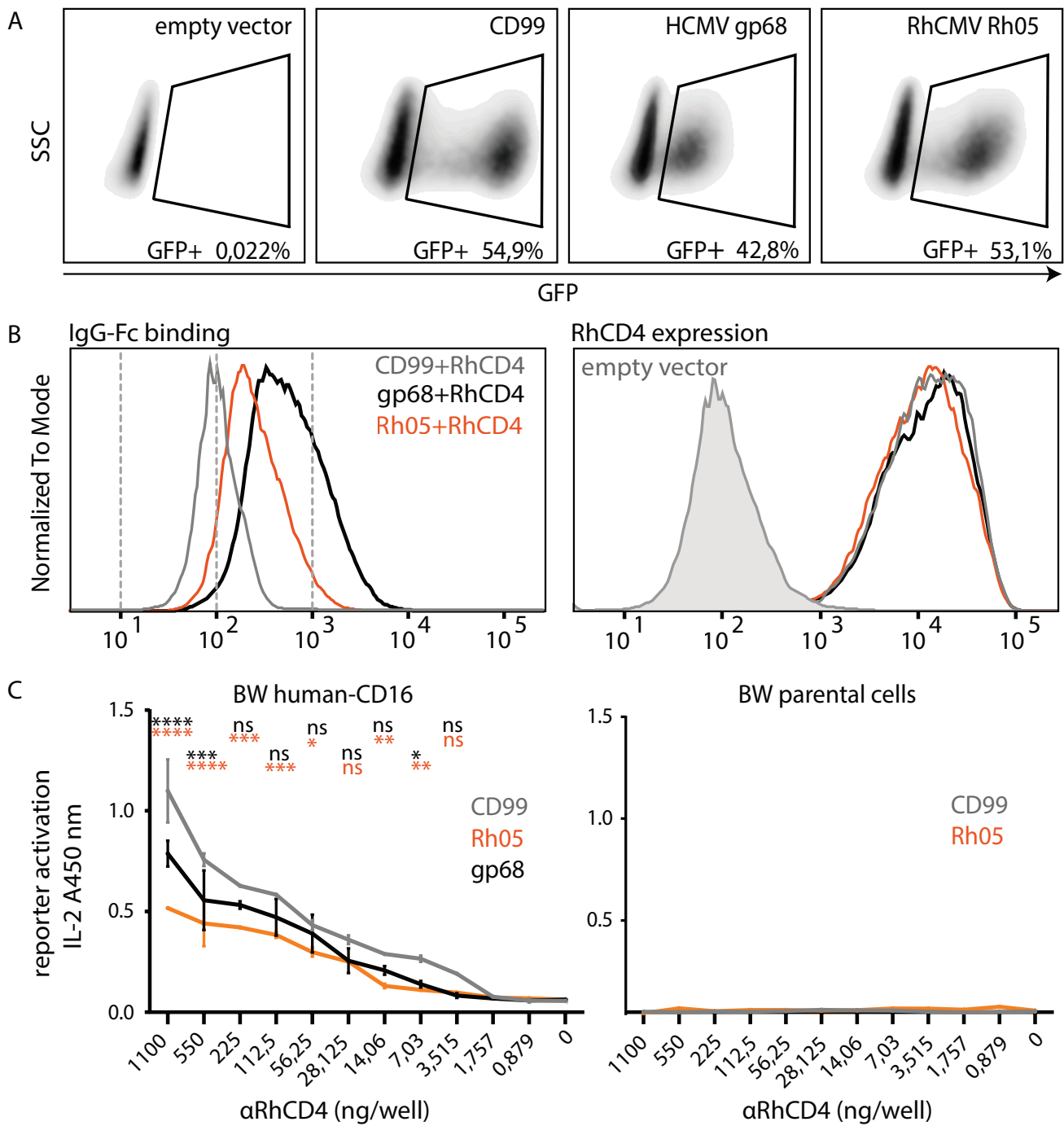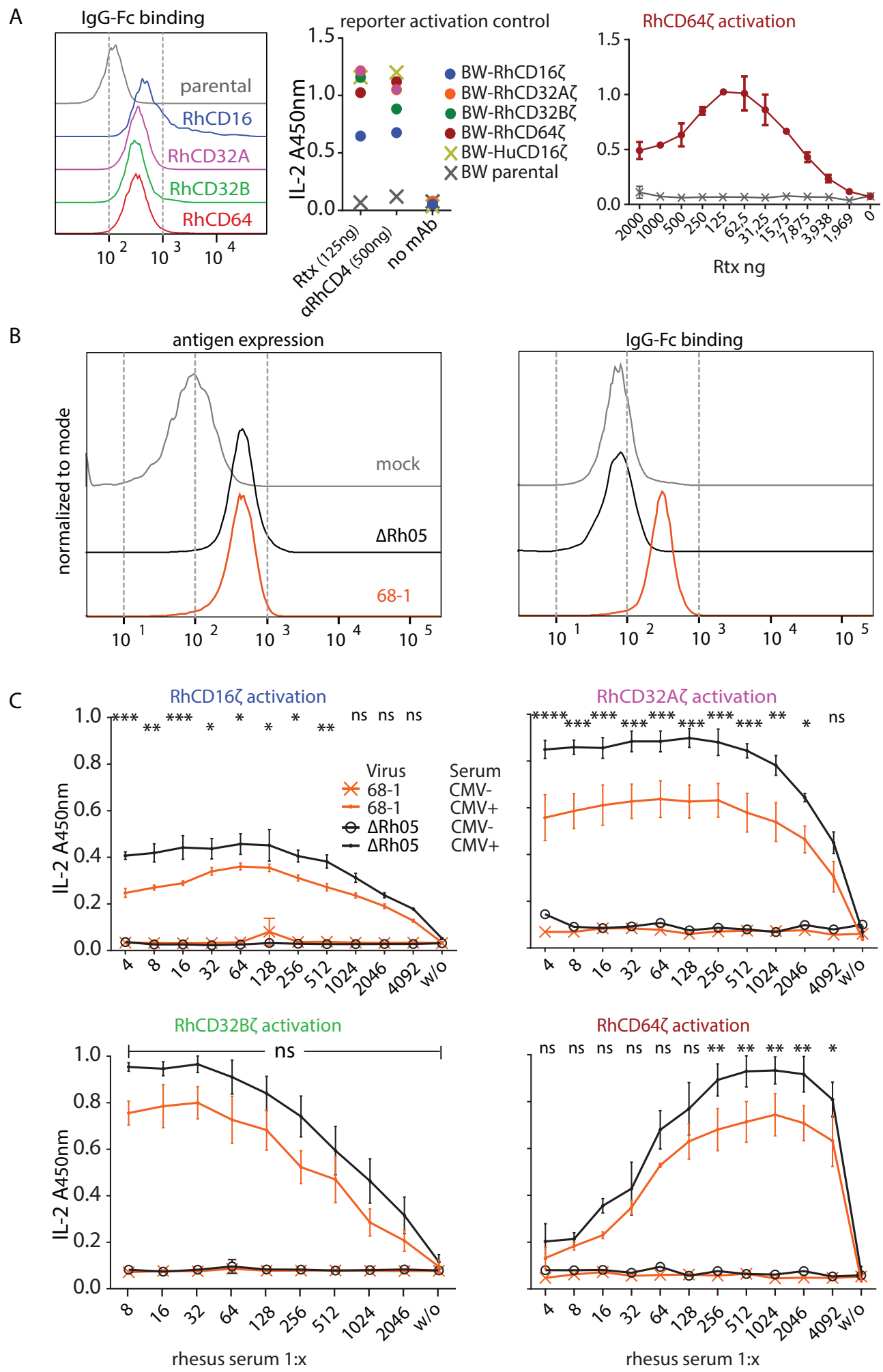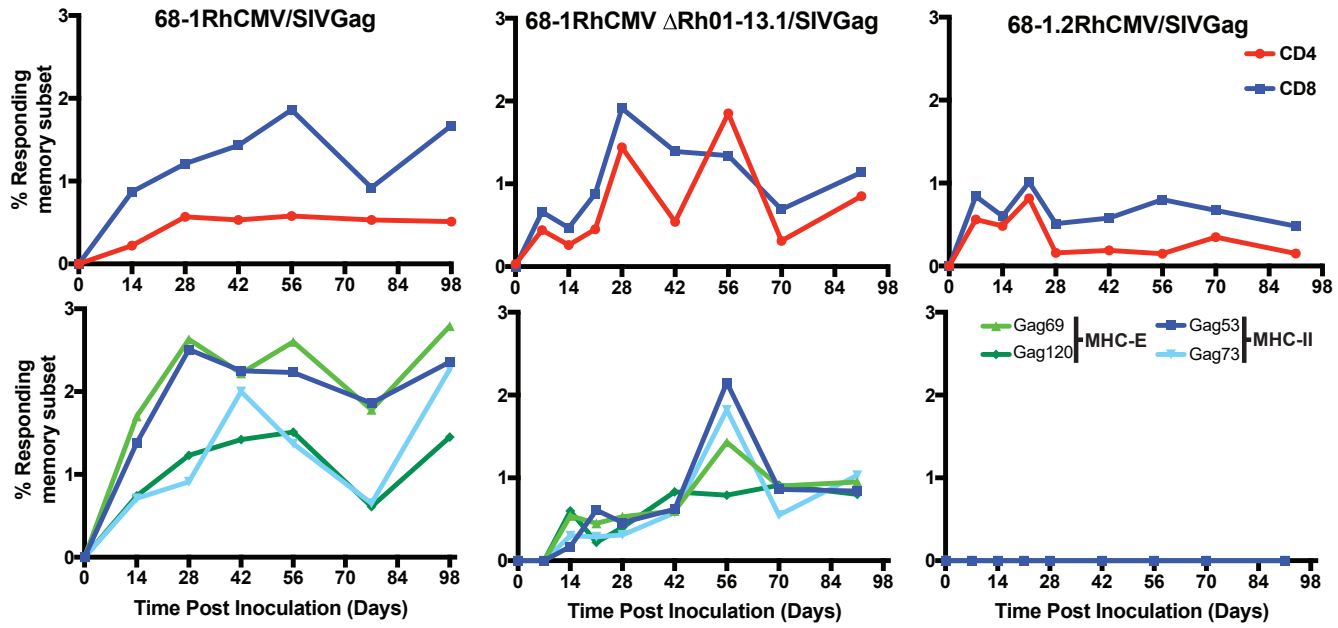
Figure 4

Figure 5

Figure 6

Figure 7