1

2   DR PHILIP STEVENSON (Orcid ID : 0000-0002-3520-5060)

3

4

6

7

Membrane association of a model CD4+ T cell vaccine antigen confers enhanced yet

incomplete protection against Murid Herpesvirus-4 infection

10

11   Joseph Yunis[1,2], Alec J Redwood[3], Gabrielle T Belz[4], Philip G Stevenson[1,5]*

12

13   [1]School of Chemistry and Molecular Biosciences, University of Queensland, Australia

14   [2]current address: John Curtin School of Medical Research, Australian National University,

15   Canberra, Australia

16   [3]Faculty of Heath and Medical Sciences, University of Western Australia, Perth, Australia

17   [4]Molecular Immunology, Walter and Eliza Hall Institute, Melbourne, Australia

18   [5]Royal Children's Hospital, Brisbane, Australia.

19

20   *Correspondence: Dr. P.G. Stevenson, School of Chemistry and Molecular Biosciences,

21   University of Queensland, St Lucia, Queensland 4072, Australia. tel. +61-7-336-52173; email

22   p.stevenson@uq.edu.au

23

27

28   **Abstract**

29   Vaccination against γ-herpesviruses has proved difficult. CD4+ T cells are essential to

30   contain infection, but how best to prime them and whether this can reduce viral loads

31   remain unclear. To address these questions, we used ovalbumin (OVA) as a model antigen,

32   delivering it with Murine cytomegalovirus (MCMV) to protect mice against OVA-expressing

33   Murine Herpesvirus-4 (MuHV-4). Membrane-associated OVA (mOVA) was more effective

34   than soluble OVA, both to prime CD4+ T cells and as an effector target. It was also a better

35   target than an OVA epitope limited to infected cells, suggesting that protective CD4+ T cells

36   recognize infected cell debris rather than infected cells themselves. While MCMV-mOVA

37   protected acutely against MuHV-4-mOVA, long-term protection was incomplete, even when

38   OVA-specific CD8+ T cells and B cells were also primed. Thus, even optimized single target

39   vaccines may poorly reduce long-term γ-herpesvirus infections.

40

41   **Introduction**

42   The capacity of γ-herpesviruses to cause cancers motivates vaccination against them.

43   Antibodies to the Epstein-Barr virus (EBV) glycoprotein (gp) 350, and CD8+ T cells specific for

44   viral latency antigens, can counter B cell transformation *in vitro*. However, neither has

45   protected by vaccination[1]. Gp350 vaccine protected tamarins against *in vitro*-type

46   transformation, but in humans EBV seems instead to colonize germinal centre-experienced

47   resting memory B cells[2], so different protection mechanisms may operate.

48   MuHV-4 reproduces in mice the germinal centre exploitation, memory B cell

49   colonization, and immune evasion characteristic of EBV[3,4]. Thus, it can be used to test

50   vaccine strategies. Whole virus vaccines limit MuHV-4 infection[3], with contributions from

51   both antibody and T cells[5,6]. Priming with recombinant gB[7] or with recombinant gp150, the

52   MuHV-4 homolog of the EBV gp350[8], reduced subsequent infection either transiently or not

53   at all. CD8+ T cell priming also protected only transiently[9,10]. While CD8+ T cell-dependent

54   control is often assumed for γ-herpesviruses, long-term MuHV-4 is more CD4+ than CD8+ T

55   cell-dependent[11,12]. Protection by whole virus vaccine also seems to be CD4+ T cell-

56   dependent[6]. This fits with EBV control in immunodeficient patients correlating better with

57   CD4+ than with CD8+ T cell reconstitution[13]; with herpesviruses harming CD4+ T cell-deficient

58   more than CD8+ T cell-deficient patients[14]; and with CD4+ T cells controlling MCMV[15]. Thus,

59   CD4+ T cell-dependent control may be a general feature of herpesviruses. With MuHV-4, it

60   reflects the impact of viral CD8+ T cell evasion[12].

61   CD4^{+} T cells have been assumed to protect by killing virus-infected cells[16], and

62   control of EBV-driven B cell proliferation was attributed to CD4^{+} T cells recognizing the

63   EBNA-1 latency antigen[17]. However such recognition remains controversial[18], and most EBV-

64   protective CD4^{+} T cells seem to recognize lytic antigens[19], suggesting that they control

65   lymphoproliferation indirectly. Immunocompromised patients accumulate EBV-infected

66   resting memory B cells rather than transformed blasts[20], arguing that most *in vivo* virus-

67   driven B cell proliferation terminates spontaneously; and increased lytic infection in these

68   patients[20] suggests that this may be a key control point.

69   Protective CD4^{+} T cells could potentially kill lytically infected cells. However, although

70   CD4^{+} T cells can kill, more usually they activate engaged presenting cells. This makes sense,

71   as most peptides reaching MHC class II derive from cell-exogenous antigens[21], so antigen^{+}

72   cells are not necessarily infected. And while CD8^{+} T cells damage their targets directly via

73   perforin and granzymes, CD4^{+} T cells kill mainly via fas signalling[22], which viruses can block[23].

74   Therefore CD4^{+} T cell cytotoxicity seems more suited to immune regulation than to

75   attacking evasive pathogens. CD4^{+} T cells control MuHV-4[24,25] and MCMV[26] via IFNγ, which is

76   more immunostimulatory than cytotoxic. They control MuHV-4 in the lungs despite infected

77   type I alveolar epithelial cells lacking MHC class II[27]; and they control MCMV in the salivary

78   glands despite infected cells degrading MHC class II[28]. These findings suggest that CD4^{+} T

79   cell-mediated infection control could be indirect.

80   If CD4^{+} T cells protect indirectly, the best target for vaccination might be an epitope

81   efficiently acquired by uninfected presenting cells, rather than one efficiently presented by

82   infected cells. To test this, we used the CD4^{+} T cell epitope of OVA as a model antigen,

83   expressed in different forms, to vaccinate against OVA-expressing MuHV-4. Long-term CD4^{+}

84   T cell priming may require persistent antigen, so we used recombinant MCMVs for vaccine

85   delivery. We expressed the OVA epitope in either membrane bound or secreted forms for

86   priming and challenge, or only in infected cells for challenge, and assessed protection in

87   each setting.

88

89   **Results**

90   **OVA expression from MCMV and MuHV-4**

91   MCMV expressing OVA from its IE2 locus via an HCMV IE1 promoter (MCMV-OVA) is

92   described[29]. We expressed mOVA the same way (MCMV-mOVA; Fig.1a). It has the N-

93   terminal 118 amino acids of the human transferrin receptor (TFR), comprising its

94   cytoplasmic tail, transmembrane domain and insertion signal, fused to the C-terminal 247

95   amino acids of OVA, which include the $OVA_{323-339}$ ($CD4^+$) and $OVA_{258-265}$ ($CD8^+$) T cell

96   epitopes. The OVA fragment of mOVA remains cell-associated rather than being secreted[30],

97   and shows enhanced immunogenicity for $CD8^+$ T cells[31]. MuHV-4 expressing OVA from an

98   ectopic viral M3 (lytic) promoter in the ORF57/ORF58 intergenic site (MHV-OVA) is

99   described[32]. We expressed mOVA the same way (MHV-mOVA; Fig.1b). We used also MuHV-

100   4 that expresses murine invariant chain with $OVA_{323-339}$ replacing the CLIP peptide (MHV-

101   IiOVA). Here expression was linked via an internal ribosome entry site to that of ORF73 - the

102   MuHV-4 episome maintenance protein, equivalent to EBV EBNA-1[32]. This arrangement

103   allows $OVA_{323-339}$ presentation by lytically and latently infected cells, without making it

104   available for presentation by uninfected cells.

105   OVA expression was identified by immunostaining infected cells with polyclonal

106   OVA-specific immune serum (Fig.1c, 1d). Western blots (Fig.1e) confirmed that mOVA was

107   solely cell-associated, while OVA was secreted (Fig.1e). We used IL-2 secretion by the

108   DO.11.10 T cell hybridoma[33] to assay IA^d-restricted $OVA_{323-339}$ presentation by BALB/c-3T3

109   fibroblasts or RAW-264 monocytes infected with recombinant MCMV or MuHV-4. Each cell

110   line was made constitutively IA^{d+} by transduction with a retroviral vector expressing the

111   human MHC class II transactivator (Fig.1f)[32]. $OVA_{323-339}$ presentation was evident for all the

112   recombinant viruses, with mOVA and IiOVA being presented better than OVA (Fig.1g). MHV-

113   mOVA but not MHV-OVA displayed OVA on the plasma membrane of infected fibroblasts

114   (Fig.1h). MCMV-infected fibroblasts showed internal OVA expression (Fig.1d), but displayed

115   neither mOVA nor new virion proteins on the plasma membrane, and so may generally

116   inhibit glycoprotein export to the plasma membrane.

117

118   **OVA expression by MCMV and MuHV-4 *in vivo***

119   MCMV-OVA and MCMV-mOVA were attenuated for replication *in vivo*, after either

120   intranasal (i.n.) (Fig.2a) or intraperitoneal (i.p.) (Fig.2b) inoculation, as described for similar

121   IE2 disruptions[29]. The attenuation was less marked after i.p. infection, so we used this for

122   immunization. MCMV-mOVA induced readily detectable OVA-specific IgG_{2a} - although little

123   IgG$_1$ - (Fig.2c). MCMV-OVA also induced OVA-specific antibody, but less. OVA$_{323-339}$-specific

124   IFNγ responses (Fig.2d) were also stronger against MCMV-mOVA than against MCMV-OVA.

125   Our MuHV-4 recombinants, in which OVA expression did not disrupt endogenous

126   viral genes, showed no significant attenuation after i.n. inoculation (Fig.3a). Again immune

127   responses to OVA were evident in infected mice, with mOVA inducing stronger antibody

128   (predominantly IgG$_{2a}$) (Fig.3b) and IFNγ responses (Fig.3c) than OVA. MHV-IiOVA induced

129   similar OVA$_{323-339}$-specific IFNγ responses to MHV-mOVA. It did not induce detectable OVA-

130   specific antibodies, as it expressed only OVA$_{323-339}$. These data established a basis for i.p.

131   vaccination with OVA-expressing MCMV to protect against i.n. OVA-expressing MuHV-4.

132

133   **Vaccinating BALB/c mice against MHV-mOVA with MCMV-mOVA**

134   MuHV-4 host colonization has 2 main components: peripheral lytic replication, and

135   latently infected B cell proliferation. Where vaccination has controlled B cell proliferation, it

136   seems to have done so by blocking lytic virus transfer to B cells[34], so reducing lytic infection

137   was our first aim. We gave MCMV-OVA or MCMV-mOVA i.p., and 6 weeks later challenged

138   the vaccinated mice or unvaccinated controls i.n. with MHV-OVA (Fig.4a). Despite inducing

139   OVA-specific immunity, neither MCMV-OVA nor MCMV-mOVA significantly reduced lytic

140   infection by MHV-OVA. Next we immunized mice with MCMV-OVA and 6 weeks later

141   challenged them i.n. with MHV-mOVA (Fig.4b). Again there was no significant reduction in

142   lytic infection, nor in virus seeding to lymphoid tissue. However when mice were primed

143   with MCMV-mOVA and challenged 6 weeks later with MHV-mOVA, there was protection

144   (Fig.4c): at day 7, both peripheral lytic replication and viral seeding to lymphoid tissue were

145   reduced. The greater efficacy of mOVA over OVA argued that for both priming and

146   challenge, CD4$^+$ T cells engage cell-associated viral antigens better than secreted antigens.

147   By day 13, lymphoid colonization in mOVA-vaccinated mice had caught up with that

148   of controls. Therefore virus-driven B cell proliferation was not blocked in mOVA-vaccinated

149   mice, and despite reduced peripheral lytic replication, enough virus reached B cells for host

150   colonization to proceed.

151

152   **Vaccinating BALB/c mice against MHV-IiOVA with MCMV-mOVA**

153   We also challenged MCMV-mOVA-primed mice with MHV-IiOVA, which loads the

154   OVA$_{323-339}$ epitope onto MHC class II of infected cells, but does not make it available for

uptake by uninfected cells. Vaccinated mice showed no reduction in peripheral lytic infection (Fig.4d). There was a small reduction in peak latent infection, but it was not sustained. Therefore despite having immunogenicity equivalent to MHV-mOVA (Fig.3c), MHV-liOVA was less efficiently controlled by primed CD4+ T cells. This result argued that MCMV-mOVA-primed CD4+ T cells did not protect by directly engaging infected targets.

Vaccinating C57BL/6 mice against MHV-mOVA with MCMV-mOVA

As mOVA includes a well-characterized, H2K^b-restricted CD8+ T cell epitope (SIINFEKL), we tested MCMV-mOVA vaccination against MHV-mOVA also in C57BL/6 (H2^b) mice (Fig.5). At 6 weeks after i.p. MCMV-mOVA, C57BL/6 mice had readily detectable OVA-specific antibodies (Fig.5a). When challenged i.n. with MHV-mOVA they showed significantly less peripheral lytic infection than did WT MCMV vaccinated controls (Fig.5b). They showed also less infection seeding to lymphoid tissue (Fig.5c). The reduction was maintained at day 14, but lost by day 21. Thus, primed OVA-specific CD8+ T cells efficiently suppressed peripheral lytic replication by MHV-mOVA, but were unable to prevent its spread to B cells and establishment of long-term latent infection. This transient protection was comparable to that obtained by priming with an immunodominant endogenous MuHv-4 epitope[9].

Vaccinating BALB/c x C57BL/6 mice against MHV-mOVA with MCMV-mOVA

C57BL/6 mice can present OVA$_{323-339}$, via IA^b. However OVA$_{323-339}$ was identified as an IA^d-restricted epitope[33], so to test protection by strong, combined CD4+ and CD8+ T cell immunity to OVA, we vaccinated BALB/c x C57BL/6 (F1) mice, which express both H2K^b and IA^d. We gave MCMV-mOVA i.p., and 6 weeks later challenged the mice i.n. with MHV-mOVA (Fig.6). OVA-specific serum IgG was evident pre-challenge (Fig.6a). At day 7 post-challenge, plaque assays showed significantly less lung and nose infection in vaccinated mice than in controls (Fig.6b).

We tested by T cell depletion prior to challenge whether this protection depended on the CD4+ or CD8+ subset. Virus titers increased significantly only if both were depleted (Fig.6c). Thus in F1 mice, MCMV-mOVA primed CD4+ and CD8+ T cells sufficiently for either to reduce peripheral MHV-mOVA replication.

186   At day 7 post-challenge, latent viral loads in mediastinal lymph nodes were also
187   significantly less in vaccinated mice then in MCMV WT infected controls (Fig.6d). Virus loads
188   in lymphoid tissue of mOVA-vaccinated mice remained significantly lower at day 13.
189   However by day 18, only their splenic viral loads were lower; and by day 22 there was no
190   significant difference between the groups. Quantitative PCR of viral DNA at day 30 and day
191   100 also showed equivalent signals between mOVA and control vaccinated mice, across a
192   range of sites. Sequencing challenge virus DNA from vaccinated mice, by ORF57/ORF58
193   locus-specific PCR, showed no loss of the OVA epitope. Therefore, while primed OVA-
194   specific CD4^{+} and CD8^{+} T cells could suppress MuHV-4 peripheral lytic infection and reduce
195   acute latency amplification in lymphoid tissue, ultimately they were unable to prevent
196   systemic infection.

197

198   **Discussion**

199   Vaccine-primed CD8^{+} T cell have protected against MuHV-4 acutely but not in the
200   long term[9,35]. CD4^{+} T cell vaccination is less well studied, and priming against an epitope in
201   gp150 has given little or no protection, even acutely[8,35]. We used OVA as a model antigen,
202   delivering it with MCMV to protect against OVA-expressing MuHV-4. MCMV-mOVA primed
203   anti-viral CD4^{+} T cells and B cells better than MCMV-OVA. MCMV degrades MHC class II in
204   infected cells[28], so we envisage that most priming was by uninfected cells, as with CD8^{+} T
205   cells[36]. Recombinant mOVA primes CD8^{+} T cells better than OVA[31], and the greater priming
206   efficacy of mOVA argued that MCMV-infected cell debris is also cross-presented better than
207   soluble protein to naive CD4^{+} T cells.

208   Primed CD4^{+} T cells protected better against MHV-mOVA than against MHV-OVA, so
209   the same preference for cell-associated antigens seemed to hold for effector CD4^{+} T cell
210   engagement. The limited capacity of mOVA-primed CD4^{+} T cells to protect against MHV-
211   IiOVA, despite its presentation of OVA$_{323\text{-}339}$, argued that they poorly kill infected cells. Thus,
212   priming against a latently expressed CD4^{+} T cell target was insufficient for protection.
213   Protective CD4^{+} T cells more effectively recognized lytically expressed mOVA. Cell type-
214   selective MHC class II disruption[37] has shown that distinct cell types drive MuHV-4-specific
215   CD4^{+} T cell priming (dendritic cell) and effector function (macrophage). Thus, we envisage

216   that primed, OVA-specific CD4^{+} T cells limited MHV-mOVA replication in the lungs by

217   engaging local macrophages that took up mOVA from infected cells.

218   Antibody helps to limit MuHV-4 replication[5,11], so CD4^{+} T cells could potentially

219   protect indirectly via B cell help. Innate immune recruitment is another possible mechanism,

220   as poor MuHV-4 control by MHC class II-deficient mice is associated with reduced NK cell

221   recruitment[37]. Antibody protects by IgG Fc receptor engagement[34,38], and NK cells mediate

222   IgG Fc receptor-dependent cytotoxicity, so there may be synergy between these pathways.

223   IFNγ activates antigen presenting cells[39], which can in turn activate and recruit NK cells[40,41],

224   so IFNγ-dependent control of MuHV-4 lung infection by CD4^{+} T cells[24] is consistent with NK

225   cell involvement. CD8^{+} T cell help by protective CD4^{+} T cells seemed less likely, as CD8^{+} T cell

226   responses to MuHV-4 are CD4^{+} T cell-independent[42], and CD8^{+} T cell depletion alone did not

227   abolish the protection of mOVA-primed mice.

228   We identified not only protection by primed anti-viral CD4^{+} T cells, but also limits on

229   their impact that were less evident in studies of B cell-deficient mice[24,25]. Specifically,

230   despite primed OVA-specific CD4^{+} T cells reducing peripheral lytic infection, they did not

231   prevent MuHV-4 colonization of lymphoid tissue, even when OVA-specific antibody and

232   CD8^{+} T cells were also primed. Anti-viral CD4^{+} T cells generally show less dramatic clonal

233   expansion than CD8^{+} T cells, and what magnitude of response vaccination should aim for is

234   unclear. OVA$_{323\text{-}339}$-specific CD4^{+} T cell priming by MCMV-mOVA was 100-fold stronger than

235   the response to recombinant gp150 in exosomes[7]; and 10-fold stronger than the primary

236   response to individual endogenous MuHV-4 epitopes[43]. Together with the evident efficacy

237   of OVA-specific CD4^{+} T cells in reducing lung infection, these results argued that CD4^{+} T cell

238   immunity to a single viral antigen is unlikely to stop γ-herpesviruses reaching B cells.

239   The consistent inadequacy of single component vaccines to prevent B cell infection

240   by MuHV-4 contrasts with the consistent efficacy of whole virus vaccines. Whole virus

241   primes CD4^{+} T cells - and other effectors - against a broad range of viral epitopes[43], so this

242   may be necessary to stop infection transfer to B cells. Possibly not enough of any single

243   epitope is presented to allow control, regardless of how many T cells have been primed

244   against it. While MuHV-4 is not EBV, they follow similar pathways of host colonization, and it

245   is unlikely that vaccination against EBV will be any easier. Therefore effective vaccination

246   against EBV may also require whole virus. Whole MuHV-4 vaccines remain effective even

247 when they lack known latency genes[44], so generating a safe live EBV vaccine may also be

248 feasible.

249

250 **Methods**

251 **Mice**

252 C57BL/6J and BALB/c mice from Animal Resource Centre, Perth, were kept at the University

253 of Queensland. Mice were infected with MCMV (10^5 p.f.u.) when 6-8 weeks old, and with

254 MuHV-4 (10^4 p.f.u.) when 6-14 weeks old, either i.n. in 30µl under isoflurane anesthesia, or

255 i.p. in 100µl. We depleted $CD4^+$ / $CD8^+$ T cells with mAbs GK1.5 / 2.43 (Bio X Cell,

256 200µg/mouse/48h, from 96h pre-infection). Flow cytometry of spleen cells confirmed that

257 depletion was >95% effective. For antibody assays, mice were bled from a tail vein (50-

258 100µl). Animal experiments were approved by the University of Queensland Animal Ethics

259 Committees in accordance with Australian National Health and Medical Research Council

260 guidelines (projects 301/13, 391/15 and 479/15). Statistical comparison was by

261 heteroscedastic Student's 2 tailed unpaired t test unless otherwise stated.

262

263 **Cells**

264 Baby Hamster Kidney (BHK-21) fibroblasts (American Type Culture Collection (ATCC) CCL-

265 10), the DO.11.10 T cell hybridoma[33], embryonic fibroblasts (MEF), NIH-3T3 cells (ATCC CRL-

266 1658), NIH-3T3-cre cells which constitutively express cre[45], BALB/c-3T3 cells (BT3) and their

267 BT3-C2TA derivative expressing the human MHC class II transactivator (C2TA) via retroviral

268 transduction[28], RAW-264 cells (ATCC TIB-71) and a similar MHC class II$^+$ RAW-C2TA

269 derivative[32], were grown in Dulbecco's Modified Eagle's Medium with 2 mM glutamine,

270 100IU/ml penicillin, 100µg/ml streptomycin, and 10% fetal calf serum (complete medium).

271

272 **Viruses**

273 All MuHV-4 recombinants were derived from a BAC-cloned genome[46], using unmutated

274 BAC-derived virus as wild-type. MHV-IiOVA co-expresses with ORF73 via an inserted internal

275 murine invariant chain with the OVA$_{323-339}$ epitope substituted for the CLIP peptide[32]. MHV-

276 OVA expresses OVA from the ORF57 / ORF58 intergenic site, via an inserted expression

277 cassette with an ectopic MuHV-4 M3 promoter[32]. MHV-mOVA was made the same way. The

278   mOVA sequence, which comprises the human transferrin receptor cytoplasmic tail and

279   transmembrane domain fused to the C-terminal 247 amino acid residues of OVA, was PCR-

280   amplified to include *Eco*RI and *Sal*I sites in the respective 5' and 3' primers and cloned into

281   *Eco*RI / *Sal*I sites between an M3 promoter (MuHV-4 genomic co-ordinates 7281–7780) and

282   a bovine growth hormone poly-adenylation site (from pcDNA3) in pSP73 (Promega)[47]. The

283   expression cassette was excised with *Bgl*II and *Xho*I, blunted with Klenow fragment DNA

284   polymerase, and cloned into a similarly blunted and phosphatased *Mfe*I site (MuHV-4

285   genomic coordinate 77176) of a *Bgl*II genomic clone (75338–78717), again in pSP73. The

286   expression cassette with its genomic flanks was then excised with *Bgl*II, cloned into the

287   *Bam*HI site of shuttle vector pST76K-SR, and recombined into BAC-cloned MuHV-4 using

288   RecA[46]. Correct insertion was checked by restriction enzyme mapping, and by DNA

289   sequencing of a PCR product spanning the genomic insertion site. Virus was reconstituted by

290   transfecting BAC DNA into BHK-21 cells with Fugene-6 (Roche Diagnostics). The loxP-flanked

291   BAC cassette of each virus was then removed by passage through NIH-3T3-cre cells, with

292   loss of the BAC-associated GFP expression cassette being evident. Virus stocks were grown

293   in BHK-21 cells. Infected cell debris was removed by centrifugation (400 × *g*, 5min), and

294   virions recovered from the cleared supernatants by ultracentrifugation (38000 × *g*, 90min).

295   All MCMVs were derived from strain K181 (Perth). MCMV-OVA, in which OVA is expressed

296   from an HCMV IE1 promoter-driven cassette inserted at the 5' end of the IE2 locus (deleting

297   its main transcription start site)[48], is described[29]. MCMV-mOVA - using the same fusion

298   protein as in MHV-mOVA - was expressed from an HCMV IE1 promoter in the same way,

299   and inserted between equivalent plasmid-cloned MCMV genomic flanks, so as to insert it

300   into the IE2 locus. Recombinants were generated by homologous recombination in NIH-3T3

301   cells, co-transfecting them with the mOVA expression cassette plus its genomic flanks, and

302   DNA from K181 MCMV with a lacZ expression cassette in the same locus. LacZ⁻ virus clones

303   were identified by β-galactosidase staining, plaque purified, and confirmed as mOVA⁺ by

304   PCR and DNA sequencing across the IE2 locus. MCMV stocks were grown in NIH-3T3 cells,

305   and concentrated from cleared supernatants by ultracentrifugation, as for MuHV-4.

306

307   **Viral infectivity assays**

308   To titer infectious MuHV-4, culture-grown stocks or freeze-thawed organ homogenates

309   were plated onto BHK-21 cell monolayers (plaque assay)[49]. To titer total reactivatable

310   MuHV-4, organs were disrupted into single cell suspensions then plated on BHK-21 cells

311   (infectious centre assay)[49]. In each case the virus-exposed monolayers were cultured for 2h,

312   overlaid with complete medium plus 0.3% carboxy-methyl-cellulose, cultured for for 4 days,

313   then fixed with 1% formaldehyde and stained with 0.1% toluidine blue for plaque counting.

314

315   **Viral Genome Quantitation**

316   MuHV-4 genomic coordinates 24832–25071 were amplified by PCR (Rotor Gene 3000,

317   Corbett Research) from 10ng DNA (Nucleospin Tissue kit, Macherey-Nagel). PCR products

318   quantified with SYBR green (Invitrogen) were compared to a standard curve of cloned

319   template amplified in parallel, and distinguished from paired primers by melting-curve.

320   Correct sizing was confirmed by electrophoresis and ethidium bromide staining. Cellular

321   DNA in the same samples was quantified by amplifying a mouse titin gene fragment.

322

323   **Immunofluorescence**

324   Cells were seeded onto glass coverslips and left to adhere (18h, 37°C, complete medium).

325   For infection, MuHV-4 or MCMV was added at the time of seeding (0.3 p.f.u. / cell). The cells

326   were cultured overnight, then fixed in PBS / 2% formaldehyde (30min, 23°C), washed in PBS

327   and blocked in PBS / 2% BSA / 0.1% Triton X-100 (30min, 23°C). Viral antigens were

328   detected with MuHV-4-specific or MCMV-specific rabbit sera, each raised by 2

329   subcutaneous inoculations of virions. The cells were washed x3 in PBS / 0.1% Tween-20.

330   OVA was detected with a rabbit polyclonal serum (AbCam). MHC class II was detected with

331   rat mAb M5/114 (BD Biosciences). Primary antibody binding was detected with Alexa488-

332   conjugated donkey anti-rat IgG pAb, Alexa488-conjugated donkey anti-rabbit IgG pAb, or

333   Alexa555-conjugated donkey anti-rabbit IgG pAb (Life Technologies). Nuclei were stained

334   with DAPI (1µg/ml). After a washing x3, the cells were mounted in ProLong Gold

335   (Invitrogen). Images were acquired on a Nikon epifluorescence microscope and analysed

336   with ImageJ.

337

338   **Immunoblotting**

339   NIH-3T3 cells infected with MCMV or MuHV-4 (1 p.f.u. / cell, 24h) were lysed in 1% Triton X-

340   100 with 150mM NacI, 50mM Tris-HCl pH=8, and protease inhibitors (Sigma Chemical Co)

341   (30min, 4°C). Cleared lysates (13,000 x g, 15 min) or infected cell supernatants were

342   denatured in Laemmli's buffer (1% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.01%

343   bromophenol blue, 100mM Tris-HCl pH=6.8) (70°C, 10min), resolved by SDS-PAGE, and

344   transferred to PVDF membranes. The membranes were blocked in 5% skimmed milk / PBS /

345   0.1% Tween-20 (1h, 23°C), probed with rabbit anti-OVA pAb (Abcam) then IRDye800 Goat-

346   anti-Rabbit Antibody IgG (LI-COR) and washed in PBS / 0.1% Tween-20. Binding was

347   detected with an Odyssey infrared imager (LI-COR).

348

349   **Flow cytometry**

350   Cells were stained for MHC class II using PerCP-Cy5.5-conjugated mAb M5/114 (BD

351   Biosciences). MuHV-4 and MCMV antigens were detected with rabbit sera raised against

352   whole virus, and OVA was detected with rabbit pAb (Abcam), each followed by Alexa-647-

353   conjugated donkey anti-rabbit IgG pAb (Invitrogen). To test CD4^{+} and CD8^{+} T cell depletions,

354   spleen cells were stained with antibodies to CD4 (fluorescein-RM4-4), and CD8β

355   (phycoerythrin-H35-17.2) (BD Biosciences). Cells were washed 2X in PBS after each

356   antibody, and analysed with an Accuri 6 flow cytometer (BD Biosciences).

357

358   **Antigen presentation assay**

359   RAW-C2TA or BT3-C2TA cells were left untreated, incubated with $OVA_{323-339}$ peptide (10μM)

360   or infected (3 p.f.u. / cell) with MuHV-4 or MCMV. After 4h the cells were washed 2x in

361   complete medium and added ($3x10^4$/well) to DO.11.10 hybridoma cells 10^5 / well). 24h later

362   culture supernatants were assayed for IL-2 content by ELISA. Nunc Maxisorp plates were

363   coated with rat anti-mouse IL-2 mAb (clone JES6–1A12, BD Biosciences) (18h, 4°C), then

364   washed x5 in PBS / 0.1% Tween-20 and blocked in PBS / 0.1% Tween-20 / 2% BSA (1hr,

365   23°C). 3-fold dilutions of culture supernatants were then added (1h, 23°C) and captured IL-2

366   was detected with biotinylated mAb JES6–5H4 (BD-Pharmingen) (1hr, 23°C), followed by

367   streptavidin-conjugated alkaline phosphatase (BD-Pharmingen), and nitrophenylphosphate

368   substrate (Sigma Chemical Co). Absorbance was read at 405nm (SPECTRA MAX 190, Bio-

369   strategy). Concentrations were calculated by comparison with a standard curve of

370   recombinant IL-2 measured in parallel.

371

372   **Antibody ELISA**

373   Nunc Maxisorp plates were coated with MCMV virion lysate, MuHV-4 virion lysate, or

374   10μg/ml OVA (Sigma Chemical Co) in 50mM $NaHCO_3$ pH=8 (18h, 4°C). The plates were

375   washed x5 in PBS / 0.1% Tween-20 and blocked with PBS / 0.1% Tween-20 / 10% skimmed

376   milk powder. 3-fold serum dilutions were incubated (1h, 23°C), then antibody binding

377   detected with alkaline phosphatase-conjugated goat anti-mouse IgG_1, IgG_{2a} or total IgG pAb

378   (Southern Biotech). Development was with nitrophenylphosphate substrate (Sigma

379   Chemical Co) and absorbance was read at 405 nm (SPECTRA MAX 190, Bio-strategy).

380

381   **IFNγ ELIspot assay**

382   Immobilon-P-bottomed plates (Merck Millipore) were coated with rat anti-mouse IFNγ mAb

383   (clone R4-6A2, BD Biosciences) (1μg/ml, 18h, 4°C). then washed x4 in PBS and blocked with

384   complete medium. As targets, naïve splenic leucocytes cells, recovered from splenic

385   homogenates by centrifugation over Ficoll (500 x g, 15min), were left untreated, infected

386   with MCMV or MuHV-4 (3 p.f.u. / cell) or incubated with $OVA_{323-339}$ peptide (10μM). After

387   incubation in complete medium (2h, 37°C) the cells were irradiated (20Gy), washed x1 and

388   added ($2x10^5$/well) to 3-fold dilutions of effector spleen cells in the αIFN-γ mAb-coated

389   plates. After incubation in complete medium (48h, 37°C) the plates were washed x3 in PBS /

390   0.1% Tween-20, and captured IFN-γ was detected with biotinylated rat anti-mouse IFNγ

391   mAb (clone XMG1.2), followed by streptavidin-conjugated alkaline phosphatase (BD

392   Biosciences). The plates were washed x6 and developed with 5-bromo-4-chloro-3-indolyl

393   phosphate / nitro-blue tetrazolium substrate (Sigma Chemical Co). Spots were counted

394   under a dissecting microscope.

395

396   **Figure Legends**

397   **Figure 1. OVA epitope expression from MCMV and MuHV-4.**

398   **a.** OVA and its membrane-bound mOVA derivative were expressed from MCMV via an

399   HCMV IE1 promoter-driven cassette, replacing a 79bp *Hpa*I fragment at the 5' end of the

400   non-coding first exon of IE2.

401   **b.** The same constructs were expressed from MuHV-4 via an M3 promoter (lytic) inserted

402   between ORFs 57/58. To express $OVA_{323-339}$ also in latency but just in infected cells, murine

403   invariant chain with $OVA_{323-339}$ replacing the CLIP peptide (IiOVA) was linked

404 transcriptionally to the ORF73 episome maintenance protein and translated via an internal

405 ribosome entry site (IRES).

406 **c.** NIH-3T3 cells either uninfected (UI) or infected (0.3 p.f.u./cell, 18h) with MHV-IiOVA,

407 MHV-mOVA or MHV-OVA were stained with antibody to OVA (αOVA) or MuHV-4 (αMuHV-

408 4). IiOVA was not detected because it includes only OVA$_{323-339}$. 2 experiments gave

409 equivalent results.

410 **d.** NIH 3T3 cells left uninfected (UI) or infected (0.3 p.f.u. / cell, 18h) with mOVA or OVA

411 expressing MCMV, were stained with antibody to OVA (αOVA) or MCMV (αMCMV). 2

412 experiments gave equivalent results.

413 **e.** Lysates and supernatants of NIH-3T3 cells either uninfected (UI) or infected with MuHV-4

414 or MCMV recombinants were immunoblotted for OVA. 2 experiments gave equivalent

415 results.

416 **f.** BALB/c 3T3 (BT3) cells and RAW-264 cells, transduced or not with retrovirus expressing

417 the human MHC class II transactivator (C2TA) were stained for MHC class II with mAb

418 M5/114. Nuclei were stained with DAPI. 2 experiments gave equivalent results.

419 **g.** MHC class II$^+$ RAW-C2TA and BT3-C2TA cells were left uninfected (UI), incubated with

420 OVA$_{323-339}$ peptide (pep) or infected with MuHV-4 or MCMV, either wild-type (WT) or

421 expressing an OVA derivative as in **a** and **b**. OVA$_{323-339}$-specific DO.11.10 cells were added,

422 and IL-2 in supernatants assayed by ELISA. 4 experiments gave equivalent results.

423 **h.** NIH-3T3 cells infected with WT, OVA or mOVA MCMV or MuHV-4 (1 p.f.u. / cell, 18h),

424 were trypsinized and assayed by flow cytometry for surface expression of viral proteins

425 (αMCMV, αMuHV-4) or OVA with polyclonal sera. MHV-mOVA but not MHV-OVA displayed

426 detectable OVA on infected cells. Despite obvious cytopathic effects, no MCMV infection

427 displayed detectable cell surface viral proteins or OVA. 3 experiments gave equivalent

428 results.

429

430 **Figure 2. *In vivo* fitness and immunogenicity of OVA-expressing MCMV.**

431 **a.** BALB/c mice were infected i.n. with WT or recombinant MCMV (OVA, mOVA). Lung and

432 salivary gland infections were quantitated by plaque assay. Symbols show individual mice (4

433 per group), crosses show means. MCMV-OVA and MCMV-mOVA titers were significantly

434 reduced in both lungs and salivary glands. 2 experiments gave equivalent results.

435   **b.** BALB/c mice were infected i.p. with WT or recombinant MCMV (4 per group). Liver,

436   spleen and salivary gland infections were quantitated by plaque assay. Again MCMV-OVA

437   and MCMV-mOVA were attenuated relative to WT, but less severely than after i.n. infection.

438   2 experiments gave equivalent results.

439   **c.** BALB/c mice were left uninfected (naive) or infected i.p. with MCMV-OVA or MCMV-

440   mOVA. MCMV-specific and OVA-specific serum IgG was measured by ELISA. MCMV-mOVA

441   induced significantly stronger OVA-specific responses than MCMV-OVA. Mean ± SD of 3

442   mice per group are shown. 3 experiments gave equivalent results.

443   **d.** BALB/c mice were infected i.p. with WT MCMV, MCMV-OVA or MCMV-mOVA. 1 month

444   later IFNγ responses of spleen cells to syngeneic splenocytes pulsed with OVA$_{323\text{-}339}$ peptide

445   (pep), infected with MCMV (vir), or left untreated (nil) were assayed by ELIspot. Bars show

446   mean ± SD of 3 mice per group. 3 experiments gave equivalent results.

447

448   **Figure 3. *In vivo* fitness and immunogenicity of OVA-expressing MuHV-4.**

449   **a.** BALB/c mice (4 per group) were infected i.n. with WT or recombinant MuHV-4 (OVA,

450   mOVA, IiOVA). Lung infection was quantitated by plaque assay and spleen infection by

451   infectious centre (i.c.) assay. The recombinant viruses showed no significant attenuation. 2

452   experiments gave equivalent results.

453   **b.** BALB/c mice left uninfected (naive) or infected i.n. with MHV-OVA or MHV-mOVA were

454   assayed for MuHV-4-specific and OVA-specific serum IgG by ELISA. MHV-mOVA induced

455   significantly stronger OVA-specific responses than MCMV-OVA. Means of 5 mice per group

456   are shown. 2 experiments gave equivalent results.

457   **c.** BALB/c mice were infected i.n. with WT or recombinant MuHV-4. 1 month later spleen

458   cell IFNγ responses to syngeneic splenocytes pulsed with OVA$_{323\text{-}339}$ peptide (pep), infected

459   with MuHV-4 (vir), or left untreated (nil) were assayed by ELIspot. Bars show mean ± SD of 5

460   mice per group. MHV-mOVA and MHV-IiOVA elicited significantly stronger OVA$_{323\text{-}339}$-

461   specific responses than MHV-OVA (p<0.01). 3 experiments gave equivalent results.

462

463   **Figure 4. Vaccination against OVA-expressing MuHV-4 by OVA-expressing MCMV.**

464   **a.** BALB/c mice left uninfected (naive) or infected i.p. with MCMV-OVA or MCMV-mOVA

465   were assayed 6 weeks later for OVA-specific serum IgG by ELISA. Bars show mean ± SD of 3

466   mice per group. The mice were then challenged i.n. with MHV-OVA and infectious virus in

467   noses and lungs plaque assayed. Symbols show individual mice, bars show means. The

468   dashed line shows the assay sensitivity limit. MCMV-OVA and MCMV-mOVA vaccine gave no

469   significant protection against MHV-OVA. 2 experiments gave equivalent results.

470   **b.** BALB/c mice (4 per group for each time point) were infected i.p. with WT MCMV or

471   MCMV-OVA. 6 weeks later they were challenged i.n. with MHV-mOVA. Infectious virus in

472   noses and lungs was measured by plaque assay. Latent virus in the superficial cervical lymph

473   nodes (SCLN), which drain the nose, and the mediastinal lymph nodes (MLN), which drain

474   the lungs, was measured by infectious centre (i.c.) assay. Neither site showed significant

475   protection. 2 experiments gave equivalent results.

476   **c.** BALB/c mice infected i.p. with WT MCMV or MCMV-mOVA were challenged i.n. 6 weeks

477   later with MHV-mOVA (5 per group for each time point). Nose and lung infections,

478   measured by plaque assay, were significantly reduced by MCMV-mOVA. At day 7, virus

479   seeding to the SCLN and MLN was also reduced. However by day 13, SCLN and MLN virus

480   loads were higher in the mOVA-primed mice, and spleen infections were equivalent to

481   controls. 2 experiments gave equivalent results.

482   **d.** BALB/c mice were infected i.p. with WT MCMV or MCMV-mOVA, and 6 weeks later

483   challenged i.n. with MHV-IiOVA (5 per group for each time point). Plaque assays showed no

484   significant protection of noses or lungs by mOVA priming. Nor did i.c. assays show less day 7

485   seeding to lymphoid tissue. At day 13 splenic viral loads were slightly lower in mOVA-primed

486   mice, but day 20 showed no difference. 2 further experiments also failed to show significant

487   protection.

488

489   **Figure 5. Protection of C57BL/6 mice against MHV-mOVA by MCMV-mOVA.**

490   **a.** C57BL/6 mice were left uninfected (naive) or infected i.p. with MCMV-mOVA or MCMV

491   WT. 6 weeks later OVA-specific serum IgG was measured by ELISA. Graphs show mean ± SD

492   of 5 mice per group. 2 experiments gave equivalent results.

493   **b.** The mice in **a** were challenged i.n. with MHV-mOVA and infectious virus in lungs

494   measured by plaque assay. Symbols show individual mice (5 per group), bars show means.

495   Dashed line = assay sensitivity limit. MCMV-mOVA induced significant protection relative to

496   WT. 3 experiments gave equivalent results.

497   **c.** I.c. assays of mice primed and challenged as in **b** (5 per group for each time point) showed

498   that the day 7 MLN and spleen infections of MCMV-mOVA-vaccinated mice were

499 significantly lower than those of controls. I.c. assays at day 14 showed that lymphoid

500 infection in MCMV-mOVA-vaccinated mice remained significantly reduced. However at days

501 day 21 and 28 there was no longer a significant difference between mOVA-vaccinated and

502 control mice. 2 experiments gave equivalent results.

503

504 **Figure 6. Protection of BALB/c x C57BL/6 mice against MHV-mOVA by MCMV-mOVA.**

505 **a.** BALB/c x C57BL/6 mice were left uninfected (naive) or infected i.p. with MCMV-mOVA or

506 MCMV WT. 6 weeks later MCMV-specific and OVA-specific serum IgG were measured by

507 ELISA (mean ± SD, 6 mice per group). 2 experiments gave equivalent results.

508 **b.** Mice immunized as in **a** were challenged i.n. with MHV-mOVA. 7 days later infectious

509 virus in noses and lungs was plaque assayed. Symbols show individual mice (6-7 per group),

510 bars show means. Dashed line = assay sensitivity limit. MCMV-mOVA protected significantly

511 relative to WT. 3 experiments gave equivalent results.

512 **c.** Mice immunized as in **a** were depleted of CD4$^+$ T cells (αCD4), CD8$^+$ T cells (αCD8) or both

513 (αCD4/8) prior to challenge (5 per group). Protection was maintained in all but the αCD4/8

514 mice. 2 experiments gave equivalent results.

515 **d.** Mice (6-8 per group for each time point, pooled from 3 experiments) were immunized

516 and challenged as in **b**. I.c. assays at day 7 showed significantly less MHV-mOVA MLN

517 infection after immunization with MCMV-mOVA. SCLN and spleen infections were not yet

518 detectable. At day 13, i.c. assays showed a significant reduction in all lymphoid infections by

519 immunization with MCMV-mOVA. However by day 18 only spleen infection was lower in the

520 immunized mice and day 22 showed no significant difference.

521 **e.** Mice (5 per group for each time point) were immunized and challenged as in **b**. Infection

522 was measured at day 30 and day 100 by quantitative PCR of viral DNA, expressed relative to

523 the cellular DNA content of each sample. MCMV-mOVA-immunized and control mice were

524 equivalent. 2 further experiments confirmed equivalent long-term MHV-mOVA DNA loads

525 between mOVA-primed and control mice.

526

527 **Acknowledgements**

530 DP190101851) and by Queensland Health. D0.11.10 cells were kindly provided by Prof. P.

531 Marrack (University of Colorado).

532

Conflict of interest statement

534

536

References

538 [1]Cohen JI, Mocarski ES, Raab-Traub N, *et al.*. The need and challenges for development of

539 an Epstein-Barr virus vaccine. *Vaccine* 2013; **31 Suppl 2:** B194-196.

540 [2]Thorley-Lawson DA, Hawkins JB, Tracy SI, *et al*. The pathogenesis of Epstein-Barr virus

541 persistent infection. *Curr Opin Virol* 2013; **3:** 227-232.

542 [3]Stevenson PG, Simas JP, Efstathiou S. Immune control of mammalian γ-herpesviruses:

543 lessons from murid herpesvirus-4. *J Gen Virol* 2009; **90:** 2317-2330.

544 [4]Barton E, Mandal P, Speck SH. Pathogenesis and host control of gammaherpesviruses:

545 lessons from the mouse. *Annu Rev Immunol* 2011; **29:** 351-397.

546 [5]Tibbetts SA, McClellan JS, Gangappa S, *et al*. Effective vaccination against long-term γ-

547 herpesvirus latency. *J Virol* 2003; **77:** 2522-2529.

548 [6]McClellan JS, Tibbetts SA, Gangappa S, *et al*. Critical role of CD4 T cells in antibody-

549 independent vaccination against γ-herpesvirus latency. *J Virol* 2004; **78:** 6836-6845.

550 [7]May JS, Stevenson PG. Vaccination with murid herpesvirus-4 glycoprotein B reduces viral

551 lytic replication but does not induce detectable virion neutralization. *J Gen Virol* 2010; **91:**

552 2542-2552.

553 [8]Ruiss R, Ohno S, Steer B, *et al.* Murine γ-herpesvirus 68 glycoprotein 150 does not

554 contribute to latency amplification in vivo. *Virol J* 2012; **9:** 107.

555 [9]Stevenson PG, Belz GT, Castrucci MR, *et al*. A γ-herpesvirus sneaks through a CD8^{+} T cell

556 response primed to a lytic-phase epitope. *Proc Natl Acad Sci USA* 1999; **96:** 9281-9286.

557 [10]Usherwood EJ, Ward KA, Blackman MA, et al. Latent antigen vaccination in a model

558 gammaherpesvirus infection. *J Virol* 2001; **75:** 8283-8288.

559 [11]Stevenson PG, Cardin RD, Christensen JP, Doherty PC. Immunological control of a murine

560 gammaherpesvirus independent of CD8^{+} T cells. *J Gen Virol* 1999; **80:** 477-483.

561 [12]Tan CSE, Lawler C, Stevenson PG. CD8^{+} T cell evasion mandates CD4^{+} T cell control of

562 chronic γ-herpesvirus infection. *PLoS Pathog* 2017; **13:** e1006311.

563 [13]Haque T, Wilkie GM, Jones MM, *et al.* Allogeneic cytotoxic T-cell therapy for EBV-positive

564 posttransplantation lymphoproliferative disease: results of a phase 2 multicenter clinical

565 trial. *Blood* 2007; **110:** 1123-1131.

566 [14]Carneiro-Sampaio M, Coutinho A. Immunity to microbes: lessons from primary

567 immunodeficiencies. *Infect Immun* 2007; **75:** 1545-1555.

568 [15]Jonjić S, Mutter W, Weiland F, *et al*. Site-restricted persistent cytomegalovirus infection

569 after selective long-term depletion of CD4+ T lymphocytes. *J Exp Med* 1989; **169:** 1199-1212.

570 [16]Stuller KA, Flaño E. CD4 T cells mediate killing during persistent gammaherpesvirus 68

571 infection. *J Virol* 2009; **83:** 4700-4703.

572 [17]Paludan C, Bickham K, Nikiforow S, *et al.* Epstein-Barr nuclear antigen 1-specific CD4^{+} Th1

573 cells kill Burkitt's lymphoma cells. *J Immunol* 2002; **169:** 1593-1603.

574 [18]Mautner J, Pich D, Nimmerjahn F, *et al*. Epstein-Barr virus nuclear antigen 1 evades direct

575 immune recognition by CD4^{+} T helper cells. *Eur J Immunol* 2004; **34:** 2500-2509.

576 [19]Mautner J, Bornkamm GW. The role of virus-specific CD4^{+} T cells in the control of Epstein-

577 Barr virus infection. *Eur J Cell Biol* 2012; **91:** 31-35.

578 [20]Babcock GJ, Decker LL, Freeman RB, *et al*. Epstein-Barr virus-infected resting memory B

579 cells, not proliferating lymphoblasts, accumulate in the peripheral blood of

580 immunosuppressed patients. *J Exp Med* 1999; **190:** 567-576.

581 [21]Unanue ER. Perspective on antigen processing and presentation. *Immunol Rev* 2002; **185:**

582 86-102.

583 [22]Shresta S, Pham CT, Thomas DA, *et al*. How do cytotoxic lymphocytes kill their targets?

584 *Curr Opin Immunol* 1998; **10:** 581-587.

585 [23]Keckler MS. Dodging the CTL response: viral evasion of Fas and granzyme induced

586 apoptosis. *Front Biosci* 2007; **12:** 725-732.

587 [24]Christensen JP, Cardin RD, Branum KC, *et al.* CD4^{+} T cell-mediated control of a γ-

588 herpesvirus in B cell-deficient mice is mediated by IFN-γ. *Proc Natl Acad Sci USA* 1999; **96:**

589 5135-5140.

590 [25]Sparks-Thissen RL, Braaten DC, Hildner K, *et al.* CD4 T cell control of acute and latent

591 murine γ-herpesvirus infection requires IFN-γ. *Virology* 2005; **338:** 201-208.

592   [26]Lucin P, Pavić I, Polić B, *et al.* γ-interferon-dependent clearance of cytomegalovirus

593   infection in salivary glands. *J Virol* 1992; **66:** 1977-1984.

594   [27]Lawler C, Milho R, May JS, *et al.* Rhadinovirus host entry by co-operative infection. *PLoS*

595   *Pathog* 2015; **11:** e1004761.

596   [28]Yunis J, Farrell HE, Bruce K, *et al.* Murine cytomegalovirus degrades MHC class II to

597   colonize the salivary glands. *PLoS Pathog* 2018; **14:** e1006905.

598   [29]Cunningham PT, Lloyd ML, Harvey NL, *et al*. Promoter control over foreign antigen

599   expression in a murine cytomegalovirus vaccine vector. *Vaccine* 2010; **29:** 141-151.

600   [30]Teasdale RD, D'Agostaro G, Gleeson PA. The signal for Golgi retention of bovine beta 1,4-

601   galactosyltransferase is in the transmembrane domain. *J Biol Chem* 1992; **267:** 4084-4096.

602   [31]Li M, Davey GM, Sutherland RM, *et al.* Cell-associated ovalbumin is cross-presented much

603   more efficiently than soluble ovalbumin in vivo. *J Immunol* 2001; **166:** 6099-6103.

604   [32]Smith CM, Rosa GT, May JS, *et al*. CD4+ T cells specific for a model latency-associated

605   antigen fail to control a γ-herpesvirus in vivo. *Eur J Immunol* 2006; **36:** 3186-3197.

606   [33]Shimonkevitz R, Colon S, Kappler JW, *et al*. Antigen recognition by H2-restricted T cells. II.

607   A tryptic ovalbumin peptide that substitutes for processed antigen. *J Immunol* 1984; **133:**

608   2067-2074.

609   [34]Glauser DL, Milho R, Lawler C, *et al*. Antibody arrests γ-herpesvirus olfactory super-

610   infection independently of neutralization. *J Gen Virol* 2019; **100:** 246-258.

611   [35]Liu L, Usherwood EJ, Blackman MA, *et al.* T-cell vaccination alters the course of murine

612   herpesvirus 68 infection and the establishment of viral latency in mice. *J Virol* 1999; **73:**

613   9849-9857.

614   [36]Busche A, Jirmo AC, Welten SP, *et al.* Priming of CD8+ T cells against cytomegalovirus-

615   encoded antigens is dominated by cross-presentation. *J Immunol* 2013; **190:** 2767-2677.

616   [37]Lawler C, Stevenson PG. A CD4+ T cell-NK cell axis of γ-herpesvirus control. *J Virol* 2020; **94:**

617   e01545-19.

618   [38]Wright DE, Colaco S, Colaco C, *et al.* Antibody limits in vivo murid herpesvirus-4 replication

619   by IgG Fc receptor-dependent functions. *J Gen Virol* 2009; **90:** 2592-2603.

620   [39]Boehm U, Klamp T, Groot M, *et al.* Cellular responses to interferon-γ. *Annu Rev Immunol*

621   1997; **15:** 749-795.

622   [40]Andrews DM, Scalzo AA, Yokoyama WM, *et al*. Functional interactions between dendritic

623   cells and NK cells during viral infection. *Nat Immunol* 2003; **4:** 175-181.

624 [41]Bihl F, Germain C, Luci C, *et al*. Mechanisms of NK cell activation: CD4^{+} T cells enter the

625 scene. *Cell Mol Life Sci* 2011; **68:** 3457-3467.

626 [42]Stevenson PG, Belz GT, Altman JD, *et al*. Virus-specific CD8^{+} T cell numbers are maintained

627 during γ-herpesvirus reactivation in CD4-deficient mice. *Proc Natl Acad Sci USA* 1998; **95:**

628 15565-15570.

629 [43]Freeman ML, Burkum CE, Cookenham T, *et al.* CD4 T cells specific for a latency-associated

630 γ-herpesvirus epitope are polyfunctional and cytotoxic. *J Immunol* 2014; **193:** 5827-5834.

631 [44]Lawler C, Simas JP, Stevenson PG. Vaccine protection against murid herpesvirus-4 is

632 maintained when the priming virus lacks known latency genes. *Immunol Cell Biol* 2020; **98:**

633 67-78.

634 [45]Stevenson PG, May JS, Smith XG, *et al.* K3-mediated evasion of CD8^{+} T cells aids

635 amplification of a latent γ-herpesvirus. *Nat Immunol* 2002; **3:** 733-740.

636 [46]Adler H, Messerle M, Wagner M, *et al*. Cloning and mutagenesis of the murine γ-

637 herpesvirus 68 genome as an infectious bacterial artificial chromosome. *J Virol* 2000; **74:**

638 6964-6974.

639 [47]May JS, Coleman HM, Boname JM, *et al*. Murine γ-herpesvirus-68 ORF28 encodes a non-

640 essential virion glycoprotein. *J Gen Virol* 2005; **86:** 919-928.

641 [48]Messerle M, Keil GM, Koszinowski UH. Structure and expression of murine

642 cytomegalovirus immediate-early gene 2. *J Virol* 1991; **65:** 1638-1643.
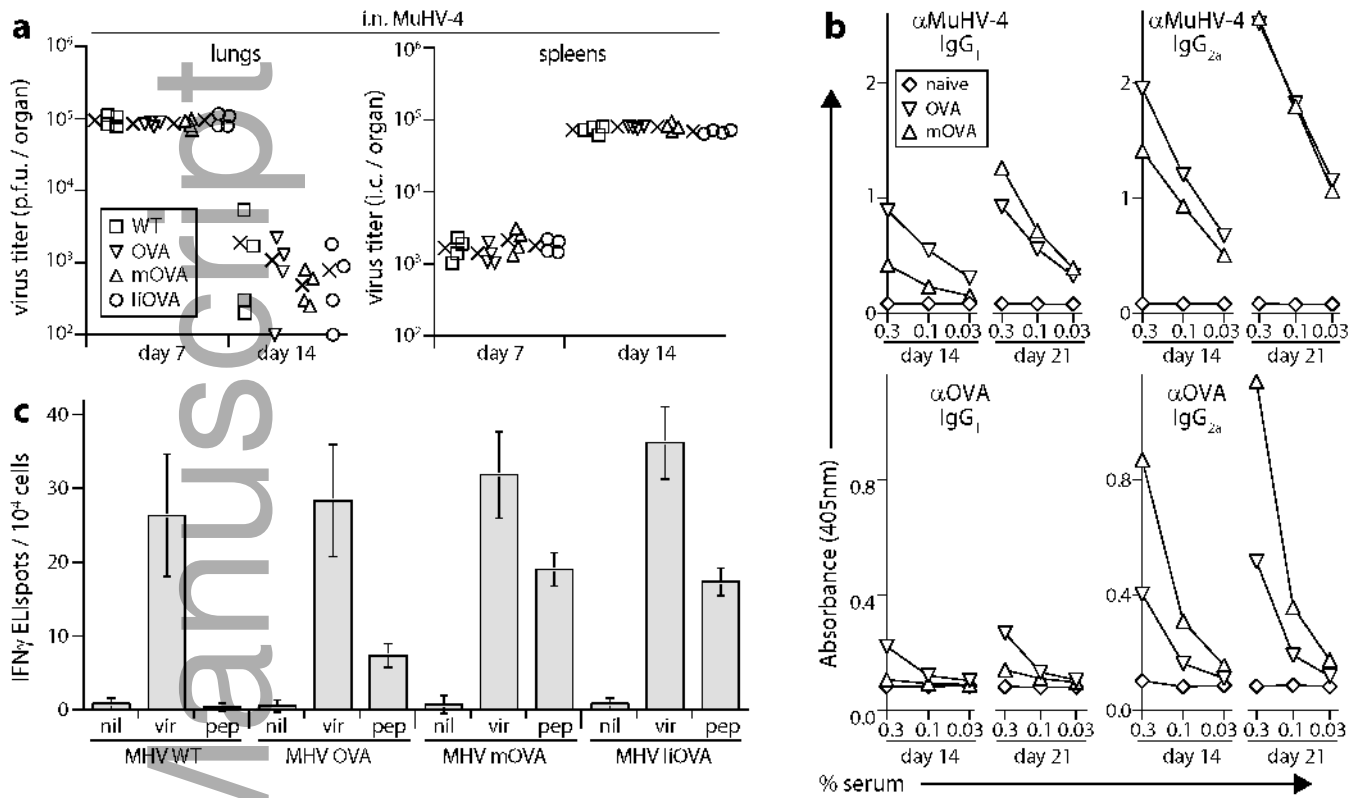
643 [49]de Lima BD, May JS, Stevenson PG. Murine γ-herpesvirus 68 lacking gp150 shows defective

644 virion release but establishes normal latency in vivo. *J Virol* 2004; **78:** 5103-5112.
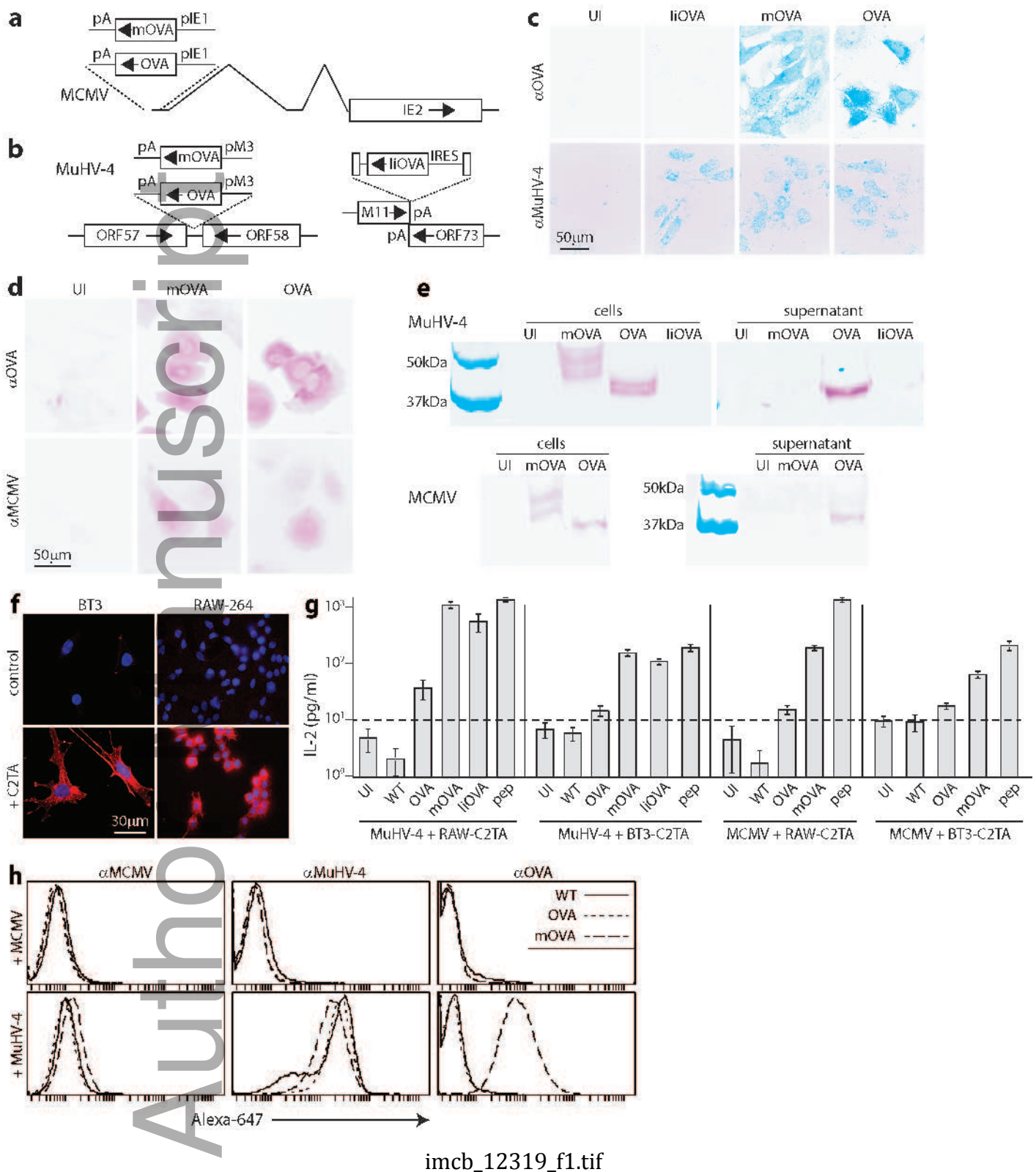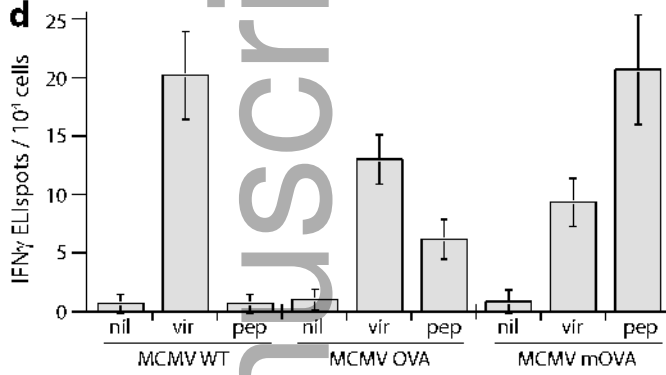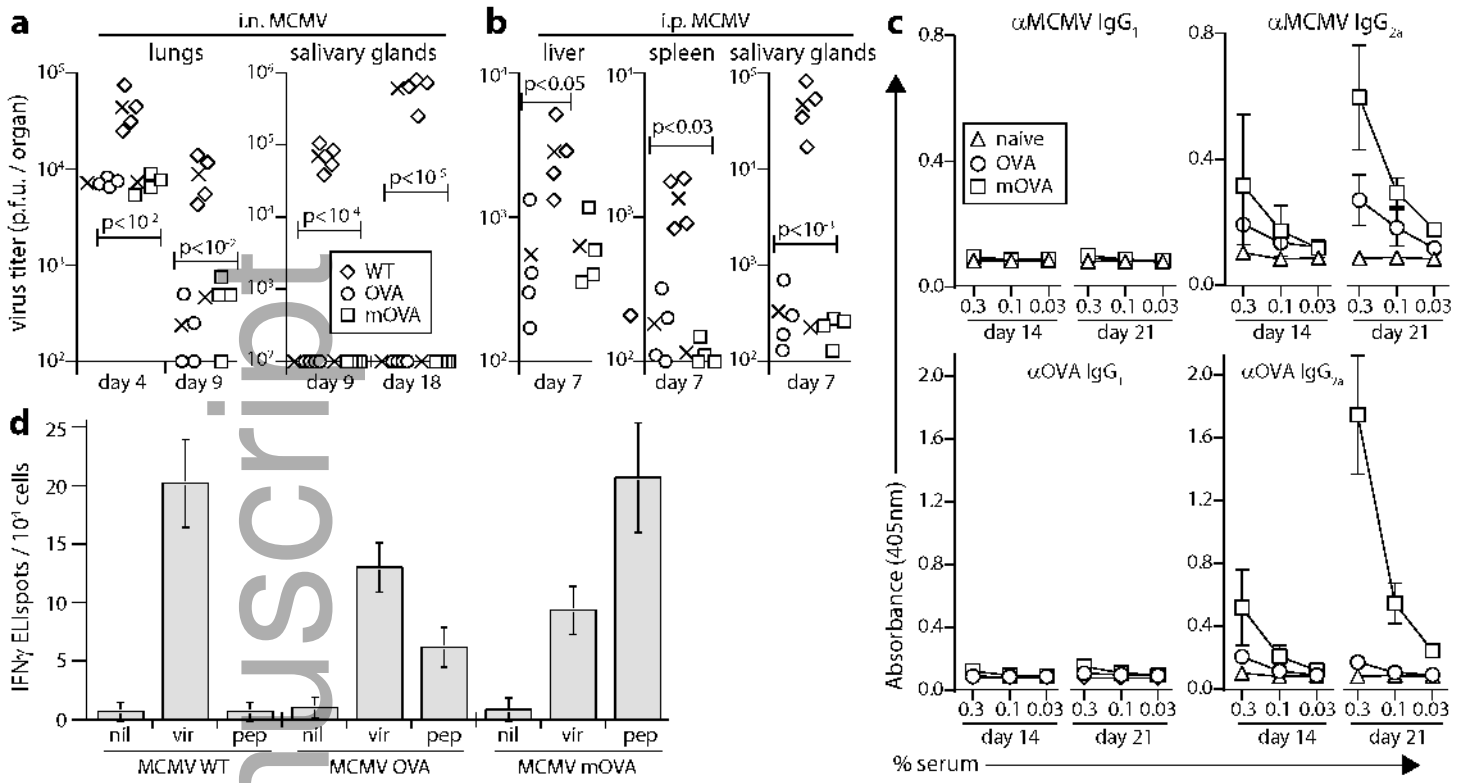
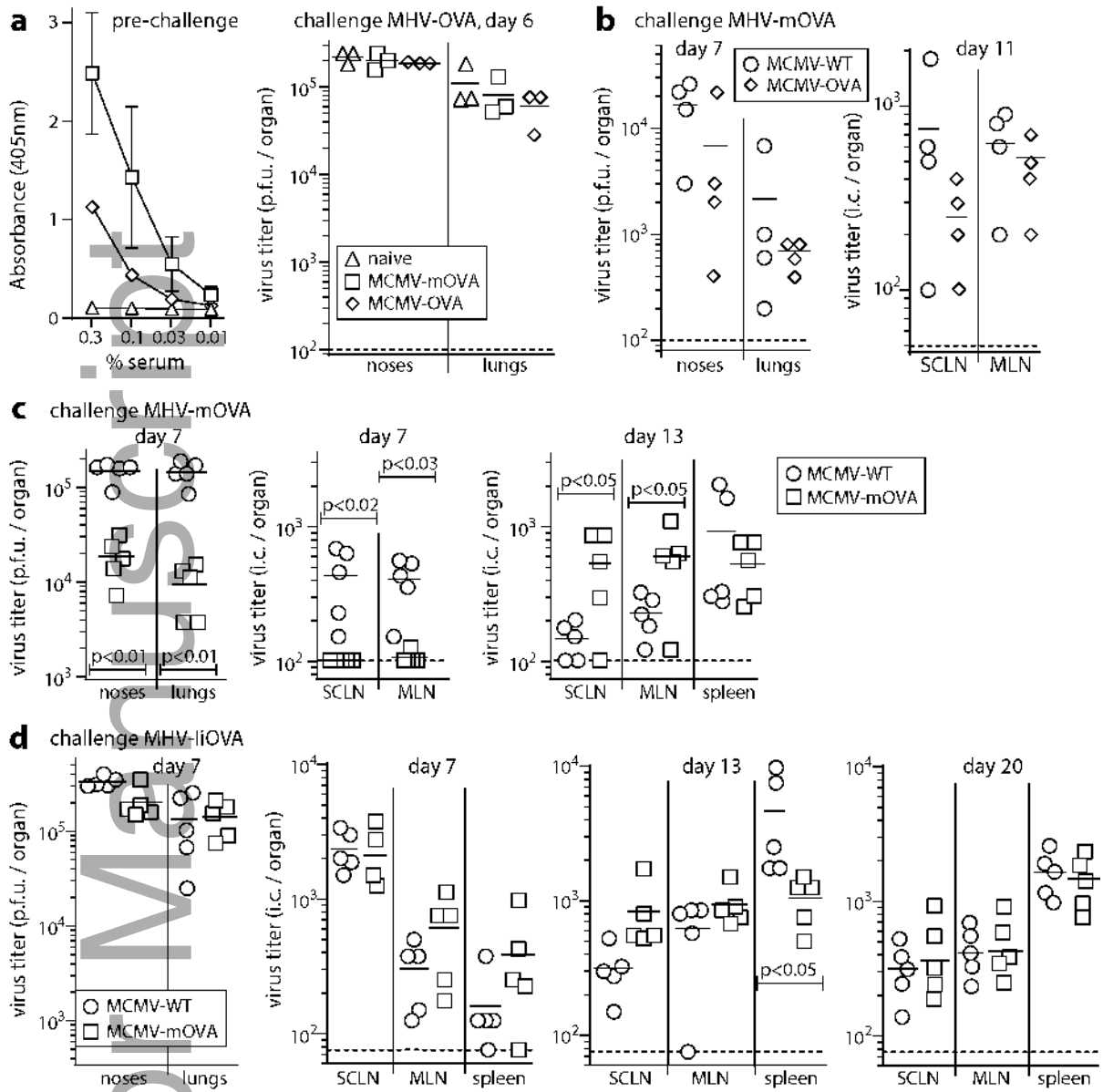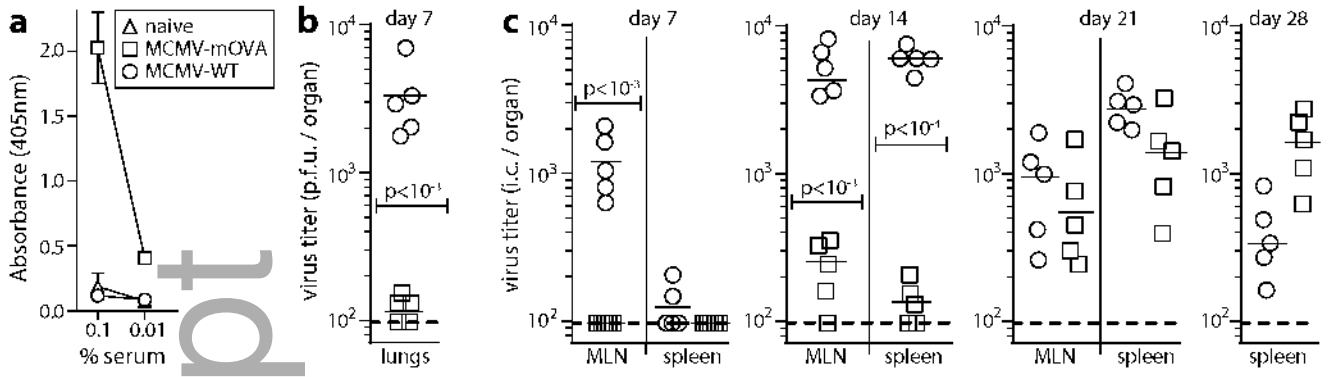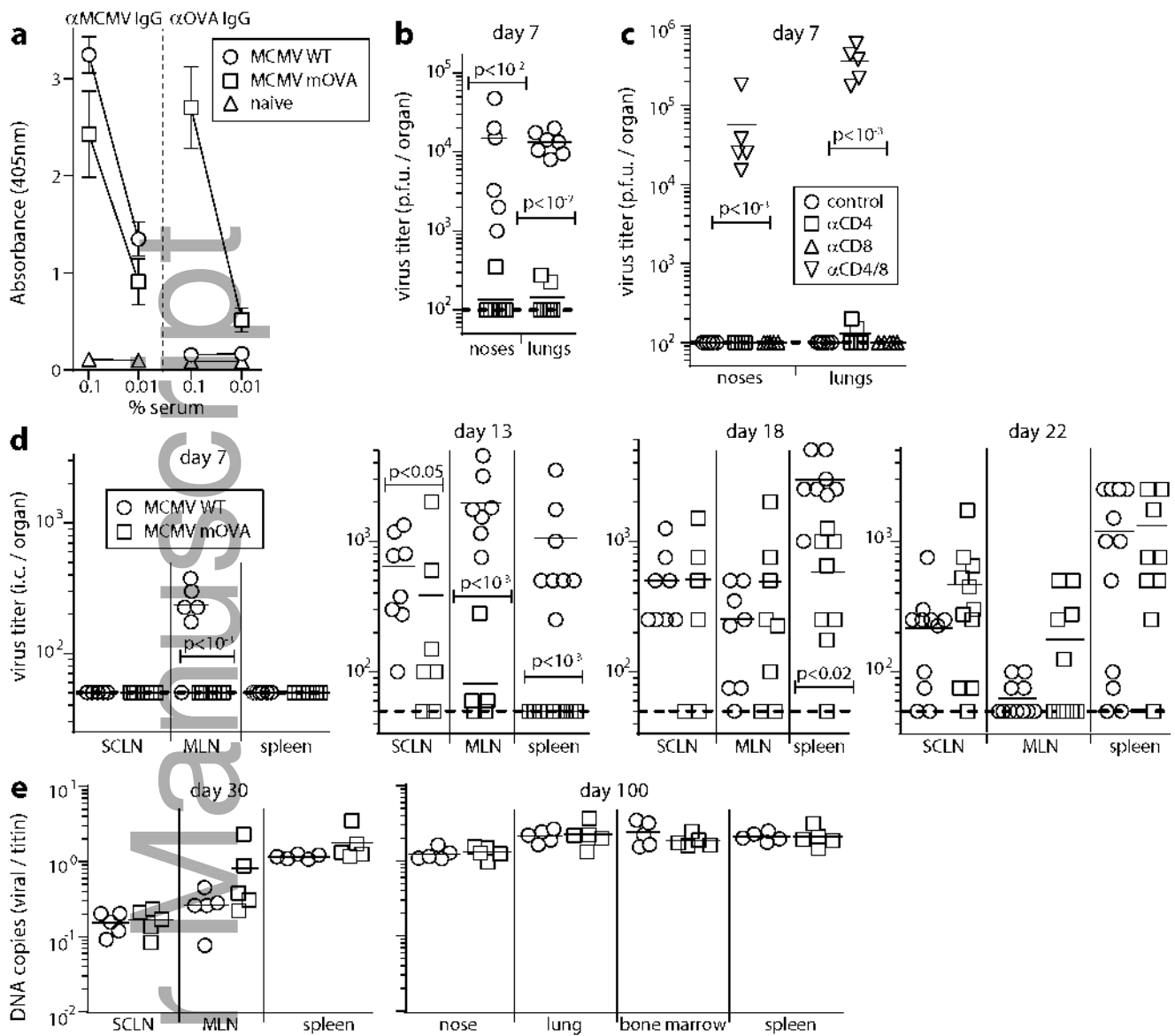imcb_12319_f1.tif

imcb_12319_f2.tif

Figure 3

imcb_12319_f3.tif

imcb_12319_f4.tif

imcb_12319_f5.tif

imcb_12319_f6.tif

Author/s:
Yunis, J; Redwood, AJ; Belz, GT; Stevenson, PG

Title:
Membrane association of a model CD4(+) T-cell vaccine antigen confers enhanced yet incomplete protection against murid herpesvirus-4 infection

Date:
2020-04-01

Persistent Link:
http://hdl.handle.net/11343/275474

File Description:
Accepted version