1   Classification: BIOLOGICAL SCIENCES: Immunology & Inflammation

2   **Protection from cytomegalovirus viraemia following glycoprotein B**

3   **vaccination is not dependent on neutralising antibodies**

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Abstract (243)

Human cytomegalovirus (HCMV) is an important pathogen in transplant patients and in congenital infection. Previously, we demonstrated that vaccination with a recombinant viral glycoprotein B (gB)/MF59 adjuvant formulation before solid organ transplant reduced viral load parameters post-transplant. Reduced post-transplant viraemia was directly correlated with antibody titres against gB consistent with a humoral response against gB being important. Here we show that sera from the vaccinated seronegative patients displayed little evidence of a neutralising antibody response against cell-free HCMV in vitro. Additionally, sera from seronegative vaccine recipients had minimal effect on the replication of a strain of HCMV engineered to be cell-associated in a viral spread assay. Furthermore, although natural infection can induce antibody dependent cellular cytotoxicity (ADCC) responses, serological analysis of seronegative vaccinees again presented no evidence of a substantial ADCC-promoting antibody response being generated de novo. Finally, analyses for responses against major antigenic domains of gB following vaccination were variable and their pattern was distinct when compared to natural infection. Taken together, these data argue that the protective effect elicited by the gB vaccine is via a novel mechanism of action in seronegative vaccinees that cannot be explained by neutralisation or the induction of ADCC. More generally, these data, which are derived from a human challenge model that demonstrated that the gB vaccine is protective, highlight the need for more sophisticated analyses of new HCMV vaccines over and above the quantification of an ability to induce potent neutralising antibody responses in vitro.

Significance Statement (114)

Conventionally, vaccines are screened for induction of a neutralising antibody response in human volunteers before proceeding to late stage clinical trials. We present results from a human cytomegalovirus (HCMV) challenge study suggesting that this paradigm may not apply universally to all viruses. Instead viruses like HCMV, which establish lifelong infections and grow both cell-free and cell-associated, may be controlled independently of a potent neutralising antibody response. Our results suggest that more detailed laboratory studies are required to identify correlates of immune protection for such viruses and failure of a vaccine to induce a neutralising antibody response should not necessarily be considered as a key go-no-go decision point in the design of future vaccine studies.

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Introduction

71 Human cytomegalovirus (HCMV) causes substantial morbidity in multiple patient

72 populations with impaired or immature immune responses (1, 2). The threat posed

73 during organ transplantation or congenital infection led to HCMV vaccine

74 development being categorised as the highest priority (3). Several vaccines against

75 HCMV (from whole virus, DNA and viral subunits) have been studied in different

76 patient cohorts establishing, in general, that a vaccination strategy targeted against

77 HCMV is a viable option with major clinical implications (4-10).

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79 One vaccine target is the viral glycoprotein B (gB) which has been shown to be

80 partially protective in three phase 2 clinical trials when presented with MF59 adjuvant

81 (6, 9, 11). The gB protein is an essential virion component required for viral entry

82 (12, 13) and represents a major target of the humoral immune response, including

83 neutralisation (14-16). Conventionally, neutralising antibody titres have been

84 considered the benchmark by which vaccines are assessed as this represents a

85 potent anti-viral mechanism. However, the humoral immune response is far more

86 complex and can produce antibodies that can drive antibody dependent cell

87 cytoxicity (ADCC), that can bind to both the pathogen directly or to the target antigen

88 expressed on the infected cell surface to recruit complement and promote pathogen

89 or cell lysis, they can promote pathogen phagocytosis as well as modulate the

90 downstream response of both the adaptive and innate immune responses (17). Here

91 we report data showing limited evidence of a neutralising antibody response as a

92 correlate of protection for the gB vaccine in a phase 2 study where transplant

4

patients were challenged with wild type HCMV (9). An inability to detect evidence of neutralisation of clinical Merlin was consistent with previous data demonstrating no effect of antibody plus complement against the laboratory strain Towne in a classical plaque assay (9).Furthermore, we provide evidence that the humoral response against gB induced in seronegatives by vaccination displays a distinct biological spectrum compared to that observed in naturally infected seropositives.

Results

Sera from seronegative vaccinated patients do not neutralise HCMV infection in single round infection assays

Note that throughout this paper the term seronegative refers to patients who were seronegative before being given gB/MF59 vaccine or placebo. We tested for evidence of neutralisation of HCMV infection using a high throughput assay that measured the establishment of a lytic infection by enumeration of immediate-early (IE) positive cells (Fig. 1). Two anti-gB monoclonal antibodies (ITC88; an anti-AD-2 antibody demonstrated to prevent gB fusion post binding (23) and 2F12; a commercial monoclonal antibody against an unspecified region of gB) inhibited HCMV infection in a concentration-dependent manner (Fig.1a). Next we tested a panel of sera from our vaccine study under the same conditions. Prior to vaccination, sera from seronegative individuals had no impact on HCMV infection. Importantly, no evidence of activity against HCMV was observed in this assay when seronegative sera post vaccination was assessed (Fig. 1b; Fig. S1a-d) even when exogenous complement was added to the sera prior to infection (Fig. S2). In contrast, sera from seropositive patients had inherent neutralising activity against HCMV prior to

117   transplantation (Fig. 1b; Fig. S1e-h) but no evidence of increased neutralising

118   capacity was observed post vaccination (Fig. 1b; Fig. S1e-h).

119

120   To address the possibility that the sera from seronegative vaccinees contained

121   antibodies capable of inducing abortive/quiescent infections, as proposed for

122   varicella infection (18), a parallel analysis was performed that measured pp28 (a viral

123   late gene) positivity (Fig. 1c,d). Unsurprisingly, pp28 positive cells were rare in the

124   ITC88 control (Fig. 1c) given that IE positive cells were rarely seen (Fig. 1b). In

125   contrast, and consistent with the IE data, no effect on pp28 positivity was observed

126   using the sera from vaccinated seronegative transplant recipients (Fig. 1c; Fig. S3).

127

128   **Sera from vaccinated seronegative patients do not inhibit spread of cell-**

129   **associated Merlin strain of HCMV in fibroblast monolayers.**

130   Our first assays measured the ability of sera to limit infection of cells with large titres

131   of cell-free virus by measuring the number of IE positive cells 24hpi. To investigate

132   whether sera had activity against cell-associated HCMV we assessed their impact on

133   the growth of HCMV in fibroblast cultures using a viral spreading assay seeded at

134   low MOI. To be able to ask this question, we utilised a Merlin-IE2-GFP virus

135   engineered to grow predominantly in a cell-associated fashion (19). The expression

136   of GFP with IE2 kinetics allows real time imaging and enumeration of the spread of

137   the virus as we visualise in real time the increase in the number of infected cells over

138   time and thus monitor the spread of the virus through the fibroblast monolayer (Fig.

139   2a). Firstly, we measured the ability of ITC88 to limit spread of this cell-associated

140   virus. The data show that ITC88 had minimal impact on spread with the number of

6

141 infected cells increasing with culture time which would be consistent with cell-
142 associated virus being resistant to neutralisation (Fig. 2b). Importantly, ITC88 could
143 effectively limit the spread of a high passage Merlin strain that grows predominantly
144 cell-free and thus is functional in this viral spreading assay (Fig. 2c). Similar data
145 were also observed with healthy donor sera whereby seropositive sera were far less
146 potent against the spread of the IE2-GFP virus whereas healthy donor seronegative
147 sera had no effect (Fig. 2d). Having established a baseline for the assay, we next
148 analysed the sera from the vaccine study. The data show that seropositive sera did,
149 on average, impact on the spread of Merlin-IE2-GFP to similar levels to those
150 observed with the control sera from natural seropositives (Fig. 2e; Fig. S4a-d). In
151 contrast, sera from seronegative individuals had no effect on viral spread in this
152 assay both prior to and post vaccination (Fig. 2e; Fig. S4e-h). Furthermore, the data
153 also demonstrated that vaccination of the seropositives did not enhance the
154 moderate inhibition of viral spread observed with the seropositive sera prior to
155 vaccination (Fig. 2e; Fig. S4a-d).

156

157 **Vaccination does not induce an antibody repertoire capable of promoting a**
158 **measurable ADCC response**

159 A lack of evidence to support potent neutralisation led us to investigate other
160 antibody effector mechanisms. ADCC involves antibody recognition of an epitope
161 and the subsequent recruitment of cellular effector functions (e.g. NK cells) to kill the
162 infected cell. To allow for a high throughput screen of our sera for any potential
163 ADCC promoting activity we developed an in vitro assay based on a previous study
164 for antibodies directed against influenza proteins (20). Recombinant vaccine gB was

165 immobilised and incubated with PBMC from healthy donors in the presence of sera.

166 We then analysed NK cells by flow cytometry for evidence of CD107a expression - a

167 classic marker of degranulation of NK cells. Validation of the assay utilised

168 PMA/Ionomycin - a potent activator of NK cell degranulation whereby, in the

169 presence of these activators, CD107a surface expression on CD56+ NK cells was

170 significantly upregulated (Fig. 3a). With this assay we could observe a differential

171 CD107a phenotype between healthy donor seropositive and seronegative sera (Fig.

172 3b; Fig. S5a,b). Having established the conditions, we tested the sera from our

173 longitudinal vaccine study. Evidence of ADCC promoting antibodies was evident in

174 the seropositive patient sera both pre and post vaccination (Fig. S5c-e). However

175 there was no evidence that vaccination boosted pre-existing responses in these

176 seropositive individuals nor were levels of ADCC promoting antibodies correlated

177 with protection from viraemia (Fig. 3c,d).

178

179 We next asked whether any effect of vaccination in seronegatives was evident. As

180 expected, no ADCC effect was evident in the seronegative samples at baseline (i.e.

181 pre-vaccination; Fig. 4a-d). The analysis of longitudinal samples post-vaccination

182 revealed no evidence that vaccination consistently elicited detectable levels of anti-

183 gB antibodies capable of inducing ADCC right up to the day of transplantation (Fig.

184 4a-d).

185

186 **Distinct antibody responses against gB epitopes in vaccinated individuals**

187 Our inability to detect evidence for neutralising or ADCC effector functions

188 associated with protection in the seronegative vaccine recipients led us to investigate

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189 the composition of the humoral response against key antigenic domains (AD) of

190 HCMV. In a parallel study of seropositive individuals we have evidence that reduced

191 viraemia post-transplant correlates with higher antibody levels against AD-2 (21)

192 consistent with this epitope being considered an important target for antibody

193 responses (22). Thus we asked whether vaccination of seronegatives induced

194 specific antibody responses against known antigenic domains of gB. ELISA assays

195 were performed on serial samples of sera from seronegatives pre and post

196 vaccination (Fig. 5). The data show that vaccination elicited limited responses

197 against the known ADs with no responses detectable at all against AD-2 (Fig. 5cd)

198 nor AD-4 (Fig. 5e,f). In contrast, AD-1 and AD-5 responses were observed in certain

199 individuals but these did not correlate with protection (Fig. 5a,b,g & h). Thus, unlike

200 for seropositives, no direct correlate of protection could be established with well-

201 defined ADs of gB.

202 .

203 **Discussion**

204 The administration of a subunit vaccine based on the key viral glycoprotein B of

205 HCMV is a potent inducer of anti-gB antibodies (6, 9, 11). Furthermore, the level of

206 these antibodies correlated with reduced viral load parameters in a randomised

207 phase 2 trial in solid organ transplant recipients (9). These data support the concept

208 that the induction of a potent humoral response against gB represents a good

209 strategy to protect from HCMV disease. However, despite this understanding of

210 improved clinical outcome, the mechanistic basis of protection is still not fully

211 understood.

212

213 Classically, the induction of potent neutralising antibody responses has been

214 considered the gold standard for evaluating any vaccine strategy (23, 24). Indeed, a

215 number of successful vaccination programmes have utilised vaccines that do exactly

216 this (24). However, in this study we could provide no supporting evidence for a

217 potent neutralising antibody response as an explanation for the success of the gB

218 HCMV vaccine. The data show that the sera of seropositive transplant recipients

219 possessed neutralising antibodies but these were not detectably enhanced by

220 vaccination with gB/MF59. Most likely, these potent antibodies are a composite of

221 anti-gB and other major glycoprotein targets including the trimer gH/gL/gO and also

222 the pentameric complex (25). Consistent with these being targets for neutralisation

223 are data that demonstrate monoclonal antibodies directed against gH or the

224 pentameric complex neutralise infection effectively (26-28). Recent work has

225 demonstrated that cell-associated HCMV growth is largely resistant to the activity of

226 Cytotect (a heterogeneous mix of anti-HCMV antibodies) presumably because the

227 physical state of the virus denies access to neutralising antibodies (19) consistent

228 with a previous report (29). Our data presented here support those observations; a

229 minor effect of seropositive sera on decreasing the rate of spread in vitro could be

230 explained by small amounts of cell-free virus made by the Merlin-IE2-GFP strain of

231 HCMV.

232

233 It is likely then that biphasic modes of growth (i.e. cell-free and cell-associated) in

234 vivo would argue that an effective vaccine against HCMV could be dependent on the

235 induction of multiple humoral effector functions. Thus, while there is still a role for a

236 vaccine that can induce neutralising antibody responses, these clinical trial data

237   argue that a vaccine against HCMV can be effective despite an inability to detect a

238   potent neutralising response associated with it. More generally, they reinforce the

239   value of assessing vaccination strategies using challenge models. A recent study in

240   mice concluded that vaccination with AD-2 was not useful because a poor

241   neutralising response was elicited. However, it was never addressed whether the

242   vaccination with AD-2 was protective against CMV challenge (30). Indeed, a recent

243   study presents data implicating a role for both neutralising and non-neutralising gB

244   antibody responses in the MCMV challenge model (31). Furthermore, this concept

245   may not be restricted to HCMV because human studies of a candidate HIV vaccine

246   reported that a major component of the anti-viral humoral response correlated with

247   ADCC (32, 33).

248

249   In contrast to acute viral infections, HCMV persists for the lifetime of the host in the

250   face of a prodigious immune response (33). HCMV encodes multiple immune

251   evasion genes to facilitate lifelong survival in the host and ability to re-infect new

252   hosts even those with pre-existing natural immunity against HCMV. This illustrates

253   the complex interactions of HCMV with the immune response and the ability of this

254   virus to persist in the face of a potent immune response may impact on the ability to

255   produce a sterilising vaccine based solely on the induction of neutralising antibodies.

256   Put simply, sera from seropositives are potently neutralising in vitro but re-infection

257   with HCMV is possible in vivo. Consequently, we investigated the ability of sera from

258   vaccinated patients to enhance antibody dependent responses. NK cells can be

259   recruited in an antibody dependent manner to promote cellular cytotoxicity. HCMV

260   encodes a number of NK immune evasion genes that suggests this is an important

261 functional interaction (34). Furthermore, the NK cell repertoire in HCMV seropositive

262 individuals is dominated by subsets of NK cells – with an implication of NK cell

263 memory (35). Whether these NK cell subsets are elite controllers of HCMV or

264 instead, reflect a virally induced reprogramming remains an important open question.

265 Clearly, seropositives invoke anti-gB responses that could direct NK cell mediated

266 ADCC based on our work. However, we could not attribute the success of the

267 vaccine to this so that, while anti-gB antibodies exist that promote ADCC, we could

268 provide no evidence that this explained the protection afforded by the vaccine. The

269 development of antibodies that promote ADCC responses may be triggered following

270 initial exposure to the pathogen or a focusing of the immune response through

271 multiple episodes of reactivation. A vaccine clearly does not deliver these additional

272 exposures to the immune system. Indeed, the vaccine delivers gB in the absence of

273 other pathogen-encoded functions and thus, potentially, presents gB in a unique

274 way. Whether this allows potent anti-HCMV responses to develop more effectively

275 than they would in the context of infection is an important question for vaccine

276 studies to address. Finally, it is important to avoid suggesting that ADCC responses

277 have no role to play. Our data show that ADCC responses directed against gB are

278 not detectable (seronegative vaccinees), boosted (seropositive vaccinees) or

279 correlate with protection (seropositive patients cohort). However, they do not rule out

280 ADCC responses against other HCMV antigens being important for control in natural

281 infection.

282

283 Although the mechanistic correlate of protection remains to be determined, it is

284 evident that the gB HCMV vaccine is protective (6, 9, 11). Interestingly, the epitope

285   analysis points towards the exciting hypothesis that a novel epitope may be

286   responsible. The vaccine gB is modified in the transmembrane domain as well

287   through the loss of the furin cleavage site and is thought to exist in a post-fusion

288   form. All these differences may result in the presentation of novel epitopes of gB not

289   normally exposed in the virion but transiently exposed during the entry process or in

290   HCMV infected cells. Studies are ongoing to test the hypothesis of novel epitopes

291   being presented by the vaccine form of gB.

292

293   In conclusion, the data in this human challenge model demonstrate that the

294   effectiveness of the gB vaccine is imparted by a novel mechanism and not wholly

295   reliant on the classic biological activity of neutralisation.

296

297   **Materials & Methods**

298   The study was approved by the UCL Research Ethics Committee and all patients

299   whose samples were investigated here gave written informed consent (9).

300   To assess sera for neutralising capacity, HCMV was pre-incubated with sera for 1

301   hour and then the whole sample used to infect HFFs. Alternatively, virus was

302   incubated with anti-gB antibody 2F12 (abcam) or anti- AD-2 monoclonal antibody

303   ITC88 (22, 36) After 24 hours cells were fixed and stained for IE gene expression

304   using anti-IE (Millipore; 1:1000) and goat anti-mouse Alexafluor 568nm (Life

305   Technologies; 1:1000). Alternatively, an anti-pp28 antibody (Santa Cruz; 1:1000)

306   was used to stain cells fixed at 72hpi and detected with the same secondary

antibody. Nuclei were counterstained with DAPI (SIGMA). Percentage infection was enumerated using Hermes WiScan instruments and software.

Either a high passage Merlin (grows cell free) or an IE2-GFP virus engineered to grow predominantly cell associated (a kind gift of Richard Stanton (19)) was used to infect HFFs at an MOI of 0.01. Cells were either fixed and stained for IE (Merlin) or visualised for GFP expression (IE2-GFP) between 1-14 days post infection. Nuclei were counterstained with DAPI (SIGMA). Percentage infection was enumerated using Hermes WiScan instruments and software.

To assay for ADCC promoting antibodies, total PBMC or purified NK cells (MACS NK cell isolation kit II; Miltenyi Biotec) from seronegative healthy donors was used. Briefly, 96 well plates were coated with gB vaccine protein (0.75ug/well) and then incubated with sera diluted in PBS as described. Either PBMC or NK cells were added to the wells and, 48 hours later, the cells harvested and stained for CD3, CD56 and CD107a expression (BD biosciences) and enumerated by Flow cytometry. Stimulation with PMA and Ionomycin was used as a positive control and healthy seronegative donor sera as a negative. Additionally, sera isolated pre-vaccination from seronegatives was used as a baseline negative response.

ELISAs for AD1,2, 4 and 5 have been described previously (15). AD1 and AD2 are non-structured epitopes and it is well established that the peptides are recognised by AD1 and AD2 antibody responses. The recombinant AD4 used has been shown to be recognised by known AD4 conformational antibodies and the structure of the AD5 antigen has been shown to have the same structure as AD5 in gB (15, 37, 38). Briefly, sera was diluted in PBS as described and then incubated with peptide coated 96 well plates. Healthy seropositive and seronegative sera were used as controls.

Anti-human IgG conjugated to HRP was used to detect CMV antibodies and visualised using TMB substrate. OD was measured at 450nm. Visit 1 (e.g. pre-vaccination of seronegative patients) was set as background/baseline.

Figure Legends

Figure 1 Vaccination does not promote neutralising antibody responses in seronegatives a) HCMV was incubated for 1 hour with different concentrations of monoclonal antibodies against gB (ITC88 and 2F12), IgG1 isotype control or media and then used to infect HFFs (MOI=0.5). Percentage IE positivity was scored 24hpi. n=3. **b)** HCMV was incubated for 1 hour with sera from seropositive or seronegative patients either vaccinated with gB or given placebo and then used to infect HFFs (MOI=1). The analysis was performed on sera isolated pre-vaccination and at day of transplant (post vaccination). Percentage IE positivity was scored 24hpi and further stratified into patients who developed viraemia. n=3. **c)** HCMV was incubated for 1 hour with sera from seronegative patients either vaccinated with gB or given placebo

354 and then used to infect HFFs (MOI=1). The analysis was performed on sera isolated

355 pre-vaccination and at day of transplant (post vaccination). Percentage pp28

356 positivity was scored 120hpi and further stratified into patients who developed

357 viraemia. n=3.

358

359 **Figure 2 Sera from vaccinated seronegatives does not control the spread of**

360 **cell associated HCMV in vitro a)** HFFs were infected with Merlin-IE2-GFP

361 (MOI=0.01) and progress of infection monitored for two weeks. Representative

362 images of GFP expression at 1, 6, 9 and 14 days post infection are shown. Cells

363 were counterstained with DAPI to show cell layers. **b-c)** HFFs infected with Merlin-

364 IE2-GFP (B) or Merlin (C) were, 24hpi, incubated with ITC88 (100ug/ml) and viral

365 spread assay 2 weeks post infection. GFP (Cell associated) or IE immunostaining

366 (Cell Free) was used to calculate percentage infection. n=3. **d)** HFFs infected with

367 Merlin (cell free) or Merlin-IE2-GFP (cell associated) were, 24hpi, incubated with no

368 sera (control), seropositive or seronegative sera. After 2 weeks GFP (Cell

369 associated) or IE immunostaining (Cell Free) was used to calculate percentage

370 infection. n=3. **e)** HFFs infected with Merlin-IE2-GFP (cell associated) were, 24hpi,

371 incubated with no sera (infected cells), healthy donor seropositive (HCMV(+)) or

372 seronegative (HCMV(-)) sera. Alternatively, they were incubated with sera from

373 either seropositive or seronegative patients given gB vaccine or placebo. Sera pre-

374 vaccination and at day of transplant (post-vaccination) was analysed. After 2 weeks

375 GFP (Cell associated) was used to calculate percentage infection. n=3. Patients

376 were further stratified into those who experienced viraemia versus those that did not.

377

Figure 3. Increased ADCC antibody responses against gB are not detected in seropositives a) Gating strategy to study evidence of ADCC activity. NK cells defined as CD56+CD3- were then assayed for CD107a expression or IFNg. PMA/Ionomycin was used as a positive control. **b)** Titration of healthy donor sera from seropositive and seronegative donors for ability to promote CD107a expression on NK cells. **c-d)** Summary of data of ADCC responses in seropositive liver and kidney organ recipients at time of transplant. Comparisons between placebo and vaccination or viraemia or no viraemia shown. n=3

Figure 4. Vaccination does not induce detectable ADCC antibody responses against gB in seronegatives a-d) Longitudinal sera samples from multiple visits were analysed for ADCC promoting activity. Samples were pre-vaccination (v#1), or 1 (v#2), 2 (v#3), 6 (v#4), 7 (v#5) months post vaccination or time of transplant (d0) or 7 days post transplant (d7). Baseline negative controls are shown using unstimulated cells or healthy donor seronegative sera and PMA/Ionomycin served as positive control.

Figure 5. Vaccination induces a pattern of epitope responses distinct from natural infection A-H) ELISA assays were performed on sera pre-vaccination (0 months) or 1,2,6 and 7 months post vaccination. Pre-vaccination represents background. ELISA ODs for anti-AD1 (a), AD2 (c), AD4 (e) and AD5 (g) responses are shown. Alternatively, data was stratified using outcome post transplant (b,d,f,h) to assess impact of responses on viraemia. Statistical significance was measured using non-parametric Mann-U Whitney test. N.S. = non significant.

References

1.   Legendre C & Pascual M (2008) Improving outcomes for solid-organ transplant recipients at risk from cytomegalovirus infection: late-onset disease and indirect consequences. *Clin Infect Dis* 46(5):732-740.

2.   Griffiths P, Baraniak I, & Reeves M (2015) The pathogenesis of human cytomegalovirus. *J Pathol* 235:288-297.

3.   Arvin AM, Fast P, Myers M, Plotkin S, & Rabinovich R (2004) Vaccine development to prevent cytomegalovirus disease: report from the National Vaccine Advisory Committee. *Clin Infect Dis* 39(2):233-239.

4.   Bernstein DI*, et al.* (2009) Randomized, double-blind, Phase 1 trial of an alphavirus replicon vaccine for cytomegalovirus in CMV seronegative adult volunteers. *Vaccine* 28(2):484-493.

5.   Plotkin SA*, et al.* (1994) Multicenter trial of Towne strain attenuated virus vaccine in seronegative renal transplant recipients. *Transplantation* 58(11):1176-1178.

6.   Pass RF*, et al.* (2009) Vaccine prevention of maternal cytomegalovirus infection. *N Engl J Med* 360(12):1191-1199.

7.   Schleiss MR (2016) Cytomegalovirus vaccines under clinical development. *J Virus Erad* 2(4):198-207.

8.   Adler SP*, et al.* (1995) Immunity induced by primary human cytomegalovirus infection protects against secondary infection among women of childbearing age. *J Infect Dis* 171(1):26-32.

9.   Griffiths PD*, et al.* (2011) Cytomegalovirus glycoprotein-B vaccine with MF59 adjuvant in transplant recipients: a phase 2 randomised placebo-controlled trial. *Lancet* 377(9773):1256-1263.

10. Schleiss MR (2009) VCL-CB01, an injectable bivalent plasmid DNA vaccine for potential protection against CMV disease and infection. *Curr Opin Mol Ther* 11(5):572-578.

11. Bernstein DI*, et al.* (2016) Safety and efficacy of a cytomegalovirus glycoprotein B (gB) vaccine in adolescent girls: A randomized clinical trial. *Vaccine* 34(3):313-319.

12. Isaacson MK, Juckem LK, & Compton T (2008) Virus entry and innate immune activation. *Curr Top Microbiol Immunol* 325:85-100.

13. Isaacson MK & Compton T (2009) Human cytomegalovirus glycoprotein B is required for virus entry and cell-to-cell spread but not for virion attachment, assembly, or egress. *J Virol* 83(8):3891-3903.

14. Rasmussen L, Matkin C, Spaete R, Pachl C, & Merigan TC (1991) Antibody response to human cytomegalovirus glycoproteins gB and gH after natural infection in humans. *J Infect Dis* 164(5):835-842.

15. Potzsch S*, et al.* (2011) B cell repertoire analysis identifies new antigenic domains on glycoprotein B of human cytomegalovirus which are target of neutralizing antibodies. *PLoS Pathog* 7(8):e1002172.

16. Banks T*, et al.* (1989) A major neutralizing domain maps within the carboxyl-terminal half of the cleaved cytomegalovirus B glycoprotein. *J Gen Virol* 70 (Pt 4):979-985.

17. Lu LL, Suscovich TJ, Fortune SM, & Alter G (2018) Beyond binding: antibody effector functions in infectious diseases. *Nat Rev Immunol* 18(1):46-61.

18. Shiraki K*, et al.* (2011) Neutralizing anti-gH antibody of Varicella-zoster virus modulates distribution of gH and induces gene regulation, mimicking latency. *J Virol* 85(16):8172-8180.

19. Murrell I*, et al.* (2017) The pentameric complex drives immunologically covert cell-cell transmission of wild-type human cytomegalovirus. *Proc Natl Acad Sci U S A* 114(23):6104-6109.
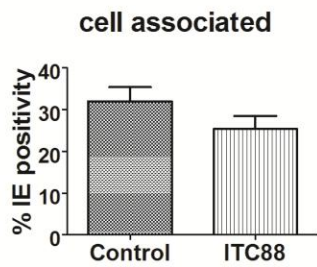
451 20. Jegaskanda S*, et al.* (2013) Cross-reactive influenza-specific antibody-dependent cellular
452   cytotoxicity antibodies in the absence of neutralizing antibodies. *J Immunol* 190(4):1837-
453   1848.
454 21. Baraniak I*, et al.* (2018) Epitope-Specific Humoral Responses to Human Cytomegalovirus
455   Glycoprotein-B Vaccine with MF59: Anti-AD2 Levels Correlate with Protection from
456   Viremia. *J Infect Dis*.
457 22. Ohlin M, Sundqvist VA, Mach M, Wahren B, & Borrebaeck CA (1993) Fine specificity of the
458   human immune response to the major neutralization epitopes expressed on
459   cytomegalovirus gp58/116 (gB), as determined with human monoclonal antibodies. *J Virol*
460   67(2):703-710.
461 23. Kwong PD, Mascola JR, & Nabel GJ (2013) Broadly neutralizing antibodies and the search
462   for an HIV-1 vaccine: the end of the beginning. *Nat Rev Immunol* 13(9):693-701.
463 24. Burton DR (2002) Antibodies, viruses and vaccines. *Nat Rev Immunol* 2(9):706-713.
464 25. Cui X, Meza BP, Adler SP, & McVoy MA (2008) Cytomegalovirus vaccines fail to induce
465   epithelial entry neutralizing antibodies comparable to natural infection. *Vaccine*
466   26(45):5760-5766.
467 26. Fouts AE*, et al.* (2014) Mechanism for neutralizing activity by the anti-CMV gH/gL
468   monoclonal antibody MSL-109. *Proc Natl Acad Sci U S A* 111(22):8209-8214.
469 27. Manley K*, et al.* (2011) Human cytomegalovirus escapes a naturally occurring neutralizing
470   antibody by incorporating it into assembling virions. *Cell Host Microbe* 10(3):197-209.
471 28. Kabanova A*, et al.* (2014) Antibody-driven design of a human cytomegalovirus
472   gHgLpUL128L subunit vaccine that selectively elicits potent neutralizing antibodies. *Proc
473   Natl Acad Sci U S A* 111(50):17965-17970.
474 29. Jacob CL*, et al.* (2013) Neutralizing antibodies are unable to inhibit direct viral cell-to-cell
475   spread of human cytomegalovirus. *Virology* 444(1-2):140-147.
476 30. Finnefrock AC*, et al.* (2016) Preclinical evaluations of peptide-conjugate vaccines targeting
477   the antigenic domain-2 of glycoprotein B of human cytomegalovirus. *Hum Vaccin
478   Immunother* 12(8):2106-2112.
479 31. Bootz A*, et al.* (2017) Protective capacity of neutralizing and non-neutralizing antibodies
480   against glycoprotein B of cytomegalovirus. *PLoS Pathog* 13(8):e1006601.
481 32. Corey L*, et al.* (2015) Immune correlates of vaccine protection against HIV-1 acquisition. *Sci
482   Transl Med* 7(310):310rv317.
483 33. Haynes BF*, et al.* (2012) Immune-correlates analysis of an HIV-1 vaccine efficacy trial. *N
484   Engl J Med* 366(14):1275-1286.
485 34. Jackson SE, Mason GM, & Wills MR (2011) Human cytomegalovirus immunity and immune
486   evasion. *Virus Res* 157(2):151-160.
487 35. Muntasell A, Vilches C, Angulo A, & Lopez-Botet M (2013) Adaptive reconfiguration of the
488   human NK-cell compartment in response to cytomegalovirus: a different perspective of
489   the host-pathogen interaction. *Eur J Immunol* 43(5):1133-1141.
490 36. Reeves MB, Breidenstein A, & Compton T (2012) Human cytomegalovirus activation of ERK
491   and myeloid cell leukemia-1 protein correlates with survival of latently infected cells. *Proc
492   Natl Acad Sci U S A* 109(2):588-593.
493 37. Wiegers AK, Sticht H, Winkler TH, Britt WJ, & Mach M (2014) Identification of a
494   neutralizing epitope within antigenic domain 5 of glycoprotein B of human
495   cytomegalovirus. *J Virol* 89(1):361-372.
496 38. Spindler N*, et al.* (2014) Structural basis for the recognition of human cytomegalovirus
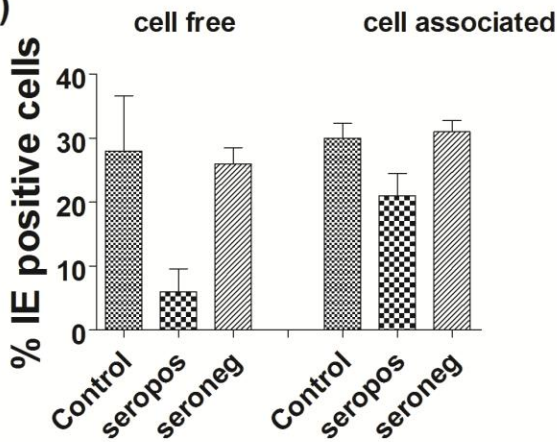497   glycoprotein B by a neutralizing human antibody. *PLoS Pathog* 10(10):e1004377.
498
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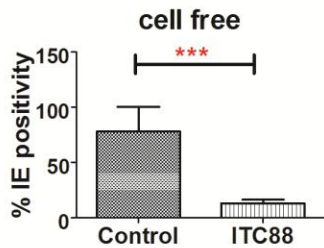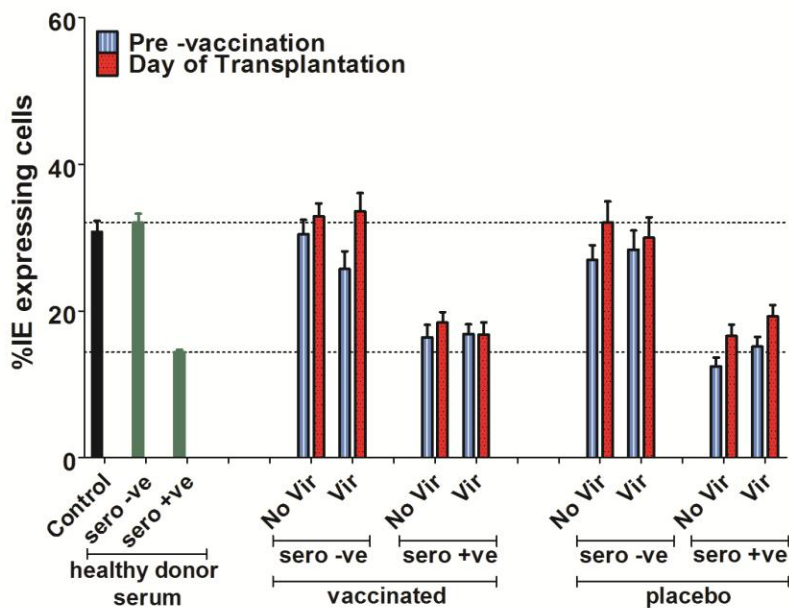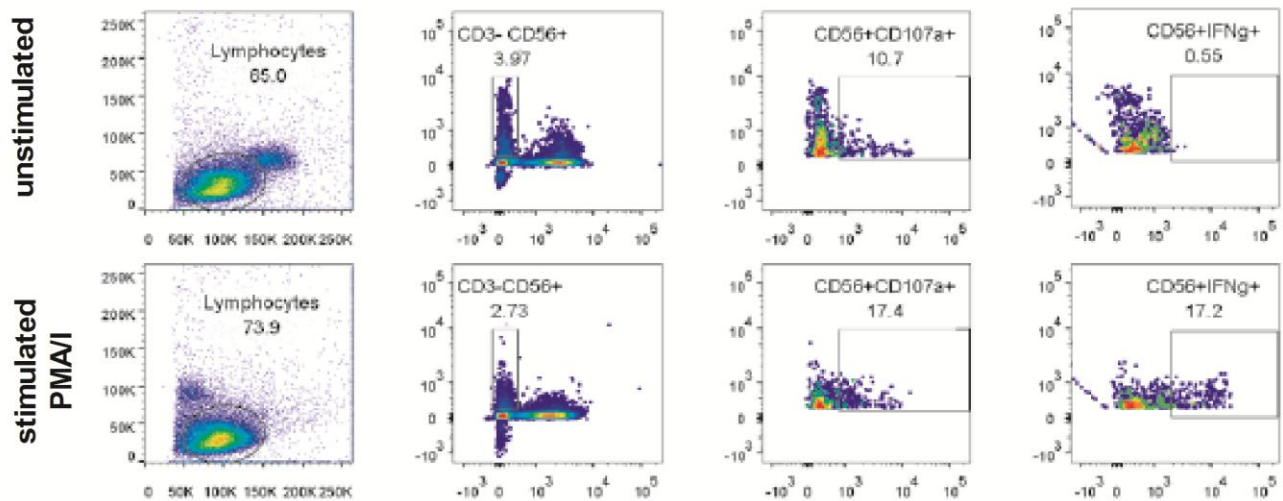
a)

b) IE Positivity

c) pp28 Positivity

a)

	1dpi	6dpi	9dpi	14dpi
DAPI staining				
GFP-IE2 expression				

b) cell associated

c) cell free

d) cell free   cell associated

e)

a)

unstimulated / stimulated PMA/I

Lymphocytes 65.0 | CD3- CD56+ 3.97 | CD56+CD107a+ 10.7 | CD56+IFNg+ 0.55

Lymphocytes 73.9 | CD3-CD56+ 2.73 | CD56+CD107a+ 17.4 | CD56+IFNg+ 17.2

b)

Serum Titration

- • HCMV seropositive donors
- □ HCMV seronegative donors

%CD3-CD56+CD107a +

neat-seropos, neat-seroneg (***), 1/5 seropos, 1/5 seroneg (**), 1/10 seropos, 1/10 seroneg (**), 1/20 seropos, 1/20 seroneg (**)

c)

Liver Transplants

%CD56+CD107+ Cells

placebo, vaccine (ns), viraemia, no viraemia (ns)

d)

Renal Transplants

%CD56+CD107a+ cells

placebo, vaccine (**), viraemia, no viraemia (ns)

A — Liver Transplant Cohort I

%CD56+CD107a+

- vaccinated
- placebo
- Unstimulated
- PMA/I

controls, v#1, v#2, v#3, v#4, v#5, dO, d7

B — Liver Transplant Cohort II

%CD56+CD107a+

- vaccinated
- placebo
- Unstimulated
- PMA/I

controls, v#1, v#2, v#3, v#4, v#5, D0, D7

C — Renal Transplants Cohort I

%CD56+CD107a+

- vaccinated
- placebo
- Unstimulated
- PMA/I
- Autologous

controls, v#1, v#2, v#3, v#4, v#5, dO, d7

D — Renal Transplants Cohort II

%CD56+CD107a+

- vaccinated
- placebo
- Unstimulated
- PMA/I
- Autologous

controls, v#1, v#2, v#3, v#4, v#5, dO, d7

A AD-1 ELISA — Anti-gB AD1 ab level, OD; months post vaccination; vaccinated: renal transplant recipient, liver transplant recipient

B AD-1 ELISA — Anti-gB AD1 ab level, OD; months post vaccination; no viraemia, viraemia; ns

C AD-2 ELISA — Anti-gB AD2 ab level, OD; months post vaccination; vaccinated: renal transplant recipient, liver transplant recipient

D AD-2 ELISA — Anti-gB AD2 ab level, OD; months post vaccination; no viraemia, viraemia; ns

E AD-4 ELISA — Anti-gB AD4 ab level, OD; months post vaccination; vaccinated: renal transplant recipient, liver transplant recipient

F AD-4 ELISA — Anti-gB AD4 ab level, OD; months post vaccination; no viraemia, viraemia; ns

G AD-5 ELISA — Anti-gB AD5 ab level, OD; months post vaccination; vaccinated: renal transplant recipient, liver transplant recipient

H AD-5 ELISA — Anti-gB AD5 ab level, OD; months post vaccination; no viraemia, viraemia; ns

Figure S1

Figure S1

Figure S2

Figure S3

Figure S4

Figure S4

Figure S5

e) Renal Transplant Patients, HCMV seropositive (II)
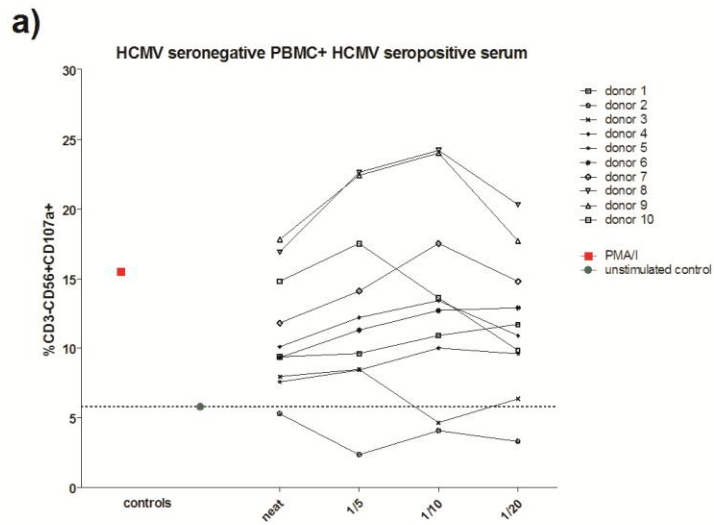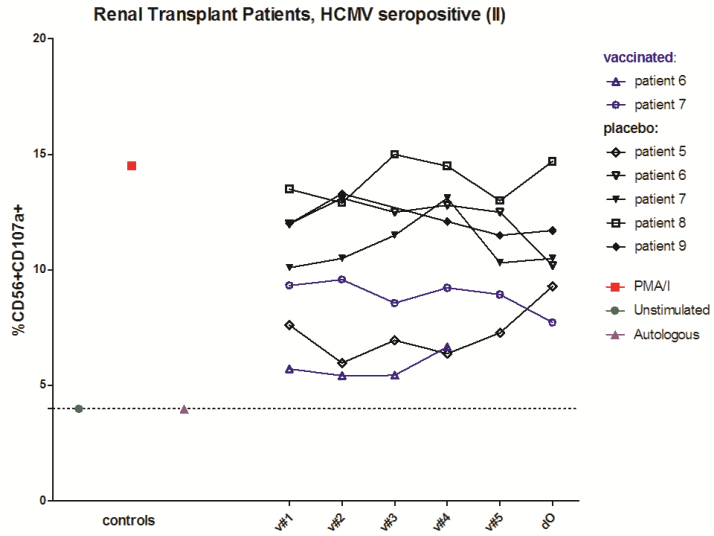
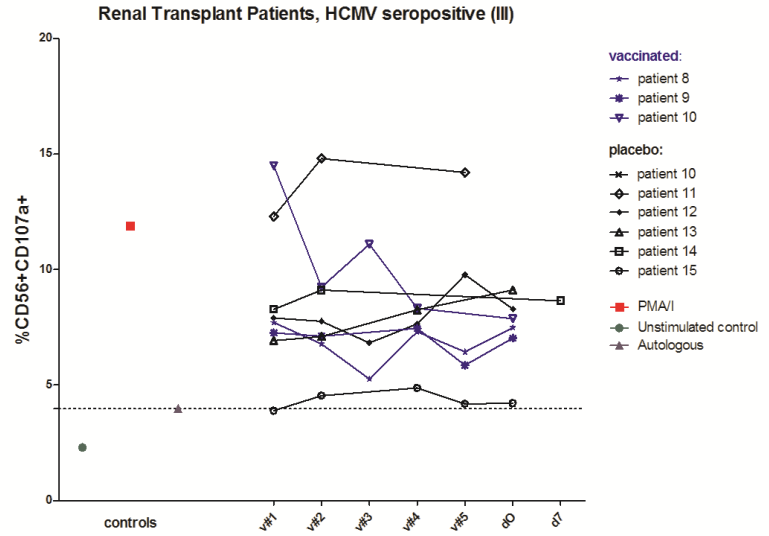**f)** Renal Transplant Patients, HCMV seropositive (III)

Figure S5

Supplementary Figure Legends

Figure S1. Vaccination does not induce detectable neutralizing antibody responses in seronegative vaccine recipients and does not boost pre-existing neutralizing antibody responses in seropositive vaccinees. Merlin was incubated with sera from seronegative (a-d) and seropositive (e-h) renal (a,b,e,f) and liver (c,d,g,h) transplant recipients or an ITC88 positive control (green bar), and used to inoculate HFFs *in vitro* (MOI=1). Infection was assayed by IE immunostaining 24hpi and the proportion of infected cells calculated by counterstaining nuclei with DAPI. Sera isolated pre-vaccination (baseline – blue bars) or post vaccination (day of transplant – red bars) was tested in triplicate. Sera from patients vaccinated with gB (a,c,e,g) or placebo (b,d,f,h) are shown and are subdivided into patients who went onto display evidence of viraemia post-transplant. The mean values of the percentage of infection are presented with the error bars indicating the standard deviation (SD).

Figure S2. Exogenous Complement does not promote neutralisation with sera from seronegative vaccinees. HCMV Merlin was incubated with media, heat inactivated sera from seronegative patients given gB vaccine (vac) or placebo and then additionally incubated with guinea pig complement (+C) at 1:2 for 3 hours. As a control, healthy donor seropositive sera was used fresh or heat inactivated and heat inactivated with the addition of complement (1:2). HFFs were then infected, immuno-stained for IE 24hpi and infection scored for % infection.

Figure S3. Pre-incubation of HCMV with seronegative sera does not routinely reduce the frequency of pp28 positive cells 96hpi.   Merlin was incubated with sera from seronegative renal transplant recipients, or an ITC88 positive control, and used to inoculate HFFs *in vitro* (MOI=1). Infection was assayed by pp28 immunostaining 96hpi and the proportion of infected cells calculated by counterstaining nuclei with DAPI. Sera isolated pre-vaccination (baseline – blue bars) or post vaccination (day of transplant – red bars) was tested in triplicate. Sera from patients vaccinated with gB, n=12 (A) or placebo, n=8 (B) are shown and are subdivided into patients who went onto display evidence of viraemia post-transplant. The mean values of the percentage of infection are presented with the error bars indicating the standard deviation (SD).

Figure S4. Vaccination **does not promote an ability of sera from seronegatives to limit cell to cell spread of HCMV in vitro.** IE2-GFP tagged Merlin was incubated with sera from seropositive liver (a,b) and renal (c,d) transplant patients and seronegative liver (e,f) and renal (g,h) transplant patients, healthy donor sera (seronegative and seropositive individual) or an ITC88 positive control, and used to inoculate HFFs in vitro (MOI=0.25). Infection was assayed by GFP positivity at 14dpi and the proportion of infected cells calculated by counterstaining nuclei with DAPI. Sera isolated pre-vaccination (baseline – red bars) or post vaccination (day of transplant – blue bars) was tested in triplicate. Sera from patients vaccinated with gB (a,c,e,g) or placebo (b,d,f,h) are shown and are subdivided into patients who went onto display evidence of viraemia post-transplant. The mean values of the

percentage of infection are presented with the error bars indicating the standard deviation (SD).

Figure S5. ADCC promoting antibodies can be detected specifically in seropositive sera from healthy donors and transplant patients but levels are not affected by vaccination. a-b) PBMC isolated from a healthy seronegative donor was incubated with sera isolated from 10 seropositive (a) or seronegative (b) donors or with PMA/Ionomycin positive control and analysed for CD107a expression on NK cells (CD3-CD56+). c-f) PBMC from a healthy seronegative donor was incubated with PMA/Ionomycin, healthy seronegative donor sera (autologous sera) or left unstimulated and NK cells analysed for evidence of CD107a surface expression by FACS. Alternatively, PBMC was incubated with longitudinal serum samples from liver (c) and renal (d-f) where v#1 is pre-vaccination, v#2 is 1 month post vaccination, v#3 is two months post vaccination, v#4 is 6 months post vaccination, v#5 is 7 months post vaccination, d0 is day of transplant and d7 is 7days post transplant.