

1 **cGAMP loading enhances the immunogenicity of VLP vaccines**

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17 **Short summary**

18 cGAMP is an innate immune signalling molecule that can be transmitted between cells by  
19 inclusion in enveloped virions. This study demonstrates enhanced immunogenicity of HIV-  
20 derived virus-like particles containing cGAMP. Viral vectors loaded with cGAMP may thus  
21 be potent vaccines.

22

23 **Abstract**

24 Cyclic GMP-AMP (cGAMP) is an immunostimulatory second messenger produced by cGAS  
25 that activates STING. Soluble cGAMP acts as an adjuvant when administered with antigens.  
26 cGAMP is also incorporated into enveloped virus particles during budding. We hypothesised  
27 that inclusion of the adjuvant cGAMP within viral vaccine vectors would promote adaptive  
28 immunity against vector antigens. We immunised mice with virus-like particles (VLPs)  
29 containing the HIV-1 Gag protein and VSV-G. Inclusion of cGAMP within these VLPs  
30 augmented splenic VLP-specific CD4 and CD8 T cell responses. It also increased VLP- and  
31 VSV-G-specific serum antibody titres and enhanced *in vitro* virus neutralisation. The superior  
32 antibody response was accompanied by increased numbers of T follicular helper cells in  
33 draining lymph nodes. Vaccination with cGAMP-loaded VLPs containing haemagglutinin  
34 induced high titres of influenza A virus neutralising antibodies and conferred protection  
35 following subsequent influenza A virus challenge. Together, these results show that  
36 incorporating cGAMP into VLPs enhances their immunogenicity, making cGAMP-VLPs an  
37 attractive platform for novel vaccination strategies.

38

39 **Running title:** Immunogenicity of cGAMP-loaded VLP vaccines

## 40      **Introduction**

41      Vaccination is a powerful strategy in the fight against infectious disease, including virus  
42      infection. Indeed, vaccination led to the global eradication of smallpox and is highly protective  
43      against some viruses including measles virus and yellow fever virus. However, the  
44      development of vaccines inducing long-lasting and broadly effective protection has been  
45      difficult for other viruses such as human immunodeficiency virus (HIV) and influenza A virus  
46      (IAV), highlighting the need for new vaccination strategies (Rappuoli et al., 2011).

47      Successful vaccines induce potent adaptive immune responses. Prophylactic vaccine-mediated  
48      protection against most virus infections is thought to be predominantly due to induction of  
49      antiviral antibody responses that prevent or rapidly control subsequent infection. Antibody  
50      responses limit virus infection and spread through several mechanisms (Pelegrin et al., 2015).

51      Neutralising antibodies directly bind virus particles and prevent them from infecting cells.  
52      Virion-bound antibodies can also trigger complement activation and virolysis. In addition,  
53      antibodies can bind to virus-infected cells and target them for lysis or viral clearance by  
54      complement or cells mediating cytolytic or viral inhibitory activity such as natural killer cells  
55      and macrophages. Following immunisation, antibodies are initially produced by short-lived  
56      extrafollicular plasmablasts. To achieve long-term protection, long-lived plasma cells and  
57      memory B cells must be generated in secondary lymphoid tissues. This process occurs in  
58      specialised structures called germinal centres (GCs) (Cyster and Allen, 2019; Linterman and  
59      Hill, 2016). In GCs, a complex interplay between follicular dendritic cells (FDCs), tingible  
60      body macrophages, CD4 T follicular helper (Tfh) cells, CD4 T follicular regulatory (Tfr) cells  
61      and B cells results in the formation of long-lived plasma cells and memory B cells producing  
62      high-affinity antibodies that confer durable protection. Tfh cells are a CD4 T cell subset  
63      specialised to provide help to B cells and are essential for GC formation. They increase the  
64      magnitude and quality of the humoral response by promoting B cell proliferation, isotype  
65      switching and plasma cell differentiation; by mediating selection of high-affinity B cells in

66 GCs; and by supporting the generation of long-lived plasma cells and memory B cells (Crotty,  
67 2019). In contrast, CD4 Tfr cells are involved in limiting GC reactions to prevent autoantibody  
68 formation. Therefore, the Tfh/Tfr ratio is important for regulation of GC responses (Sage et al.,  
69 2013).

70 Virus-specific cytotoxic T cell (CTL) responses mediate clearance of infected cells to prevent  
71 virus spread and eradicate infection. If sterilising immunity is not conferred by antibodies,  
72 CTLs can make a key contribution to prophylactic vaccine efficacy (Hansen et al., 2011), and  
73 they are critical for the control of persistent infection with viruses such as hepatitis B virus,  
74 hepatitis C virus, cytomegalovirus and HIV (Panagioti et al., 2018). CTLs exert their activity  
75 by triggering destruction of infected cells via release of perforins and granzymes; by ligation  
76 of death-domain containing receptors and/or secretion of TNF $\alpha$ ; and by producing “curative”  
77 cytokines such as IFN $\gamma$ . Both the magnitude, i.e. the number of activated cells, and  
78 polyfunctionality of the T cell response, i.e. the capacity to mediate a breadth of effector  
79 activities including production of multiple cytokines, are important determinants of CD8 T  
80 cell-based vaccine efficacy (Panagioti et al., 2018).

81 Initiation of virus-specific CD4 and CD8 T cell responses requires presentation of viral  
82 antigens to naïve T cells by professional antigen-presenting cells (APCs), principally dendritic  
83 cells (DCs). T cells need to receive three signals for activation: T cell receptor (TCR) triggering  
84 by contact with peptide-major histocompatibility complexes (MHC) (signal 1); costimulatory  
85 signals (signal 2); and inflammatory cytokines (signal 3) (Joffre et al., 2009).

86 To induce adaptive immune responses, vaccines need to contain not only appropriate antigens  
87 but also an adjuvant. Adjuvants exert a breath of effects; for example, they induce the  
88 expression of costimulatory molecules and cytokines by DCs (Coffman et al., 2010). There are  
89 only a limited number of FDA-approved adjuvants, most of which are based on aluminium  
90 salts (Shi et al., 2019). The increasing knowledge in the field of innate immunity, particularly

91 in the mechanisms underlying pathogen recognition by innate immune receptors, provides an  
92 opportunity to develop new adjuvants that specifically engage such receptors and trigger a  
93 robust response (Temizoz et al., 2018). Adjuvants targeting toll-like receptors or the cytosolic  
94 DNA sensing pathway have attracted a lot of attention (Dubensky et al., 2013). In particular,  
95 cyclic dinucleotides (CDNs) that activate stimulator of interferon genes (STING, also known  
96 as TMEM173, MPYS, ERIS and MITA) and induce a type I interferon (IFN-I) response as  
97 well as production of pro-inflammatory cytokines are being developed as adjuvants (Cai et al.,  
98 2014). CDNs facilitate both CD8 T cell and antibody responses (Blaauboer et al., 2014; Kuse  
99 et al., 2019; Li et al., 2013) and are effective as mucosal adjuvants (Blaauboer et al., 2015;  
100 Ebensen et al., 2011). 2'-3' cyclic GMP-AMP (cGAMP) is of particular interest. It is produced  
101 by cGAMP synthase (cGAS) upon DNA sensing in the cell cytoplasm (Ablasser et al., 2013;  
102 Diner et al., 2013; Sun et al., 2013). Soluble cGAMP has been employed as an adjuvant in  
103 multiple pre-clinical vaccination models and is an anti-tumour agent (Corrales et al., 2015;  
104 Demaria et al., 2015; Li et al., 2016; Li et al., 2013; Wang et al., 2017). However, cGAMP  
105 levels are likely to diminish quickly in the extracellular milieu, due to diffusion from the site  
106 of administration and degradation by phosphodiesterases such as Ectonucleotide  
107 Pyrophosphatase/Phosphodiesterase 1 (ENPP1), an enzyme degrading extracellular ATP and  
108 cGAMP (Carozza et al., 2019; Li et al., 2014). Indeed, when injected intra-muscularly, the  
109 concentration of cGAMP at the inoculation site decreases rapidly, resulting in a sub-optimal  
110 adjuvant effect (Wang et al., 2016).

111 We and others previously showed that cGAMP is packaged into nascent viral particles as they  
112 bud from the membrane of an infected cell (Bridgeman et al., 2015; Gentili et al., 2015). Upon  
113 virus entry into newly infected cells, cGAMP is released into the cytosol and directly activates  
114 STING. Building on this observation, we hypothesised that inclusion of the adjuvant cGAMP  
115 in viral vaccine vectors may enhance their immunogenicity by targeting adjuvant and antigen  
116 to the same cell and by protecting cGAMP from degradation in the extracellular environment.

117 Indeed, using HIV-derived viral-like particles (VLPs), we found that the presence of cGAMP  
118 within VLPs enhanced adaptive immune responses to VLP antigens. Antigen-specific CD4 and  
119 CD8 T cell responses were augmented, as well as neutralising antibody production. The latter  
120 was accompanied by an increase in Tfh cells in draining lymph nodes. cGAMP-loaded VLPs  
121 containing the IAV haemagglutinin protein induced neutralising antibodies and conferred  
122 protection against development of severe disease after challenge with live IAV. These results  
123 highlight the utility of cGAMP loading as a strategy to boost the immunogenicity of viral  
124 vaccine vectors.

125 **Results**

126 **cGAMP-loading of HIV-derived VLPs**

127 HIV-derived viral vectors and VLPs are routinely produced in the cell line HEK293T by  
128 transfection of plasmids encoding viral components (Milone and O'Doherty, 2018). Here, we  
129 generated VLPs by using plasmids expressing the HIV-1 capsid protein Gag fused to GFP  
130 (Gag-GFP) and the Vesicular Stomatitis Virus envelope glycoprotein (VSV-G). The resulting  
131 VLPs consist of a Gag-GFP core and a lipid membrane derived from the producer cell that is  
132 spiked with VSV-G proteins. Of note, these VLPs do not contain viral nucleic acid and can  
133 therefore not replicate in the host (Deml et al., 2005). Additional over-expression of cGAS in  
134 the VLP producer cells results in its activation, presumably by the transfected plasmid DNA,  
135 and in the presence of cGAMP in the cytosol. It is noteworthy that HEK293T cells do not  
136 express STING (Burdette et al., 2011); therefore, cGAS-overexpressing VLP producer cells do  
137 not respond to the presence of cGAMP. cGAMP is then packaged into the nascent viral  
138 particles, which are released as cGAMP-loaded VLPs (hereafter cGAMP-VLPs; Fig 1A). As a  
139 control, we produced VLPs that do not contain cGAMP (Empty-VLPs) by using a catalytically  
140 inactive version of cGAS.

141 To assess the efficiency of cGAMP incorporation into our VLPs, we extracted small molecules  
142 from VLPs as previously described (Mayer et al., 2017). cGAMP in the extract was then  
143 quantified by ELISA. While Empty-VLPs did not contain detectable levels of cGAMP,  
144 cGAMP-VLPs contained between 55 and 90 ng cGAMP per  $10^6$  infectious units (IU) of VLPs  
145 (Fig 1B). We then assessed the infectivity of the VLPs and found cGAMP-VLPs and Empty-  
146 VLPs to be equally infective (Fig 1C). To confirm that cGAMP-VLPs trigger an IFN-I  
147 response, supernatant from the STING-positive HEK293 cells used in the infectivity assay was  
148 transferred to a reporter cell line expressing firefly luciferase under the interferon-sensitive  
149 response element (ISRE) promoter (Bridgeman et al., 2015). At similar infection rates,  
150 cGAMP-VLPs induced IFN-I production while Empty-VLPs did not (Fig 1C). Taken together,

151 these results show that cGAMP can be efficiently packaged into VLPs consisting of HIV-1  
152 Gag-GFP and the VSV-G envelope.

153

154 **Immunisation with cGAMP-VLPs induces higher and more polyfunctional CD4 and CD8**  
155 **T cell responses compared to Empty-VLPs**

156 To test whether cGAMP-VLPs induce a better immune response than Empty-VLPs *in vivo*, we  
157 injected C57BL/6 mice intramuscularly with  $10^6$  infectious units of cGAMP-VLPs or Empty-  
158 VLPs or, as a control, PBS. We first assessed CD4 T cell responses in the spleen 14 days after  
159 immunisation. As we were unable to identify a specific peptide epitope within HIV Gag  
160 recognised by CD4 T cells in H-2<sup>b</sup> mice, we used bone-marrow derived myeloid cells  
161 (BMMCs) pulsed with cGAMP-VLPs for evaluation of antigen-specific CD4 T cell responses.

162 We co-cultured these cells with splenocytes for six hours before assessing IL-2, IFN $\gamma$  and  
163 TNF $\alpha$  production by CD4 T cells by intracellular cytokine staining (ICS). Compared to mice  
164 immunised with Empty-VLPs, we observed 2.7-fold increased frequencies of CD4 T cells  
165 producing each of these cytokines in response to VLP-pulsed BMMCs in mice immunised with  
166 cGAMP-VLPs (Fig 2A, gating strategy and exemplary FACS plots in Fig S1A). Moreover,  
167 cGAMP enhanced the proportion of cells that were able to co-produce two or all three cytokines  
168 (Fig 2B; 2.1- and 3.7-fold increases, respectively).

169 We next assessed CD8 T cell responses following immunisation. We screened a panel of  
170 overlapping 15-mer peptides spanning the HIV-1 Gag sequence and identified a peptide that  
171 stimulated an IFN $\gamma$  response in cells from spleen in IFN $\gamma$  ELISPOT assays (peptide p92; Fig  
172 S2A-B). We then used NetMHCpan 3.0 (<http://www.cbs.dtu.dk/services/NetMHCpan-3.0/>  
173 (Nielsen and Andreatta, 2016)) to predict the optimal epitope sequence recognised within p92,  
174 and identified a 9-mer peptide (SQVTNSATI, termed HIV-SQV) that triggered T cell  
175 recognition more efficiently than the original 15-mer peptide (Fig S2C-D). This 9-mer peptide

176 was also reported to constitute an immunodominant HIV-1 Gag epitope in H-2<sup>b</sup> mice in a prior  
177 study (Holechek et al., 2016). The HIV-SQV peptide was used for all subsequent analyses of  
178 VLP-elicited CD8 T cell responses. We evaluated responses to the HIV-SQV peptide by IFN $\gamma$   
179 ELISPOT assay and showed that, when compared to Empty-VLPs, cGAMP-VLPs induced a  
180 modest but significant increase in the magnitude of the response (Fig 2C, 1.7-fold increase).  
181 To assess whether cGAMP-loading of VLPs also enhanced the polyfunctionality of the  
182 responding CD8 T cells, we stimulated splenocytes for six hours and stained for upregulation  
183 of CD107a (LAMP-1), a degranulation marker, and for the production of IFN $\gamma$ , TNF $\alpha$  and IL-  
184 2 by ICS. Paralleling the results from the ELISPOT assay, CD8 T cells from mice immunised  
185 with cGAMP-VLPs showed a modest but significant increase in the frequency of cells  
186 upregulating CD107a (1.6-fold increase) and/or producing IFN $\gamma$  (2-fold) and/or TNF $\alpha$  (1.9-  
187 fold) (Fig 2D, gating strategy and exemplary FACS plots in Fig S1B). Furthermore, cGAMP  
188 enhanced the proportion of CD8 T cells that were able to co-produce two of the cytokines  
189 evaluated (Fig 2E; 1.9-fold increase).

190 Control of vaccinia virus infection by the immune system relies in part on CD8 T cell responses  
191 (Xu et al., 2004). As immunisation with cGAMP-VLPs increased anti-HIV Gag CD8 T cell  
192 responses, we assessed whether this resulted in increased protection against subsequent  
193 infection with a vaccinia virus expressing the same HIV Gag (vVK1 (Karacostas et al., 1989)).  
194 One month after immunisation, mice were challenged with vVK1, and five days after infection  
195 virus load in the ovaries was assessed by plaque assay. We observed no weight loss over the  
196 course of the infection (Fig S3A). Immunisation with both VLPs reduced vVK1 load, and  
197 cGAMP-VLP immunised mice showed a slight but non-significant increase in protection  
198 compared to animals immunised with Empty-VLPs (Fig S3B).

199 Taken together, these results demonstrate that cGAMP-loading of VLPs enhances  
200 polyfunctional CD4 and CD8 T cell responses to VLP antigens.

201

202 **cGAMP loading of VLPs enhances serum titres of VLP binding and neutralising**  
203 **antibodies**

204 Next, we assessed the antibody response in immunised mice. We set up ELISAs that allow  
205 detection of serum antibodies binding to any protein in the VLPs, or of antibodies specific for  
206 the VSV-G envelope or the HIV-Gag protein. In mice immunised with VLPs, we detected very  
207 strong IgG responses and lower-titre IgM responses targeting the total VLP protein pool 14  
208 days after immunisation, indicating antibody class-switching (Fig 3A and Fig S4A).  
209 Interestingly, immunisation with cGAMP-VLPs induced stronger anti-VLP antibody responses  
210 compared to the Empty-VLP immunised group, with statistically significant differences being  
211 observed in IgG2a/c, IgG2b and IgM levels. We also detected IgG antibodies targeting the  
212 VSV-G envelope, and IgG1, IgG2a/c and IgG2b titres were higher in the cGAMP-VLP  
213 immunised group (Fig 3B). Titres of antibodies recognising the intracellular antigen HIV-Gag  
214 were low or undetectable, but a similar trend was observed for a higher-magnitude response in  
215 the cGAMP-VLP immunised group (Fig 3C and S4A).

216 To test whether the anti-VLP antibodies were neutralising, we assessed the *in vitro*  
217 neutralisation capacity of sera using a VSV-G pseudotyped HIV-1-based lentivector  
218 expressing GFP (Fig 3D). The effect of pre-incubation with serum samples on the infectivity  
219 of the HIV-1-GFP virus was measured by monitoring GFP expression in HEK293 cells (Fig  
220 S4B-C). Although immunisation with both cGAMP-VLPs and Empty-VLPs induced  
221 neutralising antibodies, this response was stronger when cGAMP was present within the VLPs,  
222 and sera from cGAMP-VLP immunised mice showed a 2.5-times higher half maximal  
223 inhibitory concentration (Fig 3E-F). In summary, immunisation with cGAMP-VLPs induced  
224 an increased antibody response that targeted proteins from total VLP lysates including the  
225 VSV-G envelope protein. Moreover, cGAMP-loading enhanced production of virus  
226 neutralising antibodies.

227

228 **Incorporation of cGAMP into VLPs increases the CD4 Tfh cell response**

229 To gain insight into how immunisation with cGAMP-VLPs resulted in an increased antibody  
230 response, we investigated B and T cell populations in inguinal lymph nodes that drain the  
231 injection site. As CD4 T cell responses were increased in the spleens of cGAMP-VLP  
232 immunised mice, we first tested whether follicular CD4 T cell numbers were elevated in  
233 lymphoid tissues draining the immunisation site. We identified follicular CD4 T cells as  
234 CD4<sup>+</sup>CD44<sup>+</sup>PD1<sup>hi</sup>CXCR5<sup>hi</sup> and subdivided them into Tfh and Tfr cells by analysing FoxP3,  
235 which is expressed in Tfr cells (Fig 4A). Immunisation with VLPs led to an increase in the  
236 proportion of follicular T cells within the CD4 T cell population in the draining lymph node  
237 (Fig 4A-B). This was due to an expansion of Tfh cells, as the latter increased significantly in  
238 frequency after VLP immunisation, whereas Tfr frequencies within CD4 T cells remained  
239 unaltered. As a consequence of this, there was a profound shift in the Tfh:Tfr ratio in VLP-  
240 immunised as compared to control mice (Fig 4C). Importantly, the increase in Tfh cells was  
241 more pronounced in cGAMP-VLP immunised mice compared to Empty-VLP injected animals  
242 (Fig 4B-C; 1.6-fold increase).

243 To assess the impact of this increased Tfh response on B cell responses, we first gated on GC  
244 B cells (B220<sup>+</sup>IgD<sup>+</sup>CD95<sup>+</sup>GL7<sup>+</sup> cells; Fig 4D). Immunisation with VLPs induced a robust GC  
245 B cell response, with no difference being observed in the frequencies of GC B cells in cGAMP-  
246 VLP and Empty-VLP groups at the day 14 time-point analysed (Fig 4E). We next evaluated  
247 the generation of antibody-secreting cells (ASCs) by antigen-specific B cell ELISPOT assay  
248 on cells from both draining lymph nodes and spleens 14 days after immunisation. VLP-specific  
249 ASCs were detected in the lymph nodes of mice injected with both cGAMP-VLPs and Empty-  
250 VLPs (Fig 4F). In the spleen, VLP-specific ASCs were also observed in cGAMP-VLP  
251 immunised animals, but not in Empty-VLP immunised animals (Fig 4F). Taken together, these  
252 results suggest that immunisation with cGAMP-VLPs increased the antibody response by

253 enhancing the accumulation of Tfh cells in draining lymph nodes, thereby promoting the  
254 development of ASCs.

255

256 **cGAMP-VLPs pseudotyped with IAV haemagglutinin induce a neutralising antibody**  
257 **response and confer protection following live virus challenge**

258 As immunisation with cGAMP-VLPs induced high titres of neutralising antibodies, we  
259 explored whether they could confer protection following a live virus challenge. Protection  
260 against IAV infection correlates with serum antibody responses against the surface  
261 glycoprotein, haemagglutinin (HA) (Krammer, 2019). We therefore produced cGAMP-VLPs  
262 and Empty-VLPs incorporating HA from the mouse-adapted PR8 strain of IAV (designated  
263 cGAMP-HA-VLPs and Empty-HA-VLPs, respectively) (Fig S5A). cGAMP-HA-VLPs and  
264 Empty-HA-VLPs were equally infective, as assessed by the percentage of GFP-expressing  
265 cells observed following infection of HEK293 cells with titrated doses of VLPs (Fig S5B).  
266 Staining of infected HEK293 cells with an antibody recognising HA revealed the presence of  
267 similar percentages of HA<sup>+</sup> cells after infection with cGAMP-HA-VLPs and Empty-HA-VLPs  
268 (Fig S5B). As control, cells infected with cGAMP-VLPs without HA showed no detectable  
269 staining. These data confirmed that HA was transferred by HA-VLPs to infected cells. Finally,  
270 we verified that supernatant from cells infected with cGAMP-HA-VLPs contained IFN-I,  
271 suggesting that the presence of HA did not affect the incorporation of cGAMP into the VLPs  
272 (Fig S5C).

273 Next, we immunised mice with HA-VLPs. Two weeks after immunisation, sera were analysed  
274 for neutralising antibodies using a micro-neutralisation assay. In brief, a single cycle IAV  
275 expressing eGFP and PR8 HA was pre-incubated with sera and its infectivity was then  
276 monitored using MDCK-SIAT1 cells (Powell et al., 2012). Immunisation with both Empty-  
277 HA-VLPs and cGAMP-HA-VLPs induced neutralising antibodies, and the presence of  
278 cGAMP in the VLPs increased this response by 2.7-fold (Fig 5A). To determine if

279 immunisation conferred protection upon *in vivo* challenge with live IAV, we infected mice  
280 with  $10^4$  TCID50 of HA-matched PR8 IAV one month after immunisation. Animals  
281 immunised with  $10^6$  infectious units of both VLPs were protected against the weight loss  
282 observed between day three and four after IAV infection in PBS-treated mice, both resulting  
283 in 100% survival (Fig 5B-C). This prompted us to reduce the amount of VLPs used for  
284 immunisation. At an intermediate dose of  $2 \times 10^5$  infectious units of VLPs, cGAMP-HA-VLPs  
285 were fully protective against weight loss and disease progression to an endpoint where humane  
286 sacrifice was necessary, while immunisation with Empty-HA-VLPs delayed disease  
287 progression by about four days, resulting in 100% and 16.7% survival, respectively (Fig 5B-  
288 C). At the lowest dose of VLPs tested ( $5 \times 10^4$  infectious units), Empty-HA-VLPs were not  
289 protective whereas cGAMP-HA-VLPs protected most animals against severe disease (83%  
290 survival) (Fig 5B-C).

291 Taken together, these results show that vaccination with VLPs incorporating IAV HA induced  
292 neutralising antibodies in mice, which were protected against subsequent IAV challenge. The  
293 presence of cGAMP in HA-VLPs enhanced the antibody response and, at lower doses of VLPs  
294 used for immunisation, facilitated protection against IAV.

295 **Discussion**

296 New and more targeted adjuvants are needed for improved efficacy and safety of vaccines.  
297 There is growing interest in using adjuvants that specifically activate innate immune pathways  
298 used by cells to detect viral infections. cGAMP is one such example. cGAMP is a natural  
299 molecule produced by cells upon virus infection that specifically triggers STING and thereby  
300 induces innate and adaptive immune responses. The vaccination strategy we describe here is  
301 based on coupling the adjuvant cGAMP with antigen(s) in a single entity, namely HIV-derived  
302 VLPs. We demonstrate that cGAMP-loading of these VLPs increased CD4 and CD8 T cell  
303 responses, as well as antibody responses, against protein antigens in the VLPs. Furthermore,  
304 vaccination with VLPs containing cGAMP protected mice against disease development  
305 following infection with a virus expressing a cognate antigen.

306 HIV-derived VLPs are a flexible system that allows incorporation of proteins of choice. We  
307 demonstrate this by decorating VLPs with IAV HA, and show that upon immunisation, these  
308 VLPs induced antibodies that neutralised IAV expressing a matched HA protein. In future,  
309 other pathogen-derived proteins could be incorporated into cGAMP-loaded VLPs as a strategy  
310 to produce vaccines for a diverse breadth of pathogens. For example, multiple HA proteins  
311 from different IAV clades could be incorporated to induce broadly protective responses or  
312 envelope proteins from other viruses such as Zika or Ebola could be delivered using this  
313 approach.

314 The VLPs used here were pseudotyped with VSV-G, which has a broad tropism. It is possible  
315 to replace VSV-G with other envelope proteins that target VLPs to specific cell types. For  
316 example, the envelope protein from Sindbis virus or antibodies such as those to DEC205 target  
317 virus particles to DCs, an essential antigen presenting cell type (Trumpfheller et al., 2006; Yang  
318 et al., 2008). It will be interesting to determine whether DC-targeted VLPs containing cGAMP  
319 have a similar effect on the responses induced compared to the VSV-G pseudotyped VLPs  
320 described here. DC targeting could improve vaccine safety by restricting cGAMP delivery to

321 relevant antigen-presenting cells, thereby limiting systemic inflammation. Indeed, with the  
322 VSV-G pseudotyped VLPs used here, we observed a transient weight loss of approximately  
323 5% in cGAMP-VLP but not in Empty-VLP immunised mice (data not shown).

324 Incorporating cGAMP inside viral particles likely increases its stability at the site of injection  
325 by preventing degradation in the extracellular milieu. In addition to HIV-derived lentiviruses,  
326 other enveloped viruses also incorporate cGAMP (Bridgeman et al., 2015; Gentili et al., 2015).  
327 Therefore, our strategy of protecting the adjuvant cGAMP together with antigen in viral  
328 particles may be applicable to other viral vectored vaccines such as modified vaccinia virus  
329 Ankara (MVA).

330 Many studies are currently aimed at designing vaccines that induce antigen-specific CD8 T  
331 cells (Panagioti et al., 2018). We found that cGAMP-loading of VLPs modestly enhanced CD8  
332 T cell responses to the internal HIV-Gag antigen. The increased response in cGAMP-VLP  
333 immunised mice did not result in a significant improvement in protection against a vaccinia  
334 virus expressing the same HIV-Gag compared to that observed in animals vaccinated with  
335 Empty-VLPs. However, the immunisation strategy employed here consisted of a single dose  
336 of VLPs and may be improved by employing prime-boost strategies. In light of the neutralising  
337 antibody response induced by cGAMP-loaded VLPs, heterologous booster immunisations  
338 using non-particulate vaccines or viral particles with a different envelope protein are  
339 particularly promising.

340 Both splenic CD4 effector T cell responses as well as Tfh cell numbers in draining lymphoid  
341 tissues were enhanced by incorporation of cGAMP in VLPs. It is likely that these effects  
342 explain the increased antibody responses we observed against VLP proteins. The CD4 T cell  
343 response was skewed toward a Th1 phenotype, as indicated by robust IFN $\gamma$  and TNF $\alpha$   
344 production by CD4 T cells and enhanced IgG2a/c and IgG2b antibody responses. Both the cell  
345 type mediating antigen presentation as well as the cytokines produced at the time of T cell

346 activation are crucial for polarisation of T cell responses (Hong et al., 2018; Itano and Jenkins,  
347 2003; O'Garra, 1998). Notably, IFN-I and IL-6 production by DCs have been reported to induce  
348 the development of Tfh cells in mice (Cucak et al., 2009; Nurieva et al., 2009; Riteau et al.,  
349 2016). We previously found that cGAMP-loaded viruses induce IFN-I in bone-marrow derived  
350 macrophages *in vitro* (Bridgeman et al., 2015). Activation of STING and down-stream IRF3  
351 and NF- $\kappa$ B signalling by cGAMP *in vivo* might therefore trigger production of IFN-I and IL-  
352 6 that could underlie the potent CD4 Tfh response elicited following immunisation with  
353 cGAMP-VLPs. The specific cell types infected by VLPs *in vivo* and the cytokines induced by  
354 these cells are likely to be key aspects of the response induced by cGAMP-VLPs *in vivo* and  
355 warrant further investigation.

356 VLPs bearing IAV HA induced a neutralising antibody response and protected immunised  
357 mice against development of severe disease following challenge with live IAV. Importantly,  
358 the presence of cGAMP in VLPs enabled induction of a protective response even at low VLP  
359 doses. cGAMP-loading of viral vectored vaccines may therefore allow the vaccine dose  
360 administered to be reduced without compromising vaccine efficacy. We believe this will be  
361 advantageous in at least two ways: by increasing safety and by reducing cost of vaccine  
362 production. The latter is particularly important for lentivirus-based vectors that can typically  
363 only be produced at lower titres than other viral vectored vaccines.

364 In summary, we provide evidence that vaccination with HIV-derived VLPs containing both the  
365 adjuvant cGAMP and protein antigens constitutes an efficacious platform for induction of CD8  
366 T cell and neutralising antibody responses. This VLP-based strategy of coupling adjuvant and  
367 antigen in a single entity is therefore a promising approach for development of new and safer  
368 vaccines against a range of pathogens.

369 **Materials and methods**

370 Mice

371 All mice were on the C57Bl/6 background. This work was performed in accordance with the  
372 UK Animals (Scientific Procedures) Act 1986 and institutional guidelines for animal care. This  
373 work was approved by project licenses granted by the UK Home Office (PPL No. 40/3583,  
374 No. PC041D0AB and No. PBA43A2E4) and was also approved by the Institutional Animal  
375 Ethics Committee Review Board at the University of Oxford.

376

377 Cells

378 Cell lines (HEK293T, HEK293, 3C11, 143B, MDCK-SIAT1, MDCK-PR8) were maintained  
379 in DMEM (Sigma Aldrich) supplemented with 10% FCS (Sigma Aldrich) and 2mM L-  
380 Glutamine (Gibco) at 37°C and 5% CO<sub>2</sub>. 3C11 cells are HEK293 cells stably transduced with  
381 an ISRE-Luc reporter construct (Bridgeman et al., 2015). 143B cells were a kind gift from N.  
382 Proudfoot (University of Oxford).

383 Bone marrow cells were isolated from humanely killed adult mice by standard protocols and  
384 grown in 6-well plates for 5 days in RPMI supplemented with 10% FCS, 2mM L-Glutamine,  
385 1% PenStrep and 20ng/mL mouse GM-CSF to obtain bone marrow-derived myeloid cells  
386 (BMMCs).

387

388 Reagents and antibodies

389 See Supplementary Table 1

390

391 VLP and HIV-1 vector production

392 All VLPs were produced by transient transfection of HEK293T cells with Fugene 6 (Promega,  
393 ref E2691). HEK293T were seeded in 15-cm dishes to reach 60-70% confluence the next day  
394 and VLPs were produced by co-transfected plasmids encoding Gag-eGFP and the VSV-G

395 envelope (pGag-EGFP and pCMV-VSV-G, respectively) at a ratio of 2:1. VLPs were loaded  
396 with cGAMP by co-transfected at the same time a plasmid encoding mouse cGAS WT  
397 (pcDNA3-Flag-mcGAS). Empty-VLPs were produced as control by co-transfected a  
398 catalytically inactive mouse cGAS (cGAS AA; pcDNA3-Flag-mcGAS-G198A/S199A). One  
399 day after transfection, the medium was changed. Supernatants were collected 24, 32 and 48  
400 hours after medium change, centrifuged and filtered (Cellulose Acetate membrane 0.45 µm  
401 pore-size). At each media change VLPs were concentrated by ultracentrifugation through a  
402 20% sucrose cushion at 90,000g for 2.5 hours at 8°C using a Beckman SW32 rotor. VLPs were  
403 resuspended in PBS and subsequent harvests were resuspended using the resuspended VLPs  
404 from previous harvests to maximise titre.

405 For pseudotyping cGAMP-VLPs and Empty-VLPs with Influenza Haemagglutinin H1 (HA;  
406 pcDNA3.1-H1 (PR8)), cells were transfected as above with the following plasmids: Gag-eGFP,  
407 VSV-G, HA and cGAS WT or AA at a ratio of 2:1:1:2.

408 To produce VSV-G pseudotyped HIV-1 vectors for neutralisation assays, HEK293T cells were  
409 co-transfected with the following plasmids: HIV-1 NL4-3 ΔEnv GFP (pNL4-3-deltaE-EGFP)  
410 and VSV-G at a ratio of 2:1.

411

#### 412 VLP Titration and cGAMP incorporation assays

413 HEK293 cells were seeded at a density of  $1 \times 10^5$  cells per well in 24-well plates. The next day,  
414 cells were infected with decreasing amounts of VLPs in the presence of 8µg/mL of polybrene.  
415 24 hours after infection, cells were collected and first stained with anti-CD16/32 and Aqua  
416 fixable Live/Dead in FACS Buffer (PBS, 1% FCS, 2mM EDTA) for 15 minutes at RT. Cells  
417 used for titration of HA-VLPs were also stained for HA using a primary human anti-H1 & H5  
418 antibody in FACS Buffer for 30 minutes at 4°C. Cells were then washed twice and further  
419 stained with a secondary goat anti-human Alexa Fluor 647-conjugated antibody for 30 minutes

420 at 4°C, followed by two washes. All cells were fixed using BD Cellfix before acquisition on  
421 an Attune Nxt flow cytometer. Infection was measured by analysing GFP positive cells by flow  
422 cytometry using FlowJo version 10. VLP titres were calculated based on the number of GFP<sup>+</sup>  
423 cells compared to the number of cells in the well at the time of infection and expressed as  
424 infectious units/mL (IU/mL). Supernatants from infected cells were transferred onto ISRE  
425 reporter cells to assess IFN-I production in response to cGAMP incorporated in VLPs as  
426 described previously (Bridgeman et al., 2015). After 24 hours of incubation with supernatants,  
427 expression of the ISRE-Luc reporter was assessed using the One-Glo luciferase assay system.  
428 Small molecular extracts were prepared from VLPs as described (Mayer et al., 2017). Briefly,  
429 2x10<sup>6</sup> IU of ultra-centrifuged VLPs resuspended in PBS were lysed in X-100 Buffer (1mM  
430 NaCl, 3mM MgCl<sub>2</sub>, 1mM EDTA, 1% Triton X-100, 10mM Tris pH7.4) by adding 1/10 volume  
431 of 10X buffer for 20 minutes on ice while vortexing regularly. After centrifugation at 1,000g  
432 for 10 minutes at 4°C, supernatants were treated with 50U/mL of benzonase for 45 minutes on  
433 ice. Samples were then extracted with phenol-chloroform, and the aqueous phase was then  
434 transferred to Amicon Ultra 3K filter columns. After filtering by centrifugation at 14,000g for  
435 30 minutes at 4°C, samples were dried in a SpeedVac and resuspended in 200µL of water.  
436 cGAMP was quantified using the 2'-3' cGAMP ELISA kit following manufacturer's  
437 instructions.

438

439 IAV

440 The Influenza virus H1N1 A/Puerto Rico/8/1934 (Cambridge) (PR8) and the non-replicating  
441 S-FLU vector expressing eGFP (S-eGFP) were generated as previously described (Powell et  
442 al., 2012). Plasmids encoding the IAV Cambridge strain of A/Puerto Rico/8/34 were used to  
443 generate the wild type H1N1 A/Puerto Rico/8/1934 (Cambridge) (PR8) seed virus. The same  
444 plasmids were used to generate the PR8 S-eGFP with slight modifications: the HA coding

445 region in the plasmid expressing HA viral RNA was replaced with eGFP and an additional  
446 plasmid was included to provide a functional PR8 HA in *trans* to rescue the PR8 S-eGFP seed  
447 virus. Briefly, the plasmids were transfected into HEK293T cells using lipofectamine 2000 and  
448 supernatant containing seed virus was collected 72 hours after transfection. The wild type PR8  
449 virus and the S-eGFP vector were then propagated by infecting MDCK-SIAT1 cells or MDCK-  
450 SIAT1 stably transfected with PR8 HA (MDCK-PR8), respectively, with seed virus, followed  
451 by medium change into VGM (Viral Growth Media; DMEM, 1% BSA, 10mM HEPES buffer,  
452 1% PenStrep) containing 1 $\mu$ g/mL TPCK-treated trypsin. Viruses were harvested 48 hours later.  
453 The TCID<sub>50</sub> was determined by infecting MDCK-SIAT1 or MDCK-PR8 cells with a  $\frac{1}{2}$ -log  
454 dilution series of viruses in VGM for one hour in eight replicates using 96-well flat-bottom  
455 plates. Next, 150 $\mu$ l per well of VGM with TPCK-treated trypsin (1  $\mu$ g/mL) was added and  
456 cells were further incubated for 48 hours at 37°C. The PR8 virus and the S-eGFP vector were  
457 quantified by Nucleoprotein (NP) staining and eGFP expression respectively and TCID<sub>50</sub> was  
458 calculated using the method of Reed and Muench (Reed and Muench, 1938).

459

460 Vaccinia virus

461 Stocks of the vaccinia virus expressing HIV-1 HXB.2 Gag (vVK1) were produced by growth  
462 in 143TK-cells, and infectious virus titers were determined by plaque assay (Borrow et al.,  
463 1994).

464

465 Immunisation and viral challenge of mice

466 C57Bl/6 female mice between 6-8 weeks old were obtained from University of Oxford  
467 Biomedical Services or Envigo RMS (UK) Limited. Animals were injected intra-muscularly  
468 with 50 $\mu$ L per hindleg of PBS or 10<sup>6</sup> IU of cGAMP-VLPs or Empty-VLPs, unless otherwise  
469 stated, under inhalation isoflurane (IsoFlo, Abbott) anaesthesia. Weight was monitored every

470 day for 14 days. For immunophenotyping, mice were culled on day 14 by inhalation of carbon  
471 dioxide and cervical dislocation. For viral challenge experiments, mice were monitored every  
472 other day for an additional two weeks before challenge.

473 For IAV challenge, blood samples were acquired two weeks after immunisation for evaluation  
474 of the serum antibody response. Mice were then challenged a month after immunisation *via* the  
475 intranasal route with 10,000 TCID<sub>50</sub> of PR8 diluted in 50µL VGM under inhalation isoflurane  
476 anaesthesia. Weight was monitored daily and mice were culled by inhalation of carbon dioxide  
477 and cervical dislocation when body weight loss approached the humane end-point of 20%.

478 For vaccinia virus challenge, mice were infected *via* the intra-peritoneal route with 10<sup>6</sup> PFU  
479 vVK1 in 100µL PBS. Weight was monitored daily for five days. Animals were then culled by  
480 inhalation of carbon dioxide and cervical dislocation, and ovaries were collected for virus  
481 titration.

482

#### 483 Analysis of T cell responses by ICS and ELISPOT

484 Splenocytes were obtained by separating spleens through a 70µm strainer, and were then  
485 treated with red blood cell lysis buffer for 5 minutes, washed and resuspended in RPMI  
486 supplemented with 2% Human serum, 2mM L-Glutamine, 1% PenStrep (R2).

487 For ELISPOT assays, splenocytes were seeded in R2 at a density of 1.5x10<sup>5</sup> cells per well on  
488 ELISPOT plates pre-coated with anti-IFN $\gamma$  detection antibody. Cells were either non-treated  
489 or treated with 2µg/mL HIV-1 Gag peptide or with 10ng/mL PMA and 1µg/mL ionomycin as  
490 a control, and incubated for 48 hours at 37°C before detection according to the manufacturer's  
491 instructions (Mouse IFN $\gamma$  ELISPOT BASIC (ALP) kit).

492 For intracellular cytokine staining (ICS), cells were seeded in R2 at a density of 1x10<sup>6</sup> cells per  
493 well in a round-bottom 96 well plates. Cells were either non-treated or treated with 2µg/mL  
494 HIV-SQV 9-mer peptide or co-cultured with BMMCs pulsed overnight with cGAMP-VLP at

495 a multiplicity of infection of 1. Cells were also treated with 10ng/mL PMA and 1 $\mu$ g/mL  
496 ionomycin as a positive control. After 1 hour of incubation at 37°C, Golgi STOP was added  
497 according to manufacturer's instructions. After a further 5 hours of incubation at 37°C, cells  
498 were washed twice in FACS buffer (PBS, 1% FCS, 2mM EDTA), incubated with anti-CD16/32  
499 and Aqua or violet fixable Live/Dead in FACS Buffer for 15 minutes at RT and were then  
500 washed twice in FACS Buffer. Subsequent extracellular staining involved incubation of cells  
501 for 30 minutes at 4°C with the following antibodies: anti-CD8 BV605 and anti-CD90.2 PerCP-  
502 Cy5.5 in FACS Buffer for CD8 T cells analysis in cells stimulated with the HIV peptide, or  
503 anti-CD4 AF700, anti-CD8 BV605 and anti-MHC-II BV510 in Brilliant stain buffer for CD4  
504 T cells analysis in cells stimulated with pulsed BMMCs. Cells were then washed twice in FACS  
505 Buffer and fixed using BD Cytofix/Cytoperm buffer for 20 minutes at 4°C. After 2 washes in  
506 FACS Buffer with 10% BD Cytoperm/wash, intracellular staining was performed for using  
507 anti-TNF $\alpha$  PE, anti-IFN $\gamma$  PE-Cy7 and anti-IL2 APC in FACS Buffer with 10% BD  
508 Cytoperm/wash for 30 minutes at 4°C. After 2 washes in FACS Buffer with 10% BD  
509 Cytoperm/wash, cells were fixed for 10 minutes at RT in BD Cellfix, washed again and  
510 resuspended in FACS Buffer for acquisition on Attune NxT flow cytometers. Analysis was  
511 performed using FlowJo version 10. Gates for phenotypic markers of CD4 and CD8 T cells  
512 were based on FMO controls. Unstimulated control cells were used for other gates.  
513

#### 514 Analysis of serum antibody titres by ELISA

515 To extract protein, VLPs were lysed in PBS containing 0.5% Triton X-100 and 0.02% Sodium  
516 Azide for 10 minutes at RT. Quantity of protein extracted was quantified by BCA assay.  
517 Costar high-binding half-area flat bottom 96 well plates were coated overnight at 4°C with  
518 either 10 $\mu$ g/mL cGAMP-VLP lysates, 0.5 $\mu$ g/mL recombinant HIV-1 IIIB pr55 Gag protein or  
519 1.5 $\mu$ g/mL recombinant VSV-G protein. The next day, plates were washed twice in PBS, then

520 twice in PBS with 0.1% Tween-20 (wash buffer) and blocked in PBS with 3% BSA for 2 hours  
521 at RT. Sera collected on day 14 after immunisation were serially diluted in PBS with 0.5%  
522 BSA starting at a dilution of 1/200 and diluting 1/3. After four washes in wash buffer, serum  
523 dilutions were added to the plates in duplicates (25µl per well) and incubated for 1 hour at  
524 37°C. Plates were washed four times in wash buffer. Next, 50µl per well of HRP-conjugated  
525 antibodies recognising different antibody classes or subclasses were added using the following  
526 dilutions: goat anti-mouse IgG1 / IgG2a/c / IgG2b, 1/10,000; IgM, 1/2,000 in PBS with 0.5%  
527 BSA and incubated for 1 hour at RT. Plates were washed four times in wash buffer and 50µl  
528 of TMB substrate was added per well. Plates were incubated for approximately 30 minutes or  
529 until the signal was saturating and 50µl of STOP solution was added per well before reading  
530 absorbance at 450nm and 570nm.

531

532 Analysis of germinal centre B cells and T follicular cells in draining lymph nodes

533 Both inguinal lymph nodes were meshed through a 70µm strainer. 10<sup>6</sup> cells per animal were  
534 used for each staining.

535 For germinal centre B cell analysis, cells were first stained with anti-CD16/32 and Aqua fixable  
536 Live/Dead in FACS Buffer for 15 minutes at RT. After two washes in FACS Buffer,  
537 extracellular staining was performed using anti-B220 APC-Cy7, anti-CD95 PE, anti-IgD  
538 PerCP-Cy5.5 and GL7 AF647 in FACS Buffer for 30 minutes at 4°C. Cells were then washed,  
539 fixed for 10 minutes at RT in BD Cellfix, washed again and resuspended in FACS Buffer.

540 For T follicular cell analysis, cells were first stained with anti-CD16/32 and Aqua fixable  
541 Live/Dead in FACS Buffer for 15 minutes at RT. After two washes in FACS Buffer,  
542 extracellular staining was performed using anti-B220 BV510, anti-CD4 AF700, anti-CD44  
543 PerCP-Cy5.5, anti-CXCR5 BV421 and anti-PD-1 APC in Brilliant stain buffer for 1 hour at  
544 4°C. After two washes in FACS Buffer, cells were fixed using the eBioscience FoxP3 fixation

545 buffer for 25 minutes at RT. Cells were then washed twice in cold eBioscience Perm buffer  
546 and intracellular staining was performed using anti-FoxP3 PE-Cy7 in eBioscience Perm buffer  
547 for 40 minutes at RT. After two washes in eBioscience Perm buffer, cells were then fixed for  
548 10 minutes at RT in BD Cellfix, washed again and resuspended in FACS Buffer for acquisition  
549 on Attune NxT flow cytometers. Analysis was performed using FlowJo version 10. Gates for  
550 phenotypic markers of CD4 T cells and B cells were based on FMO controls, and gates for GC  
551 and Tfh/Tfr markers were based on PBS-immunised mice.

552

553 B cell ELISPOT

554 Cells from spleen and draining lymph nodes were collected as described above and counted.  
555 Three different amounts of cells ( $10^6$ ,  $3 \times 10^5$ ,  $1 \times 10^5$ ) were seeded in duplicate in R2 on  
556 ELISPOT plates coated overnight with 2 $\mu$ g/mL of lysates from cGAMP-VLPs (see ELISA).  
557 Plates were then incubated overnight at 37°C before detection according to manufacturer's  
558 instruction (Mouse IgG Basic ELISPOT BASIC (ALP) kit). Analysis was performed using the  
559 cell density showing the least background in PBS injected mice.

560

561 IAV micro-neutralisation assay

562 Micro-neutralisation (MN) assay was performed as described (Powell et al., 2012) with minor  
563 modifications. Briefly, a single cycle IAV expressing eGFP (S-eGFP (PR8)) containing the H1  
564 haemagglutinin was titrated to give saturating infection of  $3 \times 10^4$  MDCK-SIAT1 cells per well  
565 in 96-well flat-bottom plates, detected by eGFP fluorescence. Murine sera were heat  
566 inactivated for 30 minutes at 56°C. Dilutions of sera were incubated with S-eGFP for 2 hours  
567 at 37°C before addition to  $3 \times 10^4$  MDCK-SIAT1 cells per well. Cells were then incubated  
568 overnight before fixing in 4% formaldehyde. The suppression of infection was measured on  
569 fixed cells by fluorescence on a CLARIOstar fluorescence plate reader.

570

571 Vaccinia virus plaque assay

572 Ovaries collected in D0 (DMEM, 1% PenStrep) were homogenised using glass beads in screw  
573 cap tubes in a homogeniser (two cycles at speed 6.5 for 30 seconds). Samples were then placed  
574 on ice for 1-2 minutes and homogenisation was repeated. Samples were then subjected to three  
575 freeze-thaw cycles between 37°C and dry ice and sonicated three times for 30 seconds with 30  
576 second intervals on ice. Supernatants containing virus were collected in new tubes after  
577 centrifugation at 10,000 rpm for 3 minutes at 4°C.

578 143B cells were seeded in 12 well plates at a density of  $0.25 \times 10^6$  cells per well in 1mL D10.  
579 The next day, log serial dilutions of virus-containing samples were prepared in D0. Supernatant  
580 was replaced with 550 $\mu$ l of diluted virus-containing samples and incubated for 2 hours at 37°C,  
581 swirling plates every 30 minutes to avoid drying. Virus containing samples were then removed  
582 and cells were covered in 1.5mL of D10 containing 1% Pen/Strep and 0.5%  
583 carboxymethylcellulose (CMC). 48 hours after infection, cells were carefully washed with PBS  
584 and fixed in 4% formaldehyde for 20 minutes at RT before staining with 0.5% crystal violet.

585

586 Statistics

587 Statistical analysis was performed in GraphPad Prism v7.00 as detailed in the figure legends.

588 **Author contributions** (using the CRediT taxonomy)

589 Conceptualisation: L.C., A.B., and J.R.; Methodology: L.C., A.B., T.K.T., J.F., I.P.-P. and T.P.;  
590 Software: n.a.; Validation: L.C. and J.R.; Formal analysis: L.C. and J.R.; Investigation: L.C.,  
591 A.B., T.K.T. and J.F.; Resources: R.B. and P.B.; Data curation: L.C.; Writing – Original Draft:  
592 L.C. and J.R.; Writing – Review & Editing: all authors; Visualisation: L.C. and J.R.;  
593 Supervision: J.R., A.T., H.D. and P.B.; Project administration: L.C.; Funding acquisition: J.R.

594

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602 Expression Vector (Cat#11468) from Dr. Marilyn Resh, HIV-1 NL4-3 ΔEnv EGFP Reporter  
603 Vector from Drs. Haili Zhang, Yan Zhou, and Robert Siliciano (cat# 11100), HIV-1IIIB pr55  
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611

612 **Declaration of interests**

613 The authors have declared that no conflict of interest exists.

614

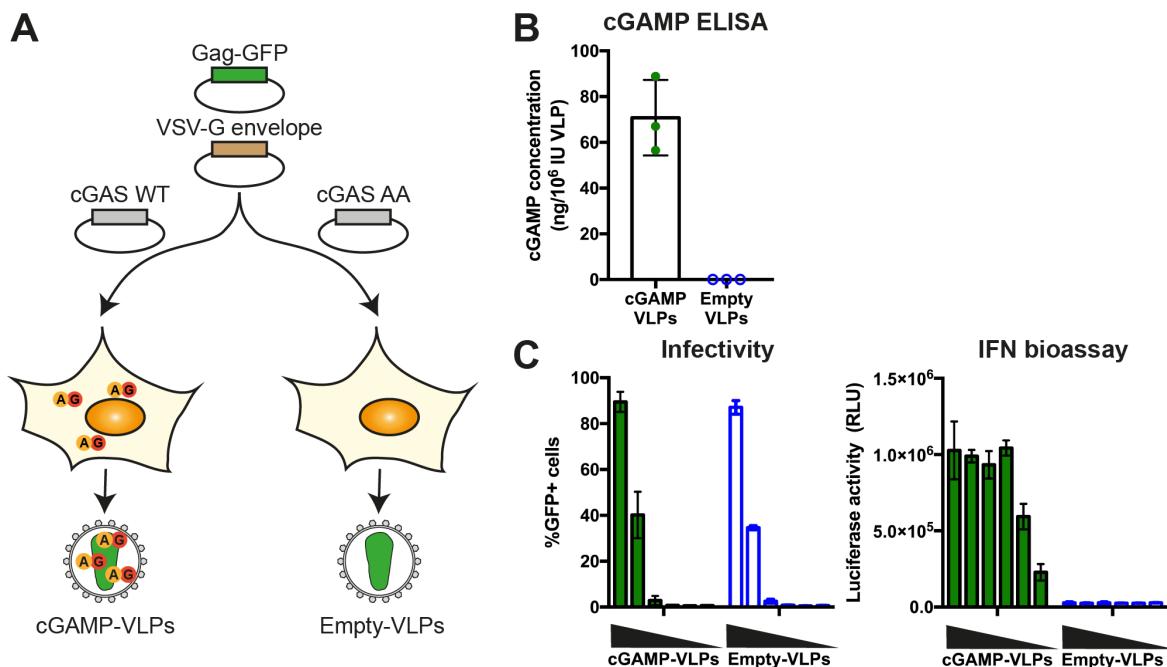
615 **Data availability statement**

616 The authors declare that all data supporting the findings of this study are available within the

617 paper and its supplementary information files.

618 **Figures and figure legends**

619

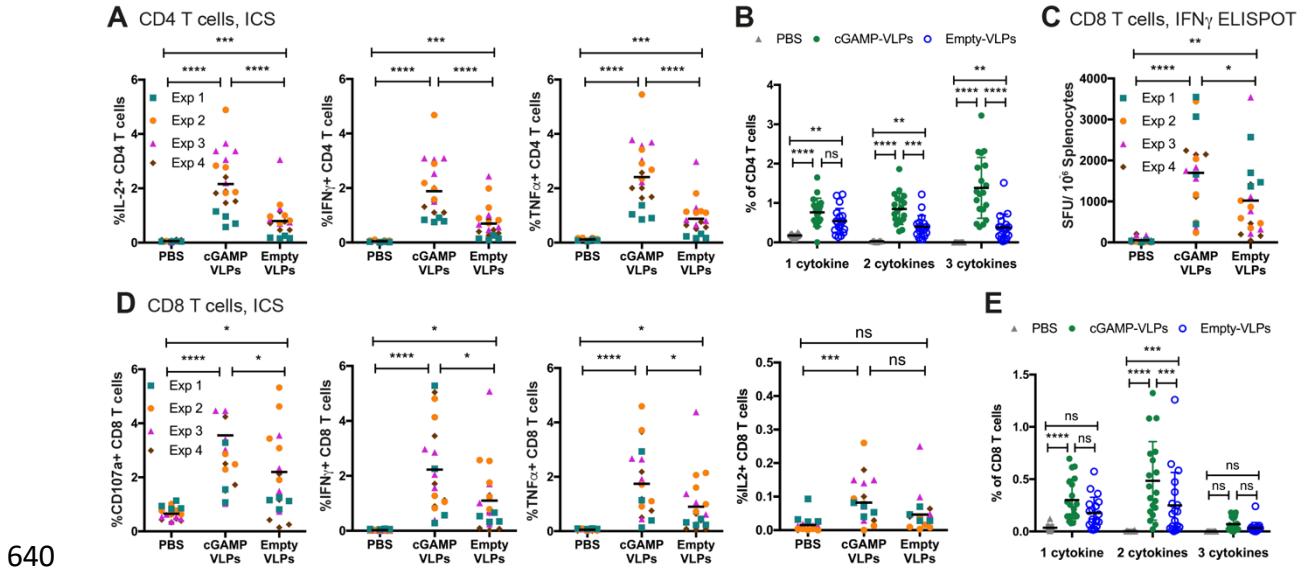


620

621 **Fig 1: cGAMP incorporated into Gag-GFP Virus-like particles (VLPs) induces IFN-I in**  
622 **infected cells.**

623 **A. Schematic representation of cGAMP- and Empty-VLP production.** HEK293T cells  
624 were transfected with plasmids encoding HIV-1 Gag-GFP and VSV-G envelope to enable VLP  
625 production. Overexpression of cGAS WT in the same cells generated cGAMP that was then  
626 incorporated into nascent VLPs (cGAMP-VLPs). As control, Empty-VLPs were produced in  
627 cells where a catalytically inactive cGAS (cGAS AA) was overexpressed. **B. cGAMP is**  
628 **incorporated into cGAMP-VLPs.** Small molecules were extracted and the cGAMP  
629 concentration was measured using a cGAMP ELISA. **C. cGAMP-VLPs induce an IFN-I**  
630 **response in target cells.** HEK293 cells were infected with decreasing amounts of cGAMP-  
631 VLPs and Empty-VLPs (1/5 serial dilutions starting at 2 $\mu$ L of VLP stocks per well) and the  
632 infection was monitored 24 hours later by quantifying GFP<sup>+</sup> cells by flow cytometry.  
633 Supernatants from the same infected cells were then transferred to a reporter cell line

634 expressing firefly luciferase under a promoter induced by IFN-I (ISRE). Luciferase activity  
635 measured 24 hours later indicated the presence of IFN-I in the supernatants.  
636 Data in (B) are pooled from three independent VLP productions. Each symbol corresponds to  
637 one VLP production and mean and SD are shown. Data in (C) are pooled from three  
638 independent VLP productions tested simultaneously in technical duplicates in infectivity and  
639 IFN-I bioassays; mean and SD are shown.

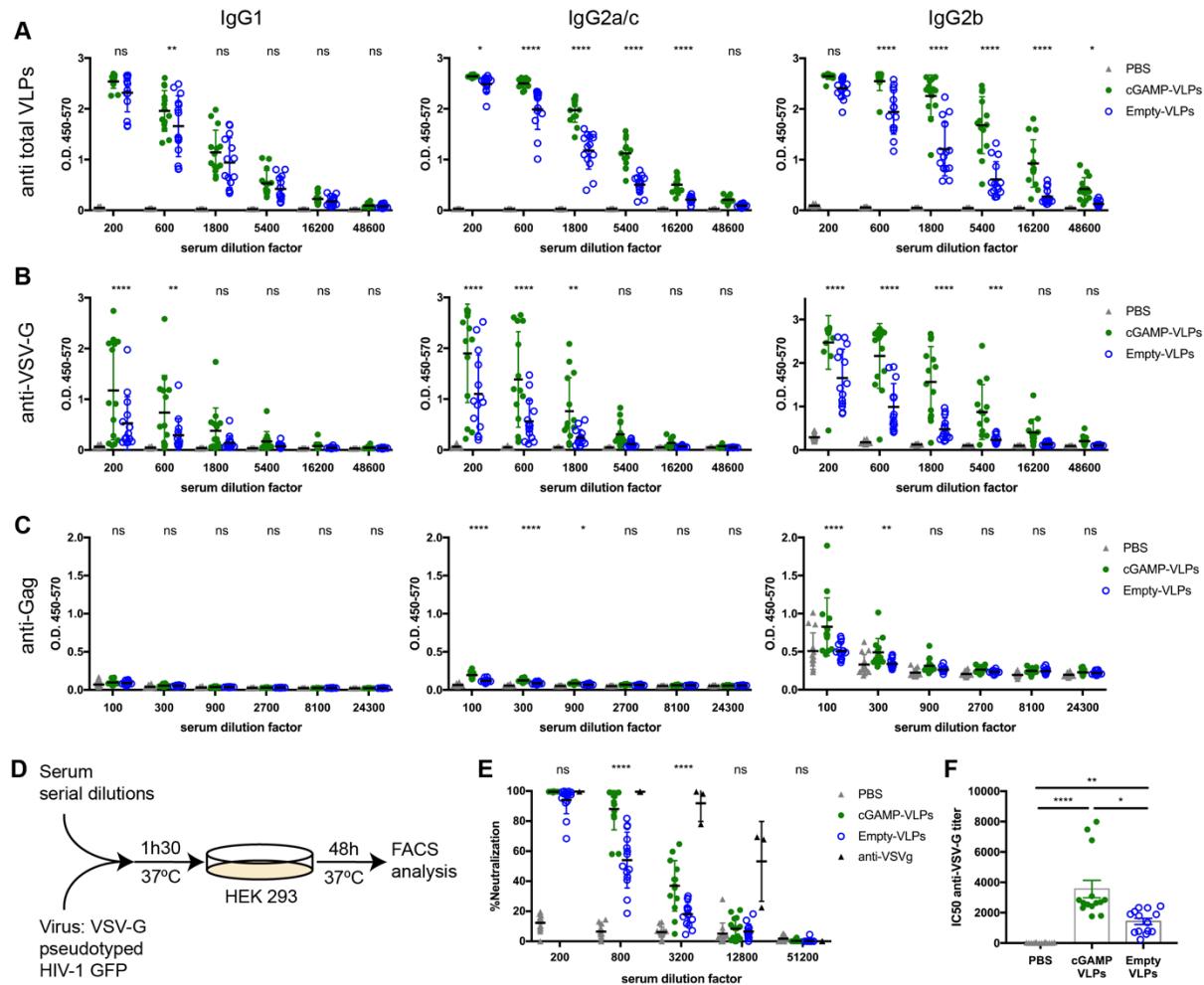


640  
641 **Fig 2: cGAMP loading of VLPs increases the magnitude of the CD4 and CD8 T cell**  
642 **responses elicited after immunisation.** C57BL/6 mice were injected with cGAMP-VLPs,  
643 Empty-VLPs, or PBS as a control *via* the intra-muscular route. 14 days later, VLP-specific T  
644 cell responses were evaluated in the spleen.

645 **A-B. Immunisation with cGAMP-VLPs enhances VLP-specific CD4 T cell responses.**  
646 BMMCs from C57BL/6 mice were pulsed overnight with cGAMP-VLPs and used to stimulate  
647 cells from spleens of immunised mice. Cells were co-cultured for 6 hours prior to evaluation  
648 of CD4 T cell responses by ICS. The percentage of total CD4 T cells producing each cytokine  
649 is shown in A and the percentage of CD4 T cells co-producing 1, 2 or 3 cytokines is shown in  
650 B. **C-E. Immunisation with cGAMP-VLPs facilitates induction of HIV-1 Gag-specific**  
651 **polyfunctional CD8 T cell responses.** Cells from spleens of immunised mice were stimulated  
652 with the HIV-SQV peptide, and responses were read out in (C) a 24-hour IFN $\gamma$  ELISPOT assay;  
653 or (D, E) a 6-hour ICS assay. (D) shows the percentage of total CD8 T cells upregulating  
654 CD107a and/or producing each cytokine, and (E) shows the percentage of CD8 T cells co-  
655 producing 1, 2 or 3 cytokines.

656 Data are pooled from four independent experiments. A total of 19 mice was analysed per  
657 condition. Symbols show data from individual animals, and in (A), (C) and (D) are colour-

658 coded by experiment. Horizontal lines indicate the mean and SD is additionally shown in (B)  
659 and (E). Statistical analyses were performed using a 2-way ANOVA followed by Tukey's  
660 multiple comparisons test. In (A), (C) and (D), data were blocked on experiments. ns  $p \geq 0.05$ ;  
661 \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; \*\*\*\* $p < 0.0001$ .

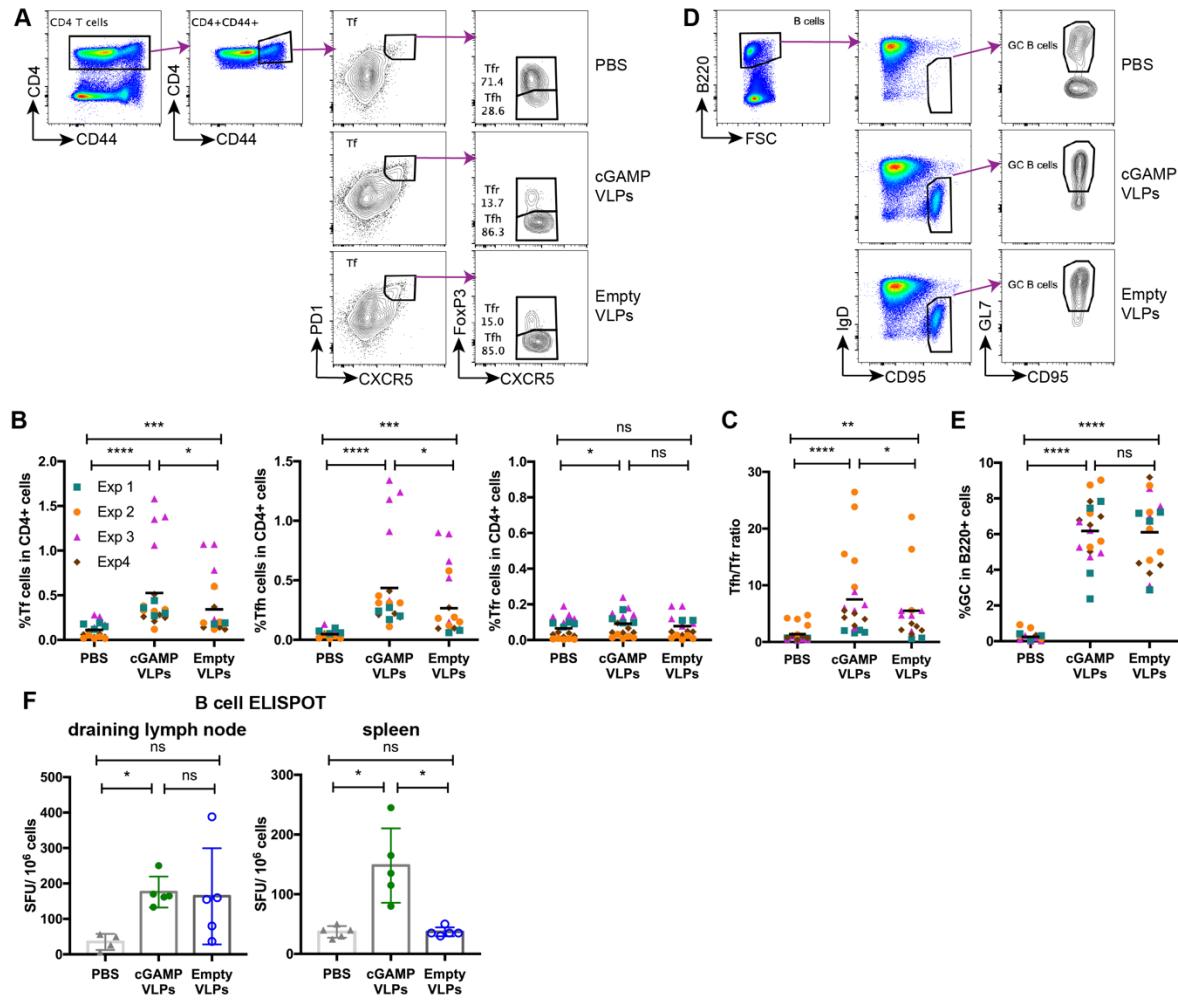


662

663 **Fig 3: Immunisation with VLPs containing cGAMP increases neutralising antibody**  
 664 **responses.** C57BL/6 mice were injected with cGAMP-VLPs, Empty-VLPs, or PBS as a  
 665 control *via* the intramuscular route. Serum antibody responses were evaluated 14 days later.  
 666 **A-C. cGAMP loading enhances IgG responses specific to VLP proteins, including VSV-**  
 667 **G.** ELISA plates were coated with lysate from cGAMP-VLPs (A), recombinant VSV-G protein  
 668 (B), or recombinant HIV-1 Gag protein (C). Antibodies of different isotypes specific for these  
 669 proteins were measured in sera from immunised mice by ELISA. The optical density at  
 670 increasing serum dilutions is shown. **D-F. Immunisation with cGAMP-VLPs enhances**  
 671 **production of neutralising antibodies.** Serial dilutions of serum samples from individual  
 672 mice were incubated with VSV-G pseudotyped HIV-1-GFP for 90 minutes at 37°C before  
 673 infection of HEK293 cells. As a control, serial dilutions of the anti-VSV-G neutralising  
 674 antibody 8G5F11 were tested in parallel. After two days, infection was measured by

675 quantifying GFP<sup>+</sup> cells by flow cytometry (D). Neutralising capacities of serum samples from  
676 individual animals were calculated as a percentage of neutralisation (calculated relative to the  
677 maximum infection in each experiment) (E) and as the half maximal inhibitory concentration  
678 (IC50) (F).

679 Data are pooled from three independent experiments. A total of 14 mice was analysed per  
680 condition. Symbols show data from individual animals, and the mean and SD are indicated.  
681 Statistical analyses were done using a 2-way ANOVA followed by Tukey's multiple  
682 comparisons test, only showing significance between cGAMP-VLPs and Empty-VLPs (A-E)  
683 or a Kruskall-Wallis test followed by Dunn's multiple comparisons test (F). ns p $\geq$ 0.05;  
684 \*p<0.05; \*\*p<0.01; \*\*\*p<0.001; \*\*\*\*p<0.0001.



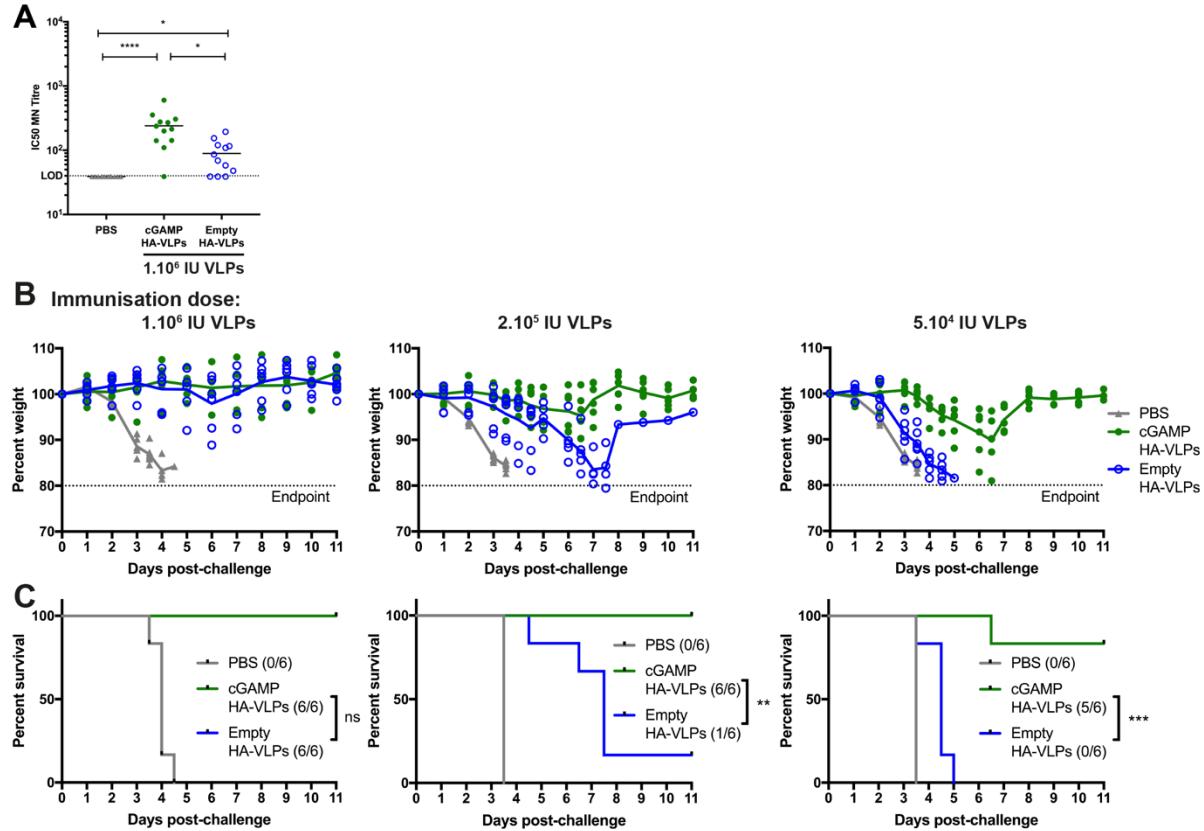
685

686 **Fig 4: cGAMP loading of VLPs enhances induction of CD4 Tfh responses.** C57BL/6 mice  
 687 were injected with cGAMP-VLPs, Empty-VLPs, or PBS as a control *via* the intra-muscular  
 688 route. 14 days later, T and B cells in the draining inguinal lymph nodes were characterised by  
 689 flow cytometry and B cell ELISPOT assays.

690 **A-C. Immunisation with cGAMP-VLPs enhances accumulation of Tfh cells in the**  
 691 **draining lymph node.** T follicular (Tf) cells were identified by flow cytometry as  
 692 CD4<sup>+</sup>CD44<sup>+</sup>CXCR5<sup>hi</sup>PD1<sup>hi</sup> cells and were further subdivided into Tfr cells (FoxP3<sup>+</sup>) and Tfh  
 693 cells (FoxP3<sup>-</sup>). The gating strategy is shown in (A) and the percentages of Tf, Tfh and Tfr cells  
 694 within CD4<sup>+</sup> cells are shown in (B). The ratio of Tfh/Tfr is shown in C. **D-E. Immunisation**  
 695 **with VLPs induces germinal centre formation.** Germinal centre B cells were identified by  
 696 flow cytometry as B220<sup>+</sup>IgD<sup>-</sup>CD95<sup>+</sup>GL7<sup>+</sup> cells. The gating strategy is shown in (D) and the  
 697 percentage of germinal centre B cells amongst B220<sup>+</sup> cells is shown in (E). **F. Immunisation**

698 **with cGAMP-VLPs increases production of antibody-secreting cells.** Cells from draining  
699 lymph nodes and spleens were seeded in ELISPOT plates coated with VLP lysates. After  
700 overnight incubation, cells producing VLP-specific IgG antibodies were identified using an  
701 anti-IgG Fc antibody.

702 In (B), (C) and (E), data were pooled from four independent experiments including a total of  
703 19 mice analysed per condition. Symbols show data from individual animals and are colour-  
704 coded by experiment. Horizontal lines indicate the mean. In (F), symbols show data from 5  
705 mice per group measured in duplicate in one experiment. Mean and SD are indicated. Statistical  
706 analyses were done using a 2-way ANOVA followed by Tukey's multiple comparisons test (B,  
707 C, E) or a Kruskall-Wallis test followed by Dunn's multiple comparisons test (F). ns  $p \geq 0.05$ ;  
708 \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; \*\*\*\* $p < 0.0001$ .



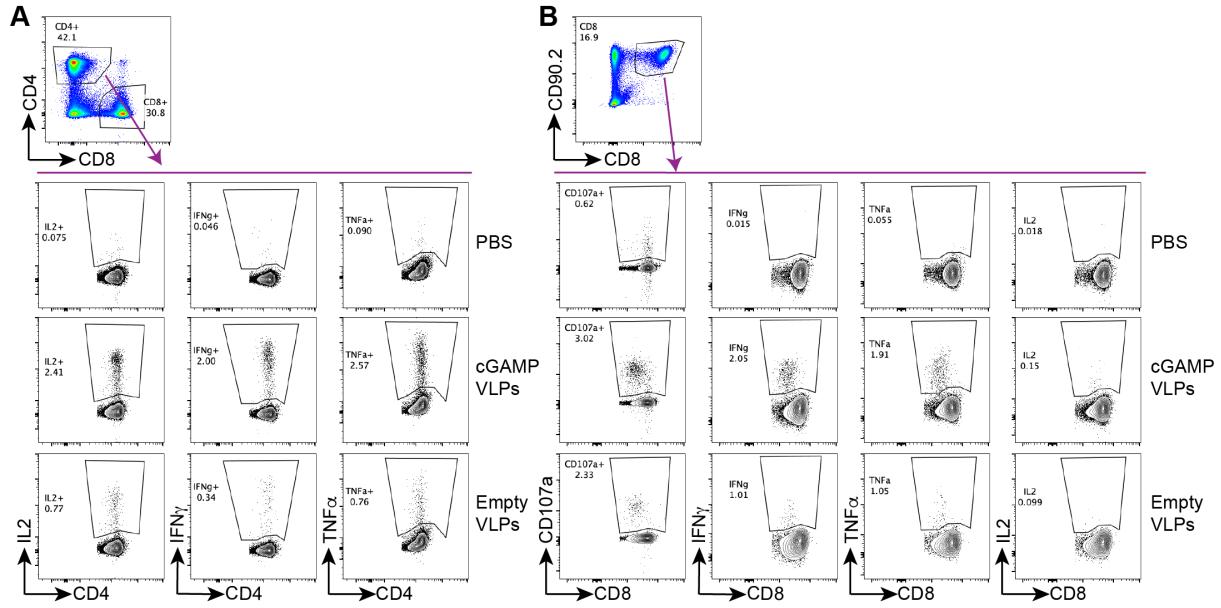
709

710 **Fig 5: cGAMP-VLPs pseudotyped with IAV HA induce neutralising antibodies and**  
711 **confer protection following IAV infection.**

712 Mice were immunised with PBS as a control, cGAMP-HA-VLPs or Empty-HA-VLPs *via* the  
713 intra-muscular route.

714 **A. VLPs pseudotyped with IAV HA induce neutralising antibodies.** Two weeks after  
715 immunisation with 10<sup>6</sup> infectious units of VLPs, sera were collected, heat-inactivated, and  
716 titres of antibodies capable of neutralising an IAV expressing a matched HA protein were  
717 determined by micro-neutralisation (MN) assay. The dotted line shows the limit of detection  
718 (LOD). **B-C. Low doses of cGAMP-HA-VLPs are able to confer protection following IAV**  
719 **challenge.** One month after immunisation with the indicated doses of VLPs, animals were  
720 infected with 10<sup>4</sup> TCID<sub>50</sub> of IAV PR8 virus. Weight loss was monitored over the following  
721 eleven days and is shown as a percentage of starting weight (B). Animals approaching the  
722 humane end-point of 20% weight loss were culled and survival to end-point curves are shown  
723 in (C).

724 In (A), data were pooled from two independent experiments including a total of 10 mice per  
725 condition. In (B) and (C), 5 mice per group were analysed for each VLP dose. In (A) and (B),  
726 symbols show data from individual animals. Statistical analyses were done using a Kruskall-  
727 Wallis test followed by Dunn's multiple comparisons test (A) or a survival analysis with the  
728 Log-rank (Mantel-Cox) test (C). ns  $p \geq 0.05$ ; \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; \*\*\*\* $p < 0.0001$ .

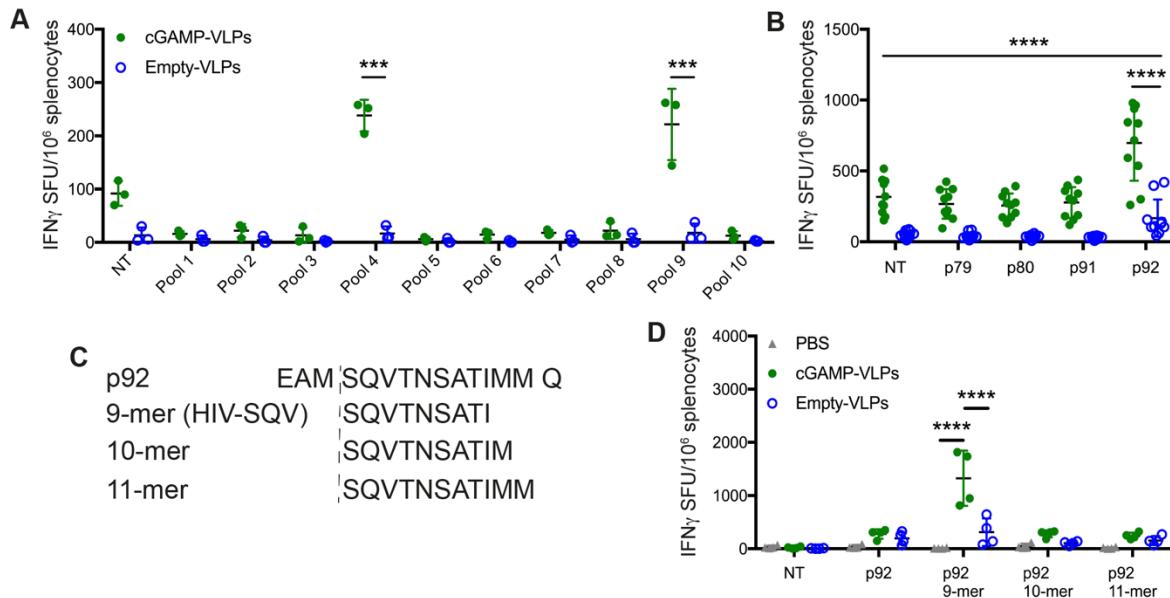


730 **Fig S1: Gating strategies for the analysis of cytokine induction by Intracellular Cytokine  
731 Staining (ICS).**

732 C57BL/6 mice were injected with PBS as a control, cGAMP-VLPs or Empty-VLPs *via* the  
733 intra-muscular route. 14 days later, antigen-specific T cell responses were assessed by  
734 intracellular cytokine staining (ICS).

735 **A.** For stimulation of CD4 T cells, BMMCs from C57BL/6 mice were pulsed overnight with  
736 cGAMP-VLPs and used to stimulate cells from spleens of immunised mice. Cells were co-  
737 cultured for six hours prior to evaluation of CD4 T cell responses by ICS. CD4 T cells were  
738 gated as live, MHC-II $^+$ , CD4 $^+$ , CD8 $^-$ . CD4 T cells expressing IL2, IFN $\gamma$  or TNF $\alpha$  were analysed  
739 as shown.

740 **B.** For stimulation of CD8 T cells, cells from spleens of immunised mice were stimulated with  
741 the HIV-SQV peptide for six hours prior to evaluation of CD8 T cell responses by ICS. CD8  
742 T cells were gated as live, CD90.2 $^+$ , CD8 $^+$ . CD8 T cells expressing CD107a, IFN $\gamma$ , TNF $\alpha$  or  
743 IL2 were analysed as shown.



744

745 **Fig S2: cGAMP-VLPs enhance T cell responses to the Gag HIV-SQV 9-mer peptide.**

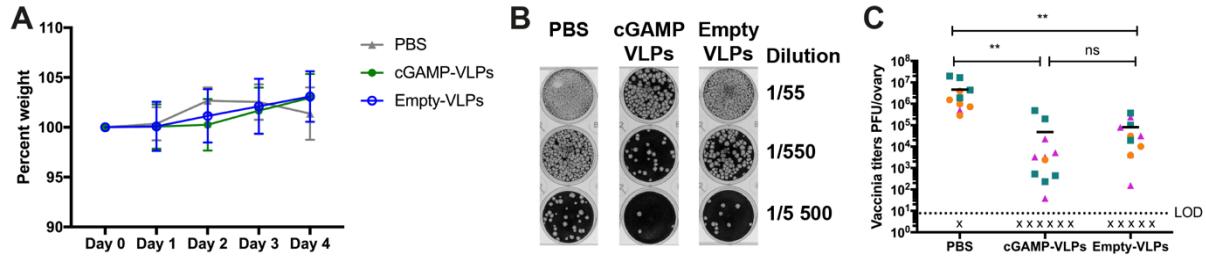
746 C57BL/6 mice were injected with cGAMP-VLPs or Empty-VLPs *via* the intra-muscular route.

747 14 days later, antigen-specific T cell responses were assessed by IFN $\gamma$  ELISPOT assay.

748 **A.** Using a panel of 100 15-mer peptides spanning the HIV-1 Gag protein, we designed ten  
 749 pools of 25 peptides so that each peptide was present in two pools and with minimal overlap  
 750 between the pools. Cells from the spleens of immunised mice were stimulated for 24 hours  
 751 with these peptide pools and responses were read out by IFN $\gamma$  ELISPOT assay. **B.** The peptides  
 752 that were common between pools 4 and 9 (p79, p80, p91, p92) were tested individually. **C.**  
 753 Using NetMHC, we identified a 9-mer, a 10-mer and an 11-mer in p92 as predicted strong  
 754 binders to H2-D<sup>b</sup>. **D.** Splenocytes from immunised mice were stimulated with the four versions  
 755 of p92 shown in (C).

756 Data in (A) and (D) are from a single experiment using three (A) or four (D) animals per group.

757 Pooled data from two independent experiments including a total of 8 mice per group are shown  
 758 in (B). In (A), (B) and (D), each symbol corresponds to one animal and mean and SD are  
 759 shown. Statistical analyses were done using a 2-way ANOVA followed by Tukey's multiple  
 760 comparisons test, showing only selected comparisons. \*\*\*p<0.001; \*\*\*\*p<0.0001.



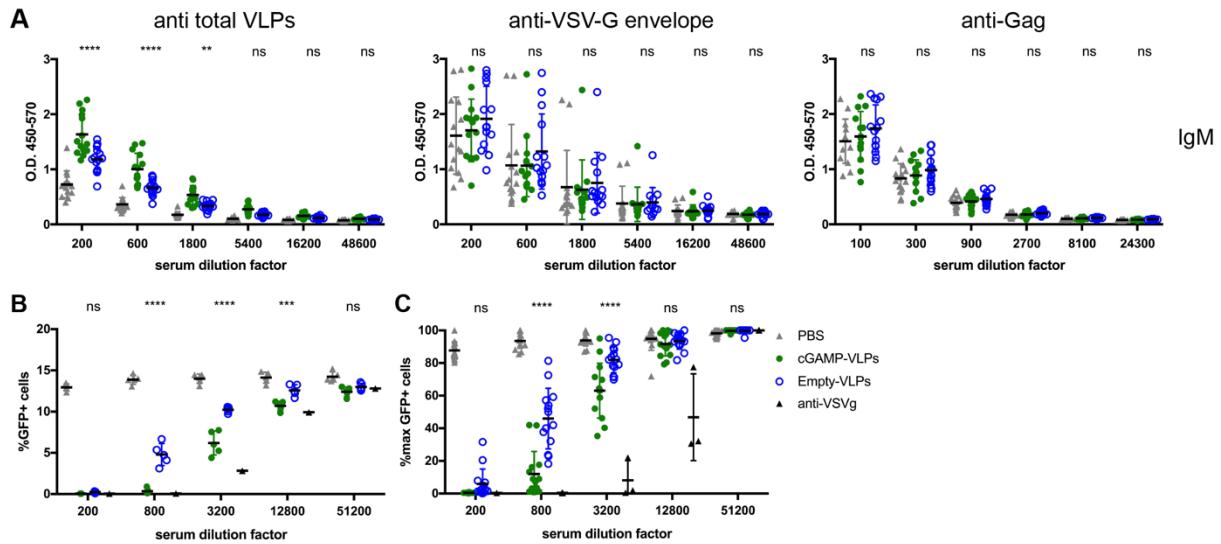
761

762 **Fig S3: Evaluation of cGAMP-VLP-elicited protection in a vaccinia virus challenge**  
763 **model.** Female C57BL/6 mice were injected with cGAMP-VLPs, Empty-VLPs, or PBS as a  
764 control *via* the intra-muscular route. One month later, mice were infected with 10<sup>6</sup> PFU of a  
765 vaccinia virus expressing HIV Gag (vVK1) by intra-peritoneal inoculation.

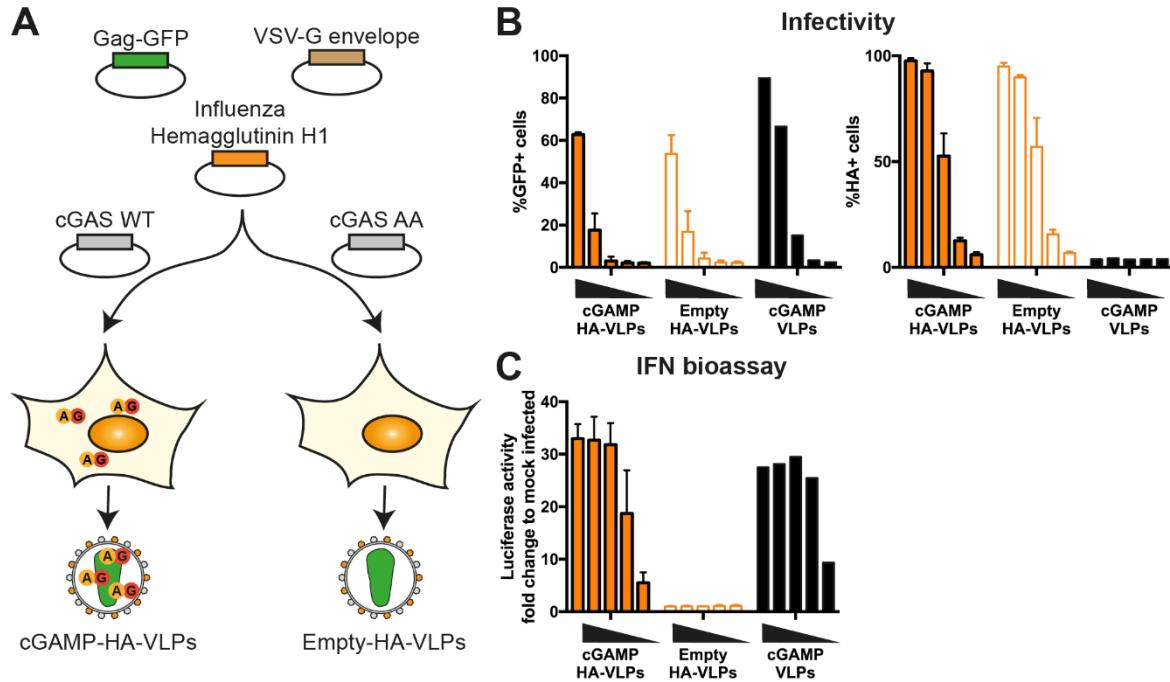
766 A. Weight loss was monitored over the course of infection and is shown as a percentage of  
767 weight prior to infection. B-C. Five days after infection, virus titres in the ovaries were  
768 quantified by plaque assay. A representative example of the plaque assay is shown in (B) and  
769 pooled data from three independent experiments including a total of 12-17 mice per group are  
770 shown in (C).

771 A total of 12 mice (PBS) and 17 mice/group (cGAMP-VLPs and Empty-VLPs) were used in  
772 3 independent experiments. In (A), mean and SD of pooled data are shown. In (C), each symbol  
773 represents data from an individual animal and colours indicate different experiments.  
774 Horizontal lines show the mean. x=sample below limit of detection (LOD).

775 Statistical analyses were done using a 2-way ANOVA followed by Tukey's multiple  
776 comparisons test. ns p≥0.05; \*\*p<0.01.



795 only showing significance between cGAMP-VLPs and Empty-VLPs. ns  $p \geq 0.05$ ; \* $p < 0.05$ ;  
796 \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; \*\*\*\* $p < 0.0001$ .



797

798 **Fig S5: Pseudotyping of cGAMP-VLPs with IAV Haemagglutinin (HA).**

799 **A. Schematic representation of cGAMP-HA-VLP and Empty-HA-VLP production.**

800 HEK293T cells were transfected with plasmids encoding HIV-1 Gag-GFP, VSV-G envelope

801 and IAV HA. cGAMP-HA-VLPs were collected from cells co-expressing cGAS WT and

802 Empty-HA-VLPs from cells co-expressing catalytically inactive cGAS AA. **B. IAV HA is**

803 present in HA-VLPs. HEK293 cells were infected with decreasing amounts of cGAMP-HA-

804 VLPs and Empty-HA-VLPs (1/5 serial dilutions starting at 2 $\mu$ L of VLP stocks per well).

805 Infection was monitored 24 hours later by quantifying GFP<sup>+</sup> and HA<sup>+</sup> cells by flow cytometry.

806 cGAMP-VLPs were used for comparison. **C. cGAMP-HA-VLPs induce a similar IFN-I**

807 **response in infected cells compared to cGAMP-VLPs.** Supernatants from infected cells

808 shown in (B) were tested for the presence of IFN-I as shown in Fig 1C.

809 Data in (B) and (C) are pooled from two independent HA-VLP productions tested

810 simultaneously in technical duplicates in infectivity and IFN-I bioassays; mean and SD are

811 shown.

812

813 **Supplementary Table 1**

814 **Reagents**

	Source	antibody clone	catalog number	additional information
<b>Peptides/proteins</b>				
HIV-1 Con B Gag Peptide Set	NIH AIDS Reagent Program		8117	
pep 92 9-mer (HIV-SQV)	Genscript			custom synthesis
pep 92 10-mer	Genscript			custom synthesis
pep 92 11-mer	Genscript			custom synthesis
recombinant HIV-1 IIIB pr55 Gag protein	NIH AIDS Reagent Program		3276	
recombinant VSV-G protein	alpha diagnostic international		VSIG15-R-10	
<b>Plasmids</b>				
pCMV-VSV-G	Addgene		plasmid # 8454	(Stewart et al., 2003)
Gag-eGFP	NIH AIDS Reagent Program		11468	(Schwartz et al., 1992)
pcDNA3-Flag-mcGAS	gift from Z Chen			(Sun et al., 2013)
pcDNA3-Flag-mcGAS-G198A/S199A	gift from Z Chen			(Sun et al., 2013)
pNL4-3-deltaE-EGFP	NIH AIDS Reagent Program		11100	(Zhang et al., 2004)
pcDNA3.1-H1 (PR8)				Original sequence from (Winter et al., 1981)
<b>Antibodies</b>				
LIVE/DEAD fixable violet dead cell stain	ThermoFischer scientific		L34955	
LIVE/DEAD fixable aqua dead cell stain	ThermoFischer scientific		L34957	
CD16/CD32 Rat anti-Mouse	eBioscience	93		
PE-Cy7 IFN $\gamma$ rat anti-mouse	eBioscience	XMG1.2		
BrilliantViolet 605 anti-mouse CD8a	Biolegend	53-6.7		
PerCP-Cy5.5 anti-mouse CD90.2	Biolegend	30-H12		
AlexaFluor 700 anti-mouse CD4	Biolegend	RM4-5		
BrilliantViolet 510 anti-mouse MHC-II (I-A/I-E)	Biolegend	M5/114.15.2		

PE anti-mouse TNF $\alpha$	Biolegend	MP6-XT22		
APC anti-mouse IL2	Biolegend	JES6-5H4		
APC-Cy7 anti-mouse B220	Biolegend	RA3-6B2		
BrilliantViolet 510 anti-mouse B220	Biolegend	RA3-6B2		
PerCP-Cy5.5 anti-mouse IgD	Biolegend	11-26c.2a		
AlexaFluor 647 GL7	Biolegend	GL7		
PerCP-Cy5.5 anti-mouse CD44	Biolegend	IM7		
BrilliantViolet 421 anti-mouse CXCR5	Biolegend	L138D7		
APC anti-mouse PD1	Biolegend	RMP1-30		
PE anti-mouse CD95	BD Bioscience	Jo2		
HRP goat anti-mouse IgG1	Bethyl laboratories		A90-205P	
HRP goat anti-mouse IgG2a/c	Bethyl laboratories		A90-207P	
HRP goat anti-mouse IgG2b	Bethyl laboratories		A90-109P	
HRP goat anti-mouse IgM	Bethyl laboratories		A90-201P	
anti-IAV Hemagglutinin H1 & H5		21-D8-5A		(Xiao et al., 2018)
<b>Buffers</b>				
Brilliant stain buffer	BD Bioscience		563794	
Fixation/Permeabilisation Solution kit with GolgiStop	BD Bioscience		554715	
eBioscience FoxP3/transcription factor staining Buffer Set	ThermoFischer scientific		00-5523-00	
BD Cellfix	BD Bioscience		340181	
<b>Others</b>				
Fugene 6	Promega		E2691	
Lipofectamine 2000	ThermoFischer scientific		11668030	
One-Glo luciferase assay system	Promega		E6120	
Amicon Ultra 3K filter columns	Millipore		UFC500396	
2'-3' cGAMP ELISA kit	Cayman chemical		501700	
Mouse IFN $\gamma$ ELISPOT BASIC (ALP) kit	Mabtech		3321-2A	

Mouse IgG Basic ELISPOT BASIC (ALP) kit	Mabtech	3825-2A	
Red blood cell lysis buffer	Sigma	R7757- 100ML	
TMB substrate	Invitrogen	00-4201-56	
TPCK-treated trypsin	Sigma	T1426	

815

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