

1 **Cellular selectivity of STING stimulation determines priming of anti-tumor T cell responses**

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16

17 **Abstract**

18 T cells that recognize tumor antigens are crucial for anti-tumor immune responses.  
19 Induction of anti-tumor T cells in immunogenic tumors depends on STING, the intracellular  
20 innate immune receptor for cyclic guanosine monophosphate-adenosine monophosphate  
21 (cGAMP) and related cyclic dinucleotides (CDNs). However, the optimal way to leverage  
22 STING activation in non-immunogenic tumors is still unclear. Here, we show that cGAMP  
23 delivery by intra-tumoral injection of virus-like particles (cGAMP-VLP) leads to differentiation  
24 of tumor-specific T cells, decrease in tumor regulatory T cells (Tregs) and anti-tumoral responses  
25 that synergize with PD1 blockade. By contrast, intra-tumoral injection of synthetic CDN leads to  
26 tumor necrosis and systemic T cell activation but no differentiation of tumor-specific T cells, and  
27 a demise of immune cells in injected tumors. Analyses of cytokine responses and genetic models  
28 revealed that cGAMP-VLP preferentially targets STING in dendritic cells at a 1000-fold less  
29 dose than synthetic CDN. Sub-cutaneous administration of cGAMP-VLP showed synergy when  
30 combined with a tumor Treg-depleting antibody to elicit systemic tumor-specific T cells, leading  
31 to complete and lasting tumor eradication. These findings show that cell targeting of STING  
32 stimulation shapes the anti-tumor T cell response and reveal a therapeutic strategy with T cell  
33 modulators.

34

## 35 **Introduction**

36 T cells that recognize tumor antigens are critical effectors of the anti-tumor immune  
37 response. Most cancer patients do not naturally mount effective T cell responses against their  
38 tumors. Immune-checkpoint blocking antibodies (ICB) led to remarkable therapeutic success  
39 albeit in a fraction of patients and tumor types. ICB require pre-existing anti-tumor T cells  
40 responses to work (Tumeh et al., 2014). The understanding of the mechanisms that efficiently  
41 generate anti-tumor T cells has the potential to expand the efficacy of ICB by enabling new  
42 classes of immunotherapeutic agents.

43 Specialized antigen presenting-cells can stimulate T cell responses from naive cells.  
44 Antigen-presenting cells are activated by innate immune signals emanating from germline-  
45 encoded pattern recognition receptors that recognize non-self or altered-self molecules. STING is  
46 an intracellular pattern recognition receptor for cyclic dinucleotides (CDNs) implicated in the  
47 response to bacteria and to intracellular DNA of foreign and altered-self origins. In mouse  
48 models, spontaneous generation of anti-tumor T cells against immunogenic tumors has been  
49 shown to rely on STING activation (Woo et al., 2014). Intra-tumoral injection of synthetic CDNs  
50 that activate STING stimulate anti-tumor responses, but the underlying mechanisms remain  
51 unclear (Corrales et al., 2015). In fact, synthetic CDNs can have contradictory immune-  
52 stimulatory and immuno-ablative effects at different doses (Sivick et al., 2018). Given that  
53 STING is broadly expressed in normal tissues and also tumors, the potential for tissue-specific  
54 activation of STING may either support protective or pathological responses (Liu et al., 2014).  
55 For example, STING activation within T cells inhibits their proliferation and, at least in mouse,  
56 triggers their death by apoptosis (Cerboni et al., 2017; Gulen et al., 2017). The optimal cell type  
57 for STING activation with the aim of priming antigen-specific anti-tumor T cell responses is  
58 unknown.

59           The STING pathway also plays an evolutionary conserved role in anti-viral immunity  
60 (Goto et al., 2020; Morehouse et al., 2020). Moreover, the natural mammalian STING agonist,  
61 2'3'-cGAMP (cGAMP) can be packaged in particles of enveloped viruses, leading to STING  
62 activation in target cells immediately after fusion of the viral particles (Bridgeman et al., 2015;  
63 Gentili et al., 2015). This represents a Trojan horse system of antiviral defense without the need  
64 to detect viral nucleic acids. Consequently, cGAMP can be packaged in non-infectious enveloped  
65 virus-like particles (VLP). These enveloped retroviral VLPs can be readily produced and  
66 purified, enabling the production of cGAMP-containing VLPs (cGAMP-VLP) (Bridgeman et al.,  
67 2015; Gentili et al., 2015). Inclusion of cGAMP enhances the immunogenicity of VLPs  
68 displaying influenza virus or SARS-CoV-2 glycoproteins (Chauveau et al., 2021).

69           Here, we leveraged the biological properties of cGAMP-VLP to investigate anti-tumoral  
70 immunity induced by STING activation. We characterized STING activation *in vivo* by cGAMP-  
71 VLP compared to established synthetic cyclic dinucleotide (CDN). Using cGAMP-VLP, we  
72 show that STING is essential in dendritic cells for the induction of tumor-specific T cell  
73 responses that respond to ICB. Finally, we identify a critical role of tumor Treg in limiting anti-  
74 tumor T cell response induced by STING activation.

75

## 76 **Results**

### 77 **Production and characterization of cGAMP-VLP**

78 cGAMP-VLP were produced by transient transfection of 293FT cells and purified through  
79 a sucrose cushion and two rounds of ultra-centrifugation. We routinely measured the  
80 concentration of cGAMP and of p24 (antigen of the structural viral protein Gag of HIV-1 used to  
81 produce the VLP) in the purified preparations. Using a nanoparticle tracker, we observed a  
82 homogenous distribution average at 158 nm, which is consistent with the size of retroviral  
83 particles (**Figure S1A**). We visualized the cGAMP-VLP by electron microscopy, which  
84 confirmed the size range (**Figure S1B**). Titration of the cGAMP-VLP on THP-1 cells induced a  
85 dose-dependent upregulation of SIGLEC-1, an IFN-stimulated gene that is upregulated in  
86 response to STING activation (**Figure S1C**). Comparison to the clinically tested CDN ADU-  
87 S100 (Corrales et al., 2015) or to synthetic 2'3'-cGAMP demonstrated that cGAMP-VLP was  
88 ~500x and ~200x more effective, respectively. We enhanced intracellular delivery of ADU-S100  
89 or 2'3'-cGAMP using lipofectamine. cGAMP-VLP was still ~9x and ~50x more effective than the  
90 lipofected ADU-S100 or 2'3'-cGAMP, respectively.

91

### 92 **Intra-tumoral injection of cGAMP-VLP induces tumor rejection**

93 To assess the anti-tumor effect of cGAMP-VLP, we used the male murine tumor MB49  
94 which can be rejected by T cell responses (Perez-Diez et al., 2007). We initiated treatment on 50  
95 mm<sup>3</sup> tumors and performed three intra-tumoral injections of cGAMP-VLP containing 50 ng  
96 cGAMP or injections of PBS, every three days (**Figure S1D**). Tumors grew continuously in the  
97 PBS group, and a minority of mice (3/8) spontaneously eliminated the tumor (**Figure S1E**). In  
98 contrast, all mice treated with cGAMP-VLP (8/8) eradicated the tumor. cGAMP-VLP induced a  
99 statistically significant anti-tumor effect (**Figure S1F**). We also measured the tumor-specific T

100 cell response in the blood in some mice. cGAMP-VLP induced a significant increase in the CD4+  
101 T cells responding to the tumor antigen DBy (**Figure S1G**). In addition, a fraction of mice treated  
102 with cGAMP-VLP showed a high level of CD8<sup>+</sup> T cell responses to the tumor antigen Uty.

103  
104 **Intra-tumoral injection of cGAMP-VLP induces T cell responses in a poorly immunogenic**  
105 **tumor model**

106 This result suggested that cGAMP-VLP has the capacity to stimulate T cell responses  
107 against tumor antigens. To investigate this effect, we switched to the murine tumor B16-OVA,  
108 which is poorly responsive to PD1 blockade (De Henau et al., 2016). We started treatment on  
109 palpable tumors and performed three intra-tumoral injections of either cGAMP-VLP, empty VLP  
110 (VLP), empty VLP with the matched dose of free 2'3'-cGAMP co-injected (VLP + equivalent  
111 cGAMP), free 2'3'-cGAMP alone, free ADU-S100 or PBS (**Figure 1A**). For cGAMP-VLP, we  
112 used an injection dose containing 33 ng of cGAMP in one experiment and 50 ng in a second  
113 experiment. For free 2'3'-cGAMP and ADU-S100, we used 50 µg per injection. To evaluate  
114 STING activation, we measured cytokines in the serum 3h after the first injection (**Figure 1B**).  
115 cGAMP-VLP, ADU-S100 and 2'3'-cGAMP induced IFN- $\alpha$ , IFN- $\beta$ , IL-6 and TNF- $\alpha$ . Empty VLP  
116 did not induce these cytokines. cGAMP-VLP induced significantly more IFN- $\alpha$ , IFN- $\beta$  and TNF-  
117  $\alpha$  than the VLP + equivalent cGAMP, consistent with the enhanced intra-cellular delivery of  
118 cGAMP contained in the VLP of cGAMP-VLP. Low (33 ng) or higher doses (50 ng) cGAMP-  
119 VLP induced similar levels of cytokines compared to 50 µg free 2'3'-cGAMP. ADU-S100 (50  
120 µg) induced higher levels of the cytokine, suggesting that STING stimulation across cell types  
121 was not saturated by cGAMP-VLP. These results show that cGAMP-VLP induces cytokine  
122 responses that require a 1000-fold less amount of cGAMP compared to the synthetic molecule.

123 We next measured tumor growth. ADU-S100 and cGAMP-VLP were tested with or  
124 without anti-PD1 to assess the impact of immune checkpoint inhibition on the response. cGAMP-  
125 VLP induced a delay in tumor growth (**Figure 1C, 1D**). Adding anti-PD1 enhanced this delay  
126 and led to complete responses in a subset of mice (**Figure 1C**). In comparison, ADU-S100  
127 induced a delay in tumor progression and some complete responses, but there was no additive  
128 effect of anti-PD1. 2'3'-cGAMP alone or co-injected with VLP induced a smaller tumor growth  
129 delay and no complete responses were observed. Empty VLP had no effect. Similar trends were  
130 observed on mouse survival (defined in this study as the time until the ethical endpoint of 2000  
131 mm<sup>3</sup> tumor size is reached) (**Figure 1E**). Specifically, anti-PD1 enhanced the survival of mice  
132 treated with cGAMP-VLP, while it had no impact when combined with ADU-S100. Furthermore,  
133 we observed that the anti-tumor effect of ADU-S100 was characterized by necrosis of all the  
134 injected tumors, while necrosis was rarely observed with cGAMP-VLP (**Figure S2A**).

135 These results suggested potential differences in T cell responses induced by cGAMP-VLP  
136 or ADU-S100. We measured the frequency of OVA-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses in  
137 blood 10 days after treatment initiation. cGAMP-VLP induced significant responses and the  
138 majority of mice showed detectable responses (**Figure 1F**). In contrast, ADU-S100 did not  
139 induce detectable T cell responses in most mice. In few mice, a T cell response was detected, but  
140 its magnitude did not reach the average response observed with cGAMP-VLP. Overall, the  
141 induction of OVA-specific T cell responses by ADU-S100 was not significant. It has been  
142 proposed that ADU-S100 ablates the T cell responses, and that at lower doses it may induce  
143 tumor-specific T cell responses in blood (Sivick et al., 2018). We performed a dose-titration of  
144 ADU-S100 in the B16-OVA model and observed a dose-response anti-tumor effect (**Figure**  
145 **S2B**). In the blood, we detected OVA-specific CD8<sup>+</sup> responses at the highest dose of ADU-S100  
146 in a subset of mice, but these were not significant (**Figure S2C**). No OVA-specific CD8<sup>+</sup>

147 response was observed at lower doses of ADU-S100, nor in CD4<sup>+</sup> T cells. Thus, lower doses of  
148 ADU-S100 do not induce tumor-specific T cell responses in blood in this model. We conclude  
149 that intra-tumoral injection of cGAMP-VLP stimulates immunogenic anti-tumor T cell responses  
150 at low doses of cGAMP.

151  
152 **Tumor specific T-cell responses elicited by intra-tumorally administered cGAMP-VLP**  
153 **translate into systemic synergy with anti-PD1**

154 We next sought to explore whether the T cell responses induced by cGAMP-VLP  
155 translate into systemic anti-tumor effect. To this end, we used a B16-OVA dual tumor model  
156 (**Figure 2A**). Intra-tumoral injection of cGAMP-VLP or ADU-S100 in one of the tumors induced  
157 IFN- $\alpha$ , IFN- $\beta$ , IL-6 and TNF- $\alpha$  in the blood (**Figure 2B**). 10 days later, significant levels of  
158 OVA-specific CD8<sup>+</sup> and CD4<sup>+</sup> T cells were detected in the blood of cGAMP-VLP treated mice  
159 (**Figure 2C**). In contrast, ADU-S100 induced T cell responses only in a minority of mice that  
160 were not statistically significant compared to the control group. We next monitored tumor growth  
161 in groups co-treated or not with anti-PD1. We confirmed that B16-OVA was resistant to anti-PD1  
162 (**Figure 2D**). cGAMP-VLP induced a delay in tumor growth in local and distant tumors, and  
163 addition of anti-PD1 extended the delay and increased the number of eradicated tumors (**Figure**  
164 **2D**). In contrast ADU-S100 induced a strong anti-tumor effect that was characterized by necrosis  
165 at the injected tumor (**Figure S2D**). At the distal tumor, ADU-S100 induced an anti-tumoral  
166 effect, but this effect was not enhanced by anti-PD1 (**Figure 2E**). Ultimately, cGAMP-VLP  
167 combined with anti-PD1 decreased the distal tumor size more potently than ADU-S100,  
168 irrespectively of its combination with anti-PD1 (**Figure 2F**). Completely responding mice were  
169 challenged at day 80 with a second round of tumor graft. Mice that eradicated their initial tumor  
170 following cGAMP-VLP treatment were more resistant to the formation of a new tumor than mice



171 that received ADU-S100 (**Figure 2G**). We conclude that cGAMP-VLP demonstrated a  
172 synergistic effect with anti-PD1, unlocking the ability of B16-OVA bearing mice to respond to  
173 immune checkpoint blockade. In contrast, the synthetic CDN ADU-S100 induces systemic anti-  
174 tumor responses that do not elicit OVA-specific T cells response and do not synergize with anti-  
175 PD1.

176

### 177 **cGAMP-VLP requires host STING and T cells to induce anti-tumor effects**

178 To understand the nature of the anti-tumor response induced by cGAMP-VLP, we tested  
179 the role of STING and T cells using *Sting1* and *Rag2* knock-out mice, respectively (**Figure 3A**).  
180 We selected a dual tumor B16-OVA model treated with intra-tumoral 50 ng cGAMP-VLP or 50  
181  $\mu$ g ADU-S100 monotherapy. Induction of IFN- $\alpha$ , IL-6 and TNF- $\alpha$  by cGAMP-VLP or ADU-  
182 S100 was lost in *Sting1*<sup>-/-</sup> mice, indicating that STING is required in host cells (**Figure 3B**). In  
183 contrast, the cytokines were still induced in *Rag2*<sup>-/-</sup> mice showing that T cells were not mediating  
184 these early response cytokines. Next, we measured the OVA-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cell  
185 response in blood. As expected, the T cell responses induced by cGAMP-VLP were not detected  
186 in *Rag2*<sup>-/-</sup> mice (**Figure 3C**). In *Sting1*<sup>-/-</sup> mice, the T cell responses induced by cGAMP-VLP  
187 were heterogeneous and not statistically significant, as compared to WT mice. Nevertheless, T  
188 cell responses were detectable in some of the mice, indicating that additional pathways contribute  
189 to the immune-stimulating activity of cGAMP-VLP. We next examined the growth of tumors.  
190 The anti-tumor effect of cGAMP-VLP and ADU-S100 on the size of injected and distal tumors  
191 was lost in *Sting1*<sup>-/-</sup> (**Figure 3D**). In *Rag2*<sup>-/-</sup> mice, the anti-tumor effect cGAMP-VLP was lost in  
192 the injected and distal tumors. In contrast, the effect of ADU-S100 was maintained in the injected  
193 tumors, but lost at the distal ones. Consistently, cGAMP-VLP and ADU-S100 increased the

194 survival of dual B16-OVA tumor bearing mice compared to PBS treated mice, and these  
195 increases were abolished in *Sting1<sup>-/-</sup>* or *Rag2<sup>-/-</sup>* mice (**Figure 3E**).

196 These results prompted us to test the relative role of CD8<sup>+</sup> T cells and NK cells in tumor  
197 elimination induced by cGAMP-VLP using depleting antibodies (**Figure S3A**). The anti-CD8 $\alpha$   
198 antibody induced a depletion of CD8<sup>+</sup> T cells at day 7 and 17, an increase in NK cells at day 17,  
199 and no effect on CD4<sup>+</sup> T cells (**Figure S3B**). In contrast the anti-NK1.1 antibody depleted NK  
200 cells and had a slight depleting effect on CD8<sup>+</sup> T cells at days 7. The antibodies had no effect on  
201 cytokine production induced by cGAMP-VLP at day 7, two days after the first round of depletion  
202 (**Figure S3C**). As expected, the anti-CD8 $\alpha$  antibody blunted the detection of OVA-specific CD8<sup>+</sup>  
203 T cells (**Figure S3D**). CD8<sup>+</sup> T cell depletion also cancelled the effect of cGAMP-VLP on mouse  
204 survival, while NK cell depletion had no effect (**Figure S3E**). We conclude that the anti-tumor  
205 effect of cGAMP-VLP requires STING in the host and CD8<sup>+</sup> T cells, but not NK cells, while the  
206 effect of ADU-S100 requires host STING but is partially independent of T cells.

207

### 208 **Immune cell composition and activation differentiates cGAMP-VLP from ADU-S100**

209 Our results suggested the following paradox: while high levels of tumor-antigen-specific  
210 T cells were detected in the blood of cGAMP-VLP treated mice but not in ADU-S100 treated  
211 mice, the abscopal anti-tumoral effect of both treatments required T cells. To resolve this  
212 paradox, we investigated the composition and activation status of immune cells in tumors and  
213 lymphoid organs (**Figure 4A**). In the injected tumors, cGAMP-VLP induced a significant  
214 increase in CD8<sup>+</sup> T cells and a decrease in CD4<sup>+</sup> Tregs and NK cells (**Figure 4B, top panel**). In  
215 contrast, ADU-S100 significantly depleted CD45.2<sup>+</sup> immune cells, in particular NK and CD4<sup>+</sup> T  
216 cells. ADU-S100 had no impact on CD8<sup>+</sup> T cells or Tregs. In the distal tumor, cGAMP-VLP  
217 induced a significant increase in CD8<sup>+</sup> T cells but Tregs levels were not affected (**Figure 4B,**

218 **bottom panel**). In contrast ADU-S100 had no significant impact on the proportion of immune  
219 cells based on the markers tested in the distal tumor. We next analyzed lymphoid organs. In the  
220 tumor-draining lymph nodes, cGAMP-VLP increased the proportion of effector memory CD4<sup>+</sup>  
221 and CD8<sup>+</sup> T cells (**Figure 4C, left panel**). In contrast, ADU-S100 decreased the frequency of  
222 central memory CD4<sup>+</sup> T cells, had no impact on effector memory CD4<sup>+</sup> T cells, and increased the  
223 proportion of effector memory CD8<sup>+</sup> T cells. In non-draining lymph nodes and in the spleen, both  
224 cGAMP-VLP and ADU-S100 increased the proportion of effect memory CD8<sup>+</sup> T cells (**Figure**  
225 **4C, middle and right panels**). It was surprising that both cGAMP-VLP and ADU-S100  
226 increased effector memory CD8<sup>+</sup> T cells in all lymphoid organs examined, but only cGAMP-  
227 VLP induced robust levels of tumor antigen-specific T cell responses. This raised the possibility  
228 that ADU-S100 might induce T cell activation independently from tumor antigens. To test this  
229 possibility, we examined the level of CD69, an early marker of T cell activation. Strikingly,  
230 ADU-S100 induces upregulation of CD69 in tumors and in all lymphoid organs tested, in both  
231 CD4<sup>+</sup> and CD8<sup>+</sup> (**Figure 4D**). This reached up to 20% and 30% of T cells in spleen and non-  
232 draining lymph nodes, a week after the last injection of ADU-S100. This systemic effect was not  
233 observed with cGAMP-VLP, which induced significant levels of CD69 in non-draining lymph  
234 nodes, but not in other organs tested. This result suggests that ADU-S100 induces a general  
235 activation of T cells, which does not appear to translate into the expansion of tumor antigen-  
236 specific T cells. In contrast, cGAMP-VLP appears to induce a specific T cell response for tumor  
237 antigens.

238

### 239 **cGAMP-VLP targets preferentially antigen-presenting cells**

240 To understand the induction of tumor antigen-specific T cells by cGAMP-VLP, we  
241 analyzed its effect *in vitro* on a set of cell types present in the tumor micro-environment, starting

242 with cell lines. We treated the tumor cell line B16-OVA, the endothelial cell line MS1, the  
243 dendritic cell line MutuDC and the macrophage cell line RAW. cGAMP-VLP induced the  
244 highest levels of IFN- $\beta$  in RAW cells, followed by MutuDC and MS1, in a dose-dependent  
245 manner (**Figure S4A, S4B**). The IFN- $\beta$  induction in B16-OVA cells was the lowest. ADU-S100  
246 also induced dose-dependent IFN- $\beta$ , but this was less cell-type selective than cGAMP-VLP.  
247 Soluble cGAMP induced detectable IFN- $\beta$  only at the highest tested dose. To gain further  
248 insights in the induction of interferons by antigen-presenting cells, we treated bone marrow  
249 derived macrophage (BMDM) and dendritic cells (BMDC), the latter obtained either with GM-  
250 CSF (which generates mainly inflammatory dendritic cells) or with FLT3L (which generates a  
251 mixed population of cDC1, cDC2 and pDCs). cGAMP-VLP and ADU-S100 induced similar  
252 levels of IFN- $\alpha$  and IFN- $\beta$  in BMDM and BMDC (with GM-CSF) (**Figure S4C**). In contrast,  
253 cGAMP-VLP induced significantly higher levels of both cytokines in BMDC (with FLT3L)  
254 (**Figure S4D**). Synthetic cGAMP induced detectable cytokines only at the highest tested dose,  
255 despite 1000-fold higher amounts than in cGAMP-VLP. These results suggested a preferential  
256 activation of STING in antigen-presenting cells by cGAMP-VLP, in particular in FLT3L-derived  
257 cells. To determine if this was associated with preferential uptake of the particles, we attempted  
258 to detect cGAMP-VLP *in vivo* in samples stained for p24, but the antibody-based detection was  
259 not sensitive enough. As a surrogate, we treated splenocytes with cGAMP-VLP and stained for  
260 p24 (**Figure S5A**). The highest levels of uptake were detected in macrophages, cDC1 and cDC2  
261 (**Figure S5B, S5C, S5D**). The particles were also detected in some lymphocytes, but only in a  
262 fraction of cells within each population. Altogether these results indicate that cGAMP-VLP  
263 targets preferentially antigen-presenting cells.

264

265 **STING is required in dendritic cells for T-cell mediated anti-tumor effects of cGAMP-VLP**

266 To decipher the contribution of STING within antigen-presenting cells, we generated  
267 STING-OST<sup>fl</sup> mice in which the first coding exon of *Sting1* was flanked by LoxP sites. We also  
268 introduced a Twin-Strep-tag (OST) at the N-terminus of STING protein. We crossed the mice to  
269 *LysM-cre* or *Itgax-cre* and confirmed preferential deletion of STING in macrophages or dendritic  
270 cells, respectively, using Strep-Tactin staining, and thus referred to these mice as STING-OST<sup>AMP</sup>  
271 and STING-OST<sup>ADC</sup>, respectively (**Figure S6A, S6B**). Following STING deletion in  
272 macrophages, the induction of IFN- $\alpha$  and IL-6 in serum by cGAMP-VLP and ADU-S100 was  
273 reduced (**Figure 5A, 5B**). However, the induction of OVA-specific T cells by cGAMP-VLP  
274 (**Figure 5C**) and the anti-tumoral effect (**Figure 5D**) were maintained. In comparison, the anti-  
275 tumor effect of ADU-S100 was partially reduced. Following STING deletion in dendritic cells,  
276 the induction of IFN- $\alpha$  and IL-6 by cGAMP-VLP was reduced, but not for ADU-S100 (**Figure**  
277 **5E**). The induction of OVA-specific T cells by cGAMP-VLP was reduced, but not completely  
278 lost (**Figure 5F**) and the anti-tumor effect of cGAMP-VLP was essentially abrogated in these  
279 mice (**Figure 5G**). In contrast, the anti-tumor effect of ADU-S100 was reduced but maintained.  
280 These results indicate that STING is specifically required in dendritic cells for the anti-tumor  
281 effect of cGAMP-VLP, while the anti-tumor effect of ADU-S100 depends partially on STING in  
282 macrophages and dendritic cells.

283

#### 284 **Systemic administration of cGAMP-VLP activates anti-tumor T cells immunity**

285 The activation of STING in dendritic cells by cGAMP-VLP raised the possibility that it  
286 could induce anti-tumor T cell responses even after injection outside of the tumor mass. We first  
287 tested the B16-OVA model combined with anti-PD1 (**Figure 6A**). Sub-cutaneous (s.c.) injection  
288 of cGAMP-VLP induced detectable levels of IFN- $\alpha$ , IFN- $\beta$ , IL-6 and TNF- $\alpha$ , albeit to lower  
289 levels than following intra-tumoral (i.t.) injection (**Figure 6B**). Tumor growth was delayed after

290 s.c. injection of cGAMP-VLP (**Figure 6C**), leading to significantly smaller tumors (**Figure 6D**).  
291 cGAMP-VLP s.c. also induced anti-OVA T cell responses (**Figure 6E**) and increased the survival  
292 of tumor-bearing mice (**Figure 6F**).

293 In these experiments, the i.t. route remained more effective than the s.c. route at inducing  
294 T cell responses and anti-tumor effects. This suggested that a negative regulator of the immune  
295 response might be eliminated locally by i.t. activation of STING. We previously noted that  
296 cGAMP-VLP induced a reduction of Tregs in the injected tumor, but not in the distal tumors  
297 (**Figure 4B**). This raised the possibility that intra-tumor Tregs might limit the anti-tumor effect of  
298 systemic STING activation by cGAMP. In order to test this hypothesis, we used an IgG2a isotype  
299 antibody against CTLA4 (anti-CTLA4-m2a), which has been shown to selectively deplete Tregs  
300 in tumors (Arce Vargas et al., 2017; Selby et al., 2013), and we confirmed this effect in the  
301 MCA-OVA tumor model (**Figure 6G, 6H**). Treatment with anti-CTLA4-m2a had no effect on  
302 the induction IFN- $\alpha$ , IL-6 and TNF- $\alpha$  by cGAMP-VLP (**Figure S7A**). In monotherapy, cGAMP-  
303 VLP s.c. or anti-CTLA4-m2a increased the frequency of OVA-specific CD8<sup>+</sup> and CD4<sup>+</sup> T cells,  
304 but no significant response to the endogenous tumor antigen p15 (**Figure 6I, S7B**). In contrast,  
305 combining cGAMP-VLP s.c. with anti-CTLA4-m2a synergized to significantly increase the  
306 levels of T cells against p15, and further increased the levels of T cells against OVA.  
307 Accordingly, combination therapy induced a near-complete reduction in tumor size (**Figure 6J,**  
308 **S7C**). Similarly, monotherapies induced an increase in survival, but only the combination therapy  
309 induced long-term survival of treated mice (**Figure 6K**). Completely responding mice were also  
310 protected from a secondary tumor challenge (**Figure S7D**). We conclude that systemic  
311 administration of cGAMP-VLP activates anti-tumor T cell immunity that synergizes with tumor  
312 Treg depletion.

313



## 315 **Discussion**

316           These results highlight the crucial importance of targeting STING activation in particular  
317 cell types, namely dendritic cells, to optimize the antigen-specific anti-tumor responses. STING  
318 was previously shown to be required in dendritic cells *in vitro* to induce an interferon response to  
319 immunogenic tumor cells or tumor DNA (Deng et al., 2014; Woo et al., 2014). *In vivo*, it was  
320 previously noted that dendritic cells are a major source of IFN- $\beta$  in tumors that induce STING-  
321 dependent immunogenic responses (Andzinski et al., 2016). Intriguingly, STING in CD11c<sup>+</sup> cells  
322 is also implicated in the negative regulation of allogeneic responses (Wu et al., 2021). Altogether,  
323 STING in dendritic cells emerges as a linchpin for the induction of antigen-specific T cell  
324 responses.

325           In contrast to cGAMP-VLP, the anti-tumor responses induced by ADU-S100 were not  
326 associated with the induction of tumor-specific T cells. It was previously proposed that the  
327 induction of antigen-specific T cells by ADU-S100 was dose-dependent (Sivick et al., 2018). We  
328 did not observe such bimodal behavior in the tumor model we tested. We noted that ADU-S100  
329 induced some level of tumor-specific T cells in experiments with in-house bred mice (**Figures**  
330 **5C, 5F**), but not with mice obtained from an external source (**Figures 1F, 2C, S2C**). This raises  
331 the intriguing possibility that housing parameters such as the composition of the microbiota, or  
332 genetic background, might affect the immunogenic properties of synthetic CDNs. We also noted  
333 that synthetic CDNs induced necrosis at the intra-tumoral injection site which was rarely seen  
334 with cGAMP-VLP. This is consistent with a role of STING activation in endothelial cells caused  
335 by synthetic CDNs as contributing to its local anti-tumor effects (Demaria et al., 2015; Francica  
336 et al., 2018; Jeong et al., 2021). The reduced dose of cGAMP in cGAMP-VLP compared to free  
337 CDN likely contributes to the reduced tissue necrosis after cGAMP-VLP treatment.



338 Multiple approaches have been proposed to optimize delivery of CDNs for use as  
339 immunomodulators in the absence of exogenous tumor antigens. Synthetic nanoparticles  
340 assembled in the presence of CDNs have been shown to enhance cytosolic delivery and activate  
341 STING-dependent anti-tumor responses (Lu et al., 2020; Wilson et al., 2018). Exosomes loaded  
342 with CDNs appear to achieve similar enhancements (Jang et al., 2021; McAndrews et al., 2021).  
343 Principles to ensure that delivery with synthetic approaches will yield tumor-specific T cell  
344 responses generated are ill-defined. A common limitation of synthetic cargos and exosomes lies  
345 in the passive delivery mechanism to target cells. In contrast, cGAMP-VLPs employ a viral  
346 fusion glycoprotein to efficiently fuse with target cells. The size of the VLPs, their lipid bilayer  
347 originating from a producer cell and the fusion triggered by VSV-G in acidic endosomes most  
348 likely contribute to the selectivity of cGAMP-VLPs for antigen-presenting cells, in particular  
349 dendritic cells. Accordingly, retroviral particles are also efficiently captured by antigen-  
350 presenting cells *in vivo* (Sewald et al., 2015). In addition, a higher expression of STING or  
351 downstream signaling proteins in antigen-presenting cells might also contribute.

352 A feature of the response to cGAMP-VLP is the decrease of tumor Tregs when it was  
353 directly injected in the tumor. We do not know if this effect is a response of Tregs to STING  
354 activation in the tumor micro-environment, or whether it is a secondary effect resulting from anti-  
355 tumor T cell stimulation. Similar to previous studies, we found that treatment with anti-CTLA4-  
356 m2a induced a partial anti-tumor response (Arce Vargas et al., 2017). Combination of s.c.  
357 cGAMP-VLP with this tumor Treg-depleting agent induced a near-complete response to  
358 treatment. These results suggest that the level of Tregs in the tumor may be an important factor to  
359 consider for clinical development of STING-targeted therapies such as cGAMP-VLP.

360 Altogether, our results establish that cell-type specific activation of STING plays a critical  
361 role in anti-tumor immunogenicity. Synthetic STING agonists appear to induce promiscuous

362 STING activation that does not necessarily entail priming of tumor-specific T cells. In contrast,  
363 cGAMP-VLP constitutes a biological product that activates STING preferentially in dendritic  
364 cells, leading to activation of tumor-specific T cells, which synergize with ICB and Treg  
365 depletion. Biological stimulation of STING with cGAMP-VLP has the potential, similar to other  
366 biological drugs such as antibodies and CAR-T cells, to contribute to a meaningful treatment  
367 regimen to induce anti-tumor immune responses in patients.

368

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378

379 **Author contributions**

380 B. Jneid performed most experiments, analyzed data and prepared figures. A. Bochnakian  
381 performed a set of in vitro experiments. F. Delisle, E. Djacoto and J. Denizeau contributed to  
382 experiments. C. Sedlik, R. Kramer, I. Walters, E. Piaggio suggested experiments and contributed  
383 to data analysis and interpretations. B. Malissen and F. Fiore conceived and developed the  
384 STING-OST<sup>fl</sup> mice. S. Carlioz developed Randmice. S. Carlioz and N. Manel conceived the  
385 study. N. Manel and B. Jneid wrote the manuscript.

386

387 **Methods**

388 **Cell culture**

389 293T cells, RAW cells and MS1 cells were cultured in DMEM GlutMAX, 10% fetal bovine  
390 serum (FBS) (Gibco), and penicillin-streptomycin (Gibco). THP-1 cells were cultured in RPMI  
391 GlutMAX medium, 10% FBS (Gibco), and penicillin-streptomycin (Gibco). B16-OVA cells were  
392 cultured in RPMI GlutMAX medium with 10% FBS (Gibco), penicillin-streptomycin (Gibco), 1

393 mM 2-mercaptoethanol, geneticin and hygromycin. MCA-OVA cells were cultured in RPMI  
394 GlutMAX medium with 10% FBS (Gibco), penicillin-streptomycin (Gibco), 1 mM 2-  
395 mercaptoethanol, and hygromycin. MB49 cells were cultured in DMEM GlutMAX medium with  
396 10% FBS (Gibco) and penicillin-streptomycin (Gibco). MutuDC were cultured as described  
397 (Kozik et al., 2020). The splenocytes were culture in RPMI GlutMAX with 10% FBS (Gibco),  
398 penicillin-streptomycin (Gibco), 1 mM 2-mercaptoethanol.

399

#### 400 **Cell differentiation from bone marrow**

401 Femurs, shin and fibula of female mice were collected immediately after sacrifice, the fat and  
402 muscle tissues were removed, the end of the bones were cut with a pair of scissors, and put in a  
403 0.5 mL tubes in which holes were made at the bottom with a needle. The 0.5 mL tube was put in  
404 a 1.5 mL tube containing 200  $\mu$ L of complete IMDM (Iscove's modified Dulbecco's medium,  
405 10% FBS, penicillin-streptomycin a 1mM 2-mercaptoethanol), and centrifugated at 11,000g for  
406 10 seconds.

407 For BMDM cells were seeded at the concentration of 1 million cells per mL in 20 mL total, in a  
408 20 cm non-tissue culture treated plates in BMDM culture media (RPMI GlutMAX, 10% FBS,  
409 penicillin-streptomycin, 1 mM 2-mercaptoethanol, 1 mM sodium pyruvate, non-essential amino  
410 acids, HEPES, 10ng/mL human M-CSF (Miltenyi Biotec). Adherent cells were detached with 5  
411 mM EDTA in PBS at day 6. Differentiation was analyzed by staining with anti-CD11b and anti-  
412 F4/80 followed by cytometry analysis.

413 For BMDC (GMCSF), cells were plated in 20 cm non-tissue culture treated plates, at a  
414 concentration of 1 million cells per mL in 20 mL, in IMDM containing conditioned supernatant  
415 from J558 cells as described (Alloatti et al., 2016). At day 4, non-adherent cells were collected,  
416 and loosely adherent cells were collected with 5 mM EDTA in PBS. Non-adherent and loosely

417 adherent cells were combined and seeded at the concentration of 0.5 million cells per mL in 20  
418 mL. At day 7, non-adherent cells were discarded, loosely adherent cells were collected with PBS-  
419 EDTA and replated at concentration of 0.5 million cells per mL in 20 mL. At day 10 non-  
420 adherent cells were discarded, loosely adherent cells were collected with PBS-EDTA.  
421 Differentiation was analyzed by staining with anti-CD11b and anti-CD11c followed by  
422 cytometry.

423 For BMDC (FLT3L), bone marrow was isolated as described above, and plated in 6-well cell  
424 culture plates at the concentration of 1.5 million cells per mL in 4mL total of complete IMDM  
425 medium supplemented with FLT3L (200ng/ml, Peprotech). At day 10 of differentiation, the  
426 loosely adherent cells were harvested using PBS/EDTA and differentiation was checked by  
427 staining for MHC-II, CD11c, B220, and CD24.

428

#### 429 **cGAMP-VLP production for *in vivo* use**

430 7.5 million 293T cells were plated in 150cm<sup>2</sup> cell culture flask and incubated overnight. One  
431 batch of cGAMP-VLP was made from 4 flasks. The following day, each flask was transfected  
432 with 13 µg of pVAX1-cGAS, 8.1 µg of HIV-1 psPAX2, 3.3 µg of pVAX1-VSVG-INDIANA2,  
433 and 50 µL of PEIpro (Ozyme reference POL115-010), according to the manufacturer's  
434 instructions. The transfection mixes were prepared in Opti-MEM (Gibco). The morning  
435 following transfection, the medium was changed with 52 mL of warm VLP production medium  
436 (293T culture medium with 10 mM HEPES and 50 µg/mL Gentamicin). One day later, the  
437 cGAMP-VLP-containing supernatant was harvested from the cells, centrifuged for 10 minutes at  
438 200 g 4°C, and filtered through 0.45 µm nylon mesh filters (Fisher 22363547). 39 mL of  
439 cGAMP-VLP-containing supernatant was gently overlaid on 6 mL of cold PBS containing 20%  
440 sterile filtered endotoxin free sucrose in 6 Ultra-Clear tubes (Beckman Coulter, ref 344058), and

441 centrifuged for 1 hour and 30 minutes at 100,000 g 4°C. The liquid phase was gently aspirated,  
442 the pellets were resuspended in cold PBS and transferred to one Ultra-Clear 13.2 mL tube  
443 (Beckman Coulter, ref 344059) and centrifuged again at 100,000g 4°C for 1 hour and 30 minutes.  
444 The PBS was gently poured out and the pellet was resuspended in 320 µl of cold PBS. Batches  
445 were split in 3 aliquotes of 100 µL for experimental use. The remaining 20 µL were diluted 1:4  
446 with 60 µL of PBS and split in 8 aliquotes of 10 µL for quality control assays. Aliquotes were  
447 stored at -80°C.

448  
449 **cGAMP quantification**  
450 2'3'-cGAMP ELISA Kit (Cayman Chemical) was used for the quantification of cGAMP in  
451 cGAMP-VLP according to the manufacturer's instructions. After performing the assay, the plate  
452 was read at a wavelength of 450 nm. Data was fitted to a 4-parameter sigmoidal curve.

453  
454 **Biological activity assay of cGAMP-VLP**  
455 50,000 THP-1 cells were plated in round bottom 96 well plates in 100 µL of medium, and  
456 stimulated with serial-dilutions of cGAMP-VLPs, soluble cGAMP or soluble ADU-S100 in 100  
457 µl. Where indicated, CDN (6 µg) were mixed with lipofectamine 2000 (6 µl) in Opti-MEM  
458 (12.75 µl each) following manufacturer's instructions. The cells were incubated for 18 to 24 hours  
459 and stained with an anti-human SIGLEC-1 (Miltenyi ref 130-098-645), fixed in PFA 1% and  
460 acquired using a BD FACSVerse cytometer.

461  
462 **Electron microscopy**  
463 cGAMP-VLP suspension was deposited on formvar/carbon-coated copper/palladium grids  
464 before uranyl/acetate contrasting and methyl-cellulose embedding for whole-mount. Images were

465 acquired with a digital camera Quemesa (EMSIS GmbH, Münster, Germany) mounted on a  
466 Tecnai Spirit transmission electron microscope (FEI Company) operated at 80kV.

467

### 468 **Nanoparticle Tracking Analysis**

469 The cGAMP-VLPs were serially diluted in PBS at room temperature and acquired on a  
470 NanoSight as previously described (Liao et al., 2019).

471

### 472 **Mice**

473 All animals were used according to protocols approved by Animal Committee of Curie Institute  
474 CEEA-IC #118 and maintained in pathogen-free conditions in a barrier facility. Experimental  
475 procedures were approved by the Ministère de l'enseignement supérieur, de la recherche et de  
476 l'innovation (APAFIS#11561-2017092811134940-v2) in compliance with the international  
477 guidelines. C57BL/6J mice were purchased from Charles River Laboratories. C57BL/6J  
478 *Rag2<sup>tm1.1Cgn</sup>* (*Rag2<sup>-/-</sup>*) mice were maintained at Centre d'Exploration et de Recherche  
479 Fonctionnelle Expérimentale. C57BL/6J *Lyz2<sup>tm1(cre)lfo</sup>* (LysM-cre), C57BL/6J *Tg(Itgax-cre)1-*  
480 *1Reiz* (Cd11c-cre), C57BL/6J *Sting<sup>gt/gt</sup>* (*Sting1<sup>-/-</sup>*) and STING-OST<sup>fl</sup> mice were maintained at  
481 Institut Curie Specific Pathogen Free facility. Mice were allowed to acclimate to the experimental  
482 housing facility for at least three days before tumor injections.

483

### 484 **Generation of STING-OST<sup>fl</sup> knock-in mice**

485 The mouse *Sting1* gene (also called Tmem173; ENSMUSG00000024349) was edited using a  
486 double-stranded HDR template (targeting vector) containing 867 and 1260 bp-long 5' and 3'  
487 homology arms, respectively. It included a loxP site and a frt-neo<sup>r</sup>-frt cassette that were both  
488 inserted in intron 2, 110 bp upstream of the start codon, a Twin-Strep-tag-coding sequence (OST;

489 (Junttila et al., 2005)) that was appended at the 5' end of the first coding exon (exon 3), and a  
490 loxP site located in intron 3, 40 bp downstream of the 3' end of exon 3. The final targeting vector  
491 was abutted to a cassette coding for the diphtheria toxin fragment A (Soriano, 1997). Two  
492 sgRNA-containing pX330 plasmids (pSpCas9; Addgene, plasmid ID 42230) were constructed. In  
493 the first plasmid, two sgRNA-specifying oligonucleotide sequences (5'-  
494 CACCGAGTAGCCCATGGGACTAGC-3' and 5'-AAACGCTAGTCCCATGGGCTACTC-3')  
495 were annealed, generating overhangs for ligation into the BbsI site of plasmid pX330. In the  
496 second plasmid, two sgRNA-specifying oligonucleotide sequences (5'-  
497 CACCGTCAAGGGTGTGATACTTGC-3' and 5'-AAAC-GCAAGTATCACACCCTTGAC-3')  
498 were annealed and cloned into the BbsI site of plasmid pX330. The protospacer-adjacent motifs  
499 (PAM) corresponding to each sgRNA and present in the targeting vector were destroyed via  
500 silent mutations to prevent CRISPR-Cas9 cleavage. JM8.F6 C57BL/6N ES cells (Pettitt et al.,  
501 2009) were electroporated with 20 µg of targeting vector and 2.5 µg of each sgRNA-containing  
502 pX330 plasmid. After selection in G418, ES cell clones were screened for proper homologous  
503 recombination by Southern blot and PCR analysis. A neomycin specific probe was used to ensure  
504 that adventitious non-homologous recombination events had not occurred in the selected ES  
505 clones. Mutant ES cells were injected into BalbC/N blastocysts. Following germline  
506 transmission, excision of the frt-neo<sup>r</sup>-frt cassette was achieved through genetic cross with  
507 transgenic mice expressing a FLP recombinase under the control of the actin promoter  
508 (Rodríguez et al., 2000). Two pairs of primers were used to distinguish the WT and edited  
509 *Tmem173* alleles. A pair of primers (sense 5'-TGTAGGATGCTATGTGCCCA-3' and antisense  
510 5'-GATCCCAGCCCAACTCAGCT-3') amplified a 501 bp-long band in the case of the wild-type  
511 *Tmem173* allele and a 722 bp-long band in the case of the mutant allele.



512 The resulting STING-OST<sup>fl</sup> mice (official name B6-*Tmem173*<sup>Tm1Ciphe</sup> mice) have been established  
513 on a C57BL/6N background. They express a multitask *Tmem173* allele in which the third exon of  
514 the *Tmem173* allele is bracketed by *loxP* sequences and a sequence corresponding to an affinity  
515 Twin-Strep-Tag (OST) is appended at the 5' end of the ORF of the *Tmem173* gene. When bred to  
516 mice that express tissue-specific Cre recombinase, the resulting offspring will have exon 3  
517 removed in the Cre-expressing tissues, resulting in cells lacking STING.

518

### 519 **Mouse randomization**

520 Mouse randomizations were performed using Randmice (<https://randmice.com>) based on tumor  
521 volume to distribute mice and homogenize the average tumor volume within the different groups.  
522 The algorithm randomly shuffles all mice between the groups and calculates the average tumor  
523 volume for each group. 10e9 iterations are performed in order to minimize the difference in  
524 tumor volume average between all groups.

525

### 526 **Tumor implantation**

527 Female mice were inoculated subcutaneously on the lower right or right and left flanks with  
528  $5 \times 10^5$  B16-OVA cells in 100  $\mu$ L of HBSS or with  $5 \times 10^5$  MB49 cells in 100  $\mu$ L of PBS . Mice  
529 were monitored for morbidity and mortality daily. Tumors were monitored twice or three times  
530 per week. Mice were euthanized if ulceration occurred or when tumor volume reached 2000  
531  $\text{mm}^3$ . Tumor sizes were measured using a digital caliper and tumor volumes calculated with the  
532 formula  $(\text{length} \times \text{width}^2)/2$ . Following tumor implantation, mice were randomized into treatment  
533 groups using the Randmice software. In some experiments, tumor-free survivors were challenged  
534 with tumor cells on the opposite, non-injected flank several weeks after the collapse of the  
535 primary tumor. Naive mice of the same age were used as controls.

536

537 ***In vivo immunotherapy***

538 Intra-tumoral (i.t.) or subcutaneous (s.c.) injections were initiated when tumors are palpable or  
539 reached close to 50 mm<sup>3</sup> (40-80 mm<sup>3</sup>), as indicated in legends. A U-100 insulin syringe or  
540 equivalent [0.33 mm (29 G) x 12.7 mm (0.5 mL)] was filled with 50 µl of samples (VLP,  
541 cGAMP-VLP or synthetic CDN diluted in PBS) and all air bubbles were removed. Mice were  
542 anesthetized with isoflurane. With the bevel facing the skin, the needle was injected shallowly  
543 into the area directly adjacent to the tumor, and the needle was moved underneath the skin until it  
544 reached the inside back of the tumor. The samples were injected slowly into the center of the  
545 tumor (for the i.t.) or under the skin, 1 cm from the border of the tumor (for the s.c.). The needle  
546 was then removed delicately to avoid reflux. Treatments consisting of 200 µg of αPD1 antibody  
547 (clone RMP1-14, BioXcell) or 200 µg isotype control antibody (Rat IgG2a, BioXcell) were  
548 diluted in PBS at 1 mg/ml and administered by intra-peritoneal (i.p.) injection at the indicated  
549 time points.

550

551 ***In vivo antibody depletion***

552 For CD8<sup>+</sup> and NK1.1 depletions studies, B16-OVA tumor bearing mice were treated with 200 µg  
553 of anti-CD8α monoclonal antibody (clone 53-6.7, BioXcell) or 200 µg of anti-NK1.1 monoclonal  
554 antibody (clone PK136, BioXcell) or 200 µg of isotype control antibody (Rat IgG2a, BioXcell)  
555 two times prior and four times after i.t. treatment with STING agonists. To confirm the cell  
556 depletion, PBMC were stained according to standard protocols before depletion, at day 7 and day  
557 17. Briefly, cells were surface-stained in 100 µL antibody-mix in FACS buffer: CD19 (clone  
558 6D5), TCR-b (clone H57-597), CD4 (clone RM4-5), CD8 (Life Technologies) and NK1.1 (clone  
559 PK136). For Treg (Foxp3+CD25+ cells) depletion, MCA-OVA tumor bearing mice were treated

560 with 200  $\mu$ g of anti-mCTLA4-mIgG2a monoclonal antibody (Invivogen) or 200  $\mu$ g of isotype  
561 control antibody (mouse IgG2a, Invivogen) three times at days 6, 9 and 12 after tumor  
562 engraftment. To confirm the Treg depletion, spleen and tumor cells were stained according to  
563 standard protocols 48 hours after the last antibody injection. Briefly, cells were surface-stained in  
564 100  $\mu$ L antibody-mix in FACS buffer: CD45.2 (clone 104), CD19 (clone 6D5), TCR-b (clone  
565 H57-597), CD4 (clone RM4-5), CD8 (Life Technologies) and CD25 (clone PC61), followed by  
566 an intracellular staining in 50  $\mu$ L with anti-Foxp3 (clone FJK-16s) and anti-Ki67 (BD  
567 Biosciences).

568

### 569 **ELISPOT Assay**

570 T cell responses were assessed by IFN- $\gamma$  ELISPOT 10 days after the first i.t. injection of  
571 cGAMP-VLP, synthetic CDNs or PBS. Mice were bled from the retro-orbital sinus. PBMCs were  
572 isolated from whole blood by lysing the red blood cells with an ammonium chloride lysis buffer  
573 (NH<sub>4</sub>Cl 1.5 M, NaHCO<sub>3</sub> 100 mM, EDTA 10 mM). 2x10<sup>5</sup> PBMCs were plated per well in the  
574 RPMI medium containing 10% FBS and 1% penicillin-streptomycin. PBMCs were stimulated  
575 overnight with media as a negative control, Dynabeads mouse T-activator CD3/CD28 (GIBCO)  
576 as a positive control, 10  $\mu$ g/mL OVA-I 257-264 peptide (SIINFEKL) or 40  $\mu$ g/mL OVA-II 265-  
577 280 peptide (TEWTSSNVMEERKIKV) or 10  $\mu$ g/mL p15E peptide (KSPWF<sup>T</sup>TTL) or 10  $\mu$ g/mL  
578 DBy 608-622 peptide (NAGFNSNRANSSRSS) or 10  $\mu$ g/mL UTy 246-254 (WMHHNMDLI).  
579 Spots were developed using mouse IFN- $\gamma$  ELISPOT antibody pair (Diaclone) according to the  
580 manufacturer's instructions. The number of spots was enumerated using an ImmunoSpot analyzer  
581 and evaluated by subtracting the specific values from the negative control spot number of each  
582 sample.

583

584 **Stimulation of cells with CDNs and cGAMP-VLP**

585 100,000 of the indicated cells were seeded in flat bottom 96-well plates in 200  $\mu$ L and incubated  
586 for few hours until attached to the plate. 100  $\mu$ L were removed and replaced with serial dilutions  
587 of ADU-S100, cGAMP, cGAMP-VLP or empty VLP. Cells were incubated for 18 hours, and  
588 IFN- $\alpha$  and IFN- $\beta$  were measured in the supernatant.

589

590 **cGAMP-VLP capture by splenocytes *in vitro***

591 Spleens were harvested from female C57BL6/J mice. Splenocytes were isolated by pressing the  
592 organ through a 40  $\mu$ m cell strainer. Red blood cells were lysed using an ammonium chloride  
593 lysis buffer as described above. 1 to 3 million cells were plated in a 96-well round bottom plate in  
594 150  $\mu$ L of medium. 50 $\mu$ L of cGAMP-VLP or PBS was added and cells were incubated overnight  
595 at 37°C 5% CO<sub>2</sub>. The following day the cells were stained with antibodies against extracellular  
596 markers (MHC-II eFluor450, eBioscience 48-5321-82; CD4 BV785, bioLegend 100552; NK1.1  
597 PerCP-Cy5.5, BD Biosciences 561111; CD11b PE, Invitrogen 12-0112-82; CD11c PETR,  
598 Invitrogen MCD11c17; CD19 PE-Cy5, Invitrogen 15-0193-82; TCR- $\beta$  PE-Cy7, bioLegend  
599 109222; CD8 APC, BD biosciences 561093; F4/80 AF700, eBioscience 56-4801-82; Fixable  
600 Viability Dye, eFluor780; eBioscience 65-0865-14), washed and permeabilized using the BD  
601 Cytotfix/Cytoperm Fixation Permeabilization Solution kit (reference 554714) according to the  
602 manufacturer's instructions. The cells were then washed with the permeabilization buffer,  
603 following by staining for 15 minutes at room temperature with a 1:100 dilution of a fluorescent  
604 anti-HIV-1 GAG antibody (KC57-FITC, Beckman Coulter reference 6604665) in  
605 permeabilization buffer. Cells were washed, resuspended in FACS buffer and acquired on a  
606 Beckman Coulter CytoFlex S analyzer. The data was analyzed using FlowJo 10.

607

608 **Immune cell composition analysis by flow cytometry**

609 All mice from the STING agonist-treated group (cGAMP-VLP and ADU-S100) and vehicle-  
610 treated group were sacrificed 24 hours after the last intratumoral injection. Spleen, draining/non-  
611 draining lymph nodes and tumors were excised. Splenocytes were isolated by pressing the spleen  
612 through a 40- $\mu$ m cell strainer, axillary or inguinal LNs were dissected, pierced once with fine tip  
613 forceps, and collected into RPMI on ice. For the splenocytes, RPMI was replaced with 2 mL  
614 enzymatic solution of CO<sub>2</sub>-independent medium containing 1 mg/mL liberase (Sigma) and 20  
615  $\mu$ g/mL DnaseI (Roche), and incubated for 30 minutes in a 37°C incubator with gentle agitation.  
616 After 30 minutes, red blood cells were lysed using an ammonium chloride lysis buffer as  
617 described above. Cells were pelleted (300 x g, 10 minutes, 4°C) and resuspended in ice cold  
618 FACS buffer containing 0.5% BSA in PBS. Excised tumors were collected in RPMI  
619 supplemented with 10 % FCS and cut into small pieces. Tumor pieces were digested with 1  
620 mg/mL liberase (Sigma) and 20  $\mu$ g/mL DnaseI (Roche) with gentle continuous agitation (using  
621 mouse tumor dissociator gentleMACS). After 40 minutes digestion at 37°C, cells were passed  
622 through a 70- $\mu$ m filter, washed by RPMI supplemented with 10 % FCS, and resuspended in  
623 FACS buffer. Single cells were stained according to standard protocols. Briefly, cells were  
624 surface-stained in 50  $\mu$ L antibody-mix in FACS buffer: CD45.2 (clone 104), CD19 (clone 6D5),  
625 TCR-b (clone H57-597), CD4 (clone RM4-5), CD8 (Life Technologies), CD62L (clone MEL-  
626 14), CD69 (clone H1.2F3), CD44 (clone IM7), CD25 (clone PC61), NK1.1 (clone PK136),  
627 Nkp46 (clone 29A1.4), CD172a (clone P84), CD11b (M1/70), CD11c (Invitrogen), MHC-2  
628 (clone M5/114.15.2), F4/80 (BM8), XCR1 (clone ZET), CD64 (clone X54-5/7.1), CD26 (clone  
629 H194-112) and CD86 (clone GL1). Dead cells were excluded using fixable viability stain  
630 according to the manufacturer's instructions. For intracellular staining, cells were fixed for 30  
631 minutes on ice using IC Fixation Buffer from Foxp3/Transcription Factor Staining Buffer Set,

632 washed with 1X permeabilization buffer, stained and resuspended in FACS buffer containing ant-  
633 Foxp3 (clone FJK-16s) and anti-Ki67 (BD Biosciences). Single-cell suspensions were then  
634 analysed by flow cytometry using FACS LSRFortessa analyzer (BD Biosciences). For the  
635 analysis of the relative amounts of OST-STING in DCs and macrophages, splenocytes were  
636 stained with antibodies directed against CD11b (M1/70) and CD64 (clone X54-5/7.1),  
637 permeabilized with BD Cytofix/Cytoperm (BD Biosciences) for 30 min at 4°C, stained with  
638 1/400 or 1/800 dilutions of Strep-Tactin APC (IBA GmbH) and analyzed by flow cytometry.

639

#### 640 **LEGENDplex Assay**

641 Serum samples were collected three hours after the first STING agonist injection and analyzed  
642 for inflammatory cytokines (IFN- $\alpha$ , IFN- $\beta$ , TNF- $\alpha$  and IL-6) using a LEGENDplex Mouse  
643 Inflammation Panel (BioLegend). For cell culture supernatants, IFN- $\alpha$  and IFN- $\beta$  concentration  
644 were measured using a LEGENDplex Mouse Type 1/2 Interferon Panel (reference 740636). Data  
645 was acquired on a FACS Verse (BD Biosciences) and analyzed with BioLegend's LEGENDplex  
646 Data Analysis Software. The standard curve regression was used to calculate the concentration of  
647 each target cytokine.

648

#### 649 **Quantification and Statistical analysis**

650 Statistical details of experiments are indicated in the figure legends, text or methods. Data were  
651 analyzed in GraphPad Prism 8 software. In Figures, \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ , \*\*\*\*  
652  $P < 0.0001$ .

653

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767

768 **Figure Legends**

769

770 **Figure 1 cGAMP-VLP induces tumor-specific T cell responses in a non-immunogenic**  
771 **tumor model**

772 **(A)** Overview of the experimental design (TW = twice weekly). Treatments were initiated on  
773 palpable tumors (15-20 mm<sup>3</sup> range).

774 **(B)** Concentrations of IFN- $\alpha$ , IFN- $\beta$ , IL-6 and TNF- $\alpha$  in the serum of B16-OVA tumor-bearing  
775 mice 3 hours after treatment (bar at mean + SEM, n = 6 to 24 mice per group, combined from 2  
776 independent experiments, Kruskal-Wallis with Dunn post-test, LLOQ = lower limit of  
777 quantification, ULOQ = upper limit of quantification).

778 **(C)** Growth curves of individual B16-OVA tumors treated as indicated. Vertical dotted line  
779 indicates the death of the last mouse in the PBS-injected group.

780 **(D)** Mean growth over time of B16-OVA tumors treated as indicated (line at mean + SEM, n = 6  
781 to 12 mice per group, combined from 2 independent experiments).

782 **(E)** Survival of B16-OVA tumor-bearing mice treated as indicated (log-rank Mantel-Cox test).

783 **(F)** Ova-specific CD8 (OVA-I) and CD4 (OVA-II) T cell responses in blood, assessed by IFN- $\gamma$   
784 ELISPOT (bar at mean + SEM, n = 6 to 12 mice per group, combined from 2 independent  
785 experiments, Kruskal-Wallis test with Dunn post-test).

786

787 **Figure 2 Tumor specific T-cell responses elicited by cGAMP-VLP translate into abscopal**  
788 **synergy with anti-PD1.**

789 **(A)** Overview of the experimental design. Treatments were initiated on palpable tumors.

790 **(B)** Concentrations of IFN- $\alpha$ , IFN- $\beta$ , IL-6 and TNF- $\alpha$  in the serum of B16-OVA dual tumor-  
791 bearing mice 3 hours after treatment (bar at mean + SEM, n = 6 to 24 mice per group, combined

792 from 2 independent experiments, Kruskal-Wallis with Dunn post-test, LLOQ = lower limit of  
793 quantification, ULOQ = upper limit of quantification).

794 (C) Ova-specific CD8 (OVA-I) and CD4 (OVA-II) T cell responses in blood, assess by IFN- $\gamma$   
795 ELISPOT (bar at mean + SEM, n = 6 to 12 mice per group, combined from 2 independent  
796 experiments, Kruskal-Wallis with Dunn post-test).

797 (D) Growth curves of individual injected and distal B16-OVA tumors treated as indicated.  
798 Vertical dotted line indicates the death of the last mouse in the PBS-injected group.

799 (E) Mean growth over time of B16-OVA injected and distal tumors treated as indicated (line at  
800 mean + SEM, n = 6 to 12 mice per group, combined from 2 independent experiments).

801 (F) Distal tumor size at the indicated days in treated mice, for groups that did not reach ethical  
802 limits (line at mean + SEM, n = 12 mice per group, combined from 2 independent experiments,  
803 Kurskal-Wallis with Dunn post-test for day 27, Mann-Whitney for day 31).

804 (G) Survival of mice after secondary challenge. In complete responding mice, B16-OVA cells  
805 were injected 80 days from the first injection of tumor cells and treatments (combined from 3  
806 experiments with single or dual tumors at the first injection, Gehan-Breslow-Wilcoxon test on  
807 cGAMP-VLP + anti-PD1 vs ADU-S100 + anti-PD1).

808

809 **Figure 3 The anti-tumor effect of cGAMP-VLP requires host STING and T lymphocytes.**

810 (A) Overview of the experimental design using B16-OVA dual tumor-bearing mice (WT, *Sting1*  
811 <sup>-/-</sup> or *Rag2*<sup>-/-</sup>). Treatments were initiated on palpable tumors.

812 (B) Concentrations of IFN- $\alpha$ , IL-6 and TNF- $\alpha$  in the serum 3 hours after the first treatment by i.t.  
813 injection of PBS, 50  $\mu$ g ADU-S100 or 50 ng cGAMP-VLP (bar at mean + SEM, n = 8 to 16 mice  
814 per group, combined from 2 independent experiments, Kruskal-Wallis with Dunn post-test,  
815 LLOQ = lower limit of quantification, ULOQ = upper limit of quantification).

816 (C) Ova-specific CD8 (OVA-I) and CD4 (OVA-II) T cell responses in blood of WT, *Sting1*<sup>-/-</sup> or  
817 *Rag2*<sup>-/-</sup> mice 17 days after tumor implantation, assessed by IFN- $\gamma$  ELISPOT (bar at mean + SEM,  
818 n = 11 to 12 mice per group, combined from 2 independent experiments, Kruskal-Wallis with  
819 Dunn post-test). Mice were randomized at day 7 and treated by i.t. injection at days 7, 10 and 13.

820 (D) Size of injected and distal tumors 16 days after tumor implantation in WT, *Sting1*<sup>-/-</sup> or *Rag2*<sup>-/-</sup>  
821 treated mice (line at mean + SEM, n = 16 mice per group except n = 15 for WT PBS group,  
822 combined from 2 independent experiments, Kruskal-Wallis with Dunn post-test).

823 (E) Survival of B16-OVA dual tumor-bearing mice (WT, *Sting1*<sup>-/-</sup> or *Rag2*<sup>-/-</sup>) treated as indicated  
824 (log-rank Mantel-Cox test).

825

826 **Figure 4 Differential T cell subset composition in response to cGAMP-VLP over ADU-S100.**

827 (A) Outline of the experiment.

828 (B) Frequency of immune cells (%CD45.2<sup>+</sup> within total live cells), NK cells (%NK1.1<sup>+</sup> within  
829 CD45.2<sup>+</sup>), TCR $\beta$ <sup>+</sup>CD4<sup>+</sup> T cells (within CD45.2<sup>+</sup>), TCR $\beta$ <sup>+</sup>CD8<sup>+</sup> T cells (within CD45.2<sup>+</sup>), Tregs  
830 (%FoxP3<sup>+</sup>CD25<sup>+</sup> within CD45.2<sup>+</sup>TCR $\beta$ <sup>+</sup>CD4<sup>+</sup>) and B cells (%CD19<sup>+</sup> within CD45.2<sup>+</sup>) in B16-  
831 OVA dual tumor-bearing mice treated as indicated at days 7, 10 and 13 and analyzed at day 14.  
832 Treatments were started on tumors of 10-20 mm<sup>3</sup> average volume per group. Data combined  
833 from groups with and without anti-PD1 (n=6 to 8 mice per group, Brown-Forsythe and Welch  
834 ANOVA test).

835 (C) Frequency of central memory (CM, gated as CD44<sup>+</sup>CD62L<sup>+</sup> within CD45.2<sup>+</sup>TCR $\beta$ <sup>+</sup>CD8<sup>+</sup> or  
836 CD4<sup>+</sup>) and effector memory (EM, gated as CD44<sup>+</sup>CD62L<sup>-</sup> within CD45.2<sup>+</sup>TCR $\beta$ <sup>+</sup>CD8<sup>+</sup> or CD4<sup>+</sup>)  
837 T cells in the indicated organs (n=8 mice per group, Brown-Forsythe and Welch ANOVA test).

838 (D) Frequency of CD69<sup>+</sup> cells within CD45.2<sup>+</sup>TCR $\beta$ <sup>+</sup>CD8<sup>+</sup> and CD45.2<sup>+</sup>TCR $\beta$ <sup>+</sup>CD4<sup>+</sup> T cells in  
839 the indicated organs (n=6 to 8 mice per group, Brown-Forsythe and Welch ANOVA test).

840

841 **Figure 5 Anti-tumor effect of cGAMP-VLP requires STING in dendritic cells**

842 **(A)** Outline of the experiment using B16-OVA dual tumor-bearing mice (STING-OST<sup>fl</sup>, STING-  
843 OST<sup>AMP</sup> or STING-OST<sup>ADC</sup>). Treatments were initiated on palpable tumors

844 **(B)** Concentrations of IFN- $\alpha$  and IL-6 in the serum of STING-OST<sup>fl</sup> or STING-OST<sup>AMP</sup> mice 3  
845 hours after the first treatment by i.t. injection of PBS, 50  $\mu$ g ADU-S100 or 50 ng cGAMP-VLP  
846 (bar at mean + SEM, n = 14 mice per group, combined from 2 independent experiments, Kruskal-  
847 Wallis with Dunn post-test, LLOQ = lower limit of quantification, ULOQ = upper limit of  
848 quantification).

849 **(C)** Ova-specific CD8 (OVA-I) and CD4 (OVA-II) T cell responses in blood of STING-OST<sup>fl</sup> or  
850 STING-OST<sup>AMP</sup> mice treated as indicated, 16 days after tumor implantation, assessed by IFN- $\gamma$   
851 ELISPOT (bar at mean + SEM, n = 12 to 14 mice per group combined from 2 independent  
852 experiments).

853 **(D)** Survival of B16-OVA dual tumor-bearing STING-OST<sup>fl</sup> or STING-OST<sup>AMP</sup> mice treated as  
854 indicated (n = 14 mice per group combined from 2 independent experiments, log-rank Mantel-  
855 Cox test).

856 **(E)** Concentrations of IFN- $\alpha$  and IL-6 in the serum of STING-OST<sup>fl</sup> or STING-OST<sup>ADC</sup> mice 3  
857 hours after the first treatment by i.t. injection of PBS, 50  $\mu$ g ADU-S100 or 50 ng cGAMP-VLP  
858 (bar at mean + SEM, n = 12 to 14 mice per group, combined from 2 independent experiments,  
859 Kruskal-Wallis with Dunn post-test, LLOQ = lower limit of quantification, ULOQ = upper limit  
860 of quantification).

861 **(F)** Ova-specific CD8 (OVA-I) and CD4 (OVA-II) T cell responses in blood of STING-OST<sup>fl</sup> or  
862 STING-OST<sup>ADC</sup> mice treated as indicated, 16 days after tumor implantation, assessed by IFN- $\gamma$

863 ELISPOT (bar at mean + SEM, n = 11 to 14 mice per group combined from 2 independent  
864 experiments).

865 **(G)** Survival of B16-OVA dual tumor-bearing STING-OST<sup>fl</sup> or STING-OST<sup>ADC</sup> mice treated as  
866 indicated (n = 12 to 14 mice per group combined from 2 independent experiments, log-rank  
867 Mantel-Cox test).

868  
869 **Figure 6 Sub-cutaneous injection of cGAMP-VLP induces anti-tumor synergy with tumor**  
870 **Treg depletion.**

871 **(A)** Outline of the experiment using B16-OVA tumors to compare i.t. and s.c. injection routes of  
872 cGAMP-VLP. Treatments were started on tumors of 50 mm<sup>3</sup> average volume per group.

873 **(B)** Concentrations of IFN- $\alpha$ , IFN- $\beta$ , IL-6 and TNF- $\alpha$  in the serum of mice 3 hours after the first  
874 treatment with PBS or 50 ng cGAMP-VLP injected by the i.t. or s.c. route (bar at mean + SEM, n  
875 = 9 to 11 mice per group, combined from 2 independent experiments, Kruskal-Wallis with Dunn  
876 post-test, LLOQ = lower limit of quantification, ULOQ = upper limit of quantification).

877 **(C)** Growth curves of individual B16-OVA tumors in mice treated as indicated (n = 18 mice per  
878 group combined from 3 independent experiments). Mice were randomized at day 7, and treated at  
879 days 7, 10 and 13 with cGAMP-VLP, and bi-weekly from day 7 for 3 weeks with anti-PD1.

880 **(D)** Size of tumor 17 days after tumor implantation in treated mice (line at mean + SEM, n = 18  
881 mice per group combined from 3 independent experiments, Kruskal-Wallis with Dunn post-test).

882 **(E)** Ova-specific CD8 (OVA-I) and CD4 (OVA-II) T cell responses in blood of mice 16 days  
883 after tumor implantation, assessed by IFN- $\gamma$  ELISPOT (bar at mean + SEM, n = 12 mice per  
884 group, combined from 2 independent experiments, Kruskal-Wallis with Dunn post-test).

885 **(F)** Survival of B16-OVA tumor-bearing mice treated as indicated (log-rank Mantel-Cox test, n =  
886 12 mice per group combined from 2 independent experiments).

887 **(G)** Outline of the experiment using MCA-OVA tumors, cGAMP-VLP and a tumor Treg-  
888 depleting antibody (anti-CTLA4-m2a). Treatments were started on tumors of 50 mm<sup>3</sup> average  
889 volume per group.

890 **(H)** Fraction of CD25<sup>+</sup>FoxP3<sup>+</sup> Tregs within CD45.2<sup>+</sup>TCRβ<sup>+</sup>CD4<sup>+</sup> cells in spleen and tumor, 48  
891 hours after last i.p. injection of αCTLA4-m2a or isotype (n=4, 2 mice from 2 independent  
892 experiments were analyzed).

893 **(I)** CD8 T cell responses against p15 antigen in blood of mice 16 days after tumor implantation,  
894 assessed by IFN-γ ELISPOT (bar at mean + SEM, n = 15 mice per group, combined from 2  
895 independent experiments, Kruskal-Wallis with Dunn post-test).

896 **(J)** Mean growth over time of MCA-OVA tumors treated as indicated (line at mean + SEM, n =  
897 15 mice per group, combined from 2 independent experiments).

898 **(K)** Survival of MCA-OVA tumor-bearing mice treated as indicated (n = 15 mice per group,  
899 combined from 2 independent experiments, log-rank Mantel-Cox test).

900

901

902



903 **Supplementary Figure Legends**

904 **Figure S1 cGAMP-VLP induces antigen-specific anti-tumor immune responses by intra-**  
905 **tumoral injection**

906 **(A)** Size distribution of purified cGAMP-VLP analyzed by Nanoparticle Tracking Analysis. Line  
907 at mean, red shading at 1 standard error of the mean (representative data of n = 21 experiments).

908 **(B)** Electron microscopy image of purified cGAMP-VLPs. Scale bars at 0.5  $\mu\text{m}$ . Arrows point to  
909 cGAMP-VLP.

910 **(C)** SIGLEC-1 induction in THP-1 by increasing concentrations of cyclic dinucleotide (CDN) in  
911 the form of cGAMP-VLP, soluble 2'3'-cGAMP or soluble ADU-S100, with or without  
912 lipofectamine. Lipofectamine 2000 alone condition is plotted at the doses equivalent to the  
913 conditions with CDN. Dotted lines indicate CDN dose at 50% SIGLEC-1<sup>+</sup> cells.

914 **(D)** Overview of the experimental design.

915 Treatments were started on tumors of 50 mm<sup>3</sup> average volume per group at day 10. Mice were  
916 treated at days 10, 13 and 16 with cGAMP-VLP or PBS injected by the i.t. route.

917 **(E)** Growth curves of individual MB49 tumors (n = 8 mice per group).

918 **(F)** Size of tumor 17 days after tumor implantation in treated mice (line at mean + SEM, n = 12  
919 mice per group combined from 2 independent experiments, Mann-Whitney test).

920 **(G)** T cell responses against UTy (class I peptide) and DBy (class II peptide) in blood of mice 20  
921 days after tumor implantation, assess by IFN- $\gamma$  ELISPOT (bar at mean + SEM, n = 6 to 8 mice  
922 per group, Mann-Whitney test).

923

924 **Figure S2 Responses to lower doses of ADU-S100 and tumor necrosis**

925 **(A)** Number of tumor necrosis events after the indicated treatments in single tumor experiments.

926 **(B)** Mean growth over time of B16-OVA tumors treated as indicated by different doses of ADU-  
927 S100 (line at mean + SEM, n = 5 to 6 mice per group).

928 **(C)** Ova-specific CD8 (OVA-I) and CD4 (OVA-II) T cell responses in blood, assess by IFN- $\gamma$   
929 ELISPOT (bar at mean + SEM, n = 5 to 6 mice per group, Kruskal-Wallis test with Dunn post-  
930 test).

931 **(D)** Number of necrosis events in the injection tumor after the indicated treatments in dual tumor  
932 experiments.

933  
934 **Figure S3 The anti-tumor effect of cGAMP-VLP requires CD8<sup>+</sup> T lymphocytes but not NK**  
935 **cells.**

936 **(A)** Evaluation of the role of CD8<sup>+</sup> T cells and NK cells, overview of the experiment. Mice were  
937 randomized at day 7 and treated by i.t. injection at days 7, 10 and 13 with PBS, 50  $\mu$ g ADU-S100  
938 or 50 ng cGAMP-VLP. Treatments were initiated on palpable tumors

939 **(B)** Fraction of CD8<sup>+</sup> T cells, CD4<sup>+</sup> T cells and NK cells in the blood at days 0, 7 and 17 after  
940 injection with isotype, anti-CD8 $\beta\alpha$  or anti-NK1.1 (bar at mean + SEM, n = 18 to 21 mice per  
941 group, Kruskal-Wallis with Dunn post-test).

942 **(C)** Concentrations of IFN- $\alpha$ , IFN- $\beta$ , IL-6 and TNF- $\alpha$  in the serum of B16-OVA dual tumor-  
943 bearing mice 3 hours after first injection of cGAMP-VLP or PBS, in mice treated with antibodies  
944 as indicated (bar at mean + SEM, n = 4 to 7 mice per group, LLOQ = lower limit of  
945 quantification, ULOQ = upper limit of quantification).

946 **(D)** Ova-specific CD8 (OVA-I) and CD4 (OVA-II) T cell responses in blood of mice treated as  
947 indicated, 17 days after tumor implantation, assess by IFN- $\gamma$  ELISPOT (bar at mean + SEM, n =  
948 5 to 6 mice per group).

949 **(E)** Survival of B16-OVA dual tumor-bearing mice treated as indicated (log-rank Mantel-Cox  
950 test).

951

#### 952 **Figure S4 Response of cell lines and dendritic cells to cGAMP-VLP**

953 **(A)** Production of IFN- $\beta$  by B16-OVA, MS1, MutuDC and RAW cell lines after stimulation with  
954 dose titration of VLPs, cGAMP, ADU-S100 and cGAMP-VLP starting at the indicated top dose  
955 (averages from n=3 independent experiments).

956 **(B)** Statistical analysis of IFN- $\beta$  at dilution 1/5 (bar at mean + SEM, n=3 independent  
957 experiments, one-way ANOVA with Tukey post-test on log-transformed data).

958 **(C)** Production of IFN- $\alpha$  and IFN- $\beta$  by BMDM, BMDC (GM-CSF) and BMDC (FLT3L) after  
959 stimulation with dose titration of VLPs, cGAMP, ADU-S100 and cGAMP-VLP at the indicated  
960 top dose (averages from n=4 or 5 independent experiments).

961 **(D)** Statistical analysis of IFN- $\alpha$  and IFN- $\beta$  at dilution 1/5 (bar at mean + SEM, n=4 or 5  
962 independent experiments, one-way ANOVA with Tukey post-test on log-transformed data).

963

#### 964 **Figure S5 Capture of cGAMP-VLP by splenocytes**

965 **(A)** Gating strategy of immune cells subsets for cGAMP-VLP capture experiments  
966 (representative of n=3 independent experiments).

967 **(B)** Anti-GAG staining and forward scatter in the indicated immune cells from splenocytes  
968 treated with PBS or cGAMP-VLP (representative of n=3 independent experiments).

969 **(C)** Overlaid anti-GAG staining in the indicated immune cells from splenocytes treated with PBS  
970 or cGAMP-VLP (representative of n=3 independent experiments).

971 **(D)** Ratio of anti-GAG mean fluorescence intensity for cGAMP-VLP over PBS (bar at mean +  
972 SEM, n=3 independent experiments).

973

974 **Figure S6 Preferential deletion of STING in macrophages or dendritic cells.**

975 (A) Representative Strep-Tactin staining in total live single cells in spleen of WT and STING-  
976 OST<sup>fl</sup> mice.

977 (B) Relative Strep-Tactin staining in CD64<sup>high</sup> and CD11c<sup>high</sup> live single cells in spleen of the  
978 indicated mouse strains (n=3 combined from 2 independent experiments, ANOVA with Tukey  
979 test).

980

981 **Figure S7 Additional results for the response to cGAMP-VLP combined with anti-CTLA-  
982 m2a.**

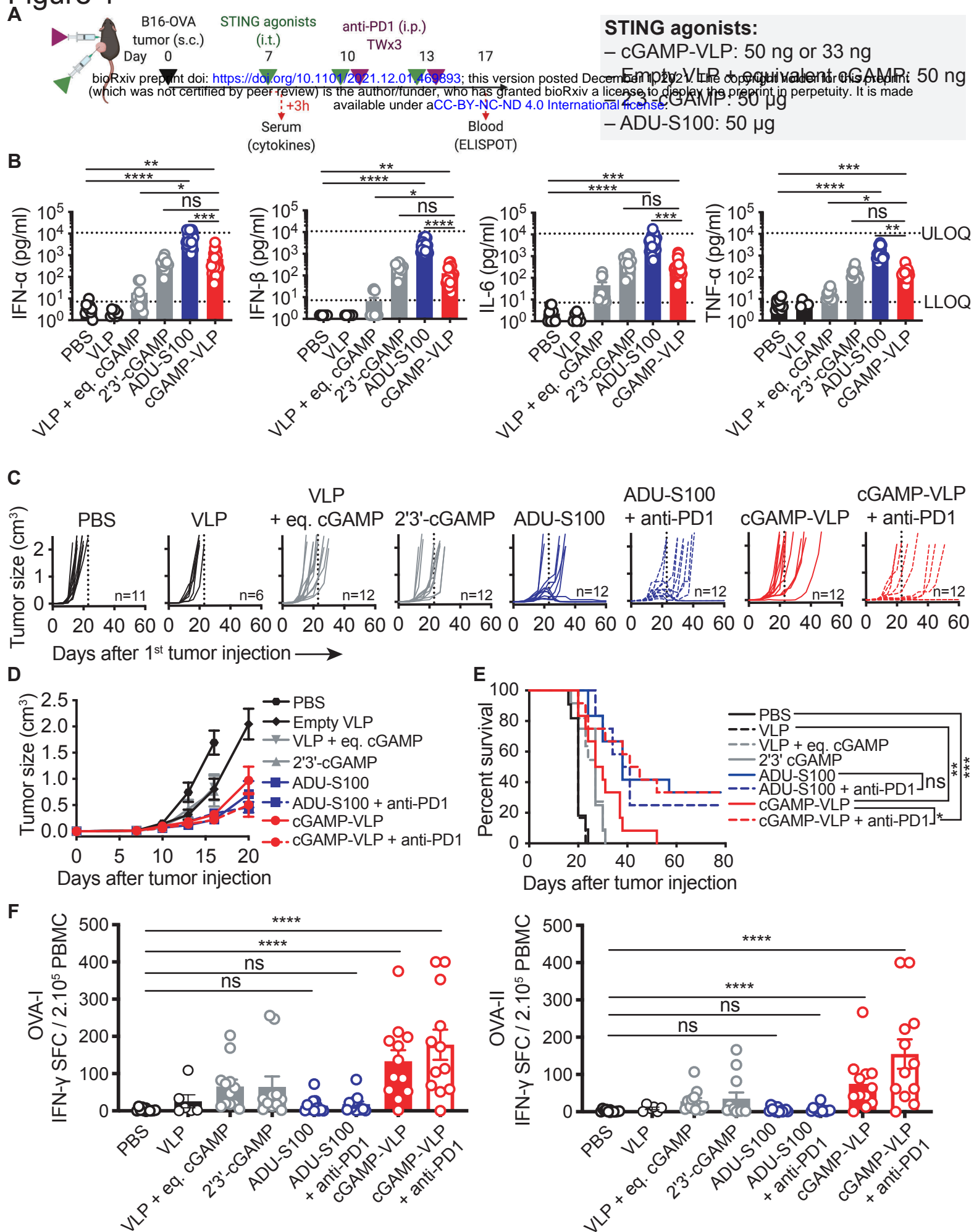
983 (A) Concentrations of IFN- $\alpha$ , IL-6 and TNF- $\alpha$  in the serum of MCA-OVA tumor-bearing mice 3  
984 hours after the first treatment with PBS or 50 ng cGAMP-VLP injected by the s.c., and i.p.  
985 injection of  $\alpha$ CTLA4-m2a or isotype. Treatments were started on tumors of 50 mm<sup>3</sup> average  
986 volume per group (bar at mean + SEM, n = 11 to 15 mice per group, combined from 2  
987 independent experiments, Kruskal-Wallis with Dunn post-test, LLOQ = lower limit of  
988 quantification, ULOQ = upper limit of quantification).

989 (B) Ova-specific CD8 (OVA-I) and CD4 (OVA-II) T cell responses in blood of mice 16 days  
990 after tumor implantation, assess by IFN- $\gamma$  ELISPOT (bar at mean + SEM, n = 15 mice per group,  
991 combined from 2 independent experiments, Kruskal-Wallis with Dunn post-test).

992 (C) Size of tumor 28 days after tumor implantation in treated mice (line at mean + SEM, n = 15  
993 mice per group combined from 2 independent experiments, Mann-Whitney test).

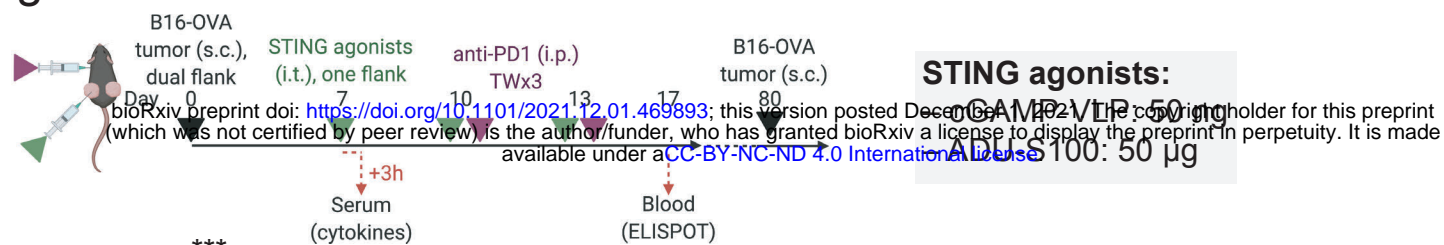
994 (D) Survival of mice after secondary challenge. In complete responding mice, MCA-OVA cells  
995 were injected 55 days from the first injection of tumor cells and treatments (combined from 2  
996 experiments, Mantel-Cox test).

# Figure 1

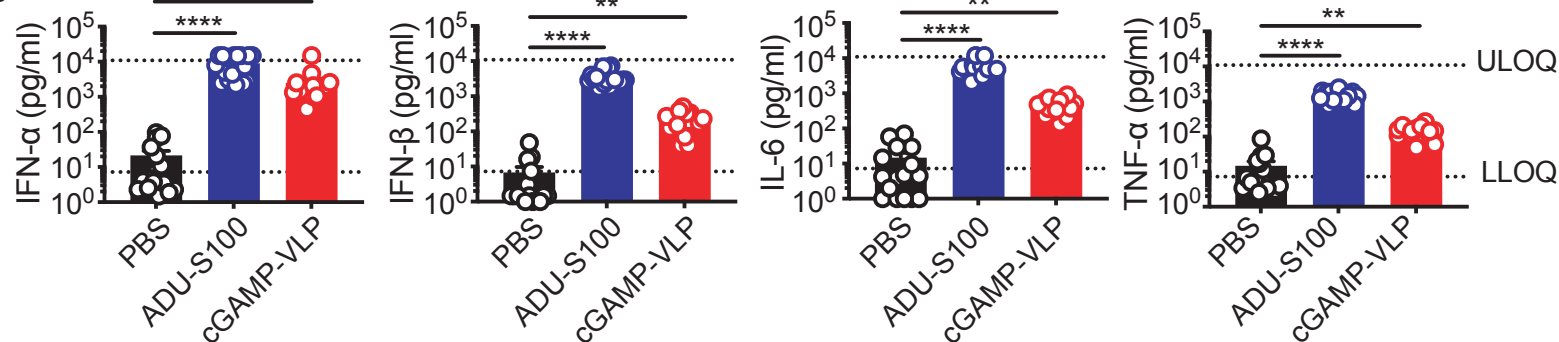


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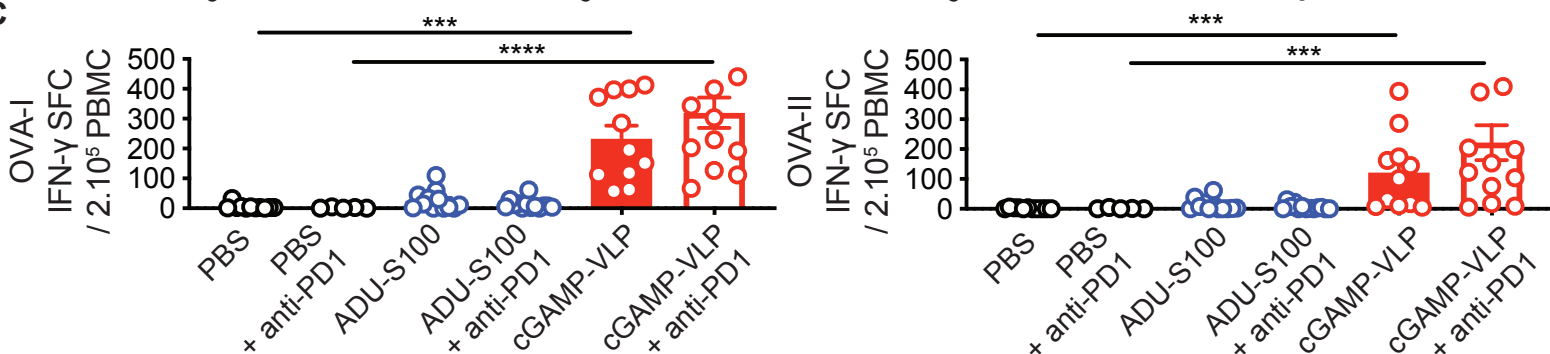
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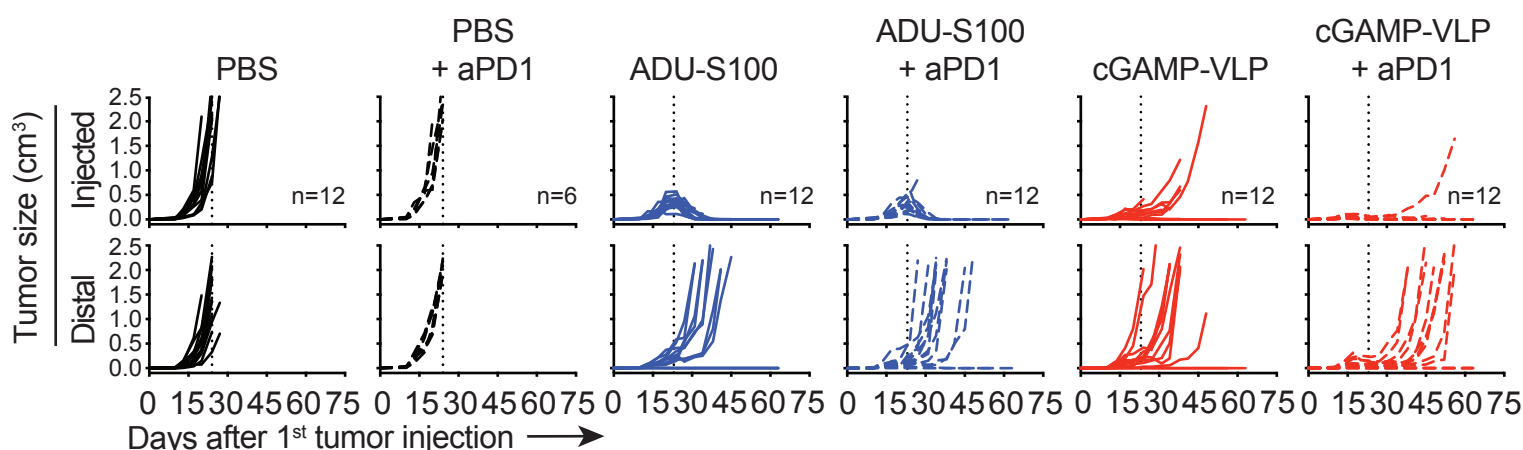
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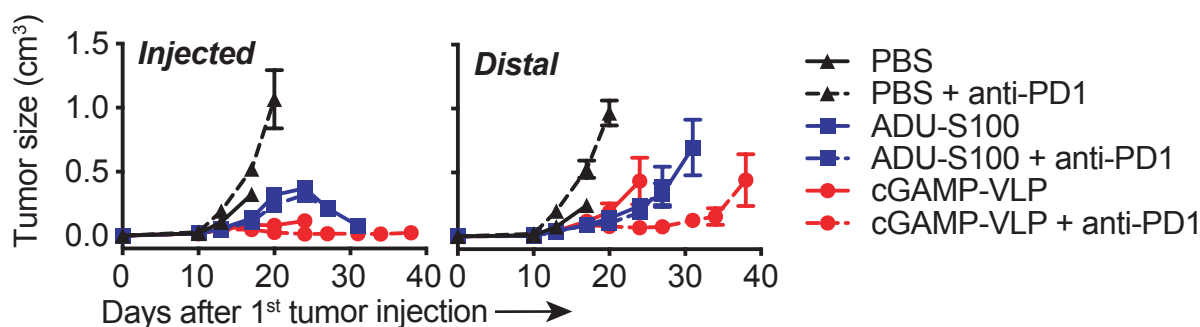
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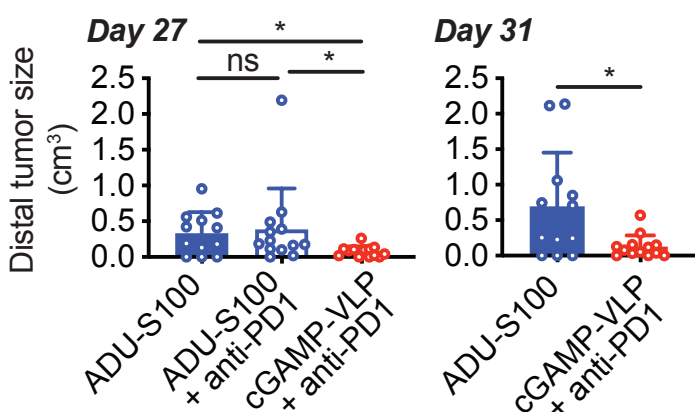
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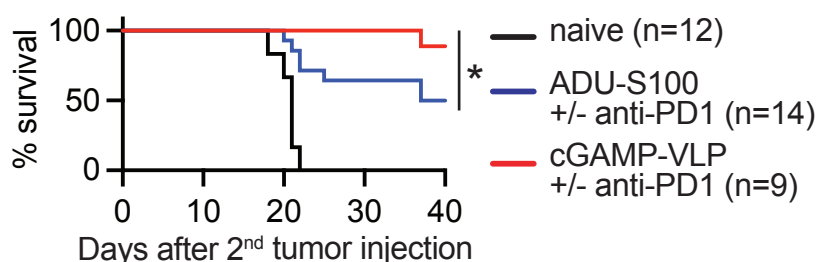
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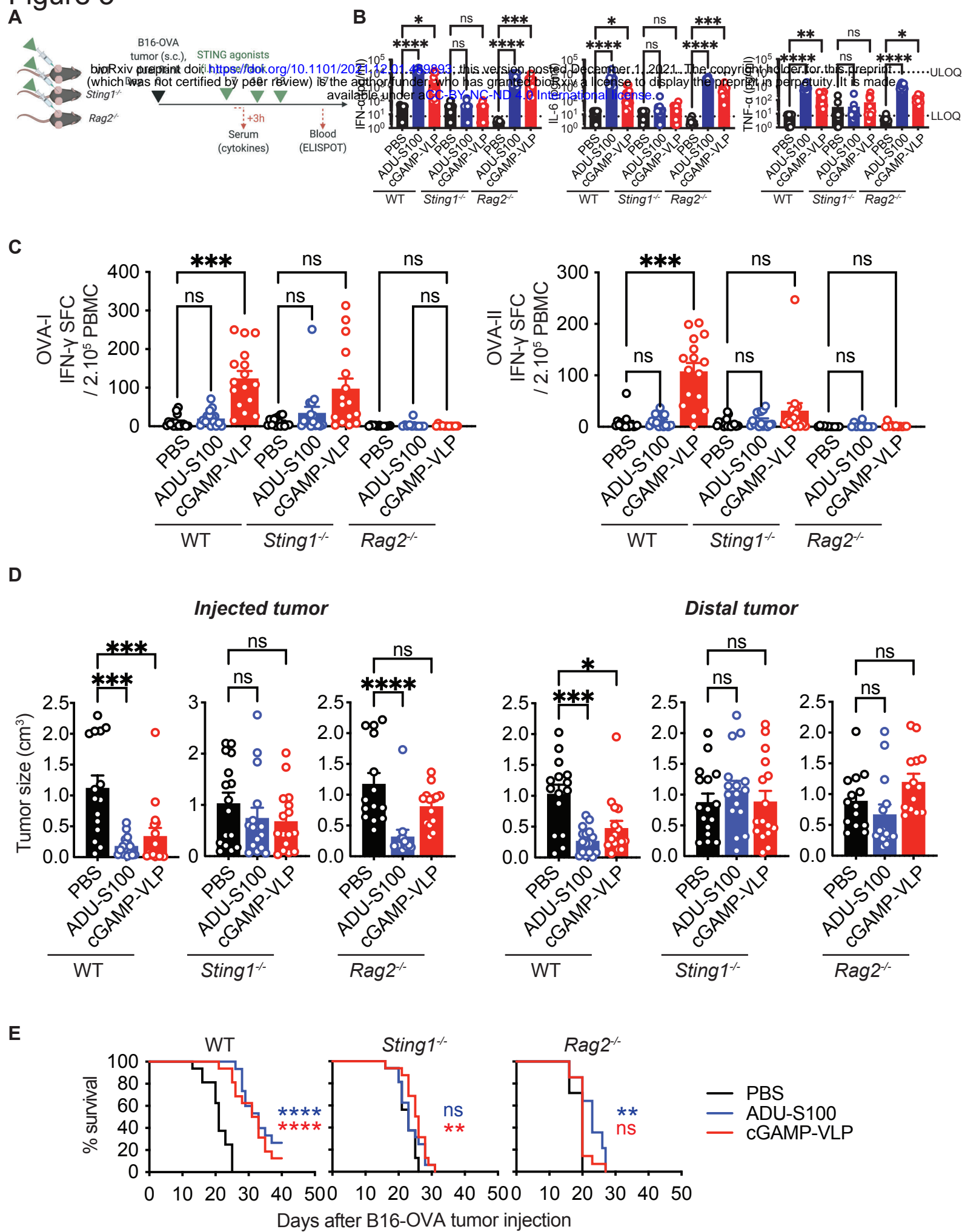
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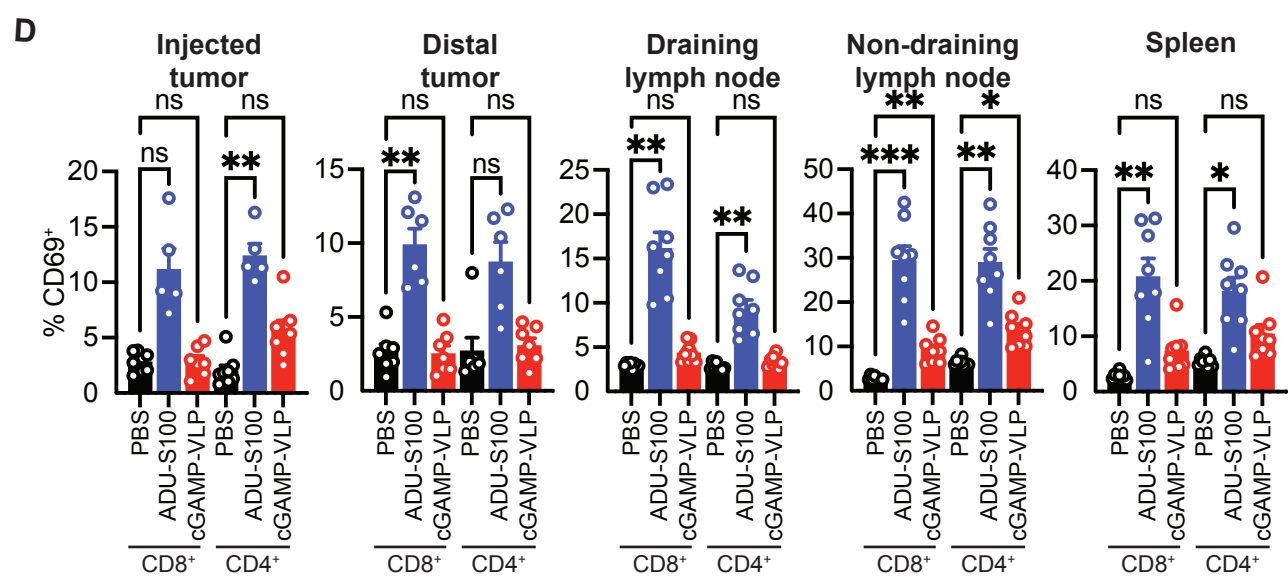
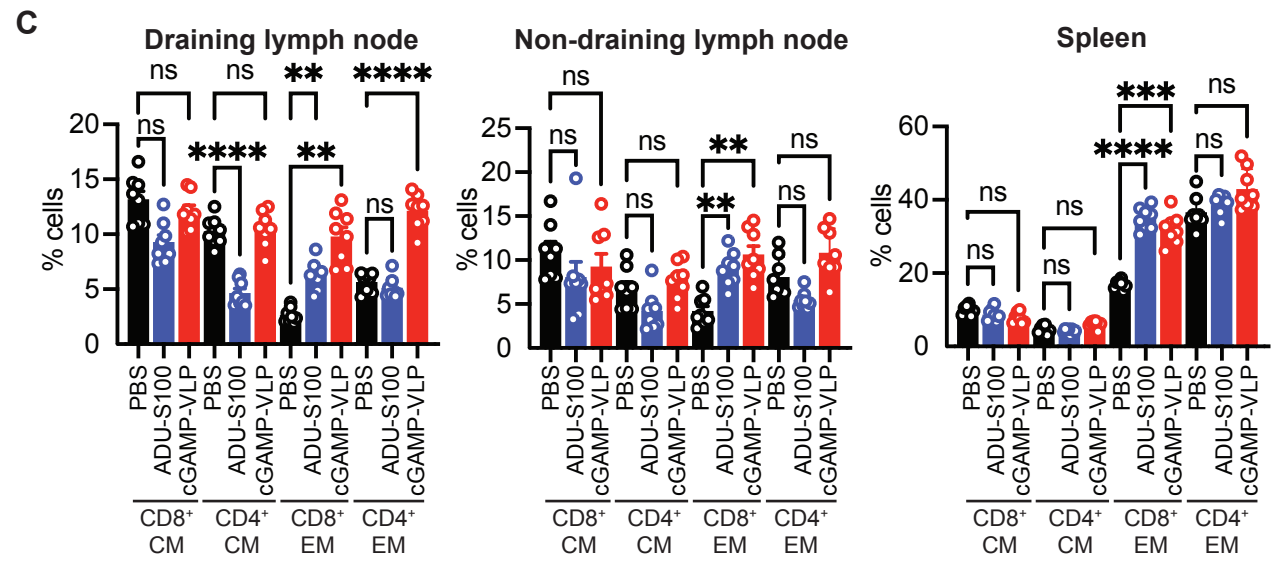
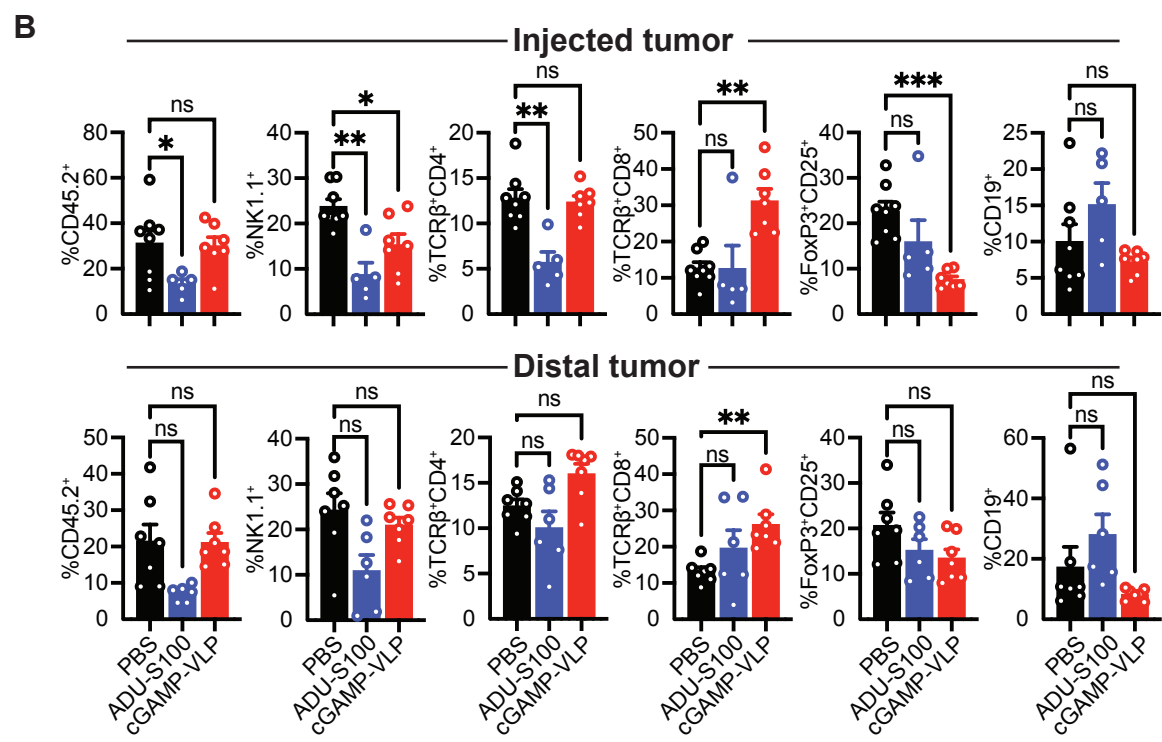
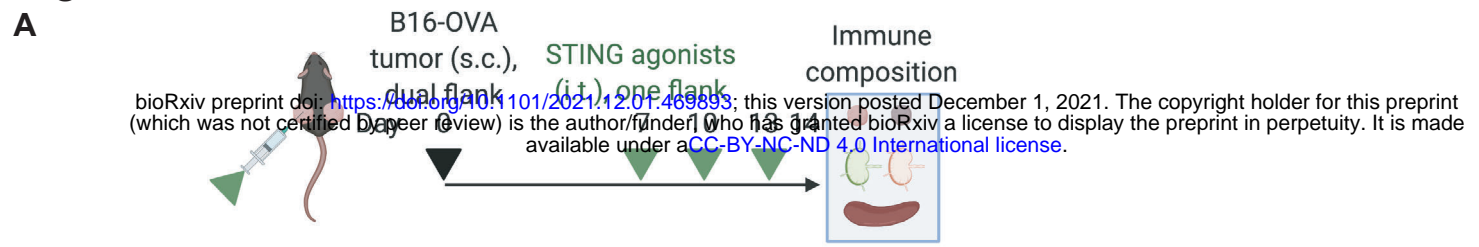
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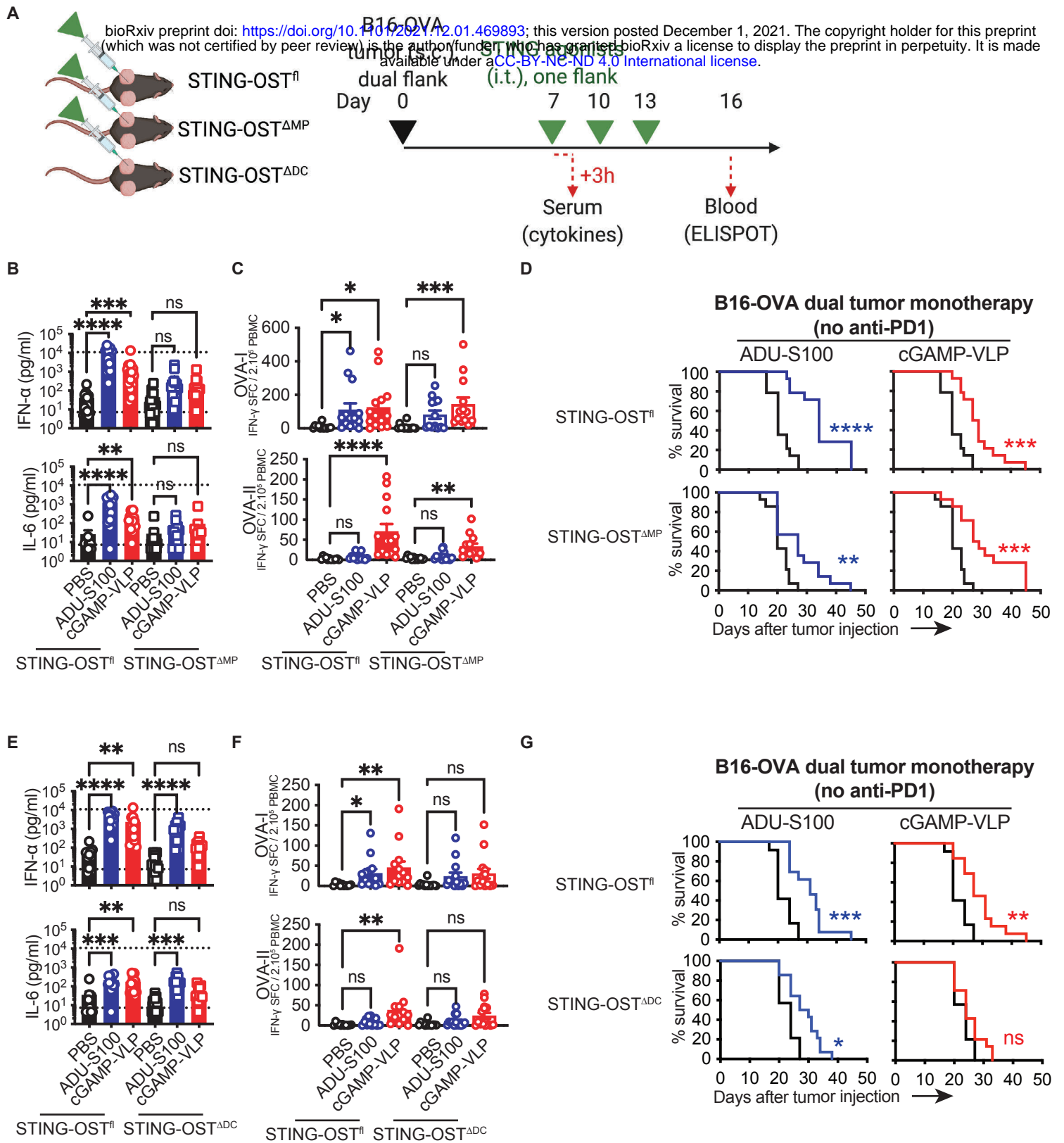


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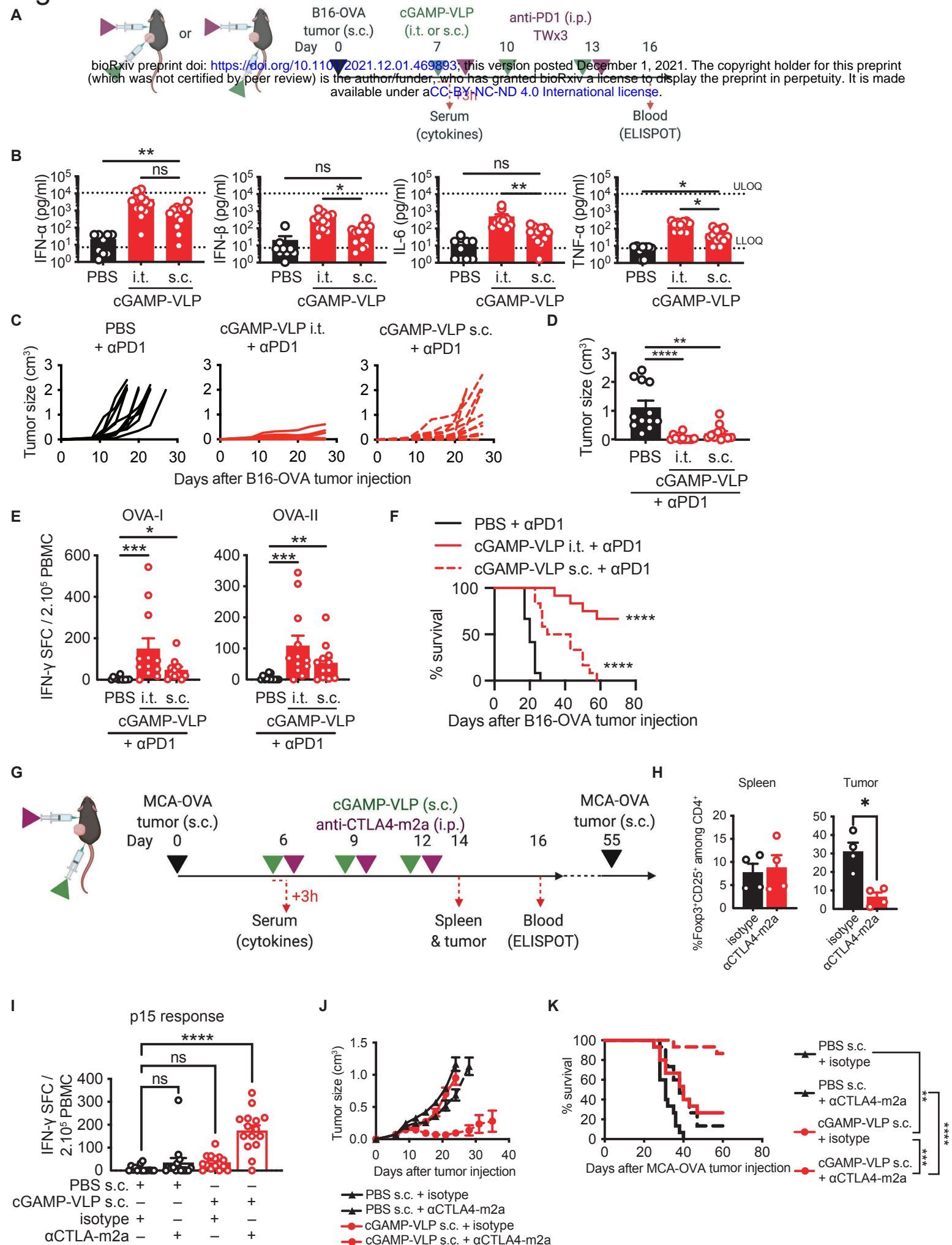




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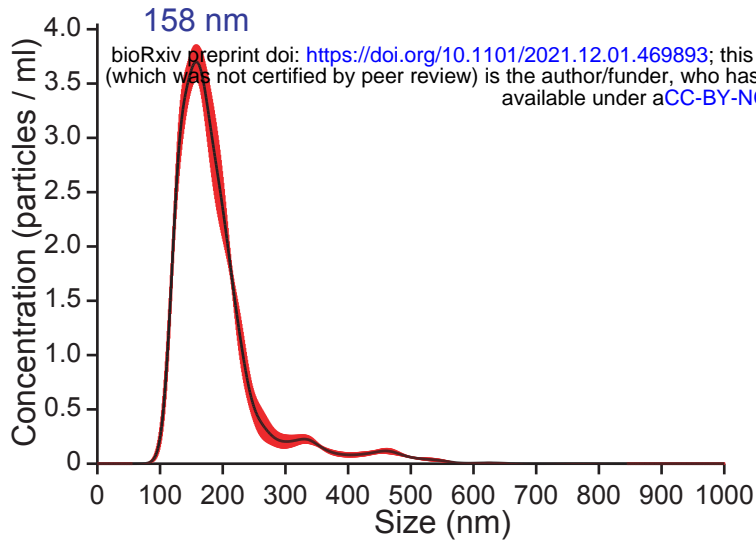


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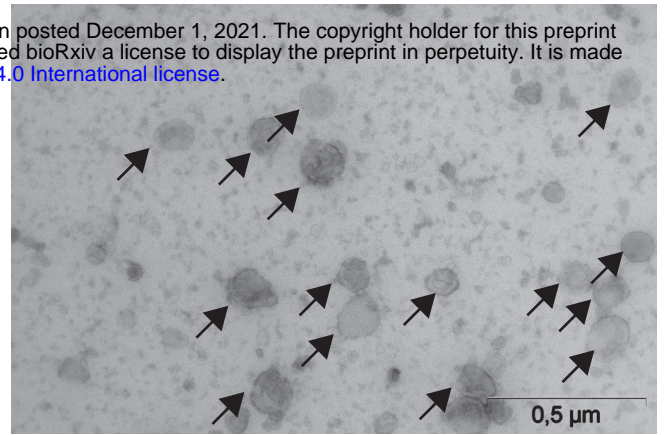


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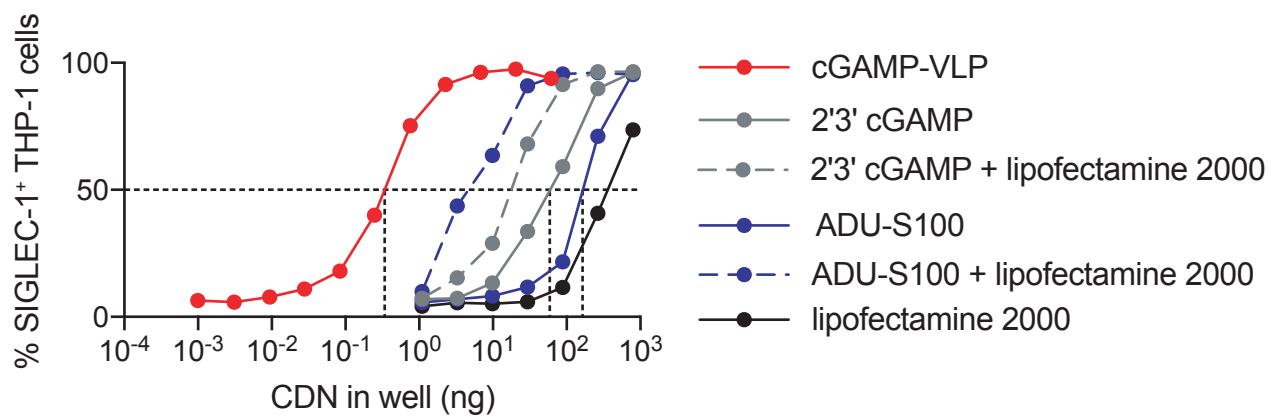
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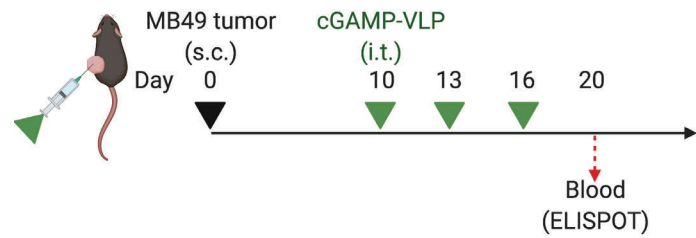
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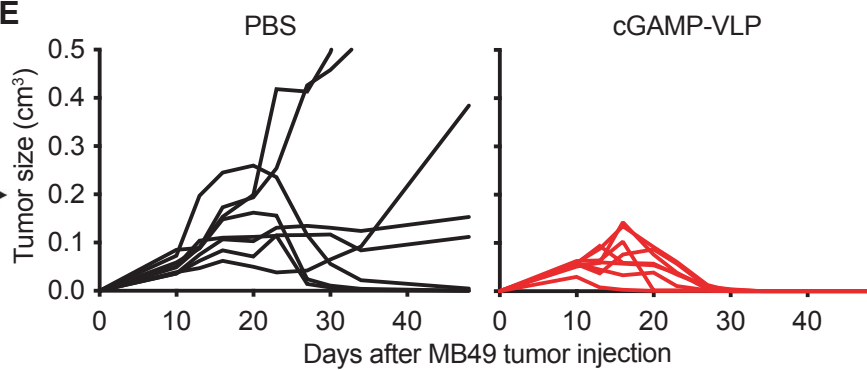
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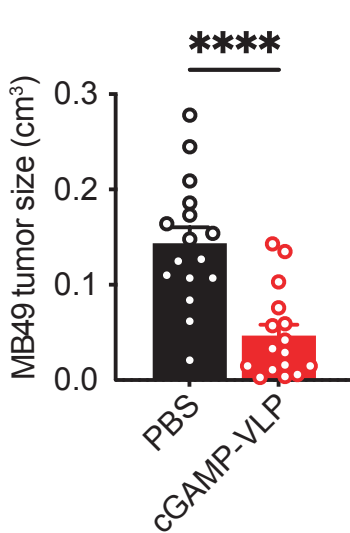
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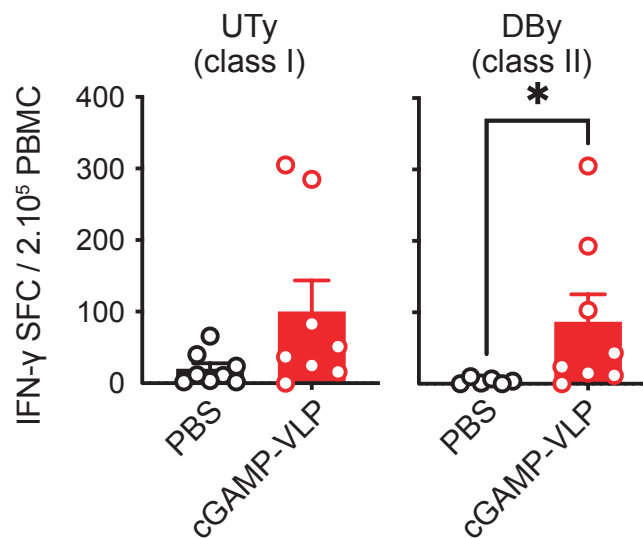
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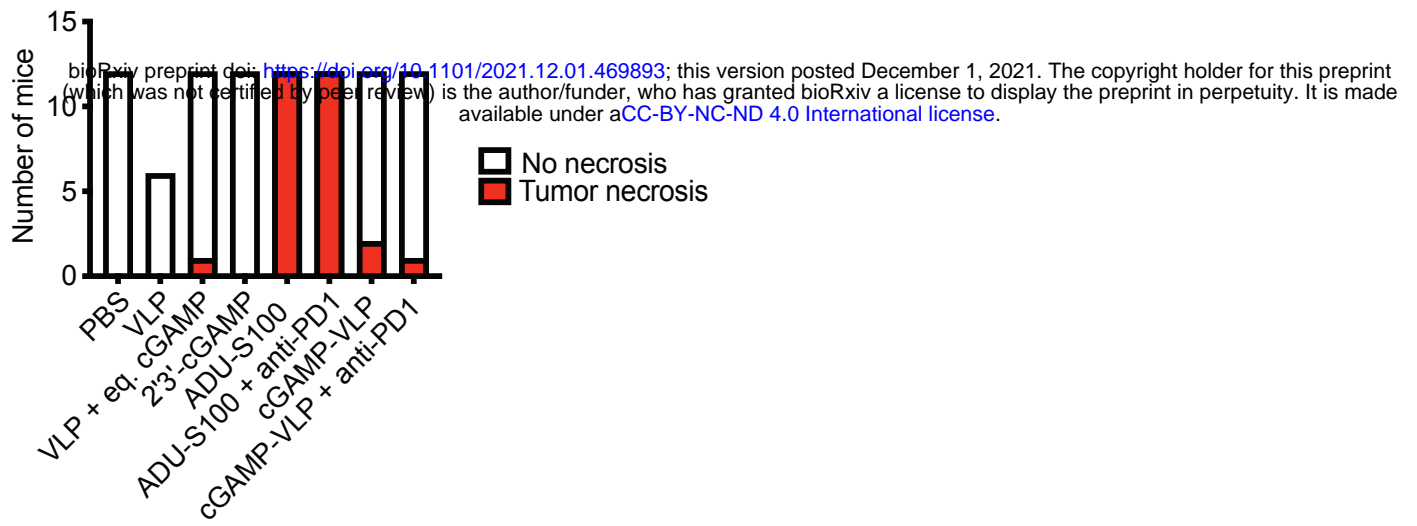


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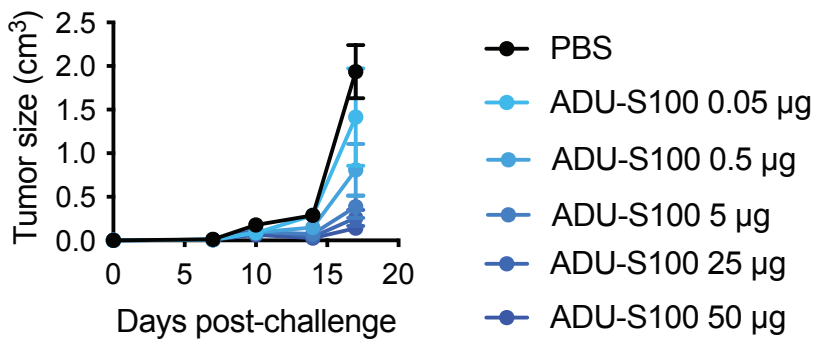


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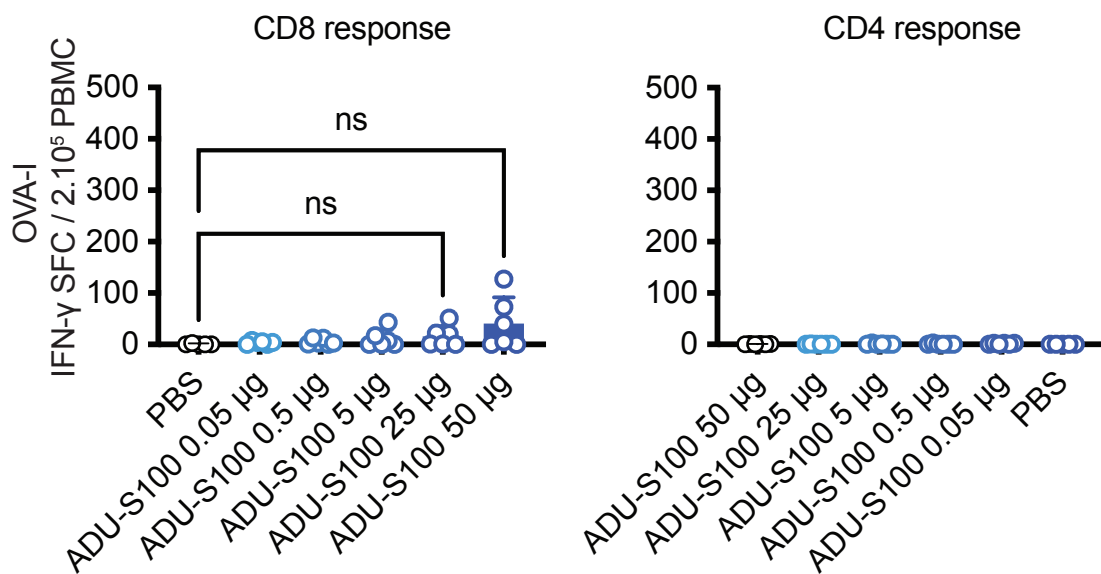
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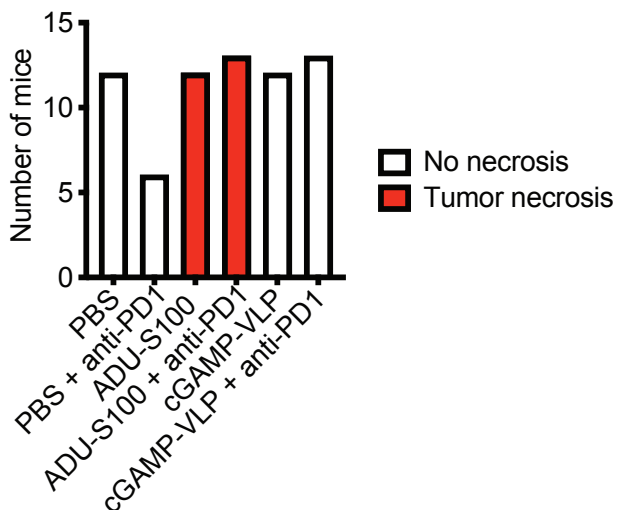
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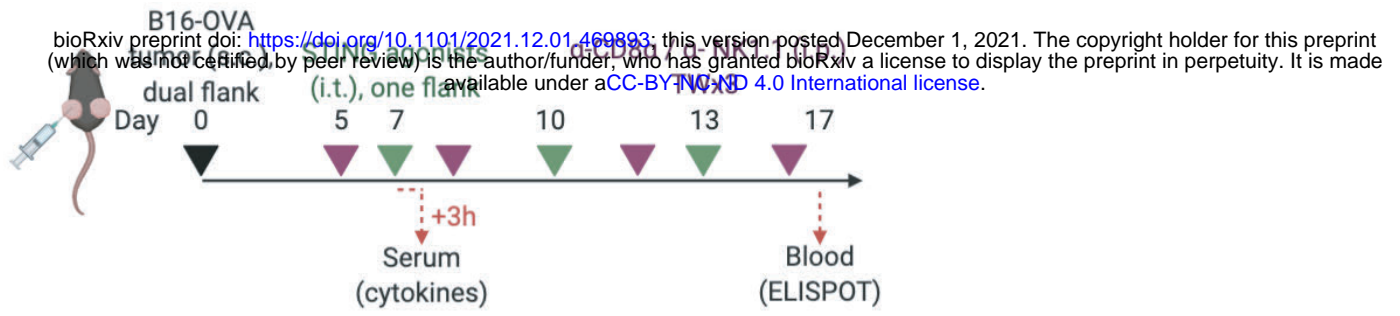


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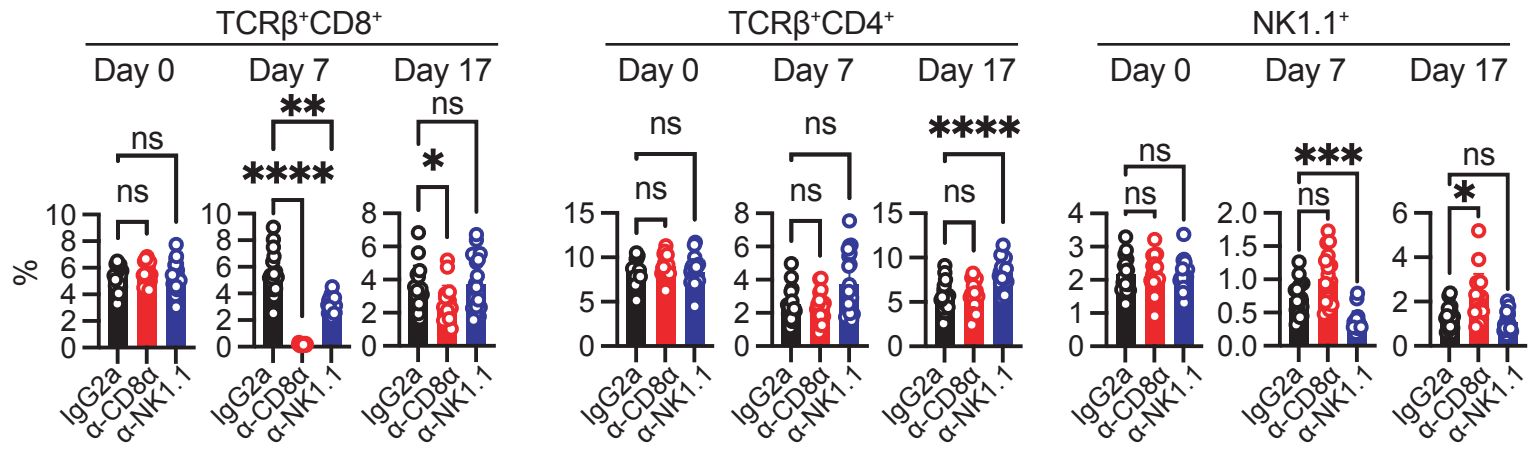


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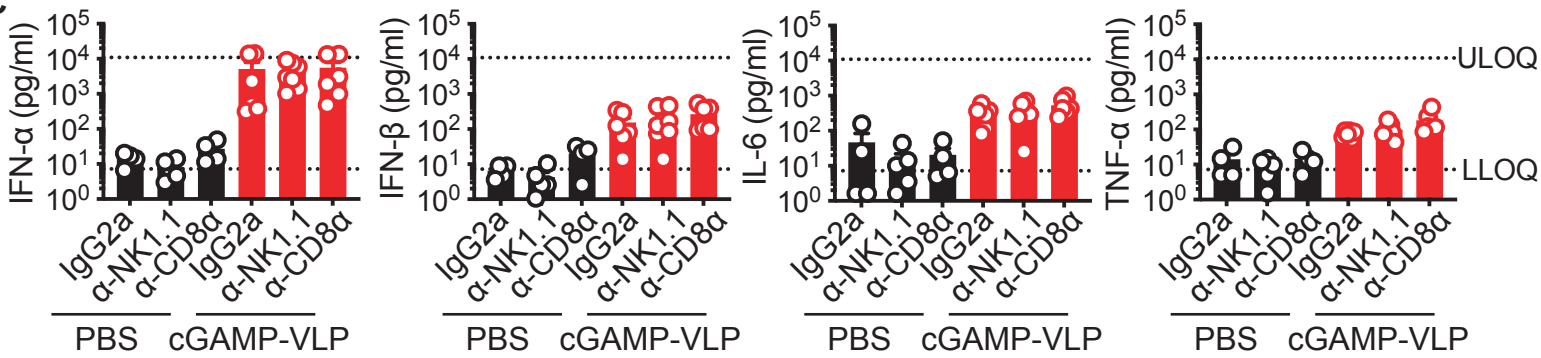
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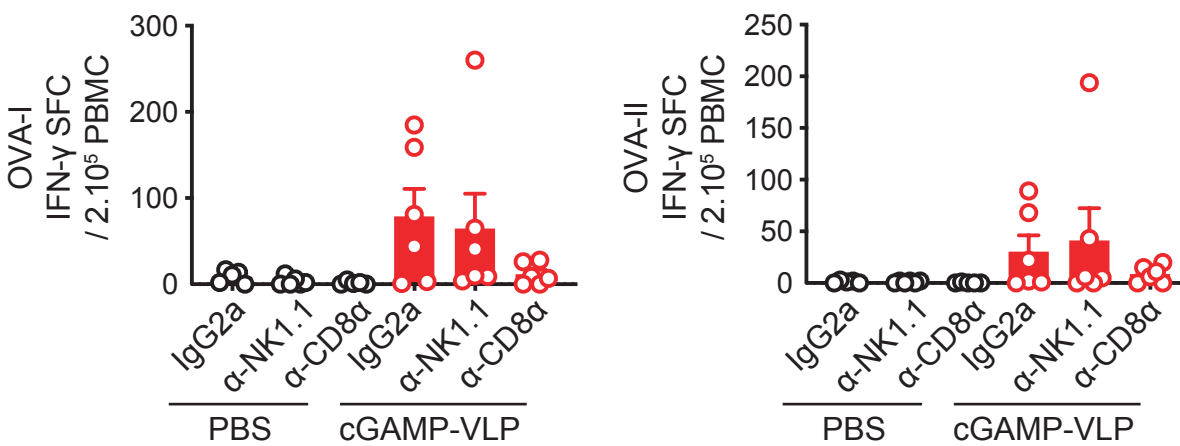
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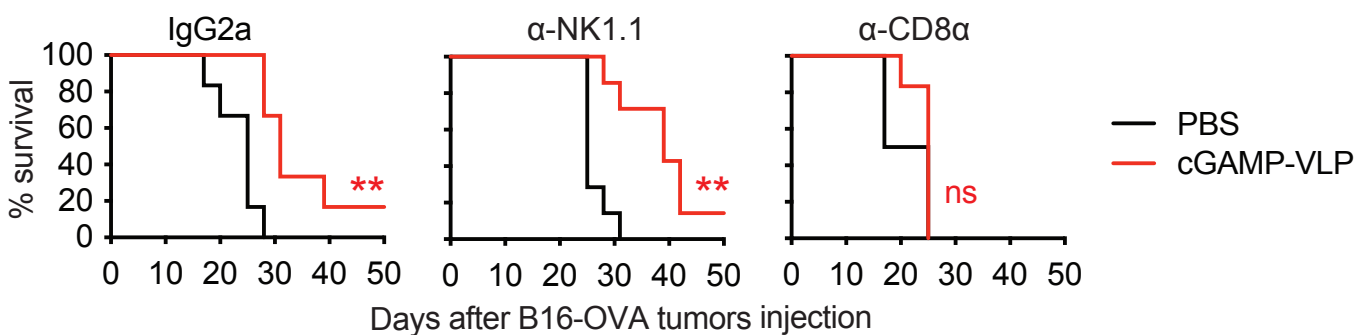
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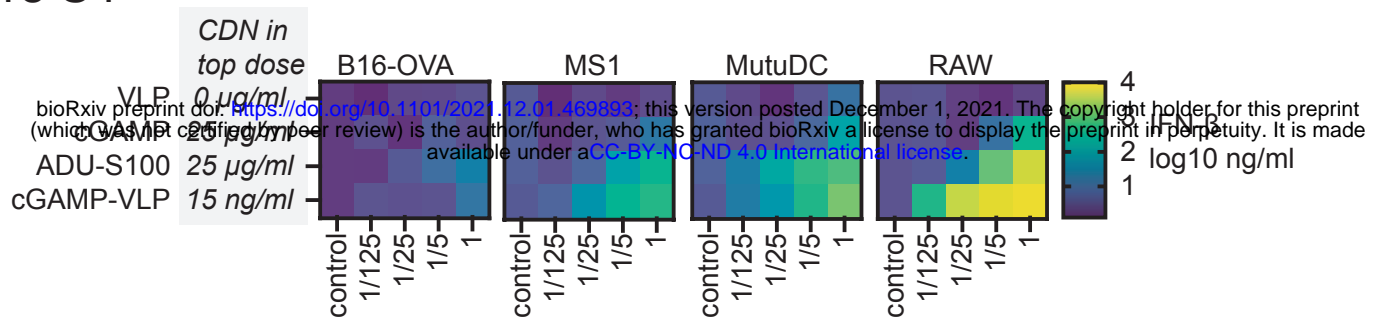


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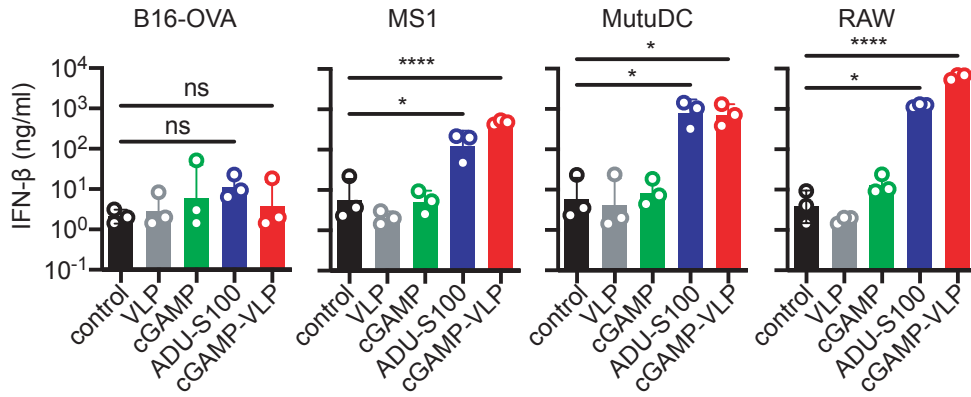


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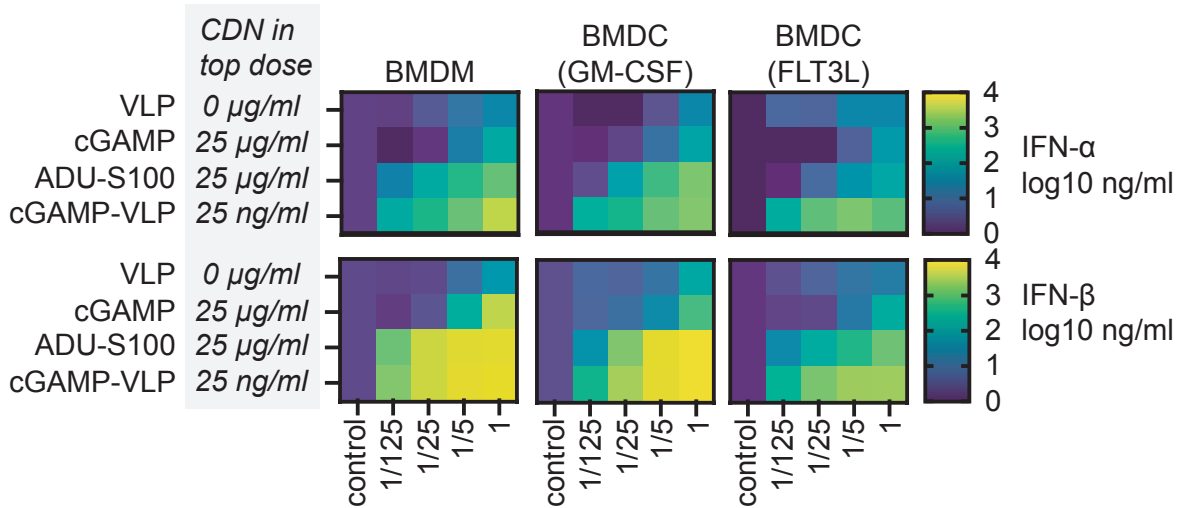
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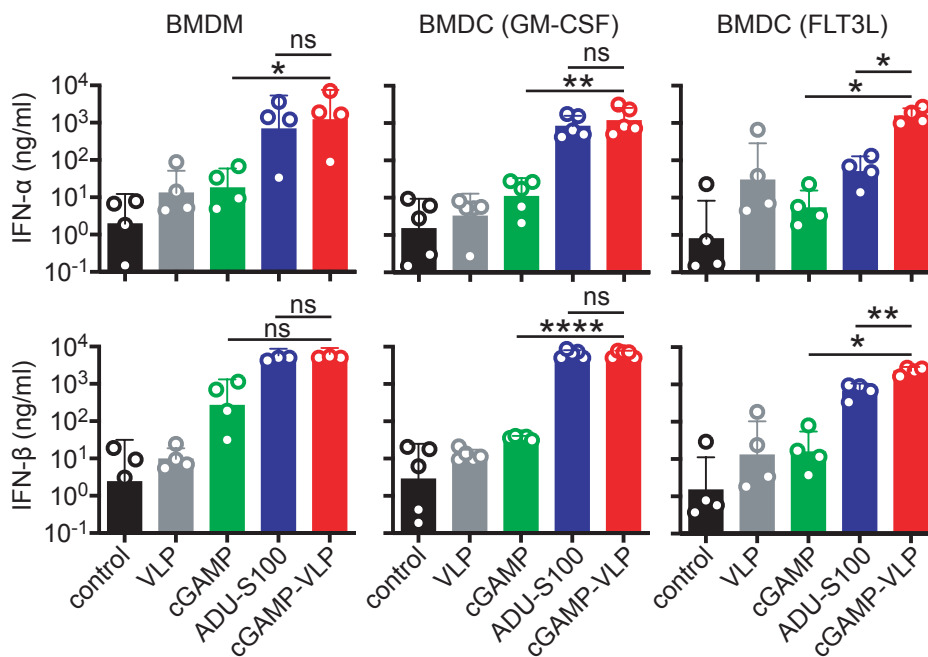
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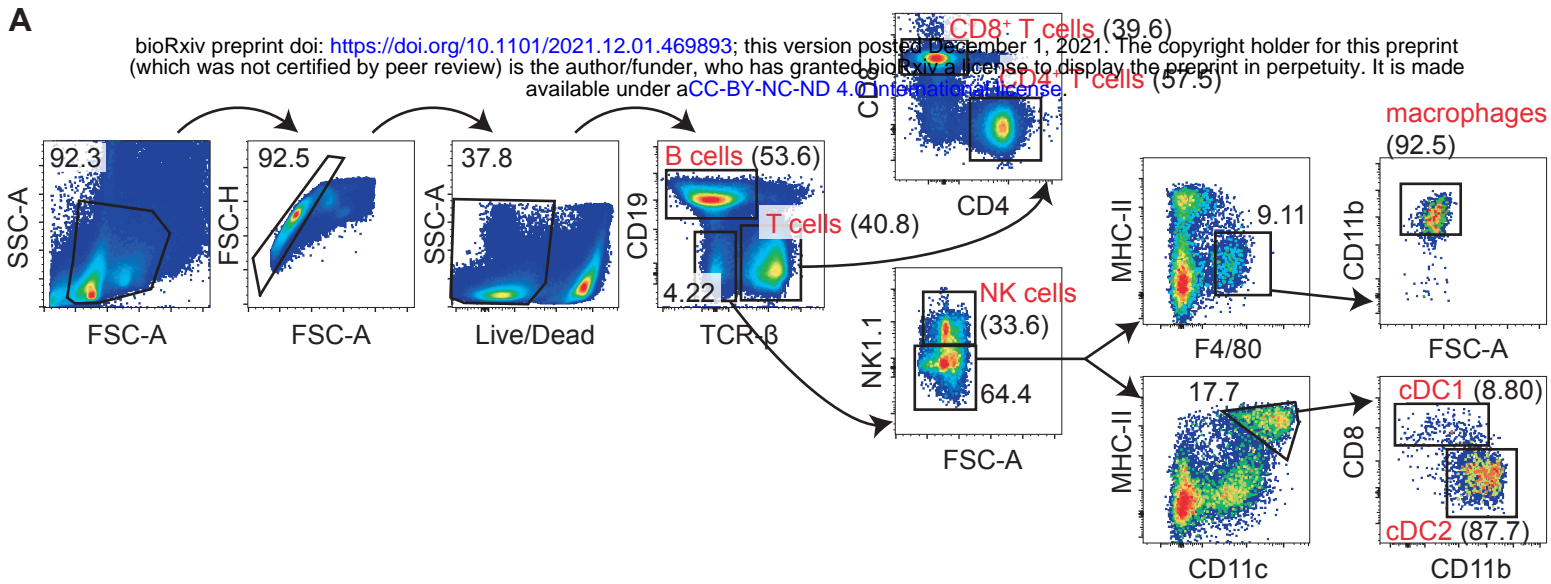
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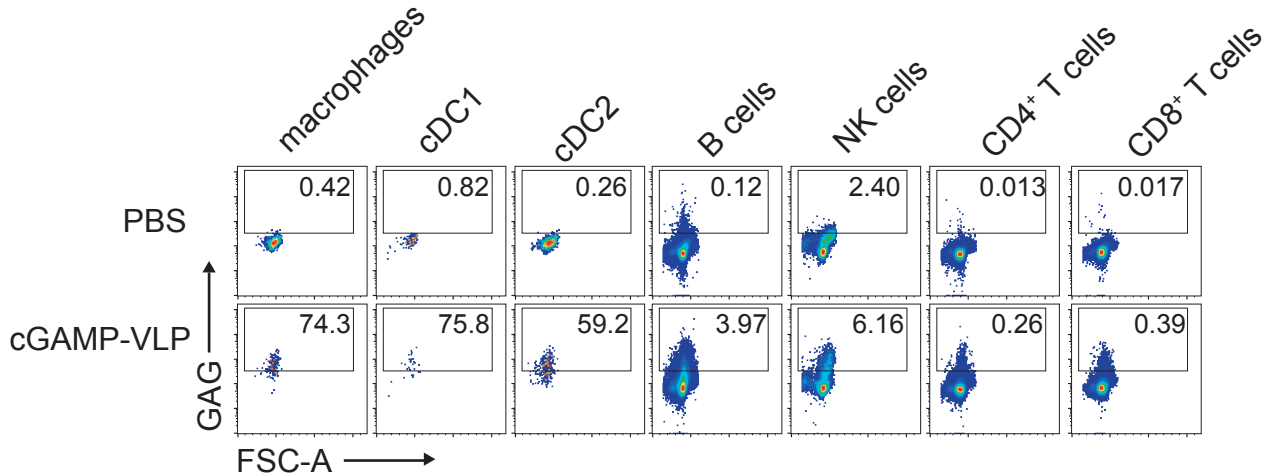
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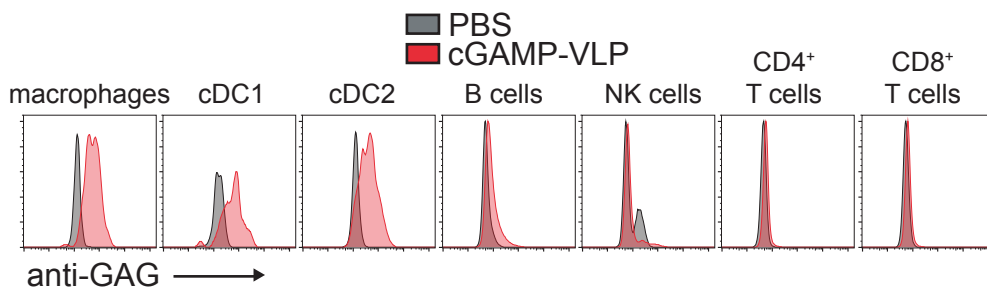
bioRxiv preprint doi: <https://doi.org/10.1101/2021.12.01.469893>; this version posted December 1, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.



**B**



**C**



**D**

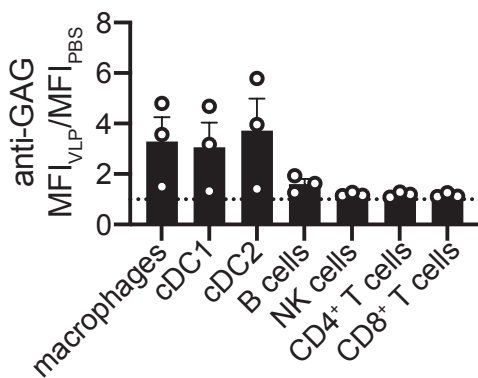
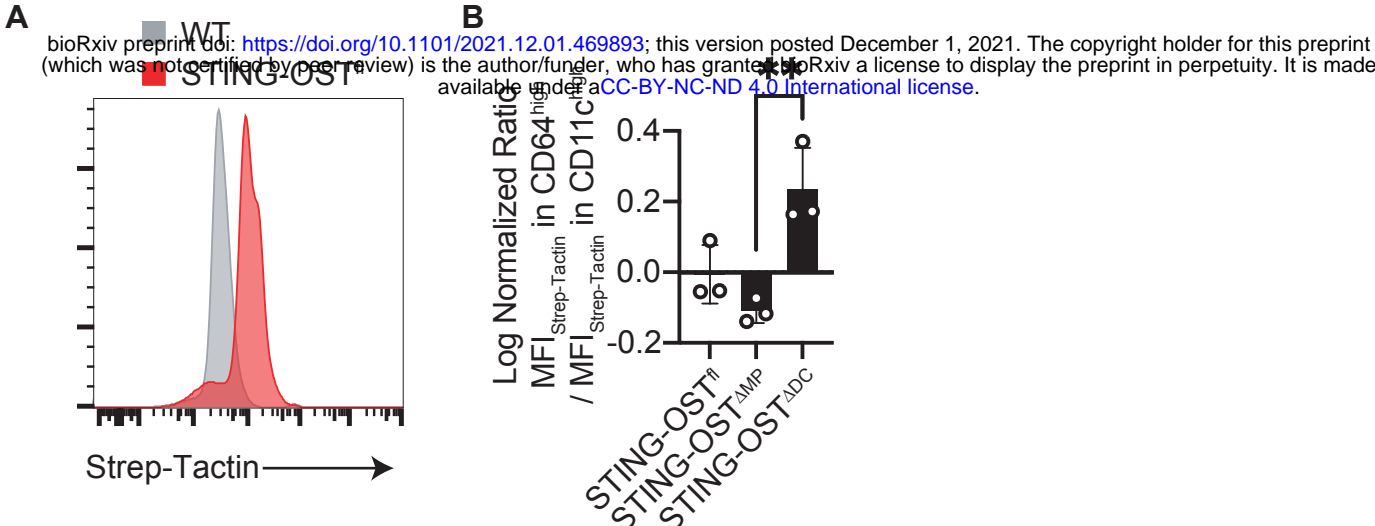


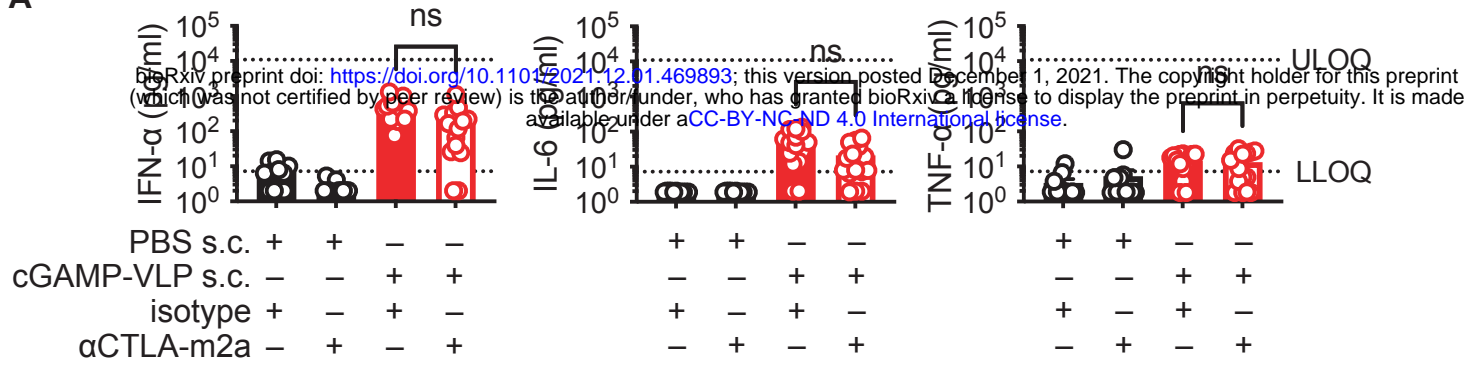
Figure S6



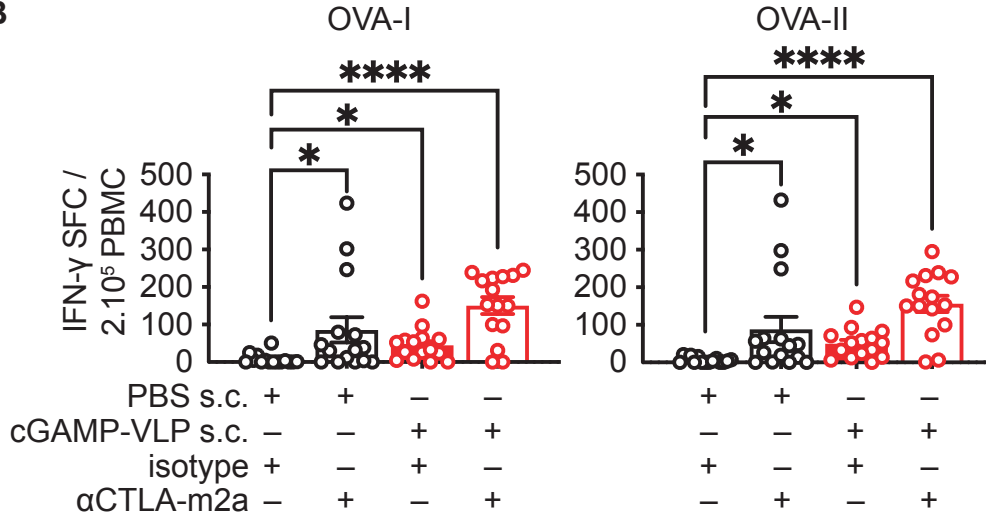


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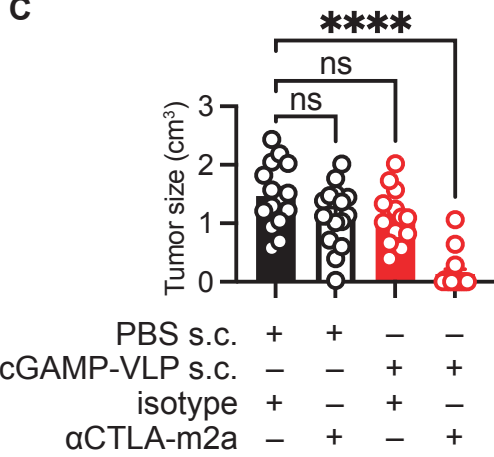
**A**



**B**



**C**



**D**

