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1 **Title:** Overcome the challenge for intratumoral injection of STING agonist for pancreatic
2 cancer by systemic administration

3

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38

39

40 **Abstract**

41 **Objective:** Due to the challenge for intratumoral administration, innate agonists have not
42 made it beyond preclinical studies for efficacy testing in most of tumor types. Pancreatic
43 ductal adenocarcinoma (PDAC) has a T-cell excluded or deserted tumor microenvironment.
44 Innate agonist treatments may serve as a T cell priming mechanism to sensitize PDACs to
45 anti-PD-1 antibody (a-PD-1) treatment.

46 **Design:** Using a transplant murine model with spontaneously formed liver metastasis and
47 also the genetically engineered KPC mouse model that spontaneously develops PDAC, we
48 compared the antitumor efficacy between intrahepatic/intratumoral and intramuscular
49 systemic administration of BMS-986301, a next-generation STING agonist. Flow cytometry,
50 Nanostring, and cytokine assays were used to evaluate local and systemic immune responses.

51 **Results:** The study demonstrated that administration of STING agonist systemically via
52 intramuscular injection is equivalent or potentially superior to its intratumoral injection in
53 inducing both effector T cell response and antitumor efficacy. Compared to intratumoral
54 administration, T cell exhaustion and immunosuppressive signals induced by systemic
55 administration were attenuated. Nonetheless, either local or systemic treatment of STING
56 agonist was associated with increased expression of CTLA-4 in the tumors. However, the
57 combination of a-PD-1 and anti-CTLA-4 antibody with systemic STING agonist
58 demonstrated the antitumor efficacy in the KPC mouse spontaneous PDAC model. Our study
59 also demonstrated the feasibility and antitumor efficacy of systemic administration of BMS-
60 986299, a new NLRP3 agonist.

61 **Conclusion:** For the first time, our study supports the clinical development of innate agonists

62 via systemic administration, instead of local administration, for treating PDAC.

63

64 **Keywords:** pancreatic ductal adenocarcinoma; stimulator of interferon genes; innate immune
65 agonist; STING; NLRP3; tumor microenvironment; immune checkpoint inhibitor;
66 immunotherapy; systemic administration; intratumoral injection.

67

68 **What is already known on this topic** – Despite promising preclinical studies, innate
69 immune agonists including STING agonists and NLRP3 agonists have not been tested in
70 most of tumor types due to the difficulties associated with their intratumoral delivery.

71 **What this study adds** – This study compared between intratumoral and systemic
72 administration of BMS-986301, a next-generation STING agonist and BMS-986299, a
73 new NLRP3 agonist in the preclinical models of pancreatic cancer. Notably, systemic
74 administration of STING agonist showed comparable or potentially superior effector T cell
75 response and antitumor efficacy compared to intratumoral administration, with attenuated T
76 cell exhaustion and immunosuppressive signals.

77 **How this study might affect research, practice or policy** – For the first time, this study
78 supports the clinical development of innate agonists via systemic administration for treating
79 pancreatic cancer. Supported by the results in this study, a phase 1 trial evaluating BMS-
80 986301 intratumoral or systemic intravenous injection as monotherapy or in combination
81 with nivolumab (PD-1 blockade) and ipilimumab (CTLA-4 blockade) in patients with
82 advanced solid cancers has been initiated (NCT03956680).

83

84 **Introduction**

85 Recent research has highlighted the crucial role of the innate immune system in tumor
86 immunosurveillance and the stimulation of antitumor immune responses[1]. This has led to
87 the development of several small-molecule innate agonists as potential immunotherapeutics
88 or vaccine adjuvants for different types of cancer[2]. However, despite promising preclinical
89 studies, these agents have not been tested in most of tumor types due to the difficulties
90 associated with their intratumoral delivery[3]. Overcoming this challenge is critical for the
91 clinical development of innate agonists. Nonetheless, the potential benefits of using innate
92 agonists as T cell priming agents continue to provide an impetus for the exploration of novel
93 strategies to overcome the challenge for their delivery.

94

95 Stimulator of interferon genes(STING) is a transmembrane protein that is expressed in
96 various endothelial, epithelial and hematopoietic cells. Upon activation at the endoplasmic
97 reticulum and subsequent translocation to the Golgi, STING recruits and activates TANK-
98 binding kinase 1(TBK1), which in turn phosphorylates and activates interferon regulatory
99 factor 3(IRF3) and NF- κ B transcriptional programs, resulting in the expression and release of
100 pro-inflammatory type I interferons(IFNs) and cytokines[4, 5, 6]. Accumulating evidence has
101 suggested that STING also possesses cell-intrinsic tumor suppressive activity[7]. The
102 mechanistic underpinnings of the cyclic GMP-AMP synthase-STING pathway make STING
103 agonists(STING-A) a promising adjuvant to cancer vaccines[8]. Numerous natural or
104 synthetic STING-A as monotherapy or in combined with other treatments have also been
105 tested in both pre-clinical studies and clinical trials across many cancer types[9, 10].

106 Synthetic cyclic dinucleotides(CDNs) were the first generation of STING-A that entered the
107 clinical trial phase of drug development due to their structural versatility and ability to bind
108 all prevalent allelic variants of human STING.

109

110 Pancreatic ductal adenocarcinoma(PDAC) is one of the most lethal malignancies due to its
111 resistance to conventional therapies. PDAC and other non-immunogenic “cold” tumors do
112 not respond to immune checkpoint inhibitors(ICIs) as monotherapy largely due to the lack of
113 tumor-infiltrating effector lymphocytes [11, 12]. Published studies have demonstrated that
114 intratumorally injected STING-A inflamed the tumor microenvironment(TME) of PDAC
115 with an effector T cell infiltration and reduced tumor burden in the mouse model of
116 PDAC[13]. However, despite these promising results at the preclinical phase, anti-tumor
117 efficacies of STING-A have not been substantiated, largely due to the challenge of
118 intratumoral delivery of such agents, which may have never reached therapeutic dose levels
119 or never been tested in the appropriate disease indications.

120

121 BMS-986301 is a novel CDN-based, next-generation STING-A and has demonstrated a
122 promising antitumor activity in the CT26 and MC38 subcutaneous tumor murine models,
123 resulting in more than 90% of tumor regression compared to only 13% with the first-
124 generation STING-A, ADU-S100. Similar results were observed with a single intratumoral
125 administration of BMS-986301 in combination with an anti-PD-1 agent [14]. However, these
126 previous studies were limited by the subcutaneously implanted tumors, which do not
127 resemble the TME of human PDAC[15]. Therefore, this present study aims to deliver this

128 next-generation CDN-based STING-A systemically and compare its anti-tumor efficacy and
129 elicited immune response with the intratumoral injection of this agent in a liver metastasis
130 model of PDAC.

131

132 **Methods**

133 **Mouse experiments**

134 The mouse study was conducted in accordance with the guidelines established by the Animal
135 Care and Use Committee of Johns Hopkins University. Female C57Bl/6 mice aged 6 to 8
136 weeks were purchased from Jackson Laboratories and maintained under the Institutional
137 Animal Care and Use Committee(IACUC) guidelines. Third-party management was
138 responsible for maintaining the IACUC mouse protocol.

139

140 STING-A(BMS986301, BMS) was dissolved into DPBS(Life Technologies) vehicle and
141 administered to tumor-bearing mice either by intratumoral or intramuscular injection once a
142 week at a dose of 5mg/kg starting on day 14, for a total of three doses. a-PD-1(BMS936558,
143 BMS) and an IgG control(ab18443, BMS) were administered intraperitoneally twice weekly
144 starting on day 14, for a total of five doses, at a dose of 10mg/kg.

145

146 **NanoString**

147 After the mouse was euthanized and the tumor was harvested, the tumor tissues were
148 submerged into RNA-later(Invitrogen) to preserve the RNA. The total RNA was extracted
149 from the whole specimen using the AllPrep DNA/RNA/Protein Mini Kit(Qiagen) according

150 to the manufacturer's instructions. The RNA was then equalized for NanoString hybridization
151 using the Formulatrix Tempest. The murine PanCancer Immune panel codeset
152 XT_PGX_MmV1_CancerImm_CSO, Cat#115000142), which contains 750 target genes
153 along with housekeeping and negative/positive control probes, was used for the NanoString
154 hybridization. The data obtained was then analyzed using NanoString nSolver 3.0 and an
155 internally developed NanoString Data Analyzer Rshiny app(BMS). The samples were run on
156 the NanoString MAX system reader, and the data was analyzed using various CRAN and
157 Bioconductor packages such as dplyr, tidyr, and reshape2 to clean, reformat and match the
158 sample annotations to the normalized data exported from NanoString nSolverV.3.0.

159

160 Additional methods are provided in the Supplemental Materials.

161

162 **Results**

163 **Develop a mouse model to resemble the intratumoral injection of STING-A in** 164 **metastatic cancer patients**

165 To examine whether systemic administration of STING-A is equivalent or superior to
166 intratumoral(IT) injection, we established two mouse models to compare these two routes of
167 innate agonist administration. We first developed a model to resemble the IT injection of
168 STING-A in patients with metastatic diseases. This liver metastasis model was developed by
169 implanting 7.5×10^5 tumor cells of the KPC tumor cell line, derived from the PDAC of the
170 Kras/p53/pdx1-Cre(KPC) mouse model, through the hemisplenectomy procedure and splenic
171 vessel injection to form liver metastases(Fig.S1A). The mice in this model would uniformly

172 die from liver metastases if not treated[17]. The changes in the sizes of the metastatic lesions
173 in the liver can be monitored by small animal ultrasonography. Liver metastases have been
174 identified as potential sites for the IT administration of the first-generation STING-A to treat
175 patients with PDAC. In this study, the liver metastases serve as the clinically relevant target
176 tumors for IT administration of STING-A(Fig.1A).

177

178 Mice were also inoculated subcutaneously with the KPC tumor cell line to form
179 subcutaneous(SubQ) tumors, not for the IT injection of STING-A, but for the abscopal effect
180 to be examined. As shown in Fig.1B, sixteen mice per group were treated by control
181 vehicles(negative control, NC), a-PD-1, STING-A, and STING-A+a-PD-1(Combo). A total
182 of five doses of a-PD-1 was administered twice a week by intraperitoneal injection(IP), while
183 the STING-A was given for three weekly doses by IT injection to mimic local treatment or
184 intramuscular(IM) injection to mimic systemic treatment. Mouse survival was followed; the
185 target liver metastatic lesion was measured by ultrasound; and the subQ tumors was
186 measured by calipers twice a week. We did not observe any obvious sign of toxicity including
187 bleeding, infection, paralysis, and weight loss as a result of IT injection of STING-A.
188 However, we noticed small areas of liver necrosis around the injection sites at the necropsy of
189 two mice(Fig.S1B). It should be noted that only approximately 35% of mice in the
190 hemisplenectomy model harbored a liver metastasis feasible for IT injection.

191

192 The results(Fig.1C) showed that the tumor growth inhibition(TGI) rate of the target liver
193 metastasis lesion was significantly increased in the STING-A and a-PD-1 combination

194 treatment group(maximum TGI=88.68±98.42%, p <0.05) and the STING-A group(maximum
195 TGI=69.38±73.94%, p <0.05) as compared to the negative vehicle group. To investigate
196 whether the local intratumoral injection of STING-A could induce the abscopal effect, we
197 implanted subcutaneous tumors on the bilateral flanks of the liver metastasis mice model to
198 mimic distant metastases. The TGI rate of bilateral SubQ tumors was also significantly
199 increased in the combo group(maximum TGI=66.38±86.00%, p <0.05) as compared with
200 negative control(Fig.1D). Interestingly, the TGI in the distant SubQ tumors is bigger than that
201 of the locally targeted liver metastatic lesion, supporting an abscopal effect from the STING-
202 A and a-PD-1 combo treatment. Moreover, we compared the survival of liver metastasis mice
203 in the above treatment groups and found that the combo treatment significantly prolonged
204 survival comparing to the control treatment and the STING-A treatment(Fig.1E).
205 Nevertheless, the combo treatment prolonged survival modestly without a statistical
206 significance comparing to the a-PD-1 treatment. It is possible that the local intratumoral
207 injection of saline may have caused inflammatory response which resulted in a small effect
208 on priming the tumor for the a-PD-1 treatment. Nevertheless, the combo treatment
209 significantly prolonged survival comparing to the control treatment and the STING-A
210 treatment(Fig.1E).

211

212 **STING-A in combination with anti-PD-1 antibody enhances effector T cells infiltration**
213 **and CD103⁺dendritic cells in both target liver metastatic lesions and non-target**
214 **metastatic lesions**

215 To understand the mechanistic basis of the enhanced anti-tumor activity of STING-A in

216 combination with a-PD-1, we performed flow cytometry analysis of the tumor infiltrating
217 leucocytes(TILs) derived from the targeted liver metastasis in the same PDAC mouse model.
218 As described in the dosing schema in Fig.1B, mice received the a-PD-1 on days 14 and 17,
219 and the STING-A on days 14. On day 21, the mice were sacrificed and the implanted SubQ
220 tumors as well as livers were harvested. TILs from the targeted liver metastatic lesion and the
221 whole liver tissue excluding the targeted lesion were compared. Note that the whole liver
222 tissue harvested on day 21 was diffusely infiltrated by non-target metastatic lesions. The
223 results showed that the STING-A+a-PD-1 combo therapy significantly increased the
224 infiltration of the CD8⁺ and CD8⁺PD-1⁺T cells, but not the CD4⁺ and CD4⁺PD-1⁺T cells in
225 the target lesion comparing to treatment controls(Fig.2A;Fig.S1C). Interestingly, a-PD-1,
226 STING-A, or their combo all decreased the MHCII⁺CD11c⁺dendritic cells(DCs); however,
227 comparing to the treatment control, a-PD-1 alone, or STING-A alone, the a-PD-1 and
228 STING-A combo significantly increased the CD11b⁻CD103⁺subtype of DCs(Fig.2B), which
229 are known to play a role in the cross-presentation of tumor antigens[20]. TILs from non-
230 target liver metastases showed that the combo treatment resulted in a significant increase in
231 CD8⁺T cells comparing not only to the treatment control, but also to a-PD-1 alone(Fig.2C).
232 The infiltration of CD8⁺PD-1⁺T cells in the combo group was comparable with the control
233 group and significantly lower than that in the STING-A alone group(Fig.S1D). Moreover,
234 CD4⁺T cells and CD4⁺PD-1⁺T cells were both significantly decreased in the combo treatment
235 group compared to the STING-A treatment group, presumably due to the treatment effect of
236 a-PD-1(Fig.2C). These results suggest that, in non-target liver metastatic lesions, CD8⁺ and
237 CD4⁺T cells both trended in the favor of anti-tumor immune response following the a-PD-1

238 and STING-A combo treatment. In non-target liver metastases, MHCII⁺CD11c⁺DCs were
239 similar among all treatment groups, suggesting that an enhanced antigen presentation was
240 originated in locally targeted lesions. However, CD11b⁻CD103⁺DCs were significantly
241 elevated in the combo treatment group in non-target lesions(Fig.2D). It is possible that
242 CD103⁺DCs trafficked from targeted lesions to non-target lesions. Taken together, these
243 results suggest that both STING-A and a-PD-1 are required to activate local immune response
244 in favor of anti-tumor response and that this immune response is extended to the non-target
245 lesions in the vicinity of the target lesion.

246

247 **STING-A in combination with anti-PD-1 antibody activate anti-tumor immunity via**
248 **innate immune response signaling pathways**

249 As the flow cytometry analysis has limitation in examining cytokine/chemokines and
250 intracellular signals involved in the innate immune response, we examined the gene
251 regulation of innate immune response following STING-A and/or a-PD-1 treatment by using
252 the NanoString assays with the murine PanCancer Immune panel. We selected differentially
253 expressed genes by comparing the STING-A and a-PD-1 combo treatment and treatment
254 controls, STING-A alone or a-PD-1 alone with a 5% false discovery rate(Fig.S1E). We first
255 examined the gene expression in the interferon(IFN) response pathways. As shown in Fig.2E,
256 the gene expression of IFN- γ (*Ifng*) was significantly increased in the combo treatment group
257 and was also accompanied by significantly increased expression of *Ifngr1*, *Ifnar2*, *Irf1*, *Irf4*,
258 *Irf5*, *Irf7*, *Irf8*, *Stat1*, *Stat2*, *Stat4*, *Ifitm1*, *Ifih1*, *Ifit1*, *Ifit2*, *Ifit3*, *Ifi35*, and *Ifi44* compared to
259 the control or single treatment groups. However, *Irf3* was significantly decreased in the

260 combo treatment group compared to the a-PD-1 treatment group(Fig.S1F). In addition, the
261 tumor necrosis factor(TNF)-response pathway genes including *Tnf*(encoding TNF- α),
262 *Lta*(encoding TNF- β), *Nfkb1*, *Nfkb2*, and *Tank* were also significantly upregulated in the
263 combo treatment group compared to the control or single treatment groups(Fig.2F). The
264 innate immune response pathways genes including *Tmem173*(*STING*), *Ddx58*, *Nlrp3*, *Nlrc5*,
265 *Myd88*, *Clec4a2*, *Clec4n*, *Clec5a*, *Clec7a*, *Nod1*, and *Nod2* were significantly increased in the
266 combo treatment group compared to the control or single treatment groups(Fig.2G). However,
267 as anticipated, the gene expression of *Jak1* and *Jak2* was similar among all treatment groups,
268 suggesting *Jak1/2* are not regulated at the RNA level(Fig.S1F).

269

270 We next investigated the expression of cytokines which are known to be involved in the
271 development and differentiation of T and B lymphocytes(Fig.3A-B;Fig.S2A-B). As shown in
272 Fig.3A, the expression of most genes in the interleukin(IL)-1 and IL-18 family except *Il18*
273 itself and *Irak1* was significantly increased in the combo treatment group compared to most
274 of other treatment groups. As demonstrated in Fig.3B, the gene expression of most of the
275 proinflammatory cytokine receptors including *Il2ra*, *Il2rb*, *Il2rg*, *Il7r*, *Il12rb1*, *Il12rb2*,
276 *Il13ra1*, *Il13ra2*, and *Il15ra* was significantly increased in the combo treatment group
277 compared to most of other treatment groups. Interestingly, the cytokines themselves including
278 *Il2*, *Il7*, *Il12*, and *Il13*, besides *Il21* which was significantly upregulated, were upregulated in
279 a non-statistically significant trend in the combo treatment group compared to the control or
280 single treatment groups(Fig.S2A). These results suggest that pro-inflammatory pathways
281 including those that mediate the inflammasome are activated broadly by the combination of

282 the STING-A and a-PD-1.

283

284 As anticipated, we found that the majority of chemokine genes were upregulated in the
285 combo treatment group compared to the a-PD-1 treatment group(Fig.S2C), likely due to the
286 innate immune response induced by the STING-A. Therefore, we focused on those associated
287 with T cell trafficking. Our results demonstrate a significantly increased expression of *Ccl1*,
288 *Ccl2*, *Ccl3*, *Ccl4*, *Ccl5*, *Ccl7*, and *Ccl8* in the combo treatment group compared to the vehicle
289 control treatment group(Fig.3C), suggesting that stimulation of innate immune response is
290 anticipated to induce myeloid cell infiltration. C-X-C motif chemokine ligand(CXCL) 9,
291 CXCL10, and CXCL11 are known to bind C-X-C motif chemokine receptor(CXCR) 3 on T
292 cells and, in response to IFN signaling to recruit memory and activated effector T cells[21].
293 We observed a significant enhancement of the expression of *Cxcl9*, *Cxcl10*, and *Cxcl11* in the
294 combo treatment group compared with the control or single treatment groups(Fig.3C)
295 although the expression of *Cxcr3* was similar among different treatment groups(Fig.S1E).
296 Interestingly, comparing to the vehicle control treatment group, the administration of a-PD-1
297 showed a statistically non-significant trend of increase whereas STING-A showed a trend of
298 decrease in the expression of *Ccl17*, which encodes a T regulatory cell(Treg) chemokine. This
299 finding is thus consistent with published studies showing that ICIs upregulate C-C motif
300 chemokine ligand(CCL) 17 expression in tumors and increase the migration of Tregs into the
301 TME of PDAC[22, 23]. Moreover, we here observed that the combo treatment led to a
302 significantly decreased expression of *Ccl17*(Fig.3C;Fig.S2D). It should be noted that the gene
303 expression results from the NanoString assay may be influenced by an influx of immune cells

304 that express the genes. Therefore, an increased expression of certain immune genes may
305 represent an increased infiltration of the relevant immune subtypes. Taken together, these
306 results suggest that STING-A may confer an antitumor effect by suppressing CCL17
307 expression or CCL17-expressing cells and thereby suppressing Treg migration into the TME.

308

309 **STING-A in combination with anti-PD-1 antibody enhances T cell activation signals**

310 Among those differentially expressed genes in the above NanoString analysis, we did further
311 statistical analysis on genes related to the activation of effector T cells. As shown in Fig.S2E
312 and as anticipated, the expression of T cell receptor/CD3 signaling pathway genes including
313 *Cd3d*, *Cd3e*, *Cd3g*, *Cd3z*, *Cd8a*, and *Lck* in the combo treatment group was significantly
314 increased[24, 25]. In addition, our results(Fig.3D) revealed that *Gzma*, *Gzmb*, *Gzmk*, and *Prfl*
315 expression was significantly elevated in the combo treatment group comparing to most of
316 other treatment groups, suggesting that the cytotoxic function of effector T cells are
317 significantly enhanced. The gene expression of T cell co-stimulatory factors including
318 *Tnfrsf9*(CD137), *Tnfrsf4*(OX40), and *Icos* were significantly increased in the combo
319 treatment group when compared to any other group and including *Cd27* when compared to
320 the vehicle treatment group(Fig.3E). However, the gene expression of co-inhibitory receptors
321 including *Pdcd1*(PD-1), *Lag3*, *Havcr2*(TIM3), and *Ctla4* and the expression of immune
322 checkpoint activators such as *Cd274*(PD-L1) and *Ido1* were significantly elevated in the
323 combo treatment group compared to any other treatment group(Fig.3F;Fig.S2F). These
324 results suggested that T cell activation in the combo treatment group may also lead to the T
325 cell exhaustion, in consistence with previously published studies[26].

326

327 **STING-A in combination with anti-PD-1 antibody enhances the infiltration and**
328 **activation of effector T cells in the distant subcutaneous tumors**

329 We next examined the immune response that mediates the abscopal effects in the distant
330 tumors. As shown in Fig.4A, we observed a significant increase in the infiltration of CD8⁺
331 and CD8⁺PD-1⁺T cells in the SubQ tumors from the combo treatment group, supporting the
332 abscopal effect. Similar to the locally targeted lesions, CD4⁺ and CD4⁺PD-1⁺T cells in the
333 SubQ tumors were not significantly changed among all treatment groups.
334 CD11b⁻CD103⁺DCs in the SubQ tumors were also similar among all treatment groups,
335 suggesting that the activation of antigen presenting cells occurred locally(Fig.4B).

336

337 We then used the same NanoString assay to assess the T cell functional status. The results
338 indicate that the expression of genes associated with the T-cell receptor CD3 complex
339 exhibited a similarly significant increase in the SubQ tumors from the combo treatment group
340 compared to other treatment groups as in the liver metastases(Fig.S3A). Similarly, genes
341 related to the cytotoxic activities of effector T cells, including *Gzma*, *Gzmb*, *Gzmk*, and *Prfl*,
342 demonstrated a significant increase in the combo treatment group compared to most of other
343 treatment groups(Fig.4C). In addition, the combo treatment group exhibited a significant
344 increase in the expression of signals related to T cell activation including *Cd27*, *Icos*,
345 *Cd274*(PD-L1), and *Ido1* comparing to most of other treatment groups(Fig.4D-E) and
346 including *Cd28*, *Cd80*, *Pdcd1*(PD-1), *Lag3*, and *Ctla4* comparing to the vehicle treatment
347 group(Fig.S3B). Consistently, genes related to chemokines for effector T cell trafficking

348 including *Cxcl9*, *Cxcl10*, and *Cxcl11* exhibited a significant increase in their expression in the
349 combo treatment group compared to most of other treatment groups(Fig.4F). *Ccl17* also
350 exhibited a significant decrease in the combo treatment group compared to the vehicle
351 treatment group. Interestingly, the expression of *Ccl28*, a T and B cell homing factor[27],
352 showed a significant increase in the combo treatment group in SubQ tumors(Fig.4F), but not
353 the target metastatic lesions(Fig.S2C). The gene expression of cytokines and cytokine
354 receptors that are relevant to the activated status of T cells was similarly increased as seen in
355 the liver metastases, again except *Il18*(Fig.4F), further supporting the abscopal effect.

356

357 In addition, the profiles of other cytokines and chemokines in the SubQ tumors were similar
358 to those in the targeted liver metastases and showed a significant increase in pro-
359 inflammatory immune responses in the IFN(Fig.4G) and TNF pathways(Fig.4H), but no
360 significant changes or a decrease in *Jak1* and *Jak2*(Fig.S3C), in the combo treatment group
361 compared to other treatment groups. Nevertheless, expression of innate agonist receptors and
362 adaptors in SubQ tumors appeared to be somewhat different from that in targeted liver
363 metastases, showing an increase in *Tlr8*, *Nlrc5*, *Nlrp3*, *Clec4n*, *Clec5a*, *Clec7a*, and *Nod1*, but
364 a decrease in *Ticam1* and *Mavs* in the combo treatment group compared to other treatment
365 groups(Fig.4I). However, the profile of chemokines and chemokine receptors that function in
366 the myeloid cell trafficking was similar between targeted liver metastases and distant SubQ
367 tumors in the combo treatment group compared to other treatment groups(Fig.S3D).

368

369 **Establish a mouse model for systemic administration of STING-A in combination with**

370 **anti-PD-1 antibody induced both systemic and intratumoral immune responses**

371 We next tested the IM administration, a systemic administrative route, of STING-A. To
372 compare the anti-tumor efficacy of IM with IT injection of STING-A, we used the same liver
373 metastasis model with implantation of SubQ tumors by following the same schema described
374 in Fig.1B. Firstly, systemic administration did not show any obvious toxicity including
375 bleeding, unhealed wound, paralysis, or weight loss, etc. All the mice following the
376 hemisplenectomy procedure were candidates for IM injection although we chose those
377 feasible for IT injection for the purpose of comparison. We then found that mice in the
378 combo(IM) group had a significantly prolonged survival when compared to other treatment
379 groups(Fig.5A). The TGI rate for SubQ tumors also exhibited a significant increase in the
380 combo(IM) group(maximum TGI=58.86 ± 49.12%, p <0.05)(Fig.5B). By combining the
381 survival data in both IT and IM experiments, we found that the majority of mice died at
382 around 3 to 4 weeks after tumor implantation whereas those who received both STING-A by
383 IT or IM and a-PD-1 may live up to 6 weeks(Fig.S4A). Mice who received STING-A
384 intramuscularly in combination with a-PD-1 reached the longest survival beyond 6 weeks
385 although, likely due to the small sample size, there was no significant survival difference
386 between the IM combo and the IT combo group.

387

388 To assess the systemic immune response induced by the IM injection of STING-A and/or a-
389 PD-1, we first measured the cytokine response in the serum samples harvested 6 hours after
390 the IM injection of STING-A. The results demonstrated that a number of cytokines especially
391 those associated with inflammation had a significantly increased level in the sera of mice

392 from the combo treatment group, including TNF- α , and IFN- γ (Fig.5C). Interestingly, several
393 T lymphocytes trafficking chemokines including CXCL9, and CXCL10 and type I cytokines
394 including IL-2[28] and IL-12[29] were significantly increased in the sera from mice in the
395 combo treatment group compared to other treatment groups, suggesting that IM injection of
396 STING-A in combination with a-PD-1 is potentially able to induce an anti-tumor systemic
397 immune response(Fig.5D). In addition, some interleukins including IL-5[30], IL-6[31], and
398 IL-10[32] that support lymphocyte growth and/or antibody production were also boosted in
399 the IM combo group(Fig.S4B). The results also indicated the increased production of
400 cytokines that participate in the recruitment of macrophages, neutrophils, and eosinophils,
401 including CCL2, CCL3, CCL4, CCL5, CXCL1, granulocyte colony stimulating factor(G-
402 CSF), and CCL11(Fig.S4C). These results suggested that the IM injection of STING-A is
403 able to induce similar systemic immune responses as evidenced by the above described
404 Nanostring analysis following the IT injection of STING-A.

405

406 Next, we assessed TILs by dissecting a single target liver metastatic lesion and a mixture of
407 non-target liver metastases, respectively, in this model treated by IM injection of STING-A.
408 The single target liver lesion was pre-selected as it would be selected for the IT treatment, but
409 without any IT treatment to be given(Fig.5E-H;Fig.S4D-E). As shown in Fig.5E-F, IM
410 injection of STING-A alone significantly enhanced the infiltration of CD8⁺ and CD4⁺T cells
411 in both the pre-selected liver metastatic lesion and other liver metastases. The enhancement
412 of the T cell infiltration was not as high in the IM combo group as the IM STING-A alone
413 group. Nevertheless, CD103⁺DCs showed a similar profile in the tumors with the IM

414 injection of STING-A(Fig.5G-H) compared to those shown above with the IT injection of
415 STING-A, suggesting that systemic IM injection of STING-A is able to activate the desired
416 antigen-presenting process in the liver metastases.

417

418 We then performed the NanoString analyses of liver metastases and SubQ tumors and
419 compared them among treatment groups. As shown in the Fig.S5A, the high-throughput
420 analysis of the NanoString results only revealed a smaller number of genes that were
421 differentially expressed among the treatment groups than those differentially expressed
422 among the treatment groups with the IT injection of STING-A(Fig.S1E). The results however
423 supported a non-significant increasing trend in the expression of genes associated with the
424 IFN response pathways in the liver metastases in the IM combo group(Fig.S5B).
425 Nevertheless, in the STING-A IM alone treatment group, the expression of T cell co-
426 stimulatory molecules including *Tnfrsf4*(OX40), *Cd27*, *Tnfrsf9*(CD137), and *Icos* exhibited a
427 trend(Fig.S5C) consistent with the results in the single STING-A IT treatment group(Fig.3E).
428 However, the IM injection of STING-A combined with a-PD-1 did not significantly increase
429 the expression of these co-stimulatory molecules, together with the above T cell infiltration
430 results(Fig.5E-F), suggesting a-PD-1 or a-PD-1 alone may not be an optimal immune
431 checkpoint inhibitor treatment strategy in combination with STING-A. Nevertheless, we
432 observed a strong trend of increased expression of genes related to the cytotoxic activities of
433 effector T cells in the IM combo group including *Gzma*, *Gzmb*, *Gzmk*, and *Prf1*(Fig.S5D).
434 Chemokines especially those involved in effector T cell trafficking including *Cxcl9*, *Cxcl10*,
435 and *Cxcl11* exhibited similar trend as those genes associated with cytotoxic activities of

436 effector T cells(Fig.S5E). In contrast, the increase in the T cell exhaustion and immune
437 checkpoint signals and myeloid cell-recruiting cytokine/chemokine signals that were
438 observed with the IT combo treatment were not observed in the IM combo group(Fig.6A).

439

440 We also examined the immune response in the SubQ tumors in the experiment with the IM
441 injection of STING-A. As shown in Fig.S6A, we observed a significant decrease in the
442 infiltration ratio of CD4⁺PD-1⁺T cells in the CD4⁺T cells in the SubQ tumors from the IM
443 combo treatment group. A similar decreasing trend of CD8⁺PD-1⁺T cells was
444 observed(Fig.S6A), demonstrating the treatment effect of a-PD-1. Next, we used the same
445 NanoString assays to assess the T cell functional status within the SubQ tumors. Interestingly,
446 unlike the results in the liver metastases, the expression of genes associated with the T-cell
447 receptor CD3 complex exhibited a significant increase(*Cd3z*) or a strong increasing trend in
448 the SubQ tumors from the combo treatment group compared to other treatment
449 groups(Fig.S6B). T cell co-stimulatory molecules including *Tnfrsf4*(OX40), *Tnfrsf9*(CD137),
450 *Cd80*, and *Icos* all exhibited an increasing trend(Fig.S6C). In addition, the IM combo
451 treatment group exhibited a significantly increased expression of *Il7r*(Fig.S6D), which has
452 been shown to play a critical role in the development of lymphocytes in the process known as
453 V(D)J recombination[33]. These results suggest that there may be an intertumoral
454 heterogeneity in the immune response to the systemic administration of STING-A. However,
455 we found that the T cell exhaustion and immune checkpoint signals exhibited an enhanced
456 expression in the IM combo treatment group compared to other treatment groups(Fig.6B).
457 These results suggest that an enhanced T cell activation status and cytotoxic function in

458 response to either IT or IM treatment of STING-A is associated with upregulation of T cell
459 exhaustion signals and CTLA-4.

460

461 The overall increasing trend of those chemokines/chemokine receptors involved in the
462 myeloid cell recruitment was less significant in both liver metastases(Fig.S5E) and SubQ
463 tumors(Fig.S6D) than that in the IT combo group, suggesting that systemic delivery of
464 STING-A does not lead to a strong induction of immunosuppressive signals. It is noteworthy
465 to mention that *Ccr5* expression in the SubQ tumors was significantly reduced by the
466 treatment of a-PD-1 compared to the vehicle treatment group, but was significantly enhanced
467 following the IM injection of STING-A in combination with a-PD-1(Fig.S6E). This result
468 appears to be in consistent with the agonistic effect of C-C motif chemokine receptor(CCR) 5
469 expression previously reported in the PDAC models[34].

470

471 In addition to above differences between tumors from IM treated groups and IT treated
472 groups, we noted that the expression of immunosuppressive myeloid cells associated-genes
473 including *ApoE*, *Trem2*, *Cxcr4*, and *Abcg1* was significantly increased in the SubQ tumors
474 from the IM combo treatment group, but not the SubQ tumors from the IT combo treatment
475 group, compared to the respective control group(Fig.6C-F). Such a difference was not
476 observed in the comparison between liver metastases from the IM combo treatment group
477 and the IT combo treatment group(Fig.S6F-I). These results suggest that systemic
478 administration of STING-A could still induce certain immunosuppressive signals that require
479 additional targeted treatments.

480

481 **STING-A in combination with immune checkpoint inhibitors significantly improved the**
482 **survival of genetically engineered KPC mice that develops invasive PDAC**
483 **spontaneously.**

484 Thus, above results suggest that systemic IM administration of STING-A led to less induction
485 of immunosuppressive signals while maintaining a systemic and intratumoral immune
486 response that favors antitumor response. These results also provide the mechanistic basis of
487 the enhanced anti-tumor activity of the IM STING-A observed in the implanted tumor
488 model(Fig.5). We next validated this antitumor activity in the genetically engineered KPC
489 mice that develops invasive PDAC spontaneously in a manner resembling human PDAC
490 pathogenesis. As CTLA-4 remains to be one of immunosuppressive signals presented in the
491 tumors treated by IM STING-A, we included anti-CTLA4 antibody to the immune
492 checkpoint inhibitor regimen. KPC mice were subjected to weekly to twice weekly ultrasonic
493 screening at age of 3 months and enrolled in the experiment once either the length, width, or
494 height of the pancreatic tumor reached 2.00 mm to ensure eligible mice had equivalent tumor
495 burdens. As shown in Fig.7A, eligible KPC mice were randomized into the three treatment
496 groups to receive a total of seven injections. Treatment toxicity and mouse survival were
497 monitored for 3 months following the first treatment. No treatment related toxicity including
498 local toxicity related to IM injection sites was observed. The STING-A monotherapy did not
499 show any antitumor activity in a later experiment(manuscript in preparation). Dual
500 checkpoint inhibitors failed to improve the overall survival of KPC mice when compared
501 with control group; however, the co-administration of IM STING-A with dual checkpoint

502 inhibitors significantly prolonged the survival of the KPC mice(Fig.7B).

503

504 **Systemic administration and intratumoral administration of an NLRP3 agonist in**
505 **combination with anti-PD-1 antibody has a similar efficacy**

506 In this study, we attempted to understand whether systemic administration can be applied to
507 another innate immune agonist. We found that the combination of IT injection of NACHT,
508 LRR, and PYD domains-containing protein 3(NLRP3) agonist and a-PD-1 had similar anti-
509 tumor efficacy as the combination of IT injection of STING-A and a-PD-1 and prolonged the
510 survival of liver metastasis mice implanted with SubQ tumors as compared to a-PD-1 alone,
511 NLRP3 agonist alone, or vehicle control(Fig.S7A). Likely due to the small sample size, the
512 combination of NLRP3 agonist and a-PD-1 failed to induce a significantly stronger tumor
513 growth inhibition on either liver metastatic lesion or the SubQ tumors than single treatment
514 groups(Fig.S7B-C). However, different routes of administration of NLRP3 agonist including
515 IT, tail-vein injection, or intra-subcutaneous tumor injection resulted in no survival difference
516 of the liver metastasis mice(Fig.S7D). Despite a small sample size, NanoString analysis
517 showed that the expression of *Gzmk*(Fig.S7E), an effector T cell cytotoxicity-associated gene,
518 was significantly increased in the NLRP3 agonist-treated tumor, but not in the STING-A-
519 treated tumor(Fig.3D), suggesting that further investigation of systemic administration of
520 other innate immune agonists such as NLRP3 agonist is warranted.

521

522 **Discussion**

523 To our knowledge, this study is one of the few exploring the effects of the STING-A in

524 combination with ICIs for the treatment of PDAC in a pancreatic liver metastasis murine
525 model. We previously used this mouse model to support the application of STING-A as an
526 adjuvant for the vaccine therapy. This mouse model resembles the spontaneous development
527 of liver metastases in human PDACs and has been used in multiple prior studies for the
528 preclinical development of rationale immunotherapy combinations[35, 36, 37]. The TME of
529 the liver metastases in this model is similar to that of orthotopically implanted KPC
530 tumors[38]. Our study demonstrated the antitumor efficacy of the combinational treatment
531 with STING-A and PD-1 blockade in this liver metastasis model as well as novel evidence
532 that demonstrated the survival benefit of the combination of a STING-A and ICIs in the
533 genetically engineered, spontaneously formed KPC tumor model. In our model, STING-A in
534 combination with a-PD-1 enhances effector T cells infiltration and CD103⁺dendritic cells in
535 both target liver metastatic lesions and non-target metastatic lesions as well as distant tumor
536 lesions that are resembled by subQ tumors. More importantly, this study demonstrated that
537 systemic administration of STING-A is equivalent to the intratumoral injection in both
538 antitumor efficacy and immune response. Neither systemic nor intratumoral administration
539 resulted in obvious toxicities; however, systemic administration was more feasible than
540 intratumoral administration and also avoided any intragenic liver injury. Our study also
541 supported the feasibility of administrating an NLRP3 agonist systemically and would
542 supports a new paradigm of the clinical development of innate immune agonists by systemic
543 administration.

544

545 Our results suggest that IM injection of STING-A is able to induce similar systemic immune

546 responses as the IT injection of STING-A. Systemic IM injection of STING-A is also able to
547 activate the desired antigen-presenting process in the liver metastases. Although the effector
548 T cell responses appear to be slightly weaker in the IM injection of STING-A, the increase in
549 the T cell exhaustion and immune checkpoint signals and myeloid cell-recruiting
550 cytokine/chemokine signals that were observed with the IT combo treatment were not
551 observed in the IM combo group.

552

553 Although this study did not demonstrate a significant difference in the treatment response
554 between IM and IT injections of STING-A, we observed that mice who received IM
555 injections of STING-A in combination with a-PD-1 survived longer than 6 weeks whereas
556 none of the mice who received IT injection of STING-A in combination with a-PD-1
557 survived longer than 6 weeks. Therefore, it might be possible to see the survival benefit of the
558 IM injection of STING-A if the sample size would potentially be larger; however, the sample
559 size in each experiment was limited by the technical difficulty of IT injection. As it would be
560 a challenge to breed a large number of the KPC transgenic mice for being randomized to
561 multiple treatment groups, we decided to test the combination of a-PD-1 and anti-CTLA-4
562 antibody instead of two ICIs by itself in the experiments with the KPC transgenic mice. Our
563 results suggest that an enhanced T cell activation status and cytotoxic function in response to
564 either IT or IM treatment of STING-A is associated with CTLA-4 upregulation. Hence, the
565 reason we included anti-CTLA-4 antibody to a-PD-1 in the experiment with the KPC
566 transgenic mice. Future studies could compare the combination of a-PD-1 and anti-CTLA-4
567 antibody with either a-PD-1 alone or anti-CTLA-4 antibody alone.

568

569 This study has a few limitations, first being the smaller sample size. As discussed above, the
570 sample size is limited by the technical complexity of IT injection and breeding KPC
571 transgenic mice. However, the sample size in the current study is, to our knowledge the
572 largest one in testing the intratumor injection of the tumors in internal organs. It is also one of
573 the largest using the KPC transgenic mice as the preclinical model for the efficacy testing.
574 However, the current sample size allowed this study to repeat most of the experiments.
575 Second, this study did not examine the effect of anti-CTLA-4 antibody separately from the
576 treatment groups testing the combination of a-PD-1 and anti-CTLA-4 antibody. Third,
577 chemotherapy, as a current standard of care treatment, was not included in the experiments in
578 this study. We were concerned that chemotherapy would complicate our comparison of the
579 antitumor efficacy and immune response between the IM and IT injection of STING-A. We
580 are studying the combination of chemotherapy and STING-A in an independent study.

581

582 To test the feasibility of systemic administration of other innate immune agonists, we
583 examined the NLRP3 agonist in this study. Although it may have been limited by the small
584 sample size, this study demonstrated that there were no significant differences in anti-tumor
585 efficacy between intratumoral and systemic administration of NLRP3 agonist. This study has
586 not performed an in-depth investigation on the NLRP3 agonist. However, with limited data,
587 this study suggests that further investigation of systemic administration of other innate
588 immune agonists such as NLRP3 agonist is warranted. The results in this study have thus
589 supported the phase 1 trial that evaluated BMS-986301 intratumoral or intravenous injection

590 as monotherapy or in combination with nivolumab(PD-1 blockade) and ipilimumab in
591 patients with advanced solid cancers(NCT03956680).

592

593 **Abbreviations:** PDAC, pancreatic ductal adenocarcinoma; TME, tumor microenvironment;
594 STING, stimulator of interferon genes; PD-1, programmed cell death protein 1; CTLA-4,
595 cytotoxic T-lymphocyte associated protein 4; KPC, Kras/p53/pdx1-Cre; NLRP3, NACHT,
596 LRR, and PYD domains-containing protein 3; TBK1, TANK-binding kinase 1; IRF3,
597 interferon regulatory factor 3; IFN, interferon; CDN, cyclic dinucleotide; ICI, immune
598 checkpoint inhibitors; TGI, tumor growth inhibition; TIL, tumor infiltrating leucocyte; DC,
599 dendritic cell; IL, interleukin; CXCL, C-X-C motif chemokine ligand; CXCR, C-X-C motif
600 chemokine receptor; CCL, C-C motif chemokine ligand; G-CSF, granulocyte colony
601 stimulating factor ; CCR, C-C motif chemokine receptor.

602

603 **Declarations**

604 **Ethics approval and consent to participate**

605 All studies and maintenance of mice were conducted in accordance with the approval of the
606 Institutional Animal Care and Use Committee (IACUC) guidelines of Johns Hopkins School
607 of Medicine (Animal Protocol: MO22M59).

608

609 **Consent for publication**

610 Not applicable.

611

612 **Availability of data and materials**

613 All data needed to evaluate the conclusions in the paper are present in the paper and the
614 Supplementary Materials. Any further information required to support our data will be
615 supplied upon request.

616

617 **Competing interests**

618 L.Z. receives grant support from Bristol-Meyer Squibb, Merck, AstraZeneca, iTeos, Amgen,
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623

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632

633 **Authors' contributions**

634 Keyu Li and Junke Wang contributed equally to this project. Concept was conceived by L.Z.
635 The strategy and the overall study were designed by K.L. and L.Z. Experiments were
636 conducted by K.L., Junke W., N.N., N.J., A.B., Jiangxin W., M.H., J.Y., D.T., B.E., C.Y., and
637 M. W. Data were collected by K.L. and Junke W. Formal analysis was conducted by K.L.,
638 Junke W, and Y.X. Genetically modified mice were generated by M.H, V.F., and M.L.
639 Original draft manuscript was written by K.L. Manuscript was reviewed and revised was by
640 A.O., B.E., M.W., and L.Z. Supervision was made by X.L., Y.W., and L.Z. The project
641 administrator is L.Z.

642

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647

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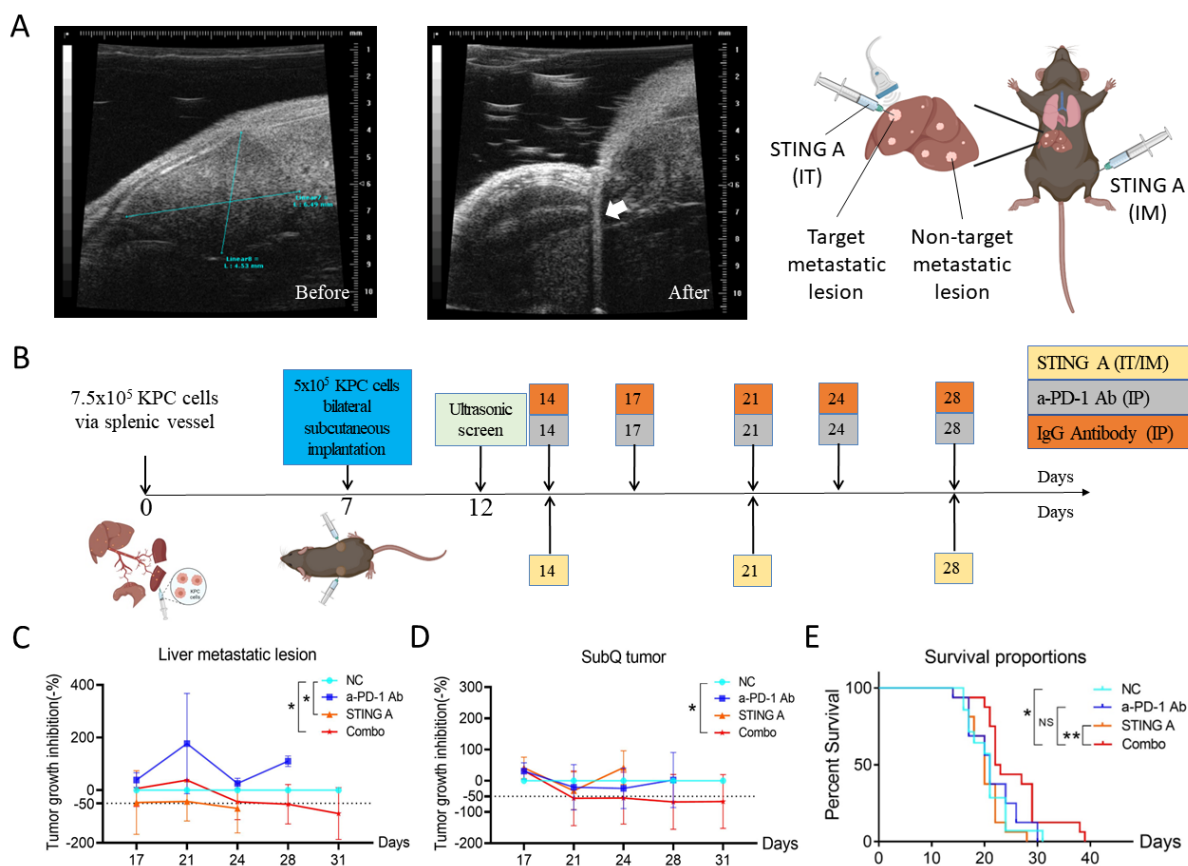
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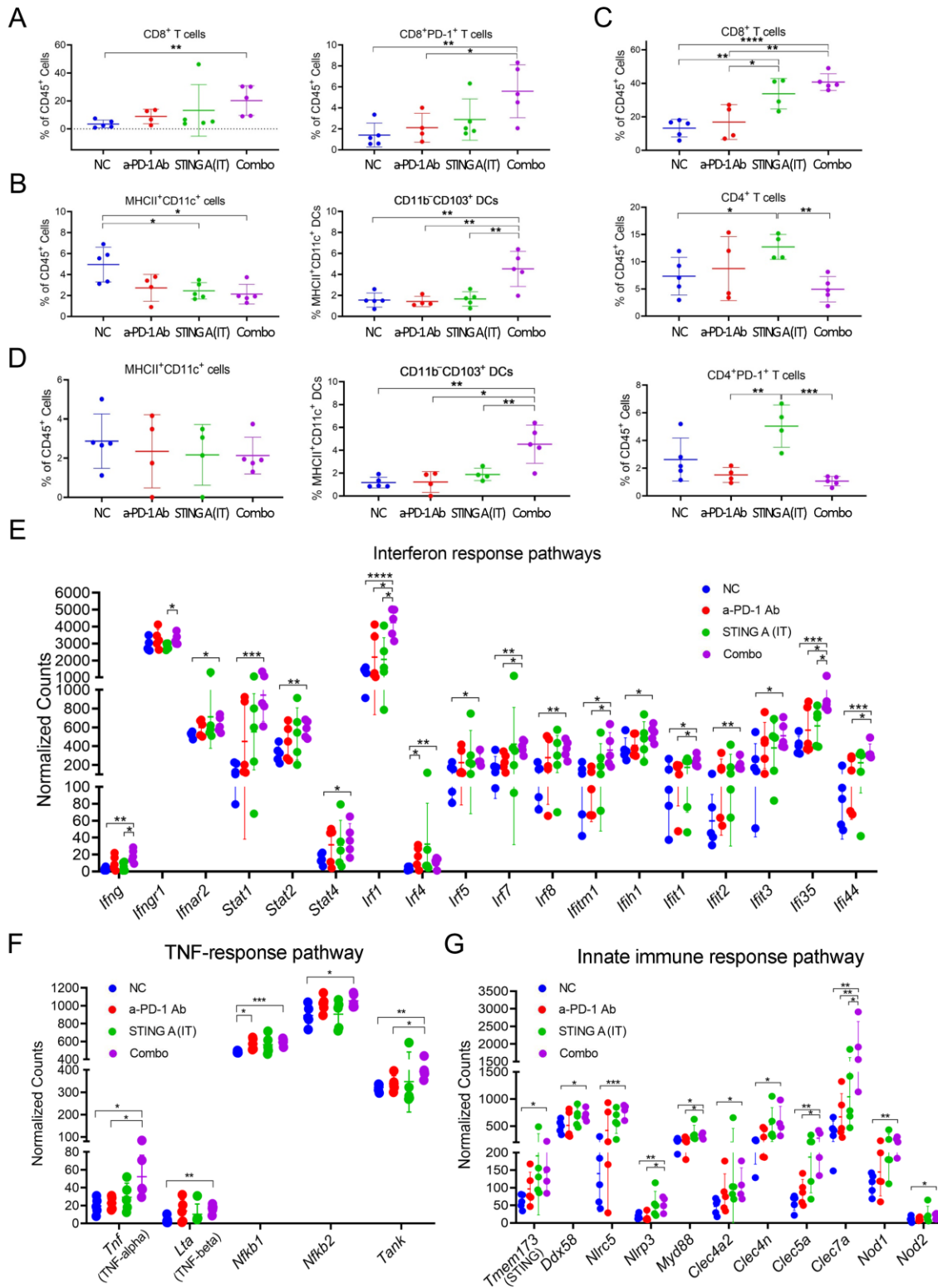
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742 **Figures and Figure legends**



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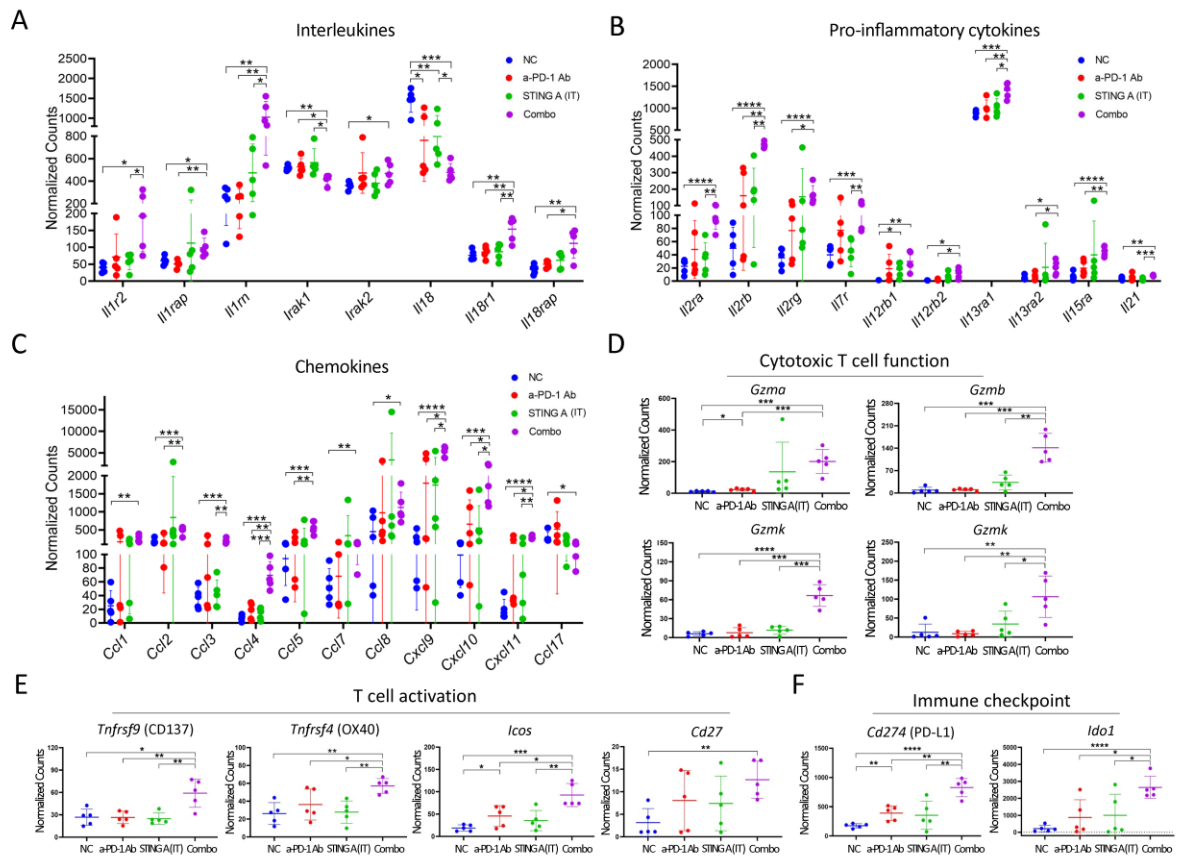
745 Figure 1. Intratumoral injection of the STING agonist in combination with anti-PD-1
746 antibody enhance local anti-tumor efficacy with abscopal effects. (A) Ultrasonographic
747 measurement for the target liver metastatic lesion before and after intratumoral injection of
748 the STING agonist. Arrow indicates the injection needle path. (B) Treatment schema. Mice
749 that met inclusion criteria were randomly assigned to each group on day 12 and monitored
750 twice a week until survival endpoints were met. (C) Tumor Growth Inhibition (TGI) of the
751 injected liver metastatic lesion during the treatment period. (D) Tumor Growth Inhibition
752 of the remote subcutaneous (SubQ) tumor during the treatment period. Dashed line at -50%
753 indicates statistically significant TGI. (E) Kaplan-Meier survival curves compare different
754 treatment groups. NC, vehicle/isotype antibody control; STING A, STING agonist; a-PD-1
755 Ab, anti-PD-1 antibody; Combo, STING A+a-PD-1 Ab; IT, intratumoral; IM, intramuscular;
756 IP, intra-peritoneal. Data shown as mean \pm SD; comparison by unpaired t test in C and D, and
757 by Log-rank test in E; * $p < 0.05$; ** $p < 0.01$; NS, not significant.
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761 Figure 2. STING agonist in combination with anti-PD-1 antibody enhances effector T cells
762 and CD103⁺dendritic cell (DC) infiltration and activates anti-tumor immunity via innate
763 immune response signaling pathways. (A) Percentages of the CD8⁺ and CD8⁺PD-1⁺T cells
764 among CD45⁺leucocytes in the target liver metastatic lesion. (B) Percentages of the
765 MHCII⁺CD11c⁺DC among CD45⁺leucocytes and the percent of CD11b⁻CD103⁺DC subtype
766 in the target liver metastatic lesion. (C) Percentages of the CD8⁺, CD4⁺, and CD4⁺PD-1⁺T
767 cells among CD45⁺leucocytes in the non-target liver metastases. (D) Percentages of
768 MHCII⁺CD11c⁺DC among CD45⁺leucocytes and the percent of CD11b⁻CD103⁺DC subtype
769 in the non-target liver metastases. (E) Expression of genes in the IFN-response pathways in
770 the target liver metastatic lesions from different treatment groups. (F) Expression of genes in
771 the TNF-response pathways in the target liver metastatic lesions from different treatment
772 groups. (G) Expression of genes in the innate immune response pathways in the target liver
773 metastatic lesions from different treatment groups. Data are shown as the mean \pm SD;
774 comparison by unpaired t test; *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.
775 Remaining comparisons are non-significant.
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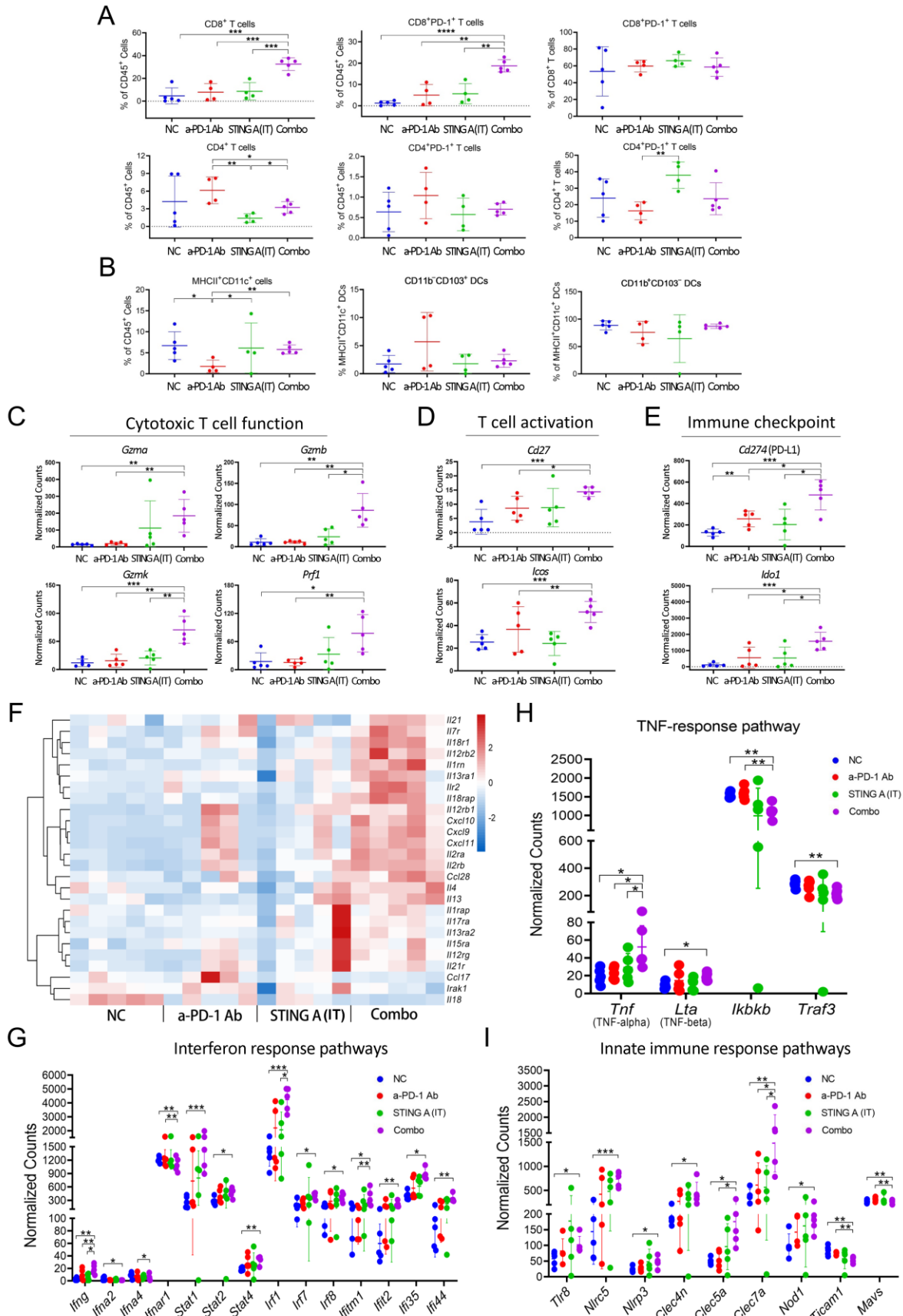


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779 Figure 3. Differentially expressed genes in the target liver metastatic lesions between
 780 treatment groups. Expression of genes, as indicated, in the gene families of cytokines (A-B),
 781 chemokines (C), the activation of effector T cells (D), the T cell co-stimulatory factors (E),
 782 and the immune checkpoint activators (F). Data shown as mean \pm SD; comparison by
 783 unpaired t test; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. Remaining
 784 comparisons are non-significant.

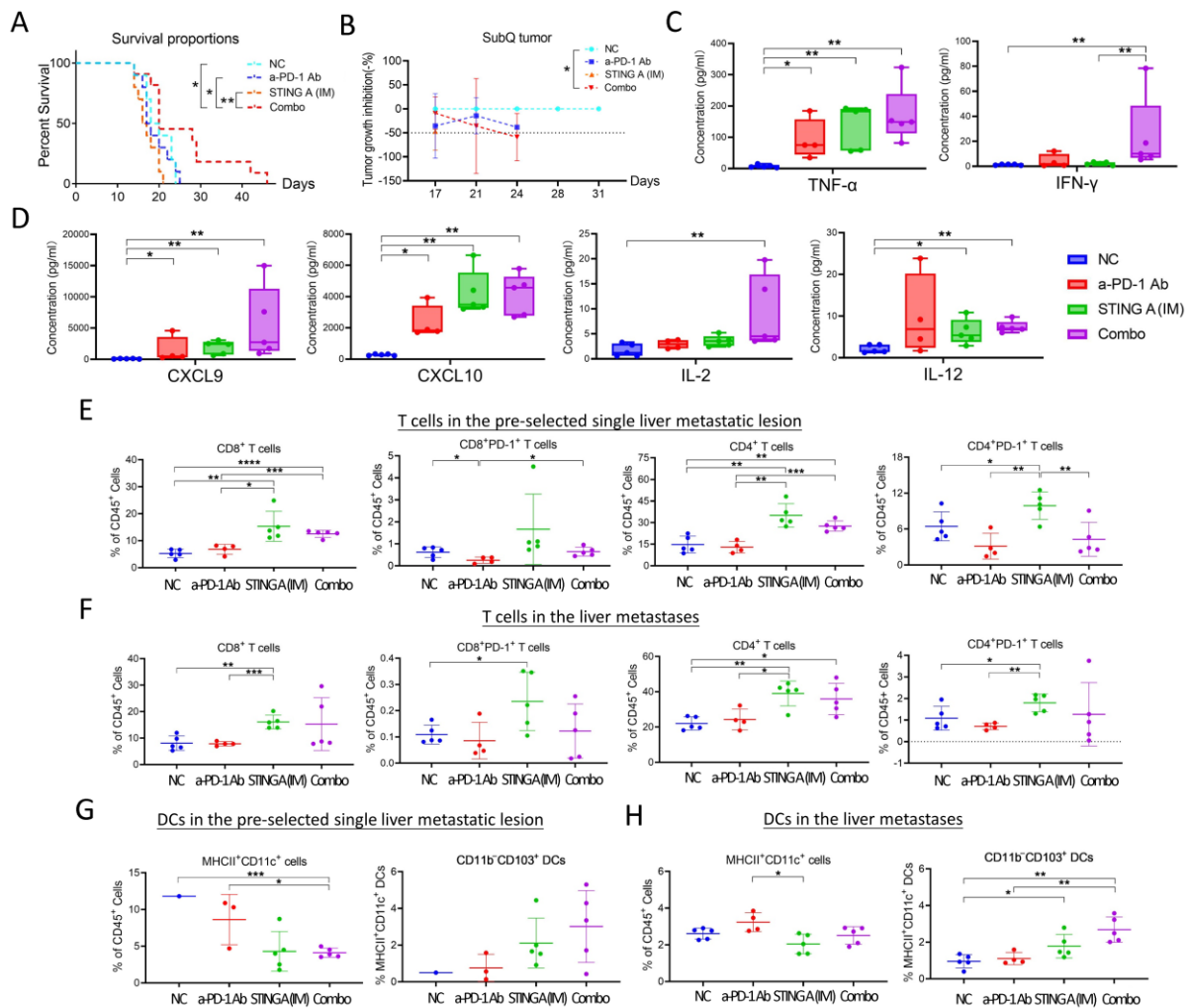
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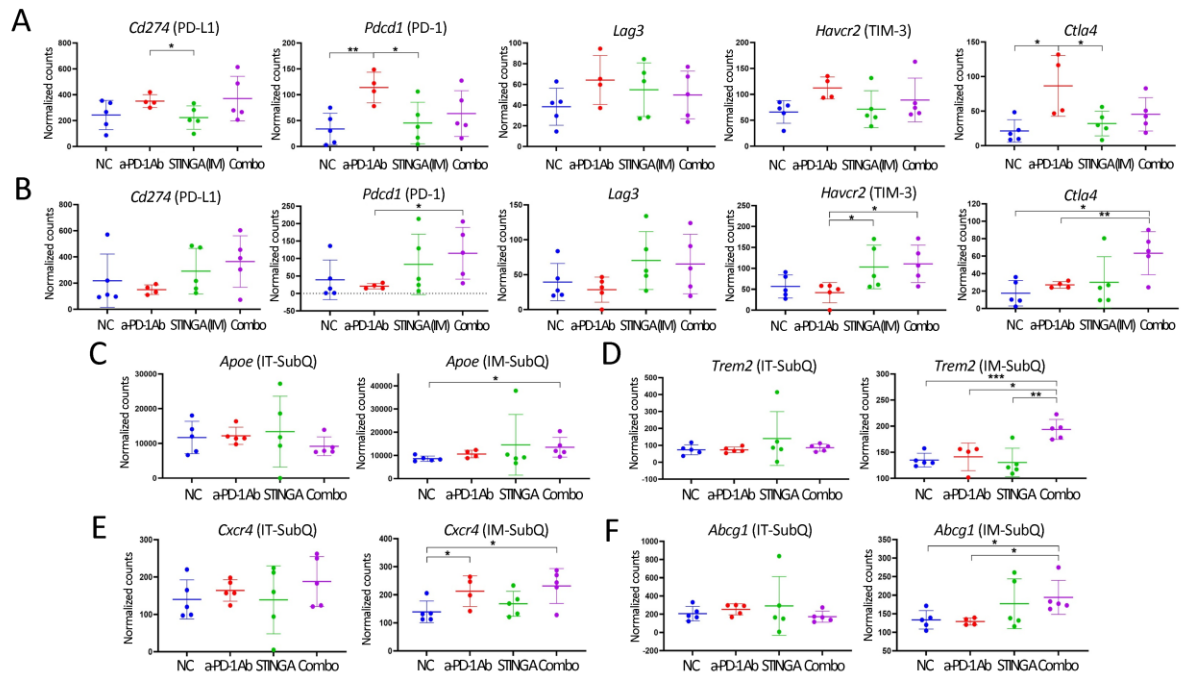
788 Figure 4. STING agonist in combination with anti-PD-1 antibody enhances the infiltration
789 and activation of effector T cells in the distant subcutaneous tumors. (A) Percentages of
790 immune cells in the remote SubQ tumors, such as the CD8⁺, CD8⁺PD-1⁺, CD4⁺, and
791 CD4⁺PD-1⁺T cells among CD45⁺leucocytes, the CD8⁺PD-1⁺ among CD8⁺T cells, and the
792 CD4⁺PD-1⁺T cells among CD4⁺T cells, respectively. (B) Percentages of the
793 MHCII⁺CD11c⁺DC, CD11b⁻CD103⁺ and CD11b⁺CD103⁻ DC subtypes DC among
794 CD45⁺leucocytes, respectively, in the distant SubQ tumors. Differentially expressed genes in
795 the distant SubQ tumors from different treatment groups, including those in the gene families
796 of cytotoxic T cell function (C), T cell activation (D), and the immune checkpoint activators
797 (E). (F) Heatmap of the interleukin family genes and chemokine genes that were significantly
798 increased in the Combo group comparing to the vehicle control treatment group. (G)
799 Expression of genes in the IFN-response pathways in SubQ tumors from different treatment
800 groups. (H) Expression of genes in the TNF-response pathways in SubQ tumors from
801 different treatment groups. (I) Expression of genes in the innate immune response pathways
802 in SubQ tumors from different treatment groups. Data shown as mean ± SD; comparison by
803 unpaired t test; *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001. Remaining
804 comparisons are non-significant.
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808 Figure 5. Intramuscular injection of STING agonist in combination with anti-PD-1 antibody
 809 prolonged the survival of liver metastasis mice and induces both systemic and intratumoral
 810 immune response. (A) Kaplan-Meier's survival curves compare the survival in different
 811 intratumoral (IM) treatment groups. (B) TGI on distant SubQ tumors. Dashed line at -50%
 812 indicates statistically significant inhibition. (C) Comparison of serum concentrations of TNF-
 813 α and IFN- γ collected 6 hours after the first IM injection between treatment groups. (D)
 814 Comparison of serum concentrations of CXCL9, CXCL10, IL-2, and IL-12 collected 6 hours
 815 after the first IM injection between treatment groups. Percentages of the CD8⁺, CD8⁺PD-1⁺,
 816 CD4⁺, and CD4⁺PD-1⁺T cells among CD45⁺leucocytes in the pre-selected, target single liver
 817 metastatic lesions (E) and non-target liver metastases (F). Percentages of the
 818 MHCII⁺CD11c⁺DC and CD11b⁺CD103⁺subtype among CD45⁺leucocytes, respectively, in
 819 the pre-selected single liver metastatic lesion (G) and non-target liver metastases (H). Data
 820 shown as mean \pm SD; comparison by Log-rank test for A and by unpaired t test for others; *p
 821 < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001. Remaining comparisons are non-
 822 significant.

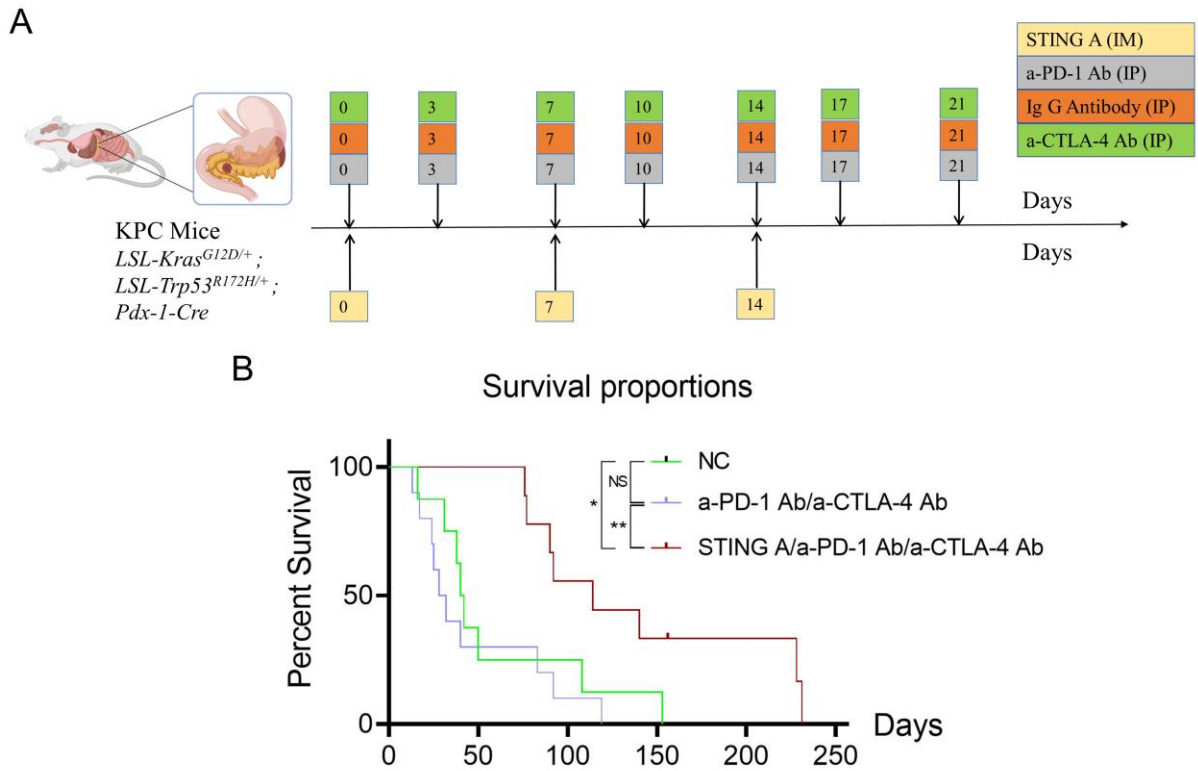


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825 Figure 6. Comparison of expressions of differentially expressed genes in tumors from
 826 systemically treated mice. Expression of genes of the checkpoint gene family in pre-selected,
 827 target single liver metastatic lesions (A) and distant subcutaneous tumors (B) from the
 828 intramuscularly treated mice. Expression of *Apoe* (C), *Trem2* (D), *Cxcr4* (E), and *Abcg1* (F)
 829 in the distant subcutaneous tumors from both intratumoral and intramuscularly treated mice.
 830 Data shown as mean \pm SD; comparison by unpaired t test; * $p < 0.05$; ** $p < 0.01$; *** $p <$
 831 0.001 . Remaining comparisons are non-significant.

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835 Figure 7. STING agonist in combination with immune checkpoint inhibitors significantly
836 improved the survival of genetically engineered KPC mice. (A) Treat schema. (B) Kaplan-
837 Meier survival curves compare overall survival between different treatment groups. NC,
838 vehicle/isotype antibody control; STING A, STING agonist; a-PD-1 Ab, anti-PD-1 antibody;
839 a-CTLA-4 Ab, anti-CTLA-4 antibody; IM, intramuscular; IP, intra-peritoneal. Data shown as
840 mean \pm SD; comparison by Log-rank test; * $p < 0.05$; ** $p < 0.01$; NS, not significant.