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1 Effects of upregulated indoleamine 2, 3-dioxygenase 1 by interferon  $\gamma$  gene transfer on interferon  
2  $\gamma$ -mediated antitumor activity

3  
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13

14 **Running title:** Effects of IDO1 on antitumor activity of IFN- $\gamma$

15

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19

20

21

22 **Abstract**

23

24 Interferon  $\gamma$  (IFN- $\gamma$ ), an anticancer agent, is a strong inducer of indoleamine 2,3-dioxygenase 1 (IDO1),  
25 which is a tryptophan-metabolizing enzyme involved in the induction of tumor immune tolerance. In  
26 this study, we investigated the IDO1 expression in organs after IFN- $\gamma$  gene transfer to mice. IFN- $\gamma$  gene  
27 transfer greatly increased the mRNA expression of IDO1 in many tissues with the highest in the liver.  
28 This upregulation was associated with reduced L-tryptophan levels and increased L-kynurenine levels in  
29 serum, indicating that IFN- $\gamma$  gene transfer increased the IDO activity. Then, Lewis lung carcinoma  
30 (LLC) tumor-bearing wild-type and IDO1 knockout (IDO1 KO) mice were used to investigate the  
31 effects of IDO1 on the antitumor activity of IFN- $\gamma$ . IFN- $\gamma$  gene transfer significantly retarded the tumor  
32 growth in both strains without any significant difference in tumor size between the two groups. By  
33 contrast, the IDO1 activity was increased only in the wild-type mice by IFN- $\gamma$  gene transfer, suggesting  
34 that cells other than LLC cells, such as tumor stromal cells, are the major contributors of IDO1  
35 expression in LLC tumor. Taken together, these results imply that IFN- $\gamma$  gene transfer-mediated IDO1  
36 upregulation in cells other than LLC cells has hardly any effect on the antitumor activity of IFN- $\gamma$ .

37

38 **Key Words:** antitumor effect; indoleamine 2, 3-dioxygenase 1; interferon- $\gamma$ ; transgene expression

39 **Introduction**

40

41 Interferon- $\gamma$  (IFN- $\gamma$ ) is a cytokine with antiproliferative and immunomodulatory activities. Therefore, it  
42 is expected to have beneficial effects in the treatment of a variety of diseases, including cancer <sup>1-3</sup>.

43 Previous studies have shown that gene delivery of IFN- $\gamma$  is an effective treatment for cancer and,  
44 furthermore, we have demonstrated success in prolonging the transgene expression of murine IFN- $\gamma$   
45 from plasmid vectors by developing ones with fewer CpG motifs <sup>4,5</sup>. A hydrodynamic injection of the  
46 newly constructed IFN- $\gamma$ -expressing plasmids resulted in prolonged therapeutic serum concentrations of  
47 IFN- $\gamma$  for more than 80 days, which significantly inhibited metastatic tumor growth and atopic  
48 dermatitis in mouse models <sup>4-7</sup>.

49

50 IFN- $\gamma$  exerts its biological activities through the Janus kinase/signal transducer and activator of  
51 transcription pathway, which modulates the expression of a variety of genes, including indoleamine 2,  
52 3-dioxygenase 1 (IDO1) <sup>8,9</sup>. It has been suggested that increased IDO activity is responsible for the  
53 protumor functions of IFN- $\gamma$  <sup>10-12</sup>. However, little information is available about how IDO1 expression  
54 and/or activity is affected after IFN- $\gamma$  gene transfer.

55

56 IDO1 is a tryptophan-metabolizing enzyme acting along the kynurenine pathway, and it is  
57 expressed in a variety of cells <sup>13,14</sup>. The induced expression of IDO1 could lead to tryptophan depletion  
58 and formation of some toxic metabolites of tryptophan, such as kynurenine, and 3-hydroxykynurenine,  
59 which can result in the impairment of T cell functions and down-regulation of immune responses <sup>15-18</sup>.

60 Various types of IDO1 inhibitors have been developed and reported to exhibit antitumor activity <sup>13,19-22</sup>.

61 However, the role of IDO1 in tumor growth is controversial so far <sup>23</sup>. Therefore, further investigation is  
62 needed to clarify the role of IDO1 in tumor development and its effects on the antitumor activity of

63 IFN- $\gamma$ .

64

65 In the present study, we first examined whether the expression of IDO1 is induced by IFN- $\gamma$   
66 gene transfer in many organs of naïve mice and in tumor tissues of solid tumor-bearing mice. The serum  
67 concentrations of L-tryptophan and L-kynurenine were used as an indicator of the IDO1 activity. Finally,  
68 IDO1-knockout mice were used to examine the involvement of IDO1 expression and activity in the  
69 antitumor effect of IFN- $\gamma$  gene transfer.

70

## 71 **Results**

72

### 73 **Induction of IDO1 expression in mouse organs after IFN- $\gamma$ gene transfer**

74

75 Figure 1a shows the mRNA levels of IDO1 in the liver, spleen, lung and kidney of mice after  
76 hydrodynamic injection of pCpG-Mu $\gamma$ , a murine IFN- $\gamma$ -expressing plasmid, or a human  
77 IFN- $\gamma$ -expressing plasmid, pCpG-Hu $\gamma$ . The plasmid doses for hydrodynamic injection were determined  
78 based on the previous study, and pCpG-Hu $\gamma$  was used as a control plasmid because human IFN- $\gamma$   
79 exhibited no significant effects in mice <sup>6</sup>. There were no significant changes in the IDO1 mRNA levels  
80 in mice receiving pCpG-Hu $\gamma$ , the control plasmid (data not shown). The IDO1 mRNA level in the liver  
81 of the pCpG-Mu $\gamma$ -injected mice was about 10,000-fold greater than that in the pCpG-Hu $\gamma$ -injected mice.  
82 The levels of IDO1 in the other organs examined were also higher in the pCpG-Mu $\gamma$  injected mice and  
83 these differences were statistically significant except in the spleen, although the magnitude of the  
84 increase in these organs (533- and 117-fold for lung and kidney, respectively) was much less than that in  
85 the liver. The mRNA expression of IDO2 and tryptophan 2,3-dioxygenase (TDO), two other  
86 tryptophan-metabolizing enzymes, was also examined. Unlike IDO1, the mRNA levels of IDO2 (Figure  
87 1b) in these organs as well as that of TDO (data not shown) in the liver were not significantly changed  
88 by IFN- $\gamma$  gene transfer.

89

### 90 **Time-courses of IDO1 expression in the liver and tryptophan catabolism after IFN- $\gamma$ gene transfer**

91  
92 To experimentally evaluate the relationship between the IDO1 expression and IFN- $\gamma$  concentration, the  
93 time-courses of mRNA expression of IDO1 in the liver and the serum concentration of IFN- $\gamma$  were  
94 measured after the injection of IFN- $\gamma$  expressing plasmids. Figure 2a shows the time-courses of the  
95 IFN- $\gamma$  serum concentration for the first 2 weeks. After hydrodynamic injection of pCMV-Mu $\gamma$ , the IFN- $\gamma$   
96 concentration reached a peak level of about 520 ng/ml at 6 h, then declined very quickly. The IFN- $\gamma$   
97 concentration in the serum at 1 day was less than 1 % of the peak level, and it had fallen below the  
98 detection limit (17 pg/ml) 3 days after injection. Figure 2b shows the mRNA expression of IDO1 in the  
99 liver. The IDO1 expression was markedly increased at 6 h, peaked at 24 h, and then declined to the  
100 baseline level ( $0.4-8.1 \times 10^{-5}/\text{GAPDH}$ ) by day 7.

101  
102 After hydrodynamic injection of pCpG-Mu $\gamma$ , the IFN- $\gamma$  concentration in the serum was  
103 maintained at above 1 ng/ml for at least 14 days (Figure 2a). The mRNA expression of IDO1 reached a  
104 peak at 6 h, then declined to the baseline level at 14 days after gene transfer (Figure 2b). On the contrary,  
105 the mRNA expression of IDO1 in the liver was not significantly increased by an intramuscular injection  
106 of pCpG-Mu $\gamma$  (Figure 2b). These results indicate that quite high IFN- $\gamma$  concentrations, for example  
107 above 4 ng/ml, are required for IDO1 induction in the liver.

108  
109 Figure 2c and 2d show the concentrations of L-tryptophan and L-kynurenine in the serum after  
110 IFN- $\gamma$  gene transfer. The L-tryptophan concentration was significantly reduced by hydrodynamic  
111 injection of pCMV-Mu $\gamma$  or pCpG-Mu $\gamma$  to about 40  $\mu\text{M}$  on day 1, and it then returned to the baseline  
112 level of about 100  $\mu\text{M}$  at day 7. In response to these changes, there was a significant increase in the  
113 serum L-kynurenine concentration of mice receiving pCMV-Mu $\gamma$  or pCpG-Mu $\gamma$ ; the concentration  
114 reached a peak at day 3 and this lasted for up to 7 days in the pCpG-Mu $\gamma$ -treated mice (Figure 2d). Then,  
115 pCpG-Mu $\gamma$  was injected into skeletal muscle to examine whether the administration route as well as the  
116 site of transgene expression is not important for the upregulation of IDO1. The plasmid dose was

117 determined based on the preliminary experiments to achieve a comparable level of serum IFN- $\gamma$   
118 concentrations to that obtained by hydrodynamic injection at 3 days after gene transfer (Figure 2a). In  
119 the case of intramuscular injection of pCpG-Mu $\gamma$ , a slight but not statistically significant increase in  
120 L-kynurenine concentration was observed on day 3. The L-kynurenine to L-tryptophan ratio calculated  
121 as an index of tryptophan degradation through the kynurenine pathway also showed a similar trend  
122 (Figure 2e). Taken together, these results confirmed that the IDO1 activity was upregulated and the  
123 L-tryptophan concentration in the serum was greatly reduced by hydrodynamic injection of  
124 IFN- $\gamma$ -expressing plasmids.

125

### 126 **Antitumor effect of IFN- $\gamma$ gene transfer in LLC tumor-bearing mice**

127

128 To confirm whether IDO1 expression inhibits IFN- $\gamma$ -induced antitumor activity, we examined the  
129 antitumor effect of IFN- $\gamma$  gene transfer in LLC tumor-bearing wild-type and IDO1 knockout (IDO1 KO)  
130 mice. Figure 3a and 3b shows the tumor growth curves of the wild-type and IDO1 KO mice,  
131 respectively, after gene transfer. There were no statistically significant differences in tumor size among  
132 the saline treated wild-type mice, the wild-type and IDO1 KO mice treated with pCpG-Hu $\gamma$ . Irrespective  
133 of the mouse strains, the hydrodynamic injection of pCpG-Mu $\gamma$  significantly retarded the tumor growth.  
134 There was no statistically significant difference in tumor size between the wild-type and IDO1 KO mice  
135 treated with pCpG-Mu $\gamma$ . In contrast, the wild-type mice treated with pCpG-Mu $\gamma$  survived longer than  
136 the IDO1 KO mice treated with the same plasmid, although the difference was not statistically  
137 significant (Figure 3c). The survival of IDO1 KO mice was hardly increased by hydrodynamic injection  
138 of pCpG-Mu $\gamma$ .

139

140 Figure 4a shows the time courses of IFN- $\gamma$  concentration in the serum after hydrodynamic  
141 injection of pCpG-Mu $\gamma$  to LLC tumor-bearing wild-type (C57BL/6) and IDO1 KO mice. Similar  
142 profiles were observed in both groups, and they were comparable to that of ICR mice (Figure 2a). To

143 assess the IDO1 activity in these tumor-bearing mice, the serum concentrations of L-tryptophan and  
144 L-kynurenine were measured. As expected, there were no changes in the concentrations of L-tryptophan  
145 and L-kynurenine in the serum of IDO1 KO mice after the injection of pCpG-Muγ, whereas a reduced  
146 L-tryptophan concentration and elevated L-kynurenine concentration were observed in the serum of  
147 wild-type mice receiving pCpG-Muγ (Figure 4b,c). The baseline level of L-kynurenine in the serum of  
148 IDO1 KO mice was significantly lower than that of the wild-type mice (Figure 4c), although the  
149 baseline levels of L-tryptophan in both strains were comparable, demonstrating a defect in L-kynurenine  
150 production in the IDO1 KO mice. The L-kynurenine to L-tryptophan ratio hardly changed in the IDO1  
151 KO mice receiving pCpG-Muγ (Figure 4d).

152

### 153 **mRNA expression of IDO1 and the concentrations of L-tryptophan and L-kynurenine in LLC** 154 **tumors**

155

156 In vitro results showed that incubation of LLC cells with 20 ng/ml murine IFN-γ for 24 h induced the  
157 expression of IDO1, although no IDO1 mRNA was detected in LLC cells under normal culture  
158 conditions (data not shown). To confirm whether IDO1 is expressed in the LLC tumors of IDO1 KO  
159 mice and is affected by IFN-γ gene transfer, the mRNA expression of IDO1 in LLC tumors was  
160 evaluated (Figure 5a). The mRNA expression of IDO1 in LLC tumors was detectable in both wild-type  
161 and IDO1 KO mice. The mRNA levels were comparable and almost constant in the control  
162 plasmid-treated group of both strains. These results suggest that LLC cells express IDO1 at low levels  
163 after inoculation into mice, and the expression of IDO1 mRNA in other cells than LLC cells is also low.  
164 The hydrodynamic injection of pCpG-Muγ significantly induced the expression of IDO1 in the tumors  
165 of the wild-type mice by up to 106-, 59-, and 74-fold at day 1, 3 and 7, respectively. On the other hand,  
166 no statistically significant changes were observed in the mRNA level of IDO1 in the tumor of the IDO1  
167 KO mice.

168



169           To evaluate the activity of IDO1 in the LLC tumors, the concentrations of L-tryptophan and  
170 L-kynurenine in the tumors were also measured. The concentrations of L-tryptophan in the LLC tumors  
171 at day 7 were comparable among all the groups (Figure 5b), probably because L-tryptophan is obtained  
172 from diet. In response to the upregulation of IDO1 mRNA expression in LLC tumors by IFN- $\gamma$ , the  
173 concentration of L-kynurenine in the LLC tumors of wild-type mice receiving pCpG-Mu $\gamma$  was  
174 significantly increased for at least the first 14 days whereas, in the IDO1 KO mice, no increase was  
175 observed in the concentration of L-kynurenine in the LLC tumors (Figure 5c). The L-kynurenine to  
176 L-tryptophan ratios in the tumors of these mice also revealed a similar trend (Figure 5d). These results  
177 indicate the comparable activity of IDO1 in the tumors of IDO1 KO mice and wild-type mice without  
178 IFN- $\gamma$  gene transfer.

179

180 **Discussion**

181

182 IFN- $\gamma$  induces IDO1 expression in various mouse and human cells<sup>24-27</sup>. In this study, we have shown  
183 that IFN- $\gamma$  gene transfer dramatically increases the IDO1 expression in a variety of mouse organs  
184 (Figure 1). Although the basal expression of IDO1 in the liver is very low<sup>14</sup>, the magnitude of the  
185 change in IDO1 expression in the liver after the hydrodynamic injection of murine IFN- $\gamma$  expressing  
186 plasmid, pCpG-Mu $\gamma$ , was much higher than that in other tissues. This may be due to the fact that the  
187 transgene is almost specifically expressed in the liver after hydrodynamic injection<sup>28</sup>. In contrast to  
188 IDO1, the TDO expression was not affected by IFN- $\gamma$  gene transfer, although it is mainly expressed in  
189 the liver. Our results are in line with a previous study demonstrating that the increased kynurenine  
190 production after an injection of LPS was caused by the induction of IDOs, but not by TDO<sup>29</sup>. The study  
191 also suggested that the induction of IDOs in tissues initiated tryptophan metabolism locally, followed by  
192 the production of kynurenine which may then be carried into the blood stream. Although the type of IDO  
193 was not identified in this study, our results showed that IDO1, but not IDO2, was upregulated in tissues  
194 by IFN- $\gamma$  gene transfer, indicating that IDO1 is the only enzyme mediating tryptophan metabolism in  
195 response to IFN- $\gamma$  gene transfer. This assumption was supported by the results of IDO1 KO mice in this  
196 study, because a dramatically increased IDO1 activity after IFN- $\gamma$  gene transfer was observed in LLC  
197 tumor-bearing wild-type mice, whereas such changes were almost undetectable in the IDO1 KO mice  
198 (Figure 4). The large differences in the L-tryptophan and L-kynurenine levels between the  
199 pCpG-Mu $\gamma$ -treated wild-type and IDO1 KO mice also support the use of these levels as indicators of  
200 IDO1 activity, despite the fact that the L-tryptophan and L-kynurenine levels can be affected by other  
201 factors than IDO1, including dietary L-tryptophan.

202

203 Irrespective of the IDO1 activity in serum, the growth rate of LLC tumors was significantly  
204 delayed by IFN- $\gamma$  gene transfer in both wild-type and IDO1 KO mice with no statistically differences in

205 the tumor size between the groups (Figure 3a, b). These results imply that IFN- $\gamma$  gene transfer-mediated  
206 IDO1 upregulation does not greatly affect the anti-tumor activity of IFN- $\gamma$ . This observation is not in  
207 good agreement with a previous report by Gasparri et al., who showed that multiple injections of a  
208 recombinant murine IFN- $\gamma$  fused with a tumor vascular homing peptide significantly induced IDO  
209 activity in tumor tissues, which consequently resulted in a lack of effective of IFN- $\gamma$  on tumor outgrowth  
210 <sup>10</sup>. This discrepancy could be explained by the different profiles of IFN- $\gamma$  and IDO1 activities; we found  
211 that high levels of IDO1 expression and activity only lasted for up to 14 days, whereas very high levels  
212 of IFN- $\gamma$  were maintained for much longer. These results also suggest a negative feedback mechanism  
213 on IFN- $\gamma$ -induced IDO1 expression <sup>30</sup>. By contrast, we also observed that the IDO1 mRNA expression in  
214 the pCMV-Mu $\gamma$ -treated mice remained high even after the serum concentration of IFN- $\gamma$  decreased to  
215 low levels (Figure 2a, b). Similar profiles of IDO1 expression were reported in a previous manuscript, in  
216 which the IDO1 expression was examined in mice receiving repeated injections of IFN- $\gamma$  <sup>31</sup>.

217  
218 An earlier study by Muller et al. demonstrated that IDO1 KO mice were resistant to skin tumor  
219 formation compared to their counterpart <sup>32</sup>. Smith et al. also reported that IDO1 KO mice showed  
220 reduced lung tumor burden and improved survival in models of primary lung carcinoma and breast  
221 carcinoma-derived pulmonary metastasis <sup>33</sup>. Initially, we supposed that the loss of intact IDO1 could  
222 delay tumor progression, even without IFN- $\gamma$  treatment. However, unexpectedly, we found no  
223 statistically significant differences in tumor growth rate and survival between the control plasmid  
224 treated-wild-type and IDO1 KO mice (Figure 3). Interestingly, it has been recently demonstrated that  
225 both IDO-competent and -deficient mice bearing IDO-deficient brain tumors exhibited longer survival  
226 compared with those bearing IDO-competent brain tumors <sup>34</sup>. Similarly, Muller et al demonstrated that  
227 there were no differences in the tumor outgrowth and response to 1-MT, between wild-type and IDO1  
228 KO mice engrafted with IDO-overexpressed primary keratinocytes <sup>35</sup>. Moreover, Blache et al showed  
229 that silencing of tumor-derived IDO1 using an IDO1-specific small hairpin RNA plasmid effectively

230 attenuated tumor growth in solid-tumor-bearing wild-type and IDO1 KO mice <sup>36</sup>. These reports strongly  
231 suggest that tumor-derived IDO1 activity is sufficient to mediate tumor immune tolerance and promote  
232 tumor progression, regardless of IDO1 expression outside the tumor. In addition, IDO1 mRNA  
233 expression could be detected in solid tumors of IDO1 KO mice bearing B16F10 melanoma <sup>36</sup>. We also  
234 detected low levels of IDO1 expression in the LLC tumor tissue of IDO1 KO mice (Figure 5a), although  
235 IDO1 expression could not be detected in cultured LLC cells under conditions without IFN- $\gamma$ . These  
236 results suggest that the IDO1 in the engrafted LLC tumor cells is upregulated by their microenvironment,  
237 including tumor-infiltrating immune cells <sup>24,37</sup>, because no other cells than LLC cells can express IDO1  
238 in the LLC-bearing IDO1 KO mice. Notably, the concentrations of L-kynurenine in the tumors of the  
239 control plasmid treated wild-type and IDO1 KO mice were comparable (Figure 5c), even though there  
240 was defective production of L-kynurenine in the serum of IDO1 KO mice.

241  
242 In the IDO1 competent mice, IDO1 could be expressed, not only in LLC cells, but also in  
243 tumor-infiltrating immune cells, such as macrophages and dendritic cells <sup>38,39</sup>. The large difference in the  
244 IDO1 mRNA expression in LLC tumor tissues after hydrodynamic injection of pCpG-Mur $\gamma$  between the  
245 wild-type and IDO1 KO mice clearly suggests that the major types of cells expressing IDO1 are the  
246 tumor-infiltrating immune cells. These findings support the role played by tumor infiltrating immune  
247 cells in modulating the IDO activity in tumor tissues, which could contribute to the T cell proliferation  
248 inhibiting effect and promote tumor outgrowth. Further studies are needed to investigate whether tumor  
249 tissue-derived IDO1 reduces the antitumor activity of IFN- $\gamma$  and, if that is the case, the strategy of  
250 silencing IDO1 expression in tumor tissues may be beneficial in increasing the IFN- $\gamma$  activity for cancer  
251 therapy <sup>36</sup>.

252  
253 In conclusion, IFN- $\gamma$  gene transfer significantly upregulates the IDO1 expression in tumor  
254 tissues and peripheral organs, but this up-regulation has only a marginal effect on both the tumor growth  
255 and antitumor activity of IFN- $\gamma$ . This could be due to the difference in the time courses of these two

256 proteins; the expression of IFN- $\gamma$  from pCpG-Mu $\gamma$ , the effective plasmid for cancer treatment, was  
257 sustained for a long period of time, whereas that of IDO1 was relatively transient. This transient  
258 up-regulation of IDO1 may also explain why sustained expression of IFN- $\gamma$  is required for antitumor  
259 activity of IFN- $\gamma$  gene transfer.

260

## 261 **Materials and methods**

262

### 263 Mice

264

265 Four-week-old male ICR mice and five-week-old male C57BL/6J mice were purchased from Japan SLC,  
266 Inc. (Hamamatsu, Japan). IDO1 knockout (IDO1 KO) mice with a C57BL/6J background were obtained  
267 from the Jackson Laboratory (Bar Harbor, ME, USA), and bred and used at five weeks of age. Mice  
268 were maintained under conventional housing conditions. The protocols for the animal experiments were  
269 approved by the Animal Experimentation Committee of the Graduate School of Pharmaceutical Sciences,  
270 Kyoto University.

271

### 272 Cell culture and treatment

273

274 Lewis lung carcinoma (LLC) cells were kindly provided by Dr. Kenichi Ogawara (Graduate School of  
275 Medicine, Dentistry and Pharmaceutical Sciences, Okayama University) and cultured in Dulbecco's  
276 modified Eagle medium (DMEM; Nissui Co. Ltd., Tokyo, Japan) supplemented with 10 % heat-inactive  
277 fetal bovine serum (FBS) at 37°C, 5 % CO<sub>2</sub>. For IFN- $\gamma$  treatment, LLC cells were seeded at a density of  
278  $1 \times 10^5$  cells/well in a 12-well plate and incubated overnight. Then, 20 ng/ml recombinant murine IFN- $\gamma$   
279 (Peprotech, NJ, USA) was added to the cells and, after a 24-hour incubation, the cells were washed with  
280 PBS and harvested for mRNA extraction.

281

282 Plasmid DNA

283

284 A CpG-free plasmid pCpG-mcs was obtained from Invivogen (San Diego, CA, USA). Murine  
285 IFN- $\gamma$ -expressing plasmids, pCMV-Mu $\gamma$  and pCpG-Mu $\gamma$ , and human IFN- $\gamma$ -expressing plasmid,  
286 pCpG-Hu $\gamma$ , were constructed as described previously<sup>5,40,41</sup>. The details of all the plasmids used in this  
287 study are summarized in Table 1.

288

289 *In vivo* gene transfer

290

291 For gene transfer to the liver, mice received a hydrodynamic tail vein injection of plasmid DNA  
292 dissolved in a large volume of saline (8 % of the body weight) over 5 sec<sup>42</sup>. In a separate group of mice,  
293 gene transfer to skeletal muscle was performed by an injection of plasmid DNA into the gastrocnemius  
294 muscle, followed by electroporation (200 V/cm, 5 ms/pulse, 12 pulses, 4Hz) using a pair of 1-cm<sup>2</sup>  
295 forceps-type electrodes connected to a rectangular direct current generator (CUY-21, Nepagene, Chiba,  
296 Japan)<sup>43</sup>.

297

298 Measurement of serum concentrations of murine IFN- $\gamma$

299

300 At indicated periods after gene transfer, 50 to 200  $\mu$ l blood samples were collected from the tail vein.  
301 The blood samples were incubated at 4 °C for 2 h to allow clotting and then centrifuged at 8,000 $\times$ g to  
302 obtain serum. The concentration of murine IFN- $\gamma$  in the serum was determined using a commercial  
303 ELISA kit (Ready-SET-Go! Mouse IFN- $\gamma$  ELISA, eBioscience, San Diego, CA, USA).

304

305 Isolation of mRNA

306

307 Total RNA was extracted from cultured cells or approximately 100 mg liver, spleen, kidney, lung or

308 tumor, using Sepasol RNA I Super (Nacalai Tesque, Kyoto, Japan). Following DNase treatment with a  
309 mixture of recombinant DNase I-RNase-free (Takara Bio, Shiga, Japan) and RNase OUT™ recombinant  
310 ribonuclease inhibitor (Invitrogen, Carlsbad, CA, USA), reverse transcription was performed using a  
311 ReverTra Ace® qPCR RT Kit (Toyobo, Osaka, Japan) according to the manufacturer's protocol to obtain  
312 total cDNA.

313

314 Quantitative real-time PCR of mRNA

315

316 Real-time PCR was carried out using total cDNA on a Light-Cycler instrument (Roche Diagnostics  
317 GmbH, Mannheim, Germany). The oligodeoxynucleotide primers used for amplification were as  
318 follows: IDO1 forward, 5'-GCCTCCTATTCTGTCTTATGCAG-3', reverse,  
319 5'-ATACAGTGGGGATTGCTTTGATT-3', IDO2 forward,  
320 5'-TGTCCTGGTGTAGCAGTCATGT-3', reverse, 5'-TGCAGGATGTGAACCTCTAACGCT-3'<sup>44</sup>;  
321 TDO forward, 5'-ATGAGTGGGTGCCCGTTG-3', reverse, 5'-GGCTCTGTTTACACCAGTTTGAG-3'  
322 <sup>45</sup> and GAPDH forward, 5'-ACGGATTTGGTCGTATTGGG-3', reverse,  
323 5'-CGCTCCTGGAAGATGGTGAT-3'. Amplification products were detected on-line via intercalation  
324 of the fluorescent dye SYBR green (LightCycler® FastStart DNA Master<sup>PLUS</sup> SYBR Green I kit, Roche  
325 Diagnostics GmbH). The cycling conditions were as follows: initial enzyme activation at 95 °C for 10  
326 min, followed by 45 cycles at 95 °C for 10 s, 60 °C for 5 s, and 72 °C for 15 s for IDO1 and TDO. The  
327 cycling conditions for IDO2 and GAPDH were the same as those for IDO1 except for the annealing  
328 temperature of 62 °C and 56 °C, respectively. The mRNA expression of target genes was normalized  
329 using the mRNA level of GAPDH.

330

331 Measurement of concentrations of L-tryptophan and L-kynurenine in mouse serum and tumors

332

333 Serum samples (50 µl) were mixed with 150 µl 3% perchloric acid, incubated at 4 °C for 1 h, and then

334 centrifuged at  $18000 \times g$  for 10 min at  $4^{\circ}\text{C}$ . The resulting supernatants were kept at  $-80^{\circ}\text{C}$  until analysis.  
335 For tumors, 50-100 mg tumor samples were homogenized in 2 volumes of KP buffer, then centrifuged at  
336  $12000 \times g$  for 10 min at  $4^{\circ}\text{C}$ . The supernatants were collected and mixed with 1 volume of 3%  
337 perchloric acid before analysis. The concentrations of L-tryptophan and L-kynurenine in the  
338 supernatants were measured by HPLC with a spectrophotometric detector (Tosoh ultraviolet-8000,  
339 Tosoh, Tokyo, Japan) or a fluorescence spectrometric detector (Hitachi, Tokyo, Japan)<sup>46</sup>. Total protein  
340 concentrations in the tumors were measured using a protein assay kit (BioRad, Tokyo, Japan). The  
341 concentrations of L-tryptophan and L-kynurenine in the tumors were expressed as  $\mu\text{mol per g tissue}$   
342 protein.

343

344 Antitumor effects of IFN- $\gamma$  gene transfer

345

346 C57BL/6J and IDO1 KO mice received inoculations of  $5 \times 10^4$  LLC cells into the dorsal skin. At 7 days  
347 after inoculation, the mice were hydrodynamically injected with pCpG-Mu $\gamma$  or pCpG-Hu $\gamma$  at a dose of  
348  $0.23 \mu\text{g}/20\text{g}$  body weight or injected with saline. The tumor size was monitored every two or three days,  
349 and the tumor volume was calculated from the equation:  $(d_1 \times d_2)^{3/2} \times (\pi/6)$ , where  $d_1$  and  $d_2$  are  
350 perpendicular tumor diameters<sup>47</sup>. The survival rate of the tumor-bearing mice was also recorded.

351

352 IDO1 expression in LLC tumors

353

354 C57BL/6J mice received inoculations of  $5 \times 10^4$  LLC cells into the dorsal skin. Seven days later, the  
355 mice were hydrodynamically injected with pCpG-Mu $\gamma$  or pCpG-Hu $\gamma$ , which was used as a control  
356 plasmid in this study, at a dose of  $0.23 \mu\text{g}/20 \text{g}$  body weight. At indicated times after gene transfer, solid  
357 tumors were dissected and the mRNA expression of IDO1 was measured as described above.

358

359 Statistical analysis



360

361 Statistical significance was evaluated by one-way ANOVA followed by Tukey`s post hoc test for  
362 multiple comparisons and Student`s *t*-test for comparisons between two given groups. The survival  
363 analysis was performed by the LogRank test using SigmaPlot® 11.0. The level of statistical significance  
364 was set at  $P < 0.05$ .

365

### 366 **Conflict of Interest**

367 The authors declare no conflict of interest.

368

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539

540

541 **Figure legends**

542

543 Figure 1. Levels of IDO1 (a) and IDO2 (b) mRNA in mouse organs after IFN- $\gamma$  gene transfer.  
544 pCpG-Mu $\gamma$  or pCpG-Hu $\gamma$  was hydrodynamically injected into mice at a dose of 0.1  $\mu\text{g}/\text{mouse}$ . At 24 h  
545 after injection, the liver, spleen, lung and kidney were sampled, and total mRNA was extracted from  
546 these organs. The IDO1 and IDO2 mRNA levels were measured by real-time PCR. The IDO mRNA  
547 levels were normalized to GAPDH mRNA as an internal control. The results are expressed as the mean  
548  $\pm$  SD of three mice. \*P<0.05 compared with the pCpG-Hu $\gamma$  injected group.

549

550 Figure 2. Time-course of IDO1 mRNA in the liver, IFN- $\gamma$ , L-tryptophan and L-kynurenine  
551 concentrations in the serum of mice after IFN- $\gamma$  gene transfer. Mice were hydrodynamically injected  
552 with 20  $\mu\text{g}$  pCMV-Mu $\gamma$ , 0.1  $\mu\text{g}$  pCpG-Mu $\gamma$  or 0.1  $\mu\text{g}$  pCpG-Hu $\gamma$  (control). Mice received intramuscular  
553 injections of pCpG-Mu $\gamma$  at a dose of 50  $\mu\text{g}$  in both sides of the legs followed by electroporation  
554 (pCpG-Mu $\gamma$  (IMEP)). Mice were sacrificed at indicated times, the livers and blood samples were  
555 collected. The IFN- $\gamma$  concentration (a), IDO1 mRNA in the liver (b), and L-tryptophan (c) and  
556 L-kynurenine (d) concentrations in the serum were measured. The L-kynurenine to L-tryptophan ratios  
557 (e) were also calculated. The results are expressed as the mean  $\pm$  SD of three mice. \*P<0.05 compared  
558 with the pCpG-Hu $\gamma$  injected group, #P<0.05 compared with the pCMV-Mu $\gamma$  injected group, §P<0.05  
559 compared with the pCpG-Mu $\gamma$  (IMEP) injected group and †P<0.05 compared with all the other groups.

560

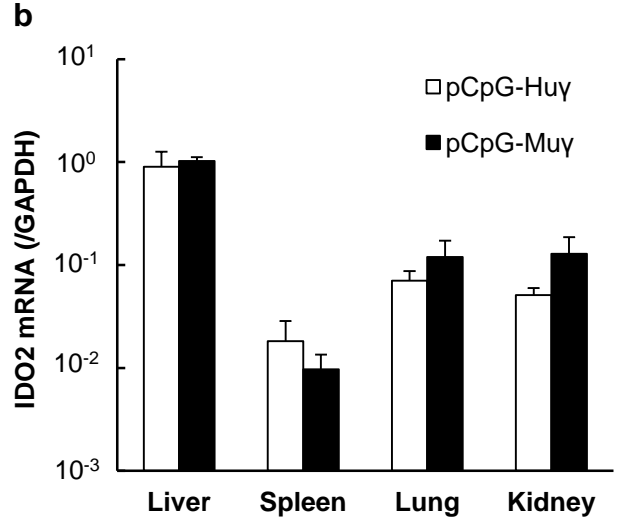
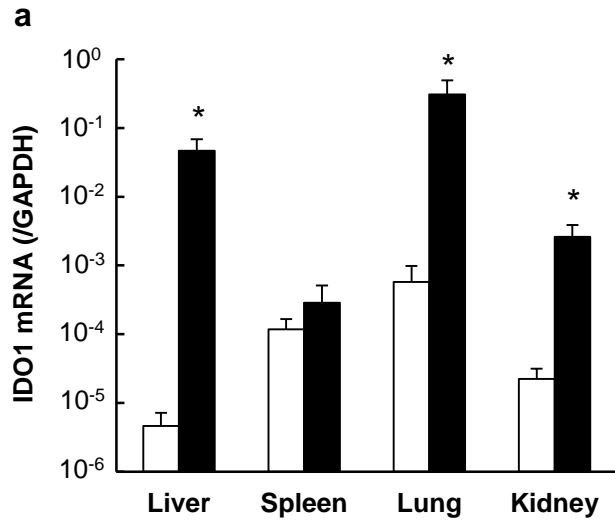
561 Figure 3. Tumor growth and survival of LLC tumor-bearing wild type and IDO1 KO mice after IFN- $\gamma$   
562 gene transfer. Wild-type (C57BL/6J) or IDO1 KO mice received intradermal injections of  $5 \times 10^4$  LLC  
563 cells in the dorsal skin. Seven days later, mice were hydrodynamically injected with pCpG-Mu $\gamma$ ,  
564 pCpG-Hu $\gamma$  or saline at a dose of 0.23  $\mu\text{g}/20\text{g}$  body weight. The tumor size of wild-type (a) and IDO1  
565 KO mice (b) was measured periodically and their survival (c) was monitored. The results are expressed  
566 as the mean  $\pm$  SD of five mice for each group of wild-type mice and seven mice for each group of IDO1

567 KO mice. \*P<0.05 compared with the pCpG-Hu $\gamma$  treated wild-type group, #P<0.05 compared with the  
568 saline treated wild-type group, and §P<0.05 compared with the pCpG-Hu $\gamma$  treated IDO1 KO group.

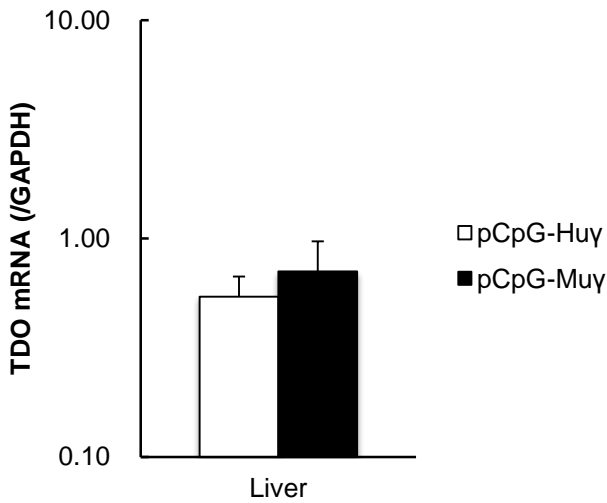
569  
570 Figure 4. Time-course of the concentrations of IFN- $\gamma$  (a), L-tryptophan (b) and L-kynurenine (c) in the  
571 serum, and the ratio of L-kynurenine to L-tryptophan (d) of tumor bearing mice after IFN- $\gamma$  gene transfer.  
572 Mouse serum samples were collected at the indicated times. The results are expressed as the mean  $\pm$  SD  
573 of five mice for each group of wild-type mice and seven mice for each group of IDO1 KO mice.  
574 \*P<0.05 compared with the pCpG-Hu $\gamma$  treated wild-type group, #P<0.05 compared with the saline  
575 treated wild-type group, §P<0.05 compared with the pCpG-Hu $\gamma$  treated IDO1 KO group and †P<0.05  
576 compared with all the other groups.

577  
578 Figure 5. Levels of IDO1 mRNA, and concentrations of L-tryptophan and L-kynurenine in LLC tumor  
579 of tumor-bearing mice after IFN- $\gamma$  gene transfer. LLC cells ( $5 \times 10^4$  cells per mouse) received  
580 intradermal injections in the dorsal skin. Seven days later, mice were hydrodynamically injected with  
581 pCpG-Mu $\gamma$  or pCpG-Hu $\gamma$  at a dose of 0.23  $\mu$ g/20 g body weight. The tumors were collected at indicated  
582 times, the levels of IDO1 mRNA (a) and the concentrations of L-tryptophan (b) and L-kynurenine (c) in  
583 the tumors were measured. The levels of IDO1 mRNA were normalized to GAPDH mRNA as an  
584 endogenous control. The concentrations of L-tryptophan and L-kynurenine in the tumor were  
585 standardized per g total protein. The L-kynurenine to L-tryptophan ratios (d) were also calculated. The  
586 results are expressed as the mean  $\pm$  SD of four mice for the levels of IDO1 mRNA and three mice for the  
587 concentrations of L-tryptophan and L-kynurenine. †P<0.05 compared with all the other groups.

Figure 1  
(Revised)



TDO expression (data not shown in figure)



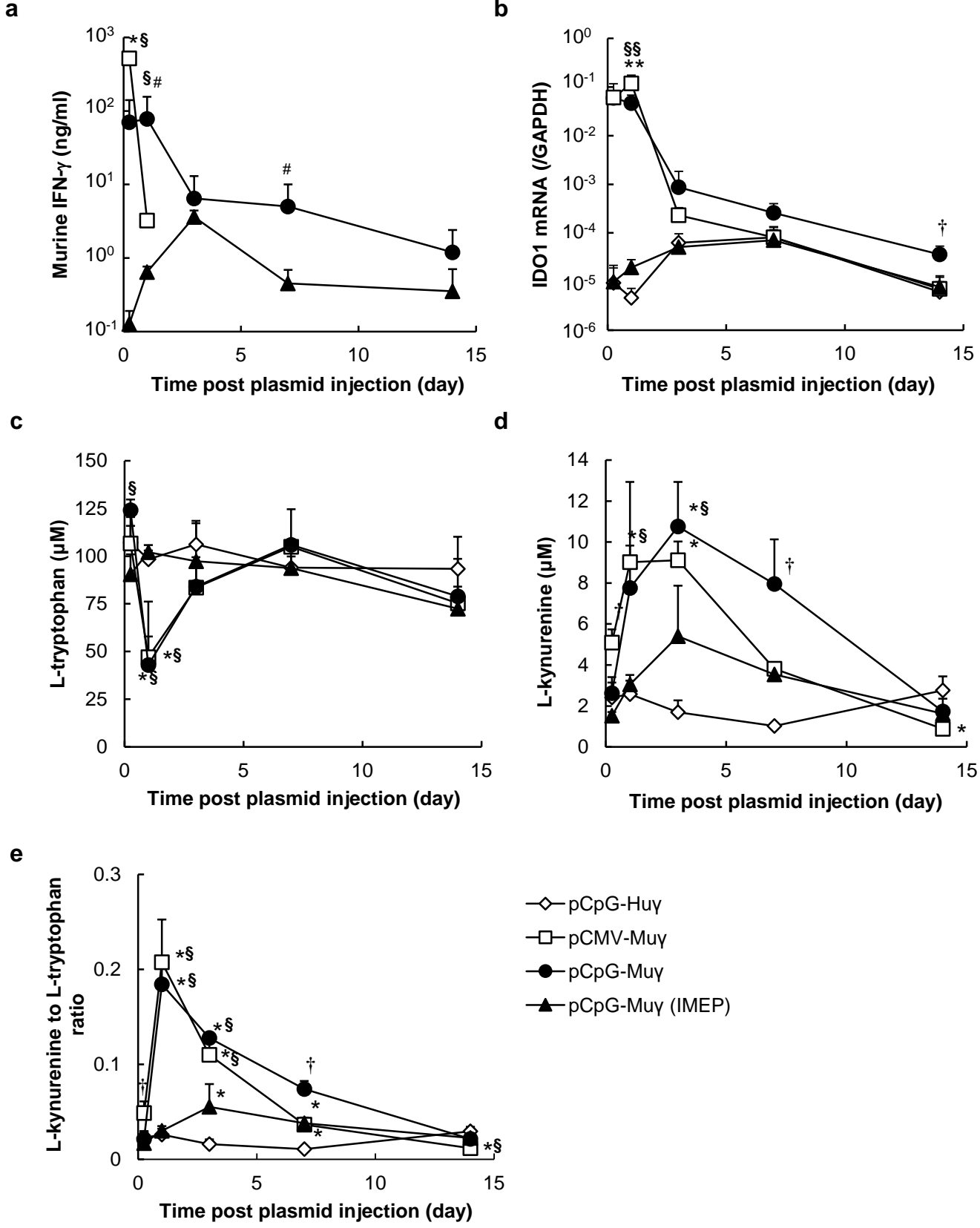


Figure 2

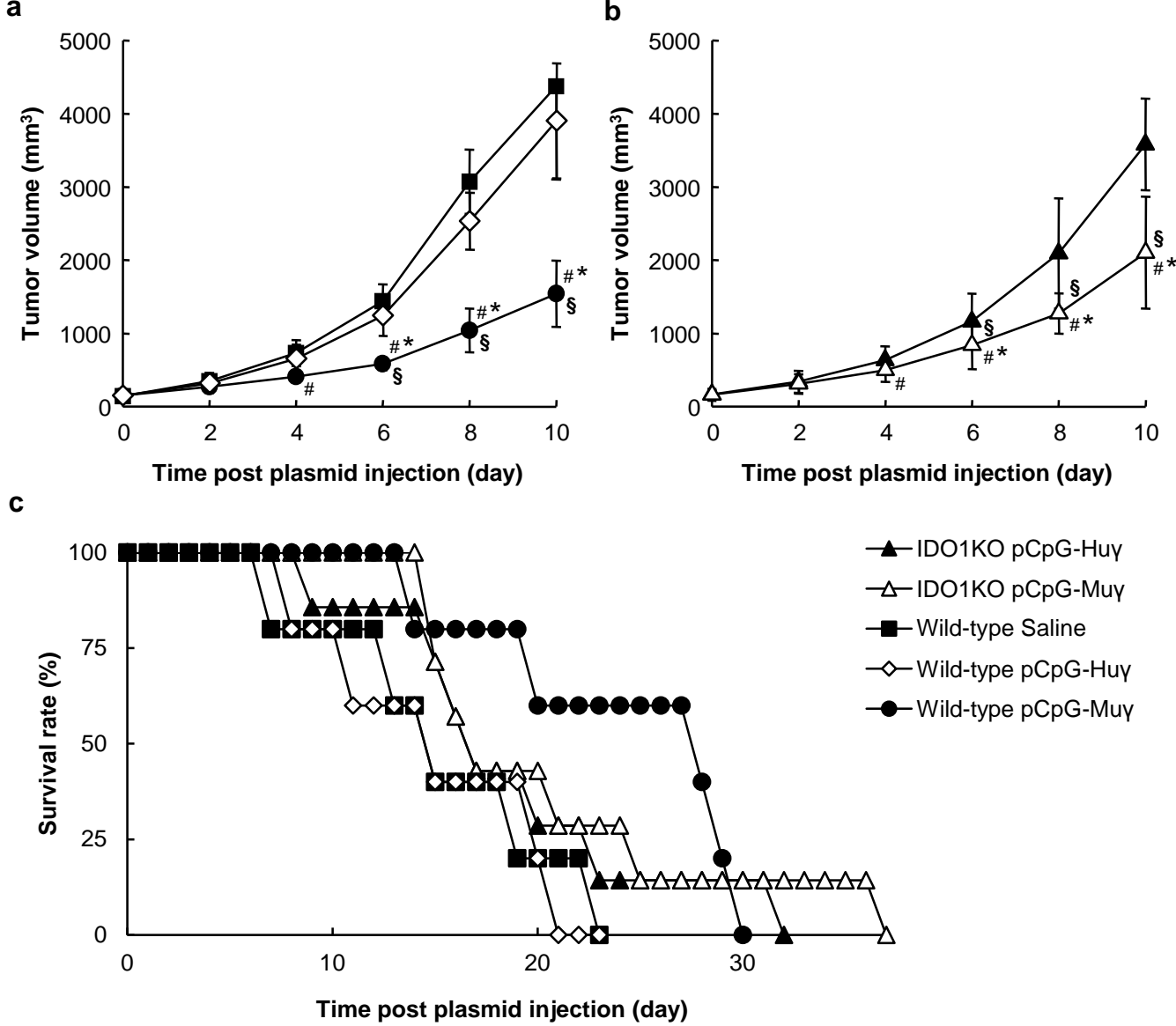


Figure 3  
(Revised)

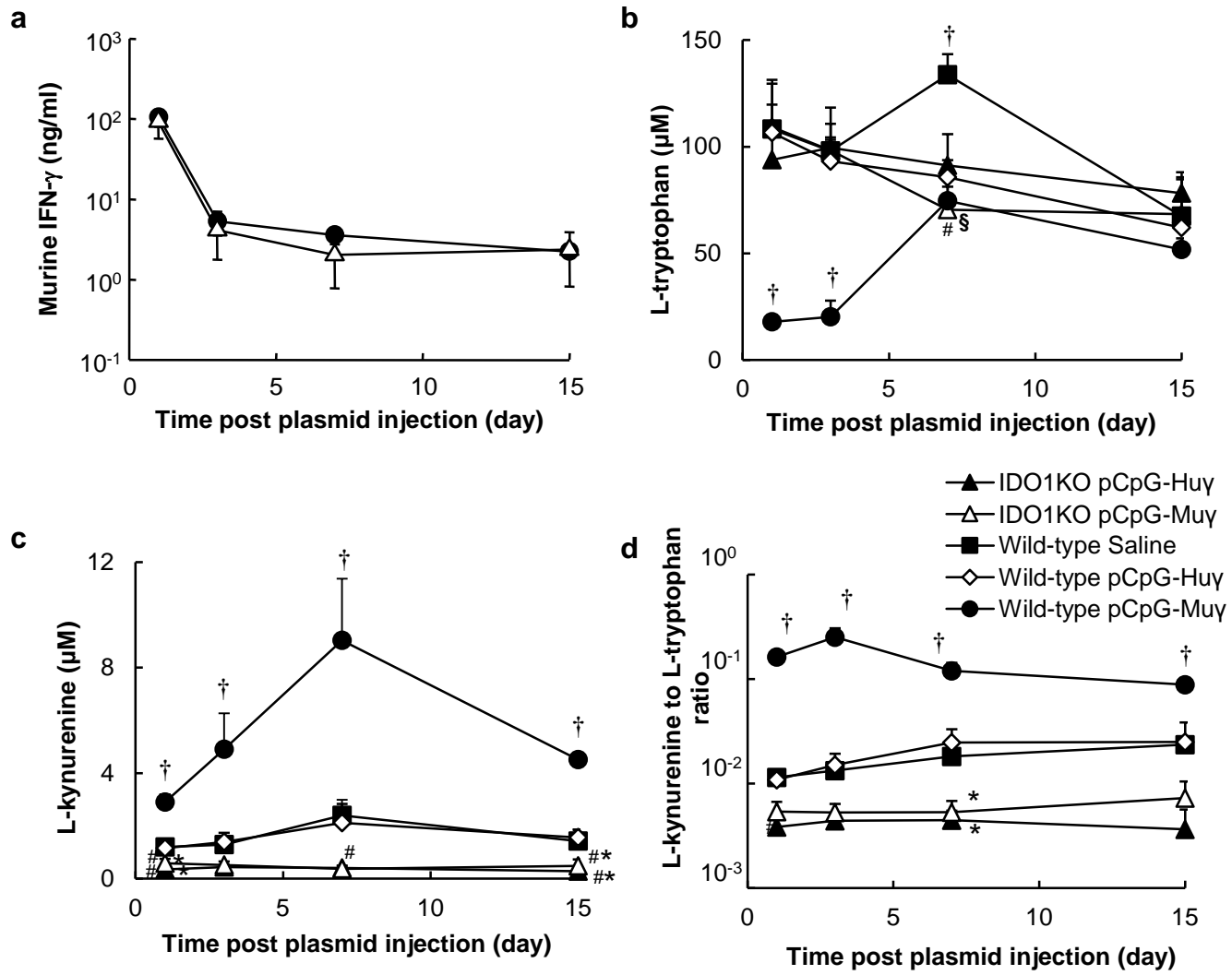


Figure 4  
(Revised)



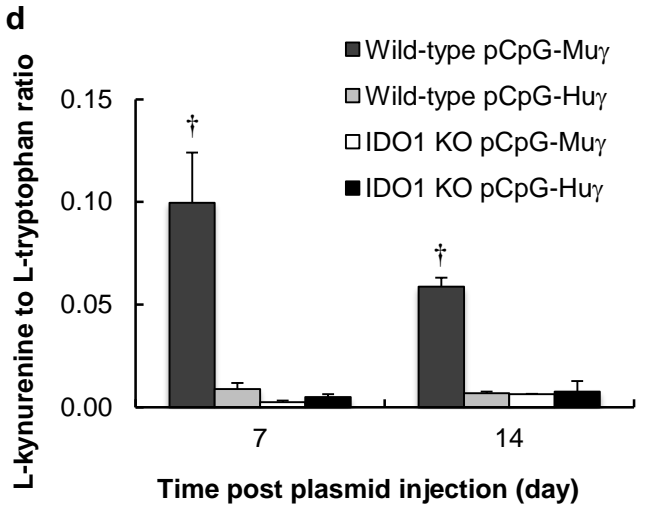
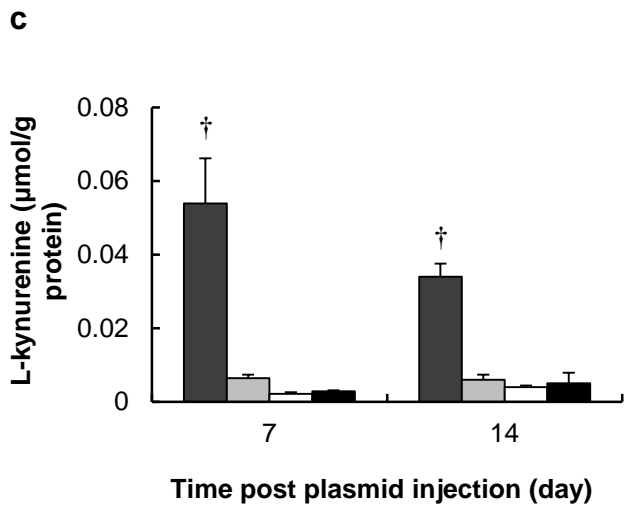
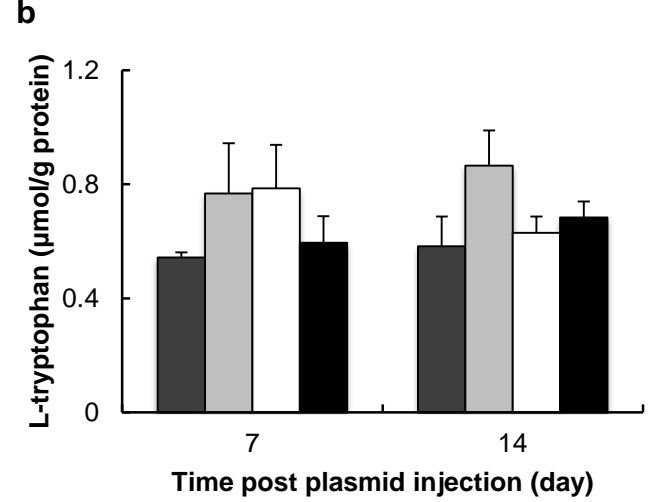
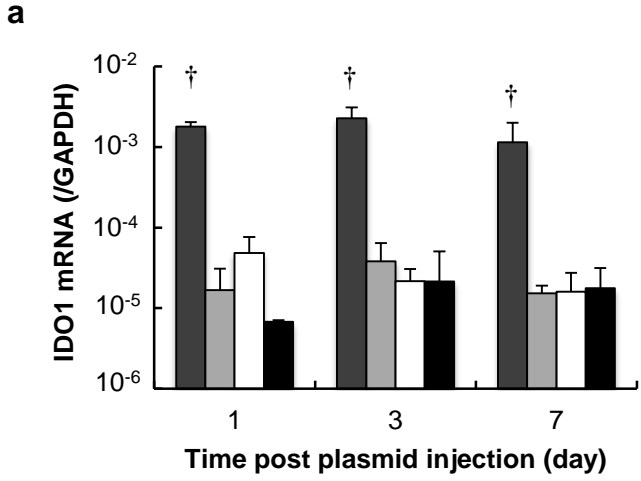


Figure 5  
(Revised)

**Table1** Plasmid DNA used in this study.

Plasmid	Promoter	Backbone	cDNA
pCMV-Mu $\gamma$	CMV	pcDNA3.1	Murine IFN- $\gamma$
pCpG-Mu $\gamma$	EF1	pCpG-mcs	Murine IFN- $\gamma$
pCpG-Hu $\gamma$	EF1	pCpG-mcs	Human IFN- $\gamma$