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

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Abstract

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

Key Words: antitumor effect; indoleamine 2, 3-diooxygenase 1; interferon-γ; transgene expression

Introduction

Interferon-γ (IFN-γ) is a cytokine with antiproliferative and immunomodulatory activities. Therefore, it is expected to have beneficial effects in the treatment of a variety of diseases, including cancer [1-3]. Previous studies have shown that gene delivery of IFN-γ is an effective treatment for cancer and, furthermore, we have demonstrated success in prolonging the transgene expression of murine IFN-γ from plasmid vectors by developing ones with fewer CpG motifs [4,5]. A hydrodynamic injection of the newly constructed IFN-γ-expressing plasmids resulted in prolonged therapeutic serum concentrations of IFN-γ for more than 80 days, which significantly inhibited metastatic tumor growth and atopic dermatitis in mouse models [4-7].

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

IDO1 is a tryptophan-metabolizing enzyme acting along the kynurenine pathway, and it is expressed in a variety of cells [13,14]. The induced expression of IDO1 could lead to tryptophan depletion and formation of some toxic metabolites of tryptophan, such as kynurenine, and 3-hydroxykynurenine, which can result in the impairment of T cell functions and down-regulation of immune responses [15-18]. Various types of IDO1 inhibitors have been developed and reported to exhibit antitumor activity [13,19-22]. However, the role of IDO1 in tumor growth is controversial so far [23]. Therefore, further investigation is needed to clarify the role of IDO1 in tumor development and its effects on the antitumor activity of IFN-γ.

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

Results

Induction of IDO1 expression in mouse organs after IFN-γ gene transfer

Figure 1a shows the mRNA levels of IDO1 in the liver, spleen, lung and kidney of mice after hydrodynamic injection of pCpG-Muγ, a murine IFN-γ-expressing plasmid, or a human IFN-γ-expressing plasmid, pCpG-Huγ. The plasmid doses for hydrodynamic injection were determined based on the previous study, and pCpG-Huγ was used as a control plasmid because human IFN-γ exhibited no significant effects in mice [6]. There were no significant changes in the IDO1 mRNA levels in mice receiving pCpG-Huγ, the control plasmid (data not shown). The IDO1 mRNA level in the liver of the pCpG-Muγ-injected mice was about 10,000-fold greater than that in the pCpG-Huγ-injected mice. The levels of IDO1 in the other organs examined were also higher in the pCpG-Muγ injected mice and these differences were statistically significant except in the spleen, although the magnitude of the increase in these organs (533- and 117-fold for lung and kidney, respectively) was much less than that in the liver. The mRNA expression of IDO2 and tryptophan 2,3-dioxygenase (TDO), two other tryptophan-metabolizing enzymes, was also examined. Unlike IDO1, the mRNA levels of IDO2 (Figure 1b) in these organs as well as that of TDO (data not shown) in the liver were not significantly changed by IFN-γ gene transfer.

Time-courses of IDO1 expression in the liver and tryptophan catabolism after IFN-γ gene transfer

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

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

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

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

Antitumor effect of IFN-γ gene transfer in LLC tumor-bearing mice

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

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

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

mRNA expression of IDO1 and the concentrations of L-tryptophan and L-kynurenine in LLC tumors

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

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

Discussion

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

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

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

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

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

In the IDO1 competent mice, IDO1 could be expressed, not only in LLC cells, but also in tumor-infiltrating immune cells, such as macrophages and dendritic cells [38,39]. The large difference in the IDO1 mRNA expression in LLC tumor tissues after hydrodynamic injection of pCpG-Muγ between the wild-type and IDO1 KO mice clearly suggests that the major types of cells expressing IDO1 are the tumor-infiltrating immune cells. These findings support the role played by tumor infiltrating immune cells in modulating the IDO activity in tumor tissues, which could contribute to the T cell proliferation inhibiting effect and promote tumor outgrowth. Further studies are needed to investigate whether tumor tissue-derived IDO1 reduces the antitumor activity of IFN-γ and, if that is the case, the strategy of silencing IDO1 expression in tumor tissues may be beneficial in increasing the IFN-γ activity for cancer therapy [36].

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

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

Materials and methods

Mice

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

Cell culture and treatment

Lewis lung carcinoma (LLC) cells were kindly provided by Dr. Kenichi Ogawara (Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama University) and cultured in Dulbecco's modified Eagle medium (DMEM; Nissui Co. Ltd., Tokyo, Japan) supplemented with 10 % heat-inactive fetal bovine serum (FBS) at 37°C, 5 % CO_2. For IFN-γ treatment, LLC cells were seeded at a density of 1×10^5 cells/well in a 12-well plate and incubated overnight. Then, 20 ng/ml recombinant murine IFN-γ (Peprotech, NJ, USA) was added to the cells and, after a 24-hour incubation, the cells were washed with PBS and harvested for mRNA extraction.

282 Plasmid DNA

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284 A CpG-free plasmid pCpG-mcs was obtained from Invivogen (San Diego, CA, USA). Murine
285 IFN-γ-expressing plasmids, pCMV-Muγ and pCpG-Muγ, and human IFN-γ-expressing plasmid,
286 pCpG-Huγ, were constructed as described previously [5,40,41]. The details of all the plasmids used in this
287 study are summarized in Table 1.

288

289 *In vivo* gene transfer

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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 [42]. 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^2
295 forceps-type electrodes connected to a rectangular direct current generator (CUY-21, Nepagene, Chiba,
296 Japan) [43].

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298 Measurement of serum concentrations of murine IFN-γ

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300 At indicated periods after gene transfer, 50 to 200 μl 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×g to
302 obtain serum. The concentration of murine IFN-γ in the serum was determined using a commercial
303 ELISA kit (Ready-SET-Go! Mouse IFN-γ ELISA, eBioscience, San Diego, CA, USA).

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305 Isolation of mRNA

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

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

Quantitative real-time PCR of mRNA

Real-time PCR was carried out using total cDNA on a Light-Cycler instrument (Roche Diagnostics GmbH, Mannheim, Germany). The oligodeoxynucleotide primers used for amplification were as follows: IDO1 forward, 5'-GCCTCCTATTCTGTCTTATGCAG-3', reverse, 5'-ATACAGTGGGGATTGCTTTGATT-3', IDO2 forward, 5'-TGTCCTGGTGCTTAGCAGTCATGT-3', reverse, 5'-TGCAGGATGTGAACCTCTAACGCT-3' [44]; TDO forward, 5'-ATGAGTGGGTGCCCGTTG-3', reverse, 5'-GGCTCTGTTTACACCAGTTTGAG-3' [45] and GAPDH forward, 5'-ACGGATTTGGTCGTATTGGG-3', reverse, 5'-CGCTCCTGGAAGATGGTGAT-3'. Amplification products were detected on-line via intercalation of the fluorescent dye SYBR green (LightCycler® FastStart DNA Master^{PLUS} SYBR Green I kit, Roche Diagnostics GmbH). The cycling conditions were as follows: initial enzyme activation at 95 °C for 10 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 cycling conditions for IDO2 and GAPDH were the same as those for IDO1 except for the annealing temperature of 62 °C and 56 °C, respectively. The mRNA expression of target genes was normalized using the mRNA level of GAPDH.

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

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

centrifuged at 18000 × g for 10 min at 4 °C. The resulting supernatants were kept at -80°C until analysis. For tumors, 50-100 mg tumor samples were homogenized in 2 volumes of KP buffer, then centrifuged at 12000 x g for 10 min at 4 °C. The supernatants were collected and mixed with 1 volume of 3% perchloric acid before analysis. The concentrations of L-tryptophan and L-kynurenine in the supernatants were measured by HPLC with a spectrophotometric detector (Tosoh ultraviolet-8000, Tosoh, Tokyo, Japan) or a fluorescence spectrometric detector (Hitachi, Tokyo, Japan) [46]. Total protein concentrations in the tumors were measured using a protein assay kit (BioRad, Tokyo, Japan). The concentrations of L-tryptophan and L-kynurenine in the tumors were expressed as μmol per g tissue protein.

Antitumor effects of IFN-γ gene transfer

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

IDO1 expression in LLC tumors

C57BL/6J mice received inoculations of 5×10^4 LLC cells into the dorsal skin. Seven days later, the mice were hydrodynamically injected with pCpG-Muγ or pCpG-Huγ, which was used as a control plasmid in this study, at a dose of 0.23μg/20 g body weight. At indicated times after gene transfer, solid tumors were dissected and the mRNA expression of IDO1 was measured as described above.

Statistical analysis

Statistical significance was evaluated by one-way ANOVA followed by Tukey`s post hoc test for multiple comparisons and Student's *t*-test for comparisons between two given groups. The survival analysis was performed by the LogRank test using SigmaPlot® 11.0. The level of statistical significance was set at $P < 0.05$.

Conflict of Interest

The authors declare no conflict of interest.

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Figure legends

Figure 1. Levels of IDO1 (a) and IDO2 (b) mRNA in mouse organs after IFN-γ gene transfer. pCpG-Muγ or pCpG-Huγ was hydrodynamically injected into mice at a dose of 0.1 μg/mouse. At 24 h after injection, the liver, spleen, lung and kidney were sampled, and total mRNA was extracted from these organs. The IDO1 and IDO2 mRNA levels were measured by real-time PCR. The IDO mRNA levels were normalized to GAPDH mRNA as an internal control. The results are expressed as the mean ± SD of three mice. *P<0.05 compared with the pCpG-Huγ injected group.

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

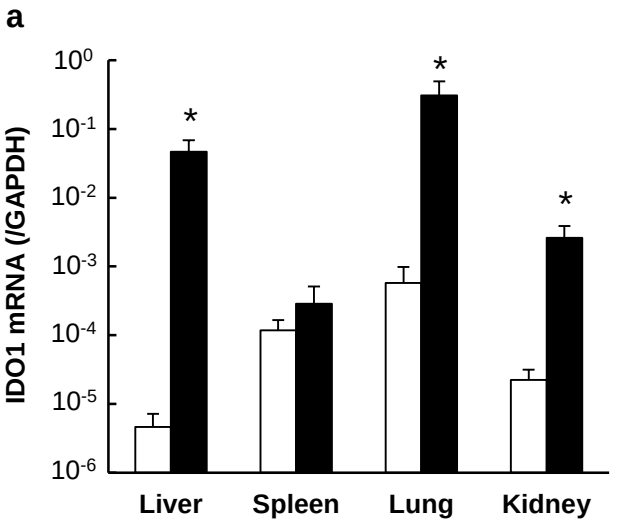
Figure 3. Tumor growth and survival of LLC tumor-bearing wild type and IDO1 KO mice after IFN-γ gene transfer. Wild-type (C57BL/6J) or IDO1 KO mice received intradermal injections of 5×10^4 LLC cells in the dorsal skin. Seven days later, mice were hydrodynamically injected with pCpG-Muγ, pCpG-Huγ or saline at a dose of 0.23 μg/20g body weight. The tumor size of wild-type (a) and IDO1 KO mice (b) was measured periodically and their survival (c) was monitored. The results are expressed as the mean ± SD of five mice for each group of wild-type mice and seven mice for each group of IDO1

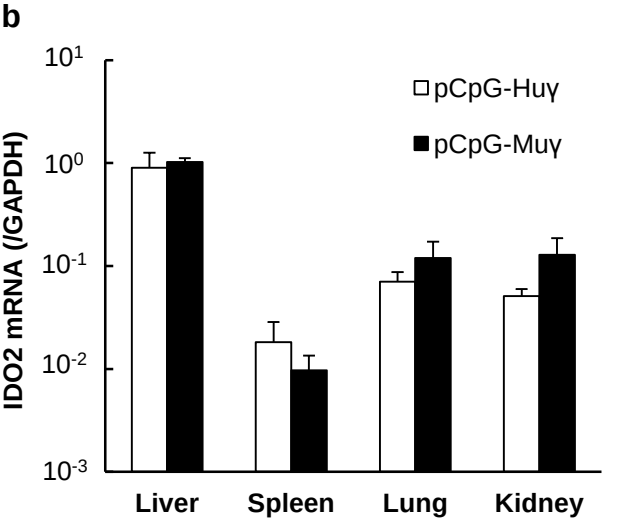
KO mice. *P<0.05 compared with the pCpG-Huγ treated wild-type group, #P<0.05 compared with the saline treated wild-type group, and §P<0.05 compared with the pCpG-Huγ treated IDO1 KO group.

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

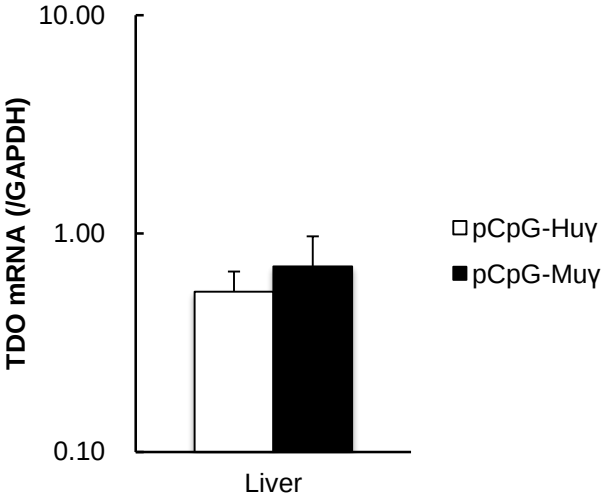
Figure 5. Levels of IDO1 mRNA, and concentrations of L-tryptophan and L-kynurenine in LLC tumor of tumor-bearing mice after IFN-γ gene transfer. LLC cells (5×10^4 cells per mouse) received intradermal injections in the dorsal skin. Seven days later, mice were hydrodynamically injected with pCpG-Muγ or pCpG-Huγ at a dose of 0.23 μg/20 g body weight. The tumors were collected at indicated times, the levels of IDO1 mRNA (a) and the concentrations of L-tryptophan (b) and L-kynurenine (c) in the tumors were measured. The levels of IDO1 mRNA were normalized to GAPDH mRNA as an endogenous control. The concentrations of L-tryptophan and L-kynurenine in the tumor were standardized per g total protein. The L-kynurenine to L-tryptophan ratios (d) were also calculated. The results are expressed as the mean ± SD of four mice for the levels of IDO1 mRNA and three mice for the 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)



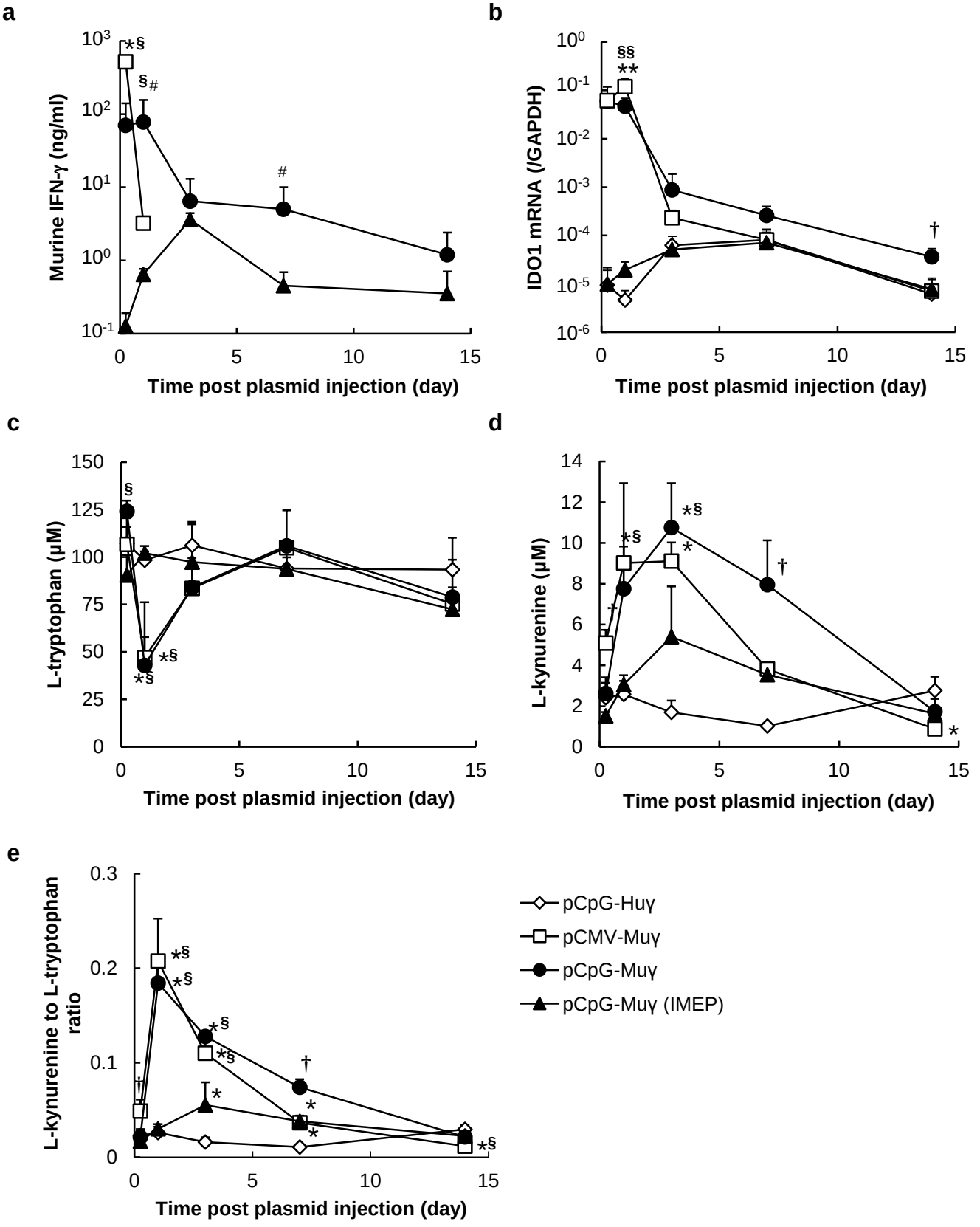

a
Murine IFN-γ (ng/ml)
Time post plasmid injection (day)
b
IDO1 mRNA (/GAPDH)
Time post plasmid injection (day)
c
L-tryptophan (μM)
Time post plasmid injection (day)
d
L-kynurenine (μM)
Time post plasmid injection (day)
e
L-kynurenine to L-tryptophan ratio
Time post plasmid injection (day)
pCpG-Huγ
pCMV-Muγ
pCpG-Muγ
pCpG-Muγ (IMEP)

Figure 2

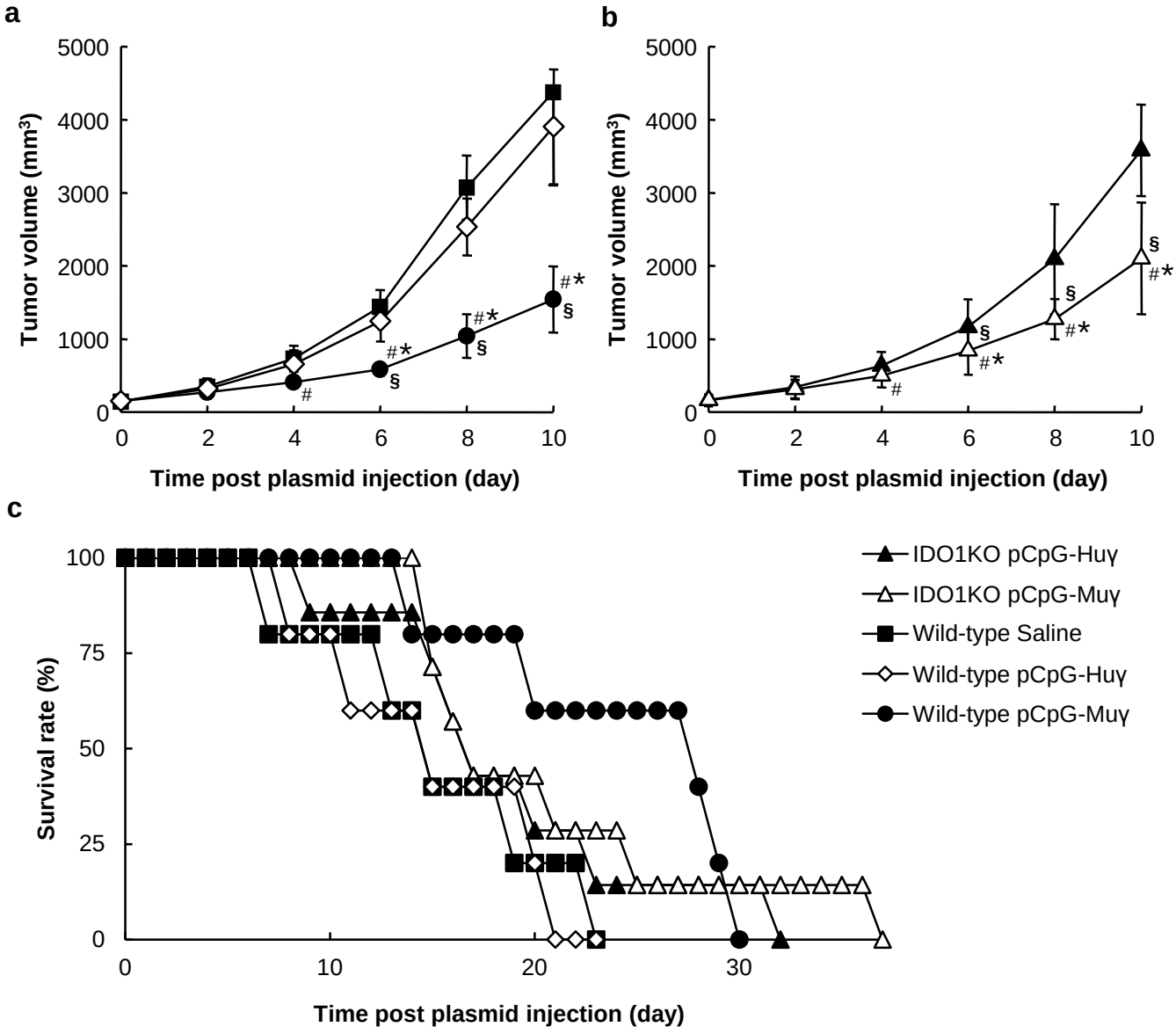


Figure 3
(Revised)

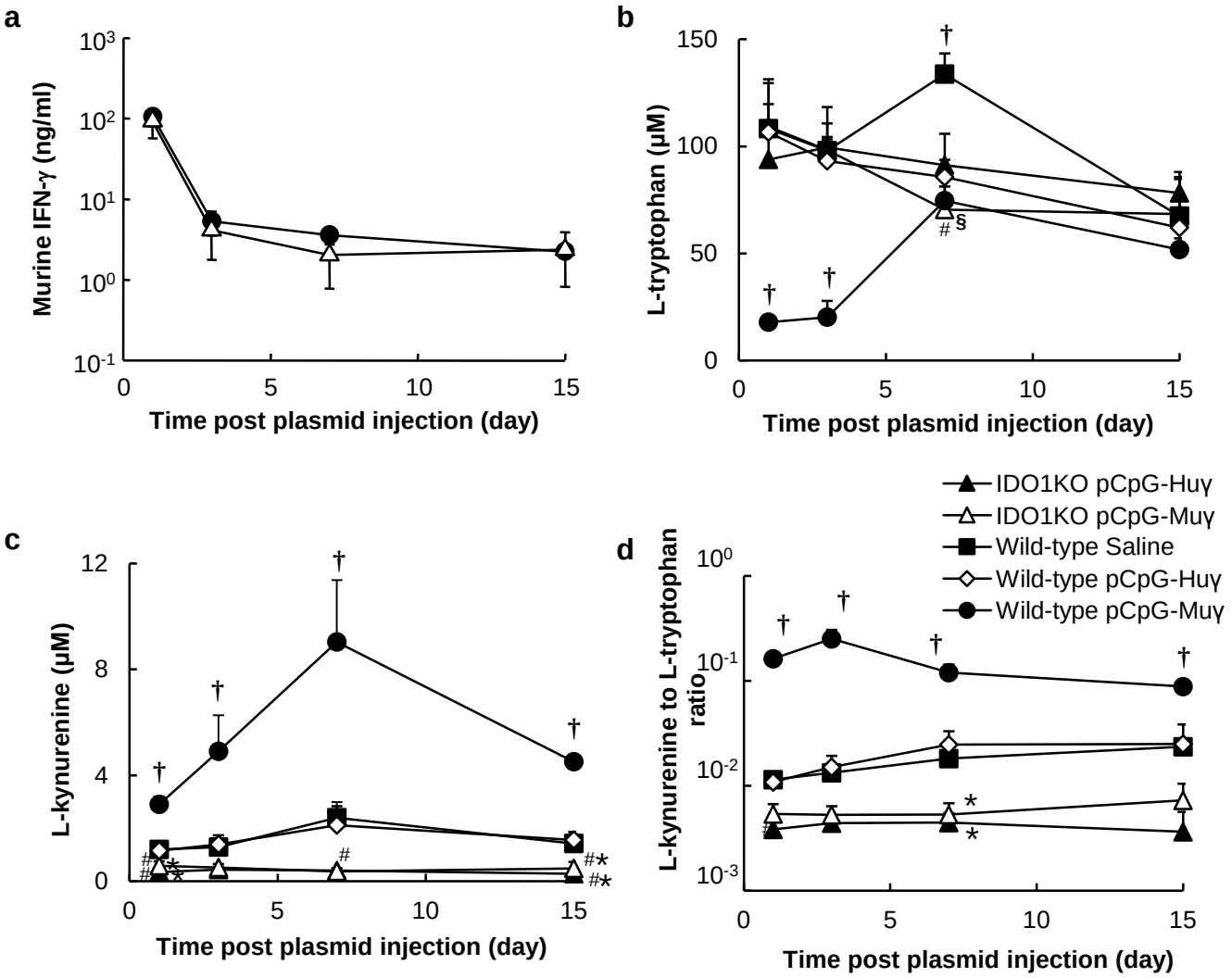


Figure 4
(Revised)

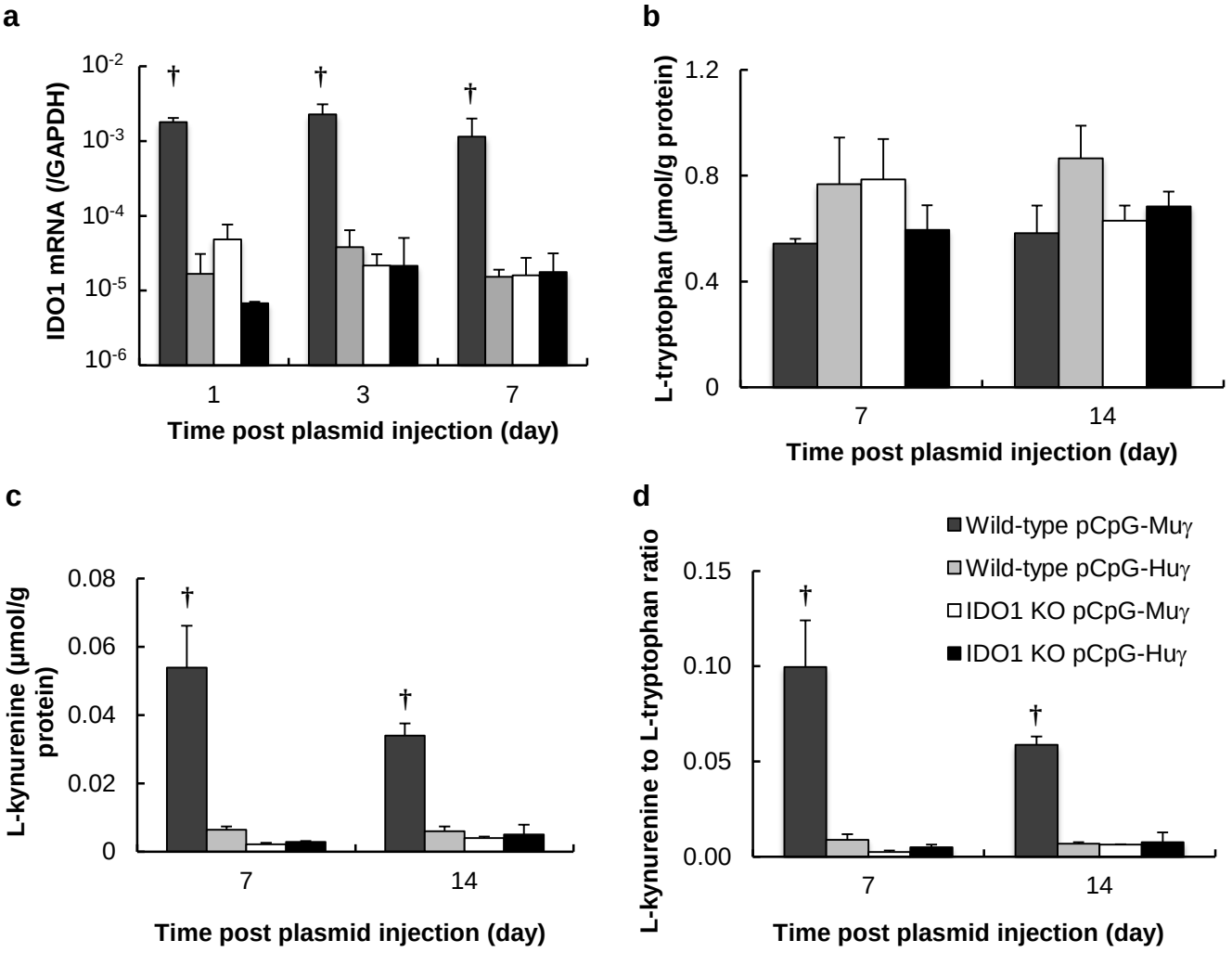


Figure 5
(Revised)

Table1 Plasmid DNA used in this study.

Plasmid	Promoter	Backbone	cDNA
pCMV-Muγ	CMV	pcDNA3.1	Murine IFN-γ
pCpG-Muγ	EF1	pCpG-mcs	Murine IFN-γ
pCpG-Huγ	EF1	pCpG-mcs	Human IFN-γ