Cancer Science
Japanese Cancer Association JCA
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Inhibition of indoleamine 2,3-dioxygenase 1 expression alters immune response in colon tumor microenvironment in mice

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Key words

cancer immunity, colorectal cancer, indoleamine 2,3-dioxygenase, microenvironment, tumorigenesis

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Funding Information
No sources of funding were declared for this study.

Received December 24, 2014; Revised May 11, 2015; Accepted May 25, 2015

Cancer Sci **106** (2015) 1008–1015

doi: 10.1111/cas.12705

Indoleamine 2,3-dioxygenase (IDO), an enzyme that degrades the essential amino acid L-tryptophan along the kynurenine pathway, exerts immunomodulatory effects in a number of diseases. IDO expression is increased in tumor tissue and in draining lymph nodes; this increase is thought to play a role in tumor evasion by suppressing the immune response. A competitive inhibitor of IDO is currently being tested in clinical trials for the treatment of relapsed or refractory solid tumors, but the efficacy of IDO inhibition in colorectal tumors remains to be fully elucidated. In this study, we investigated the effect of IDO deficiency on colon tumorigenesis in mice by genetic deletion and pharmacological inhibition. *Ido1*-deficient$^{(-/-)}$ mice were crossed with *Apc*$^{Min/+}$ mice or were administered azoxymethane with or without dextran sodium sulfate. *Ido1* deficiency did not lead to significant differences in the size and number of colon tumors. Similarly, the pharmacological inhibition of IDO using 1-methyltryptophan (1-mT) also resulted in no significant differences in tumor size and number in *Apc*$^{Min/+}$ mice. However, *Ido1* deficiency altered the immune response in the tumor microenvironment, showing a significant increase in mRNA expression of pro-inflammatory cytokines and a significant decrease in the number of Foxp3-positive regulatory T cells in the colon tumors of *Ido1*$^{(-/-)}$ mice. Importantly, 1-mT treatment also significantly altered cytokine expression in the colon tumor tissues. These results suggest that IDO inhibition alone cannot sufficiently suppress colon cancer development in mice despite its immunomodulatory activity in the tumor microenvironment.

Indoleamine 2,3-dioxygenase (IDO) is an intracellular enzyme that degrades essential amino acid L-tryptophan to *N*-formylkynurenine, which is the first and rate-controlling step in the kynurenine pathway.[1] IDO has two paralogs, IDO1 and IDO2. Both enzymes catalyze the degradation of tryptophan but their distribution differs among organs.[2] IDO exerts immunosuppressive effects by reducing the local concentration of tryptophan and increasing the production of immunomodulatory tryptophan metabolites, which have various effects on immune cells.[3,4] For instance, the metabolites suppress proliferation and promote apoptosis of T lymphocytes,[2–4] and induce differentiation of naive T cells into regulatory T cells (Tregs).

IDO has attracted considerable attention as a novel target for the development of cancer therapeutics.[5] Some human tumors express higher IDO levels than normal tissues; this may contribute to their escape from attack by the host immune system.[6] Treatment with an IDO inhibitor reduces the volume of tumors that were transplanted into mice preimmunized with the tumor antigen.[6] This suggests that IDO inhibitors may exert antitumor effects by suppressing immune tolerance. In keeping with this hypothesis, a competitive inhibitor of IDO, 1-methyltryptophan (1-mT), is currently in clinical trials for the treatment of some relapsed or refractory solid tumors.[5] Because IDO expression has been reported in human colorectal cancer (CRC),[7–9] it is believed that CRC could be potentially treated by using IDO inhibitors. However, the clinical significance of IDO expression in CRC still seems to be a controversial topic. Previous studies have indicated that, unlike in other tumors such as endometrial carcinoma,[10] ovarian carcinoma[11] and hepatocellular carcinoma,[12] in CRC, the IDO expression levels are not correlated with the overall survival of patients.[7,9] However, one recent study shows that tumoral IDO1 expression at the invasive front was significantly associated with overall survival.[8]

The role of IDO expression in the tumor microenvironment of CRC remains largely unknown. The association of IDO

expression and the infiltrating T lymphocytes in human CRC has been evaluated in previous studies, but consistent results have not been obtained.[7,8] While an initial study showed that higher IDO expression was significantly associated with a reduction of CD3+ infiltrating lymphocytes in human CRC,[7] a more recent study indicated the lack of a significant correlation between IDO1 expression levels and the number of CD3+ or CD8+ T lymphocytes.[8] Because the effect of IDO expression on other immune regulatory factors such as cytokine expression is yet to be elucidated, further studies are needed to examine the role of IDO in the tumor microenvironment.

Chang *et al.* (2011) and Thaker *et al.* (2013) examine the effect of IDO inhibition on colon carcinogenesis in rodent models.[13,14] In models of colitis-associated colon tumor, Thaker *et al.* demonstrate the suppression of colon tumor development in *Ido1* gene-deficient$^{(-/-)}$ mice and in mice that were administered a pharmacological inhibitor of IDO1.[14] However, the study by Chang *et al.* give contradictory results, showing an increased development of colitis-associated colon tumors in *Ido1*$^{(-/-)}$ mice.[13] Therefore, further studies are needed to conclusively demonstrate the role of IDO1 in the development of colitis-associated colon tumors. In addition, because IDO inhibition has been reported to severely exacerbate colitis,[15–18] the effect of IDO1 inhibition on colon tumorigenesis should be examined separately from colitis.

In this study, we investigated the effect of genetic deletion of *Ido1* on colon tumorigenesis in an *Apc*$^{Min/+}$ mouse model as well as chemically-induced colon tumor models by using azoxymethane (AOM) with or without dextran sulfate sodium (DSS). The changes in cytokine expression and subset composition of infiltrating lymphocyte in the tumor microenvironment were also examined in colon tumors. In addition, we used pharmacologic approaches with IDO inhibitors in *Apc*$^{Min/+}$ mice to examine the effects of these compounds in colon tumors.

Materials and Methods

Animals. *Ido1*$^{(-/-)}$ mice of the C57BL/6J background and *Apc*$^{Min/+}$ mice of the C57BL/6J background were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). The mice were maintained under controlled conditions (23 ± 2°C and 12-h light:dark cycle). All animals were handled in accordance with the regulations for animal experiments at Gifu University.

Colon tumorigenesis. Animal models of AOM-induced colon tumorigenesis were generated according to previous studies with slight modification.[19,20] Five-week-old *Ido1*$^{(-/-)}$ or *Ido1*-wild-type$^{(+/+)}$ mice received weekly i.p. injection of 10 mg/kg AOM (Sigma-Aldrich, St. Louis, MO, USA) for 5 weeks. The mice were maintained without further treatment and killed 10 weeks after the last dose of AOM. In addition, a colitis-associated colon tumorigenesis model was produced as previously described.[21] In the colitis-associated model, the 5-week-old *Ido1*$^{(-/-)}$ or *Ido1*$^{(+/+)}$ mice received a single AOM injection (10 mg/kg i.p.) followed by administration of 2% DSS (molecular weight 36 000–50 000; MP Biomedicals, Santa Ana, CA, USA) in drinking water for 7 days. Fifteen weeks after the first AOM injection, all surviving mice were killed and analyzed.

To investigate the effects of *Ido1* deficiency in the *Apc*$^{Min/+}$ mouse model, *Ido1*$^{(-/-)}$ mice were crossed with *Apc*$^{Min/+}$ mice to generate *Apc*$^{Min/+}$;*Ido1*$^{(-/-)}$ and *Apc*$^{Min/+}$;*Ido1*$^{(+/+)}$ offspring for analyses. The mice were maintained without treatment and were killed at the age of 20 weeks.

1-mT treatment. Thirteen-week-old *Apc*$^{Min/+}$ mice were assigned to three groups: 1-L-mT treatment, 1-D-mT treatment and vehicle treatment (control) groups. The mice were given 1-L-mT or 1-D-mT (both purchased from Sigma-Aldrich) in their drinking water (5 mg/mL in alkaline water [pH 11.0]) or alkaline water adjusted to pH 11.0 (control group) for 7 weeks. All surviving mice were killed at 20 weeks of age and analyzed.

In the preliminary examination of the inhibitory effect of 1-mT on IDO activity *in vivo*, 13–17-week-old *Apc*$^{Min/+}$ mice were administered 1-L-mT or 1-D-mT in drinking water at the same concentration for 7 days, and the serum concentrations of L-kynurenine and L-tryptophan were measured using HPLC as previously reported[22] to calculate the kynurenine-to-tryptophan ratio.

Macroscopic analysis and sample preparation. The colon was opened longitudinally and examined for tumor number and size. Digital images were used to measure precise tumor dimensions, and the greatest dimension was adopted as the tumor size for each specimen. Macroscopically identified tumors of sufficient size were cut in half in the direction of the long axis. One half was fixed in 10% buffered formalin for 24 h at room temperature and processed for histological and immunohistological analyses. The other half of each colon tumor was analyzed by quantitative PCR. Smaller tumors were collected for either analysis.

Histological and immunohistochemical analyses. Paraffin-embedded sections (3-μm-thick) of the colon wall were prepared and stained with H&E for histological analysis. Cross-sections of colon wall were routinely cut in *Apc*$^{Min/+}$ mice and AOM/DSS-treated mice. Horizontal sections of colon wall were prepared in AOM alone-treated mice and the number of intramucosal microadenomas was counted on the H&E-stained section.[23] Immunohistochemical analyses were performed as described previously using the following primary antibodies: rabbit polyclonal anti-IDO (provided by K. Saito, Kyoto University),[24] rabbit polyclonal anti-Foxp3 (Abcam, Cambridge, UK), rabbit polyclonal anti-CD3 (DAKO, Glostrup, Denmark) and rat monoclonal anti-CD45R/B220 (clone RA3-6B2; BD Biosciences, San Jose, CA, USA). Double immunostaining for IDO and CD11c was performed as described previously with a slight modification.[16] Ethical approval for use of archival tissues of human CRC was provided by the Ethics Committee of Gifu University School of Medicine.

Quantitative real-time RT-PCR. Quantitative real-time RT-PCR of eight genes of interest (*Ifng*, *Tnf*, *Il-10, Tgfb*, *Foxp3*, *Cd3e*, *Ido1* and *Ido2*) was performed as previously described.[25] The PCR primers are listed in Table S1. The expression of each gene was normalized to β-actin (*Actb*) expression by using the standard curve method.

Statistical analysis. All data are presented as the mean ± SD. For comparisons between the two groups, the data were analyzed using the unpaired Student's *t*-test with Welch's correction or paired *t*-test. For comparisons between more than two groups, we used the Kruskal–Wallis test followed by Steel's test for comparison with controls. $P < 0.05$ was considered statistically significant.

Results

IDO expression is significantly increased in colon tumor tissue. We first tested whether IDO is expressed in mouse colon tumors as well as in human CRC[7–9] by means of immunohistochemistry. We examined colon tumors and intramucosal microadenomas (i.e. colonic pre-neoplastic lesions) that developed

in three different $Apc^{Min/+}$ mice (n = 7 and 122, respectively). The cytoplasmic IDO expression was significantly increased in the colon tumor tissues compared with that in the adjacent non-tumor tissues, whereas the expression remained at normal or slightly elevated levels in most of the microadenomas (24.6 and 63.1%, respectively) (Fig. 1a). In addition, IDO was highly expressed in the inflammatory cells infiltrating the tumor stromas, including CD11c-positive dendritic cells (Figs 1a and S1). A similar expression pattern of IDO was observed in human CRC (n = 5, Fig. 1b), as previously reported.[7–9]

***Ido1* deficiency does not significantly affect colon tumor development.** To investigate the role of IDO1 in colon tumorigenesis, the effects of *Ido1* deficiency were examined in various mouse models. First, based on the immunostaining data (Fig. 1a), we quantified the colon tumors that developed in $Apc^{Min/+}$;$Ido1^{(-/-)}$ and $Apc^{Min/+}$;$Ido1^{(+/+)}$mice. Macroscopic analysis revealed that there was no significant difference in the number and size of the tumors between these genotypes (Fig. 2a). In addition to the $Apc^{Min/+}$ mouse model, chemically induced colon tumorigenesis models were used. In the mice administered AOM alone, a small number of tumors were identified macroscopically (Fig. 2a); therefore, the number and size of intramucosal microadenomas were analyzed on the horizontal section of colon mucosa and no significant differences were observed between $Ido1^{(-/-)}$ and $Ido1^{(+/+)}$ mice (Fig. 2c).

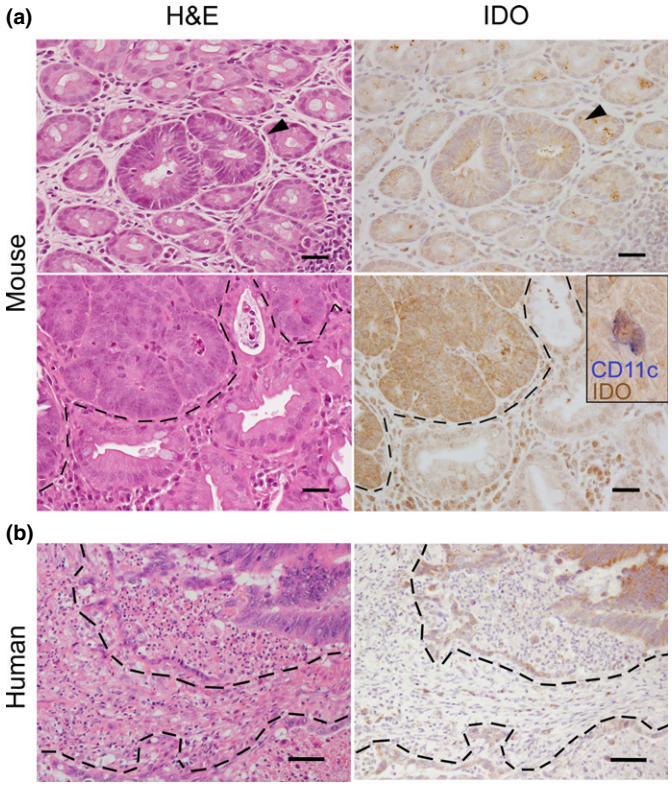

Fig. 1. Immunohistochemical staining for indoleamine 2,3-dioxygenase (IDO) in the colon lesions of $Apc^{Min/+}$ mice and human colorectal cancer (CRC). (a) Intramucosal microadenoma (upper) and adenocarcinoma (lower) developed in the colons of $Apc^{Min/+}$ mice. The inset in the lower panel shows a double positive cell for IDO (brown) and the dendritic cell marker CD11c (blue) in the tumor stroma (see also Fig. S1). (b) Human CRC. Arrowheads indicate microadenoma and the dashed line delineates the border between tumor tissues and non-tumor tissues. Scale bars = 70 μm.

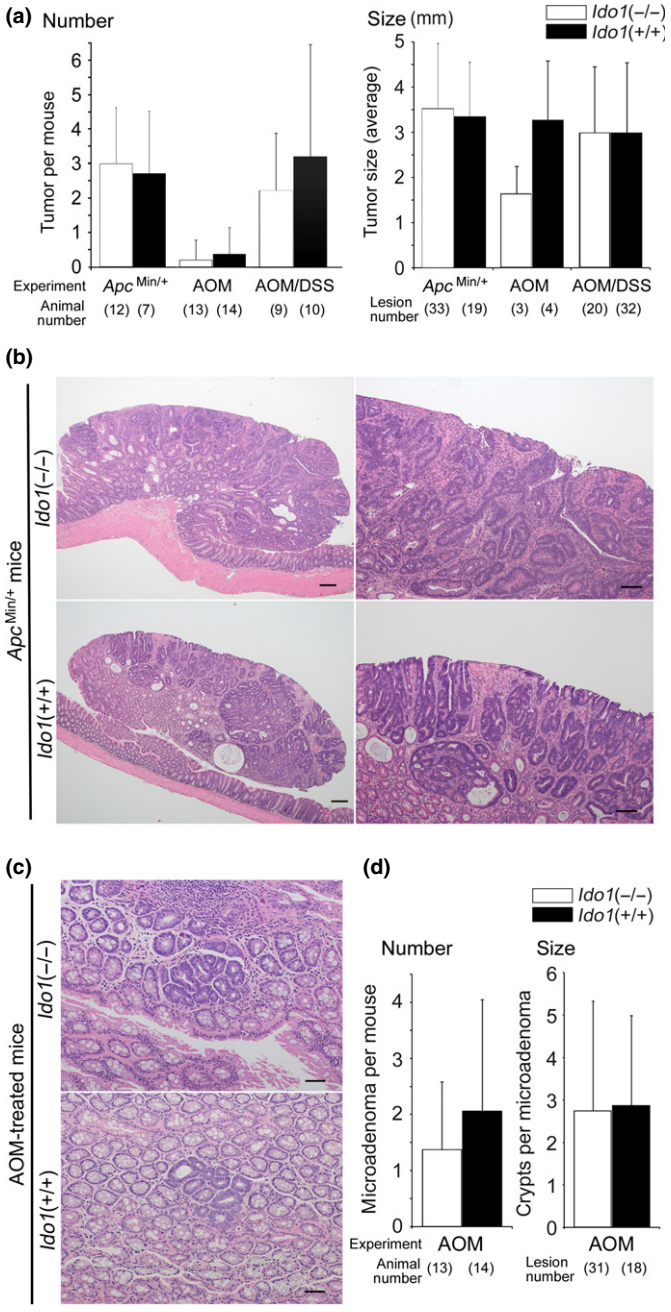

Fig. 2. Effects of *Ido1* deficiency on colon tumorigenesis in mice. (a) Average number and size of colon tumors in $Apc^{Min/+}$, azoxymethane (AOM)-treated, and AOM/dextran sulfate sodium (DSS)-treated mice. $Apc^{Min/+}$ mice were examined at the age of 20 weeks and AOM-treated and AOM/DSS-treated mice were examined 20 weeks after the first AOM injection. (b) Representative images of colon adenocarcinomas in $Apc^{Min/+}$ mice with the $Ido1^{(-/-)}$ and $Ido1^{(+/+)}$ genotypes. Scale bars: 200 μm (left panels) and 100 μm (right panels). (c) Representative images and the number and size (the number of aberrant crypts in each lesion) of intramucosal microadenomas in AOM-treated mice. Scale bars: 50 μm (left panels). Data are presented as the means ± SD.

Because the administration of AOM alone failed to induce a sufficient number of tumors, we used the colitis-associated tumorigenesis model in which mice were given the potent tumor promoter DSS following AOM administration. In the colitis-associated tumorigenesis model, the number of macroscopically identified colon tumors increased as expected and showed a tendency to be smaller in $Ido1^{(-/-)}$ mice than in

Table 1. Summary of microscopic analysis of colon tumors

	Number of tumors examined	Incidence (%)		Histological grade of adenocarcinoma (%)				
				Degree of differentiation			Degree of invasiveness	
		Adenoma	Adenocarcinoma	Well	Moderate	Poor	Mucosal invasion	Non-invasive
Apc$^{Min/+}$ mouse								
Ido1 (−/−)	21	7 (33.3)	14 (66.7)	5 (35.7)	9 (64.3)	0 (0.0)	6 (42.9)	8 (57.1)
Ido1 (+/+)	11	2 (18.2)	9 (81.8)	5 (55.6)	4 (44.4)	0 (0.0)	2 (22.2)	7 (87.8)
AOM-treated mouse								
Ido1 (−/−)	2	0 (0.0)	2 (100)	0 (0.0)	2 (100)	0 (0.0)	1 (50.0)	1 (50.0)
Ido1 (+/+)	3	1 (33.3)	2 (66.7)	0 (0.0)	2 (100)	0 (0.0)	1 (50.0)	1 (50.0)
AOM/DSS-treated mouse								
Ido1 (−/−)	13	2 (15.4)	11 (84.6)	7 (63.6)	4 (36.4)	0 (0.0)	6 (46.2)	5 (38.5)
Ido1 (+/+)	14	2 (14.3)	12 (85.7)	4 (33.3)	8 (66.7)	0 (0.0)	6 (42.9)	6 (42.9)
1-mT-treated *Apc*$^{Min/+}$ mouse								
1-L-mT treatment	31	5 (16.1)	26 (83.9)	11 (42.3)	15 (57.7)	0 (0.0)	12 (46.2)	14 (53.8)
1-D-mT treatment	32	2 (6.3)	30 (93.8)	11 (36.7)	19 (63.3)	0 (0.0)	18 (60.0)	12 (40.0)
Control	35	5 (14.3)	30 (85.7)	11 (36.7)	19 (63.3)	0 (0.0)	16 (53.3)	14 (4.7)

Ido1$^{(+/+)}$ mice, but this difference was not statistically significant owing to individual variability (Fig. 2a). All the colon tumors had similar sizes in the colitis-associated model (Fig. 2a). Subsequently, the tumors were microscopically analyzed in detail, but the *Ido1* deficiency did not affect the incidences of adenomas and adenocarcinomas or the histological grade of the tumors, including the degrees of invasiveness and differentiation, in any model (Table 1, Figs 2b and S2). These results indicated that *Ido1* deficiency does not significantly affect colon tumor development in mice.

***Ido1* deficiency alters cytokine expression in colon tumor microenvironment.** IDO is mainly expressed in the dendritic cells in tumor stroma and tumor draining lymph nodes where it suppresses immune reactions. In the present study, IDO-expressing dendritic cells were also observed in tumor stroma in mouse colon tumors (Fig. 1a, inset in the lower right panel and Fig. S1). Therefore, to investigate the effect of IDO1 deficiency on the production of pro-inflammatory and anti-inflammatory cytokines, we examined the mRNA expression levels of *Ifng*, *Tnf*, *Il-10* and *Tgfb* in the mouse colon tumor tissue. In both *Apc*$^{Min/+}$ and AOM/DSS-treated mice, the expression of *Ifng* in the colon tumor tissue was significantly higher in *Ido1*$^{(-/-)}$ mice than in *Ido1*$^{(+/+)}$ mice (Fig. 3a,b). *Ido1* deficiency also led to a significantly higher expression of *Tnf* in the colon tumors of *Apc*$^{Min/+}$ mice (Fig. 3a), while no significant difference was observed between mice with the *Ido1*$^{(-/-)}$ and *Ido1*$^{(+/+)}$ genotypes in AOM/DSS-treated mice (Fig. 3b). In contrast, there were no significant differences in the expression of *Il-10* and *Tgfb* between mice with the two genotypes in both *Apc*$^{Min/+}$ and AOM/DSS-treated mice (Fig. 3a,b). These results indicate that IDO1 alters the expression of pro-inflammatory cytokines in colon tumor tissue. In addition, *Ido2* expression was detected in mouse colon tumor tissues, similar to that reported in human CRC;[26] however, no significant difference was observed between mice with the *Ido1*$^{(-/-)}$ and *Ido1*$^{(+/+)}$ genotypes (Fig. 3a,b).

***Ido1* deficiency decreases regulatory T cells in colon tumor microenvironment.** IDO promotes the differentiation of naive T cells into Tregs and the migration of Tregs, which is thought to be one of the key mechanisms of immunosuppression.[27] Interestingly, a recent study showed the accumulation of regulatory T cells in intestinal tumors of *Apc*$^{Min/+}$ mice.[28] To clarify the effect of IDO deficiency on Treg infiltration in mouse colon tumors, we counted the number of lymphocytes that were positive for Foxp3 (a marker of Tregs) in tumor stroma by immunohistochemical analysis; and we then calculated the cellular density per unit area. In addition, expression of *Foxp3* and *Cd3e* mRNA in the homogenized tumor tissue was assessed by real-time PCR to calculate the *Foxp3/Cd3e* ratio, which serves as a molecular indicator of the proportion of Tregs to total T-cell content. Immunohistochemically, Foxp3-positive lymphocytes were scattered in tumor stroma (Fig. 4a). The density of Foxp3-positive lymphocytes in tumor stroma was significantly lower in *Ido1*$^{(-/-)}$ mice than in *Ido1*$^{(+/+)}$

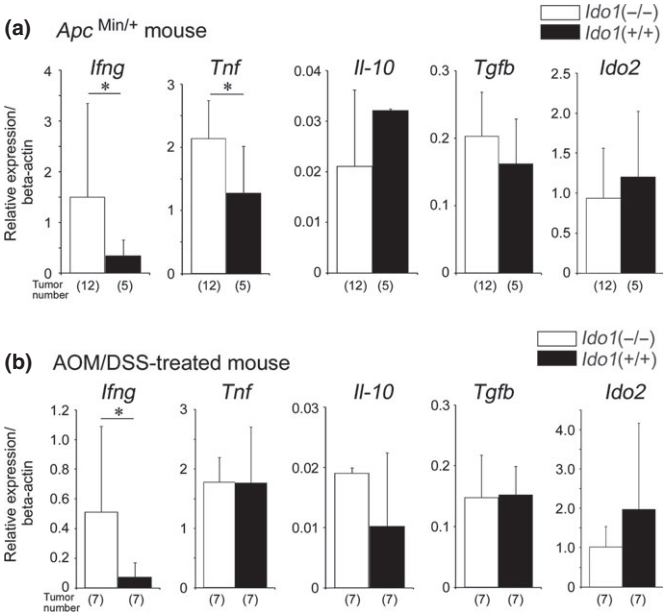

Fig. 3. Relative mRNA expression of pro-inflammatory and anti-inflammatory cytokines and *Ido2*, in colon tumor tissues in *Apc*$^{Min/+}$ (a) and AOM/DSS-treated mice (b). Data are presented as the means ± SD. **P* < 0.05 by Student's *t*-test.

mice, as determined after AOM/DSS treatment, whereas there were no significant differences between genotypes in the density of CD3-positive cells (Fig. 4c). The ratio of Foxp3/CD3-positive cells was slightly lower in *Ido1*$^{(-/-)}$ mice than in *Ido1*$^{(+/+)}$ mice after treatment with AOM/DSS and the *Foxp3/Cd3e* mRNA expression ratio was lower, but the differences were not statistically significant (Fig. 4c). Similarly, in *Apc*$^{Min/+}$ mice, although no significant differences were observed, the Treg density was lower in *Ido1*$^{(-/-)}$ mice than in *Ido1*$^{(+/+)}$ mice (Fig. 4b). Although there were fewer CD45R-positive cells than CD3-positive cells in the tumors, the density of CD45R-positive cells was significantly lower in *Ido1*$^{(-/-)}$ mice than in *Ido1*$^{(+/+)}$ mice after AOM/DSS treatment (Fig. 4c).

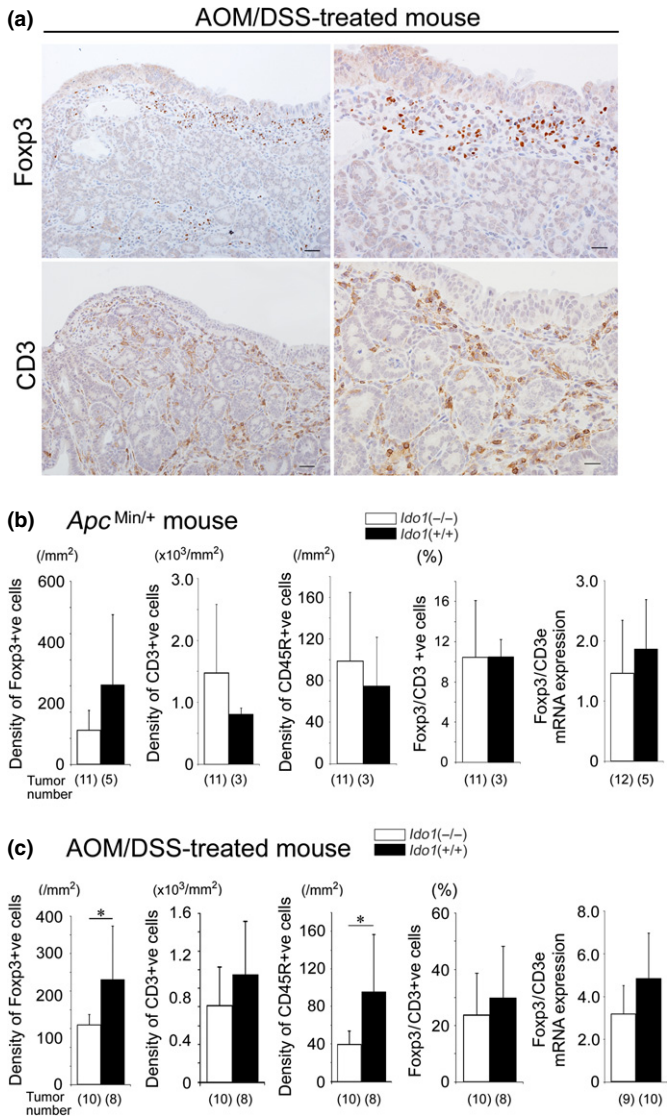

Fig. 4. Effects of *Ido1* deficiency on tumor-infiltrating lymphocytes in colon tumor tissues. (a) Immunohistochemical staining for Foxp3 (upper panels) and CD3 (lower panels) in colon tumors of *Apc*$^{Min/+}$ mice. Scale bars: 100 μm (left panels) and 20 μm (right panels). (b,c) The densities of Foxp3-positive cells (Tregs), CD3-positive cells (T cells) and CD45R-positive cells (B-cells), the ratio of Foxp3-positive cells to CD3-positive cells assessed by immunohistochemistry (IHC), and the ratio of *Foxp3/Cd3e* mRNA expression assessed by quantitative PCR in tumor tissues of *Apc*$^{Min/+}$ mice (b) and AOM/DSS-treated mice (c). Data are presented as the means ± SD. *$P < 0.05$ by Student's *t*-test.

These results indicate that IDO1 deficiency can suppress the differentiation into Tregs and/or migration of Tregs, and, thus, reduce the Treg population in colon tumor tissue.

IDO inhibitors do not reduce colon tumor burden, but alter cytokine expression in tumor microenvironments. The competitive IDO inhibitor 1-mT has two isomers, 1-L-mT and 1-D-mT.[29] In the preliminary assessment of the inhibitors, the serum kynurenine-to-tryptophan ratio decreased significantly only after 1-L-mT administration in *Apc*$^{Min/+}$ mice (Fig. 5a). However, considering that the 1-mT form actually used in clinical trials is 1-D-mT, and recent studies have suggested that its effect is unlikely to be dependent on inhibition of the enzymatic activity of IDO,[30,31] the longer-term effects of both isomers were investigated in the colon tumors of *Apc*$^{Min/+}$ mice. Macroscopically, the average number and size of colon tumors were similar in 1-L-mT-treated, 1-D-mT-treated and control groups (Fig. 5b). In addition, the microscopic examination revealed that there were no significant differences in histological grades of the colon tumors and the degree of inflammation among 1-L-mT, 1-D-mT and control groups (Table 1). In contrast, although it was not possible to simply define the effects of the IDO inhibitors as immunopotentiating, the cytokine expression in the colon tumor tissues changed significantly following 1-L-mT or 1-D-mT treatment (Fig. 5c). The 1-L-mT treatment significantly decreased the expression of *Il-10* and *Tgfb*, consistent with the immunosuppressive role of IDO, but also significantly decreased *Tnf* expression (Fig. 5c). Similarly, the 1-D-mT treatment significantly changed the expression of *Il-10* and *Tnf* (Fig. 5c). The expression of *Ido1* and *Ido2* was also examined to investigate whether IDO expression was changed in response to IDO inhibitors, but it decreased in the colon tumors of 1-L-mT-treated and 1-D-mT-treated mice. These results indicate that IDO inhibitors do not affect colon tumor development but can alter cytokine expression in colon tumor tissues in mice.

Discussion

Increased IDO expression has been reported in human CRC,[7–9] but its biological significance is yet to be fully elucidated. Because previous rodent studies were conducted in a chemically-induced colitis-associated model,[13,14] we used an *Apc*$^{Min/+}$ mouse model to evaluate the role of IDO in colon tumorigenesis separately from colitis. In the present study, by immunohistochemistry, we show that IDO is expressed in mouse colon tumors as well as in human CRC, suggesting that IDO has some roles in mouse colon tumorigenesis. However, our results show that *Ido1* deficiency did not lead to any changes in the number, size and histological grade of the colon tumors developed in *Apc*$^{Min/+}$ mice. Consistent with this result, the pharmacological inhibition of IDO also did not have any effect on colon tumor development in *Apc*$^{Min/+}$ mice. These results suggest that IDO inhibition alone is not sufficient to affect colon cancer development. Although we did not use the colitis-associated tumorigenesis model, intratumoral inflammation would still be involved in the development of colon tumors. IDO inhibition would have a promoting effect on colon tumorigenesis through intratumoral inflammation enhancement, but would also exert a suppressing effect by enhancing the anti-tumor immune response. Therefore, the lack of an effect of IDO inhibition on colon tumor development might reflect a balance of the promotion and suppression effects. In contrast, in human CRC, higher IDO expression was significantly correlated with the frequency of liver metastasis,[7] and IDO1 expression at the invasive front

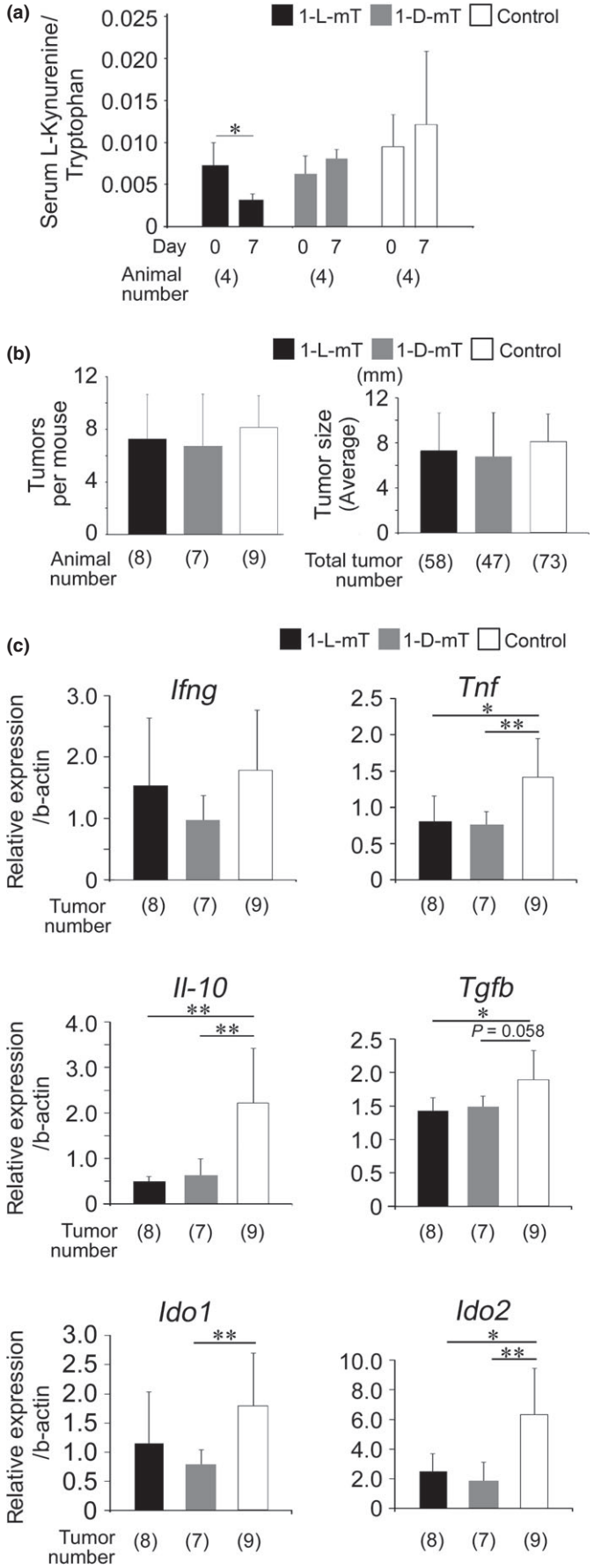

Fig. 5. Effects of indoleamine 2,3-dioxygenase (IDO) inhibitors in colon tumors of $Apc^{Min/+}$ mice. (a) Serum kynurenine-to-tryptophan ratio of $Apc^{Min/+}$ mice treated with 1-L-mT or 1-D-mT for 7 days. Data are presented as the means ± SD. $^*P < 0.05$ by paired *t*-test. (b) Average number and size of colon tumors in $Apc^{Min/+}$ mice treated with 1-L-mT or 1-D-mT. $Apc^{Min/+}$ mice were examined at the age of 20 weeks. (c) Relative mRNA expression of cytokines and IDO in colon tumor tissues of 1-L-mT-treated and 1-D-mT-treated mice. Data are presented as the means ± SD. $^*P < 0.05$ by Student's *t*-test.

was significantly associated with overall survival, suggesting a role for IDO in tumor invasion.[8] The findings from human CRC suggest the possibility that IDO may be involved in tumor progression at more advanced stages; however, it is difficult to evaluate this in mouse models owing to the lack of available models that reproduce highly invasive and metastatic colon tumors. Furthermore, although the enzymatic activity of IDO2 has been reported to be far less than that of IDO1[30,31] and the compensatory increases in *Ido2* mRNA expression were not observed in colon tumors of $Ido1^{(-/-)}$ mice, we cannot completely exclude the possibility that IDO2 may play a complementary role in IDO1-deficient conditions. In addition, a recent study revealed the novel function of IDO2 as a negative regulator of IDO1.[32] While IDO1 expression suppressed cell growth with downregulation of cell survival-related proteins, IDO2 co-expression alleviated IDO1-dependent cell growth suppression.[32] Considering the expression of both *Ido1* and *Ido2* in human CRC,[26] inhibition of IDO2 rather than IDO1 can lead to tumor growth suppression. Further investigations are needed to elucidate the contribution of IDO2 expression to colon carcinogenesis.

Genetic deficiency of *Ido1* has been reported to significantly affect the development of colitis-associated colon tumors in mice. However, two previous studies gave opposite results by showing a significant increase and decrease in colon tumors in $Ido1^{(-/-)}$ mice, respectively.[13,14] In the present study, no statistically significant difference was observed in AOM/DSS-treated mice, but the colon tumor number was smaller in the $Ido1^{(-/-)}$ mice than in the $Ido1^{(+/+)}$ mice. This supports the hypothesis that IDO1 inhibition suppresses the development of colitis-associated tumors. However, considering the relationship between inflammation and CRC,[33] the application of IDO-targeting therapy to patients with colitis-associated tumors seems to be impractical. It has been reported that IDO inhibition does not increase the severity of DSS-induced colitis if DSS is administered at low doses,[34,35] as in the colitis-associated tumor model used in previous studies[14] and in the present study. In contrast, it has been repeatedly demonstrated that IDO expression is elevated in animal models of colitis, and that IDO inhibition leads to significantly more severe colitis.[15,16,35] Similarly, IDO1 is highly upregulated in human colon in inflammatory conditions, such as inflammatory bowel disease; this upregulated IDO1 expression is thought to be responsible for suppressing excessive inflammatory reactions.[17,18] These results imply that IDO inhibition could pose a risk of promoting tumors via worsening of inflammation in patients with colitis-associated tumors.

The role of IDO in the colon tumor microenvironment remains largely unknown. The most important finding of the present study is that IDO inhibition led to significant changes in the inflammatory response within the tumors, although it did not affect the colon tumor development in mice. In the $Ido1^{(-/-)}$ mice, the expression of pro-inflammatory cytokines increased significantly in the colon tumors of $Apc^{Min/+}$ and

AOM/DSS-treated mice. Because the kynurenine pathway suppresses the differentiation of T helper type 1 (Th1) cells,[3] IDO1 deficiency may cause increased expression of the Th1-type cytokine IFN-γ in local tissues. It should be mentioned that, while not only genetic deletion but also pharmacological inhibition of IDO altered the expression of cytokines, there was no consistent expression change pattern. IDO inhibitors significantly reduced the expression of anti-inflammatory cytokines. In particular, *Il-10* expression was markedly decreased following both 1-L-mT and 1-D-mT treatments. However, because the decreased *Tnf* expression was observed in both 1-L-mT-treated and 1-D-mT-treated mice, it is not possible to describe the effect of IDO inhibitors in colon tumors as immunopotentiating. 1-L-mT and 1-D-mT were previously thought to selectively inhibit IDO1 and IDO2, respectively.[29] However, recent studies have shown that 1-L-mT inhibits not only IDO1 but also IDO2, although it is more effective for IDO1 than for IDO2.[30,31] Interestingly, it has been shown that *Ido2*$^{(-/-)}$ mice displayed significantly lower cytokine induction, including the induction of IFN-γ, and TNF-α, in a contact hypersensitivity model whereas these cytokine levels were elevated in *Ido1*$^{(-/-)}$ mice.[36] Therefore, the decrease in the expression of *Ifng* and *Tnf* in 1-L-mT-treated mice might be due to IDO2 inhibition, while the significant elevation in the expression of *Ifng* and *Tnf* in *Ido1*-deficient mice was caused by IDO1 deficiency. The cytokine expression change pattern was similar in 1-L-mT-treated and 1-D-mT-treated mice, but the underlying mechanism is likely to be different. In a previous study using recombinant mouse IDO, 1-D-mT has been shown to be a poor inhibitor of both mouse IDO1 and IDO2 without a detectable inhibition of IDO2 activity, thereby suggesting that its immunomodulatory effect can be independent of the inhibition of IDO enzymes in mice.[30] Consistent with this finding, the serum kynurenine-to-tryptophan ratio was not changed after 1-L-mT administration in *Apc*$^{Min/+}$ mice.

IDO promotes the differentiation into Tregs and migration of Tregs, and, thus, plays an important role in suppressing T-cell-mediated inflammation.[27,37] Previous studies revealed that IDO expression correlated with increased Tregs infiltration in several human tumors, including glioma[38] and breast cancer.[39] In line with these findings, we found that *Ido1* deficiency resulted in a significant decrease in the number and proportion of Tregs in colon tumor stroma in the AOM/DSS-treated mice. The accumulation of Tregs in local tissues can also contribute to the immune evasion of tumors.[40–42] It has also been reported that elimination of Tregs resulted in a decrease in the number of skin papillomas, with an improved antitumor immune response in mice.[43] Therefore, it is possible that the decreased number of Tregs in colon tumors contributed to a reduced tumor burden in AOM/DSS-treated mice. A recent study showed that various lymphocyte subsets infiltrate mouse colon tumors using flow cytometry;[44] therefore, further studies are needed to comprehensively examine the changes in lymphocyte subsets following IDO inhibition.

In mouse colon tumors, IDO expression was observed not only in tumor cells but also in tumor infiltrating cells, including dendritic cells. Considering the previous findings in human tumors, both cell types are likely to contribute to IDO-dependent immune regulation in the tumor microenvironment. In human CRC, higher levels of IDO expression in tumor cells were associated with a significant reduction in tumor-infiltrating T cells.[7] Alternatively, although IDO was identified only in tumor-infiltrating cells and not in tumor cells in human hepatocellular carcinomas, IDO mRNA levels correlated significantly with the cytokine gene expression,[45] thereby suggesting that IDO-expressing tumor-infiltrating cells play an independent role in cytokine regulation.

In summary, we have demonstrated that genetic deletion or pharmacological inhibition of IDO1 alters immune responses, such as cytokine expression, and the number of Tregs in the colon tumor microenvironment, but does not significantly affect mouse colon tumor development. These results suggest that IDO inhibition alone would not be sufficient to suppress colon cancer development in mice despite its immunomodulatory activity in the tumor microenvironment.

Acknowledgments

The authors thank Kyoko Takahashi, Ayako Suga and Masayoshi Shimizu for technical assistance, Hideki Nikami at Gifu University for supply of the mice and Yasuko Yamamoto at Kyoto University for providing anti-IDO antibody.

Disclosure Statement

The authors have no conflict of interest to declare.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1. Double-immunostaining for indoleamine 2,3-dioxygenase (IDO) and the dendritic cell marker CD11c in colon tumors of $Apc^{Min/+}$ mice.

Fig. S2. Representative images of colon lesions in azoxymethane (AOM)/dextran sulfate sodium (DSS)-treated and AOM-treated mice with $Ido1^{(-/-)}$ and $Ido1^{(+/+)}$ genotypes.

Table S1. Primers used for quantitative real-time PCR.