The absence of LPA$_2$ attenuates tumor formation in an experimental model of colitis-associated cancer

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Abstract

Background & Aims—Chronic inflammation is a risk factor for colon cancer (CC). Lysophosphatidic acid (LPA), a naturally produced phospholipid, mediates multiple effects that are vital to disease process, including inflammation and cancer. The expression of LPA receptor 2 (LPA$_2$) is up-regulated in several types of cancer, including ovarian and colon cancer, but the importance of LPA and LPA$_2$ in the development and progression of CC is unclear. In this study, we sought to determine whether LPA and LPA$_2$ regulate the progression of CC *in vivo*.

Methods—We examined the potential role of LPA in CC progression by administering LPA to Apc$^{Min/+}$ mice. We determined the loss of LPA$_2$ function in tumorigenesis in the colon by treating mice with genetic deletion of LPA$_2$ (LPA$_2^{-/-}$) with azoxymethane (AOM) and dextran sulfate sodium (DSS).

Results—We found that LPA increased tumor incidence in Apc$^{min/+}$ mice. LPA$_2^{-/-}$ mice showed reduced mucosal damage and fewer tumors than wild-type (WT) mice. Reduced epithelial cell proliferation and decreases in β-catenin, Krüppel-like factor 5 (KLF5), and cyclooxygenase-2 (COX-2) expression were observed in LPA$_2^{-/-}$ mice. Unlike WT mice, induction of monocyte chemoattractant protein-1 (MCP-1) and macrophage migration inhibitory factor (MIF) was significantly attenuated in LPA$_2^{-/-}$ mice with reduced infiltration by macrophages.

Conclusion—These results show that LPA is capable of promoting tumorigenesis in the colon. The absence of LPA$_2$ attenuates several effects that contribute to cancer progression *in vivo* and, hence, the current study identifies LPA$_2$ as an important modulator of CC.

INTRODUCTION

CC is the fourth most common cancer and inflammation is an established risk factor for CC [1]. Patients with inflammatory bowel disease are at increased risk for CC and the mortality in patients diagnosed with CC in the setting of IBD is higher than for sporadic colorectal cancer [1].

LPA is an extracellular lipid mediator that evokes multiple growth factor-like effects in almost every cell type [2, 3]. LPA mediates its effects primarily by coupling to a family of G protein-coupled receptors, LPA_1~LPA_5 [4]. The initial indication that LPA could contribute to tumorigenesis came from studies showing that LPA increases cell proliferation and motility [5]. Subsequently, over-expression of LPA_2 in ovarian cancer cells has suggested that aberrant LPA-LPA_2 signaling axis might have a tumor promoting activity in ovarian cancer [6, 7]. Recent evidence shows that deregulation of LPA_2 or LPA_3 is more commonly found in other types of cancer [8–10].

Mice deficient in individual LPA receptors are available [11–13]. Whereas mice with targeted deletion of LPA_1 or LPA_3 show receptor-specific defects such as craniofacial deformity or delayed implantation of embryos [11, 13], genetic ablation of LPA_2 in mice ($LPA_2^{-/-}$) does not cause any obvious phenotypic defect [12]. Moreover, transgenic expression of LPA_2 in ovaries did not result in ovarian malignancy in mice [14]. Although the absence of tumors in LPA_2 transgenic mice implies a non-essential or redundant role of LPA_2 in tumorigenesis, a body of *in vitro* experimental evidence tends to suggest the insufficiency of LPA-LPA_2 signaling axis to induce malignancy in ovary [14]. Therefore, the significance of LPA_2 in tumorigenesis remains unclear and specific pathways affected are not known.

AOM, a metabolite of 1,2-dimethylhydrazine, has been used widely to induce the formation of precancerous epithelial lesions, aberrant crypt foci (ACF) [15]. Together with AOM, repeated oral administration of DSS is widely employed to induce acute inflammatory reaction and ulceration in the entire colon, and accelerates and increases the incidence of colon carcinogenesis [16]. The potential role of LPA in inflammation has extensively been studied [17] and chronic inflammation increases the risk of CC [1]. In this study we investigated the loss of LPA_2 function in colitis-associated cancer (CAC). Our study revealed that the extent of colon carcinogenesis was markedly decreased in $LPA_2^{-/-}$ mice with decreased epithelial cell proliferation and infiltration of inflammatory leukocytes.

METHODS

Mice

$LPA_2^{-/-}$ mice [12] were bred into C57BL/6 background for at least 10 generations. $LPA_2^{+/-}$ mice were crossbred to derive WT (i.e., $LPA_2^{+/+}$), $LPA_2^{+/-}$, and $LPA_2^{-/-}$ littermates and these littermates were used in all studies. Experiments with animals were carried out under approval by the Institutional Animal Care and Use Committee of Emory University.

LPA treatment

Six weeks old male C57BL/6 and $Apc^{Min/+}$ mice (Jackson Laboratory) were given 1 μg/kg LPA suspended in 0.1% BSA containing PBS (~0.06 μmole per mouse; Avantis) by placing LPA into the stomach using a 22-gauge gavage needle once every three days for one month. Control animal received the same volume of PBS/0.1 % BSA. Animals were killed one month after the last LPA administration. The small intestine was removed, flushed with ice-cold PBS, cut open longitudinally along the main axis, and examined under a dissecting microscope for the presence of adenomas.

AOM and DSS treatment

WT, $LPA_2^{+/-}$, and $LPA_2^{-/-}$ littermates of 6 to 8 week-old age were injected with 10 mg/kg of AOM (Midwest Research Institute) intraperitoneally at the beginning of the experiment. After 14 day, mice were given 3% DSS (Sigma-Aldrich) in drinking water for 7 days, followed by a 2 week-period of recovery with normal water. This was followed by another 1-week 3% DSS treatment and 2-weeks of normal water. Body weight of each mouse was measured and recorded daily. Mice were killed on day 36 or day 56. Colon was removed, flushed with ice-cold PBS, cut open longitudinally, and examined under a dissecting microscope for the presence of tumors as previously described [16]. Colonic tissues were fixed in 10% buffered formalin overnight for histological analysis. Paraffin embedded sections were stained with hematoxylin and eosin (H&E) for microscopic assessment of colitis.

AOM treatment

$LPA_2^{-/-}$ and WT littermates of 5–6 weeks old age received 4 weekly intraperitoneal injections of AOM at 10 mg/kg body weight. Four weeks after the last AOM injection, all mice were killed, and their colons were removed and fixed in 4% paraformaldehyde. Fixed tissues were stained with methylene blue and ACF were visualized by stereo microscopy. ACF were identified using a published set of criteria [18].

Immunohistochemistry

See Supplementary Materials.

Myeloperoxidase Activity

See Supplementary Materials.

Quantitative RT-PCR (qRT-PCR)

See Supplementary Materials.

Statistic Analysis

Data are expressed as means ± SE. Statistical significance was determined by one-way ANOVA. *p*-values <0.05 were considered significant.

RESULTS

Effect of LPA on the development of adenomas in Apc^Min/+ mice

Previous studies have shown that LPA_2 expression is elevated in human CC patients and cell lines [9, 10]. qRT-PCR analysis showed that LPA_2 mRNA level was elevated 3.5 ± 0.78 fold (n = 6) in intestinal adenomas of Apc^Min/+ mice compared to normal intestinal tissue from WT controls (Figure 1A). There was a small statistically significant increase in LPA_1 in Apc^Min/+ mice (P < 0.05) as well, but LPA_3 expression was not different.

LPA plays a vital role in intestinal wound healing, cell proliferation, cell survival, cytokine induction, and regulation of ion transport [9, 10, 19–22]. We initially tested whether LPA regulates tumorigenesis in the colon by orally administering LPA to Apc^Min/+ mice. Although the stability of orally administered LPA may be a concern [23], a previous study has shown that orally administered LPA is effective in inhibition of fluid secretion in the ileum, demonstrating the bioavailability of orally administered LPA in the intestine [22]. Administration of LPA increased the number of adenomas (60 ± 5.2, n = 9, P < 0.001) in Apc^Min/+ mice compared to Apc^Min/+ mice that received carrier (32 ± 6.7, n = 9) (Figure 1B). This result shows that LPA

may potentiate tumorigenesis in the colon, although whether this effect was mediated by LPA_2 is unclear.

Reduced tumor incidence in $LPA_2^{-/-}$ mice

We next sought to determine the importance of LPA_2 in colon tumorigenesis by characterizing the loss of LPA_2 function *in vivo*. To this end, we used a CAC model, which combines the treatment of mice with AOM and DSS (Figure 2A). AOM and DSS induced colitis in WT, $LPA_2^{+/-}$, and $LPA_2^{-/-}$ littermates as evidenced by the weight loss (Figure 1B) and appearance of soft stool, but a significant difference in weight loss between WT and $LPA_2^{-/-}$ mice was observed. Similarly, the clinical score, which quantifies the appearance of occult blood and diarrhea, was significantly lower in $LPA_2^{-/-}$ than WT littermates (Supplementary Figure 1A). Weight loss in $LPA_2^{+/-}$ mice was similar to that in WT. Moreover, 3 each of WT and $LPA_2^{+/-}$ mice died by day 56 as compared to none among $LPA_2^{-/-}$ mice (Figure 2C). WT and $LPA_2^{+/-}$ mice developed multiple polypoid tumors in the middle to distal colon, whereas tumors were fewer and smaller in $LPA_2^{-/-}$ mice (Figure 2D). The colons of WT and $LPA_2^{+/-}$ mice were heavier than that of $LPA_2^{-/-}$ mice, indicating occurrence of edema in these mice (Supplementary Figure 1B). There was no difference in macroscopic or microscopic features among untreated mice.

The mean number of tumors in the colon of $LPA_2^{-/-}$ mice was 0.7±0.2 at day 36 vs. 1.5±0.6 for WT and 1.8±0.5 for $LPA_2^{+/-}$ (Figure 3A) (n = 8, P < 0.05). At day 56, an average of 13.8 ±1.8 and 13.1±1.1 adenomas per animal was found in WT and $LPA_2^{+/-}$ mice, respectively (n = 10, P < 0.01). In contrast, $LPA_2^{-/-}$ mice developed 8.1±1.6 lesions per animal (Figure 3B). Furthermore, the numbers of tumors larger than 2 mm were significantly less in $LPA_2^{-/-}$ mice than in WT or $LPA_2^{+/-}$ mice (Figure 3C; 2.8±0.7 for $LPA_2^{-/-}$ vs. 6.8 ± 0.8 for WT and 7.3 ± 0.5 for $LPA_2^{+/-}$ mice). Histological examination of colonic sections showed no notable difference between untreated WT and $LPA_2^{-/-}$ littermates (Figure 3D). By day 36, tubular adenomas were present in WT and $LPA_2^{+/-}$ mice, but the numbers and size of lesions were comparatively smaller in $LPA_2^{-/-}$ mice. At day 56, the numbers and size of lesions were progressively increased in all mice, but the polyps were flatter and smaller in $LPA_2^{-/-}$ mice compared to those in WT or $LPA_2^{+/-}$ mice. Together these results show that the absence of LPA_2 protected animals from the combination treatment of AOM and DSS and suggest a crucial role of LPA_2 in the progression of tumor in the colon. Heterozygosity of LPA_2, however, was not sufficient for the protection and we will not display data from $LPA_2^{+/-}$ mice hereafter.

Decreased Cell Proliferation in $LPA_2^{-/-}$ mice

The difference in tumor size between WT and $LPA_2^{-/-}$ mice suggests that there may be an underlying difference in the rate of cell proliferation. Epithelial cell proliferation was examined by immunohistological staining of Ki67. In untreated mice, Ki67 labeling was confined to the nuclei of epithelial cells located at the base of colonic crypts in WT and $LPA_2^{-/-}$ mice and no observable difference was noted between WT and $LPA_2^{-/-}$ mice (Figure 4A and D). Following AOM/DSS treatment, WT mice had a large population of proliferating cells in non-tumorous crypts as well as in tumors (Figure 4B and C). Ki67 labeling extended from the base of the crypt to more than half way of the crypt height and elongation of the crypt height compared to untreated animals was evident (Figure 4B). On the contrary, Ki67-positive epithelial cells were largely limited to the base region of the crypt in $LPA_2^{-/-}$ mice (Figure 4E and F), suggesting that LPA_2 is involved in proliferation of epithelial cells in the crypts following AOM and DSS treatment.

β-catenin and KLF5 expression in $LPA_2^{-/-}$ mice

Previous studies have linked the proliferation of CC cells by LPA to the regulation of β-catenin and KLF5 [19, 20]. To determine whether differential expression of β-catenin contributes to the

cell proliferation, we examined β-catenin expression in WT and LPA$_2^{-/-}$ mice. Figure 5A shows immunohistological staining of β-catenin in epithelial cells of colonic section. In treated WT mice, β-catenin labeling was more evident in the cytoplasm and nuclei of epithelial cells in adenomas (Figure 5B–c and b vs. a). In comparison, up-regulation of β-catenin in LPA$_2^{-/-}$ mice was substantially lower and less nuclear staining was observed, demonstrating that LPA$_2$ regulates activation of β-catenin *in vivo* [19, 20].

Figure 5B shows that KLF5 protein expression was significantly up-regulated in treated WT mice. Immunohistological staining of KLF5 shows that all epithelial cells in the crypts of WT and LPA$_2^{-/-}$ mice were positive for KLF5 expression (Figure 5C-a and d). In treated WT mice (Figure 5C-b), the staining of KLF5 was more intense in the base of the crypt and KLF5 labeling extended to the epithelial cells near the surface. In comparison, a small increase in KLF5 expression was observed in AOM/DSS-treated LPA$_2^{-/-}$ mice (Figure 5B) and the neck of the colon crypts were less populated with KLF5-positive cells in these mice relative to WT mice (Figure 5C–e and f). Together, these results suggest that the differential expression of β-catenin and KLF5 might provide the molecular basis for the decreased cell proliferation in AOM and DSS treated LPA$_2^{-/-}$ mice.

Reduced COX-2 expression in LPA$_2^{-/-}$ mice

An increase in COX-2, which generates prostaglandin E$_2$ (PGE$_2$) from arachidonic acid, has been associated with the progress of CC [24]. Hence, we examined the expression of COX-2 proteins by Western blot and immunohistochemical analysis. The expression level of COX-2 protein was slightly lower in untreated LPA$_2^{-/-}$ mice than untreated WT mice (Figure 5D). This difference can also be seen in immunohistochemical staining (Figure 5E, a vs. d). AOM/ DSS treatment resulted in a significant increase in COX-2 protein expression in WT mice. Epithelial expression of COX-2 in colonic crypt of WT mice was observed (Figure 5E-b), but COX-2 expression was largely found in lamina propria cells (Figure 5E-c). Induction COX-2 expression in LPA$_2^{-/-}$ mice was markedly lower compared with WT mice (Figure 5D, 5E-e and f) and clearly fewer COX-2 expressing lamina propria cells were found in LPA$_2^{-/-}$ mice.

Cytokine induction in LPA$_2^{-/-}$ mice

Previous studies have shown that LPA can induce secretion of cytokines and chemokines from cancer cells, including CC cells [10, 25, 26]. We contemplated whether there was a difference in the expression of cytokines and chemokines between WT and LPA$_2^{-/-}$ mice. The expression levels of various cytokines and chemokines were determined by qRT-PCR and the results are shown in Figure 6A. There was no apparent difference in TNF-α, INF-γ, IL-1β, and IL-6 expression in untreated WT and LPA$_2^{-/-}$ mice, and AOM and DSS markedly stimulated expression of these cytokines in both mice. Although the expression levels of TNF-α, INF-γ, IL-1β, and IL-6 in treated LPA$_2^{-/-}$ mice were lower compared to WT mice, they did not reach statistical significance. In contrast, we observed a significant difference in the expression of MCP-1 and MIF between WT and LPA$_2^{-/-}$ mice. Whereas the expression levels of MCP-1 and MIF were elevated more than 3-fold in treated WT mice, small increases were observed in LPA$_2^{-/-}$ mice. Moreover, the basal expression levels of MCP-1 and MIF were lower in LPA$_2^{-/-}$ than WT mice. The induction of MCP-1 and MIF was confirmed *in vitro* by treating Caco-2 cells with LPA (Supplemental Figure 2). Together, these results suggest LPA$_2$-mediated signaling specifically regulates the expression of pro-inflammatory factors such as MCP-1 and MIF.

Infiltration of inflammatory cells

Early during tumor formation, stromal cells and tumor-infiltrating leukocytes provide signals that regulate cancer cell growth and differentiation. Because MCP-1 mRNA level was markedly decreased in LPA$_2^{-/-}$ mice, we next determined whether the lack of LPA$_2$ affected

infiltration of inflammatory cells. Histological analysis showed massive infiltration of neutrophils in AOM/DSS-treated mice and quantification of neutrophil infiltration is shown in Figure 6C. Marked increases in MPO activity were observed in WT and $LPA_2^{-/-}$ mice, but with no difference between the two groups, implying that infiltration of neutrophils was not affected by the absence of LPA_2. On the contrary, immunohistochemical analysis using an anti-Mac-3 antibody showed extensive infiltration of macrophages in WT mice, whereas a reduction in the number of macrophages in the lamina propria of $LPA_2^{-/-}$ mice was evident. These results show that a decrease in macrophage infiltration is a part of the protective mechanisms rendered by the absence of LPA_2.

AOM-induced neoplasia

The combination treatment of AOM and DSS relies on inflammatory response evoked by DSS intake, which hasten the development of adenocarcinomatous lesions [16]. To determine whether enhanced inflammatory response is needed to observe the protective effect of loss of LPA_2 function, we subjected WT and $LPA_2^{-/-}$ mice with AOM alone. WT and $LPA_2^{-/-}$ mice both developed ACF, but significantly smaller numbers of ACF were found in the colons of $LPA_2^{-/-}$ mice than in WT control mice (Figure 7). The mean number of ACF in WT mice was 24.3 ± 2.1 vs. 11.6 ± 1.5 for $LPA_2^{-/-}$ mice (n = 9, P < 0.01). No ACF was found in saline-treated WT or $LPA_2^{-/-}$ mice (data not shown). These results show that the loss of LPA_2 function impeded the formation of early neoplastic lesions in the colon without onset of inflammation.

DISCUSSION

The prominent production of LPA and the effects of LPA in most ovarian and prostate cancer cells along with over-expression of LPA_2 have suggested the autocrine system of LPA-LPA_2 axis promotes growth and invasion of such cancers [3, 6, 7]. More recently, over-expression of LPA_2 in colon cancer patients has been shown and suggests a potential role of LPA_2 in colon cancer [9, 10]. However, the absence of tumor in ovaries of LPA_2 transgenic mice has left the role of LPA_2 in tumorigenesis uncertain [14]. In this study, we determined the importance of LPA_2 in CC by characterizing the loss of LPA_2 function in mice. By challenging $LPA_2^{-/-}$ mice with agents that induce dysplasia in the colon, we were able to reveal the pathological importance of LPA_2 in CC. Our data herein demonstrate that the absence of LPA_2 results in a marked decrease in tumor incidence and progression of colon adenocarcinomas. The amelioration of CC in $LPA_2^{-/-}$ mice was accompanied by a decrease in both cell proliferation and chemokine expression, providing insights into the protection against CC in $LPA_2^{-/-}$ mice. Although our study was focused on the characterization of a CAC model, the propensity to protect animals was consistent in the study using AOM alone. To our knowledge, the current study is the first direct evidence demonstrating the importance of LPA_2 in colon carcinogenesis *in vivo* and our data suggest LPA_2 as a potential target for preventive strategies for CC.

The absence of tumors in the transgenic LPA_2 ovaries suggests incomplete capacity of the LPA-LPA_2 signaling axis to initiate cancer and it has been suggested that LPA_2 might increase the susceptibility to develop cancer or hasten cancer progress when other genetic factors are present [14]. This is in part supported by our current study in which LPA increased the number and size of adenomas in $Apc^{Min/+}$ mice that are predisposed to forming adenomas in the small intestine [27]. However, our study does not specifically address whether the increase in adenomas in $Apc^{min/+}$ mice by LPA resulted from signaling by LPA_2 despite upregulation of LPA_2 expression in these mice. A further study based on deletion of LPA_2 in $Apc^{Min/+}$ background is needed to fully recapitulate this aspect.

The Wnt/β-catenin cascade plays a single most dominant role in controlling cell fate along the crypt-villus axis. Once the Wnt/β-cascade is mutationally activated, the adenoma cells maintain

their progenitor status populating entire villi [28]. LPA had been shown to inactivate glycogen synthase kinase-3 in NIH3T3 and HEK293 cells although nuclear accumulation of β-catenin by LPA was not reported in these cells [29]. On the other hand, Yang et al. [19] have shown that LPA activates the β-catenin pathway in HCT116 and LS174T human CC cells, resulting in an accumulation of β-catenin in the nucleus. However, we previously reasoned that LPA might be able to influence proliferation of CC cells independent of the Wnt/β-catenin cascade as the Wnt/β-catenin cascade is often mutated in CC cells [20]. This led us to identify KLF5 as an additional target of LPA-elicited signaling that regulates proliferation of CC cells [20]. KLF5, a member of a family of zinc finger-containing transcription factors, is predominantly expressed in the crypt compartment of intestine where cells rapidly replicate [30]. Our data show a number of features demonstrating LPA_2-dependent proliferation of epithelial cells in the crypts. AOM and DSS induced hyperplasia, as evidenced by the expansion of Ki67-positive cells and concurrent up-regulation of β-catenin and KLF5 expression in WT mice. In contrast, the crypt height of $LPA_2^{-/-}$ mice was not only shorter but proliferating cells were largely confined to the crypt base along with significantly lower presence of nuclear β-catenin and KLF5. β-catenin and KLF5 are transcriptional factors, which can activate targets such as c-Myc, cyclin D, cyclin B1, and Cdc2 [28, 30]. In addition, LPA has previously been shown to induce the generation of reactive oxygen species and subsequent activation of downstream genes [31]. Hence, this ability of LPA to regulate oncogenes and tumor suppressor genes suggests a potential role of LPA as a mutagen.

Whereas our study demonstrates the pro-proliferative effect of LPA_2-mediated signaling on intestinal epithelial cells, increased epithelial cell proliferation alone is insufficient to cause cancer. LPA, however, is capable of inducing effects that potentially contribute to cancer progress. Herein we showed that COX-2 induction following AOM and DSS administration was markedly reduced in $LPA_2^{-/-}$ mice. The link between COX-2 and CC is supported by a variety of studies [24, 32]. Studies have shown that COX-2 expression is elevated in human CC patient and $Apc^{Min/+}$ mice and epidemiological studies have demonstrated the efficacy of COX-2 inhibitors in reducing the tumor incidence in CC patients [24, 32]. In AOM and DSS-treated $LPA_2^{-/-}$ mice, significantly fewer COX-2 positive cells was detected compared with WT mice. Moreover, the basal COX-2 expression in $LPA_2^{-/-}$ mice was lower in untreated $LPA_2^{-/-}$ mice than WT, suggesting a role of LPA_2 in maintenance of basal COX-2 expression. These findings are consistent with previous studies that LPA is an inducer of COX-2 expression in ovarian cancer cells [33, 34] and that $LPA_3^{-/-}$ mice have reduced levels of COX-2 and PGE_2/PGI_2 that result in a defect in implantation [13].

LPA is a potent inducer of cytokines and angiogenic factors [10, 25, 35]. LPA_2 transgenic mice secreted increased amounts of vascular endothelial growth factor and urokinase-type plasminogen activator [14]. The down-regulation of MCP-1 and MIF in $LPA_2^{-/-}$ mice is especially significant as this represents one potential mechanism where the absence of LPA_2 ameliorates CC progress. MCP-1 is a chemoattractant for monocytes, endothelial cells, and a subset of T lymphocytes, and its expression correlates with macrophage infiltration and tumor vessel density in human colorectal cancer [36]. The reduced MCP-1 expression is in part thought to be responsible for the decrease in the number of infiltrating macrophages in $LPA_2^{-/-}$ mice. Macrophages are one of the predominant stromal cells that are found in cancers and studies have correlated the macrophage infiltration and the prognosis of cancers [37]. Macrophages contribute to tumor progression by producing matrix metalloproteinases as well as COX-2 and PGE_2 [37, 38]. Previously, F4/80-positive macrophages and to a lesser extent neutrophils were shown to be the major COX-2 producing leukocytes in the colon of AOM and DSS treated mice [39]. Hence, our finding of reduced MCP-1 expression in $LPA_2^{-/-}$ mice parallels the decreased infiltration of macrophages and COX-2 expression. Our *in vitro* study showed that LPA can stimulate MCP-1 secretion from human CC cells and, hence, we suggest that LPA-LPA_2 signaling axis is a major contributor of MCP-1 in the intestine and that under the

pathological conditions, such as cancer, MCP-1 secreted by the epithelial cells induces transepithelial migration of macrophages, which adds to tumor progression.

Another effect observed in $LPA_2^{-/-}$ mice was the induction of MIF. Over-expression of MIF has been shown in several neoplasms, including prostate and colorectal cancer [40, 41]. In addition, increased expression of MIF in human and $Apc^{min/+}$ mouse adenomas has been reported and homozygous deletion of *mif* gene in $Apc^{min/+}$ mice decreased the number and size of adenomas, providing direct evidence for its function in colon carcinogenesis [42]. Multiple effects have been ascribed to MIF, including tumor invasion, angiogenesis, and down-regulation of the tumor suppressor p53, suggesting that increased expression of MIF might exacerbate tumor progression by suppressing p53-mediated growth arrest and apoptosis [42–44]. MIF is abundantly expressed in intestinal epithelial cells, but during inflammatory processes as well as cancer, macrophages are thought to be a major producer of MIF [45]. The mechanisms underlying the up-regulation of MIF expression during the early stage of intestinal tumorigenesis is unclear, but our data suggest that the LPA-LPA_2 signaling axis is a significant contributor to this regulation.

In summary, we demonstrated that the loss of LPA_2 significantly attenuated the progression of CC. During the recent years, several inhibitors targeting LPA receptors have been synthesized [46–48], but these inhibitors target LPA_1 or LPA_3, but not LPA_2. Recently, LPA_2-specific compound was reported, but this compound was not available to examine the efficacy of LPA_2-specific inhibition as a therapeutic strategy for CC prevention. However, our current study show that inhibiting LPA_2-mediated signaling has potential to restrict tumor progression and targeting LPA_2 might be a new strategy for CC prevention and treatment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Abbreviations

LPA

lysophosphatidic acid

AOM

azoxymethane

DSS

dextran sulfate sodium

CAC

colitis-associated cancer

Apc

adenomatous polyposis coli

ACF

aberrant crypt foci

COX-2

cyclooxygenase-2

PG

prostaglandin

MCP-1

monocyte chemoattractant protein 1

MIF

Macrophage migration inhibitory factor

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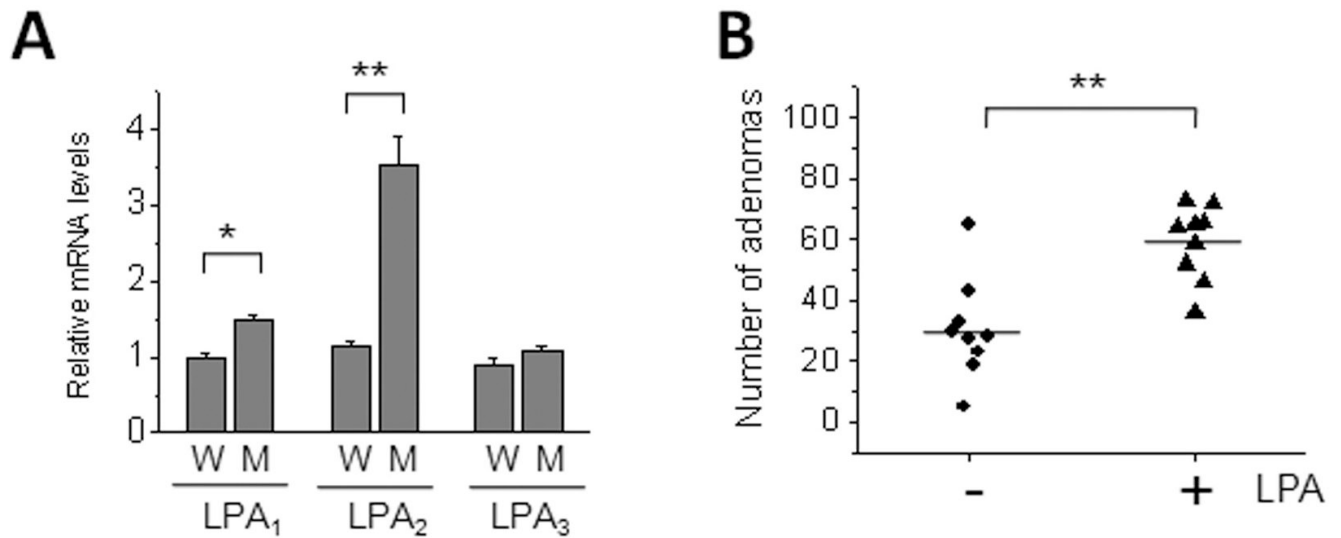
Figure 1.
The effect of LPA on tumor incidence in Apc$^{Min/+}$ mice

(A) The expression of LPA receptors in adenomas of Apc$^{Min/+}$ mice was determined by quantitative RT-PCR. W: WT, M: Apc$^{Min/+}$. n = 6. *, $P < 0.05$. **, $P < 0.01$. (B) The effect of LPA on colonic tumorigenesis was determined by oral administration of LPA to Apc$^{Min/+}$ mice. Six weeks old male C57BL/6 and Apc$^{Min/+}$ mice were given 10 μg/kg LPA suspended in 0.1% BSA containing PBS by gavage once every three days for one month. Control animal received the same volume of PBS+BSA. Animals were killed one month after the last LPA administration and the small intestine was examined under a dissecting microscope for the presence of adenomas. Each data point represents the number of adenomas found in the entire intestine of each mouse. Horizontal bars show the median of each group. n = 9. **, $P < 0.001$.
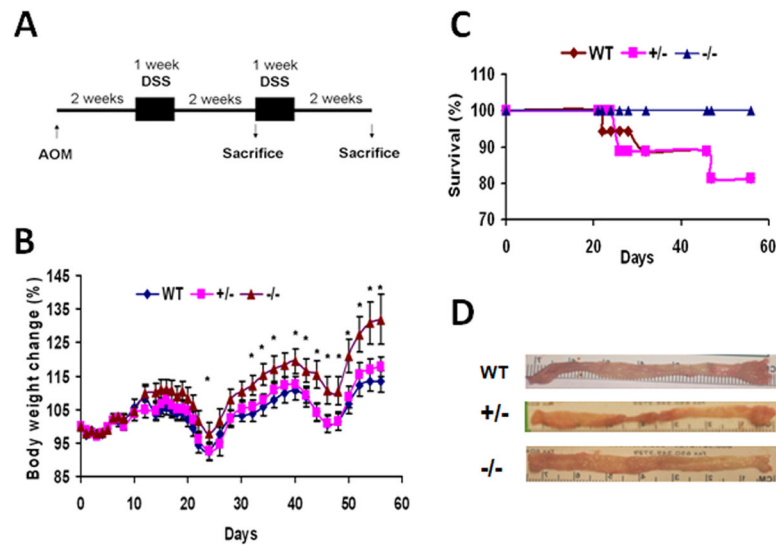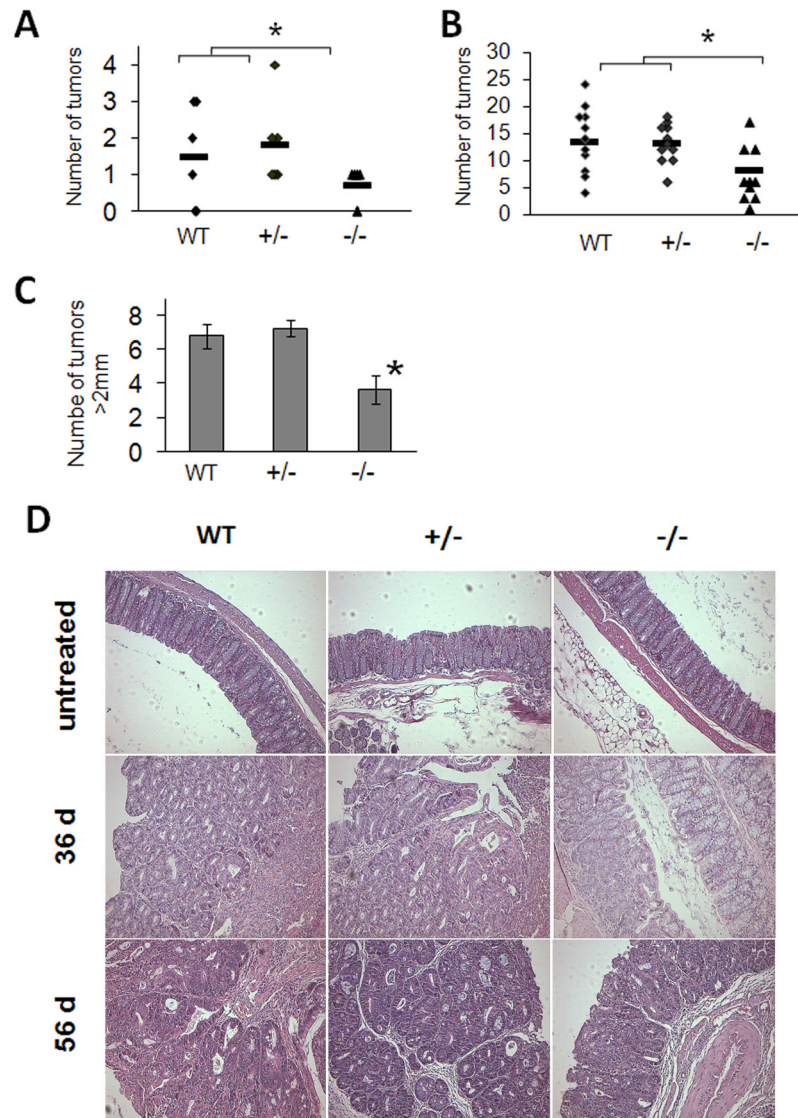
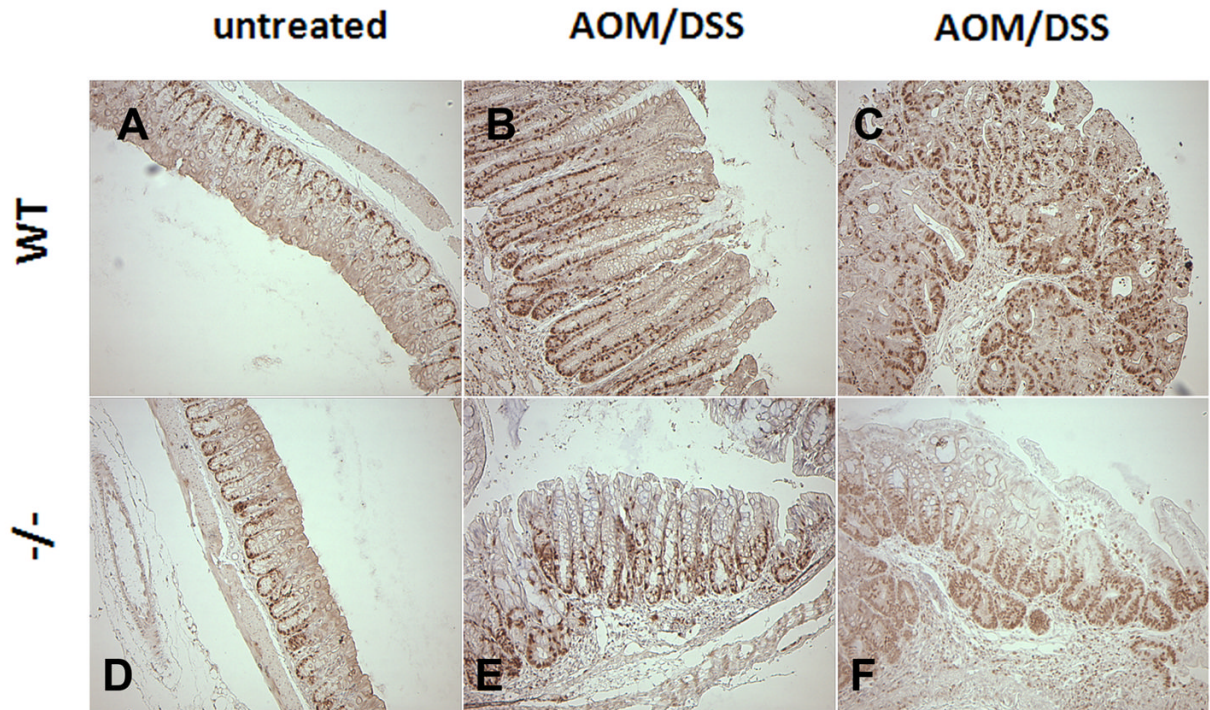**Figure 2.**
Tumor formation induced by AOM and DSS treatment
(A) Schematic presentation of AOM and DSS treatment (B) Body weight changes represented as mean ± SE during the course of AOM and DSS treatment. *, $P < 0.05$ compared with WT. (C) Mortality were followed during the course of AOM and DSS treatment. (D) Representative gross appearance of colon of WT, $LPA_2^{+/-}$, and $LPA_2^{-/-}$ littermates.

Figure 3.

Reduced tumors in LPA$_2$$^{-/-}$ mice

Numbers of tumors at (A) day 36 or (B) day 56 in the colons of WT, LPA$_2$$^{+/-}$, and LPA$_2$$^{-/-}$ mice were counted under the microscope. Each point represents the number of tumors in the colon of each mouse. Horizontal bars represent the mean. n $\geq$ 8. *, $P < 0.01$. (C) The number of tumors larger than 2 mm in diameter at day 56 is shown. Each value represents the mean $\pm$ SE. n=10. *, $P < 0.01$. (D) Representative colon tissues stained with H&E. n = 6. Magnification: x100.
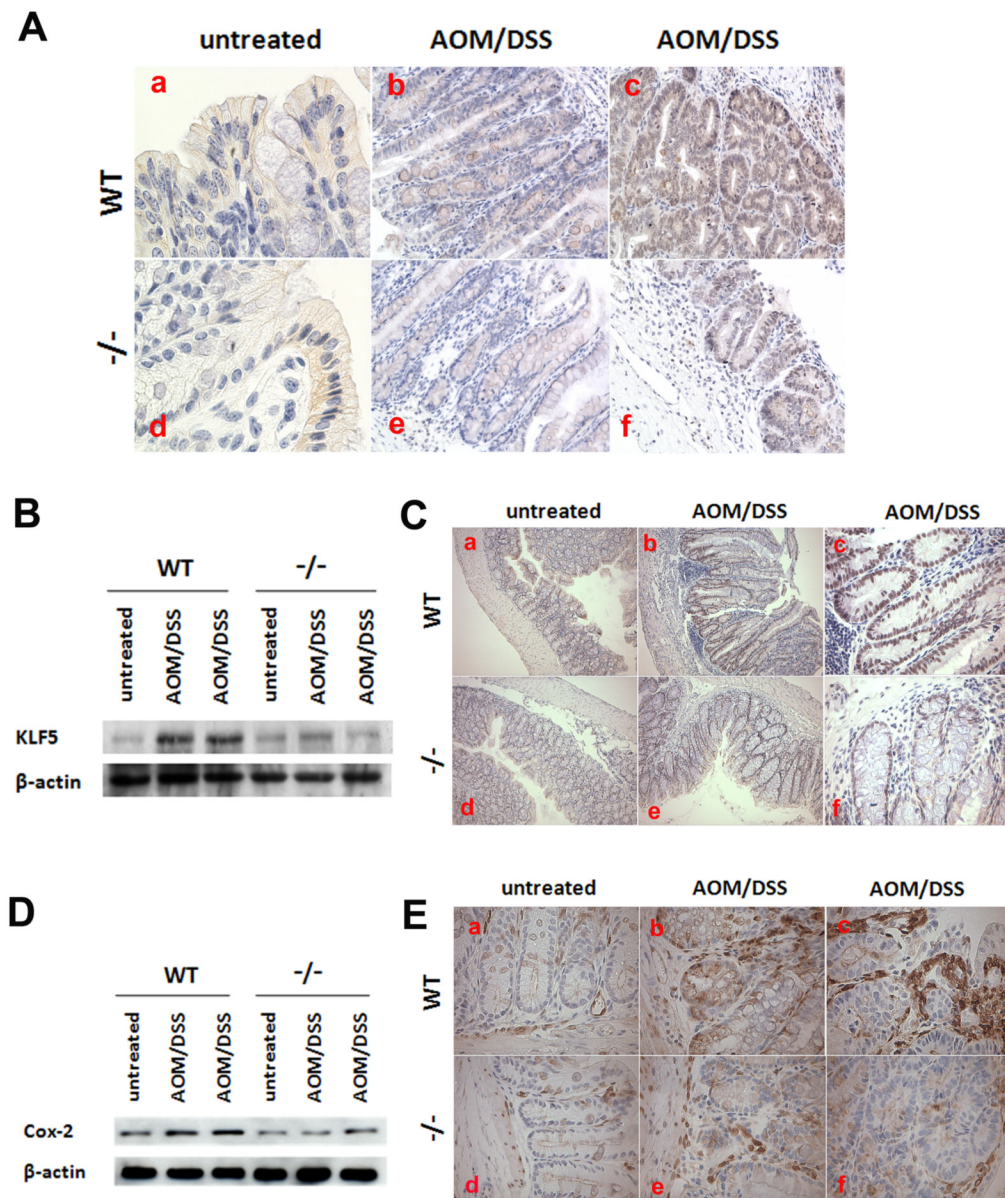
Figure 4.
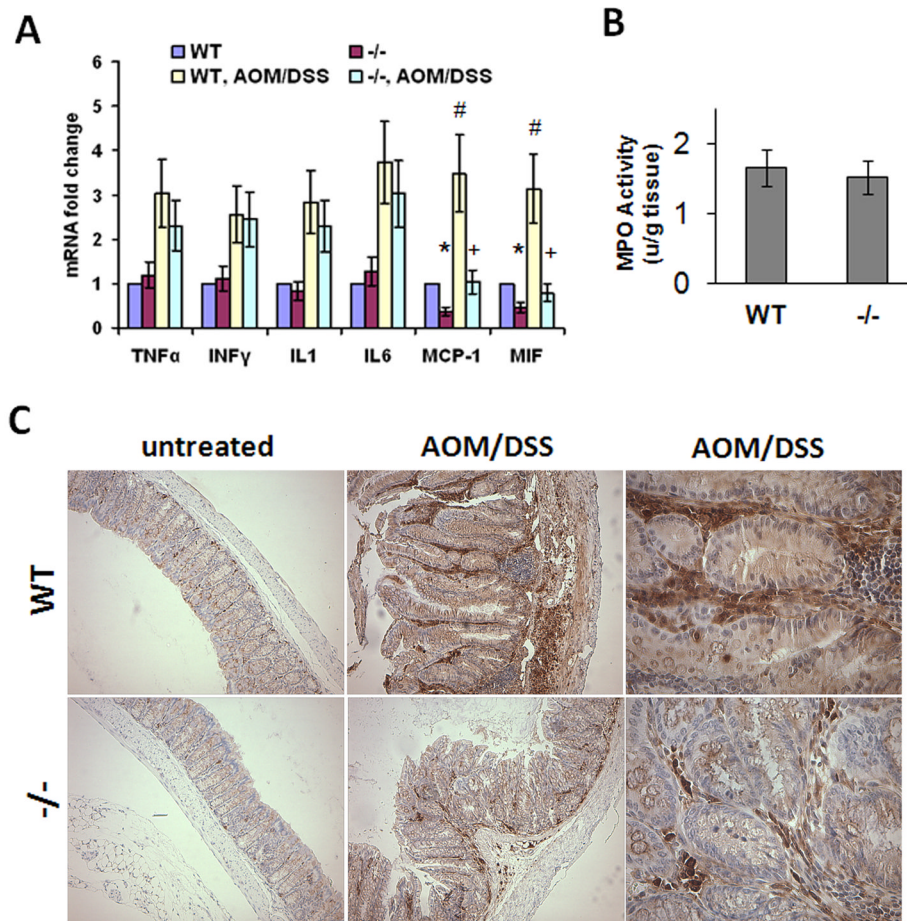Reduced cell proliferation in LPA$_2$$^{-/-}$ mice
Paraffin embedded colonic sections from untreated (A and D) and AOM and DSS treated WT
(B-C) and LPA$_2$$^{-/-}$ (E-F) mice were deparaffinized and immunolabeled using an antibody
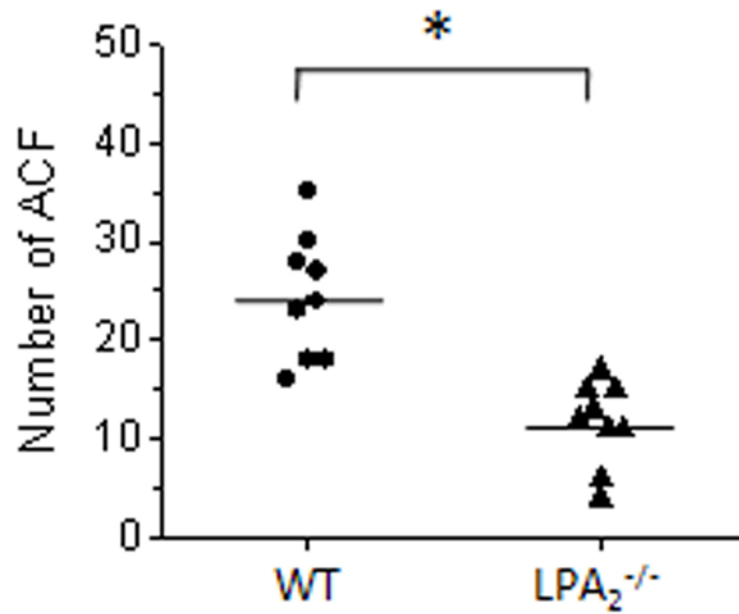against Ki67. n = 5. Magnification at x100.

Figure 5.
Expression of β-catenin, KLF5, and COX-2

(A) Colon tissues of untreated and AOM and DSS-treated WT and LPA$_2^{-/-}$ mice were stained with antibodies against β-catenin. WT: a–c; LPA$_2^{-/-}$: d–f. n=5. Magnification: a and d at x630; b–c and e–f at x100 (B) Western blot shows KLF5 protein expression in the colon of WT and LPA$_2^{-/-}$ mice, n = 3. (C) Representative immunohistologic staining of colonic tissues for KLF5 are shown (n = 5). WT: a–c; LPA$_2^{-/-}$: e–f. Magnification: a–b and d–e at x100; c and f at x400. (D) COX-2 protein expression in protein extracts from the colons of WT and LPA$_2^{-/-}$ mice was determined by Western immunoblotting. n = 3. (B) Immunohistological analysis of COX-2 expression in colon tissues from WT and LPA$_2^{-/-}$ mice. n = 5. Magnification; x400.

Figure 6.
Cytokine gene expression in the colon

(A) Quantitative RT-PCR was performed on total RNAs from the colons of WT and LPA$_2^{-/-}$ mice before and after treatment with AOM and DSS. Data are presented as mean ± SE from at least 6 mice from each group. *, $P < 0.05$; #, $P < 0.001$ compared to untreated WT. +, $P < 0.05$ compared to untreated LPA$_2^{-/-}$. (C) MPO activity was measured in the colons of WT and LPA$_2^{-/-}$ mice. n ≥ 8 mice from each group. (D) immunohiostological analysis of Mac-3-positive macrophage in colon tissues from WT and LPA$_2^{-/-}$ mice. n = 5.

Figure 7.
ACF formation by AOM treatment
WT and LPA$_2$$^{-/-}$ mice were given repeated intraperitonial injection of AOM. The number of
ACF was determined in methylene blue stained colons. Each data point represents the number
of ACF in each mouse. n = 9 per group. *, $P < 0.001$.