Mast cell 5-Lipoxygenase activity promotes intestinal polyposis in APCΔ468 mice

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Precis: Findings suggest that the established role of arachadonic acid biosynthesis in colon tumorigenesis may be driven to a large degree from mast cells that support myeloid-derived suppressor cells, an important component of the tumor microenvironment thought to drive immune escape.
Arachidonic acid metabolism has been implicated in colon carcinogenesis, but the role of hematopoietic 5-lipoxygenase (5LO) that may impact tumor immunity in development of colon cancer has not been explored. Here we show that tissue-specific deletion of the 5LO gene in hematopoietic cells profoundly attenuates polyp development in the APCΔ468 murine model of colon polyposis. In vitro analyses indicated that mast cells in particular utilized 5LO to limit proliferation of intestinal epithelial cells and to mobilize myeloid-derived suppressor cells (MDSCs). Mice lacking hemapoietic expression of 5LO exhibited reduced recruitment of MDSCs to the spleen, mesenteric lymph nodes, and primary tumor site. 5LO deficiency also reduced the activity in MDSCs of arginase-1, which is thought to be critical for MDSC function. Together, our results establish a pro-tumorigenic role of hematopoietic 5LO in the immune microenvironment and suggest 5LO inhibition as an avenue for future investigation in treatment of colorectal polyposis and cancer.

Introduction
5-Lipoxygenase (5LO), an essential enzyme in the arachidonic acid pathway, produces leukotriene B4 (LTB4) and the cysteinyl leukotrienes, a group composed of leukotriene C4 (LTC4), leukotriene D4 (LTD4), and leukotriene E4 (LTE4) (1-3). Several studies have implicated a role for 5LO in the development of lung, pancreatic, and in particular, colon cancer (4-12). In colon cancer, our previous studies and others have investigated the direct effects of 5LO byproducts in normal and tumor epithelial cells alone (10-12). However, there exists the distinct and as yet unexplored possibility that 5LO may exert its tumorigenic effect through the hematopoietic system.

The mast cell is a hematopoietic component which we have recently shown to have an important role in intestinal tumor development (13). In particular, we found that functional depletion of mast cells led to a regression of murine intestinal polyposis with decreased proliferative indices. We also showed that mast cells alter the tumor immune response (14). The immune system is believed to mediate the body’s recognition and rejection of tumor cells. Conversely, dysfunction of the immune system allows for tumors to escape recognition and grow uninhibited. There are an abundance of candidate factors through which mast cells may exert their tumorigenic and immunomodulating effects, including TNF-α, IL-1, IL-6, and TGF-β (15).

Mast cells have also been shown to be carriers of the 5LO enzyme and produce significant amounts of 5LO byproducts (16). 5LO metabolism has been shown to be directly mitogenic to colon cancer cell types (10-12). LTB4 also chemoattracts multiple hematopoietic cell lineages involved in the tumor immune response (16-18). It is therefore possible that 5LO mediates the mast cell’s direct effect on tumor growth as well as its effects on the immune response to tumor.

The myeloid-derived suppressor cell (MDSC) is a hematopoietic mediator of immunologic dysfunction that has been shown to contribute to tumorigenesis. MDSCs directly inhibit the tumor-specific response by CD8+ T cells (19-21). Furthermore, studies have shown that inhibition of MDSC tumor recruitment results in an increased tumor-specific CD8+ T cell-mediated response (22, 23). Much attention has thus been given to identifying the chemokines responsible for MDSC recruitment. However, the list of known chemotactic factors for MDSCs is limited and includes stem cell factor, S100, PGE2, and CCL-2 (22, 24-26). 5LO byproducts have been shown to recruit several myeloid lineages that share a similar...
derivation as MDSCs, including neutrophils and eosinophils (17, 18). It is therefore possible that 5LO byproducts also recruit MDSCs.

Utilizing a murine, APC-driven model of polyposis, we present the novel finding that genetic deletion of 5LO and more specifically hematopoietic 5LO, profoundly abrogates polyp burden. Mast cells isolated from 5LO-deficient mice, which are incapable of producing 5LO byproducts, have a significantly reduced capacity in promoting intestinal epithelial proliferation. We also show that LTB4 chemoattracts MDSCs and that 5LO-deficient mast cells have a diminished ability to recruit MDSCs when compared to mast cells with the 5LO enzyme. Correspondingly, we observed a significant decrease in MDSC polyp infiltration with 5LO deficiency. Furthermore, we found that MDSCs from 5LO-deficient mice had diminished immunosuppressive arginase-1 activity. Our study demonstrates that hematopoietic 5LO, and particularly mast cell 5LO, promotes polyposis through its direct mitogenic effects on murine intestinal epithelium as well as through its recruitment of MDSCs. These results provide evidence that hematopoietic 5LO plays a key role in early tumorigenesis.

Materials and Methods

**APCΔ468 and 5LO−/− mice** All animal work was approved and conducted under the guidelines of Northwestern University’s Animal Care and Use Committee. APCΔ468/5LO+/− mice were crossed with 5LO−/− mice to generate APCΔ468/5LO−/− mice. APCΔ468 were used as positive controls. All mice were of the C57BL/B6 genetic background. Offspring were genotyped by PCR. Mice were sacrificed at 4 months of age.

**Bone Marrow Reconstitution** APCΔ468 mice were lethally irradiated at 6 weeks of age. Bone marrow-derived mononuclear cells were harvested from either B6 or 5LO−/− donors and lineage-depleted using the Multenyi MS columns according to manufacturer’s instruction. APCΔ468 mice were then reconstituted with mononuclear cells from one of the two donor types. Mice were sacrificed at 4 months of age.

**Cysteinyl Leukotriene and Prostaglandin E Metabolite Detection** Cysteinyl leukotriene and prostaglandin E metabolite levels were measured using Cayman Chemical enzyme immunoassay kits,
according to manufacturer’s instruction.

**Immunofluorescence Analysis** 5µm thick sections of frozen or formalin-fixed paraffin embedded intestine were cut. Formalin-fixed sections were deparaffinized and rehydrated in graded alcohol. Antigen retrieval was performed using 1X Target retrieval solution (DAKO), whereas the frozen sections were fixed in ice-cold methanol for 15 minutes. Frozen and formalin-fixed sections were incubated with 1% bovine serum albumin for 30 min at room temperature. Subsequently, sections were incubated either with anti-mouse Mac3 (BD Pharmingen) and biotinylated anti-mouse Gr1 (BD Pharmingen) or anti-mouse CD4 (Abcam) and anti-mouse FoxP3 (eBioscience) or anti-mouse CD8 (Santa Cruz Biotechnology) and anti-mouse FoxP3 or anti-mouse CD11c (BD Pharmingen) and anti-mouse IAb (Abcam) or anti-mouse CD11b (BD Pharmingen)/CD3 (BD Pharmingen) and 5LO (Abcam) overnight at 4°C. Primary antibodies were washed twice the next day with PBS and incubated with appropriate Alexafluor-488 and Alexaflour-594 secondary antibodies (Invitrogen) for 1 hour. Finally, sections were washed twice with PBS and incubated for 15 minutes with 4',6-diamidino-2-phenylindole (Sigma), washed twice with PBS and mounted with anti-fade mounting medium. Images were acquired using TissueGnostics Tissue/Cell High Throughput Imaging and Analysis System.

**BrdU Incorporation** Mice were injected with 400 µl of 10 mg/ml BrdU (Sigma) 2 hours prior to cull. Paraffin sections (4 µm) were stained for BrdU using the BD Pharmingen™ BrdU in situ detection kit.

**Apoptosis** The ApopTag Red in situ apoptosis detection kit from Chemicon International (catalog no. S7185) was used according to the manufacturer’s instructions.

**Preparation of Intestinal MNCs** MNCs were prepared as previously described (27). Briefly, homogenized intestine was agitated in a solution containing collagenase type 4 (Worthington Biochemical Corporation) in RPMI medium 1640 at 37°C for 20 min. Cells were isolated using a 40-60% Percoll gradient and washed twice.
**MCp Assay** Frequencies of MCp in MNCs were measured as described previously (27). MNCs were suspended in 100 ml of RPMI medium consisting of 20 ng/ml of stem cell factor (SCF), 20 ng/ml of IL-3, and 100,000 irradiated spleen cells per well (3,000 rad). 10,000 MNCs were plated per well in a 96-well plate and diluted serially in subsequent rows. After 15 days incubation at 37°C with 5% CO2, colonies of matured MC were scored.

**Mouse BMMC culture** Murine BMMCs were obtained by culturing femoral BM cells at 5 x 10^5 cells/ml in complete RPMI 1640 with 5 ng/ml murine IL-3 and 12.5 ng/ml SCF. Media and flask were changed twice a week for 6 weeks and were maintained at 37°C in 5% CO2. At 6 weeks, conditioned media was obtained by replating cells at the same 5 x 10^5 cells/ml density in serum-free RPMI with the same concentrations of IL-3 and SCF.

**Thymidine Incorporation** IMCE cells were kindly provided by Dr. Robert Whitehead of Vanderbilt University (28). IMCE cells were plated at a density of 5 x 10^4 cells/mL in a 96-well plate (33° permissive temperature, 5% CO2). Cells were treated with BMMC conditioned media from either APC^Δ468 or APC^Δ468/5LO^−/− mice. Both conditioned medias were supplemented with IFN-γ (5U/mL). 1 μCi ³H-thymidine was added for a 14-hour incubation at 24, 48, and 72-hour time points. Incorporation was measured using a Perklin-Elmer MicroBeta plate counter.

**Antibody staining and FACS** FACS acquisition was performed with BD FACSCanto. Data analyses used FlowJo software (Tree Star). Gr-1-biotin, Gr-1-APC, CD11b-FITC, CD11b-PE, Ly6C-PerCP, and Ly6G-APC were purchased from BD Biosciences. L/D was purchased from Invitrogen.

**MDSC Chemotaxis** 96-well chemotaxis plates (Neuroprobe) were used as previously described (29) with the following modifications: MDSCs were suspended at 2 x 10^6 cells/mL of serum-free RPMI and 50 μL were applied to the top wells. 300 μL of serum-free RPMI at varying concentrations of LTB4, LTD4, and LTE4 were added to the lower wells. In some experiments, lower wells were plated with BMMC conditioned media from APC^Δ468 or APC^Δ468/5LO^−/− mice. For rescue experiments, 10nM LTB4 was added.
to $\text{APC}^{\Delta 468}/\text{LO}^{-}$ BMMC conditioned media. Plates were incubated for 3h at 37°C and migrated cells were counted using inverted microscopy. Assays were performed in duplicate in three separate experiments.

**Arginase-1 and NOS detection** $10^6$ Gr1$^+$ cells were plated for 18h in the presence of IFN-γ and LPS. Supernatant was used for NOS detection (Cayman Chemical) according to manufacturer’s instruction. Cells were suspended in 500 μl PBS, lysed using sonication, and centrifuged. Supernatant was used for arginase assay (BioAssay Systems), according to manufacturer’s direction.

**ROS detection** Upon oxidization, hydroethidine intercalates with cellular DNA and emits at approximately 600nm. MNCs were incubated with 1 μM hydroethidine (Invitrogen) at room temperature for 30 minutes, then washed with PBS. Cells were then analyzed using flow cytometry.

**Results**

**5LO is upregulated in APC$^{\Delta 468}$ mice**

Our lab has previously shown that 5LO is upregulated in human colon polyps and cancer as compared to normal colon tissue (10). In order to further investigate these findings, we utilized the APC$^{\Delta 468}$ mouse, which bears a truncated APC gene and develops a severe polyposis by 4 months (30). We hypothesized that 5LO would be increased in the intestines of APC$^{\Delta 468}$ mice as it is in human colon polyps. Analogous and supplemental to our previous findings in human colon tissue, we found both a local and systemic elevation of 5LO activity in the APC$^{\Delta 468}$ mouse.

5LO was upregulated in the intestinal lymphoid tissue of APC$^{\Delta 468}$ intestine compared to wild-type controls (Fig. 1a). Furthermore, enzyme immunoassays (EIA) were performed for the 5LO byproduct group, cysteinyl leukotrienes. These were elevated in the sera of APC$^{\Delta 468}$ mice compared to wild type controls (Fig. 1a v). In order to further delineate which cells were responsible for systemic elevation of 5LO metabolites, we stained for the CD11b and CD3 markers to evaluate for the myeloid and lymphoid populations, respectively. We found that T-cells (CD3$^+$) expressed 5LO while myeloid cells (CD11b$^+$) did not co-localize for 5LO in the intestinal lymphoid tissue and spleens of APC$^{\Delta 468}$ mice (Fig. 1b - c).
5LO deficiency suppresses intestinal polyposis in APC\(^{Δ468}\) mice

Given the observed upregulation of 5LO in the APC\(^{Δ468}\) mouse, we examined the effects of 5LO gene knockouts on this same model. 5LO\(^{-/-}\) mice were bred to APC\(^{Δ468}\) mice and their phenotypes were evaluated at 4 months. 5LO null mutation led to a dramatic reduction in the number and size of intestinal polyps (Fig. 2a and b). The APC\(^{Δ468}\) group developed 79 ± 4 polyps with a median polyp diameter of 2.7mm (Fig. 2i and j). In contrast, APC\(^{Δ468}/5LO^{−/-}\) mice had 46 ± 6 polyps with a median diameter of 1.2mm, an approximately 40% (P < 0.005) and 44% (P < 0.0001) reduction, respectively (Fig. 2i and j). The APC\(^{Δ468}\) group also had increased mitotic indices and decreased apoptosis compared to the APC\(^{Δ468}/5LO^{−/-}\) group (Fig. 2c – f, k and l). Based on these observations, we concluded that 5LO significantly contributes to intestinal tumor development.

5LO deficiency suppresses polyposis via the hematopoietic system

As 5LO was increased specifically in the intestinal lymphoid tissue of APC\(^{Δ468}\) mice (Fig. 1a), we hypothesized that hematopoietic 5LO was primarily responsible for the observed tumorigenesis. To test our hypothesis, we performed adoptive transfer experiments using APC\(^{Δ468}\) littermates as recipients and reconstituted them with bone marrow from either healthy wild-type (wt BM) mice or healthy 5LO\(^{-/-}\) (5LO BM) mice. At 4 months of age, mice were evaluated for phenotype. The wt BM group had a similar degree of polyposis as the parental APC\(^{Δ468}\) group with a mean of 112.8 ± 9.075 polyps (Fig. 2g and m). In contrast, 5LO BM mice had a profound reduction in their polyp burden by approximately 91%, with 9.8 ± 2.871 polyps (P < 0.001) (Fig. 3h and m). The degree to which polyposis was reduced in the 5LO BM mice precluded any statistically significant evaluation of polyp diameter (Supplementary Fig. S1), mitotic indices, or apoptosis.

Mast cells potentiate the proliferation of murine intestinal epithelial cells via 5LO metabolism.

Our next goal was to identify a hematopoietic lineage through which 5LO was tumorigenic. We first quantified total intestinal mast cell presence in the 5LO-deficient groups compared to their controls. We
found that mast cells were significantly reduced in the polyps of the APCΔ468/5LO−/− and 5LO BM groups compared to their respective APCΔ468 and wt BM controls (Fig. 3). We also observed a decrease in polyp infiltration by Mac3+ and Gr1+ cells (Fig. 3). As our lab had previously found the mast cell to have a critical role in polyposis, we further explored its potential tumorigenic role in the context of 5LO deficiency.

In addition to a quantitative difference of mast cells being present between the four mouse groups, we also explored the possibility of a qualitative difference. We harvested conditioned media from mast cells derived from APCΔ468 and APCΔ468/5LO−/− mice, and compared the effects of media treatment from these respective groups on the proliferation of Immorto-Min colonic epithelial (IMCE) cells. Harboring an APC min mutation, IMCE is an immortalized intestinal epithelial cell line (28). Measuring thymidine incorporation, APCΔ468 mast cells potentiated IMCE proliferation to a significant degree over APCΔ468/5LO−/− mast cells at 24, 48 and 72 hours (Fig. 4a). Furthermore, APCΔ468 mast cells increased proliferation compared to standard non-conditioned media at 48 and 72 hours (Fig. 4a).

**LTB4 chemoattracts bone marrow-derived MDSCs**

We next evaluated if 5LO modulates the tumor immune response. The 5LO byproduct LTB4 has been specifically shown to chemoattract a variety of hematopoietic lineages, including neutrophils and eosinophils, which share similar myeloid derivation as MDSCs (16, 17). We hypothesized that MDSCs might be similarly recruited to tumor by LTB4. MDSCs were isolated from APCΔ468 bone marrow using Gr-1 column selection, which yielded > 90% purity for Gr-1+CD11b+ MDSCs. 96-well chemotaxis plates were used with MDSCs plated in the top wells. LTB4, LTD4, and LTE4 were applied to the lower wells in concentrations of 1nM, 10nM, and 100nM. LTB4 induced migration of MDSCs in a concentration-dependent manner up to 10nM (Fig. 4b). LTD4 and LTE4 did not chemoattract MDSCs in any concentration (Fig. 4b).

**Mast cells chemoattract MDSCs via 5LO metabolism.**

Mast cells have been shown to produce LTB4 in a significant concentration of 10.6nM *ex vivo* (16). We therefore evaluated if the mast cell affects the tumor immune response through an ability to chemoattract
MDSCs via LTB4. We compared mast cells with and without the 5LO enzyme and evaluated their respective capacities to chemoattract MDSCs. Bone marrow derived mast cells (BMMCs) were isolated from APC\(^{Δ468}\) and APC\(^{Δ468}/5LO^{-/-}\) mice and grown in culture for 8 weeks with SCF and IL-3. Conditioned media was harvested and re-plated into the lower wells of chemotaxis plates. MDSCs were plated in the top wells. Indeed, conditioned media from APC\(^{Δ468}/5LO^{-/-}\) BMMCs chemoattracted MDSCs to a significantly lower degree than did conditioned media from APC\(^{Δ468}\) BMMCs (Fig. 4c). Furthermore, addition of LTB4 (10nM) to the APC\(^{Δ468}/5LO^{-/-}\) media resulted in a rescue of the chemoattraction of MDSCs (Fig. 4c).

5LO deficiency mitigates MDSC polyp infiltration

If LTB4 recruits MDSCs, deficiency in 5LO would therefore mitigate MDSC recruitment, thus enhancing the anti-tumor response and decreasing polyposis. To evaluate the capacity of 5LO to recruit MDSCs to tumor, \textit{in vivo}, we analyzed Gr-1\(^+\)CD11b\(^+\) MDSC presence in the polyp, mesenteric lymph nodes, spleen, and bone marrow of APC\(^{Δ468}/5LO^{-/-}\) and APC\(^{Δ468}\) mice. To ensure that any potential reduction in MDSC recruitment in 5LO deficient mice was not solely a function of their diminished tumor burden, polyps were microdissected from both groups and compared. With polyp-to-polyp comparison, Gr-1\(^+\)CD11b\(^+\) MDSCs were still significantly reduced by approximately four fold (Fig. 5a and b).

We also found that 5LO expands MDSCs in peripheral lymphoid organs. There was a significant reduction in Gr-1\(^+\)CD11b\(^+\) MDSCs isolated from the mesenteric lymph nodes and spleens of APC/5LO\(^{-/-}\) mice, by approximately three fold, respectively (Fig. 5a and b). Interestingly, there was a trend of near significance (\(P = 0.09\)) towards increased Gr-1\(^+\)CD11b\(^+\) MDSCs in the bone marrow compartment of APC/5LO\(^{-/-}\) mice. This may suggest a buildup of unrecruited MDSCs (Fig. 5a and b) that remain at their point of origin, the bone marrow. 5LO BM mice showed a similar reduction in Gr-1\(^+\)CD11b\(^+\) MDSC recruitment in the gut (66%), mesenteric lymph node (53%), and spleen (47%) (Fig. 5c). These findings suggest that MDSC recruitment to the tumor site, mesenteric lymph nodes, and spleen, is 5LO-dependent.

5LO deficiency reduces granulocytic MDSC polyp infiltration and arginase-1 activity

Two distinct subsets of MDSCs were found during the analysis of Gr-1\(^+\)CD11b\(^+\) cells, consistent with previously published literature (31, 32). The predominant subpopulation was the granulocytic MDSC
which is identified by its CD11b^+Ly6G^-Ly6C^low phenotype (Fig. 6a). Monocytic MDSCs, with the epitopes CD11b^-Ly6G^-Ly6C^hi, composed the second subtype and was generally present to a lesser degree than granulocytic MDSCs from the same mouse (Fig. 6a). Only the granulocytic subpopulation was reduced in APC^Δ468/5LO^-/- mice and 5LO BM mice compared to their APC^Δ468 and wt BM controls (Fig. 6b and c). Monocytic MDSCs, on the other hand, were not reduced to significant degree, except in the gut of 5LO BM mice. As there were not enough polyps in the 5LO BM intestine to analyze their MDSCs, the majority of intestine evaluated was grossly normal from this group. We therefore compared these MDSCs to MDSCs isolated from adjacent normal intestine in the wt BM group. In the gut, we found a distinct subpopulation with the epitopes, CD11b^-Ly6G^-Ly6C^hi (Fig. 6a). This population was reduced in the 5LO BM group in similar fashion as the granulocytic MDSCs from the spleen and mesenteric lymph nodes.

We further analyzed the activity of arginase, ROS, and NOS by the wt BM and 5LO BM groups. The wt BM group had a statistically significant greater degree of arginase-1 activity than did the 5LO BM group (Fig. 7a). However, we did not find a significant difference in ROS or NOS production (Fig. 7b and c). 5LO appears to play a significant role in the recruitment of granulocytic MDSCs and in the activity of arginase-1 in MDSCs.

At the time of this report, a study implicated the IL-17 pathway in the mobilization of MDSCs (33). We have previously reported finding elevated IL-17 in polyposis and suggested that polyposis is a Th17-driven disease (14, 34). Both CD4^+ T-cells and antigen presenting cells contribute to long term Th17 inflammation. Therefore, in order to understand the impact of 5LO deficiency on the T-cell compartment and on antigen presenting cells, we evaluated the infiltration of polyps by CD4^+, CD4^+FoxP3^+, CD11c^-IA^-^b^+, and CD8^-^cells in the APC^Δ468 and APC^Δ468/5LO^-/- experimental groups. There was no statistically significant difference observed in the infiltration by CD8^-^cells between these two experimental groups (Supplementary Fig. S3). However, CD4^+, CD4^+FoxP3^+, and CD11c^-IA^-^b^+ cell infiltration was significantly reduced in the polyps of 5LO-deficient mice (Supplementary Fig. S3).

Discussion
There are a limited number of studies that have evaluated the role of 5LO in intestinal tumorigenesis. Of these studies, all have focused on the effects of 5LO activity on the epithelial cell alone. The 5LO byproduct, LTD₄, was shown to increase proliferation and cell survival in human intestinal epithelial cells (35). We have previously shown that 5LO inhibitor treatment of mice xenografted with human colon cancer significantly reduced tumor volume (10). In the current study we have observed diminished tumor burden specifically through 5LO genetic deletion and have provided evidence for a hematopoietic mechanism.

Recent studies show that signals from myeloid cells, mast cells in particular, are critical determinants of tumor behavior regulating growth and the tumor immune response (13, 14). APCΔ₄₆₈ mice reconstituted with 5LO-deficient bone marrow experienced a profound abrogation in their polyposis, even though the intestinal epithelial compartment of these mice had a constitutive APC mutation and were 5LO-proficient. We found that the mast cell is an important effector of the tumorigenic properties of hematopoietic 5LO. Previous studies have shown that LTB₄ is a potent chemoattractant of mast cells (16). In line with these prior findings, we observed a significant decrease in mast cell polyp infiltration in the APCΔ₄₆₈/5LO⁻/⁻ and 5LO BM groups, compared to their respective controls. 5LO deficiency also resulted in a decrease in the polyp infiltration by Mac3⁺ cells, which have also been shown to utilize 5LO for many of their effector functions (36, 37). Our findings further indicate that homing and expansion of mast cells and macrophages are 5LO-dependent.

We showed that the mast cell population from APCΔ₄₆₈ mice potentiated proliferation of IMCE murine enterocytes to a significantly greater degree at three different time points than mast cells from the APCΔ₄₆₈/5LO⁻/⁻ group. When LTD₄ was originally shown to increase intestinal epithelial cell proliferation, it was thought to be part of an autocrine system. The epithelial cells were believed to produce their own LTD₄, providing for their own survival and proliferation. Our results show that mast cells may alternatively have a paracrine growth effect, wherein mast cells produce 5LO byproducts that potentiate the proliferation of the intestinal epithelium.

The mast cell is also tumorigenic through its effect on the tumor immune response. By being a hematopoietic source for LTB₄ in the APCΔ₄₆₈ model, mast cells recruit MDSCs to the tumor site. At the
time of this report, a separate group described a capacity of mast cells to potentiate the accumulation of MDSCs in a xenograft model for hepatocellular carcinoma (33). They suggested that this regulation was IL-17-mediated. We herein show that mast cells specifically chemoattract MDSCs, and that a significant portion of this recruitment is 5LO-mediated. BMMCs produce LTB4 at 10nM ex vivo, the concentration that we found was optimal for chemoattracting MDSCs (16). In fact, MDSCs appear to become less reactive to LTB4 with concentrations higher than 10nM. This exquisite sensitivity separates MDSCs from other myeloid lineages like neutrophils and mast cells which require LTB4 at a peak concentration of 100nM (16, 38). Furthermore, 5LO deficiency in mast cells significantly reduced their ability to chemoattract MDSCs.

*In vivo*, 5LO deficiency markedly reduced MDSC presence in the spleen, mesenteric lymph nodes, and gut. In response to cancer, murine MDSCs are mobilized from the bone marrow to the peripheral lymphoid organs and tumor site (39). We found that 5LO contributes to an expanded presence of MDSCs in the secondary lymphoid organs and polyp. 5LO appears to play a role in the chemokinesis specifically of granulocytic MDSCs, which were significantly reduced in the spleen and mesenteric lymph nodes of 5LO-deficient mice. Monocytic MDSCs, on the other hand, were not changed to a statistically significant degree, with the exception of monocytes infiltrating the intestine of APCΔ468 mice with 5LO-deficient BM.

We also found that within the gut, granulocytic MDSCs appeared to shift towards a CD11b^Ly6G^Ly6C^med phenotype, similar to that of inflammatory peritoneal neutrophils (Fig. 6a) (40). This population was present more in the polyp than in adjacent normal intestine in APCΔ468 mice. Furthermore, 5LO-deficient APCΔ468 mouse intestine had a lower percentage of CD11b^Ly6G^Ly6C^med cells when compared to the APCΔ468 group, consistent with the reductions observed with splenic and mesenteric lymph node granulocytic MDSCs. This epitope shift reflects increasing evidence that MDSCs have phenotypic and functional overlap with other established myeloid lineages and emphasizes the plasticity of MDSCs. It is also important to acknowledge that eicosanoids and MDSC physiology have been previously linked in the context of COX-2 metabolism (41). This prior study showed that the COX-2 metabolite, PGE2, induced the differentiation of MDSCs. However, we did not find any evidence that the COX-2 pathway was affected in our model, confirming that 5LO affects MDSC physiology in a mechanism distinctly separate from COX-2 metabolism (Supplementary Fig. S4). In summary, 5LO
appears to play a significant role in the mast cell’s recruitment of granulocytic and CD11b+Ly6G+Ly6Cmed MDSCs.

We also found that 5LO deficiency directly decreases arginase-1 activity in MDSCs. Utilizing various mechanisms, MDSCs suppress the CD8+ T-cell-mediated response to tumor. Granulocytic MDSCs produce large amounts of reactive oxygen species (ROS) whereas the other subtype, monocytic MDSCs, produce nitric-oxide synthase (NOS) (32, 42). Both subtypes produce arginase-1 which catabolizes L-arginine, depleting it from the microenvironment thereby inhibiting T-cell proliferation through several known mechanisms (43). We did not observe a difference in ROS or NOS production with 5LO deficiency. Thus, 5LO deficiency inhibits the mast cell-mediated recruitment of MDSCs as well as the immunosuppressive activity of arginase in MDSCs.

Interestingly, 5LO BM mice experienced a greater degree of polyp suppression than did the APCΔ468/5LO-/- mice, which are deleted for 5LO in both the epithelial and hematopoietic compartments. There is the possibility that 5LO serves a protective role at the intestinal epithelium, whereby deleting 5LO would remove that protective mechanism. A corollary example may be found in the gastroduodenal mucosa, where arachidonic acid metabolism has a protective role against helicobacter-induced preneoplasia (44). In other words, baseline 5LO activity may be protective in healthy mice while too much activity in cancer is pathogenic. In our APCΔ468/5LO-/- cross, we may have the benefit of eliminating 5LO overactivity but then also suffer from loss of baseline protection. Nonetheless, polyposis was still significantly suppressed in the APCΔ468/5LO-/- mouse as reflected by polyp number, median polyp diameter, decreased mitotic activity, and increased apoptosis.

We did not observe a difference in CD8+ cell infiltration in the intestines of the APCΔ468 and APCΔ468/5LO-/- experimental groups. It is important to note, however, that CD8+ is upregulated in gut-infiltrating intraepithelial lymphocytes, which are in a tolerized state (45, 46). The infiltration by tumor-infiltrating CD4+FoxP3- and CD4+FoxP3+ cells were significantly decreased in APCΔ468/5LO-/- mice compared to APCΔ468 controls. While CD4+FoxP3+ cells have been shown to recruit mucosal mast cells (47) and promote inflammation, regulatory T-cells (Tregs) normally have anti-inflammatory properties. However, we recently reported that in tumor-bearing mice, Tregs can switch functions and contribute to the escalation in cancer-associated inflammation (34). Indeed, our data suggests that T-cells are a major source
of 5LO activity. This is in line with a pro-inflammatory function of gut-infiltrating lymphocytes in our model. Thus, along with the observed decrease in MDSCs, the drop in CD4+ cells could imply a second mechanism through which 5LO contributes to tumor growth.

Our findings indicate that hematopoietic 5LO significantly contributes to polyp development. In particular, the mast cell utilizes 5LO to potentiate the growth of the intestinal epithelium as well as recruit MDSCs to the polyp site. 5LO also increases the activity of the immune suppressive enzyme arginase-1 in the MDSC. Based on these findings, we propose that targeting 5LO may be an effective therapeutic strategy for polyposis and potentially colorectal cancer.

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References


**Figure Legends**

**Figure 1.** 5-LO is upregulated in the APCΔ468 mouse. a. (i – v) 5-LO IHC in intestinal lymphoid tissue. (i) healthy B6 control (control) (Magnification, ×200) (ii) high power view of inset in panel a (×1000) (iii) APCΔ468 mouse (APC) (×200) (iv) high power view of inset in panel c (×1000) (v) Median cysteinyl leukotriene serum levels (Cyst LTs) in healthy B6 controls (control, 48.43 ± 5.1, n=5) versus APCΔ468 mice (APC, 95.92 ± 7.562, n=5) (**, P < 0.001). b. Representative immunofluorescent micrographs of intestinal lymph node and splenic tissue from control and APC mice. CD11b+ cells stained in red in upper panels and indicated by white triangle. CD3+ cells stained in red in lower panels. 5LO+ cells stained in green and indicated by white pentagon. CD3+5LO+ cells co-localized and stained in yellow, indicated by white arrow. Scale bar represents 50 μm, (x 200). c. Frequency of CD3+5LO+ cells were significantly increased in the intestinal lymph nodes (1.589 ± 0.2237, control versus 7.471 ± 0.7683, APC, *P < 0.05) and spleens (1.011
control versus 5.035 ± 0.4306, APC, *P < 0.05) of APC mice. Values are a percent of double positives per total DAPI+ cells per high-powered field. All means shown are ± SEM. To determine statistical significance, t tests were used.

**Figure 2.** 5-LO deficiency suppresses intestinal polyposis. Representative images of intestines from (a) APC^Δ468^ (APC) and (b) APC^Δ468^/5LO^−/−^ (APC/5LO) mice (Magnification, x 10). IHC for BrdU in adenomas from (c) APC and (d) APC/5LO mice (magnification, x 400). IHC for TUNEL in adenomas from (e) APC versus (f) APC/5LO mice, (x 400). Intestines of APC mice reconstituted with (g) wt (wt BM) and (h) 5LO^−/−^ (5LO BM) BM (Magnification, x 10). Note: polyps pictured of 5LO BM were the only polyps found in that mouse. (i) Mean polyp numbers in APC (n=13) versus APC/5LO (n=7) mice (APC, 79 ± 4; APC/5LO, 46 ± 6; **, P < 0.05). (j) Mean polyp diameters (APC, 2.7mm ± 0.09; APC/5LO 1.2mm ± 0.08; ***, P < 0.0001). (k) Mean mitotic indices of adenomas in APC (17.19 ± 1.13) versus APC/5LO mice (6.93 ± 0.56; ***, P < 0.0001). (l) Mean apoptotic indices of adenomas in APC (1.48 ± 0.21) versus APC/5LO (3.3 ± 0.4) mice. **, P < 0.0005. (m) Mean polyp numbers from wt BM (112.8 ± 9.075, n=6) and 5LO BM (9.8 ± 2.871, n=5) groups (***, P < 0.001). Means shown are ± SEM. To determine statistical significance, t tests were used.

**Figure 3.** Myeloid cell infiltration in APC^Δ468^ (APC), APC^Δ468^/5LO^−/−^ (APC/5LO), APC^Δ468^ with B6 bone marrow (wt BM) chimeras and APC^Δ468^ with 5LO^−/−^ bone marrow chimeras (5LO BM). (a) Representative images of infiltration of CAE^+^ mast cells, Mac3^+^, and Gr1^+^ cells in the APC, APC/5LO, wt BM, and 5LO BM groups. (b) Frequencies of CAE^+^ mast cells, Mac3^+^, and Gr1^+^ cells in the APC, APC/5LO, wt BM, and 5LO BM groups. CAE^+^ mast cells are stained in red, black and white bars represent 50 µm, *P <0.05. All means shown are ± SEM. To determine statistical significance, t tests were used.

**Figure 4.** Mast cells increase proliferation of intestinal epithelial cells and chemoattract MDSCs via 5-LO. (a) IMCE cells were treated with conditioned media from BMMCs derived from APC^Δ468^ (APC) or APC^Δ468^/5-LO^−/−^ (APC/5LO) mice or non-conditioned RPMI supplemented with IFN-γ (Control). Proliferative effects are shown by thymidine incorporation at three separate time points (*, P < 0.05, **, P < 0.005). APC conditioned media increased proliferation compared to Control at 48h and 72 h (P < 0.05). (b) LTB4 chemoattracted MDSCs maximally at 10nM. LTD4 or LTE4 did not chemoattract MDSCs at any concentration (c) Chemotaxis assay of BMMC conditioned media from APC versus APC/5LO mice. Addition of LTB4 to APC/5LO conditioned media (APC/5LO + LTB4) partially restored chemoattraction of MDSCs (*, P < 0.05, **, P < 0.005). Means shown are ± SEM. To determine statistical significance, t tests were used.

**Figure 5.** 5LO deficiency mitigates MDSC recruitment. (a) Representative flow cytometry data of Gr-1^−^CD11b^−^ MDSCs from polyps, mesenteric lymph nodes, spleen, and bone marrow. Flow cytometry results expressed as fold increase or decrease of Gr-1^−^CD11b^−^ MDSCs from APC^Δ468^ (APC) or APC^Δ468^/5-LO^−/−^ (APC/5LO) mice as compared to their respective APC^Δ468^ and wt BM controls (MDSC yields from controls set as 0). Results are from minimum 3 separate mouse experiments (*, P < 0.05). To determine statistical significance, t tests were used.

**Figure 6.** 5LO deficiency mitigates recruitment of granulocytic but not monocytic subtypes of MDSCs to the intestine. (a) Representative flow cytometry data gated on CD11b^−^ live cells: Ly6G^−^Ly6C^−^ (granulocytic) and Ly6G^+^Ly6C^+^ (monocytic) MDSCs. MDSCs derived from spleen and gut, APC^Δ468^ (APC), APC^Δ468^/5LO^−/−^ (APC/5LO). APC polyp-derived MDSCs further attained an Ly6G^−^Ly6C^+^ phenotype. (b) Fold change of granulocytic and monocytic MDSCs in APC/5LO mice compared to APC controls. (c) Fold change of MDSCs in APC mice reconstituted with 5LO^−/−^ BM (5LO BM) compared to APC mice reconstituted with wt BM as controls. MDSC yields from controls in panels b and c set as 0. Results are from minimum of 3 separate mouse experiments (**, P < 0.01; ***, P < 0.001). G-MDSCs, granulocytic MDSC; M-MDSCs, monocytic MDSC. To determine statistical significance, t tests were used.
Figure 7. Mechanisms of suppression by MDSCs. APCΔ468 mice reconstituted with 5LO⁺ BM (5LO BM) had a significant decrease in their (a) arginase activity (wt BM, 2.052 ± 0.2349 U/10⁶ cells versus 5LO BM, 1.403 ± 0.1005 U/10⁶ cells; *, P < 0.05). There was no difference seen in the production of (b) ROS or (c) NOS. Results are from minimum four separate mice per group. Means shown are ± SEM. To determine statistical significance, t tests were used.

Supplementary Figure S1. The effects of deletion of hematopoietic 5LO on polyp diameter. No statistically significant difference was found between wt BM (2.512 ± 0.09592, n=6) and 5LO BM (2.268 ± 0.2446, n=5) groups (P = 0.3368). Means shown are ± SEM. To determine statistical significance, t tests were used.

Supplementary Figure S2. Murine BMMCs were grown in 6-week culture in presence of SCF and IL-3. Purity was confirmed using FACS analysis. Mast cell (Sca-1+FceR1+) purity was >90%.

Supplementary Figure S3. Infiltration of other inflammatory subsets in APCΔ468 (APC), APCΔ468/5LO⁻ (APC/5LO), APCΔ468 with B6 bone marrow (wt BM) chimeras and APCΔ468 with 5LO⁻ bone marrow chimeras (5LO BM). (a) Representative images of infiltration of CD4⁺, CD4⁺FoxP3⁺, CD8⁺ and CD11c⁺IAb⁺ cells in the APC, APC/5LO, wt BM, and 5LO BM mouse groups. (b) Frequencies of CD4⁺, CD4⁺FoxP3⁺, CD8⁺ and CD11c⁺IAb⁺ cells in the APC, APC/5LO, wt BM, and 5LO BM mouse groups. White bars represent 50 µm, *P <0.05. All means shown are ± SEM. To determine statistical significance, t tests were used.

Supplementary Figure S4. No statistically significant difference was found between PGE M levels between the APC and APC/5LO groups (P = .3725) as well as the wt BM versus 5LO BM groups (P = .8666). Means shown are ± SEM. To determine statistical significance, t tests were used.
# Mast cell 5-Lipoxygenase activity promotes intestinal polyposis in APC Δ468 mice

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