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Apolipoprotein A-I mimetics mitigate intestinal inflammation in COX2-dependent inflammatory bowel disease models

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Abbreviations: 3F(14) (Ac-D-W-L-K-A-F-Y-D-K-V-A-E-K-F-K-E-A-F-NH2); 4F, (Ac-D-W-F-K-A-F-Y-D-K-V-A-E-K-F-K-E-A-F-NH2); D-4F, 4F synthesized from all D-amino acids; 6F, (D-W-L-K-A-F-Y-D-K-F-F-E-K-F-K-E-F-F without end blocking groups); APOA1, apolipoprotein A-I; BHT, butylated hydroxytoluene; BL6 mice, C57BL/6 mice; BP, barrier permeability; CX, celecoxib; CCHF, cholate-containing high fat diet; COX2, cyclooxygenase 2; Cox2 MKO, Cox2 myeloid knock-out; Cox2 TKO, Cox2 total knock-out; DUOX2, dual oxidase 2; FDR, false discovery rate; HDL, high density lipoprotein; IL1β, interleukin 1 beta; IL6,
interleukin 6; **IL10**, interleukin 10; **KoDiAPC**, 1-palmitoyl 2-(5-keto-6-octene-dioyl) phosphatidylcholine; **LPS**, lipopolysaccharide; **MRM**, multiple reaction monitoring; **LC-MS/MS**, liquid chromatography-mass spectrometry/mass spectrometry; **LDL**, low density lipoprotein; **oxPAPC**, oxidized PAPC; **PAC**, piroxicam accelerated; **PAPC**, palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine; **PON**, paraoxonase-1; **POVPC**, 1-palmitoyl, 2-oxo-valeroyl phosphatidylcholine; **PX**, piroxicam; **SPE**, solid phase extraction; **SPR**, surface plasmon resonance; **TC**, total cholesterol; **TG**, triglyceride; **Tg6F**, transgenic tomatoes expressing the 6F peptide; **TLR4**, toll-like receptor 4; **TNF**, tumor necrosis factor. (For abbreviations of additional LC-MS/MS analytes, please see Supplemental Materials.)
Cyclooxygenase 2 (Cox2) total knockout and myeloid knockout (MKO) mice develop Crohn’s-like intestinal inflammation when fed cholate-containing high fat diet (CCHF). We demonstrated that CCHF impaired intestinal barrier function and increased translocation of endotoxin, initiating TLR/MyD88-dependent inflammation in Cox2 KO but not WT mice. Cox2 MKO increased pro-inflammatory mediators in LPS-activated macrophages, and in the intestinal tissue and plasma upon CCHF challenge. Cox2 MKO also reduced inflammation resolving lipoxin A4 (LXA4) in intestinal tissue, while administration of an LXA4 analog rescued disease in Cox2 MKO mice fed CCHF. The apolipoprotein A-I (APOA1) mimetic 4F mitigated disease in both the Cox2 MKO/CCHF and piroxicam-accelerated Il10−/− models of inflammatory bowel disease (IBD) and reduced elevated levels of pro-inflammatory mediators in tissue and plasma. APOA1 mimetic Tg6F therapy was also effective in reducing intestinal inflammation in the Cox2 MKO/CCHF model. We further demonstrated that APOA1 mimetic peptides: i) inhibited LPS and oxidized 1-palmitoyl-2-arachidonoyl-sn-phosphatidylcholine (oxPAPC) dependent pro-inflammatory responses in human macrophages and intestinal epithelium; and ii) directly cleared pro-inflammatory lipids from mouse intestinal tissue and plasma. Our results support a causal role for pro-inflammatory and inflammation resolving lipids in IBD pathology and a translational potential for APOA1 mimetic peptides for the treatment of IBD.
Introduction

Inflammatory bowel disease (IBD), inclusive of Crohn’s disease (CD) and ulcerative colitis (UC), is a chronic and relapsing inflammatory disorder of the gastrointestinal tract. IBD consists of a dysregulated mucosal immune response to gut microbiota, brought about by host and/or environmental triggers in genetically susceptible individuals (1). However, there exists no single pathogenic mechanism for IBD. Several genetic susceptibility loci have been identified for IBD (2). The candidate genes broadly implicate several distinct functional categories, including epithelial barrier function, innate immunity, T cell signaling, and autophagy; but even within a category, the underlying pathways are largely distinct (2, 3). The range of environmental factors associated with the development of IBD is also complex (4). In the absence of a single pathogenic mechanism, investigation into the etiology and pathogenesis of IBD requires careful elucidation of the disparate causal contributions of the many susceptibility-linked genes and disease-associated environmental factors.

IBD exhibits high morbidity, but there are currently few effective pharmacological interventions. For example, between 70% and 90% of all CD patients eventually undergo surgery within their lifetime, and approximately 40% will require repeated surgery (5). Anti-TNF therapies including both infliximab and adalimumab offer a treatment option for IBD that is refractory to conventional therapies including corticosteroids and immunomodulators (6). However, treatment failures with anti-TNF therapies are common (7). There exists a pressing need for new IBD therapies.

Cyclooxygenase 2 (COX2) is the inducible form of cyclooxygenase and is responsible for the rate limiting step in the conversion of arachidonic acid to prostanoids (prostacyclins, prostaglandins, and thromboxanes). Prostanoids have been well-characterized as pro-inflammatory mediators, and their biosynthesis is blocked by non-steroidal anti-inflammatory drugs including selective COX2 inhibitors.
However, COX2 can play a role in the resolution of inflammation, partially through its induction of the pro-resolving eicosanoid lipoxin A4 (LXA4) (8, 9).

Multiple genetic susceptibility studies have linked COX2 to an altered risk of developing IBD (2, 10, 11), but the exact nature of the link is not clear due to conflicting associations when utilizing data from COX2 polymorphisms. Nevertheless, it has been shown that COX2 and COX2-dependent prostaglandin E2 (PGE2) help maintain adaptive immune tolerance to dietary antigens in the proximal small intestine (12, 13). Consistent with the observations of Newberry et al., we previously reported that both Cox2 total knock-out (Cox2 TKO) and Cox2 myeloid-specific knock-out (Cox2 MKO) mice or COX2-inhibited (COX2i) mice develop severe CD-like inflammation in their ileo-ceco-colic junctions when challenged with a cholate-containing or cholate-containing high fat diet (CCHF) (14, 15). Our prior findings suggested that COX2 plays an anti-inflammatory role at the ileo-ceco-colic junction in mice. In the present study, we investigated the molecular mechanisms by which COX2 deficiency can contribute to the development of IBD.

Apolipoprotein A-I (APOA1) mimetic peptides including both 4F and 6F are short (18 amino acids) sequences that recapitulate the secondary structure and partial function of APOA1, the main structural protein of high density lipoprotein (HDL) (16). 4F and transgenic 6F (Tg6F; extract from tomatoes expressing the 6F peptide) are protective in numerous animal models of inflammatory diseases, including atherosclerosis (16). We have previously reported that circulating 4F selectively targets the small intestine, where it is transported into the intestinal lumen, is reabsorbed by the intestinal mucosa, and mediates the trans-intestinal efflux of cholesterol (17). We have also demonstrated that both oral and subcutaneous administered 4F reduces the levels of pro-inflammatory fatty acid metabolites in enterocytes of LDLR-null mice on western diet (18). We thus sought further to determine whether orally administered 4F and Tg6F could inhibit the development of intestinal inflammation in animal models of IBD including the Cox2 KO/CCHF model.
Our results indicate that: 1) myeloid COX2 generates an anti-inflammatory/pro-resolving lipid and pro-inflammatory check on TLR-dependent mural inflammation that can drive intestinal inflammation in IBD when compromised; 2) pro-inflammatory lipids play a causal role in IBD pathology, while anti-inflammatory/pro-resolving lipids play a protective role; and 3) APOA1 mimetic peptides hold promise as therapies for IBD, in part through their capacities to inhibit both LPS and oxidized 1-palmitoyl-2-arachidonoyl-sn-phosphatidylcholine (oxPAPC) signaling and to promote luminal clearance of mural pro-inflammatory lipids.
Results

Dysregulated response to TLR-dependent signaling drives intestinal inflammation in the Cox2 KO/CCHF models of IBD.

Consistent with our prior reports (15), Cox2 TKO and COX2i but not control mice developed severe chronic inflammation in their ileo-ceco-colic junctions when fed CCHF for 2 weeks (Figure 1A). Disease was characterized by inflammatory lesions in both the submucosa and muscularis propria, sporadic foci of mucosal ulceration, and thickening of the intestinal wall.

Bile acids pose a risk of cellular toxicity (19), while disrupted barrier function (20) or epithelial damage itself (21) can cause intestinal inflammation. We thus sought to determine if altered barrier function or epithelial damage preceded the development of inflammation in the COX2 models. C57BL/6J (BL6) mice were fed CCHF or CCHF with the COX2 inhibitor celecoxib (CX). We assessed intestinal epithelial damage (22), changes in whole intestinal barrier permeability (23), translocation of endotoxin into portal vein serum, and ileo-ceco-colic inflammation over 2 weeks (Figure 1B). CCHF significantly increased whole intestinal permeability equally in both CX-treated and control mice, as assessed by urinary excretion of sucralose (Figure 1B, top left). We also observed that CCHF comparably increased intestinal permeability in wild-type (WT) and Cox2 TKO mice (Supplemental Figure 1), indicating that off-target effects of celecoxib had not confounded our prior result (24).

The increase in permeability was accompanied by a significant increase in endotoxin translocated into portal vein serum in CX-treated and control mice (Figure 1B, bottom left). The increases in both permeability and endotoxin preceded histological evidence of inflammation in the ileoceco-colic junctions of CX-treated mice (Figure 1B, top right). Nonetheless, despite similar increases in both permeability and endotoxin levels, only CX-treated but not control mice developed severe
intestinal inflammation. Epithelial ulceration was observed in CX-treated mice at day 14 only (Figure 1B, bottom right), after the initiation of inflammation in these mice. Since ulceration followed the development of inflammation, ulceration itself was likely a consequence rather than a cause of the inflammation.

We pursued the hypothesis that CCHF initiates intestinal inflammation by causing loss of barrier function and translocation of pathogen associated molecular patterns (PAMP) including lipopolysaccharide (LPS). We thus determined the dependence of the intestinal inflammation on gut microbiota. We pre-treated Cox2 TKO and BL6 mice for 1 week with antibiotics (AB) to eliminate intestinal bacteria (25). We challenged the mice with CCHF or CCHF + CX for 2 weeks, respectively, while continuing AB. AB treatment abrogated inflammation in the ileo-ceco-colic junctions of Cox2 TKO and CX-treated mice, as assessed by gross pathology (Figure 1C) and histological disease score (Supplemental Figure 2). (For this and subsequent histological measures of disease, we employed a 0-12 point scoring system that incorporated assessments of both inflammation and epithelial damage/altered mucosal architecture; see Supplemental Methods.) To exclude the possibility that AB treatment inhibited inflammation by blocking the bacterial conversion of cholate to the more cytotoxic deoxycholate (26), we determined the effects of cholate and deoxycholate on barrier permeability in AB-treated TKO mice, which treatment prevented the conversion of cholate to deoxycholate. Cholate and deoxycholate significantly increased barrier permeability, but we observed no difference between their effects (Supplemental Figure 3).

LPS induces the production of pro-inflammatory mediators by stimulating the LBP/CD14/MD-2/TLR4 cell surface formation. This complex then signals through the adaptor protein MyD88 to trigger the nuclear translocation of NFκB and the transcription of pro-inflammatory target genes (27). We inhibited MyD88 with the small molecule T6167923 (28) in Cox2 TKO mice fed CCHF. As with AB
treatment, MyD88 inhibition abrogated gross pathological (Figure 1D) and histological (Supplemental Figure 4) evidence of ileo-ceco-colic inflammation.

If LPS/PAMP translocation and TLR/MyD88 signaling initiates disease in our models, there must exist a differential response to bacteria-dependent TLR ligands in COX2 deficient versus control mice. Cox2 myeloid conditional knock-out (MKO) mice (Cox2^{fl/fl}; LysM^{cre/+}) developed ileo-ceco-colic inflammation comparable to that of Cox2 TKO and COX2i mice, upon 7 or 10 week CCHF (Supplemental Figure 5). Elevated levels of neutrophils and macrophages but not CD4+ or CD8+ T-cells characterized the inflammatory lesions (Supplemental Figure 6) (15). CCHF diet significantly increased intestinal barrier permeability through 3 weeks in Cox2 MKO and floxed control (FLOX) mice as assessed by FITC-dextran 4 kD (29) (Supplemental Figure 7). The LysM promoter of these mice is expressed in almost 100% of non-splenic macrophages and approximately 60% of neutrophils (30). We conducted a pilot study using neutrophil-specific Cox2 KO mice (Cox2^{fl/fl}; Mrp8^{cre/+}), whose promoter is unexpressed in macrophages (30). Following 7 wk CCHF, we did not observe any evidence of ileo-ceco-colic inflammation (data not shown). As a result, we restricted our investigation into differential responses to LPS/PAMP to the macrophage compartment. We generated bone-marrow derived macrophages (BMDM) from Cox2 MKO and FLOX mice (Supplemental Figure 8). We activated the macrophages with LPS and determined the expression of NFκB pro-inflammatory target genes using qPCR through 24 hours. We had validated the loss of COX2 activity by measuring COX2 metabolites including PGE2, PGD2, PGJ2, and 15dPGJ2 via LC-MS/MS (Supplemental Figure 9). We observed that the expressions of pro-inflammatory genes including Tnf and Il1b were significantly higher in Cox2 MKO BMDM (Figure 1E). Dysregulation of TLR/MyD88-dependent pro-inflammatory signaling within the macrophage compartment could thus partially explain the differential response to CCHF between control and COX2 deficient mice.
Cox2 MKO mice fed CCHF exhibit elevated levels of pro-inflammatory lipid mediators in intestine and plasma, while the loss of inflammation resolving LXA4 in intestine appears partially causal for disease.

Cox2 MKO enhanced the acute inflammatory response and inhibited its resolution in the carrageenan hind paw model (Supplemental Figure 10). COX2 can modulate the production of pro-resolving lipid mediators including LXA4 (8), an eicosanoid that promotes resolution in part by stimulating efferocytosis (31). Macrophage COX2 can directly mediate intracellular production of LXA4 through LPS-dependent generation of 15HETE (32). Loss of macrophage COX2 can also shunt arachidonic acid (AA) through the 5-lipoxygenase (5LOX) and 12- or 15-lipoxygenase (12/15LOX in mice) pathways, leading to the production of pro-inflammatory eicosanoids (32). We thus hypothesized that Cox2 MKO mice fed CCHF would exhibit higher pro-inflammatory and lower pro-resolving lipid mediator levels compared to FLOX controls.

We developed an MRM LC-MS/MS method to measure a panel of pro-inflammatory, anti-inflammatory, and pro-resolving lipid mediators (see Supplemental Methods for details). The method quantitatively evaluates most of the bioactive signaling metabolites of arachidonic acid (AA), DHA, and EPA in the COX and LOX pathways, together with pathway markers and stable end products (Table 1 and Supplemental Table 1). We validated the method for intestine, plasma, and cell culture lysate and media (Supplemental Tables 2-5 and Supplemental Figure 11).

We fed Cox2 MKO and FLOX mice either chow with CCHF for 8.5 weeks then determined the levels of the above mediators in the ileo-ceco-colic junction (Figure 2A and Supplemental Table 6) and plasma (Figure 2B and Supplemental Table 7). (See Supplemental Tables 8-10 for application of Benjamini-Hochberg procedure (33) to all lipidomic data sets for the control of false discovery rate (FDR) at level α = 0.05). On CCHF, Cox2 MKO significantly increased the intestinal levels of several pro-inflammatory signals compared to FLOX controls (Figure 2C): the pro-inflammatory LOX mediators
12HETE, 15HETE, and 13HODE; and the pro-inflammatory COX-dependent PGI2 degradation product
6ketoPGF1α. In plasma, Cox2 MKO significantly increased the pro-inflammatory LOX signals 12HETE,
5HETE and 15HETE; and the pro-inflammatory COX signal PGF2α together with its degradation product
13,14-dihydro-15-PGF2α, and TXB2 (Figure 2D). Cox2 MKO also significantly increased in both tissue and
plasma the total level of all LOX and COX pro-inflammatory mediators; and in plasma, PGE2 (Figure 2E).

We investigated whether these elevated pro-inflammatory signals in plasma might constitute
biomarkers for IBD. In an independent experiment, we determined the levels of pro-inflammatory LOX
mediators in plasma of Cox2 MKO and FLOX mice on chow, and 3 and 8.5 weeks CCHF (Supplemental
Figure 12). Pro-inflammatory LOX signals including 12- and 5HETE were significantly elevated in Cox2
MKO mice with 8.5 weeks CCHF. The differential plasma levels of these signals correlated with the
degree of intestinal inflammation in these mice. We also observed that a significant increase in the
systemic inflammation marker serum amyloid A (SAA) in FLOX mice did not associate with elevated
signals (Supplemental Figure 13). Both results together suggest that the plasma levels of these pro-
inflammatory LOX products possess at least prima facie specificity with respect to IBD. The significant
but modest increase in SAA in FLOX mice, which can be induced by IL1 and IL6 but also by LPS (34),
provides further evidence of the otherwise modest effect of CCHF in normal mice.

Cox2 MKO also significantly reduced pro-resolving LOX signals in the ileo-ceco-junctions of
CCHF-fed mice (Figure 2F). Both LXA4 and the total of 5LOX specialized pro-resolving mediators (SPM)
(LX + RvD series) were significantly decreased. Moreover, the ratio of 5LOX-associated SPM to pro-
inflammatory LTB4 and its product 6trans12epiLTB4 was significantly decreased (see “5LOX SPM/Pro-INF
LOX” in Figure 2F).

We had observed the co-presence of neutrophils and macrophages in the inflammatory lesions
of Cox2 MKO mice fed CCHF (see Supplemental Figure 6). This co-presence suggested that the
resolution of inflammation, which involves the efferocytosis of neutrophils by macrophages, failed (35).
LXA4 can block neutrophil migration and stimulate efferocytosis (36). We thus hypothesized that the significant reduction of LXA4 in the ileo-ceco-colic junctions of Cox2 MKO + CCHF mice was partially causal for the chronic inflammation in these mice. We administered a stable analog of LXA4 (BML111) to Cox2 MKO mice across the course of an 8.5 week CCHF (37). BML111 treatment significantly improved several measures of intestinal inflammation, including gross pathology (Figure 2G, left) and histological disease score (Figure 2G, right). (See Supplemental Figure 14 for additional measures and representative images.)

The APOA1 mimetic peptides 4F and Tg6F inhibit the development of intestinal inflammation in the Cox2 MKO/CCHF model of IBD.

Gene expression of APOA1 is reduced in the ilea of patients with ileal or colonic CD (38). Colonic APOA1 is also protective in mouse models of UC (39). We determined both APOA1 protein and gene expression in the proximal colons and terminal ilea of MKO and FLOX mice fed 8.5 wk CCHF or chow (Supplemental Figure 15). Gene expression was significantly reduced in the proximal colons of Cox2 MKO mice fed CCHF.

We co-treated Cox2 MKO mice fed CCHF with the APOA1 mimetic D-4F (500 μg/ml drinking water) for 7 and 10 weeks. 4F protected against ileo-ceco-colic inflammation in Cox2 MKO mice at both time points. 4F treatment blocked the increase in ileo-ceco-colic thickness (Figure 3A); significantly improved disease score (Figure 3B); and blocked the increase in muscularis thickness (Figure 3C). 4F treatment significantly inhibited the influx of F4/80+ macrophages and Ly6G+ neutrophils into the ileo-ceco-colic junctions (Figures 3D-E). 4F-treated mice also expressed significantly less Tnf but more Il10 in their ileo-ceco-colic junctions (Figure 3F).
To determine whether the protective effect of 4F in this model extended to other APOA1 mimetic peptides, we co-treated Cox2 MKO mice fed CCHF with 0.06% Tg6F (w/w) for 10 weeks (40). To assess whether Tg6F could inhibit the progression of existing disease, we delayed the start of treatment with Tg6F in another group until the mice had been fed CCHF for 5 weeks. Both early and late onset Tg6F therapy rescued gross pathology and improved histology (Figure 3G-H and Supplemental Figure 16). These results suggest that APOA1 mimetic peptides as a class may be protective against intestinal inflammation in the Cox2 KO/CCHF model of IBD even after the disease has been well established.

4F treatment improves the balance of pro-inflammatory and inflammation resolving lipid mediators in Cox2 MKO mice fed CCHF, while suppressing the pro-inflammatory phenotype of LPS-treated macrophages.

We determined the effect of oral D-4F therapy on the levels of lipid inflammatory mediators in the ileo-ceco-colic junctions and plasmas of Cox2 MKO mice fed CCHF for 8.5 weeks (Figures 4A-B; and see Supplemental Tables 6-7). 4F treatment rescued the intestinal levels of total pro-inflammatory LOX products as well as 12HETE and 13HODE (Figure 4C). In plasma, 4F significantly reduced the level of every pro-inflammatory LOX mediator elevated by Cox2 MKO together with most additional mediators in this class (Figure 4D). 4F also significantly reduced in plasma most pro-inflammatory COX mediators as well as PGE2 (Figure 4H).

We determined the effect of 4F on plasma paraoxonase-1 (PON1) activity as a measure of HDL and on plasma total cholesterol (TC) (Supplemental Figure 17) (41). 4F significantly reduced TC, consistent with our prior reports that 4F can enhance the trans-intestinal efflux of cholesterol (17). 4F also significantly improved PON1 activity. We last determined the effect of 4F on broad classes of plasma lipids in Cox2 MKO mice fed CCHF. We employed ion mobility MS/MS shotgun lipidomics (42)
through the SCIEX Lipidyzer platform to determine the concentrations of 1103 lipid species from 13 lipid classes. We observed significant reductions in total triacylglycerols, diacylglycerols, cholesterol esters, and lyso-phosphatidylethanolamines (Supplemental Figure 18).

Last, we investigated the protective mechanism of 4F by asking whether 4F could inhibit the pro-inflammatory effects of LPS upon mouse macrophages. We co-treated LPS-activated BMDM with 4F (15 μg/ml) and assessed the expression of NFκB pro-inflammatory target genes. 4F significantly inhibited expression of *Il1b* (Figure 5A) and *Tnf* (Figure 5B) in Cox2 MKO and FLOX BMDM through 24 hours. We also assessed the effect of 4F on lipid inflammatory mediators in BMDM cell lysates. 4F significantly reduced the levels of LPS-induced COX metabolites including PGE2 (Figure 5C) and PGD2 (Figure 5D). Comparing the mono-hydroxylated COX and LOX AA-metabolites 11HETE and 15HETE, respectively, we observed that 4F treatment significantly lowered the levels of 11- but not 15HETE (Figure 5E). This differential effect on structurally comparable lipids suggests that 4F did not lower the levels of COX metabolites by binding and clearing these lipid species. Rather, these results altogether suggest that 4F inhibits the effects of LPS by directly interfering with LPS-dependent signaling.

**4F inhibits the LPS-mediated pro-inflammatory response of human macrophages and intestinal epithelium.**

We stimulated human THP-1 macrophages with LPS and co-treated with 4F across 24 hours. As with mouse macrophages (see Figure 5), we observed that 4F significantly inhibited the induction of PGE2 (Figure 6A) and other prostanoids (Supplemental Figure 19). We examined by western blot the LPS-dependent degradation of the NFκB inhibitor IκBα across 60 minutes. 4F significantly prevented the degradation of IκBα at both 20 and 30 minutes (Figure 6B and Supplemental Figure 20). We obtained comparable results using Cox2 MKO peritoneal macrophages (data not shown). Macrophages do not
internalize 4F (GM Ananthamariah, unpublished data), and 4F can bind to oxidized and non-oxidized fatty acids with high affinity (43). We hypothesized that 4F inhibits the LPS-dependent activation of the NFκB pathway by binding to the lipid A moiety of LPS, thereby preventing lipid A from activating LBP/CD14/MD-2/TLR4. We employed surface plasmon resonance analysis to determine the binding affinity between 4F and both LPS (Figure 6C, left) and lipid A (Figure 6C, right). 4F bound to both with high affinity (K_D’s of approximately 1 and 17 nM, respectively), supporting our hypothesis.

We next investigated the effect of 4F on the LPS-dependent pro-inflammatory response of human intestinal epithelium ex vivo. We isolated small intestinal crypts from human surgical samples and cultured them in Matrigel (see Supplemental Methods). Under these conditions, the crypts expand into small intestinal epithelial structures known as enteroids (Figure 6D) that recapitulate intestinal epithelial physiology (44). We examined the way that small intestinal epithelium might amplify the pro-inflammatory response of macrophages. We collected conditioned media (CM) from THP-1 macrophages that had been treated with LPS or LPS + 4F for either 4 or 12 hours. We exposed the human enteroids to CM for 12 hours and determined pro-inflammatory gene expression by qPCR. CM from LPS-activated macrophages significantly induced gene expression of TLR4, COX2, IL1B, IL6, and TNF, with 12 hr CM significantly increasing expression over 4 hr CM (Figure 6E-I). By contrast, CM from macrophages treated with LPS + 4F did not increase expression of these genes. The magnitude of the pro-inflammatory response of the enteroids to CM from LPS-activated macrophages highlights the potential amplifying effect of pro-inflammatory stimuli in the lamina propria by intestinal epithelium. The degree of the inhibition of this epithelial response by 4F suggests a protective mechanism by which 4F not only inhibits the primary pro-inflammatory response of resident macrophages but also interrupts the positive feedback loops that propagate and amplify this response within the intestinal mucosa.
Oxidized PAPC is elevated in the plasma and ceca of Cox2 MKO + CCHF mice, while 4F reduces oxPAPC in vivo and inhibits the oxPAPC-dependent pro-inflammatory response of human macrophages.

Lipid peroxidation markers are elevated in plasma (45) and affected intestinal tissue (46) of CD patients, and lipid peroxides may be pathogenic for CD (47). POVPC, PGPC, and KOdiAPC are all phospholipids in which the polyunsaturated arachidonoyl group at the sn2 position of PAPC oxidatively truncates following a peroxidation step at one of its double bonds (48). Because PAPC is a common and representative phospholipid, these oxPAPC species are considered biomarkers of lipid peroxidation (49).

We determined the levels of POVPC, PGPC, and KOdiAPC (Supplemental Methods) in the ileo-ceco-colic junction (Figure 7A) and plasma (Figure 7B) of Cox2 MKO and FLOX mice fed chow or 8.5 wk CCHF. We observed that these species were significantly elevated in both compartments of Cox2 MKO mice fed CCHF. We further determined oxPAPC levels in the proximal colon and terminal ileum of these mice, as well as in the ileo-ceco-colic junctions of FLOX and Cox2 MKO mice fed CCHF for 3 weeks. Elevated oxPAPC levels correlated both spatially and temporally with advanced intestinal inflammation (Supplemental Figure 21). In contrast to human CD, the increase in oxPAPC species did not associate with an increase in expression of dual oxidase 2 (Duox2) in the terminal ileum or proximal colon (Supplemental Figure 22) (38).

We investigated the effect of 4F upon these oxPAPC species in both Cox2 MKO and FLOX mice fed CCHF. After only 3 weeks of therapy, oral D-4F had significantly reduced oxPAPC in the cecum (Figure 7C) and plasma (Figure 7D) of both groups.

Oxidized PAPC species are themselves biologically active and can be pro-inflammatory (48, 50). We considered the hypotheses that oxPAPC helps amplify intestinal inflammation, while 4F inhibits this effect. POVPC significantly increased IL1B gene expression in THP-1 macrophages by 3 hours (Figure 7E).
4F co-treatment blocked this induction, while 3F(14)—an 18 amino acid peptide that lacks the affinity of 4F for oxidized lipids (43)—did not.

**4F can directly clear lipid pro-inflammatory mediators from intestinal tissue and plasma.**

4F reduced the levels of pro-inflammatory LOX but not COX mediators in Cox2 MKO mice fed CCHF (Figure 8A; cf. Figure 4C). This selective reduction of disease-elevated mediators weighs against the possibility that 4F only indirectly lowers these mediators by otherwise inhibiting disease. We hypothesized that 4F could directly clear pro-inflammatory LOX mediators from intestinal tissue. We previously employed Ussing chambers to study the effect of 4F on trans-intestinal cholesterol efflux (TICE) ex vivo (17). We now mounted in Ussing chambers small intestinal explants from Cox2 MKO mice fed 8.5 week CCHF. Mucosal/luminal side D-4F significantly increased the clearance into the mucosal media of most pro-inflammatory LOX mediators (Figure 8B).

We previously reported that pro-inflammatory LOX mediators associate with lipoproteins including HDL (51, 52). We also demonstrated that 4F enhances trans-intestinal cholesterol efflux (TICE), while showing ex vivo that luminal 4F can increase the trans-intestinal efflux of lipoprotein-associated cholesterol (17). We now asked whether luminal 4F could also affect the trans-intestinal transport of lipoprotein-associated pro-inflammatory mediators. We combined deuterated 15HETE, 12HETE, and 13HODE with HDL and LDL and loaded the mixture into the serosal media of Ussing chambers in which we mounted explants from the proximal SI. We determined by LC-MS/MS that trans-intestinal transport of these tagged lipids occurred even at baseline (Figure 8C). There thus may exist trans-intestinal transport of lipid species besides cholesterol in vivo. Further, mucosal-side 4F significantly increased the trans-intestinal transport of 15HETE-d and 12HETE-d (Figure 8D). Luminal 4F
might directly enhance trans-intestinal lipid transport, and thereby possible clearance, of lipoprotein-associated inflammatory mediators.

**4F treatment inhibits development of colitis in the piroxicam-accelerated Il10−/− model**

4F binds to cholesterol with high affinity ($K_D = \text{approximately } 11 \text{ nM}$) (43). We considered the possibility that 4F is protective in the Cox2 KO/CCHF models by binding and inactivating dietary cholate. We determined the binding affinity between D-4F and cholate, observing only low affinity binding (**Supplemental Figure 23**). 4F also did not inhibit the early effect of CCHF on barrier permeability (**Supplemental Figure 24**), further indicating that 4F does not inactivate CCHF.

We nonetheless assessed the efficacy of 4F therapy in a second mouse model of IBD, the piroxicam accelerated (PAC) Il10−/− model (53). Il10−/− mice that are fed the NSAID piroxicam (PX) for 9 days develop colitis that is characterized by hyperplasia and focal transmural inflammation reminiscent of human CD. In the PAC Il10−/− model, disease is assessed at day 14 (5 days post-PX) by determining colon length, colitis severity, and histopathological score (53).

Oral D-4F (500 ug/ml drinking) improved several markers of disease in PAC Il10−/− mice. PX significantly decreased colon length while 4F therapy rescued this effect (**Figure 9A**). Colitis severity was determined on a 4-point scale by assessing the thickening of the mucosa, vascularity, granularity, and feces consistency (53). 4F therapy significantly improved colitis disease score (Figure 9A). 4F therapy also significantly improved histological disease score, as assessed by the degree of lesions, hyperplasia, ulceration, and % area involved (53) (**Figure 9B**).

The PAC Il10−/− model depends upon the presence of gut microbiota to initiate disease (53). Given the off-target epithelial toxicity of PX, it has been conjectured but not demonstrated that PX accelerates disease by impairing barrier function (54). We thus assessed the effect of PX on whole
intestinal permeability in $Il10^{-/-}$ mice across 9 days of PX challenge. We observed a biphasic response, with an early increase in permeability (day 0-3) followed by a late increase (day 7-9) that coincides with increased disease (Figure 9C, left panel). We also observed increased endotoxin in portal vein serum at day 9 (Figure 9C, right panel).

4F inhibited the effect of LPS on $Il10^{-/-}$ peritoneal macrophages treated with PX (Figure 9D). We also determined the levels of lipid inflammatory mediators in colon from $Il10^{-/-}$ and PAC $Il10^{-/-}$ mice +/- 4F. Multiple pro-inflammatory mediators were increased in the colon of PAC $Il10^{-/-}$ mice (Figure 9E and Supplemental Table 11). 4F therapy rescued the increase of all pro-inflammatory LOX products, without inhibiting the significant increase in the sole elevated COX product TXB2 (Figure 9F).

Finally, we determined the effect of 4F on plasma lipid inflammatory mediators in these mice (Supplemental Table 12). 4F therapy significantly reduced the levels of most pro-inflammatory LOX and COX mediators. The broad nature of the effects of 4F on plasma lipids suggests a broad clearance mechanism capable of affecting all plasma-associated mediators together.
Discussion

We have investigated the pathogenic mechanisms of the Cox2 KO/CCHF models of IBD together with the protective mechanisms of APOA1 mimetic peptides (Figure 10). CCHF increased barrier permeability and initiated TLR/MyD88-dependent intestinal inflammation, while the loss of myeloid COX2 enhanced the pro-inflammatory response of LPS-activated macrophages; elevated the levels of lipid pro-inflammatory mediators in intestinal tissue and plasma; and reduced the level of pro-resolving LXA4 in intestinal tissue. An LXA4 analog (BML111) rescued disease in the Cox2 MKO/CCHF model. 4F therapy inhibited the pro-inflammatory effects of LPS and oxPAPC on macrophages (and intestinal epithelium), and it reversed the disease-dependent increase of lipid pro-inflammatory mediators while enhancing luminal clearance of mural pro-inflammatory lipids. These observations support the correlate hypotheses that dysregulated levels of inflammatory mediators are pathogenic for disease in the Cox2 MKO model, and that rescue of these levels is protective.

Disease in the Cox2 MKO/CCHF model overlaps with human CD in several respects. The inflammation is localized to the ileo-colic junction and is transmural in nature (see Figure 1A). Apoa1 expression is inhibited in this region (see Supplemental Figure 14), as occurs in human CD (38). Lipid peroxidation markers are elevated in plasma and inflamed intestine (see Figure 6), as in human CD (55, 56). However, unlike human CD (38), expression of Duox2 was not increased in the terminal ilea of diseased mice (see Supplemental Figure 22). Furthermore, the reduction of Apoa1 expression in the colon was offset by lack of change in the ileal and plasma compartments.

Increased intestinal permeability has been proposed as a primary etiologic factor in IBD (57). However, the inflammatory process itself can impair barrier function (58). There remains debate whether changes in barrier permeability are early events in pathogenesis or mere secondary phenomena (59). Our results indicate that alteration in barrier function is an important initiating event in both the Cox2 KO/CCHF and PAC Il10−/− models (see Figures 1 and 9C). Nonetheless, we did observe
delayed increases in barrier permeability in both the Cox2 MKO/CCHF and PAC II10\(^{-/-}\) models, indicative of an inflammation-dependent secondary effect (see Supplemental Figure 7 and Figure 9C).

Loss of barrier function alone was not sufficient to cause intestinal inflammation in Cox2 MKO mice fed CCHF. Barrier permeability increased along the whole intestine (see Figure 1B and Supplemental Figure 1), but inflammation was localized to the ileo-ceco-colic junction. We originally considered the possibility that disease depended upon the conversion of cholate to deoxycholate within this compartment, but we observed no difference between the functional effects of these bile salts (see Supplemental Figure 3). However, disease in this model did depend upon TLR/MyD88-specific ligands (see Figure 1), whose concentrations are greatest in the ceca of mice.

Cox2 MKO enhanced the pro-inflammatory phenotype of LPS-activated BMDM (see Figure 1E) (cf. (60, 61)). Cox2 MKO also prevented production of PGJ2 and 15d-PGJ2 in these macrophages (see Supplemental Figure 9). The cyclopentenone prostaglandins PGJ2 and 15d-PGJ2 can act as negative regulators of NFκB (36). It is possible that a loss of negative feedback control of NFκB partially explains the pro-inflammatory effects of Cox2 MKO in LPS-activated macrophages. Because additional PAMPs including lipoteichoic acid (LTA) and peptidoglycan (PG) also can signal through NFκB (27), we speculate that Cox2 MKO also enhances the pro-inflammatory effects of these additional PAMPs.

The pro-inflammatory LOX and COX mediators 15HETE, 12HETE, 5 HETE, 11 HETE, PGE2, and TXB2 are elevated in the colons of human UC patients (62). We observed comparable increases in pro-inflammatory LOX and COX mediators in the intestinal tissues of both the Cox2 MKO and PAC II10\(^{-/-}\) models (see Figures 2 and 9). The overlap between the pro-inflammatory eicosanoid signatures of these mouse models with that of human UC suggests that the common elevations may constitute a more general signature for IBD. Intestinal inflammation also correlated with elevated plasma lipid pro-inflammatory mediators including both LOX metabolites and oxPAPC (see Figures 2, 7, and 9; and
Supplemental Figures 12 and 21). These plasma-associated inflammatory mediators may thus be of interest as possible IBD biomarkers.

Pro-inflammatory lipid mediators may be causative for disease in both IBD models. 4F therapy rescued the increase of pro-inflammatory LOX mediators in the intestinal tissue without affecting the levels of pro-inflammatory COX mediators (see Figures 4C, 8A, and 9F). 4F can also directly clear pro-inflammatory LOX mediators from intestinal tissue (see Figure 8). Together, these observations support a causal role for the LOX mediators. Of note, 12HETE enhances vascular permeability and neutrophil recruitment (63), and elevated 12HETE has been associated with human IBD (62). Furthermore, disease associated with elevated oxPAPC in intestine, and oxPAPC induced a pro-inflammatory response in macrophages. 4F lowered intestinal oxPAPC and inhibited the direct effect of oxPAPC on macrophages (see Figure 7). These further observations support a role for oxPAPC in amplifying disease in Cox2 MKO mice fed CCHF.

Loss of pro-resolving lipid mediators may be causative for disease in the Cox2 MKO/CCHF model. Macrophage COX2 can mediate intracellular production of LXA4 (9), and Cox2 MKO lowered the level of LXA4 compared to FLOX controls (see Figure 2F). LXA4 can trigger anti-inflammatory and pro-resolving functions including blocking neutrophil migration and stimulating efferocytosis (36). LXA4 formation is lower in colonic mucosal biopsies from patients with active UC versus healthy control (64), while stable analogs of LXA4 were protective in DSS and TNBS models of UC (65, 66). The stable LXA4 analog BML111 inhibited disease in Cox2 MKO mice fed CCHF (see Figure 2G), supporting the hypothesis that COX2-dependent loss of LXA4 is partially causative of disease in this model. By contrast, LTB4 is a potent pro-inflammatory mediator involved in neutrophil recruitment (67) whose production can be enhanced by loss of COX2 (9). Altered balance of 5LOX-associated resolving mediators to LTB4 has been associated with plaque instability in atherosclerosis (68) and could further explain the chronic inflammation of this model.
We have concluded that myeloid COX2 constitutes a check on TLR-dependent intestinal inflammation in the ileo-colic junction. Our account thus extends earlier work that had also demonstrated a role for COX2 in intestinal immune tolerance. Newberry et al. showed that COX2 and PGE2 help maintain adaptive immune tolerance to dietary antigens in the proximal small bowel (12), which function they further attributed to constitutive Cox2 expression and PGE2 production by stromal cells in the lamina propria (13). Despite the general overlap, however, our current account differs from this prior work in at least one respect. Contrary to Newberry et al., we did not observe an expansion of the T lymphocyte population in the inflammatory lesions of the Cox2 MKO/CCHF model (see Supplemental Figure 5). Rather, the immunosuppressive effect of myeloid COX2 in our model appears to bear primarily on innate immunity. However, Newberry et al. identified stromal-derived PGE2 as the essential mediator of COX2-dependent adaptive immune suppression. By contrast, myeloid Cox2 KO did not result in a decrease in PGE2 within the ileo-ceco-colic junctions of Cox2 MKO mice fed CCHF (see Figure 2E), indicating complimentary production of PGE2 by other cell types. Our results thus appear consistent with the earlier work of Newberry et al. on COX2 and intestinal immune tolerance.

The APOA1 mimetic peptides 4F and Tg6F inhibited intestinal inflammation in our mouse models. In the Cox2 MKO/CCHF model, 4F directly suppressed LPS-dependent signaling and appeared to interrupt many of the amplifying pro-inflammatory feedback loops present in disease. In particular, 4F blocked the pro-inflammatory effect of LPS-activated macrophages on intestinal epithelium; it lowered oxPAPC while inhibiting its pro-inflammatory effects; and it cleared mural pro-inflammatory lipids. These results thus extend earlier reports on the efficacy of mimetic peptides against DSS-induced colitis (39, 69). APOA1 mimetic peptides offer several advantages over existing IBD therapies. These drugs are not immune suppressive; and unlike current biologics, they can be taken orally and are not antigenic (70-72).
4F therapy reduced the plasma levels of lipid pro-inflammatory mediators (see Figures 4, 7, and 8). We demonstrated ex vivo that lipid inflammatory mediators can be transported across intestinal explants from lipoproteins into mucosal media, indicating that there may exist trans-intestinal lipid transport (TILT) of lipid species over and above cholesterol. 4F can enhance this trans-intestinal transport (see Figure 8D). It is thus possible that TILT mediates the effect of 4F on plasma lipid inflammatory mediators in vivo. We previously demonstrated that 4F can enhance the trans-intestinal efflux of cholesterol (17), and 4F lowered total cholesterol in the plasmas of Cox2 MKO/CCHF mice (see Supplemental Figure 17). 4F may mediate the reduction of plasma cholesterol and plasma pro-lipid inflammatory mediators through comparable trans-intestinal mechanisms (see Figure 10). It is also possible that 4F may act like APOA1 to increase macrophage expression of the anti-oxidant APOE (73), which effect could further account for the anti-oxidant properties of 4F.

The current study contains several limitations. First, inflammation is a dynamic process but we sampled lipid inflammatory mediators at only a few time points. Second, we do not fully elucidate the mechanisms by which loss of myeloid COX2 results in the dysregulated lipid mediator levels that we observe. Third, we do not fully establish the causal significance of most of the dysregulated lipid inflammatory mediators.

Nonetheless, the present study identifies a role for myeloid COX2 in limiting TLR-dependent intestinal inflammation. It highlights the potential importance of pro-inflammatory and pro-resolving lipids as modulators of IBD. Finally, we offer further evidence of the possible translational value of APOA1 mimetics as therapy for IBD, while adding to our understanding of their protective mechanisms.
Materials and Methods

Mice. Cox2 MKO (Cox2\(^{fl/fl}\); LysM\(^{Cre/+}\)), Cox2 FLOX (Cox2\(^{fl/fl}\)), Cox2 TKO (Cox2\(^{luc/luc}\)), Cox2\(^{fl/fl}\); Mrp8\(^{Cre/+}\), and Il10\(^{-/-}\) mice were all generated, bred, and maintained as described {Watanabe, 2010 #2; Ishikawa, 2006 #203; Holgersen, 2014 #20; Abram, 2014 #37}. C57BL/6J mice were from Jackson Labs. Equal numbers of male and female mice were used in all in vivo studies.

APOA1 mimetic peptides. D-4F and 3F(14) peptides were synthesized as previously described (17, 75). For in vivo studies, D-4F was supplied as 500 μg/ml in drinking water, and the mice were allowed to drink ad libitum. For cell culture studies, D-4F or 3F(14) were added at 15 μg/ml to the cell culture media. Tg6F extracts were prepared as described previously (40, 76). For studies involving Tg6F, the final diets contained 0.06% of Tg6F extract by weight (40).

Ethics Statement. All human tissues used in this study were obtained from de-identified and discarded surgical specimens following clinical surgical pathology evaluation. The UCLA Institutional Review Board approved procurement and use of surgical samples, which waived the requirement for informed consent for tissues obtained from the UCLA Translational Pathology Core Laboratory (IRB #11-002504). The Animal Research Committee at UCLA approved the animal protocols used in this study and the methods were carried out in accordance with the approved guidelines.

Statistics. For lipidomic analyses (see Figures 2, 4, and 9), FDR corrections were performed by application of the Benjamini-Hochberg procedure to p-values determined for each analyte by one-way ANOVA, with FDR controlled at level \( \alpha = 0.05 \). For each analyte within a data set deemed a discovery/significant, post hoc analyses consisted of Tukey’s multiple comparisons test. For all other
multiple comparisons, one-, two-, or three-way ANOVA was followed by Tukey’s or Dunnett’s multiple comparisons test. For comparisons involving one independent variable with two groups, we performed Student’s t-tests; and for studies consisting of more than one Student’s t-test, we performed Holm-Sidak’s correction for multiple tests. In all cases of multiple comparisons/tests, we adjusted p-values in accord with the appropriate correction. Statistical significance was set at $p < 0.05$ with respect to final (adjusted) $p$-values. Variation is reported as SEM.

For all other Materials and Methods, please refer to Supplemental Materials.

Author Contributions: David Meriwether designed studies, conducted experiments, analyzed data, and wrote the manuscript. Dawoud Sulaiman conducted the Ussing chamber experiments. Carmen Volpe, Anna Dorfman, and Hannah Trost helped conduct the animal studies and maintain the animal colonies. Victor Grijalva performed macrophage studies. Nasrin Dorreh did western blots and lipid extractions. Sergio Solorzano and Jifang Wang helped perform the human enteroid studies. Ellen O’Connor and Jeremy Papesh helped develop the LC-MS/MS lipid analysis methods. Muriel LaRouche provided the Ussing chambers and training for their use. Mayakonda N. Palgunachari and G.M. Anantharamaiah synthesized the APOA1 mimetic peptides D-4F and 3F(14). Harvey R. Herschman generated and provided the Cox2 TKO and neutrophil-specific knockout mice and reviewed the experimental protocols and results. Martin G. Martin and Alan M. Fogelman provided guidance, feedback, supervision, and financial support. Srinivasa T. Reddy provided guidance and feedback regarding the overall study and individual experiments; edited the manuscript; and provided laboratory and financial support.
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Figure 1. Dysregulated response to TLR-dependent signaling drives intestinal inflammation in the COX2KO/CCHF models of IBD. (*, p < 0.05; **, p < 0.01; *** p < 0.001; ****, p < 0.0001) A: Both Cox2 TKO (upper panel) and BL6 + celecoxib (CX) treated mice (lower panel) develop transmural and sporadically ulcerating inflammation in their ileo-ceco-colic junctions when fed CCHF for 2 weeks (representative images) (scale bar = 250 um). B: C57BL/6J fed CCHF diet with or without CX were assessed at multiple time points (n = 4 mice/group/time point). CCHF significantly increased whole intestinal barrier permeability across 0-7 days independently of CX (Upper left panel). Endotoxin in portal vein serum significantly increased in both groups by 7 days (lower left panel). Inflammation significantly increased in CX-treated mice by 11 days (upper right panel), while epithelial damage did not significantly increase until day 14 (lower right panel) (histological assessments, 0-4 pt scales). C: Mice were pre-treated with vancomycin, ampicillin, neomycin, and metroniadazole for 7 days and continued on antibiotics (AB) for 14 days of CCHF or CCHF + CX (n = 4/group). AB abrogated intestinal inflammation in both COX2 TKO (upper panel) and C57BL6 + CX (BL6 + CX) treated (lower panel) mice as assessed by ileo-ceco-colic thickness. D: Cox2 TKO and WT mice were treated 3x/wk i.p. with the MyD88 inhibitor T6167923 (0.25 mg/injection) across 2 weeks CCHF diet (n = 5/group). MyD88 inhibition (MyD88i) significantly inhibited intestinal inflammation in Cox2 TKO mice. E: Cox2 MKO significantly enhanced RNA expression of Il1b (upper panel) and Tnf (lower panel) in LPS-activated bone marrow derived macrophages compared to FLOX controls (n = 3/group) (fold change vs FLOX 0 hr) (statistics shown only for FLOX vs MKO). (Statistics: two-way ANOVA and Tukey’s multiple comparisons test with adjusted p values.)
Figure 2. Cox2 MKO (MKO) mice challenged with CCHF exhibit elevated levels of lipid pro-inflammatory lipid mediators in intestine and plasma, while the loss of inflammation resolving LXA4 in intestine appears partially causal for disease in MKO mice fed CCHF.

(*, p < 0.05; **, p < 0.01; ***, p < 0.001; ****, p < 0.0001)

A-F: Lipid inflammatory mediators were determined by LC-MS/MS in the ileo-ceco-colic junctions (A) and plasmas (B) of MKO and FLOX mice fed CCHF or chow for 8.5 weeks (n = 5-7/group) (*ms* = mouse). All significant differences between MKO and FLOX on CCHF are presented: pro-inflammatory (Pro-INF) LOX and COX mediators in the intestines (C) and plasmas (D); PGE2, PGD2, and associated metabolites in both intestine (Int) and plasma (Pl) (E) (*Tt* = total). In intestine, Cox2 MKO significantly reduced the levels of both the specialized pro-resolving mediator (SPM) LXA4 and total 5LOX SPM, together with the ratio of total 5LOX SPM to LTB4 + 6trans12epiLTB4 (*Tt LTB4*) (F). G: A stable analog of LXA4 (BML111) administered twice weekly i.p. significantly inhibited markers of disease including ileo-ceco-colic thickness (n = 10/group) (left) and H&E score (n = 3-5/group) (right). (Statistics. A-F: Benjamini-Hochberg procedure applied to one-way ANOVA for each lipidomic analyte with FDR at level α = 0.05, followed by Tukey’s multiple comparisons test with adjusted p-values. G: One-way ANOVA with Tukey’s multiple comparisons test and adjusted p-values.)
Figure 3. APOA1 mimetics inhibit the development of intestinal inflammation in the Cox2 MKO and CCHF model of IBD. (*, p < 0.05; **, p < 0.01; ***, p < 0.001; ****, p < 0.0001) A-F: Cox2 MKO (MKO) mice fed CHOW, or CCHF for 7 and 10 weeks and treated with oral D-4F (500 ug/ml drinking water). (A) 4F significantly inhibited thickening of the ileo-ceco-colic junctions. (B) (left) Representative images of MKO mice fed CHOW, CCHF, or CCHF+4F for 10 weeks (scale bar = 250 um); (right) 4F significantly inhibited the H&E disease score (0-12 points). (C) 4F significantly reduced CCHF-dependent cecal muscularis thickening. (D-E) 4F treatment significantly reduced infiltration of macrophages (F4/80+) and neutrophils (Ly6G+) into the ileo-ceco-junctions of MKO mice on CCHF diet (left panels, representative 7 wk images; scale bars = 200 um). (F) 4F significantly altered expression of Tnf and Il10 in the ileo-ceco-colic junctions of MKO + 7 wk CCHF mice. G-H: MKO mice fed CCHF for 10 weeks were treated with Tg6F for the full 10 weeks or the last 5 weeks. Both treatments significantly suppressed ileo-ceco-colic thickening (G) while also improving histopathology (H; representative images; scale bar = 200 um). (Statistics: (A-C, G): one-way ANOVA with Tukey’s multiple comparisons test and adjusted p-values. (D-F): Student’s t-tests with Holm-Sidak correction for multiple tests and adjusted p-values.)
Figure 4. 4F treatment improves the inflammatory mediator profile of mouse intestinal tissue and plasma. (*, p < 0.05; **, p < 0.01)

A-E: The effect of 4F on the intestinal and plasma lipid mediator profile of Cox2 MKO (MKO) and FLOX mice fed CHOW or CCHF (8.5 wks) was determined by LC-MS/MS (n = 5-7/group). Heatmaps of lipid mediator changes in intestine (A) and plasma (B) are shown (*ms* = mouse). Significant reductions of intestinal pro-inflammatory LOX (C) and plasma pro-inflammatory LOX (D) and COX (D) mediators (*Tt* = total). (Statistics: Benjamini-Hochberg procedure applied to one-way ANOVA for each lipidomic analyte with FDR at level α = 0.05, followed by Tukey’s multiple comparisons test with adjusted p-values.)
Figure 5. 4F treatment improves the inflammatory mediator profile of mouse macrophages. (*, p < 0.05; **, p < 0.01; *** p < 0.001; ****, p < 0.0001) A-C: LPS-activated (25 ng/ml) BMDM from FLOX and MKO mice were co-treated with D-4F (15 mg/ml) across 24 hrs (n = 3/group). 4F significantly suppressed Il1b (A) and Tnf (B) expression in both FLOX and MKO BMDM (fold change versus 0 hr). LPS significantly increased prostanoid production in FLOX but not MKO mice, while 4F significantly reduced the levels of PGE2 (C) and PGD2 (D) in FLOX lysate from 10 hours. In FLOX lysate, 4F also selectively suppressed the COX pathway marker 11HETE without reducing the lipoxygenase product 15HETE (E). (Statistics. (A,B): For each of FLOX +/- 4F and MKO +/- 4F, two-way ANOVA with Tukey’s multiple comparisons test and adjusted p-values. (C, D): Three-way ANOVA with Tukey’s multiple comparisons test and adjusted p-values. (E): For each of the two analytes, two-way ANOVA with Tukey’s multiple comparisons test and adjusted p-values.)
Figure 6. 4F inhibits the LPS-mediated pro-inflammatory response of human macrophages and intestinal epithelium. (#, p < 0.1; *, p < 0.05; **, p < 0.01; ***, p < 0.001; ****, p < 0.0001) A-B: Human THP1 macrophages were treated with 4F (15 μg/ml), LPS (20 ng/ml), or LPS + 4F. 4F significantly inhibited total PGE2 in cell lysates and media across 24 hours (A) (n=3/group). 4F (“4”) significantly inhibited LPS (“L”) dependent IκBα degradation at 30 minutes, as determined by western blots (NT = no treatment) (left: representative blot; right: densitometric analysis of 3 experiments) (B). C: 4F binds both LPS (left; Kd = 0.86 nM) and lipid A (right; Kd = 17.5 nM) with high affinity, as determined by surface plasmon resonance analysis (C). D-I: Crypts were isolated from human small intestine and grown into enteroids in matrigel (D; representative image; scale bar = 200 μm). Conditioned media from THP-1 cells treated with LPS or LPS + 4F for 4 (“LPS 4h”; “LPS + 4F 4h”) or 12 hrs (“LPS 12h”; “LPS + 4F 12h”) was added to the enteroids, and pro-inflammatory gene expression at 12 hours was determined by qPCR (n = 3/group) (NT = no treatment; “Media” = THP1 media only) (Fold change vs NT) (E-I). (Statistics: one (B, E) or two way (A) ANOVA with Tukey’s multiple comparisons test and adjusted p-values)
Figure 7  Oxidized PAPC is elevated in the plasma and ceca of Cox2 MKO + CCHF-fed mice, while 4F both reduces oxidized PAPC in vivo and inhibits oxidized PAPC-dependent pro-inflammatory response in human macrophages. (*, p < 0.05; **, p < 0.01; ***, p < 0.001) Levels of oxPAPC products (viz., POVPC, PGPC, and KOdiAPC) were determined in the ceca (n = 7) (A) and plasmas (n = 10) (B) of Cox2 MKO (MKO) and FLOX mice fed CCHF or chow for 8.5 weeks. Independently, the effect of 4F on oxPAPC in the ceca (C) and plasma (D) of MKO and FLOX mice fed CCHF for 3 weeks was determined. Lastly, human THP-1 macrophages were treated with 1 uM POVPc for 3 hours, with and without the apoA-I mimetic peptides 4F and 3F(14); and expression of IL1B was determined by qPCR (n = 3-6) (fold change vs no-treatment (NT)) (E). After 8.5 weeks, MKO + CCHF diet significantly increased oxPAPC species in both ceca and plasma. 4F significantly lowered levels of oxPAPC in vivo, and 4F but not 3F(14) significantly inhibited the pro-inflammatory effect of POVPc on macrophages. (Statistics: (A-D): For each analyte, two-way ANOVA with Tukey’s multiple comparisons test and adjusted p-values. (E): one-way ANOVA with Tukey’s multiple comparisons test and adjusted p-values.)
Figure 8. 4F can directly clear lipid pro-inflammatory mediators from intestinal tissue and plasma. (*) p < 0.05)  
A: 4F fails to significantly suppress the disease-elevated inflammatory COX mediators in the intestinal tissue of 8.5 wk CCHF-challenged Cox2 MKO (MKO) mice, in contrast to its differential effect on disease-elevated inflammatory LOX mediators (cf. Figure 4D). B: Pairs of matching intestinal explants from 8.5 wk CCHF-challenged MKO mice (1 pr/mouse) were mounted in Ussing chambers. As determined by LC-MS/MS, lumen-side D-4F (50 mg/ml) significantly increased the pair-wise clearance of pro-inflammatory LOX mediators from the intestinal tissue into mucosal media.  
C-D: Deuterated pro-inflammatory LOX mediators (15HETE-d, 12HETE-d, 13HODE-d) were combined with HDL and LDL (1 mg oxFA-d/(2.5mg HDL + 5 mg LDL)) and added to the serosal sides of pairs of matching BL6 intestinal explants mounted in Ussing chambers. There existed a baseline trans-intestinal transport of these tagged lipids (C, representative LC-MS/MS chromatogram). Mucosal-side 4F (50 mg/ml) significantly increased the pair-wise trans-intestinal transport of both 13HODE-d and 15HETE-d (D) (n = 3-6/group) (Statistics.  
(A): Benjamini-Hochberg procedure applied to one-way ANOVA for each lipidomic analyte with FDR at level α = 0.05, followed by Tukey’s multiple comparisons test and adjusted p-values (cf. Figure 2). (B,D): Student’s t-tests with Holm-Sidak correction for multiple tests and adjusted p-values.
Figure 9. 4F treatment inhibits the development of colitis in the piroxicam-accelerated Il10-/- model of IBD. (*, p < 0.05; **, p < 0.01; ***, p < 0.001; ****, p < 0.0001) A-B: Il10-/- mice were treated with and without piroxicam (PX) and 4F (n = 4-7/group). 4F partially rescued the effect of PX on colon length and colitis score (A). 4F also improved disease histopathology: representative H&E images (black scale bars = 200 μm) (B, left); H&E disease score (B, right). C: PX increased both whole intestinal barrier permeability of Il10-/- mice in a biphasic manner, as determined by LC-MS/MS of urinary excretion of sucralose (left); as well as the translocation of endotoxin into portal vein serum at day 9 (right). D: 4F inhibited the production of prostanoids including PGE2 (shown here) in LPS-activated Il10-/- BMDM +/- PX (n = 3/group). E-F: The lipid inflammatory mediator profile in the colons of the mice from (A-B) was determined by LC-MS/MS (cf. Supplemental Table 8) (n = 4-8 mice/group). Heatmap of changes (“ms” = mouse) (E); all significant differences are represented (F). 4F significantly inhibited the disease-dependent increase of pro-inflammatory LOX mediators (“Tt” = total). (Statistics. One-way (A, B), repeated measures one-way (C, left), or three-way (D) ANOVA with Tukey’s multiple comparisons test and adjusted p-values. (C, right): Student’s t-test. (E-F): Benjamin-Hochberg procedure applied to one-way ANOVA for each lipidomic analyte with FDR at level α = 0.05, followed by Tukey’s multiple comparisons test and adjusted p-values.)
Figure 10. Proposed pathogenic mechanisms of Cox2 MKO and CCHF-dependent intestinal inflammation together with the protective mechanisms of APOA1 mimetic peptides. A: Cholate increases barrier permeability, initiating both bacteria and MyD88-dependent inflammation in Cox2 KO mice. i) Cox2 KO macrophages in the lamina propria are more pro-inflammatory in response to PAMP/LPS activation, perhaps through loss of negative feedback control of NFκB. In turn, intestinal epithelium amplifies the pro-inflammatory stimuli of macrophages, resulting in a dysregulated pro-inflammatory positive feedback loop. Elevated oxPAPC levels further amplify the pro-inflammatory response. ii) Cox2 KO macrophages are less inflammation resolving, through loss of LXA4. iii) The APOA1 mimetic peptide 4F interrupts the positive feedback loops by inhibiting the LPS-dependent activation of macrophages; blocking pro-inflammatory signal amplification by intestinal epithelium; and reducing the levels of oxPAPC while directly blocking their effects upon macrophages. B: Upon CCHF challenge, Cox2 MKO elevates lipid pro-inflammatory mediators in intestinal tissue and plasma while reducing pro-resolving mediators in tissue. 4F reduces the levels of pro-inflammatory LOX mediators in ceca (right), possibly through direct clearance. 4F also reduces the levels of pro-inflammatory lipid mediators in plasma (left), possibly through enhancement of the transintestinal lipid transport (TILT) of lipoprotein-associated lipid inflammatory mediators into the lumen of the proximal SI—in a manner comparable to the effect of 4F upon the trans-intestinal cholesterol efflux (TICE) of free cholesterol (FC) (17).
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<tr>
<td>Δ12-PGJ2</td>
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<tr>
<td>[PGI2]</td>
<td>Vasodilation</td>
</tr>
<tr>
<td>[PGF2alpha]</td>
<td>Pro-INF in rheumatoid arthritis, atherosclerosis</td>
</tr>
<tr>
<td>[TXA2]</td>
<td>Microvascular constriction, PMN adherence</td>
</tr>
<tr>
<td>TXB2</td>
<td>PMN chemotaxis and adherence</td>
</tr>
<tr>
<td>11HETE</td>
<td>Leukocyte regulation; COX activity marker</td>
</tr>
<tr>
<td>PGE3</td>
<td>Induce COX2 and IL6 in macrophage</td>
</tr>
<tr>
<td>TXB3</td>
<td>Induce COX2 and IL6 in macrophage</td>
</tr>
<tr>
<td>LTB4</td>
<td>Vascular leakage; leukocyte chemotaxis and adhesion</td>
</tr>
<tr>
<td>LTC4</td>
<td>Vascular permeability</td>
</tr>
<tr>
<td>5HETE</td>
<td>PMN chemotaxis and degranulation</td>
</tr>
<tr>
<td>12HETE</td>
<td>Neutrophil chemotaxis and adhesion</td>
</tr>
<tr>
<td>15HETE</td>
<td>Activator of leukotriene biosynthesis; lipoxin pathway marker</td>
</tr>
<tr>
<td>5oxoETE</td>
<td>PMN chemotaxis and degranulation</td>
</tr>
<tr>
<td>13HODE</td>
<td>PMN chemotaxis</td>
</tr>
<tr>
<td>9HODE</td>
<td>PMN chemotaxis</td>
</tr>
<tr>
<td>LXA4</td>
<td>Promote M1 to M2; NFκB negative regulator; inhibit neutrophil chemotaxis and adhesion</td>
</tr>
<tr>
<td>LX4</td>
<td>Promote M1 to M2; NFκB negative regulator; inhibit neutrophil chemotaxis and adhesion</td>
</tr>
<tr>
<td>MaR−1</td>
<td>Tissue regeneration; M1 to M2; inhibit neutrophil migration; increase efferocytosis</td>
</tr>
<tr>
<td>RV1</td>
<td>Limit neutrophil infiltration</td>
</tr>
<tr>
<td>RV2</td>
<td>Enhance survival in sepsis; protect against colitis</td>
</tr>
<tr>
<td>RV3</td>
<td>Limit leukocyte migration, enhance macrophage efferocytosis</td>
</tr>
<tr>
<td>RV4</td>
<td>Limit leukocyte migration, enhance macrophage efferocytosis</td>
</tr>
<tr>
<td>RV5</td>
<td>Enhance bacterial containment and phagocytosis</td>
</tr>
<tr>
<td>PDx</td>
<td>Inhibit neutrophil infiltration and platelet aggregation</td>
</tr>
<tr>
<td>RVE1</td>
<td>Reduce PMN infiltration; promote resolution in colitis model</td>
</tr>
</tbody>
</table>

a Anti-inflammatory cyclooxygenase products
b Pro-inflammatory/pro-resolving cyclooxygenase products
c Pro-inflammatory cyclooxygenase products
d Pro-inflammatory lipoxygenase products
e Pro-resolving lipoxygenase products
f Analytes in brackets are measured indirectly via stable metabolic or degradation products