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Protectin $D1_{n\text{-}3\ DPA}$ and Resolvin $D5_{n\text{-}3\ DPA}$ are novel effectors of intestinal protection.

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ABSTRACT

The resolution of inflammation is an active process orchestrated by specialized pro-resolving lipid mediators (SPM) that limit the host response within the affected tissue; failure of effective resolution may lead to tissue injury. Since persistence of inflammatory signals is a main feature of chronic inflammatory conditions including inflammatory bowel diseases (IBDs), herein we investigated expression and functions of SPM in intestinal inflammation. Targeted liquid chromatography-tandem mass spectrometry-based metabololipidomics was used to identify SPMs from n-3 polyunsaturated fatty acids (PUFA) in human IBD colon biopsies, quantifying a significant upregulation of the resolvin and protectin pathway as compared to normal gut tissue. Systemic treatment with $PD1_{n\text{-}3\ DPA}$ or $RvD5_{n\text{-}3\ DPA}$ protected against colitis and intestinal ischemia/reperfusion-induced inflammation in mice. Inhibition of 15-lipoxygenase activity reduced $PD1_{n\text{-}3\ DPA}$ and augmented intestinal inflammation in experimental colitis. Intra-vital microscopy of mouse mesenteric venules demonstrated that $PD1_{n\text{-}3\ DPA}$ and $RvD5_{n\text{-}3\ DPA}$ decreased the extent of leukocyte adhesion and emigration following ischemia-reperfusion. These data were translated by assessing human neutrophil-endothelial interactions under flow: $PD1_{n\text{-}3\ DPA}$ and $RvD5_{n\text{-}3\ DPA}$ reduced cell adhesion onto TNF-α-activated human endothelial monolayers. In conclusion we propose that innovative therapies based on n-3 DPA derived mediators could be developed to enable anti-inflammatory and tissue protective effects in inflammatory pathologies of the gut.

SIGNIFICANT STATEMENT

In this work we provide evidence for a functional role of novel bioactive lipid mediator of the docosapentaenoic acid (DPA) metabolome in experimental intestinal inflammation. Supported by detecting changes in DPA-derived mediators in colon biopsies from inflammatory bowel diseases, we studied the pharmacological properties of two mediators. Exogenous administration of $PD1_{n\text{-}3\ DPA}$ or $RvD5_{n\text{-}3\ DPA}$ in mice markedly reduced DSS-induced colitis though a mechanism at least in part linked to decreased leukocyte-endothelial interaction and reduced granulocyte trafficking, as assessed by intravital microscopy. The translational impact of these data was determined by the ability of $PD1_{n\text{-}3\ DPA}$ and $RvD5_{n\text{-}3\ DPA}$ to reduce human neutrophil adhesion onto TNF-α-activated human endothelial monolayers. We propose that n-3 DPA-derived mediators could represent the basis for innovative therapeutic strategies in settings of intestinal inflammation.

INTRODUCTION

Inflammatory bowel diseases (IBDs), the main forms being Crohn's disease (CD) and ulcerative colitis (UC), are chronic inflammatory conditions of unknown etiology that primarily affect the gastrointestinal tract(1). IBD affects an estimated 1.5 million Americans, 2.2 million people in Europe, and several hundred thousands more world-wide(2). Irrespective of the etiopathogenesis, it is accepted that the chronic nature of IBDs is caused by inflammatory signals (i.e. cytokines, proteases) leading to overt pathology(1). Healthy intestinal mucosa relies on a complex equilibrium: when this is disrupted a robust and persistent inflammatory response develops characterised by mucosal injury, increased epithelial permeability, invasion of bacteria into *lamina propria* and marked neutrophils recruitment(1). This paradigm has driven research in the field informing current treatments including biologics (i.e. anti-TNF-α). However, these treatments are immunosuppressive (hence plagued by side-effects), expensive and ineffective in a good proportion of patients(3). The increasing incidence of IBD and the inadequacy of current treatments makes imperative to develop new therapeutic approaches.

The ultimate remit of the inflammatory response is to protect the host from exogenous or endogenous dangers: following removal of the noxious stimuli the inflammatory process is finely programmed to self-resolve to avoid harmful injury(4),(5). The last step of this physiological multi-component process is tissue repair with return to tissue functionality, instructing the adaptive immune response and altogether regaining homeostasis(6, 7). However, if not modulated, inflammatory mechanisms induce self-harm leading to tissue injury as in chronic diseases including IBDs(8, 9). This beneficial profile of the inflammatory response is ensured with the turning on of resolution programs and the biosynthesis of pro-resolving mediators that counter-regulate the action of inflammation-initiating mediators(10). Within the effectors of resolution, an important role is emerging for specialized pro-resolving lipid mediators or SPMs: these are families of mediators formed via the stereospecific conversion of essential polyunsaturated fatty acids (PUFAs) by enzymes including the lipoxygenases(10).

During inflammation, increased local vascular permeability leads to oedema which supplies PUFAs from blood to the site of injury, together with elevated numbers of immune cells which carry the biosynthetic enzymes required for the local SPM production(11). These mediators, in turn, interact with specific receptors to temper leukocyte reactivity, dampen inflammatory pain and promote tissue repair and regeneration(12).

Recently, we reported that n-3 docosapentaenoic acid (n-3 DPA), an intermediary product between eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), is converted to novel SPMs by both human and murine leukocytes(13). Production of these novel SPM was temporally regulated in self-limited inflammatory exudates. In addition, n-3 DPA-derived 10R,17S-dihydroxydocosa-7Z,11E,13E,15Z,19Z-pentaenoic acid ($PD1_{n\text{-}3\ DPA}$) and 7R,14S-dihydroxydocosa-8E,10E,12Z,16Z,19Z-pentaenoic acid ($MaR1_{n\text{-}3\ DPA}$) exert potent pro-resolving actions stimulating human macrophage phagocytosis and efferocytosis(13, 14). Herein we identified presence of n-3 DPA-derived SPM in human colon and characterised their potent anti-inflammatory, pro-resolving and tissue protective actions of two DPA-derived mediators, denoted as $PD1_{n\text{-}3\ DPA}$ and $RvD5_{n\text{-}3\ DPA}$ (7S,17S-dihydroxydocosa--8E,10Z,13Z,15E,19Z-pentaenoic acid) in settings of intestinal inflammation.

RESULTS

Identification of n-3 DPA pro-resolving mediators in colon biopsy from control and IBD patients and mice

Using targeted LC/MS/MS based LM metabololipidomics we profiled the arachidonic acid (AA), EPA, DHA and n-3 DPA metabolomes with human intestinal biopsies from control and IBD patients (see supplementary Table 1 for demographics). In the presence of overt inflammation, as typified by marked damage of the mucosal architecture displayed by biopsies of IBD colon samples (Figure 1A), levels of leukotriene B_4, prostaglandin E_2 and thromboxane B_2 (further metabolite of the potent

platelet-agonist thromboxane A_2) were significantly increased as compared to tissue biopsies from control. In these biopsies we also identified and quantified SPM from all four of the major bioactive metabolomes (supplementary Table 2). Of note, we identified mediators from the n-3 DPA bioactive metabolome in both biopsies from control and IBD patients. These mediators were identified in accordance with published criteria, matching retention times and at least 6 characteristic ions in the MS-MS spectrum and included n-3 DPA resolvins ($RvD1_{n\text{-}3\ DPA}$, $RvD2_{n\text{-}3\ DPA}$, $RvD5_{n\text{-}3\ DPA}$), protectins ($PD1_{n\text{-}3\ DPA}$, 10S,17S-diHDPA) and maresins ($MaR1_{n\text{-}3\ DPA}$) (Figure 1B-C and supplementary Table 2). Quantification using multiple reaction monitoring demonstrated that $RvD5_{n\text{-}3\ DPA}$, $PD1_{n\text{-}3\ DPA}$ and the protectin pathway marker 10S,17S-diHDPA were augmented in tissue biopsies from IBD patients compared to those from control (Figure 1D-F). Together these findings identify SPM including n-3 DPA derived SPM in human colonic tissue. In addition these results indicate an up-regulation of the resolvin and protectin pathways from the n-3 DPA metabolome in IBD.

We next tested whether inhibition of endogenous SPM production would impact disease activity. Using targeted LC/MS/MS based LM metabololipidomics we profiled the AA, EPA, DHA and n-3 DPA bioactive metabolomes in colon tissues from naive mice, mice receiving DSS, with or without a 15-LOX inhibitor. In colon tissues from naive mice we identified mediators from all four bioactive metabolomes including the n-3 DPA derived $PD1_{n\text{-}3\ DPA}$ (Figure 2A-B and Supplemental Table 3). Administration of DSS lead to upregulation of several protective pathways including both DHA and n-3 DPA derived protectins as well as the E-series resolvins and the DHA derived RvD5 suggesting the activation of host protective mechanisms during ongoing inflammation. Administration of a 15-LOX inhibitor reduced tissue concentrations of $PD1_{n\text{-}3\ DPA}$ and increased levels of several pro-inflammatory eicosanoids including PGD_2 and PGE_2 (Supplemental Table 3). This modulation of SPM profiles was associated with a decreased colon length, increased macroscopic tissue damage and neutrophil infiltration (Figure 2D-F). These results suggest that inhibition of endogenous $PD1_{n\text{-}3\ DPA}$ leads to a failure to counter regulate colonic inflammation leading to increased tissue damage.

$PD1_{n\text{-}3\ DPA}$ and $RvD5_{n\text{-}3\ DPA}$ protect mice against DSS-induced colitis.

Given that the enzymatic inhibition of n-3 DPA-derived SPMs is deleterious in colitis and that the levels of these mediators are regulated in human colon biopsies from IBD patients (Figure 1 and 2 and supplementary Table 2 and 3) we tested the pharmacological actions of $PD1_{n\text{-}3\ DPA}$ and $RvD5_{n\text{-}3\ DPA}$ in DSS-induced colitis (Figure 2A). Five days of oral DSS administration to mice induced severe colon inflammation as evidenced by significant colon shortening, increased wall thickness and macroscopic damage score (Figure 2B). Systemic treatment with either $PD1_{n\text{-}3\ DPA}$ or $RvD5_{n\text{-}3\ DPA}$ (0.3 µg/mouse, given i.p. daily) prevented colon length reduction. Treatment with $PD1_{n\text{-}3\ DPA}$ significantly reduced colon wall thickness and macroscopic colon damage (Figure 2C). In DSS-induced intestinal inflammation, tissue damage is predominantly mediated by infiltrating neutrophils(15), thus we assessed the actions of $PD1_{n\text{-}3\ DPA}$ and $RvD5_{n\text{-}3\ DPA}$ on this parameter by measuring tissue myeloperoxidase (MPO) activity (Figure 2C). Against a marked increase in colonic MPO activity in vehicle-treated mice, animals receiving $PD1_{n\text{-}3\ DPA}$ or $RvD5_{n\text{-}3\ DPA}$ displayed reduced MPO values (Figure 2C). Analyses of both pro- and anti-inflammatory cytokines in intestinal samples showed a pronounced increase in colonic TNF-α, IL-1β and IL-6 levels, with a partial decrease in IL-10, in vehicle-treated DSS mice. $PD1_{n\text{-}3\ DPA}$, but not $RvD5_{n\text{-}3\ DPA}$, significantly reduced pro-inflammatory cytokines levels, without correcting IL-10 (Figure 2D). Notably, both compounds restored the histological architecture of the mucosa decreasing mucosal ulceration when compared to DSS-treated vehicle group (Figure 2E). These findings indicate that $PD1_{n\text{-}3\ DPA}$ and $RvD5_{n\text{-}3\ DPA}$, elicit anti-inflammatory and tissue protective actions in intestinal inflammation.

We then assessed whether $PD1_{n\text{-}3\ DPA}$ was also protective when administered using a therapeutic treatment paradigm. Mice were given DSS for 7 days followed by 3 days of water. Pharmacological treatment with either $PD1_{n\text{-}3\ DPA}$ (0.3 µg/mouse, i.p.) or vehicle commenced at first signs of disease (day 5, beginning of weight loss; not shown): $PD1_{n\text{-}3\ DPA}$ treated group displayed significant protection from DSS-induced colonic inflammation as measured by protection from colon shortening, decreased macroscopic damage, MPO activity and inflammatory cytokine levels (Figure 3F).

$PD1_{n\text{-}3\ DPA}$ and $RvD5_{n\text{-}3\ DPA}$ prevent local and systemic inflammation following intestinal ischemia/reperfusion (I/R).

Given the association for intestinal ischemia in the pathogenesis of IBD we tested whether $PD1_{n3\ DPA}$ and $RvD5_{n\text{-}3\ DPA}$ regulated host responses following gut ischemia(16). Occlusion of the superior mesenteric artery for 50 min (ischemia) followed by 5h reperfusion provoked a robust granulocyte infiltration in the gut with marked histological damage as characterised by fragments of mucosa detected in the lumen, damage to the villi and disintegration of the *lamina propria* (Figure 3). Post-ischemic administration of $PD1_{n\text{-}3\ DPA}$ (most effective at 0.1 µg/mouse) and $RvD5_{n\text{-}3\ DPA}$ (0.1 and 1 µg/mouse) significantly prevented granulocyte infiltration (Figure 3A-B). Administration of $PD1_{n\text{-}3\ DPA}$ preserved the structure and length of the villi, $RvD5_{n\text{-}3\ DPA}$ was less effective (Figure 3C). At the dose of 0.1 µg, $PD1_{n\text{-}3\ DPA}$ significantly reduced secondary granulocyte recruitment into the lungs and plasma IL-1β levels (Figure 3D-E).

To establish the potential impact of endogenous n-3 DPA derived mediators in gastro-intestinal protection we assessed whether in animals with reduced expression of fatty acid elongase 2 (ELOVL2)(17), the enzyme that converts n-3 DPA to DHA (Supplementary Figure 1A). Administration of siRNA to mice reduced expression of ELOVL2 in peripheral tissues (Supplementary Figure 1B). Mice were then supplemented daily with EPA and subjected to I/R mediated injury. Using lipid mediator profiling we found that plasma levels of n-3 DPA derived SPM including $RvD5_{n\text{-}3\ DPA}$ and $PD1_{n\text{-}3\ DPA}$ were significantly increased (Supplementary Figure 1C-E and Supplementary Table 4). These increases were associated with reduced systemic levels of LTB_4 and 12S-HHT wen compared with mice given the mock plasmid alone (Supplementary Figure 1F). Of note, MPO levels in ELOVL2 knock down, but not mock siRNA-treated, animals supplemented with EPA were also significant reduced when compared to levels found in WT mice (Supplementary Figure 1G).

$PD1_{n\text{-}3\ DPA}$ and $RvD5_{n\text{-}3\ DPA}$ regulate neutrophil-endothelial interactions.

Given that neutrophil trafficking is a key step in the etiopathology of gastro-intestinal damage in IBD, we investigated the process of leukocyte trafficking in more detail. Initially a model of zymosan peritonitis was tested, followed by direct observation of post-capillary venules by intravital microscopy. Administration of $PD1_{n\text{-}3\ DPA}$ and $RvD5_{n\text{-}3\ DPA}$ to mice treated with zymosan, markedly reduced leukocyte numbers in the exudates, as compared to the vehicle-treated mice. Low doses of 0.1 and 1 µg $RvD5_{n\text{-}3\ DPA}$ decreased neutrophil counts by 38 and 60%, respectively. $PD1_{n\text{-}3\ DPA}$ was more effective at the lower dose of 0.1µg, which yielded ~45% reduction in the neutrophil response (Supplementary Figure 2).

Intravital microscopy of mesenteric post-capillary venules, inflamed by clamping of the superior mesenteric artery for 30 min followed by 90 min reperfusion, allowed monitoring of the processes of leukocyte rolling, adhesion and emigration(18). The dose of 0.1 µg was selected and administered prior to reperfusion. Treatment with $PD1_{n\text{-}3\ DPA}$ or $RvD5_{n\text{-}3\ DPA}$ decreased the number of adherent leukocytes onto the post-capillary endothelium (Figure 5A), while not affecting the intermittent contacts typical of the rolling process (not shown). Equally, a significant reduction in the extent of leukocytes emigrated into the sub-endothelial tissue was quantified (Figure 5B) Representative images are in Figure 5C.

To establish the translational relevance of these findings, we assessed the actions of these two mediators on human neutrophil-endothelial interactions under flow. Pre-incubation of human neutrophils with $PD1_{n\text{-}3\ DPA}$ or $RvD5_{n\text{-}3\ DPA}$ drastically reduced neutrophil adhesion and transmigration onto TNF-α-activated endothelial monolayers (Figure 5D), with activity at a concentration range between 10 pM and 100 nM. No effect was observed on the number of human neutrophils rolling on the endothelium. These results made us to dwell on human neutrophil functions and determined if these novel n-3 DPA mediators could modulate their reactivity; to reduce manipulation, a whole blood protocol was used.

Incubation of whole blood with TNF-α (10 ng/ml) up-regulated cell surface expression of both total CD11b as quantified by flow cytometry. Addition of $PD1_{n\text{-}3\ DPA}$ or $RvD5_{n\text{-}3\ DPA}$ prior to TNF-α led to a significant reduction of total CD11b (~30%) immunostaining (Figure 5E). There was some degree of selectivity in these responses since the two mediators did not modulate basal CD62L expression nor TNF-α induced CD62L shedding (data not shown). This is contract to what observed with PAF stimulation, with an effect of $PD1_{n\text{-}3\ DPA}$ or $RvD5_{n\text{-}3\ DPA}$ on CD62L shedding (Figure 5E).

DISCUSSION

In the present study we report the tissue protective actions of two novel n-3 DPA derived mediators, denoted as $PD1_{n\text{-}3\ DPA}$ and $RvD5_{n\text{-}3\ DPA}$. These mediators: i) reduced inflammation following DSS-induced colitis; ii) prevented local and systemic inflammation following intestinal I/R and iii) regulated neutrophil-endothelial interactions, which may occur downstream modulation of adhesion molecule activation. Of translational relevance, we could identify n-3 DPA SPM in human colon biopsies. These results suggest a relevant profile for these SMPs as an innovative therapeutic approach to control intestinal inflammation like in IBD.

Two decades of research now support the concept that resolution of inflammation is an active process brought about by specific mediators and receptors(12). We studied here resolution, or its lack, in the persistent and unresolved inflammation typical of IBD(1). Of relevance the gut is in a state of underlying inflammation and it is plausible that endogenous checkpoints are properly operative to avoid progression to chronic conditions(19). Moreover, the relapsing and remitting nature of IBD together with the spontaneous resolution that sometime is observed(20) suggests existence of contrasting endogenous processes enacted by pro-inflammatory and pro-resolving mediators during the pathogenesis of the disease. SPM are produced by leukocytes, including neutrophils and macrophages, via both transcellular biosynthesis as well as in a cell autonomous manner (21, 22). Other cell types including epithelial cells (23) and platelets (21) may also contribute to the SPM production since they carry some of the

initiating enzymes in the biosynthesis of these molecules. Many of these cell types including macrophages and epithelial cells are present in human naïve (healthy) colon biopsies and their numbers are increased in IDB (24). In the present study we found an increase in both pro-inflammatory initiating inflammation and a decrease in the pro-resolving n-3 PUFA mediators including $PD1_{n\text{-}3\ DPA}$. Of note, the identification of these mediators is within their bioactive concentration (fM to nM)(13, 14), a finding aligned with recent findings where SPM at bioactive concentrations were identified in human tissues including spleen, lymph nodes, plasma(25) and also milk(26). The endogenous protective role of the SPM was further tested using a 15-lipoxygenase inhibitor which decreased the formation of $PD1_{n\text{-}3\ DPA}$ and that was associated with an increase in the production of pro-inflammatory molecules and colon inflammation.

SPMs derived from n-3 PUFA such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) mediators have been linked to protective actions in intestinal inflammation(27). This is in line with findings made with both EPA and DHA derived pro-resolving mediators. Administration of resolvin E1 (RvE1), resolvin D2, 17(*R*)-hydroxy docosahexaenoic acid, aspirin-triggered resolvin D1 and Maresin 1 resulted in an improved disease activity score, reduced colonic damage and lower PMN infiltration in several models of colitis(27). In the present study we report that administration of $PD1_{n\text{-}3\ DPA}$ or $RvD5_{n\text{-}3\ DPA}$ was effective in preventing the hallmark of intestinal inflammation after DSS administration to mice, including an effect on colon length, microscopic damage score and granulocyte recruitment. These tissue markers could be secondary to the regulation exerted by DPA mediators onto pro-inflammatory cytokine production, as in the case of $PD1_{n\text{-}3\ DPA}$. The clinical efficacy of anti-TNF therapy in IBD(3) supports this mechanism. Of interest, both $PD1_{n\text{-}3\ DPA}$ and $RvD5_{n\text{-}3\ DPA}$ did not to regulate colonic levels of IL-10, setting these mediators apart from AA-derived LXA_4 (28) or other pro-resolving mediators like Annexin-A1(29, 30) and alpha-melanocyte stimulating hormone(31). To substantiate the physio-pathological impact of this new pathway, it was important to observe that inhibition of the enzyme responsible for conversion of DPA to DHA led to an increase in tissue level of n-3 DPA-derived

SPM, a biochemical effect linked to a reduction in systemic eicosanoid levels during i/R injury supporting the host protective actions of these novel mediators.

IBD is characterized by marked infiltration of inflammatory cells and elevated concentration of inflammatory mediators both within the gut and in the circulation(32). DSS-induced colitis is a useful model to study the early initiating event in IBD such as the neutrophil recruitment to the intestinal wall and the enhanced production of pro-inflammatory cytokines that typifies the inflammatory cascade(33, 34). Among the properties of n-3 PUFA-derived mediators(10, 13), $PD1_{n\text{-}3\ DPA}$ or $RvD5_{n\text{-}3\ DPA}$ displayed potent bio-actions that are relevant to the pathophysiology of IBD including their ability to regulate neutrophil recruitment (*vide infra*). Each of these mediators also displayed characteristic regulation of inflammatory cytokines whereby $PD1_{n\text{-}3\ DPA}$ regulated TNF-α, IL- 1β and IL-6 whereas $RvD5_{n\text{-}3\ DPA}$ only partially reduced IL- 1β levels. Thus, these findings suggest that these two mediators may activate distinct protective responses potentially through engagement of different receptors.

At doses as low as 0.1 µg (equivalent to ~90 nmol), $PD1_{n\text{-}3\ DPA}$ and $RvD5_{n\text{-}3\ DPA}$ significantly reduced neutrophil recruitment in zymosan peritonitis and intestinal ischemia reperfusion injury in mice. As the latter model results in secondary organ injury(35), we could complement the pharmacological properties of these two compounds by establishing their ability to confer both intestinal tissue protection and reduce excessive granulocyte recruitment into the lungs together with decreased level of plasma IL-1β.

The series of molecular and cellular events that lead to neutrophil recruitment are well characterised(36). Using intra-vital microscopy of mesenteric venules we investigated the actions of these n-3 DPA mediators on specific processes within the leukocyte recruitment cascade in vivo finding that both $RvD5_{n\text{-}3\ DPA}$ and $PD1_{n\text{-}3\ DPA}$ specifically regulated cell adhesion while leaving cell rolling unaffected. This observation was also replicated with human primary cells, using the flow chamber system. The potent actions of $PD1_{n\text{-}3\ DPA}$ and $RvD5_{n\text{-}3\ DPA}$ on TNF-α-induced neutrophil recruitment under shear

conditions indicate that the endogenous conversion of n-3 DPA into pro-resolving mediators can counterbalance the actions of pro-inflammatory mediators. Single cell analyses with human neutrophils identified at least one of the mechanisms by which these mediators could regulate inflammatory responses. Both molecules modulated adhesion molecule expression on the neutrophil cell surface, with the intriguing observation that the specific target (CD11b or CD62L) may be stimulus dependent.

In conclusion, the present study offers evidence that endogenous lipid mediators derived from the n-3 DPA biosynthetic pathway exert beneficial actions on leukocyte reactivity and cytokine production, regulating the outcome of intestinal inflammation. Thus, the potent actions and structures of resolvin D5 and protectin D1 derived from n-3 DPA can be used as guidance for the development of innovative therapeutic strategies to control the imbalanced inflammatory status typical of IBD and other inflammatory pathologies.

MATERIALS AND METHODS

An extended version of Materials and Methods is presented as supplementary file.

All procedures were performed under the UK Animals (Scientific Procedures) Act, 1986. Human cells were prepared according to an approved protocol (East London & the City Local Research Ethics Committee; no. 06/ Q605/40; P/00/029 ELCHA). The Ethics Committee approved the human research protocol (ClinicalTrials.gov Identifier: NCT01990716) to collect biopsies and clinical information.

Sample extraction and lipid mediator metabololipidomics.

Human and mouse gastrointestinal tissues extraction and lipid mediator metabololipidomics was conducted as described(37).

Induction and assessment of DSS-induced colitis

Mice were provided access ad libitum to water containing 2.5% DSS over a 5-day period. Every other day, the DSS solution was replenished. On Day 5, DSS was replaced with normal drinking water for 3 days; on Day 8 the animals were euthanized. Control mice received only drinking water. $RvD5_{n\text{-}3\ DPA}$, $PD1_{n\text{-}3\ DPA}$ or vehicle (0.3 µg/100 µl in saline 0.01% EtOH) were administered i.p. once per day (from day 1 to day 5). The same model of DSS-induced colitis was performed treating mice with a 15-lipoxygenase inhibitor PD 146176 (Cayman Chemical). The drug, dissolved in DMSO/water was administered daily by gavage (100 mg/kg every day until day 8).

The therapeutic properties of $PD1_{n\text{-}3\ DPA}$ were assessed with a different protocol for DSS-induced colitis. Mice were provided access ad libitum to water containing 2.5% DSS over a 7-day period. Every other day, the DSS solution was replenished. On Day 7, DSS was replaced with normal drinking water for 3 days. $PD1_{n\text{-}3\ DPA}$ or vehicle (0.3 µg/100 µl in saline 0.01% EtOH) were administered i.p. daily (from day 5 to day 10).

At the end of day 8 or 10, the colon was removed, measured and examined for the consistency of the stool found within as well as the gross macroscopic appearance and

length, as described(38, 39). Samples harvested for histology were analyzed by light microscopy and scored(38, 39). MPO activity was performed as previously described(40). Intestinal levels of cytokines were assayed using ELISA kit purchased from Neogen Corporation according manufacturer's specifications. Colonic samples were harvested for metabololipidomics analysis as previously described(37).

Intestinal ischemia/reperfusion injury

Mice were kept under anesthesia during the all period of surgery. After abdominal laparotomy, the superior mesenteric artery (SMA) was occluded using a micro-vascular clip to cause ischemia. After 50 minutes, the clip was gently removed, allowing reperfusion and mice were treated with 0.1 or 1 µg $PD1_{n\text{-}3\ DPA}$, $RvD5_{n\text{-}3\ DPA}$ or vehicle (PBS 0.01% EtOH). After recovering from anesthesia, animals were returned to their cages. After 5 hours, mice were sacrificed. For myeloperoxidase (MPO) activity jejunal-ileal tissue and lungs were excised and processed. MPO activity was measured as an index of blood-borne PMN infiltration as described(40). Following overnight fixation in 10% neutral buffered formalin, tissues were embedded in paraffin. Sections (5 µm) were stained with hematoxylin and eosin. Microscopic histological damage score was evaluated by a person unaware of the treatments and based on a semi-quantitative scoring system. The scores for each feature were then summed with a maximum possible score of 5 as described(41). Plasma levels of cytokines were assayed using ELISA kit. Specimens of the jejunum-ileum were also collected for intestinal lipid extraction and LC-MS/MS measurements.

Using in vivo-JetPEI™ transfection reagent as a carrier for delivering plasmids *in vivo* we administered 40 µg of mock or mouse ELOVL2 siRNA lentiviral vectors. Mice were transfected with either 40 µg mock or ELOVL2 siRNA and given EPA (every other day for 6 days) or vehicle. ELOV2 expression in the liver was determined after 6 days by Western blot analysis. ELOVL2 siRNA or control animals were subjected to 50 min intestinal ischemia followed by a 5 hours reperfusion as above.

Intravital microscopy of the mouse mesenteric microcirculation.

Intestinal ischemia reperfusion injury was performed in mice as described above. At the end of the ischemic phase, animals were treated i.v. with 0.01 or 0.1 μg $PD1_{n\text{-}3\ DPA}$, $RvD5_{n\text{-}3\ DPA}$ or vehicle (PBS 0.01% EtOH). After 90 minutes reperfusion, the mouse microcirculation was monitored by intravital microscopy with quantification of the extent of cell rolling, adhesion and emigration(18). The vascular bed was exposed and positioned under the microscope; recording started after a 5 min equilibration period, followed by off-line analyses. These were made in 1 to 3 randomly selected post-capillary venules (diameter, 20-40 μm; visible length ≥100 μm) for each mouse.

AUTHOR CONTRIBUTIONS

Study concept and design of the experiments: TG, JD, CNS, MP. Acquisition of data: TG, RAC, DFC. Analysis and interpretation of data: TG, JD, RAC, DFC. Drafting of the manuscript: TG, JD, MP. Critical revision of the manuscript for important intellectual content: CNS, NV, CD. Contributed reagents: CNS, TVH, MA. Colon Biopsies Support: DB, LA, CD.

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Figure 1: Altered LM-SPM profiles in human colon biopsies from IBD patients. Colon biopsies were obtained from control and IBD patients (supplementary Table 1 for demographic information). (A) H&E staining of human colon biopsies. (B-C) SPM profiles were obtained using LC-MS-MS based lipid mediator profiling. (B) Multiple reaction monitoring (MRM) chromatograms for identified mediators. (C) Representative MS-MS spectra employed for the identification of n-3 DPA resolvin D5 ($RvD5_{n\text{-}3\ DPA}$, 7S,17S-dihydroxydocosa--8E,10Z,13Z,15E,19Z-pentaenoic acid), n-3 DPA protectin D1 ($PD1_{n\text{-}3\ DPA}$, 10R,17S-dihydroxydocosa-7Z,11E,13E,15Z,19Z-pentaenoic acid) and 15-epi Lipoxin A_4 (5S,6R,15R-trihydroxyeicosa-7E,9E,11Z,13E-tetraenoic acid). Results are representative of n=30 colon biopsies (supplementary Table 2). (D-F) Quantification of $RvD5_{n\text{-}3\ DPA}$, $PD1_{n\text{-}3\ DPA}$ and 10S,17S-diHDHA in the tissue samples.

Figure 2: Inhibition of 15-lipoxygenase decreased colon $PD1_{n\text{-}3\ DPA}$ levels and was associated with increased tissue damage. Mice received PD 146176 (100 mg/kg per os daily). On day 8, tissues were harvested and lipid mediators were identified and quantified using lipid mediator metabololipidomics. (A) Schematic illustrating treatment regime. (B) Multiple reaction monitoring (MRM) chromatograms for identified mediators. (C) Representative MS-MS spectra employed for the identification of $PD1_{n\text{-}3\ DPA.}$ Results for B and C are representative of n=14 mice. (D) Colon length, (E) macroscopic damage and (F) intestinal MPO activity. Data are reported as means ± SEM of 6 mice per group. $^{*}P < 0.05$ *vs.* control (C). $^{+}P < 0.05$ *vs.* DSS vehicle-treated group (V), one-way ANOVA followed by Dunnett's post-hoc test.

Figure 3. $PD1_{n\text{-}3\ DPA}$ and $RvD5_{n\text{-}3\ DPA}$ protect mice against DSS-induced colitis. (A) Mice had access to drinking water with or without 2.5% DSS for 5 days, then switched to normal water for further 3 days prior to colon collection for analyses (Day 8). Animals were treated i.p. with vehicle (100 µl PBS 0.01% EtOH) or with 0.3 µg $PD1_{n\text{-}3\ DPA}$ or $RvD5_{n\text{-}3\ DPA}$ daily from day 0 to 5. Control mice (CTL) received only drinking water. Day 8 analyses: (B) colon length, wall thickness and macroscopic damage. (C) Intestinal MPO activity. (D) Cytokines (TNF-α, IL-1β, IL-6 and IL-10) levels as determined in colon homogenates using ELISA. (E) Histological score with representative images of colon

sections. (F) In another set of experiments, mice received DSS as above, but 0.3 µg $PD1_{n\text{-}3\ DPA}$ was administered daily from day 5. Day 10 analyses included colon length, macroscopic damage score, MPO activity and tissue cytokine levels. In all cases, data are reported as means ± SEM of 6 mice per group. *$P < 0.05$ versus CTL. $^{+}P < 0.05$,$^{++}P < 0.01$ versus DSS vehicle-treated group, one-way ANOVA followed by Dunnett's post-hoc test.

Figure 4. Exogenous administration of $PD1_{n\text{-}3\ DPA}$ or $RvD5_{n\text{-}3\ DPA}$ prevents local and systemic ischemia/reperfusion injury. Mice were subjected to intestinal ischemia (30 min) followed by 5 h reperfusion (I/R) or sham operation. I/R mice were given vehicle (100 µl; PBS 0.01%EtOH), $PD1_{n\text{-}3\ DPA}$ or $RvD5_{n\text{-}3\ DPA}$ (0.1 µg i.v.) prior to reperfusion. After 5 h reperfusion, multiple markers were quantified as indicated below: (A) Gut MPO activity, (B) intestinal histological score on colon sections stained with H&E, (C) representative images of colon sections, (D) lungs MPO activity and (E) plasma IL-1β levels. Values are mean ± SEM of 6–8 mice per group. *$P < 0.05$, **$P < 0.01$ *vs.* sham; $^{+}P < 0.05$ *vs.* vehicle group, one-way ANOVA followed by Dunnett's post-hoc test.

Figure 5. $PD1_{n\text{-}3\ DPA}$ and $RvD5_{n\text{-}3\ DPA}$ reduce neutrophil-endothelial interactions. Mice were subjected to intestinal ischemia (30 min) followed by reperfusion (90 min) (I/R). Animals were treated i.v. prior to reperfusion with 0.01 or 0.1 µg $PD1_{n\text{-}3\ DPA}$, $RvD5_{n\text{-}3\ DPA}$ or vehicle (100 µl PBS 0.01% EtOH). Post-capillary venules were imaged and recorded for offline quantitation of white blood cell interaction with the endothelium. (A) Number of adherent cells to the post-capillary venule endothelium and (B) emigrated cells in the sub-endothelial space. (C) Representative light microscopy images are shown for vehicle, $PD1_{n\text{-}3\ DPA}$ and $RvD5_{n\text{-}3\ DPA}$ (0.1 µg/mice). Black arrows identify leukocytes adherent to post-capillary venules. Data are mean ± SEM of 6 mice per group. **$P < 0.01$ *vs.* respective vehicle value, one-way ANOVA followed by Dunnett's post-hoc test. (D) Human neutrophils were incubated with vehicle (PBS 0.1% EtOH), $PD1_{n\text{-}3\ DPA}$ or $RvD5_{n\text{-}3\ DPA}$ (0.01 to 100 nM) for 15 min at 37°C. Cells were then perfused over TNF-α-stimulated endothelial cell monolayers for 8 min, and the extent of cell capture, adhesion, rolling and transmigration were quantified. Results are mean ± SEM

of 6 distinct cell donors, *P < 0.05, **P < 0.01, ***P < 0.001 *vs.* vehicle, one-way ANOVA followed by Dunnett's post-hoc test. (E) Whole blood from healthy volunteers was incubated with vehicle (PBS 0.1% EtOH), $PD1_{n\text{-}3\ DPA}$ or $RvD5_{n\text{-}3\ DPA}$ (100 nM) for 15 min at 37°C and then stimulated with TNF-α or PAF. Histograms of CD11b and CD62L expression on human neutrophils are shown (data are representative from 4 distinct blood donors).