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

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The resolution of inflammation is an active process orchestrated by specialized proresolving lipid mediators (SPM) that limit the host response within the affected tissue; failure of effective resolution may lead to tissue injury. Because persistence of inflammatory signals is a main feature of chronic inflammatory conditions, including inflammatory bowel diseases (IBDs), herein we investigate 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 in human IBD colon biopsies, quantifying a significant upregulation of the resolvin and protectin pathway compared with normal gut tissue. Systemic treatment with protectin (PD)$1_{n\text{-}3\ DPA}$ or resolvin (Rv)$D5_{n\text{-}3\ DPA}$ 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. Intravital 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 antiinflammatory and tissue protective effects in inflammatory pathologies of the gut.

inflammation resolution | specialized proresolving mediators | omega-3 fatty acids | intravital microscopy | neutrophils

Inflammatory bowel diseases (IBDs), the main forms being Crohn's disease and ulcerative colitis, are chronic inflammatory conditions of unknown etiology that primarily affect the gastrointestinal tract (1). IBD affects ∼1.5 million Americans, >2 million people in Europe, and several hundred thousands more worldwide (2). Irrespective of its etiopathogenesis, the chronic nature of IBDs is caused by inflammatory signals (i.e., cytokines, proteases) leading to overt pathology (1). Healthy intestinal mucosa rely on a complex equilibrium: when this is disrupted a robust and persistent inflammatory response develops characterized by mucosal injury, increased epithelial permeability, invasion of bacteria into lamina propria, and marked neutrophil recruitment (1). This paradigm has driven research in the field informing current treatments, including biologics like 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 multicomponent 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 proresolving mediators that counter-regulate the action of inflammation-initiating mediators (10). Within the effectors of resolution, an important role is emerging for specialized proresolving lipid mediators (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 edema, which supplies PUFAs from blood to the site of injury, together with elevated numbers of immune cells that carry the biosynthetic enzymes required for the local SPM production (11). These mediators, in turn, interact with specific receptors to temper leukocyte reactivity,

Significance

We provide evidence for a functional role of bioactive lipid mediators of the docosapentaenoic acid (DPA) metabolome in intestinal inflammation. Supported by changes in DPA-derived mediators in colon biopsies from inflammatory bowel diseases, we studied the pharmacological properties of two mediators. Exogenous administration of protectin (PD)$1_{n\text{-}3\ DPA}$ or resolvin (Rv)$D5_{n\text{-}3\ DPA}$ in mice reduced dextran sulfate sodium-induced colitis though a mechanism partly 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.

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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 SPMs 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 ($MaR1_{n\text{-}3\ DPA}$) exerted potent proresolving actions stimulating human macrophage phagocytosis and efferocytosis (13, 14). Herein we identified the presence of n-3 DPA-derived SPM in human colon and characterized their antiinflammatory 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 Proresolving Mediators in Colon Biopsy from Control and IBD Patients as Well as Mice. Using targeted LC-MS/MS based lipid mediator (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 Table S1 for demographics). In the presence of overt inflammation, as typified by marked damage of the mucosal architecture displayed by biopsies of IBD colon samples (Fig. 1*A*), levels of leukotriene (LT) B_4, prostaglandin (PG) E_2, and thromboxane (TX) B_2 (further metabolite of the potent platelet-agonist TXA_2) were significantly increased compared with tissue biopsies from control. In these biopsies we also identified and quantified SPM from all four of the major bioactive metabolomes (Table S2). 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

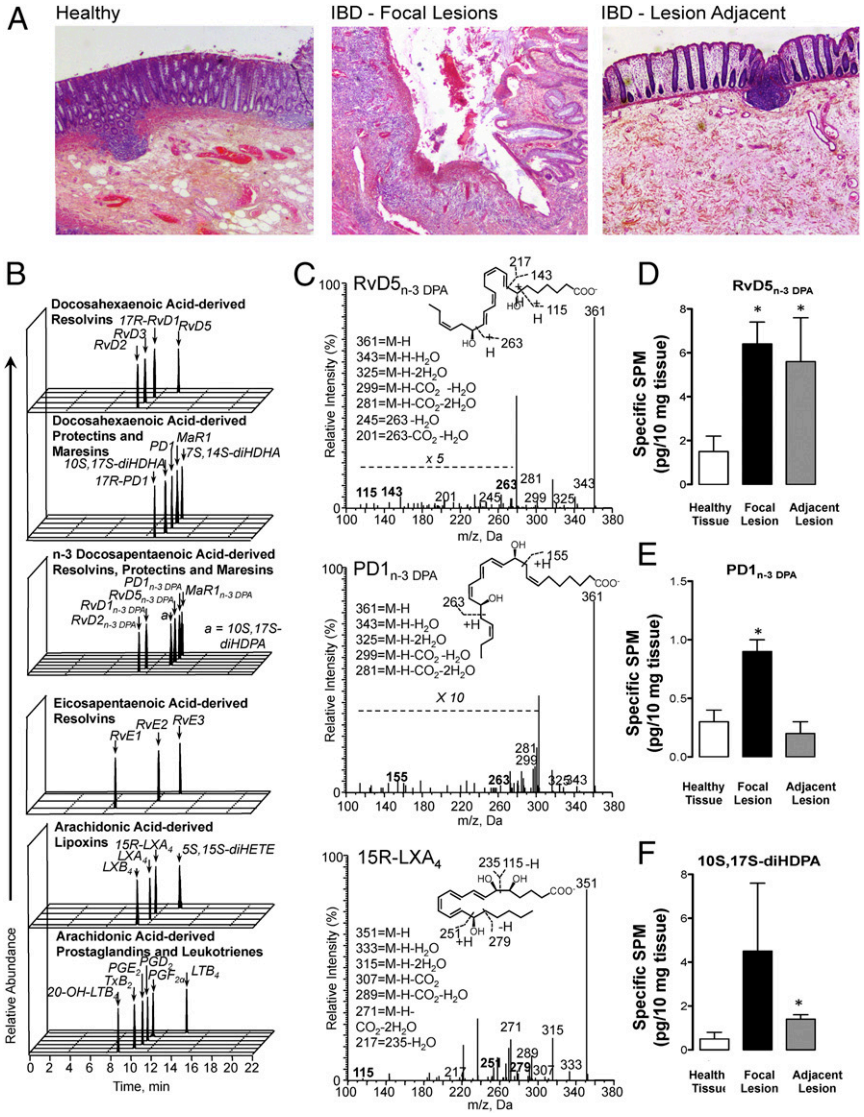

Fig. 1. Altered LM-SPM profiles in human colon biopsies from IBD patients. Colon biopsies were obtained from control and IBD patients (see Table S1 for demographic information). (*A*) H&E staining of human colon biopsies. (Magnification: 20×.) (*B* and *C*) SPM profiles were obtained using LC-MS/MS based LM profiling. (*B*) Multiple reaction monitoring (MRM) chromatograms for identified mediators. (*C*) Representative MS/MS spectra used 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 (Table S2). (*D–F*) Quantification of $RvD5_{n\text{-}3\ DPA}$, $PD1_{n\text{-}3\ DPA}$ and 10S,17S-diHDHA in the tissue samples. *$P < 0.05$ vs. healthy tissue.

published criteria, matching retention times and at least six 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 ($MaR1_{n\text{-}3\ DPA}$) (Fig. 1 *B* and *C* and Table S2). 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 with those from control (Fig. 1 *D–F*). Taken 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 if 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 dextran sulfate sodium (DSS), with or without a 15-LOX inhibitor treatment. In colon tissues from naive mice, we identified mediators from all four bioactive metabolomes, including the n-3 DPA-derived $PD1_{n\text{-}3\ DPA}$ (Fig. 2 *A* and *B* and Table S3). Administration of DSS leads to up-regulation 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 proinflammatory eicosanoids, including PGD_2 and PGE_2 (Table S3). Modulation of SPM profiles was associated with a decreased colon length, increased macroscopic tissue damage, and neutrophil infiltration (Fig. 2 *B–D*). 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 levels of these mediators are regulated in human colon biopsies from IBD patients (Figs. 1 and 2 and Tables S2 and S3), we tested the pharmacological actions of $PD1_{n\text{-}3\ DPA}$ and $RvD5_{n\text{-}3\ DPA}$ in DSS-induced colitis (Fig. 3*A*). Five days of oral DSS administration to mice induced severe colon inflammation, evident through significant colon shortening, increased wall thickness, and macroscopic damage score (Fig. 3*B*). Systemic treatment with $PD1_{n\text{-}3\ DPA}$ or $RvD5_{n\text{-}3\ DPA}$ (0.3 μg per mouse daily) prevented colon length reduction. Treatment with $PD1_{n\text{-}3\ DPA}$ significantly reduced colon wall thickness and macroscopic colon damage (Fig. 3*B*). 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 (Fig. 3*C*). Against a marked increase in colonic MPO activity, animals receiving $PD1_{n\text{-}3\ DPA\ or}$ $RvD5_{n\text{-}3\ DPA}$ displayed reduced MPO values (Fig. 3*C*). Analyses of cytokines 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 proinflammatory cytokines levels without correcting IL-10 (Fig. 3*D*). Both compounds restored the histological architecture of the mucosa, decreasing mucosal ulceration compared with the DSS-treated vehicle group (Fig. 3*E*).

We then assessed if $PD1_{n\text{-}3\ DPA}$ was also protective when given using a therapeutic treatment. Mice were given DSS for 7 d, followed by 3 d of water. 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): $PD1_{n\text{-}3\ DPA}$-treated group displayed significant protection as measured by reduced colon shortening, decreased macroscopic damage, MPO activity, and inflammatory cytokine levels (Fig. 3*F*).

$PD1_{n\text{-}3\ DPA}$ and $RvD5_{n\text{-}3\ DPA}$ Prevent Local and Systemic Inflammation Following Intestinal Ischemia/Reperfusion. Given the association for intestinal ischemia in the pathogenesis of IBD, we tested if $PD1_{n3\ DPA}$ and $RvD5_{n\text{-}3\ DPA}$ regulated host responses following gut ischemia (16). Occlusion of the mesenteric artery (ischemia) followed by reperfusion provoked a robust granulocyte infiltration in the gut with marked histological damage, as characterized by fragments of mucosa detected in the lumen, damage to the villi, and disintegration of the lamina propria (Fig. 4). Postischemic administration of $PD1_{n\text{-}3\ DPA}$ (most effective at 0.1 μg per mouse) and $RvD5_{n\text{-}3\ DPA}$ (0.1–1 μg per mouse) significantly reduced granulocyte infiltration (Fig. 4 *A* and *B*). Administration of $PD1_{n\text{-}3\ DPA}$ preserved structure and length of the villi, whereas $RvD5_{n\text{-}3\ DPA}$ was less effective (Fig. 4*C*). At the dose of 0.1 μg, $PD1_{n\text{-}3\ DPA}$ reduced secondary granulocyte recruitment into the lungs and plasma IL-1β levels (Fig. 4 *D* and *E*).

To establish the impact of endogenous n-3 DPA-derived mediators in gastrointestinal protection, we assessed the profiles in animals with reduced expression of fatty acid elongase 2 (ELOVL2) (17), the enzyme that converts n-3 DPA to DHA (Fig. S1*A*). Administration of siRNA to mice reduced ELOVL2 expression in peripheral tissues (Fig. S1*B*). Mice were then supplemented daily with EPA and subjected to ischemia/reperfusion (I/R) injury. LM profiling identified that plasma levels of n-3 DPA-derived SPM, including $RvD5_{n\text{-}3\ DPA}$ and $PD1_{n\text{-}3\ DPA}$, were significantly increased (Fig. S1 *C–E* and Table S4). These increases were associated with reduced systemic levels of LTB_4 and 12S-HHT when compared with mice given the mock plasmid alone (Fig. S1*F*). Of note, MPO levels in ELOVL2 knockdown, but not mock siRNA-treated animals supplemented with EPA, were significantly reduced compared with levels found in control mice (Fig. S1*G*).

$PD1_{n\text{-}3\ DPA}$ and $RvD5_{n\text{-}3\ DPA}$ Regulate Neutrophil–Endothelial Interactions. Next, we focused on the process of leukocyte trafficking by using a model of zymosan peritonitis, followed by direct observation of postcapillary 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, compared with the vehicle-treated mice. Low doses of $RvD5_{n\text{-}3\ DPA}$ decreased neutrophil counts by >40% (Fig. S2). $PD1_{n\text{-}3\ DPA}$ was more effective at the lower dose of 0.1 μg.

I/R of the mesenteric microcirculation followed by intravital microscopy allowed monitoring of the processes of leukocyte rolling, adhesion, and emigration (18). The dose of 0.1 μg was selected and administered before reperfusion. Treatment with $PD1_{n\text{-}3\ DPA}$ or $RvD5_{n\text{-}3\ DPA}$ decreased the number of adherent leukocytes onto the postcapillary endothelium (Fig. 5*A*) but did not affect the intermittent contacts typical of the rolling process

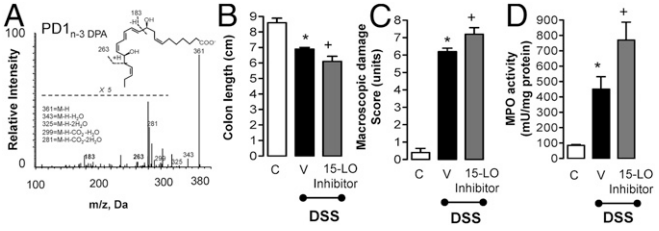

Fig. 2. 15-lipoxygenase inhibition decreased colon $PD1_{n\text{-}3\ DPA}$ levels and increased tissue damage. Mice received PD 146176 (100 mg/kg orally daily). On day 8, tissues were harvested and LM were identified and quantified using LM metabololipidomics. (*A*) Representative MS/MS spectra used for the identification of $PD1_{n\text{-}3\ DPA}$. (*B*) Colon length, (*C*) macroscopic damage, and (*D*) intestinal MPO activity. Data are means ± SEM of six mice per group. $^{*}P < 0.05$ vs. control. $^{+}P < 0.05$ vs. DSS vehicle-treated group (V), one-way ANOVA, followed by Dunnett's post hoc test.

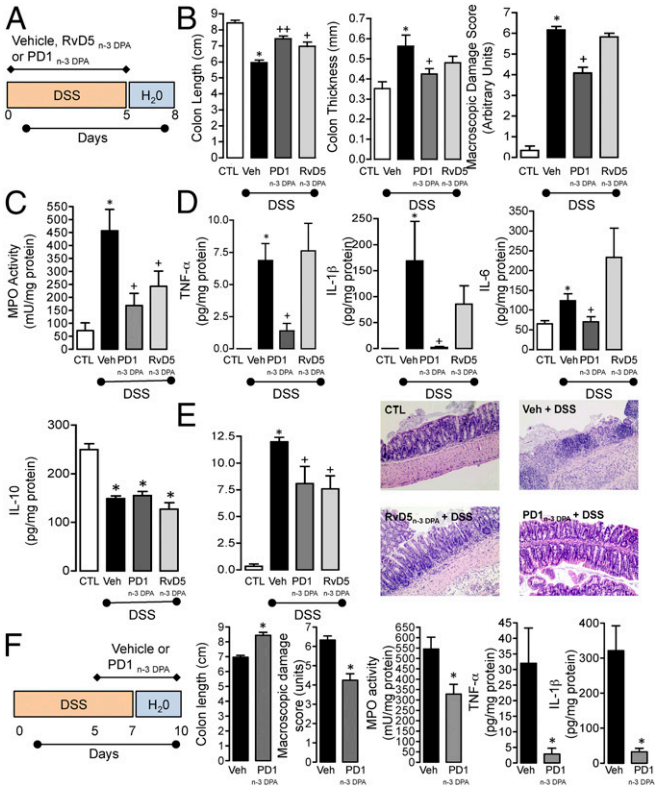

Fig. 3. $PD1_{n\text{-}3\ DPA}$ and $RvD5_{n\text{-}3\ DPA}$ protect mice against DSS-induced colitis. (*A*) Mice had access to drinking water with or without 2.5% DSS for 5 d, then switched to normal water for an additional 3 d before colon collection for analyses (day 8). Animals were treated intraperitoneally 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 days 0–5. 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. (Magnification: 50×.) (*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 means ± SEM of six mice per group. $^{*}P < 0.05$ versus CTL. $^{+}P < 0.05$, $^{++}P < 0.01$ vs. DSS vehicle-treated group, one-way ANOVA, followed by Dunnett's post hoc test.

(95 ± 13% of the response in vehicle-treated animals; not significant). A significant reduction in the extent of leukocytes emigrated into the subendothelial tissue was also quantified (Fig. 5*B*). Representative images are in Fig. 5*C*.

Preincubation of human neutrophils with $PD1_{n\text{-}3\ DPA}$ or $RvD5_{n\text{-}3\ DPA}$ drastically reduced cell capture (Fig. 5*D*) compounded by an effect on cell adhesion and transmigration onto TNF-α–activated endothelial monolayers (Fig. S3), with activity between 10 pM and 100 nM. No effect was observed on the number of human cell rolling on the endothelium (Fig. S3). These results made us dwell on human neutrophil functions. Incubation of whole blood with TNF-α up-regulated cell surface expression of total CD11b. Addition of $PD1_{n\text{-}3\ DPA}$ or $RvD5_{n\text{-}3\ DPA}$ before TNF-α led to a 32 ± 4% and 28 ± 4% of reduction for total CD11b immunostaining ($n = 4$ donors, $P < 0.05$) (representative histograms in Fig. 5*E*). There was some degree of selectivity in these responses because the two mediators did not modulate basal CD62L expression nor TNF-α–induced CD62L shedding (102 ± 6% with respect to appropriate control; $n = 4$ donors, not significant). This finding is in contrast to what observed with PAF stimulation, with an effect of $PD1_{n\text{-}3\ DPA}$ or $RvD5_{n\text{-}3\ DPA}$ on CD62L shedding (~50 ± 6% reduction of PAF response for either mediator, $n = 4$ donors, $P < 0.05$) (representative histogram in Fig. 5*E*).

Discussion

In the present study, we report the tissue-protective actions of two 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 herein 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 sometimes is observed (20), suggest existence of contrasting endogenous processes enacted by proinflammatory and proresolving mediators during the pathogenesis of the disease. SPM are produced by leukocytes, including neutrophils and macrophages, via transcellular biosynthesis and in a cell-autonomous manner (21, 22). Other cell types, including epithelial cells (23) and platelets (21), may also contribute to the SPM production because 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 IBD (24). In the present study we found an increase in both proinflammatory mediators, and a decrease in the proresolving n-3

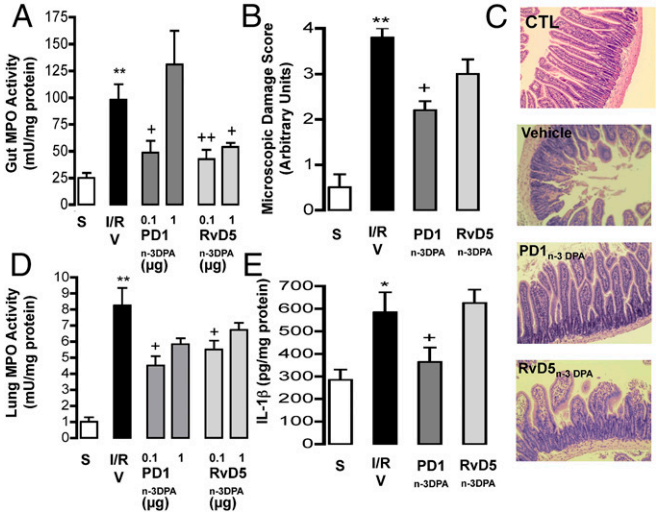

Fig. 4. Exogenous administration of $PD1_{n\text{-}3\ DPA}$ or $RvD5_{n\text{-}3\ DPA}$ prevents local and systemic I/R injury. Mice were subjected to intestinal ischemia (30 min) followed by 5-h reperfusion (I/R) or sham operation (S). Animals were given vehicle (100 μL), $PD1_{n\text{-}3\ DPA}$, or $RvD5_{n\text{-}3\ DPA}$ (0.1 μg intravenously, unless otherwise specified) before reperfusion. After 5-h reperfusion, multiple markers were quantified: (*A*) Gut MPO activity; (*B*) intestinal histological score on colon sections stained with H&E; (*C*) representative images of colon sections (magnification: 50×); (*D*) lung MPO activity; (*E*) plasma IL-1β levels. Data are mean ± SEM of 6–8 mice per group. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. sham; $^{+}P < 0.05$, $^{++}P < 0.01$ vs. vehicle group, one-way ANOVA, followed by Dunnett's post hoc test.

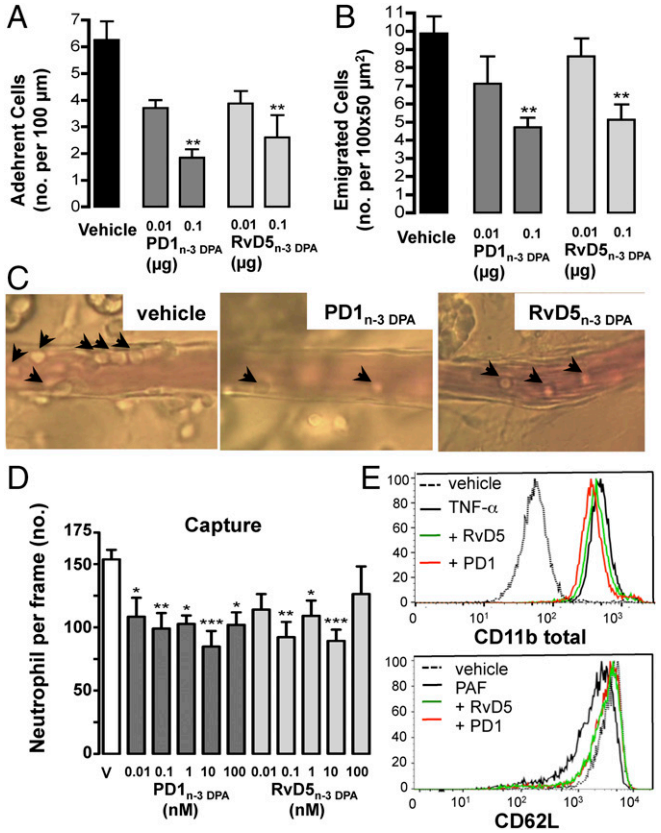

Fig. 5. $PD1_{n\text{-}3\ DPA}$ and $RvD5_{n\text{-}3\ DPA}$ reduce neutrophil–endothelial interactions. Mice were subjected to intestinal ischemia (30 min) followed by reperfusion (90 min) (*I/R*). Animals were treated intravenously before reperfusion with 0.01 or 0.1 µg $PD1_{n\text{-}3\ DPA}$, $RvD5_{n\text{-}3\ DPA}$, or vehicle (100 µL PBS 0.01% EtOH). Postcapillary venules were imaged and recorded for offline quantitation of white blood cell interaction with the endothelium. (*A*) Number of adherent cells to the postcapillary venule endothelium and (*B*) emigrated cells in the subendothelial space. (*C*) Representative light-microscopy images are shown for vehicle, $PD1_{n\text{-}3\ DPA}$, and $RvD5_{n\text{-}3\ DPA}$ (0.1 µg per mice). Black arrows identify leukocytes adherent to postcapillary venules. (Magnification: 400×.) Data are mean ± SEM of six mice per group. $^{**}P < 0.01$ vs. respective vehicle, one-way ANOVA followed by Dunnett's post hoc test. (*D*) Human neutrophils were incubated with vehicle, $PD1_{n\text{-}3\ DPA}$, or $RvD5_{n\text{-}3\ DPA}$ (0.01–100 nM) prior perfusion over TNF-α–stimulated endothelial cell monolayers for 8 min, and the extent of cell capture was quantified. Results are mean ± SEM of six 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; indicated as PD1 or RvD5) 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 (representative from four distinct blood donors).

PUFA mediators, including $PD1_{n\text{-}3\ DPA}$. Of note, levels of these mediators were within their bioactive concentration (femtomolar to nanomolar) (13, 14), a feature 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 protective role of endogenous SPM was tested using a 15-lipoxygenase inhibitor that decreased the formation of $PD1_{n\text{-}3\ DPA}$ and that was associated with an increase in the production of proinflammatory molecules and colon inflammation.

SPM derived from n-3 PUFA-like DHA and EPA have been linked to protective actions in intestinal inflammation (27). Administration of resolvin E1, resolvin D2, 17(*R*)-hydroxy DHA, aspirin-triggered resolvin D1, and Maresin 1 resulted in an improved disease activity score, reduced colonic damage, and lower polymorphonuclear 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 effects could be secondary to the regulation of proinflammatory 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 regulate colonic levels of IL-10, setting these mediators apart from LXA_4 (28) or other proresolving mediators, like Annexin-A1 (29, 30) and α-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 increased tissue levels of n-3 DPA-derived SPM, an effect linked to a reduction in systemic eicosanoid levels during I/R injury, supporting the host protective actions of these molecules.

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 proinflammatory 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 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. Because 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 characterized (36). Using intravital 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 conversion of n-3 DPA into proresolving mediators can counterbalance the actions of proinflammatory mediators. Single-cell analyses with human neutrophils identified at least one mechanism 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) might be stimulus-dependent.

In conclusion, the present study offers evidence that endogenous LMs derived from the n-3 DPA biosynthetic pathway exert beneficial actions on leukocyte reactivity and cytokine production, regulating the outcome of intestinal inflammation. Thus, $PD1_{n\text{-}3\ DPA}$ and $RvD5_{n\text{-}3\ DPA}$ 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 in *SI Materials and Methods*. All procedures were performed under the ethical approval both for animal and human cell work. For studies involving mice, all procedures were performed under the United Kingdom 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. Written informed consent was received from participants before inclusion in the study.

Sample Extraction and LM Metabololipidomics. Human and mouse gastrointestinal tissues extraction and LM metabololipidomics was conducted as described previously (25).

Induction and Assessment of DSS-Induced Colitis. Mice were provided access ad libitum to water containing 2.5% DSS over a 5-d period. Every other day, the DSS solution was replenished. On day 5, DSS was replaced with normal drinking; on day 8 the animals were killed. Control mice received only drinking water. From days 1–5, $RvD5_{n\text{-}3\ DPA}$, $PD1_{n\text{-}3\ DPA}$, or vehicle (0.3 μg/100 μL in saline 0.01% EtOH) were administered intraperitoneally daily. In other cases, the 15-lipoxygenase inhibitor PD 146176 (Cayman Chemical) was used at 100 mg/kg.

For the therapeutic protocol, mice were provided access to water containing 2.5% DSS over a 7-d period. On day 7, DSS was replaced with normal drinking water for 3 d. $PD1_{n\text{-}3\ DPA}$ or vehicle (0.3 μg/100 μL in saline 0.01% EtOH) were administered intraperitoneally 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 previously (37, 38). Samples harvested for histology were analyzed by light microscopy and scored (37, 38). MPO activity was performed as previously described (39). Intestinal levels of cytokines were assayed using ELISA kit. Colonic samples were harvested for metabololipidomics analysis, as previously described (25).

Intestinal I/R Injury. In anesthetized mice, the superior mesenteric artery (SMA) was occluded for 50 min; on reperfusion, $PD1_{n\text{-}3\ DPA}$, $RvD5_{n\text{-}3\ DPA}$, or vehicle was given, and tissues collected at 5 h. MPO activity was measured as an index of neutrophil infiltration (39). Tissue sections (5 μm) were stained with H&E. Microscopic histological damage score was evaluated blindly (40). Cytokines were assayed by ELISA. Specimens of the jejunum-ileum were also collected for intestinal lipid extraction and LC/MS/MS measurements. In other experiments, mice were transfected with either 40 μg mock or ELOVL2 siRNA and given EPA (every other day for 6 d) or vehicle. ELOV2 expression in the liver was determined after 6 d by Western blot analysis. ELOVL2 siRNA or control animals were subjected to 50-min intestinal ischemia followed by a 5-h reperfusion, as described above.

Intravital Microscopy of the Mouse Mesenteric Microcirculation. I/R was performed with 30-min ischemia and 90-min reperfusion; intravital microscopy of the mesentery was conducted as described previously (18). One to three postcapillary venules were analyzed for the extent of cell rolling, adhesion, and emigration.

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