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Novel n-3 Immunoresolvents: Structures and Actions

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Resolution of inflammation is now held to be an active process where autacoids promote homeostasis. Using functional-metabololipidomics and in vivo systems, herein we report that endogenous n-3 docosapentaenoic (DPA) acid is converted during inflammation-resolution in mice and by human leukocytes to novel n-3 products congenerous to D-series resolvins (Rv), protectins (PD) and maresins (MaR), termed specialized pro-resolving mediators (SPM). The new n-3 DPA structures include 7,8,17-trihydroxy-9,11,13,15E,19Z-docosapentaenoic acid (RvD1$_{n-3\ DPA}$), 7,14-dihydroxy-8,10,12,16Z,19Z-docosapentaenoic acid (MaR1$_{n-3\ DPA}$) and related bioactive products. Each n-3 DPA-SPM displayed protective actions from second organ injury and reduced systemic inflammation in ischemia-reperfusion. The n-3 DPA-SPM, including RvD1$_{n-3\ DPA}$ and MaR1$_{n-3\ DPA}$, each exerted potent leukocyte directed actions in vivo. With human leukocytes each n-3 DPA-SPM reduced neutrophil chemotaxis, adhesion and enhanced macrophage phagocytosis. Together, these findings demonstrate that n-3 DPA is converted to novel immunoresolvents with actions comparable to resolvins and are likely produced in humans when n-3 DPA is elevated.

T he resolution of acute inflammation is an active process temporally orchestrated by local-acting mediators that limit further neutrophil recruitment to sites of inflammation[1–4]. During the onset of inflammation, chemical mediators, including the arachidonic acid-derived eicosanoids (e.g. leukotriene (LT) B$_4$ and prostaglandin (PG) E$_2$), mount initiation and propagation of inflammation[5,6], actions that are actively counter-regulated and orchestrated by pro-resolution agonists[1,2,4]. These pro-resolving autacoids also stimulate the clearance of debris, apoptotic cells and bacteria, promoting homeostasis[1,2,7].

One of the key steps during resolution of inflammation is an increase in local vascular permeability leading to edema[6,8] and the transport of n-3 essential fatty acids (EFA) from blood to the site of inflammation[9]. n-3 EFA are linked with protective actions in a number of inflammatory conditions including rheumatoid arthritis[10], neurological disorders[11] and cardiovascular disease[12]. At the site of inflammation, n-3 EFA are converted to novel potent mediators by exudate leukocytes that promote inflammation-resolution[1,2,13].

The E-series resolvins, e.g. resolvin (Rv) E1, are produced from 5Z,8Z,11Z,14Z,17Z-eicosapentaenoic acid (EPA)[14]. The D-series resolvins, which include Resolvin D1 (7S, 8R, 17S-trihydroxy-4Z, 9E, 11E, 13Z, 15E, 19Z-docosahexaenoic acid; RvD1) and Resolvin D2 (7S, 16R, 17S-trihydroxy-4Z, 8E, 10Z, 12E, 14E, 19Z-docosahexaenoic acid; RvD2), the protectins[15] and maresins[16] are biosynthesized from 4Z,7Z,10Z,13Z,16Z,19Z-docosahexaenoic acid (DHA). These potent autacoids were initially identified using temporal lipidomics with self-resolving exudates[14,15] and are now appreciated for their ability to stereo-selectively promote inflammation-resolution by tempering leukocyte responses[16,17,13]. The resolvins, protectins and maresins are coined specialized pro-resolving mediators (SPM) that by definition limit further neutrophil recruitment to the site of inflammation and promote macrophage clearance of debris, apoptotic cells and bacteria[1,2,7]. In addition, the SPM exert potent actions in promoting wound repair and tissue regeneration as well as dampening inflammatory pain[16,18]. Targeted lipid mediator metabololipidomics of tissues obtained from a number of species ranging from primordial organisms such as planaria[16] to humans[19,20] indicates that SPM production, including RvD1, RvE1[19,20] and maresin 1[2,14], is evolutionarily conserved.

In mammals, alpha-linolenic acid (9Z, 12Z, 15Z-octadecatrienoic acid; ALA) is converted via elongation and desaturation to EPA and subsequently to DHA. An intermediate in the conversion of EPA to DHA is n-3 docosapentaenoic acid (7Z,10Z,13Z,16Z,19Z-docosapentaenoic acid; n-3 DPA)[10,12,21]. n-3 DPA carries 22 carbons and contains five double bonds, with the first double bond being found on carbon 7. The structural differences of n-3 DPA from EPA, DHA and n-6 docosapentaenoic acid (4Z, 7Z, 10Z, 13Z, 16Z,-docosapentaenoic acid; n-6 DPA), a biochemically distinct form of DPA where the first double bond is found on carbon 4, are

thought to confer unique biophysical properties that are of functional relevance, for example in neuronal systems[22]. In humans, genome-wide association studies demonstrate that elevation in circulating levels of n-3 DPA and a concomitant decrease in DHA levels are associated with single nucleotide polymorphisms in the gene encoding for the fatty acid elongase 2 (*ELOVL2*)[21]. n-3 DPA is present in a number of mammalian tissues including plasma, brain, retina and heart at levels comparable to those of EPA and DHA[23]. Since circulating levels of n-3 DPA in human cohorts of European, African, Hispanic and Chinese ancestry with mutations in the *ELOVL2* gene are elevated[21], we addressed herein whether n-3 DPA is a precursor to novel bioactive molecules. We report that endogenous n-3 DPA is converted to novel bioactive products, determined their structures and established their anti-inflammatory and pro-resolving actions.

Results

n-3 docosapentaenoic acid products exert potent anti-inflammatory and tissue-protective actions in vivo.

Since even minor changes in the structural properties of EFA are of functional significance[22], we first investigated whether n-3 DPA, products that contain one fewer double bond than those derived from DHA (Fig. 1a), produced by exudate leukocytes exerted protective actions during acute inflammation. For this purpose, we employed a model of surgery-induced second organ injury, the murine hind limb ischemia reperfusion model (Fig. 1b)[9]. Administration of an isolated mixture obtained via solid-phase extraction (see Methods) of the n-3 DPA products 10 min prior to onset of reperfusion led to protection from secondary organ injury as evidenced by reduction in lung tissue damage (Fig. 1c) and decrease in the number of infiltrated leukocytes into the lungs ($\sim$45%, $p < 0.05$). These actions were comparable to protection afforded by the DHA-derived pro-resolving mediator RvD1 (Fig. 1c, d).

Assessment of whole blood neutrophil-platelet aggregates in these mice, a marker of systemic inflammation[24], following administration of n-3 DPA products or RvD1 gave a significant reduction (55–65%) in the levels of platelet-leukocyte aggregates found 2 h post reperfusion (Fig. 1e).

Using targeted lipid mediator metabololipidomics, we next assessed whether these n-3 DPA products also regulated pro-inflammatory eicosanoid biosynthesis following ischemia reperfusion. Administration of these products led to a significant reduction in plasma prostanoid levels including PGE_2 ($\sim$75%) and thromboxane B_2 (TxB_2) ($\sim$80%; Fig. 1F). Here we also found a significant reduction in plasma LTB_4 ($\sim$60%) levels along with a decrease in levels of its double di-oxygenation isomer 5S,12S-diHETE ($\sim$75%; Fig. 1g). Of note the n-3 DPA products displayed equal or higher potency at regulating plasma eicosanoid levels when compared to RvD1 (Fig. 1f, g). These results demonstrate that n-3 DPA products possess potent anti-inflammatory and tissue-protective actions, regulating leukocyte recruitment, pro-inflammatory mediator biosynthesis and systemic inflammation.

Targeted metabololipidomics of plasma following ischemia reperfusion injury.

Having found that n-3 DPA products display potent actions during ischemia reperfusion, we next investigated the role of endogenous n-3 DPA derived in the control of acute inflammation. First we assessed the plasma levels of unesterified arachidonic acid (AA), eicosapentaenoic acid (EPA), n-3 docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA) in mice that were not subjected to an inflammatory stimulus. Plasma AA levels were 573.0 ng/ml, EPA levels were 116.2 ng/ml, n-3 DPA levels were 66.3 ng/ml and DHA levels were 146.1 ng/ml. Following ischemia reperfusion injury, circulating values for all 4 PUFA were elevated, with levels for n-3 DPA increasing $\sim$12 times to those found in uninjured mice (Fig. 2a).

We next sought evidence for the conversion of n-3 DPA to bioactive mediators during acute inflammation. Using lipid mediator metabololipidomics and monitoring the precursor ion m/z 345 in Q1 and the product ion m/z 327 in Q3, we found three major products eluting in LC-peaks (Fig. 2b). Analysis of the tandem mass spectra (MS-MS) for the products under each peak demonstrated that peak *I*, at retention time (R_T) = 17.1 min, corresponded to 17-HDPA (Supplementary Fig. 1a), the peak at R_T = 17.3 min (peak *II*) to 14-HDPA (Supplementary Fig. 1b) and peak *III* at R_T = 17.6 min corresponded to 7-HDPA (Supplementary Fig. 1c). Quantification of these novel products by multiple reaction monitoring (MRM) in the plasma of uninjured mice and mice subjected to ischemia reperfusion demonstrated that all three products were elevated following ischemia reperfusion injury (Fig. 1c). These results demonstrate that during acute inflammation, systemic n-3 DPA levels are elevated, and this n-3 EFA is converted to novel oxygenated products.

Chiral metabololipidomics of endogenous n-3 DPA products.

In order to elucidate the biosynthetic origins of these novel n-3 DPA-derived products we designed chiral metabololipidomic profiling of the n-3 DPA products generated in vivo. Reverse-phase chiral LC-MS-MS metabololipidomics of plasma samples obtained from mice subjected to ischemia reperfusion injury achieved baseline separation of 7R/S-HDPA, 14R/S-HDPA and 17R/S-HDPA (Supplementary Fig. 2). Quantification of the two isomers for each of the products identified demonstrated that the R to S ratio for all of the monohydroxy products identified was $\sim$20% : $\sim$80%. These results indicated that the conversion of n-3 DPA to these novel products is enzymatically regulated, since mammalian lipoxygenases are known to insert molecular oxygen in predominantly the S configuration[5].

Targeted metabololipidomics during onset and resolution of acute inflammation uncovers novel n-3 docosapentaenoic acid products.

Having found that n-3 DPA is converted in vivo to yield monohydroxy acids that predominantly possess the S chirality, we next investigated whether these n-3 DPA monohydroxy products were precursors and/or pathway markers for the biosynthesis of bioactive mediators. To this end, we employed targeted LM metabololipidomics with plasma from mice subjected to ischemia reperfusion injury. Multiple reaction monitoring of m/z 377 in Q1 and m/z 143 in Q3 yielded two peaks, the first at R_T = 11.6 min and the second at R_T = 12.1 min (Fig. 3a). Inspection of the MS-MS spectrum for the product eluting in peak at R_T = 11.6 min demonstrated that this material corresponded to RvD2$_{n-3\ DPA}$ with the following characteristic ions assigned: m/z 307, m/z 279, m/z 249, m/z 233, and m/z 143 (c.f. Supplementary Fig. 3a, b); while assessment of the MS-MS spectrum for the products at R_T = 12.1 min demonstrated that this material corresponded to RvD1$_{n-3\ DPA}$ (Fig. 3b). Multiple reaction monitoring of m/z 361 in Q1 and m/z 263 in Q3 yielded three peaks, one at R_T = 13.6 min, the second peak with R_T = 13.7 min and the third peak R_T = 14.4 min (Fig. 3a). Assessment of MS-MS spectra for the product with R_T = 13.6 min gave characteristic fragmentation corresponding to RvD5$_{n-3\ DPA}$ (Fig. 3c). MS-MS fragmentation for the material at R_T = 13.7 min demonstrated characteristic fragmentation corresponding to PD1$_{n-3\ DPA}$ (Fig. 3d). The peak eluting at R_T = 14.4 min was identified as PD2$_{n-3\ DPA}$ with the following characteristic ions assigned: m/z 233, m/z 247, m/z 189 (c.f. Supplementary Fig. 4c, d). These findings demonstrate that n-3 DPA is converted to novel products that are cognate to pro-resolving mediators from DHA; therefore, we employed the nomenclature from the DHA bioactive metabolome to describe each of these new structures.

Since vascular leakage during inflammation supplies the site of inflammation with PUFA[9], we next investigated tissue levels of n-3 DPA products during the onset and resolution of inflammation. For this purpose, we applied a self-limited model of inflammation where, following the administration of a pro-inflammatory stimulus into the
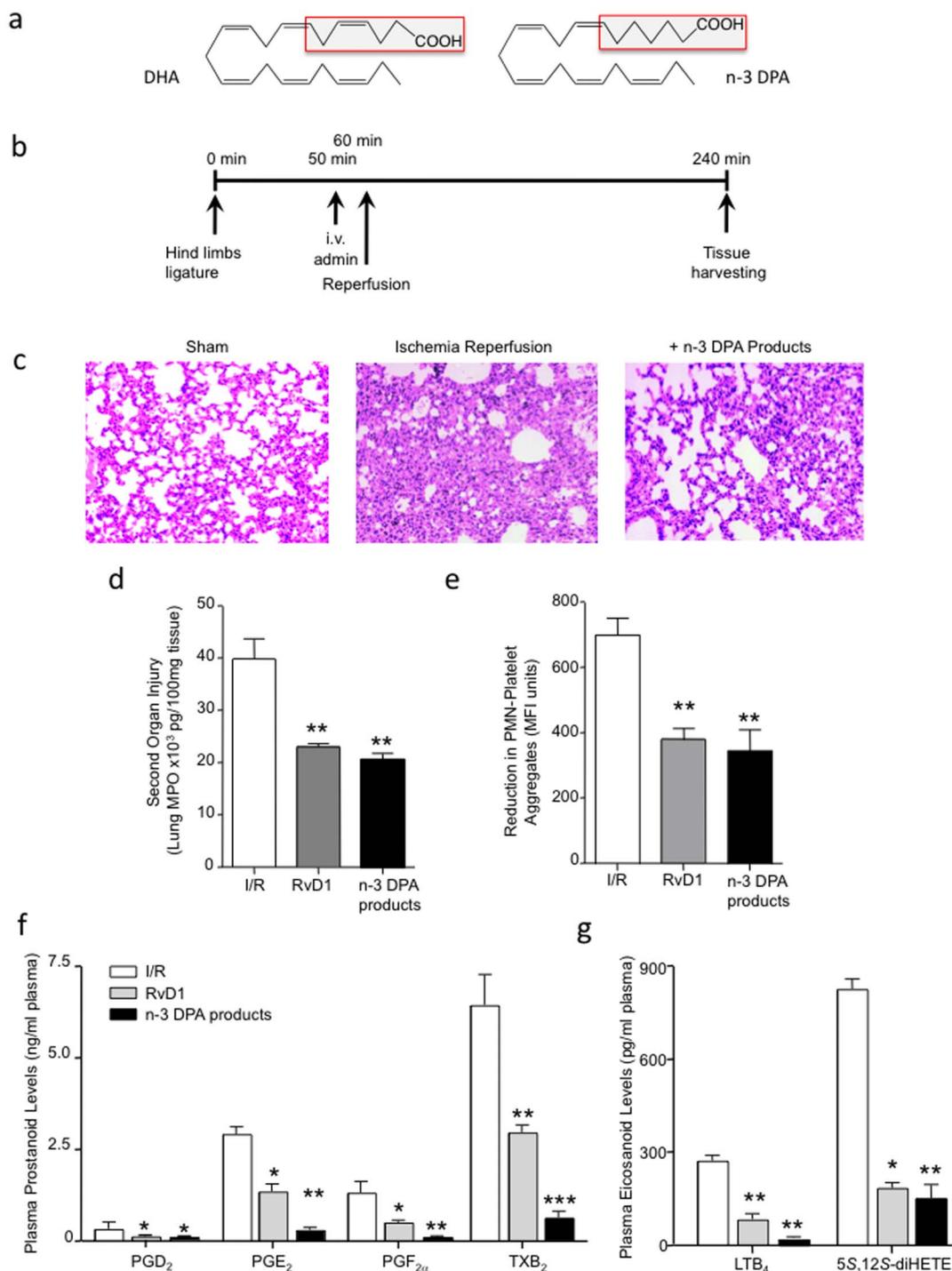
Figure 1 | n-3 DPA-derived products display potent anti-inflammatory and tissue protective actions *in vivo* that are comparable to RvD1.
(a) Structures of DHA and n-3 DPA. (b) Ischemia was induced by applying tourniquets to the hind limb of 6-8-week-old male FvB mice. After 1 h, tourniquets were removed and reperfusion ensued for 3 h. 10 min prior to reperfusion, vehicle (saline containing 0.1% EtOH), RvD1 (500 ng) or a mixture of n-3 DPA-derived products (see Methods for details) were administered intravenously. At the end of reperfusion, lungs were collected; (c) tissue histology by H&E staining (x200) and (d) MPO levels were assessed. (e) Blood was collected, incubated with rat anti-mouse Ly6G and rat anti-mouse CD41 antibodies and neutrophil leukocyte aggregates were assessed by flow cytometry. (f) Plasma prostanoid and (g) leukotriene levels were assessed by lipid mediator metabololipidomics. Results c are representative n = 4. Results d–e are mean ± SEM. n = 4. * $P < 0.05$, ** $P < 0.01$ vs. vehicle mice.

mouse peritoneum, there is a rapid recruitment of neutrophils into the site that peaks at around 6 h (T_{max}; Fig. 4a). This is followed by a decline in neutrophil numbers over the next 18 h. The time difference between T_{max} and the point where neutrophil numbers reach 50% of maximum (T_{50}) is defined as the resolution interval (R_i)[25]. Using LM metabololipidomics, we profiled the levels of LTB_4 and

PGE_2 that were rapidly produced during the initiation phase of the inflammatory response concomitant with neutrophil infiltration into the tissue (Fig. 4b). Maximal LTB_4 levels coincided with peak neutrophil infiltration, whereby LTB_4 levels rapidly subsided over the next 8 h. PGE_2 levels were also elevated early in the initiation phase of the response, with levels for this mediator persisting into the
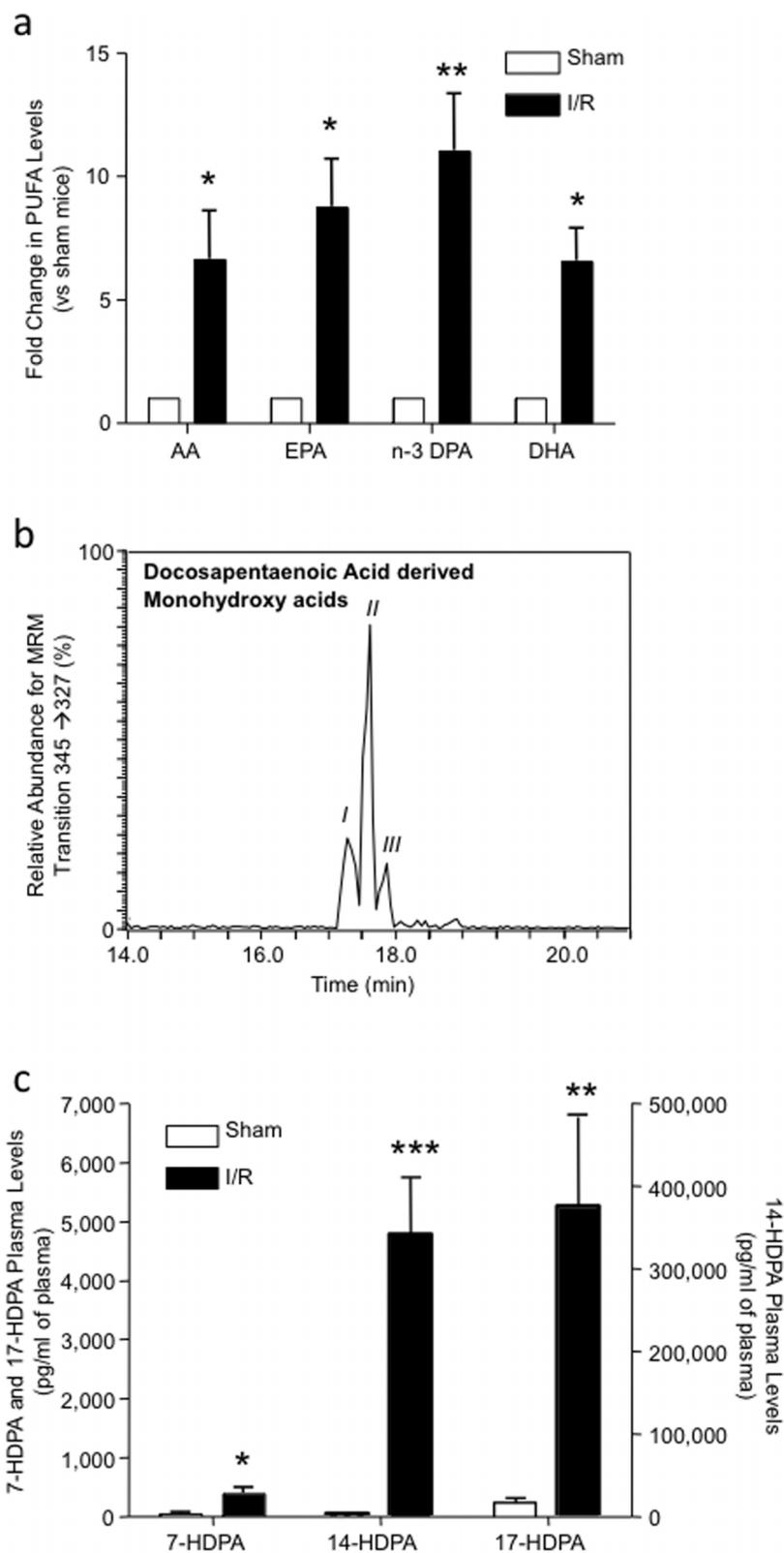
Figure 2 | n-3 DPA levels increase in acute inflammation and is converted to novel products *in vivo*. Mice were subjected to ischemia reperfusion injury (see Methods for details) at 2 h of reperfusion, blood was collected via cardiac puncture and plasma was obtained by centrifugation, products were extracted and monohydroxy n-3 DPA levels were assessed by lipid mediator metabololipidomics. (a) Plasma polyunsaturated fatty acid levels; (b) Representative chromatographs obtained by Multiple Reaction Monitoring (MRM) of the parent ion (Q_1) m/z 345 and a diagnostic daughter ion (Q_3) m/z 327. (c) Monohydroxy-containing levels in plasma of sham mice and mice subjected to I/R. Results for a and c are mean ± SEM. n = 4. Results for b are representative of n = 4. *$P < 0.05$; **$P < 0.01$; ***$P < 0.01$ vs. sham mice.
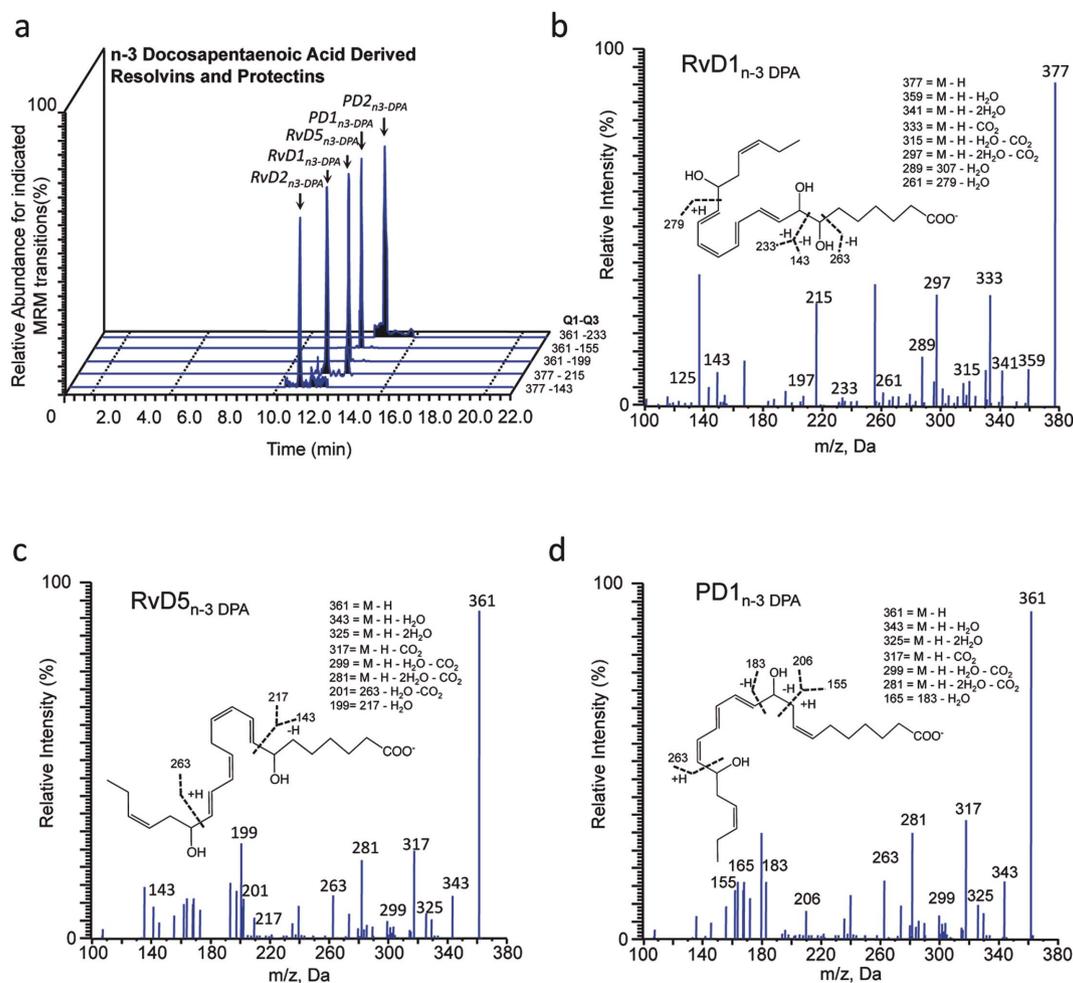
Figure 3 | Identification of novel endogenous n-3 DPA pro-resolving mediators. Mice were subjected to ischemia reperfusion injury (see Methods and Fig. 2 for details). Two h into reperfusion, blood was collected and lipid mediators identified by lipid mediator metabololipidomics. (a) Representative chromatographs obtained by Multiple Reaction Monitoring of the parent ion (Q_1) and a diagnostic daughter ion (Q_3) in the MS-MS of n3-DPA resolvins, protectins and maresins. Representative MS-MS spectra used for identification of (b) $RvD1_{n-3\ DPA}$, (c) $RvD5_{n-3\ DPA}$, and (d) $PD1_{n-3\ DPA}$. Results are representative of n = 4.

resolution phase. In this experimental setting, which characterizes the initiation and resolution phases of the inflammatory response[25], we determined the profile of novel n-3 DPA products in order to temporally stage each of these new products within the self-limited inflammatory response. Here we found that endogenous production of $RvD1_{n-3\ DPA}$ and $PD2_{n-3\ DPA}$ displayed a bi-phasic profile, reaching a maximum during peak neutrophil infiltration and late into resolution (Figure 4c, d). $PD1_{n-3\ DPA}$, $MaR2_{n-3\ DPA}$ and $MaR3_{n-3\ DPA}$ levels were each found to reach a maximum at the 4h interval and gradually decreased over the next 20 h (Figure 4d, e). The peak in exudate $RvD2_{n-3\ DPA}$ levels coincided with the onset of resolution (the point where PMN levels reach ~50 of T_{max}). $RvD5_{n-3\ DPA}$ levels were found to gradually increase over the course of inflammation-resolution, with a maximum being reached late in the resolution phase. The n-3 DPA product corresponding to $MaR1_{n-3\ DPA}$ gave levels that were elevated in the peritoneum of naive mice, where upon challenge with zymosan these levels drastically decreased. Also, $MaR1_{n-3\ DPA}$ accumulated late during resolution (Figure 4e). These results establish the endogenous production of novel n-3 DPA resolvins, protectins and maresins during acute inflammation and stage their formation primarily within the self-limited inflammatory response.

Specialized pro-resolving mediators from n-3 DPA exert potent anti-inflammatory actions in vivo. We next tested whether these

novel structures carried bioactivity. Intravenous administration of 100 ng of $RvD1_{n-3\ DPA}$ and $RvD2_{n-3\ DPA}$ significantly reduced neutrophil recruitment into the peritoneum (A; ~45%; Fig. 5a). In these experiments, we also found that the novel tri-hydroxy-containing n-3 DPA products significantly reduced exudate levels of the pro-inflammatory cytokines Interleukin (IL) 6 (~55%; Fig. 5b) and monocyte chemoattractant protein (MCP)-1 (~55%; Fig. 5c). Administration of the di-hydroxy-containing n-3 DPA products from both 17-hydroperoxy-DPA (HpDHA; $RvD5_{n-3\ DPA}$ and $PD1_{n-3\ DPA}$; B; ~47%) and 14-HpDPA ($MaR1_{n-3\ DPA}$ and $MaR2_{n-3\ DPA}$; C; ~50%) also significantly reduced PMN recruitment and pro-inflammatory cytokine levels in these exudates (Fig. 5a–c).

Human leukocytes produce n-3 DPA immunoresolvents. Having found that these products are produced in murine systems, we next sought evidence for their production by human leukocytes. Assessment of methyl formate fractions obtained from activated human peripheral blood neutrophils incubated with n-3 DPA by lipid mediator metabololipidomics profiling gave products that displayed chromatographic and MS-MS spectra consistent with resolvins, protectins and maresins that carry the n-3 DPA backbone (Fig. 5a). Assessment of the UV absorbance spectra and MS-MS fragments for each of the products gave fragment ions that
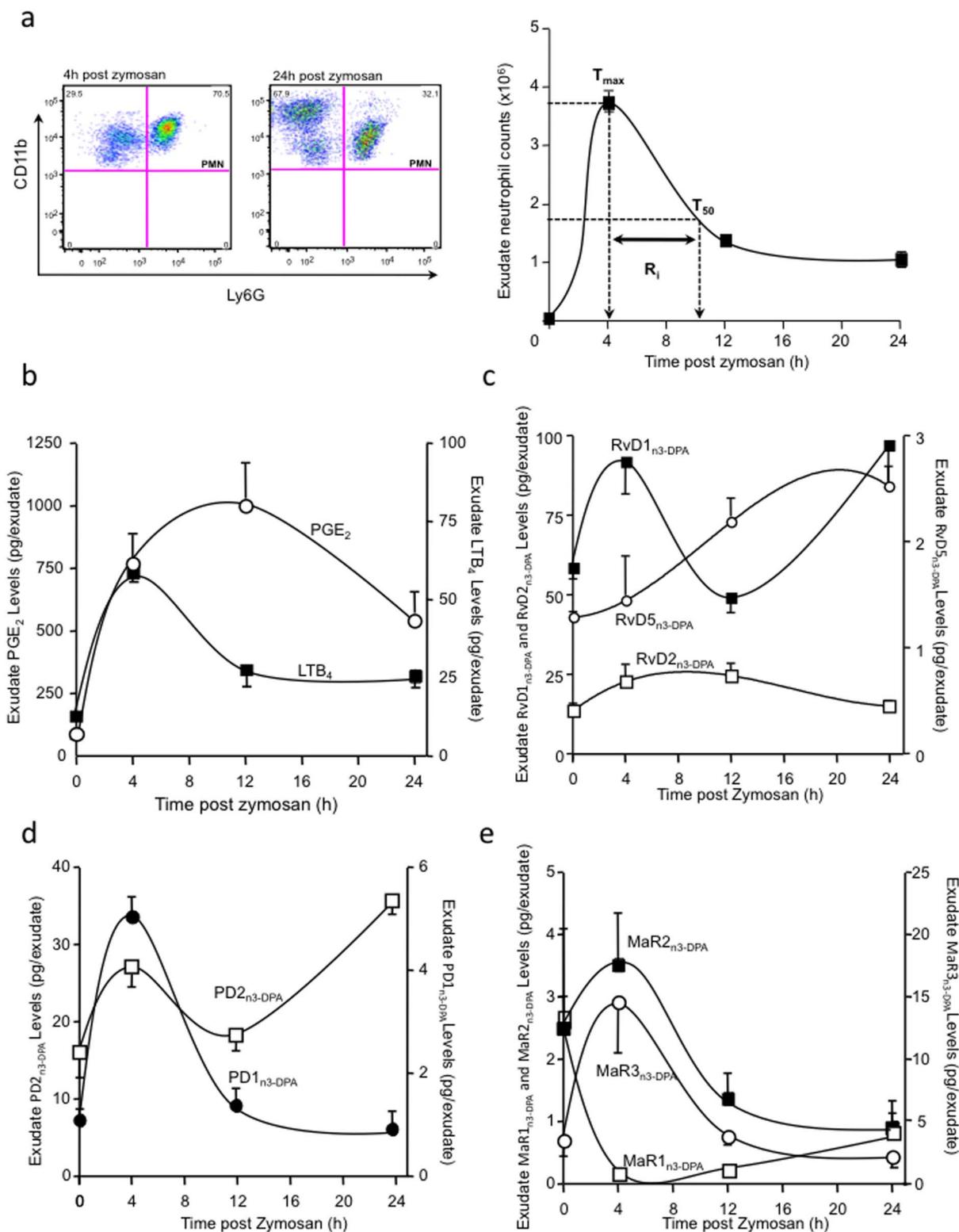
Figure 4 | Self-limited inflammation: Endogenous formation of novel immunoresolvents from n-3 DPA. Mice were treated with 0.1 mg zymosan i.p.; after the indicated time intervals peritoneal exudates were collected. (a) Exudate leukocyte counts obtained by light microscopy and flow cytometry. Exudate levels for (b) prostaglandin (PG) E_2 and leukotriene (LT) B_4; (c) resolvins, (d) protectins and (e) maresins were measured by lipid mediator metabololipidomics. Results are mean ± SEM. n = 4 mice per time point.

were consistent with $RvD2_{n-3\ DPA}$ with m/z 377 [M-H], m/z 359 [M-H-H_2O] and m/z 333 [M-H-CO_2] (Fig. 5a and Supplementary Fig. 3a, b). Additional diagnostic ions were identified at m/z 247, m/z 143 and m/z 279 consistent with the presence of hydroxy groups at carbon positions 7, 16 and 17. In the tri-hydroxy chromatographic

regions, we also identified $RvD1_{n-3\ DPA}$ as demonstrated by its characteristic fragmentation pattern and UV absorbance spectra, consistent with hydroxyl groups at the carbon 7, 8 and 17 positions (Fig. 3a, b and Supplementary Fig 3c, d). Assessment of the UV absorbance and MS-MS spectra in the di-hydroxy region
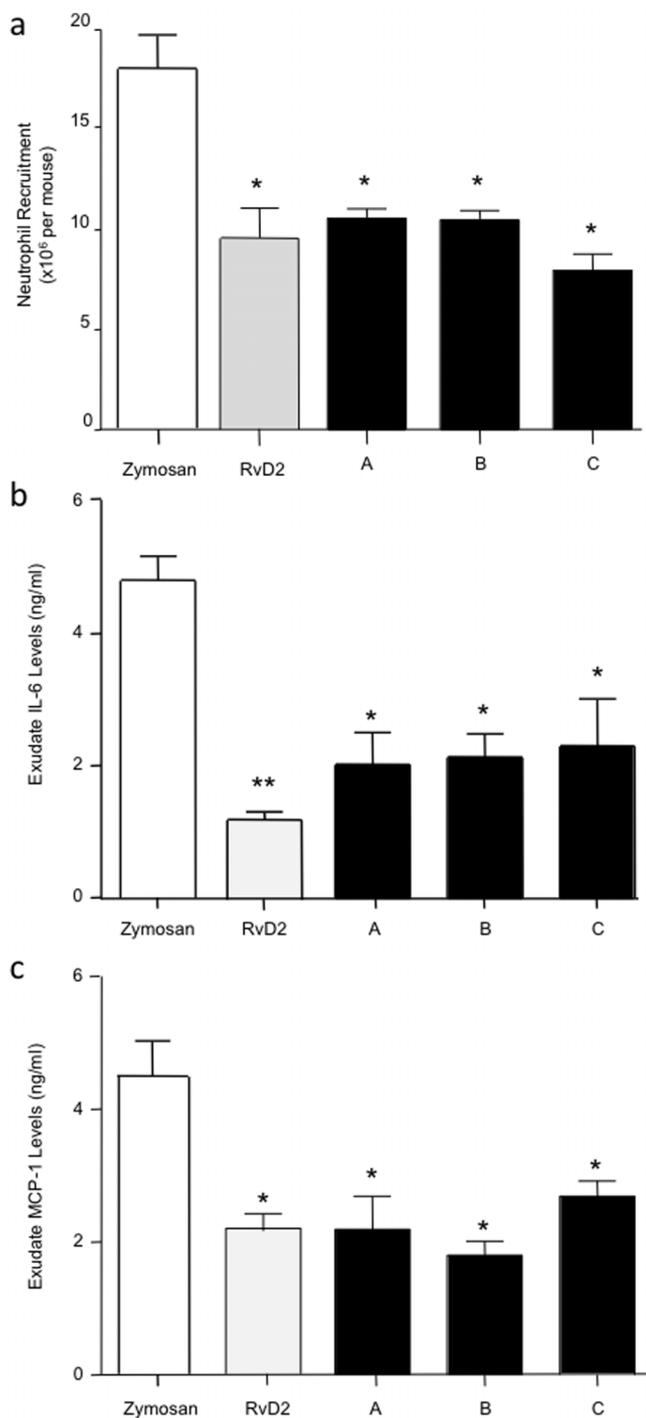
Figure 5 | n-3 DPA immunoresolvents display potent anti-inflammatory actions *in vivo*. The indicated n-3 DPA-derived product mixtures were administered by intravenous injection 10 min prior to the intraperitoneal administration of zymosan (0.1 mg, 500 µl PBS) to 6–8-week-old male FvB mice. At 4 h, peritoneal exudates were collected and the (a) number of infiltrated neutrophils was assessed by light microscopy and flow cytometry. Exudate levels for the pro-inflammatory mediators (b) IL6 and (c) MCP-1 were determined by cytokine array. The ratio of RvD1$_{n-3\ DPA}$ to RvD2$_{n-3\ DPA}$ was ~3 : 1 (A), the ratio of MaR1$_{n-3\ DPA}$ to MaR2$_{n-3\ DPA}$ was ~4 : 1 (B); the ratio of RvD5$_{n-3\ DPA}$ to PD1$_{n-3\ DPA}$ was ~9 : 1 (C). Results are mean ± SEM. n = 4. * $P < 0.05$, ** $P < 0.01$ vs. zymosan mice.

revealed the presence of RvD5$_{n-3\ DPA}$ (Fig. 3a, c and Supplementary Fig 3e, f), PD1$_{n-3\ DPA}$ (Fig. 3a and Supplementary Fig. 5a, b) and MaR3$_{n-3\ DPA}$ (Fig. 3a and Supplementary Fig. 5e, f). Using LM metabololipidomics we also found that human monocyte-derived

macrophages produce resolvins, protectins and maresins from endogenous n-3 DPA (n = 3). These findings demonstrate that human leukocytes can convert both exogenous as well as endogenous n-3 DPA to novel n-3 DPA immunoresolvents.

n-3 DPA products exert anti-inflammatory and pro-resolving actions on human leukocytes. We next investigated whether these novel n-3 DPA products retained their leukocyte directed anti-inflammatory and pro-resolving actions when incubated with human leukocytes. A key step in neutrophil recruitment to the site of inflammation is firm adhesion to the vascular endothelium[26]. Incubation of neutrophils with RvD1$_{n-3\ DPA}$ and RvD2$_{n-3\ DPA}$, (A; ~30%), RvD5$_{n-3\ DPA}$ and PD1$_{n-3\ DPA}$, (B; ~25%) or PD1$_{n-3\ DPA}$ and PD2$_{n-3\ DPA}$ (C; ~22%) led to a significant reduction in neutrophil adhesion to TNF-α activated endothelial cells to a similar extent as RvD2 (~30%; Fig. 7a), a potent pro-resolving mediator[27].

Neutrophil-endothelial cell adhesion is mediated by adhesion molecules expressed on both the endothelial and neutrophil surface that are up-regulated during inflammation[26]. One of these adhesion molecules is Intercellular Adhesion Molecule 1 (ICAM-1/CD54) expressed on endothelial cells[28]. Incubation of each of the n-3 DPA products with endothelial cells prior to incubation with TNF-α also led to a significant reduction in endothelial cell ICAM-1 expression (~25–40%; Fig. 5b).

We next questioned whether the n-3 DPA pro-resolving mediators regulated human peripheral blood leukocyte recruitment to a chemotactic stimulus. Incubation of human leukocytes with RvD2 led to a significant reduction in neutrophil (~42%) recruitment towards an IL-8 gradient. When human neutrophils were incubated with RvD1$_{n-3\ DPA}$ and RvD2$_{n-3\ DPA}$ (1 nM), we also found a significant reduction in neutrophil chemotaxis (~45%) towards an IL-8 gradient (Fig. 7b). Incubation of human neutrophils with 1 nM of the di-hydroxy containing products (i.e. RvD5$_{n-3\ DPA}$ and PD1$_{n-3\ DPA}$, C; or MaR1$_{n-3\ DPA}$ and MaR2$_{n-3\ DPA}$, D) also led to a significant reduction in neutrophil chemotaxis (~40–75%) towards IL8 (Fig. 7b).

Because macrophage clearance of cellular debris and apoptotic cells is a critical process in promoting the resolution of acute inflammation[1], we next incubated human monocyte-derived macrophages with RvD2, which led to a significant increase in the uptake of opsonized zymosan particles (~50%; Fig. 7c). Addition of RvD5$_{n-3\ DPA}$ and PD1$_{n-3\ DPA}$, (1 nM) to macrophages each also led to a significant increase (~70%) in macrophage phagocytosis of fluorescently labeled zymosan (Fig. 7c). Macrophage incubations with MaR1$_{n-3\ DPA}$ and MaR2$_{n-3\ DPA}$ (~55%) or RvD1$_{n-3\ DPA}$ and RvD2$_{n-3\ DPA}$ (~45%) also gave significant increases in macrophage uptake of fluorescently labeled zymosan (Fig. 7c). These results demonstrate that the novel n-3 DPA mediators exert potent anti-inflammatory and pro-resolving actions with human leukocytes limiting human neutrophil recruitment and macrophage phagocytosis, key processes in promoting resolution and by definition those assigned to pro-resolving mediators[1,2].

Discussion

In the present report, using lipid mediator metabololipidomics, we identified novel n-3 DPA-derived products and staged their biosynthesis during self-limited inflammation in inflammatory exudates. With murine models of acute inflammation and human leukocytes, we determined their anti-inflammatory, pro-resolving and tissue protective actions, defining these novel n-3 DPA products as immunoresolvents.

In humans, circulating n-3 DPA levels do not appear to be directly associated with dietary intake, unlike other n-3 EFA including DHA and EPA[21,29], thus suggesting in humans a primary endogenous metabolic origin for n-3 DPA. Along these lines, a recent genome wide association study with more then 8500 participants from various ethnicities demonstrated that elevated plasma n-3 DPA levels are

associated with single nucleotide polymorphisms in the genes encoding for the fatty acid elongase 2 (*ELOVL2*) and glucokinase regulatory protein (*GCKR*)[21]. This increase in n-3 DPA levels is also associated with a reduction in circulating DHA levels. Of interest, circulating n-3 DPA has been associated with protection from cardiovascular disease[30–32]. Hence, our present findings indicating that n-3 DPA is a precursor to new potent bioactive products may have wide implications in individuals carrying elevated circulating levels of n-3 DPA.

Ischemia-reperfusion injury is of considerable consequence in the pathology of many diseases including periodontal disease, arthritis and stroke as well as being of relevance during surgical procedures, in particular those involving extremities, causing aberrant leukocyte activation that results in local and remote tissue and organ damage[33]. Neutrophils in these settings play pivotal roles in the perpetuation of reperfusion injury giving rise to second organ injury[33]. However, phagocytes, including neutrophils when appropriately activated, are also instrumental in orchestrating resolution processes via their temporal production of pro-resolving mediators in resolving exudates[14,15,34,35]. Hence our

findings indicating that n-3 DPA products obtained from phagocytes collected from self-resolving exudates display potent protective actions from second organ injury during ischemia reperfusion have implications in a wide range of human pathologies. Indeed these products markedly reduced both local tissue damage and neutrophil infiltration into the lungs (Fig. 1), a hallmark of second organ injury, to a similar extent as the DHA-derived resolvin D1, a known potent pro-resolving mediator[9].

The formation of platelet-leukocyte aggregates in the vasculature is also a component of many inflammatory disorders including stroke, sepsis and hypertension. Formation of these microcellular aggregates enhances the production of a number of pro-inflammatory cytokines including IL-8 and MCP-1 as well as increases the levels of platelet aggregating factor, a potent pro-inflammatory lipid mediator (for a review, see ref. 24). In this context, elevated levels of circulating platelet-leukocyte aggregates are proposed as an early marker for acute myocardial infarction and are increasingly regarded as a cardiovascular risk factor[36]. In the present studies, administration of phagocyte-derived n-3 DPA products led to a reduction in circulating platelet-neutrophil aggregates following ischemia
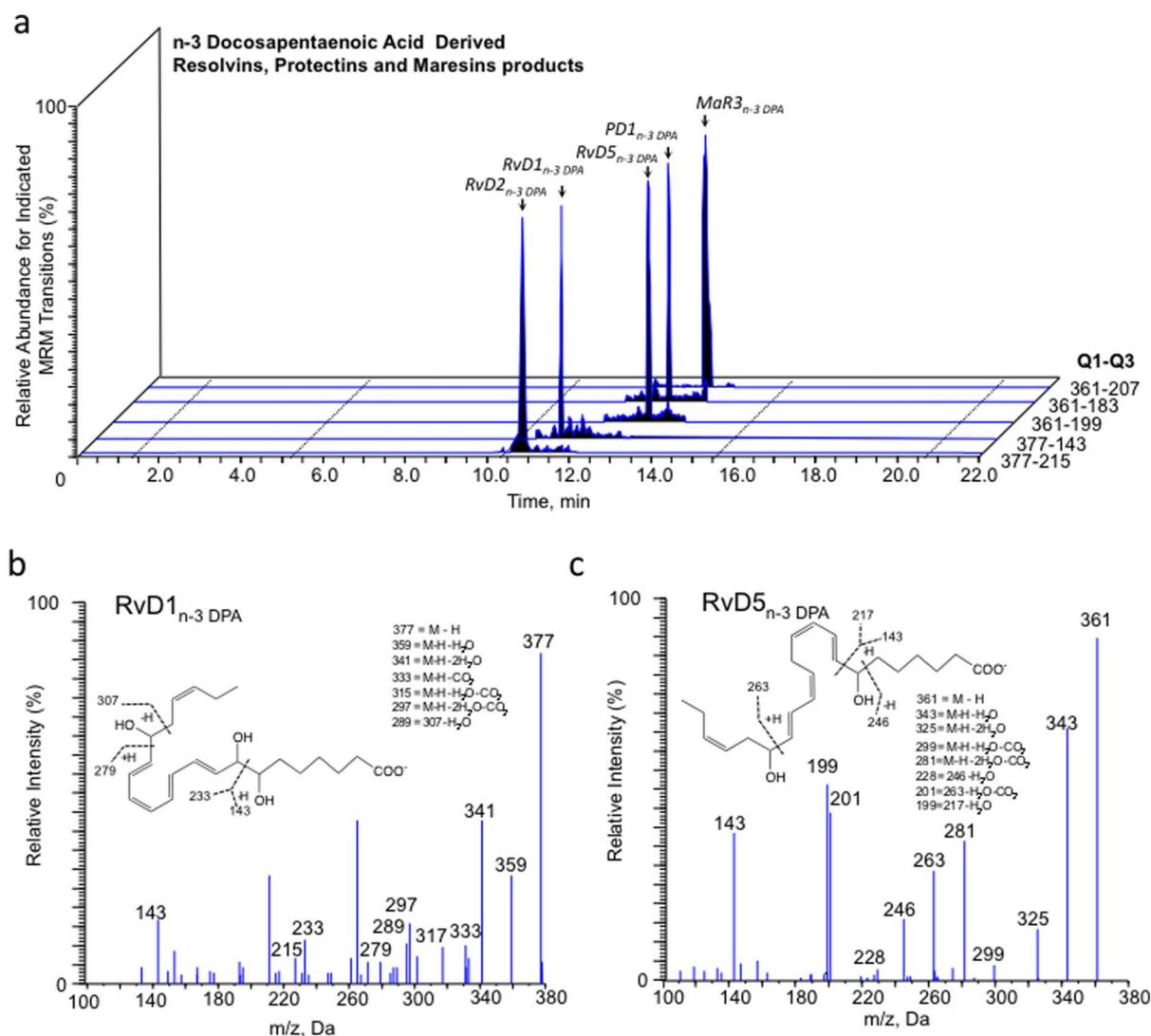

Figure 6 | Human neutrophils produce novel n-3 DPA-derived immunoresolvents. Human neutrophils were prepared from peripheral blood (see Methods for details), suspended in DPBS (80 × 10⁶/ml) and incubated with serum treated zymosan (0.1 mg) and n-3 DPA (1 µM, 30 min, 37°C, pH 7.45); incubations were stopped with ice-cold methanol and products assessed by lipid mediator metabololipidomics. (a) Representative chromatographs obtained by Multiple Reaction Monitoring of the parent ion (Q_1) and a diagnostic daughter ion (Q_3) in the MS/MS. Representative MS/MS spectra used for identification of (b) RvD1$_{n-3\ DPA}$ and (c) RvD5$_{n-3\ DPA}$. Results are representative of n = 4.
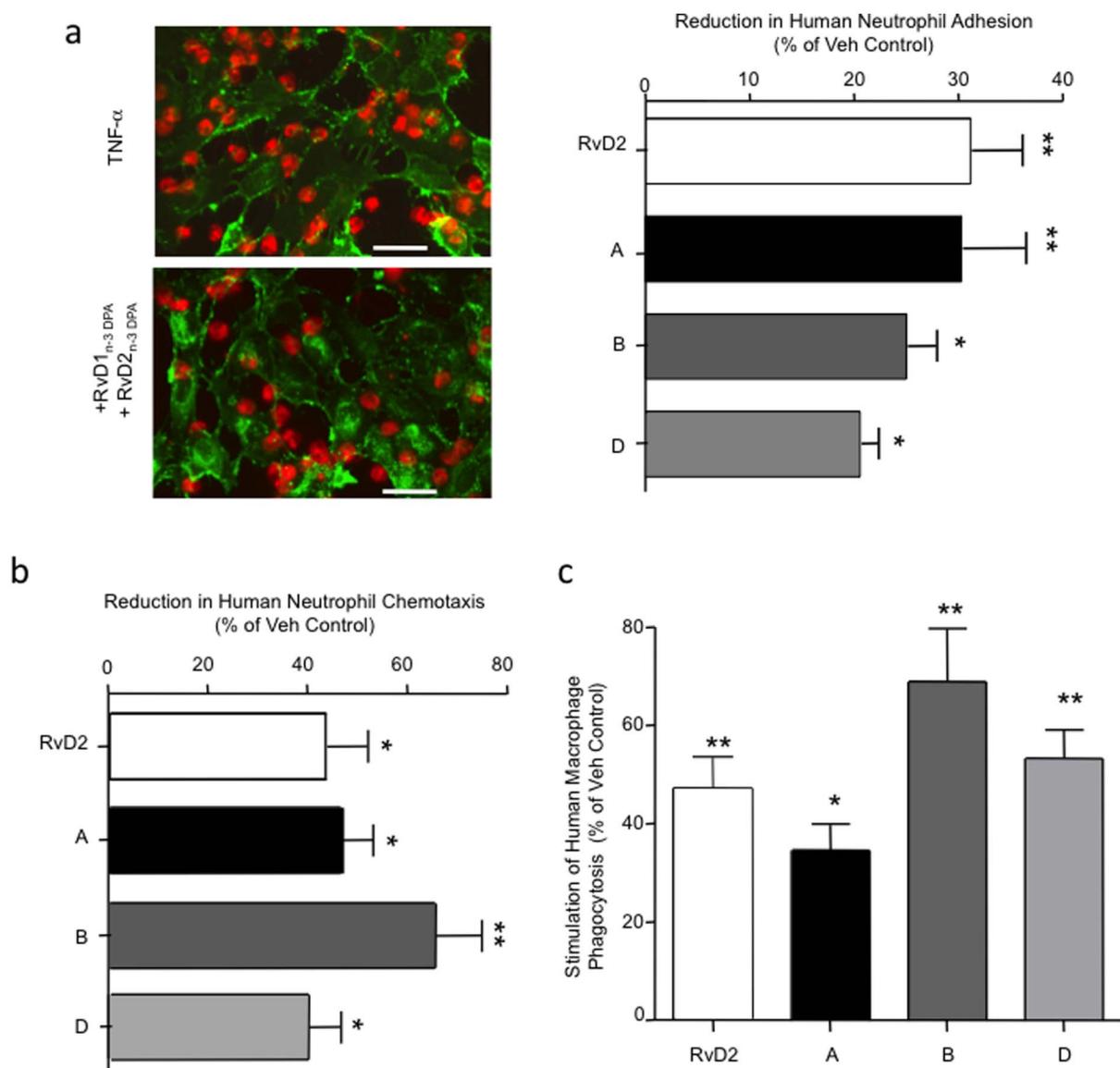
Figure 7 | Reduction in human neutrophil chemotaxis, neutrophil-endothelia cell adhesion and stimulation of macrophage phagocytosis by n-3 DPA-derived immunoresolvents. (a) *Left panel*: micrographs depict PKH26-labeled neutrophils adherent to WGA-Alexafluor® 488-labeled HUVEC stimulated with TNF-α (10 ng/ml, 4 h, 37°C, 0.1% FCS) with or without n-3 DPA resolvins (1 nM, 15 min, 37°C, pH7.45; ×40 magnification). *Right panel*: Fluorescently labeled human neutrophils were incubated with vehicle (0.1% EtOH in PBS) or n-3 DPA products. These were then added to TNF-α-stimulated HUVEC and incubated for 30 min (37°C), non-adherent cells were washed and extent of neutrophil adhesion assessed using a SpectraMax M3 Plate reader. (b) Neutrophils were incubated with vehicle (0.1% EtOH in PBS) or n-3 DPA products (1 nM, 15 min, 37°C, pH7.45) prior to loading on ChemoTx chambers and assessing chemotaxis towards IL-8 (100 ng/ml, 90 min, 37°C, pH7.45). (c) Macrophages were incubated with vehicle (0.1% EtOH in PBS) or n-3 DPA products (1 nM, 15 min, 37°C, pH7.45) prior to addition of fluorescently labeled zymosan (1 : 10 macrophages to zymosan). After 60 min (37°C, pH7.45), the incubation was stopped, extracellular fluorescence quenched using trypan blue and phagocytosis assessed using a SpectraMax M3 Plate reader. The ratio of $RvD1_{n-3\,DPA}$ to $RvD2_{n-3\,DPA}$ (A) was ~3 : 1; the ratio of $RvD5_{n-3\,DPA}$ to $PD1_{n-3\,DPA}$ (B) was ~9 : 1; the ratio of $PD1_{n-3\,DPA}$ to $PD2_{n-3\,DPA}$ (C) was ~1 : 5; the ratio of $MaR1_{n-3\,DPA}$ to $MaR2_{n-3\,DPA}$ (D) was ~4 : 1. Results are mean ± SEM. n = 4 independent neutrophil and macrophage preparations (*$P < 0.05$; **$P < 0.05$ vs. vehicle incubated cells). Bar = 50 μm.

reperfusion injury. In addition, we also found a substantial reduction in plasma pro-inflammatory eicosanoid levels including LTB_4 and TxB_2 (Fig. 1), actions that were comparable to those afforded by DHA-derived RvD1. Therefore, these results demonstrate that n-3 DPA products display potent systemic anti-inflammatory and tissue protective actions.

Phagocytes carry lipoxygenase enzymes that are involved in the biosynthesis of pro-resolving mediators[14,15,35]. These enzymes convert their substrate in a stereospecific manner, inserting molecular oxygen predominantly in the *S* configuration[5,34]. In the present study we found that the systemic levels of n-3 DPA and the new n-3 DPA products (i.e. 17-HDPA, 14-HDPA and 7-HDPA) were each elevated during acute inflammation in vivo (Fig. 2). In addition, chiral lipidomics of these n-3 DPA products demonstrated that the hydroxy groups in these products were predominantly in the *S* configuration suggesting that each was produced via lipoxygenases (Supplementary Fig. 2). Targeted metabololipidomics demonstrated that n-3 DPA is further oxygenated, in both murine in vivo and isolated human leukocytes, to products that are congenerous of the DHA-derived pro-resolving mediators (Figs. 3, 6). In addition, endogenous levels of these novel n-3 DPA mediators were temporally regulated during self-limited inflammation suggesting that each may possess distinct roles in the regulation of inflammation-resolution and in organ protection (Fig. 4).
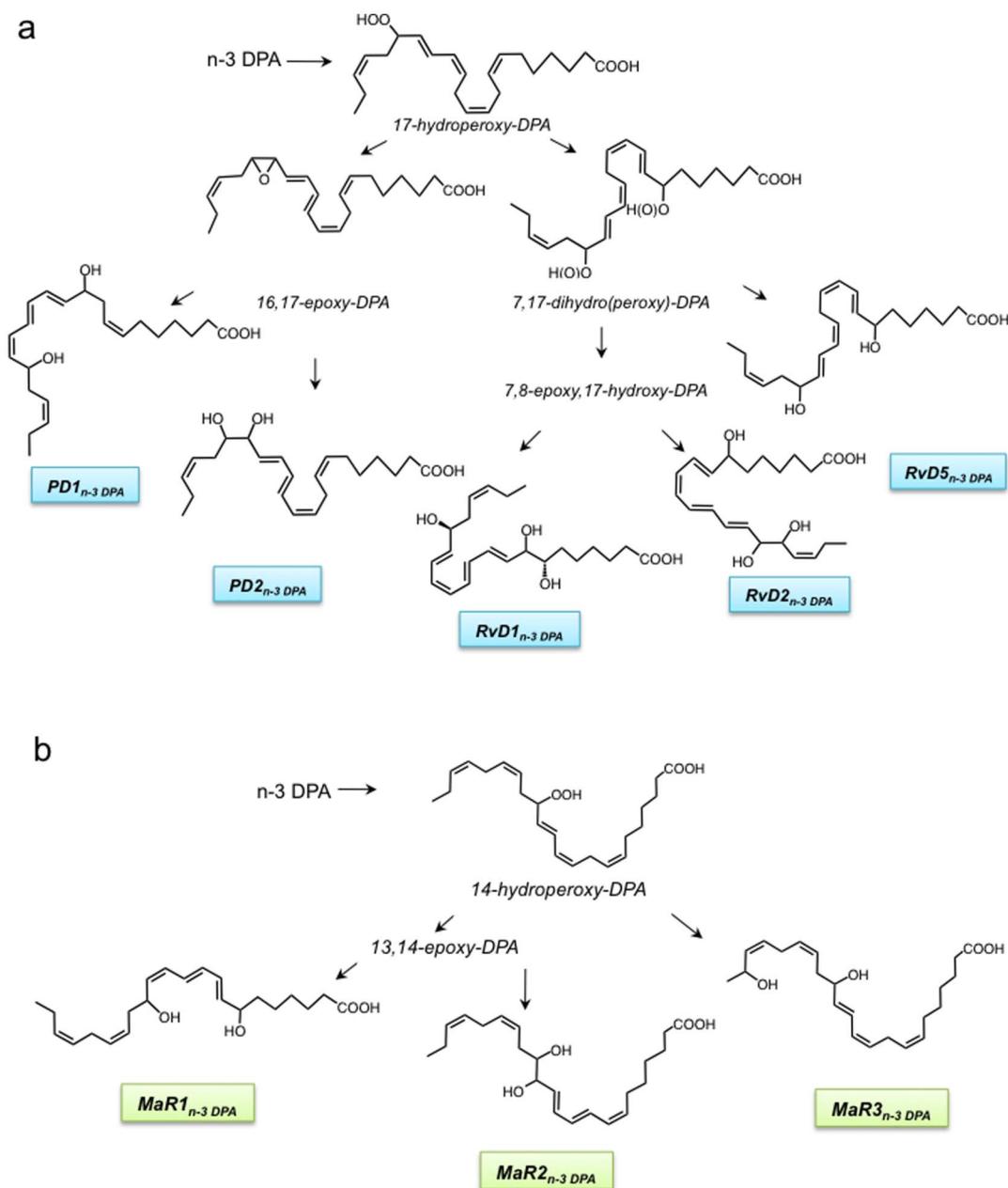
Figure 8 | Biosynthetic schemes proposed for novel n-3 docosapentaenoic acid products and their actions. At the site of injury, n-3 DPA is converted to (a) 17-HpDPA that undergoes further conversion by lipoxygenation to the n-3 DPA resolvins. 17-HpDPA is also a substrate for enzymatic conversion to an epoxide intermediate that is next enzymatically hydrolyzed to the n-3 DPA protectin structures. (b) n-3DPA is also converted to 14-lipoxygenation to yield 14-HpDPA that is further converted to an epoxide intermediate and then enzymatically hydrolyzed to MaR1$_{n-3\,DPA}$ and/or MaR2$_{n-3\,DPA}$. 14-HpDPA can also undergo a second oxygenation at the omega −1 position to yield the MaR3$_{n-3\,DPA}$. Note that each product is depicted in the 17S and 14S configuration based on the results obtained from chiral lipidomics that indicated S as the predominate form of each but may also carry 17R as well as 14R chirality from lipoxygenase reactions as these lesser components (see Supplementary Fig. 2 and text for details). The complete stereochemistries of these novel mediators remain to be established and are depicted in their likely configuration based on biogenic synthesis (see Supplementary Figures 3–5 for retention times, UV and MS-MS for each of these mediators).

In light of the results from the present studies in conjunction with earlier mechanisms proposed for the biosynthesis of the D-series resolvins, protectins[15,34] and maresins[35], the pathways for the novel n-3 DPA immunoresolvent are illustrated in Fig. 8. In this proposed scheme, n-3 DPA is first converted via 17 lipoxygenation to 17-hydroperoxy-8Z,10Z,13Z,15E,19Z-docosapentaenoic

acid (17-HpDPA). This intermediate can next undergo a second lipoxygenation by 5-lipoxygenase-like reaction to yield the 7,8, 17-trihydroxy-9,11,13,15E,19Z-docosapentaenoic acid (RvD1$_{n-3DPA}$), 7, 16, 17-trihydroxy-8,10,12,14E,19Z-docosapentaenoic acid (RvD2$_{n-3DPA}$) and/or 7,17-trihydroxy-8E,10,13,15E,19Z-docosapentaenoic acid (RvD5$_{n-3DPA}$). In addition, 17-HpDPA can undergo

enzymatic conversion to an epoxide intermediate that is next enzymatically hydrolyzed to either 10,17-dihydroxy-7Z,11,13,15,19Z-docosapentaenoic acid (PD1$_{n-3DPA}$) or 16,17-dihydroxy-7Z,10,13,14,19Z- docosapentaenoic acid (PD2$_{n-3DPA}$). In a parallel pathway, the arachidonate 12-lipoxygenase coverts n-3 DPA to 14-hydroperoxy-7Z,10 Z,12E,16Z,19Z-docosapentaenoic acid (14-HpDPA) that is further converted to an epoxide intermediate and then enzymatically hydrolyzed to yield 7,14-dihydroxy-8,10,12,16Z,19Z-docosapentaenoic acid (MaR1$_{n-3 DPA}$) or 13,14-dihydroxy-7Z,9,11,16Z,19Z-docosapentaenoic acid (MaR2$_{n-3 DPA}$). Alternatively, this 14-HpDPA can undergo a second oxygenation at the omega -1 position to yield 14, 21-dihydroxy-7Z,10Z,12E,16Z,19Z-docosapentaenoic acid (MaR3$_{n-3 DPA}$). Of note, since we identified the S isomer of both 17-HDPA and 14-HDPA, the reduction products of 17-HpDPA and 14-HpDPA, as the major products in inflamed tissues (Supplementary Fig. 2), it is highly likely that the stereochemistry at these positions is retained in the biosynthesis of the novel n-3 DPA-derived resolvins, protectins and maresins. Of note, it is conceivable that the R-containing diastereomers of the n-3 DPA resolvins, protectins and maresins may also be of biological relevance in inflammation-resolution, since they were also obtained via lipoxygenation reaction albeit to lesser proportions than their corresponding R-containing products (see Supplementary Fig. 2).

Excessive neutrophil activation and infiltration to the inflamed site can be detrimental since it may lead to further tissue damage and propagation of the inflammatory response[4,13]. When administered in vivo, RvD1$_{n-3 DPA}$, RvD2$_{n-3 DPA}$, RvD5$_{n-3 DPA}$, PD1$_{n-3 DPA}$, MaR1$_{n-3 DPA}$, and MaR2$_{n-3 DPA}$ each reduced neutrophil infiltration in murine peritonitis (Fig. 5). These mediators also demonstrated potent cytokine counter-regulatory actions reducing exudate levels of IL-6 and MCP-1 (Fig. 5) to levels comparable with the pro-resolving mediator RvD2[27]. The novel n-3 DPA immunoresolvents were also found to exert potent anti-inflammatory actions with human neutrophils and endothelial cells regulating central steps in the leukocyte recruitment cascades, reducing neutrophil chemotaxis and adhesion to endothelial cells (Fig. 7) as well as expression of the adhesion molecule ICAM-1 by endothelial cells (Supplementary Fig. 6). Importantly, these mediators were found to enhance macrophage phagocytosis, a key process in accelerating the onset or resolution (Fig. 7)[1,4,37]. Thus, the actions for each of these novel n-3 DPA products are in accord with the key characteristic that define an immunoresolvent, a property shared with their DHA resolution metabolome SPM counterparts.

Since mammals lack enzymes that can insert double bonds in either the n-3 or n-6 position to polyunsaturated fatty acids, the precursor molecules for the production of n-3 and n-6 essential fatty acids, namely alpha linolenic acid (ALA) and linoleic acid (9Z,12Z-octadecadienoic acid), must be obtained via dietary intake[12]. In mammalian tissues, ALA is converted by elongation and desaturation to EPA and DHA. Linoleic acid on the other hand is converted, in a parallel pathway in humans, to arachidonic acid and subsequently to n-6 DPA[10,12]. Thus, while n-3 DPA and n-6 DPA each share the carbon 22 and five unsaturated double bonds, their structures are different because they are produced via separate biosynthetic pathways from chemically dissimilar precursors and each possesses distinct biophysical properties[22]. Earlier, oxygenated products obtained from n-6 DPA were found to reduce neutrophil recruitment in a model of dermal inflammation[38], enhance macrophage phagocytosis[39], reduce ear swelling in a model of delayed type hypersensitivity[38] and protect against intestinal damage in a mouse model of intestinal inflammation[39], albeit displaying lower potency then the n-3 pro-resolving mediators[1] as well as compared to the new n-3 DPA mediators described herein. This is in accord with the finding that even subtle differences in the stereochemistry of lipid mediators can result in dramatic changes in their potency[34].

In summary, herein we established the structures of new n-3 DPA-derived products, their formation from endogenous sources, staged

the production of each of these novel mediators during-inflammation resolution and determined their anti-inflammatory, pro-resolving and tissue protective properties. These actions are characteristic of specialized pro-resolving mediators[37] and define these novel n-3 DPA products as immunoresolvents. In view of the role of lipid mediators in inflammation and its timely resolution[1,4,37], the n-3 DPA metabolome documented herein may mediate some of the beneficial actions associated with probiotic dietary supplementation[40]. Moreover, these new n-3 immunoresolvents may also serve as a compensatory mechanism in people with elevated n-3 DPA levels and lower circulating DHA levels[21] to compensate for losses in DHA SPM-regulated leukocyte-mediated tissue damage and timely resolution of acute inflammation.

Methods

Materials. Zymosan A, bovine serum albumin (BSA), Roswell Park Memorial Institute media 1640 (RPMI 1640), DPBS and Histopaque 1077-1 were purchased from Sigma-Aldrich. Rat anti-mouse Ly6G (clone 1A8; BD Biosciences); rat anti-mouse F4/80 (clone: BM8), CD11b (clone: Mac-1) and CD41 (clone: eBioMWReg30) were from eBioscience. Human recombinant granulocyte-monocyte colony stimulating factor (GM-CSF) and LC grade solvents were purchased from Fisher Scientific; Agilent Eclipse C18 (4.6 mm × 100 mm × 1.8 μm; 4.6 mm × 50 mm × 1.8 μm) column; C18 SPE columns (Biotage); fluorescently conjugated Zymosan A (Invitrogen); LC-MS-MS quantification and deuterated internal standards (d$_8$-5S-hydroxyeicosatetraenoic acid (d$_8$-5S-HETE), d$_4$-LTB$_4$, d$_5$-lipoxin A$_4$ (d$_5$-LXA$_4$), d$_4$-PGE$_2$, RvD1, RvD2; Cayman Chemicals).

Animals. All animals used in the present study were male FVB mice (Charles River Laboratories) that were 6–8 weeks old (weighing 20–25 g). They were maintained in a temperature-and light-controlled environment and had unlimited access to water and food (laboratory standard rodent diet 5001 (Lab Diet)), containing 1.5% eicosapentaenoic acid, 1.9% DHA of total fatty acids. Experiments were performed in accordance with the Harvard Medical School Standing Committee on Animals guidelines for animal care (Protocol 02570).

n-3 DPA-derived mediator preparations. Exudates were obtained from mice 12 h after zymosan administration and incubated with n-3 DPA in DPBS (1 μM, 45 min, 37°C, pH 7.45); the incubations were stopped using 2 volumes of ice-cold methanol and products extracted as outlined in the *sample extraction and lipid mediator metabololipidomics* section below.

14S-HpDPA was prepared from n-3 DPA (~15 μM) incubated with 5.4 U/ml isolated 12-lipoxygenase (LOX) (porcine) (0.05 M phosphate buffer, 0.02% Tween 20, pH 7.4). 14S-HpDHA was isolated via RP-HPLC (1100 Series; Agilent Technologies) using a C18 column and a mobile phase consisting of methanol/water (60 : 20, vol/vol) at 0.5 ml/min that was ramped up to 98 : 2 vol/vol over for 20 min. Reduction with NaBH$_4$ yielded 14S-HDPA used for mass spectrometry standard. In determined incubations 14S-HpDPA was incubated with human macrophages (40 × 10^6/ml) or neutrophils (80 × 10^6/ml) in PBS (containing calcium and magnesium) and serum treated zymosan (StZ, 0.1 mg/ml); the incubations were stopped after 45 min and products extracted.

17S-HpDPA was prepared from n-3 DPA (~15 μM) incubated with 100 U/ml isolated soybean-LOX (Borate buffer, 4°C, pH 9.2). 17S-HpDHA was isolated via RP-HPLC. Reduction with NaBH$_4$ yielded 17S-HDPA used for mass spectrometry standard. Biogenic synthesis of the di- and tri- dioxygenation products was performed with 5-LOX enzyme (200 U/ml) incubated with 17S-HpDPA. In determined incubations, 17Hp-DPA was added to human macrophages (40 × 10^6/ml) or neutrophils (80 × 10^6/ml) in PBS (containing calcium and magnesium) and cells were then incubated with StZ (0.1 mg/ml), incubations were stopped after 45 min and products extracted. These were scaled up for direct comparison of biological and physical properties with other novel compounds isolated from peripheral blood leukocytes or inflammatory exudates.

Ischemia reperfusion injury. Mice were anesthetized by intraperitoneal injection of a mixture of xylazine (80 mg/Kg) and ketamine (10 mg/Kg). Hind-limb ischemia was initiated using tourniquets consisting of a rubber band placed on each hind limb as in[9]. Ten min prior to the initiation of reperfusion n-3 DPA products (obtained as described above) or Resolvin D1 (0.5 ng) were administered by intravenous injection and compared to vehicle alone. At the end of this reperfusion period (2 h), mice were euthanized and blood collected via cardiac puncture, lungs harvested, frozen in liquid nitrogen and stored at −80°C or stored in 10% (v/v) buffered formalin and processed for histology by the Children's Hospital Boston Core Histology Facility. The frozen lungs were homogenized from individual mice, centrifuged and the tissue myeloperoxidase (MPO) levels were determined using a mouse MPO ELISA (R&D Systems.).

To investigate platelet leukocyte aggregates in murine whole blood after ischemia reperfusion, blood was collected 2 h post reperfusion by cardiac puncture and incubated with PE conjugated rat anti-mouse Ly6G and FITC conjugated rat anti-mouse CD41 or relevant isotype controls for 30 min at 4°C. Red blood cells were lysed

using ice-cold red-blood cell lysis buffer (BD Biosciences) and cells fixed with 1% formalin prior to analysis by a BD Canto II. Data was analyzed using FlowJo (TreeStar Inc.).

Sample extraction and lipid mediator metabololipidomics. All samples for LC-MS-MS analysis were extracted on SPE columns as in Ref. 41. Prior to extraction, 500 pg of deuterium-labeled internal standards d_8-5S-HETE, d_4-LTB$_4$, d_5LXA$_4$ and d_4PGE$_2$ were added to facilitate quantification of sample recovery.

The LC-MS-MS system, QTrap 5500 (ABSciex), was equipped with an Agilent HP1100 binary pump and diode-array detector (DAD). An Agilent Eclipse Plus C18 column (100 mm × 4.6 mm × 1.8 μm) was used with a gradient of methanol/water/acetic acid of 60:40:0.01 (v/v/v) to 100:0:0.01 at 0.4 ml/min flow rate. To monitor and quantify the levels of the various LM, a multiple reaction monitoring (MRM) method was developed with signature ion fragments for each molecule. Identification was conducted using published criteria[17] with at least six diagnostic ions. Calibration curves were obtained using synthetic LM mixture (d_8-5S-HETE, d_4LTB$_4$, d_5LXA$_4$, d_4PGE$_2$, TXB$_2$, PGD$_2$, PGF$_{2\alpha}$, RvD1, RvD2, RvD5, Protectin (PD)1, Maresin 1 (MaR1), 17-hydroxydocosahexaenoic acid (17-HDHA), 14-hydroxydocosahexaenoic acid (14-HDHA) and 7-hydroxydocosahexaenoic acid (7-HDHA) at 1, 10, 100, 275 pg. Linear calibration curves for each were obtained with r^2 values in the range 0.98–0.99. Quantification was carried out based on peak area of the Multiple Reaction Monitoring (MRM) transition and the linear calibration curve for each compound. Where calibration curves for a structurally related DHA-derived product were not available (14,21-diHDPA, 13,14-diHDPA and 16,17-diHDPA), levels were monitored using a compound with similar physical properties.

For chiral lipidomic analysis, a Chiralpak AD-RH column (150 mm × 2.1 mm × 5 μm) was used with isocratic methanol/water/acetic acid 95:5:0.01 (v/v/v) at 0.15 ml/min. To monitor isobaric monohydroxy docosapentaenoic acid levels, a multiple reaction monitoring (MRM) method was developed using signature ion fragments for each molecule.

Zymosan peritonitis. Zymosan (0.1 mg) was injected intraperitoneally (i.p.) in 1 ml of sterile saline. Exudates were collected at 0, 4, 12 and 24 h post zymosan. Leukocyte numbers and differential counts in the peritoneal exudates were determined as in[16]. In designated experiments, mice were administered intravenously (i.v): Vehicle (saline containing 0.1% EtOH), or the indicated mixture of n-3 DPA-derived products at 100 ng/mouse 5 min prior to i.p. zymosan administration (0.1 mg). After 4 h the exudates were collected and the number of extravasated neutrophils determined using Turks solution and flow cytometry as above.

Neutrophil isolation and chemotaxis. Peripheral blood neutrophils were obtained from healthy volunteers as in Ref. 17. Briefly, neutrophils were prepared following density separation by layering on Ficoll-Histopaque 1077-1. The cells were then centrifuged at 300 g (30 min, 4°C), and contaminating red blood cells were lysed by hypotonic lyses. These were then suspended at 1×10^6 cells/ml in DPBS containing 0.1% BSA and incubated with vehicle (0.1% EtOH in DPBS), RvD2 (1 nM) or the indicated mixtures of n-3 DPA-derived products (1 nM) for 15 min at 37°C. 2.5×10^4 cells were then added to the upper chamber of the ChemoTx System (3 μm pore size filter) and chemotaxis towards IL8 (100 ng/ml) was assessed (90 min, 37°C, 5% CO$_2$). The number of cells migrated into the bottom chamber was determined using Alama Blue following manufacturer's instructions on a Spectra Max M3 microplate reader (Molecular Devices Inc., Sunnyvale, CA).

Human neutrophil-endothelial cell adhesion. HUVEC were purchased from Lonza and cultured to passage 4. The cells were plated onto 96-well plates (Costar) coated with 1% gelatin and incubated overnight. The HUVEC were then incubated with TNF-α (10 ng/ml, 4 h, 37°C) in media containing 0.1% fetal bovine serum. Human peripheral blood neutrophils were isolated as described above and labeled with CFDA as in Ref. 17. These were then suspended in DPBS and incubated with vehicle (0.1% EtOH) or the indicated mixture of n-3 DPA products (1 nM, 37°C, pH 7.45). After 15 min the PMN (1×10^5) were added to the HUVEC and incubated for 60 min (37°C). The plates were then washed with DPBS to remove non-adherent cells and the number of adherent neutrophils assessed using a Spectra Max M3 microplate reader.

Endothelial cell adhesion molecule expression. HUVEC were plated onto 1% gelatin coated 12-well plates and incubated overnight. Cells were then incubated with vehicle (0.1% EtOH in DPBS) or the indicated mixture of n-3 DPA products for 15 min. The HUVEC were then incubated with TNF-α (10 ng/ml, 37°C) for 4 h. At the end of these incubations, ICAM-1 levels were assessed by flow cytometry following staining with fluorescently conjugated mouse anti-human ICAM-1 antibody (Clone HCD54; BioLegend) using the staining protocol[17].

Macrophage preparation and phagocytosis. Macrophages were prepared and phagocytosis was assessed as in Ref. 16. Briefly, cells were incubated with vehicle (0.1% EtOH in DPBS), RvD2 (1 nM) or the indicated n-3 DPA products (1 nM) for 15 min at 37°C, then FITC-labeled zymosan was added and cells incubated 60 min at 37°C. Phagocytosis was assessed using an M3 SpectraMax plate reader.

Statistics. All results are expressed as means ± SEM. Differences between groups were compared using Student's t test (2 groups) or 1-way ANOVA (multiple groups) followed by post hoc Bonferroni test. The criterion for statistical significance was $P < 0.05$.

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Author contributions

J.D. designed and carried out experiments, analyzed data, and contributed to manuscript and figure preparations; R.A.C. carried out experiments, analyzed data, and contributed to manuscript and figure preparations; C.N.S. carried out overall experimental design, conceived the research plan, and contributed to manuscript and figure preparations.

Additional information

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