

# Novel Proresolving Aspirin-Triggered DHA Pathway

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## SUMMARY

Endogenous mechanisms in the resolution of acute inflammation are of interest because excessive inflammation underlies many pathologic abnormalities. We report an aspirin-triggered DHA metabolome that biosynthesizes a potent product in inflammatory exudates and human leukocytes, namely aspirin-triggered Neuroprotectin D1/Protectin D1 [AT-(NPD1/PD1)]. The complete stereochemistry of AT-(NPD1/PD1) proved to be 10*R*,17*R*-dihydroxydocosa-4*Z*,7*Z*,11*E*,13*E*,15*Z*,19*Z*-hexaenoic acid. The chirality of hydroxyl groups and geometry of the conjugated triene system essential for bioactivity were established by matching biological materials with stereochemically pure isomers prepared by organic synthesis. AT-(NPD1/PD1) reduced neutrophil (PMN) recruitment in murine peritonitis in a dose-dependent fashion whereby neither a  $\Delta^{15}$ -trans-isomer nor DHA was effective. With human cells, AT-(NPD1/PD1) decreased transendothelial PMN migration as well as enhanced efferocytosis of apoptotic human PMN by macrophages. These results indicate that AT-(NPD1/PD1) is a potent anti-inflammatory proresolving molecule.

## INTRODUCTION

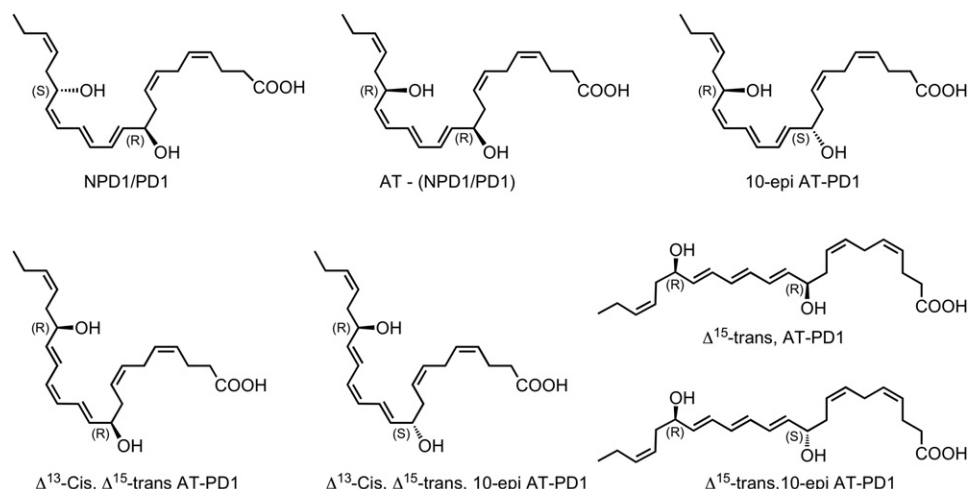
Neutrophils (PMN) are first to arrive in the acute inflammatory response and play an important protective role in innate immunity and host defense. However, excessive accumulation of PMN within tissues can lead to tissue damage, amplification of the inflammatory response, injury from within, and prolongation of the signs of inflammation (Majno et al., 1982). The control of neutrophil infiltration is of wide interest, as new anti-inflammatory agents are needed to control excess neutrophil responses that can give rise to chronic inflammatory diseases (Dinarello, 2010). Along these lines, evidence was sought for the endogenous mechanism(s) controlling PMN infiltration and natural tissue resolution, because protective PMN (i.e., acute inflammatory responses) are programmed to be self-limited and tightly controlled (Serhan et al., 2002; Serhan and Savill, 2005). Lipid mediators such as prostaglandins and leukotrienes play pivotal roles in the initiation of acute inflammation (Samuelsson, 1983),

whereas resolvins and protectins promote and stimulate active resolution (Bazan et al., 2010; Serhan et al., 2002; Serhan and Savill, 2005). In excess, prostaglandins and leukotrienes are proinflammatory (Samuelsson, 1983).

Unlike many of the current anti-inflammatory agents, which delay complete resolution and are considered toxic to this vital process (i.e., resolution toxic) (Gilroy et al., 1999; Schwab et al., 2007), aspirin is unique in that it jump-starts resolution by novel previously unrecognized mechanisms that involve the biosynthesis of aspirin-triggered (AT) lipid mediators (Gilroy and Perretti, 2005; Serhan, 2007). For example, aspirin-triggered lipoxins were the first aspirin-triggered lipid mediators uncovered (Clària and Serhan, 1995). Aspirin is well appreciated for its ability to inhibit COX-1 and inactivate this enzyme, blocking both prostaglandin and thromboxane production in cells that possess these biosynthetic pathways (Samuelsson, 1982; Vane, 1982). The mechanism of aspirin's action involves acetylation of COX within the enzyme's catalytic region. This prevents alignment of the substrate arachidonic acid for oxygenation within the catalytic center that produces the prostaglandin endoperoxide intermediate (PGG<sub>2</sub>), which is required for the biosynthesis of both thromboxanes and prostaglandins via COX-1.

Aspirin's action within cells that possess COX-2 is different. The catalytic region of COX-2 is larger than that of COX-1, and when it is acetylated by aspirin, the biosynthesis of endoperoxide is blocked. Yet, unlike COX-1, acetylated COX-2 remains active, producing lipoxygenase-like products such as 15-HETE from arachidonic acid, but with the oxygen insertion in the *R* configuration rather than *S* as is the case with lipoxygenases (Clària and Serhan, 1995; Rowlinson et al., 2000). The COX-2-produced 15*R*-HETE is converted to the potent bioactive aspirin-triggered lipoxins that retain the carbon 15 position alcohol in the *R* configuration, as in AT-15*R*-(*epi*)-lipoxin A<sub>4</sub>, which is produced in vivo in humans (Chiang et al., 2004). AT-LXA<sub>4</sub> is longer acting in vivo than LXA<sub>4</sub> because the 15*R* alcohol is less efficiently enzymatically converted to the inactive 15-oxo-LXA<sub>4</sub> metabolite (Serhan et al., 1993). This aspirin-triggered pathway was recently demonstrated in humans, where low-dose aspirin is anti-inflammatory and triggers the endogenous biosynthesis of AT-LXA<sub>4</sub>, which, in turn, stops PMN infiltration in skin blisters (Morris et al., 2009 and references within).

Docosahexaenoic acid (DHA), an omega-3 fatty acid, is enriched in neural tissues (Bazan, 2006; Lands, 2005; Salem et al., 2001) and is also converted to potent mediators, including resolvins and protectins, in resolving inflammatory exudates (Serhan et al., 2002). One of the protectins, 10,17-docosatriene,



**Figure 1. Synthetic Protectins Prepared for Lipidomics and Matching**

Neuroprotectin D1/protectin D1, aspirin-triggered neuroprotectin D1/protectin D1, and related isomers are shown.

is produced in murine ischemic stroke and is a potent regulator of PMN infiltration, reducing stroke-mediated tissue damage (Marcheselli et al., 2003; Serhan et al., 2002). Given its potent protective actions in the retina and brain, we initially termed this DHA-derived mediator neuroprotectin D1 (NPD1) (Bazan et al., 2010; Mukherjee et al., 2004; Stark and Bazan, 2011). Because this potent chemical mediator has a broader range of activities in the immune, cardiovascular, and renal systems, for nonneurological local biosynthesis and actions, we used the name protectin D1 (PD1). The complete stereochemistry and anti-inflammatory actions of NPD1/PD1 (10*R*,17*S*-dihydroxy-docosa-4*Z*,7*Z*,11*E*,13*E*,15*Z*,19*Z*-hexaenoic acid) were unambiguously established (Serhan et al., 2006), and its immunoregulatory roles were demonstrated (Ariel et al., 2005, 2006; Serhan and Savill, 2005). Of interest, recent results indicate that NPD1/PD1 is also renoprotective (Hassan and Gronert, 2009), induces corneal nerve regeneration (Cortina et al., 2010), and stimulates cardiac and neural stem cell differentiation at nanomolar potencies (Yanes et al., 2010).

Aspirin-triggered resolvins and related  $\omega$ -3-derived mediators were first identified in self-limited resolving murine exudates as well as brain, and their production was documented with isolated human cells (i.e., leukocytes, microglia, and vascular cells) (Serhan et al., 2002) and in stroke (Marcheselli et al., 2003). The complete stereochemistry and bioactions of aspirin-triggered 10,17-docosatriene remained to be established. Here, we report the complete stereochemistry of the aspirin-triggered protectin pathway from murine exudates and human PMN and report that AT-(NPD1/PD1) displays potent protective bioactions comparable to NPD1/PD1 in vitro and in vivo, reducing both PMN infiltration and enhancing the removal of apoptotic PMN by macrophages.

## RESULTS

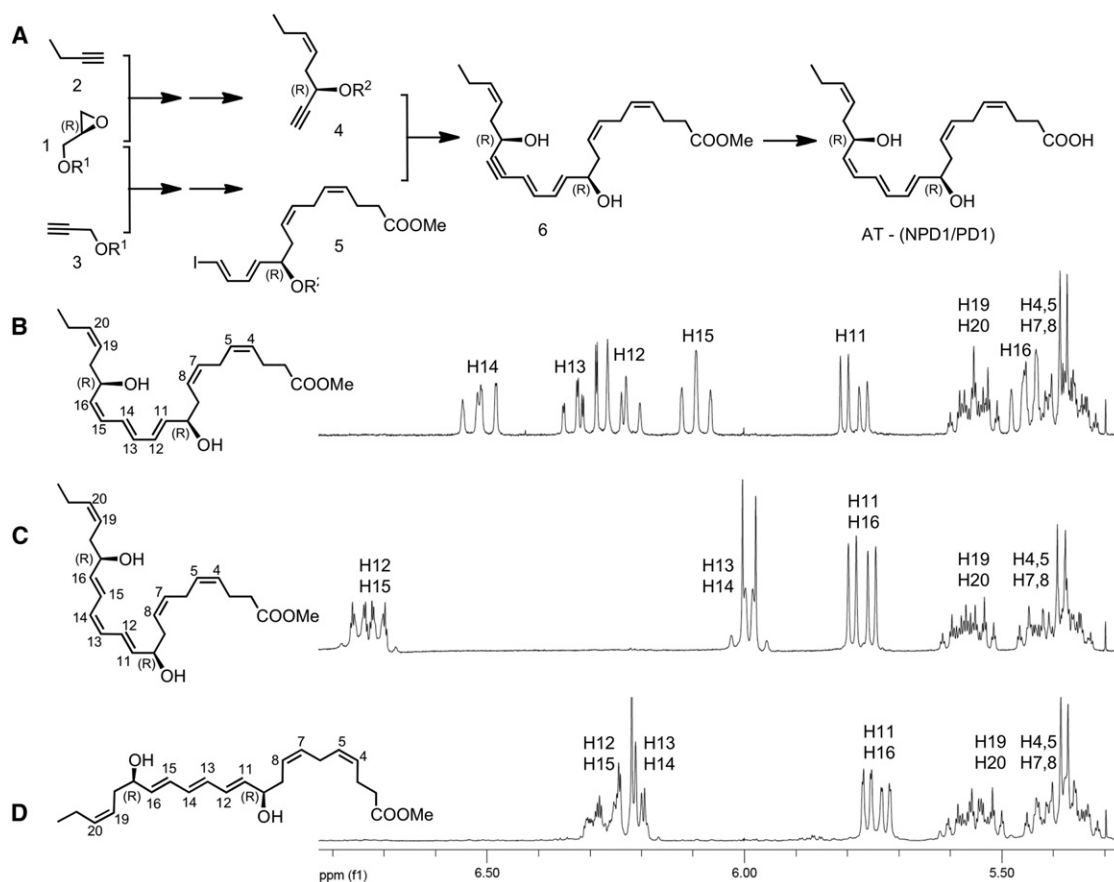
### Stereochemical Characterization of Synthetic AT-(NPD1/PD1) Isomers

The structure and stereochemistry of each of the synthetic AT-(NPD1/PD1) stereoisomers (Figure 1) was fully confirmed by

NMR analysis (Figure 2). Because the hydroxyl group chirality was directly incorporated from an enantiomerically pure starting material, the final R/S assignments at the C-10 and C-17 positions were made unambiguously (Figure 2A). The isomeric purity and double bond geometry for each stereoisomer was confirmed by NMR spectroscopy. The chemical shifts and coupling constants for each stereoisomer showed a distinct and characteristic profile in the NMR olefinic region in the NMR (Figures 2B–2D).

### Stereochemical Assignment of AT-(NPD1/PD1)

To determine the complete stereochemical assignment and bioactions of AT-(NPD1/PD1), we directly compared the physical and biological properties of DHA-derived AT-(NPD1/PD1) and related 10,17 dihydroxy-docosatriene stereoisomers produced by leukocytes to those prepared in stereochemically pure form by total organic synthesis. Figure 3A is a representative MRM analysis of 10*R*,17*R*-dihydroxydocosa-4*Z*,7*Z*,11*E*,13*E*,15*Z*,19*Z*-hexaenoic acid (denoted as synthetic AT-(NPD1/PD1)), 10*S*,17*R*-dihydroxydocosa-4*Z*,7*Z*,11*E*,13*E*,15*Z*,19*Z*-hexaenoic acid (denoted as 10*S*,17*R*-PD1 isomer), and NPD1/PD1. Synthetic AT-(NPD1/PD1) and NPD1/PD1 were separated in these liquid chromatography conditions (for details, see Experimental Procedures), with retention times at 8.2 and 12.0 min respectively, whereas the 10*S*,17*R*-PD1 isomer coeluted with NPD1/PD1. This 10*S*,17*R*-PD1 isomer was found to be biologically inactive (Serhan et al., 2006) and did not coelute with the aspirin-triggered product; therefore, we next focused on determining the complete stereochemistry of AT-(NPD1/PD1). As expected, spectroscopic properties of synthetic AT-(NPD1/PD1),  $\Delta^{15}$ -trans-AT-(NPD1/PD1), and NPD1/PD1 are essentially identical (Figures 3B–3D, respectively). The UV spectra of synthetic AT-(NPD1/PD1),  $\Delta^{15}$ -trans-AT-(NPD1/PD1), and NPD1/PD1 displayed essentially the same triplet bands of absorbance with  $\lambda_{\text{max}}^{\text{MeOH}}$  at  $\sim 271$  nm. Also, tandem mass spectra of these three compounds shared essentially the same fragmentation patterns. The major ions of three tandem mass spectra were assigned as following:  $m/z$  341, 323, 315, 297, and 279 were neutral loss



**Figure 2. Strategy for the Total Organic Synthesis of Isomerically Pure Protectin Isomers and Their Stereochemical Assignment by NMR Spectroscopy**

(A) The total synthesis of AT-(NPD1/PD1) started with the epoxide opening of the R-glycidol derivative 1 by metallated derivatives of alkynes 2 and 3, leading after several steps to the synthesis of key intermediates 4 and 5, which were subjected to cross-coupling to form the acetylenic precursor 6, which after selective hydrogenation and ester hydrolysis afforded AT-(NPD1/PD1).

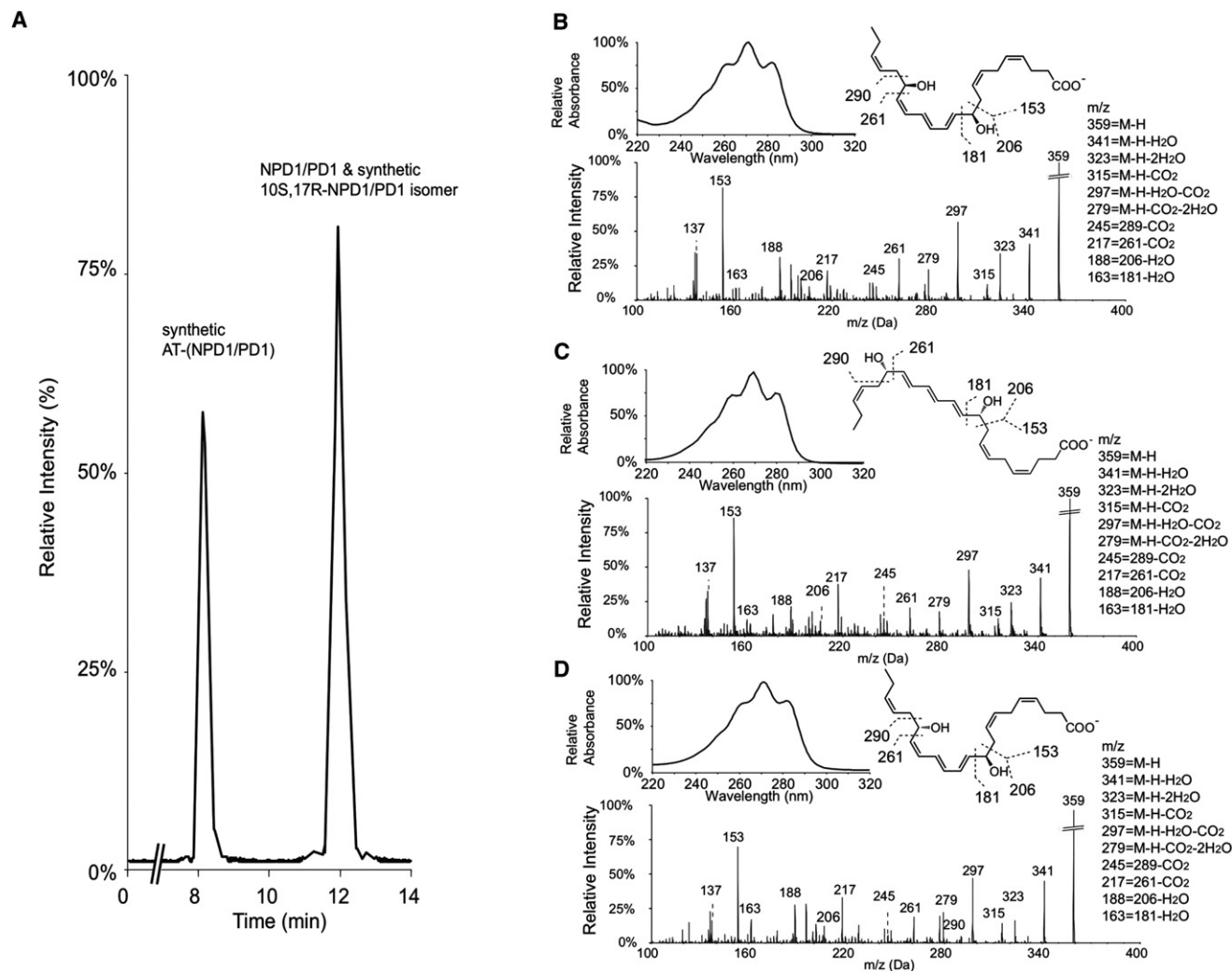
(B–D) The NMR assignments of the olefinic region of three AT-(NPD1/PD1) stereoisomers show the anticipated chemical shifts and coupling constants for each isomer.

ions, which were consistent with two hydroxyl groups and one carboxyl group of each compound (see figure insets);  $m/z$  153, 181, 206, and their corresponding neutral loss ions,  $m/z$  163 and 188, originated from fragmentations around C-10; and the cleavages between C-16 and C-17 generated  $m/z$  261 and its neutral loss  $m/z$  217. These results are consistent with earlier findings (Hong et al., 2003; Serhan et al., 2006) and also further confirmed the chemical integrity of synthetic AT-(NPD1/PD1). Together, synthetic AT-(NPD1/PD1) and NPD1/PD1 were able to be discriminated using their reverse-phase liquid chromatography retention times, which were different (Figure 3A), whereas their UV and mass spectra were virtually indistinguishable, as was that of the biologically inactive AT-(NPD1/PD1) isomer using LC-UV-MS-MS retention times (*vide infra*).

#### Matching of Biologic AT-(NPD1/PD1) with Synthetic AT-(NPD1/PD1) Candidates

AT-(NPD1/PD1) was identified in resolving murine exudates treated with aspirin (Serhan et al., 2002). Hence, biologic AT-(NPD1/PD1) was obtained from resolving murine exudates *in vivo*.

Briefly, peritonitis was initiated via intraperitoneal (i.p.) administration of zymosan A (1 mg/mouse), and exudates were harvested 24 hr after injection (i.e., within the resolution phase; Bannenberg et al., 2005). Exudates were subject to solid phase extraction and were analyzed using LC-UV-MS-MS-based mediator lipidomics. Representative MRM ( $m/z$  359 > 153) is shown in Figure 4A. Among the candidates, synthetic AT-(NPD1/PD1) matched peak I with retention time at 8.2 min (Figure 4B), which was confirmed by coinjection (Figure 4C). Synthetic AT-(NPD1/PD1) gave a UV spectrum and tandem mass spectrum that was essentially identical to the material beneath Peak I (Figures 4D and 4E), which further confirmed the matching. AT-(NPD1/PD1) was also isolated and identified from activated human leukocytes, namely, aspirin-treated human PMN. A representative tandem mass spectrum of the human PMN aspirin-triggered product is shown in Figure 4F. Collectively, by matching biologic materials from both human and murine exudates AT-(NPD1/PD1) with synthetic candidates, these results established the complete stereochemistry of endogenous AT-(NPD1/PD1) as 10R,17R-dihydroxydocosa-4Z,7Z,10E,12E,14Z,19Z-hexaenoic acid.



**Figure 3. LC-MS-MS Lipidomics and Properties of Synthetic AT-(NPD1/PD1), NPD1/PD1, and Their Isomers**

(A) MRM chromatogram (m/z 359 > 153) of coinjection of synthetic AT-(NPD1/PD1), 10S,17R-AT-(NPD1/PD1) isomer, and NPD1/PD1 is shown. AT-(NPD1/PD1) and NPD1/PD1 separate in this LC system, with retention times of 8.2 and 12.0 min.

(B–D) Representative tandem mass and UV spectra of synthetic AT-(NPD1/PD1) (B),  $\Delta^{15}$ -trans-NPD1/PD1 (C), and NPD1/PD1 (D) are shown.

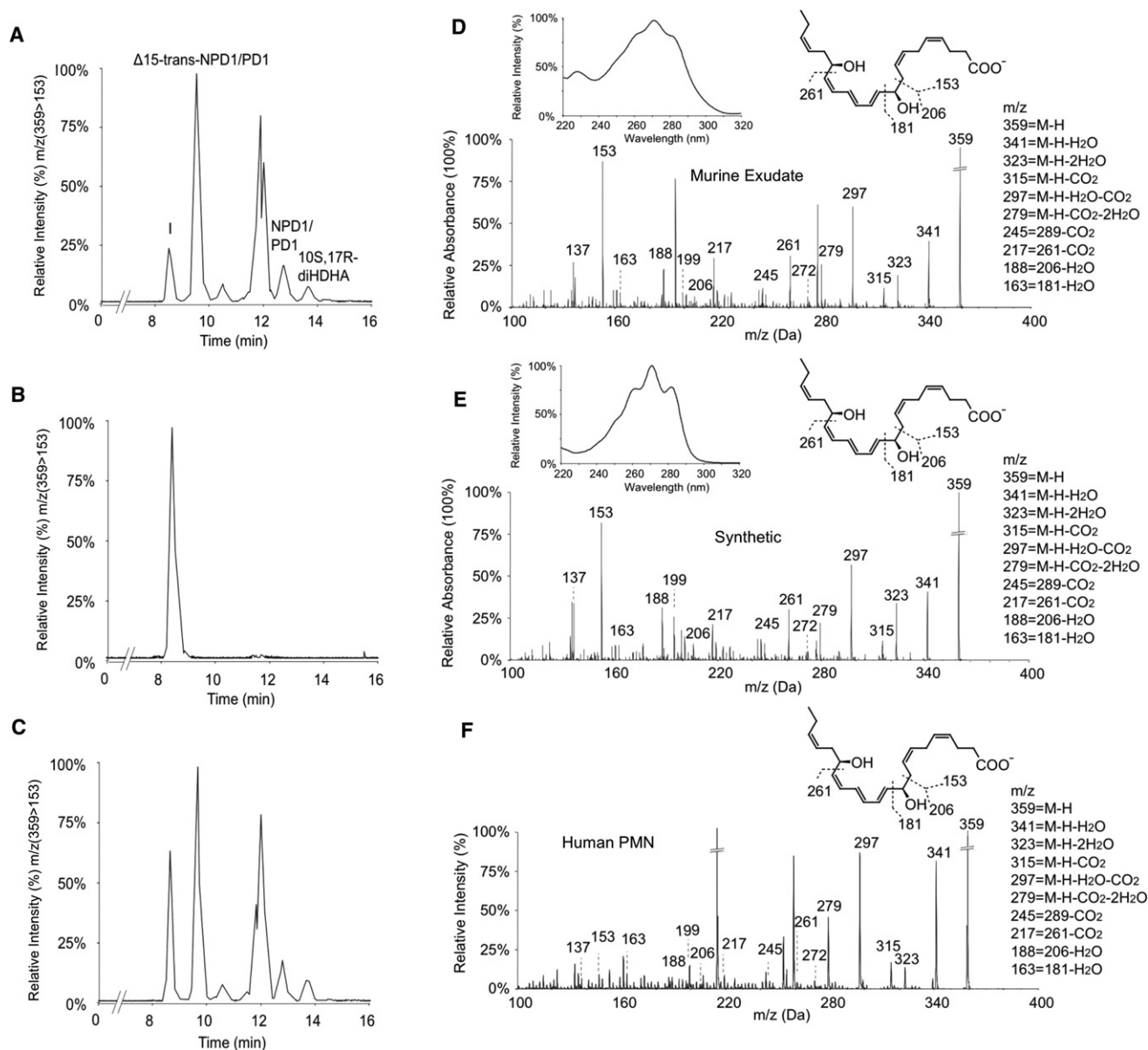
### AT-NPD1/PD1 Limits Neutrophil Infiltration In Vivo

The complete stereochemical assignment for AT-(NPD1/PD1) also relied on determining in parallel its biological actions. Earlier results indicated that NPD1/PD1 exerted potent anti-inflammatory actions regulating leukocyte trafficking in murine systems (Hong et al., 2003; Serhan et al., 2002, 2006). Hence, we sought to compare directly the bioactions of NPD1/PD1 to that of AT-(NPD1/PD1) performed in parallel with the physical matching experiments (Figures 3 and 4). Synthetic AT-(NPD1/PD1) limited PMN infiltration into the peritoneum in TNF- $\alpha$ -stimulated peritonitis (Figure 5). Both NPD1/PD1 (0.1–10 ng) and AT-(NPD1/PD1) (0.01–10.0 ng) proved to be significant regulators of TNF- $\alpha$ -stimulated leukocyte infiltration into the peritoneum. AT-(NPD1/PD1) reduced total leukocyte population of the exudate, including PMN infiltrates (Figures 5A and 5B), reaching a maximal reduction at 1 ng/mouse by as much as 50.4%  $\pm$  8.8%. The PMN population was also reduced (Figures

5C and 5D), with AT-(NPD1/PD1) reaching a maximal PMN reduction at 1 ng/mouse (62.2%  $\pm$  7.8%). Representative flow cytometry dot plots obtained with murine exudates indicated a reduction in the Ly6G<sup>+</sup>CD11b<sup>+</sup> population as compared to TNF- $\alpha$  alone (Figure 5C, inset). Of note, the  $\Delta^{15}$ -trans-AT-(NPD1/PD1) isomer did not reduce either the total exudate leukocyte population or PMN infiltration (Figures 5B and 5D, upper panels).

Recently, we found that NPD1/PD1 limited PMN infiltration in zymosan A-initiated peritonitis (Serhan et al., 2006). Therefore, we determined whether AT-(NPD1/PD1) also reduced PMN infiltration in zymosan A-stimulated peritonitis. Indeed, AT-(NPD1/PD1) (0.1–100.0 ng) significantly reduced total leukocyte (Figure 5E), as well as PMN infiltration (Figure 5F), reaching a maximal reduction of 47.8%  $\pm$  10.0% and 49.1%  $\pm$  11.9%, respectively. In comparison to AT-(NPD1/PD1) (1.0 and 10.0 ng), equal doses of either the precursor DHA or  $\Delta^{15}$ -trans-AT-NPD1/PD1 did not





**Figure 4. LC-MS-MS Matching: Comparisons for Biologic and Synthetic AT-(NPD1/PD1)**

(A) Graph shows representative MRM analysis ( $m/z$  359 > 153) of incubations of cells from zymosan A-induced (1 mg/mouse) peritonitis with exudate taken at 24 hr. Cells ( $50 \times 10^6$ /ml) were treated with aspirin (2 mM, 20 min, 37°C), and then incubated with DHA (5  $\mu$ g/ml) and A23187 (5  $\mu$ M) for 20 min at 37°C.

(B and C) Panels (B) (methyl formate fraction obtained from mouse peritonitis exudates) and (C) are representative MRM analyses ( $m/z$  375 > 153) of synthetic AT-(NPD1/PD1) and coinjection of synthetic AT-(NPD1/PD1).

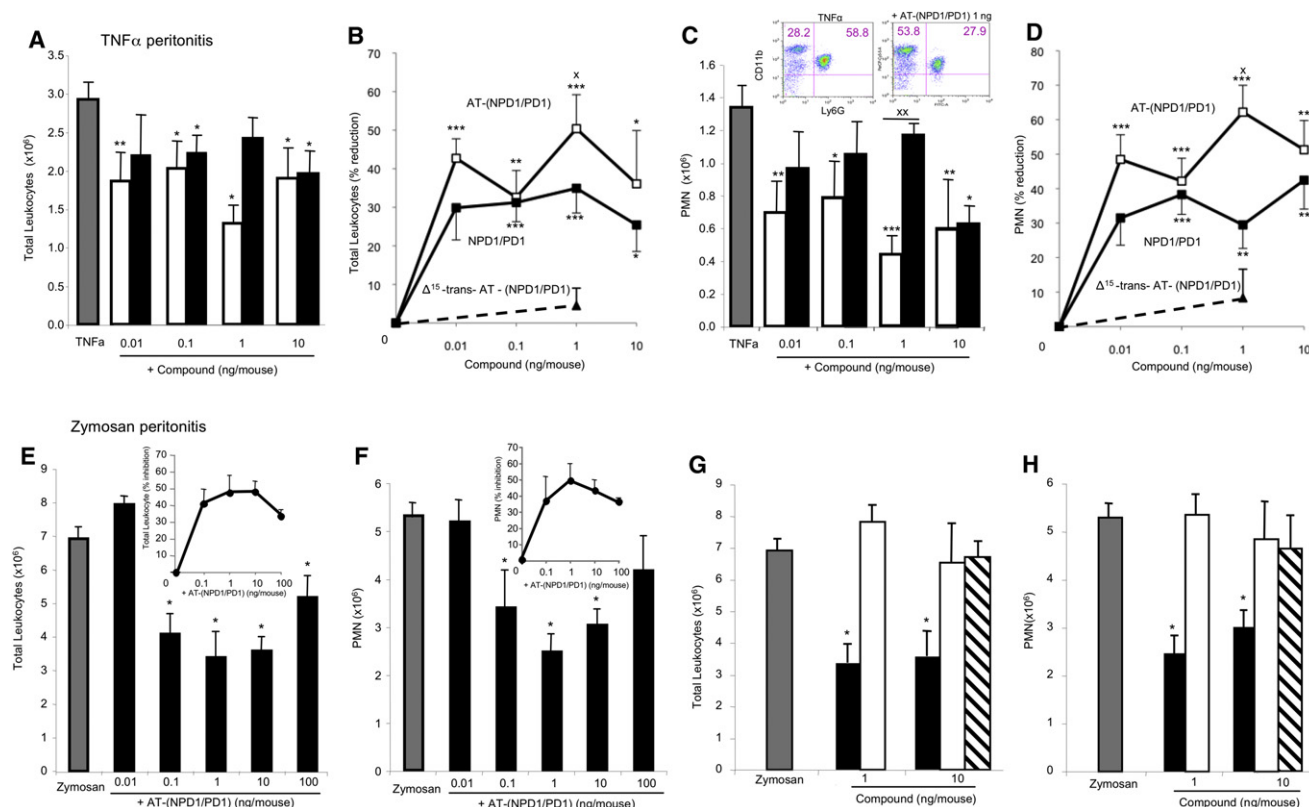
(D and E) Graphs are representative online UV (insets) and tandem mass spectra of exudate AT-(NPD1/PD1) from mouse peritonitis exudates (D) and synthetic AT-(NPD1/PD1) (E).

(F) Graph is representative tandem mass spectrum of AT-(NPD1/PD1) obtained from incubations of isolated human PMN. Human PMN ( $50 \times 10^6$ /ml) were incubated with TNF- $\alpha$  (100 ng/ml, 4 hr, 37°C) followed by aspirin (2 mM), DHA (5  $\mu$ g/ml), and A23187 (5  $\mu$ M) for 20 min at 37°C ( $n = 3$ ).

reduce either total leukocyte infiltration or PMN infiltration (Figures 5G and 5H). Together, these results indicate that AT-(NPD1/PD1) regulates inflammatory responses induced by the proinflammatory cytokine TNF- $\alpha$  and the TLR ligand, zymosan A. Moreover, these results indicate that the actions of AT-(NPD1/PD1) and NPD1/PD1 are stereospecific in limiting PMN accumulation in vivo in both murine types of inflammation.

#### AT-NPD1/PD1 Reduced Human PMN Transendothelial Migration and Enhanced Efferocytosis

Because this aspirin-triggered compound reduces PMN infiltration in murine systems, we next questioned whether it affects PMN transendothelial migration using isolated human cells because this is the first committed step of leukocytes in acute inflammation. AT-(NPD1/PD1) and NPD1/PD1 (0.1–10.0 nM)



**Figure 5. AT-(NPD1/PD1) Reduces Leukocyte Infiltration in Peritonitis: Direct Comparison to NPD1/PD1**

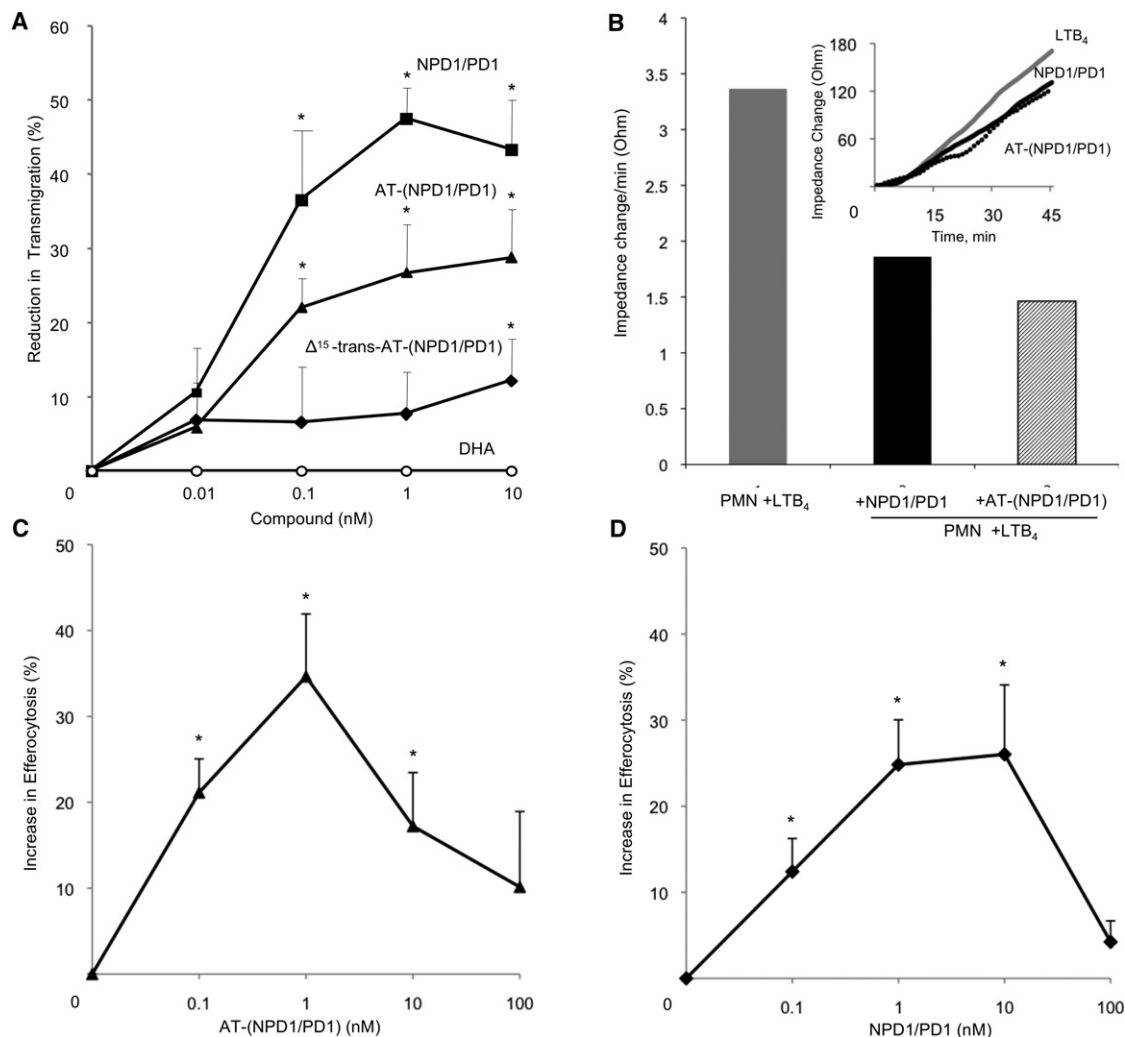
(A–D) Peritonitis was initiated by peritoneal injection of 500 ng of TNF- $\alpha$  alone, TNF- $\alpha$  plus synthetic AT-(NPD1/PD1) (0.01–10.0 ng), or NPD1/PD1 (0.01–10 ng). Peritoneal lavages were obtained at 4 hr and leukocytes were enumerated (see [Experimental Procedures](#)). Shown are total leukocyte numbers (A), dose-response: percentage reduction of total leukocytes (B), total PMN numbers (C), and dose-response: percentage reduction of PMN infiltration (D). Representative flow cytometry dot plots are shown (C, inset). TNF- $\alpha$ , gray bars; AT-(NPD1/PD1), black bars; NPD1/PD1, white bars. Values are mean  $\pm$  SEM of  $n = 7$ . (E–H) AT-(NPD1/PD1) dose response and direct comparison to DHA. Zymosan A alone (100  $\mu$ g/mouse), zymosan A plus AT-(NPD1/PD1) (0.01–100 ng/mouse), or zymosan A plus DHA (1 and 10 ng/mouse) were injected (i.p.) and lavages were obtained at 4 hr. Total exudate leukocyte (PMN, monocyte, lymphocyte) numbers (E and G), and total PMN (F and H) are shown. Insets in (E) and (F) show percentage reduction in total leukocytes and PMN. Zymosan A, gray bars; AT-(NPD1/PD1), black bars; DHA, white bars;  $\Delta^{15}$ -trans-AT-(NPD1/PD1), striped bars. All values are mean  $\pm$  SEM of  $n = 3$ . \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  versus vehicle;  $\chi^2 p < 0.05$ ,  $\chi^2 p < 0.01$  AT-(NPD1/PD1) versus NPD1/PD1. Using one-way and two-way ANOVA indicated that AT-NPD1 and NPD1/PD1 were not significantly different from each other ( $p > 0.05$ ).

significantly reduced ( $\sim 30\%$  and  $\sim 50\%$ , respectively) PMN transendothelial migration induced by LTB $_4$  (Figure 6A). In comparison, equal concentrations of the  $\Delta^{15}$ -trans isomer of AT-(NPD1/PD1), where the conjugated triene portion of the molecule was in the *trans* rather than *cis* configuration, did not significantly reduce PMN transendothelial migration. Again in this system, the precursor DHA did not reduce LTB $_4$ -stimulated PMN-transendothelial migration (Figure 6A). To corroborate these findings, we next used an electric cell-substrate impedance sensing system (ECIS) that sensitively quantitates cellular responses in two cell systems by real-time monitoring of barrier impedance (Tsikitis et al., 2004). Both AT-(NPD1/PD1) and NPD1/PD1 (1 nM) decreased LTB $_4$ -stimulated PMN-transendothelial migration by  $\sim 40\%$  and  $30\%$ , respectively (Figure 6B). AT-(NPD1/PD1) also enhanced the uptake of apoptotic human PMN by human macrophages at concentrations as low as 0.1 nM, as did NPD1/PD1 when compared directly (Figures 6C and 6D). The response was bell shaped and was consistent with the dose-response relationship observed for efferocytosis

and proresolving lipid mediators such as RvE1 (Hong et al., 2008).

## DISCUSSION

In this report, we present evidence for the formation, complete stereochemistry, and anti-inflammatory and proresolving actions of a novel aspirin-triggered DHA metabolome that produces AT-(NPD1/PD1). On the basis of physical and biological properties in matching results with material from human cells, murine exudates, and total organic syntheses, the complete stereochemistry of AT-(NPD1/PD1) proved to be 10*R*,17*R*-dihydroxydocosa-4*Z*,7*Z*,11*E*,13*E*,15*Z*,19*Z*-hexaenoic acid (Figures 1–4). The new aspirin-triggered product demonstrated potent regulatory actions with leukocytes in vivo (Figure 5) and reduced human PMN transendothelial migration and enhances efferocytosis (Figure 6). This matching approach was deemed necessary because typically less than nanogram amounts of a given lipid mediator are produced in vivo, and  $>100 \mu$ g of



**Figure 6. AT-(NPD1/PD1) Limits Human PMN Transmigration Across Human Endothelial Cells and Enhances Human Macrophage Efferocytosis of Apoptotic Human PMNs**

(A) Neutrophils ( $10^6$  cells/monolayer) were exposed to vehicle containing buffer or indicated concentrations of NPD1/PD1 (square), AT-(NPD1/PD1) (triangle),  $\Delta^{15}$ -trans-AT-NPD1/AT-PD1 (diamond), or DHA (circle) (15 min, pH 7.45, PBS<sup>-/-</sup>, 37°C). Transmigration was initiated with addition of  $10^{-8}$  M LTB<sub>4</sub> (90 min, 37°C). Results are mean  $\pm$  SEM obtained from 3–5 separate PMN donors, each point in triplicate.

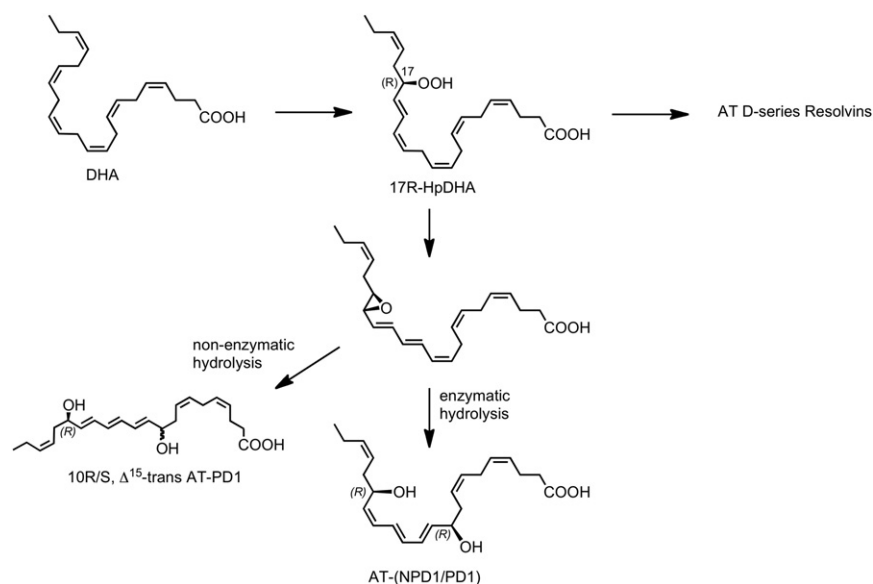
(B) HUVECs were plated ( $0.2 \times 10^6$  cells/well) in an ECIS chamber for 24 hr. After 24 hr, PMN were isolated from human donors and incubated with AT-(NPD1/PD1) or NPD1/PD1 (1 nM) for 15 min at 37°C. LTB<sub>4</sub> (10 nM) and PMN ( $10^6$  cells) were then added to the HUVECS. Impedance changes were monitored using Applied Biophysics ECIS software. Inset shows representative real-time tracing of impedance changes. Results are mean  $\pm$  SEM of  $n = 3$  separate donors and HUVEC preparations, \* $p < 0.05$ . Using a two-way ANOVA, NPD1/PD1 was not significantly different from AT-NPD1/PD1 in reducing PMN transendothelial migration ( $p > 0.05$ ).

(C and D) Human macrophages ( $10^5$  cells/well) were exposed to vehicle containing buffer or indicated concentrations of AT-(NPD1/PD1) (black triangles; C) or NPD1/PD1 (black squares; D) (15 min, pH 7.45, PBS<sup>-/-</sup>, 37°C). Uptake was initiated with addition of CFDA-labeled human apoptotic PMNs ( $3 \times 10^5$  cells/well, 60 min, 37°C). Increase in efferocytosis was determined by monitoring total fluorescence from PMNs; mean  $\pm$  SEM, \* $p < 0.05$  versus vehicle,  $n = 3$  healthy subjects.

isolated product is required for direct NMR determinations of conjugated double bonds that might give assignments for stereochemistry (Serhan et al., 2006; Spite et al., 2009; Sun et al., 2007).

Similar to the inversion of stereochemistry observed in the aspirin-triggered lipoxin and resolvins pathways, we anticipated that AT-(NPD1/PD1) was likely to possess the carbon 17R stereochemistry (Serhan et al., 2002), rather than the 17S chirality present in NPD1/PD1 produced via a lipoxygenase reaction

(Bazan et al., 2010). Aspirin acetylation of COX-2 gives rise to a catalytic activity that converts DHA to 17R-hydroxy-containing products (Serhan et al., 2002). It is noteworthy to point out that the chirality at the C-10 position and the double bond geometry of the triene system could not be reliably predicted on the basis of LC-MS-MS lipidomics alone. As proposed for other potent polyunsaturated fatty acid-derived local mediators, it is likely that the aspirin-triggered protectin pathway involves the conversion of the hydroperoxide 17R-HpDHA to an epoxide intermediate



**Figure 7. Proposed Biosynthesis Scheme for Aspirin-Triggered Protectins and AT-(NPD1/PD1)**

The stereochemistry and anti-inflammatory pro-resolving bioactions of the members of this pathway are determined (see text for details). The stereochemistry of the epoxide intermediate is shown in tentative configuration.

AT-(NPD1/PD1) differs from that of NPD1/PD1 only at carbon C-17, so the two are diastereomers. Results from earlier investigations have shown that the differences in the MS and UV spectra between lipid mediator diastereomers are minimal, if any (Clària and Serhan, 1995; Spite et al., 2009; Sun et al., 2007), which is consistent with the present results. For example, tandem mass spectra of the AT-17R-containing product and NPD1/PD1 were essentially identical, and both

(Figure 7). Given the significant conformational differences anticipated for these DHA-derived products, the stereochemical mode of the enzymatic epoxide opening and the resulting triene geometry needed to be verified by matching with isomers of known stereochemistry. In order to achieve the complete stereochemical assignments for the bioactive product(s), therefore, we prepared several geometric isomers of the 10,17R-dihydroxydocosa-4Z,7Z,11,13,15,19Z-hexaenoic acid, with specific changes in R/S and Z/E configurations, and confirmed their stereochemistry using NMR spectroscopy (Figures 1 and 2). These synthetic materials were also used for the direct matching and comparison with biologically produced AT-(NPD1/PD1).

This aspirin-triggered pathway was first identified in resolution phase inflammatory exudates and brain tissues of mice treated with aspirin, and its basic structure was proposed (e.g., 10,17R-dihydroxydocosa-4Z,7Z,11,13,15,19Z-hexaenoic acid) (Marcheselli et al., 2003; Serhan et al., 2002). To confirm this structural elucidation as well as to establish its complete stereochemistry, it is essential to determine its physical and spectroscopic properties and compare them with those of candidates with known stereochemistries using LC-UV-MS-MS (Yang et al., 2011). Among these, 10S,17R-dihydroxydocosa-4Z,7Z,11E,13E,15Z,19Z-hexaenoic acid (denoted as 10S,17R-NPD1/PD1 isomer in Figure 3) is the nonsuperimposable mirror image of NPD1/PD1 (10R,17S-dihydroxydocosa-4Z,7Z,11E,13E,15Z,19Z-hexaenoic acid) (Serhan et al., 2006). Therefore, the two are enantiomers, and as expected they display essentially identical chemical and physical properties in a symmetric environment (Carey and Sundberg, 2007). Indeed, 10S,17R-NPD1/PD1 isomer was indistinguishable from NPD1/PD1 in terms of chromatographic behavior (regular reverse phase liquid chromatography retention times), UV, and tandem mass spectrum patterns (Figure 3), yet proved biologically inactive (Serhan et al., 2006) and did not coelute with the authentic human- or mouse-derived AT material with LC-MS-MS (Figure 3A).

The present matching results indicate that, in this aspirin-triggered DHA metabolome, the stereochemical configuration of

compounds displayed essentially the same triplet-band of absorbance-shaped UV spectra with  $\lambda_{\text{max}}^{\text{MeOH}}$  at 271 nm (Figures 3B and 3C), characteristic of “ol-triene-ol” structures (Borgeat and Samuelsson, 1979; Serhan et al., 2006). Some diastereomers can possess different physical properties (Carey and Sundberg, 2007) by which they can be physically differentiated. Indeed, NPD1/PD1 and its aspirin-triggered isoform were well separated using reverse phase lipid chromatography conditions (Figure 3A). Diastereomers can give different biological properties, such as LTB<sub>4</sub> and its natural isomer 12-epi-6-trans-LTB<sub>4</sub>, which is log orders of magnitude less active (Borgeat and Samuelsson, 1979; Lewis et al., 1981). Along these lines, the aspirin-triggered diastereomer of NPD1/PD1 separated in chromatographic retention time directly compared to NPD1/PD1 but shared its potent biological action (Figures 5 and 6).

We confirmed the actions of this aspirin-triggered response with synthetic AT-(NPD1/PD1), which was potent, giving activity at picogram amounts per mouse (Figure 5). TNF- $\alpha$  is a well-known cytokine for its role in host defense; however, aberrant or uncontrolled TNF- $\alpha$  responses are associated with several inflammatory disorders (Dinarello, 2010). Although anti-TNF therapies are widely available and in clinical use, it is becoming increasingly clear that a complete blockade of TNF- $\alpha$  may promote adverse effects (Antoni and Braun, 2002), thus indicating limitations in this approach (Lin et al., 2008). Importantly, AT-(NPD1/PD1) is a potent regulator of PMN infiltration in vivo yet did not completely block PMN recruitment, and hence is not immunosuppressive. This is consistent with the unique counter-regulatory actions of resolvins and specialized proresolving mediators that do not compromise host defense via immune suppression of effector cell function (Spite et al., 2009). Of interest, with human PMN transmigration, 1–10 nM amounts of AT-(NPD1/PD1) reduced LTB<sub>4</sub>-stimulated PMN transmigration, and the  $\Delta^{15}$ -trans isomer of AT-(NPD1/PD1) was dramatically less potent, underscoring the stereoselectivity of AT-(NPD1/PD1) and the counterregulatory yet not immunosuppressive actions of proresolving mediators. Along these lines, NPD1/PD1



promotes corneal epithelial wound healing (Gronert et al., 2005) and regulates adiponectin (González-Pérez et al., 2009). Given the present results, these processes may also be amenable to AT-(NPD1/PD1) regulation.

In the biosynthesis of protectins, two endogenous forms of the pathway exist, namely the aspirin-dependent as documented here, and the lipoxygenase-initiated pathway for NPD1/PD1 (Bazan, 2006; Bazan et al., 2010; Hong et al., 2003; Mukherjee et al., 2004; Serhan et al., 2002). Hence, NPD1/PD1 joins the other specialized anti-inflammatory–proresolving mediators that are biosynthesized in both aspirin-dependent COX-2 routes (i.e., 17R dominant) as well as lipoxygenase-initiated (17S-dominant chirality) routes (Serhan, 2007). COX-2 is induced in human PMN (Pouliot et al., 1998), and, in the presence of aspirin, the acetylated enzyme converts DHA to a 17R-hydroperoxy-containing DHA-derived intermediate 17R-HpDHA identified as predominantly 17R-hydroxy-containing for its chirality at the carbon 17 position (Serhan et al., 2002). Next, this hydroperoxide intermediate of DHA is converted to both 17R-AT D-series resolvins and/or AT-(NPD1/PD1), as outlined in Figure 7. The biosynthesis of active mediators carrying potent selective actions involves conversion of the 17-hydroperoxy intermediate to a 16R,17R-epoxide that stereoselectively opens with an apparent enzymatic production of AT-(NPD1/PD1) with the E,Z,Z triene configuration (Figure 7). This double bond configuration proved critical for potent bioactivity, because the  $\Delta^{15}$ -trans-AT-(NPD1/PD1) isomer was essentially inactive.

Taken together, the results presented here confirm that the structure of AT-(NPD1/PD1) is 10R,17R-dihydroxy-docosa-4Z,7Z,11E,13E,15Z,19Z-hexaenoic acid. Notably, this assignment implies that the postulated enzyme-catalyzed opening of the 16R,17R epoxide precursor of AT-(NPD1/PD1) would proceed, in theory, via the introduction of the C-10 hydroxyl from the same side as the epoxide oxygen atom to give the 10R,17R configuration, likely from water. This addition would be opposite of the observed opening of the 16S,17S epoxide-containing intermediate precursor of NPD1/PD1 that leads to the 10R,17S configuration. This difference suggests that the C-10 hydroxyl group is introduced via an enzyme-catalyzed process and that the enzyme involved has a strong preference for the formation of the 10R isomer, rather than the stereochemical inversion of the epoxide precursor. These observations are consistent with the formation of a rigid cationic-type intermediate at the enzyme active site, which undergoes stereospecific attack by a water molecule from the same direction to afford the 10R product. Also, the well-known antarafacial hydrogen abstraction in LTA<sub>4</sub> biosynthesis in this case to produce the 16,17-epoxide-containing intermediate from DHA might be similar to the biosynthetic mechanism of LTA<sub>4</sub> from 5-HpETE as carried out by the 5-LOX (Shimizu et al., 1984; Wetterholm et al., 1995). It is possible in the present scheme (Figure 7) that the 17-hydroperoxide intermediate is converted to an epoxide at C16-C17 position that could give to *cis* or *trans* epoxides that require enzymatic processing in situ to give the potent active stereochemical structure of AT-(NPD1/PD1). It is presently not known whether 15-LOX, 12-LOX, or other lipoxygenases use the same reaction mechanism as 5-LOX to produce *cis*-epoxide-containing LTA<sub>4</sub>-like intermediates or whether unanticipated new enzymatic mechanisms are involved.

In humans, low-dose aspirin triggers 15-epi-lipoxin production, which reduces PMN infiltration following challenge (Morris et al., 2009) and is formed in healthy subjects (Chiang et al., 2004) as well as demonstrates reduced mortality from colorectal cancer in long-term studies with daily aspirin (Rothwell et al., 2010). Omega-3 EFA (EPA and DHA) at ~1 g/day reduce cardiovascular mortality (Skulas-Ray et al., 2011). Thus, the combination of aspirin with omega-3 EFA and the aspirin-triggered metabolome (i.e., AT-LX, AT-Rv) can have a beneficial therapeutic impact in many diseases associated with ongoing inflammation and host-mediated local tissue injury. Indeed, in a recent clinical study, the combination of both aspirin (81 mg/day) and omega-3 EPA and DHA significantly reduced periodontal disease in patients with chronic periodontitis (El-Sharkawy et al., 2010), presumably by jump-starting resolution of periodontal inflammation via production of aspirin-triggered mediators and inhibition of prostanoids (Van Dyke and Serhan, 2006). Hence, it is likely that the aspirin inhibition of prostanoids and concomitant biosynthesis of aspirin-triggered lipid mediators such as AT-lipoxins, AT-resolvins, and AT-protectins, including the new AT-(NPD1/PD1) structure established herein, may each contribute to some of these beneficial outcomes.

## SIGNIFICANCE

**The results of the present report establish the stereochemistry of AT-(NPD1/PD1) and several of its natural AT-isomers generated by human leukocytes and murine tissues during inflammation. Moreover, they demonstrate the potent stereoselective anti-inflammatory and proresolving actions of AT-(NPD1/PD1) in regulating PMN tissue accumulation and provide new avenues to mark the impact of DHA and its endogenous anti-inflammatory, proresolving mediators (Serhan, 2007) that can be evoked with aspirin treatment. Reducing PMN excess accumulation in tissues by these new strategies can both shorten the resolution interval of acute inflammation (Schwab et al., 2007; Serhan and Savill, 2005) and affect the transition from innate to adaptive immunity without compromising host defense.**

## EXPERIMENTAL PROCEDURES

### Materials

DHA was from Cayman Chemicals (Ann Arbor, MI). Calcium ionophore, A23187, and aspirin (ASA) were purchased from Sigma (St. Louis, MO). Zymosan A and 5-carboxyfluorescein diacetate, acetoxymethyl ester (CFDA) were from Invitrogen (Carlsbad, CA). Recombinant murine TNF- $\alpha$  was from Peprotech (Rocky Hill, NJ). PerCp-Cy5.5 anti-mouse CD11b (clone M1/70) and FITC-anti-mouse-Ly6G (clone RB6-8C5) were purchased from eBiosciences (San Diego, CA). Costar 5.0- $\mu$ m transwells were from Corning (Corning, NY). Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza Walkersville Inc. (Walkersville, MD).

### LC-MS-MS-Based Mediator Lipidomics

LC-UV-MS-MS-based mediator lipidomics was performed using linear ion trap quadrupole mass spectrometer (3200 QTRAP, Applied Biosystems, Foster City, CA) equipped with two HPLC pumps (LC20AD, Shimadzu, Columbia, MD) coupled to a reverse phase column (Lunar C18, 150 mm  $\times$  2 mm  $\times$  3  $\mu$ m, Phenomenex, Torrance, CA). The mobile phase consisted of methanol:water:acetic acid 70:30:0.01 (v/v/v) with flow rate at 0.2 ml/min; after 25 min, the mobile phase was ramped to 100:0:0.01 over next 5 min, and kept at 100:0:0.01 for 10 min to wash column. All intact cell incubations and in vivo

exudates were stopped with 2 volumes of cold methanol and kept at  $-20^{\circ}\text{C}$  for at least 30 min. Samples were extracted using C18 solid phase extraction (SPE) and further analyzed using LC-MS-MS. Detailed procedures and LC-MS-MS instrument settings as well as isolation, quantitation, and structural determination of AT-(NPD1/PD1) and related lipid-derived mediators used in the present experiments for elucidation of novel products were as reported elsewhere (Hong et al., 2003; Serhan et al., 2002, 2006).

#### Preparation of Biologic AT-(NPD1/PD1)

Biologic AT-(NPD1/PD1) was isolated using murine exudates from zymosan-initiated peritonitis as well as human PMN as described elsewhere (Serhan et al., 2006). Briefly, peritonitis was initiated by intraperitoneal (i.p.) administration of 1 mg of zymosan A in 1 ml of sterile saline using 6–8-week-old FVB male mice (Charles River Laboratories, Wilmington, MA) that were fed Lab Diet 5001. After 24 hr, mice were sacrificed, and peritoneal exudates were harvested. All animal experiments were performed in accordance with the Harvard Medical School Standing Committee on Animals guidelines for animal care (Protocol 02570). Exudate cells ( $50 \times 10^6$  cells/ml) were suspended in 1 ml of DPBS $^{+/-}$  and were treated with aspirin (2 mM, 20 min,  $37^{\circ}\text{C}$ ) followed by incubation with HPLC-purified DHA (5  $\mu\text{g}$ , 5 min,  $37^{\circ}\text{C}$ ), and were stimulated with A23187 (5  $\mu\text{M}$ , 20 min,  $37^{\circ}\text{C}$ ).

Human neutrophils (PMN) were isolated by dextran–Histopaque (1077) double gradient from whole blood from healthy volunteers (deidentified) who denied taking medications 2 weeks before donation (Partners Human Research Committee Protocol no. 88-02642) and were anticoagulated with heparin (1 U/ml) (Krishnamoorthy et al., 2010). Informed consent was obtained from all donors. To induce COX-2, PMN ( $50 \times 10^6$  cells/incubation) were stimulated with recombinant human TNF- $\alpha$  (10 ng/ml) for 4 hr at  $37^{\circ}\text{C}$  as described elsewhere (Pouliot et al., 1998). Afterward, aspirin (2 mM) was added (20 min,  $37^{\circ}\text{C}$ ) followed by A23187 (5  $\mu\text{M}$ ) and DHA (5  $\mu\text{g}/\text{ml}$ ) and incubated for 20 min at  $37^{\circ}\text{C}$ . Incubations were stopped with 2 volumes of cold methanol and were kept at  $-20^{\circ}\text{C}$  for at least 30 min as above.

#### Total Synthesis and Stereochemical Characterization of AT-(NPD1/PD1)

Several isomerically pure stereoisomers of NPD1/PD1 having either the 10R,17R or the 10S,17R stereochemistry were prepared by total synthesis, by utilizing enantiomerically pure starting materials and by employing highly stereocontrolled processes (see results in Figures 1 and 2). The hydroxyl group stereochemistry at both the C-10 and C-17 positions was secured from the corresponding protected glycidol derivative. The following isomers were synthesized: 10R,17R-dihydroxy-docosa-4Z,7Z,11E,13E,15Z,19Z-hexanenoic acid, 10R,17R-dihydroxy-docosa-4Z,7Z,11E,13Z,15E,19Z-hexanenoic acid, 10R,17R-dihydroxy-docosa-4Z,7Z,11E,13E,15E,19Z-hexanenoic acid, 10S,17R-dihydroxy-docosa-4Z,7Z,11E,13E,15Z,19Z-hexanenoic acid, 10S,17R-dihydroxy-docosa-4Z,7Z,11E,13Z,15E,19Z-hexanenoic acid, and (VI) 10S,17R-dihydroxy-docosa-4Z,7Z,11E,13E,15E,19Z-hexanenoic acid. The methyl ester of each isomer was fully characterized by high field NMR spectroscopy that unambiguously confirmed its double bond geometry.

#### Acute Inflammatory Exudates: Murine Peritonitis

Peritonitis was performed as described above. Briefly, the methyl ester of each new synthetic stereoisomer or DHA was administered via intraperitoneal injection (i.p.) with either zymosan A (100  $\mu\text{g}/\text{ml}$ ) or TNF- $\alpha$  (500 ng/ml) in 1 ml of saline. After 4 hr, the mice were sacrificed in accordance with the Harvard Medical Area Standing Committee on Animals (Protocol no. 02570). Peritoneal lavages were harvested immediately, cells were enumerated, cell viability was determined using Trypan blue exclusion, and differential cell counts were monitored by Wright-Giemsa staining and flow cytometry (BD FACS Canto). PMN were identified based on LY6G $^{+}$ CD11b $^{+}$  staining and analyzed via FlowJo Software.

#### Human Neutrophil Transmigration

HUVEC confluent monolayers were grown on polycarbonate membranes (5.0- $\mu\text{m}$  pore size, 0.1% gelatin) for 24 hr as described elsewhere (Gimbrone et al., 1974; Serhan et al., 2006).

PMN were isolated as described above and were fluorescently labeled with CFDA for 20 min at room temperature. Excess CFDA was removed (1000 rpm,

10 min, RT), and PMN were incubated with either vehicle-containing buffer or compound for 15 min at  $37^{\circ}\text{C}$ . A chemotactic gradient was established by placing the transwell inserts in media containing 10 nM LTB $_4$ . PMN ( $10^6$  cells) were added in the upper chambers, and transmigration was performed at  $37^{\circ}\text{C}$  for 90 min. Transmigrated PMN were quantified via fluorescence using a Victor3 plate reader.

#### Electric Cell-Substrate Impedance Sensing

Human PMN and human endothelial interactions were assessed using changes in electric cell-substrate impedance sensing (ECIS) 1600R system (Applied Biophysics). ECIS measures real-time cellular responses by monitoring impedance changes of cells placed atop specific electrodes (Tsikitis et al., 2004; Wang et al., 2010). HUVECs ( $\sim 0.2 \times 10^6$  cells/well) were cultured to confluence (24 hr) on an 8W10E+ electrode array. Next, fresh human PMN were isolated ( $10^6$  cells/well) following venipuncture and exposed to either vehicle containing buffer (EMB serum free media), AT-(NPD1/PD1) (1 nM), or NPD1/PD1 (1 nM) for 15 min at  $37^{\circ}\text{C}$ . PMN were added to each well and impedance baseline was set at 4 kHz. Cells were then exposed to LTB $_4$  (10 nM) and changes in impedance reflecting transendothelial PMN migration were monitored in real time via ECIS software (v1.2.35.0) for 45 min, at  $37^{\circ}\text{C}$  in 0.5% CO $_2$ .

#### Efferocytosis: Uptake of Apoptotic PMN by Human Macrophages

Monocytes were isolated from human whole blood and cultured in RPMI with 10 ng/ml human GM-CSF (R&D Systems) at  $37^{\circ}\text{C}$  for 7 days (Geissler et al., 1989; Krishnamoorthy et al., 2010). For efferocytosis of apoptotic PMNs, isolated human PMNs were allowed to undergo apoptosis in RPMI ( $2 \times 10^6$  cells/ml) for 16–18 hr then labeled with carboxyfluorescein diacetate (10  $\mu\text{M}$ , 30 min at  $37^{\circ}\text{C}$ ; Molecular Probes). Macrophages ( $1.0 \times 10^5$  cells/well) were incubated with either AT-(NPD1/PD1), NPD1/PD1 or vehicle (15 min at  $37^{\circ}\text{C}$ ). Apoptotic PMNs were added at 1:3 (Macrophage:PMN), and incubations carried out at  $37^{\circ}\text{C}$  for 60 min. Supernatants were aspirated and trypan blue (0.03% in DPBS $^{+/-}$  for  $\sim 60$  s) was added to quench extracellular fluorescence. Fluorescence was measured using a M3 SpectraMax plate reader (Molecular Devices).

#### Statistical Analysis

The significance of differences between groups was evaluated using the two-tailed Student's *t* test. In addition, one-way and two-way ANOVA were carried out for evaluating significance of difference among more than two groups and for dose-response curves; *p* values of less than 0.05 were considered statistically significant.

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#### REFERENCES

- Antoni, C., and Braun, J. (2002). Side effects of anti-TNF therapy: current knowledge. *Clin. Exp. Rheumatol.* 20 (6, Suppl 28), S152–S157.
- Ariel, A., Li, P.-L., Wang, W., Tang, W.-X., Fredman, G., Hong, S., Gotlinger, K.H., and Serhan, C.N. (2005). The docosatriene protectin D1 is produced

- by  $T_H2$  skewing and promotes human T cell apoptosis via lipid raft clustering. *J. Biol. Chem.* **280**, 43079–43086.
- Ariel, A., Fredman, G., Sun, Y.-P., Kantarci, A., Van Dyke, T.E., Luster, A.D., and Serhan, C.N. (2006). Apoptotic neutrophils and T cells sequester chemokines during immune response resolution through modulation of CCR5 expression. *Nat. Immunol.* **7**, 1209–1216.
- Bannenberg, G.L., Chiang, N., Ariel, A., Arita, M., Tjonahen, E., Gotlinger, K.H., Hong, S., and Serhan, C.N. (2005). Molecular circuits of resolution: formation and actions of resolvins and protectins. *J. Immunol.* **174**, 4345–4355.
- Bazan, N.G. (2006). Cell survival matters: docosahexaenoic acid signaling, neuroprotection and photoreceptors. *Trends Neurosci.* **29**, 263–271.
- Bazan, N.G., Calandria, J.M., and Serhan, C.N. (2010). Rescue and repair during photoreceptor cell renewal mediated by docosahexaenoic acid-derived neuroprotectin D1. *J. Lipid Res.* **51**, 2018–2031.
- Borgeat, P., and Samuelsson, B. (1979). Arachidonic acid metabolism in polymorphonuclear leukocytes: effects of ionophore A23187. *Proc. Natl. Acad. Sci. USA* **76**, 2148–2152.
- Carey, F.A., and Sundberg, R.J. (2007). *Advanced Organic Chemistry, Part A: Structure and Mechanisms* (New York: Springer Science).
- Chiang, N., Bermudez, E.A., Ridker, P.M., Hurwitz, S., and Serhan, C.N. (2004). Aspirin triggers antiinflammatory 15-*epi*-lipoxin  $A_4$  and inhibits thromboxane in a randomized human trial. *Proc. Natl. Acad. Sci. USA* **101**, 15178–15183.
- Clària, J., and Serhan, C.N. (1995). Aspirin triggers previously undescribed bioactive eicosanoids by human endothelial cell-leukocyte interactions. *Proc. Natl. Acad. Sci. USA* **92**, 9475–9479.
- Cortina, M.S., He, J., Li, N., Bazan, N.G., and Bazan, H.E. (2010). Neuroprotectin D1 synthesis and corneal nerve regeneration after experimental surgery and treatment with PEDF plus DHA. *Invest. Ophthalmol. Vis. Sci.* **51**, 804–810.
- Dinarello, C.A. (2010). Anti-inflammatory agents: present and future. *Cell* **140**, 935–950.
- El-Sharkawy, H., Aboelsaad, N., Eliwa, M., Darweesh, M., Alshahat, M., Kantarci, A., Hasturk, H., and Van Dyke, T.E. (2010). Adjunctive treatment of chronic periodontitis with daily dietary supplementation with omega-3 fatty acids and low-dose aspirin. *J. Periodontol.* **81**, 1635–1643.
- Geissler, K., Harrington, M., Srivastava, C., Leemhuis, T., Tricot, G., and Broxmeyer, H.E. (1989). Effects of recombinant human colony stimulating factors (CSF) (granulocyte-macrophage CSF, granulocyte CSF, and CSF-1) on human monocyte/macrophage differentiation. *J. Immunol.* **143**, 140–146.
- Gilroy, D.W., and Perretti, M. (2005). Aspirin and steroids: new mechanistic findings and avenues for drug discovery. *Curr. Opin. Pharmacol.* **5**, 405–411.
- Gilroy, D.W., Colville-Nash, P.R., Willis, D., Chivers, J., Paul-Clark, M.J., and Willoughby, D.A. (1999). Inducible cyclooxygenase may have anti-inflammatory properties. *Nat. Med.* **5**, 698–701.
- Gimbrone, M.A., Jr., Cotran, R.S., and Folkman, J. (1974). Human vascular endothelial cells in culture: growth and DNA synthesis. *J. Cell Biol.* **60**, 673–684.
- González-Pérez, A., Horrillo, R., Ferré, N., Gronert, K., Dong, B., Morán-Salvador, E., Titos, E., Martínez-Clemente, M., López-Parra, M., Arroyo, V., and Clària, J. (2009). Obesity-induced insulin resistance and hepatic steatosis are alleviated by omega-3 fatty acids: a role for resolvins and protectins. *FASEB J.* **23**, 1946–1957.
- Gronert, K., Maheshwari, N., Khan, N., Hassan, I.R., Dunn, M., and Laniado Schwartzman, M. (2005). A role for the mouse 12/15-lipoxygenase pathway in promoting epithelial wound healing and host defense. *J. Biol. Chem.* **280**, 15267–15278.
- Hassan, I.R., and Gronert, K. (2009). Acute changes in dietary omega-3 and omega-6 polyunsaturated fatty acids have a pronounced impact on survival following ischemic renal injury and formation of renoprotective docosahexaenoic acid-derived protectin D1. *J. Immunol.* **182**, 3223–3232.
- Hong, S., Gronert, K., Devchand, P.R., Moussignac, R.-L., and Serhan, C.N. (2003). Novel docosatrienes and 17S-resolvins generated from docosahexaenoic acid in murine brain, human blood, and glial cells: autacoids in anti-inflammation. *J. Biol. Chem.* **278**, 14677–14687.
- Hong, S., Porter, T.F., Lu, Y., Oh, S.F., Pillai, P.S., and Serhan, C.N. (2008). Resolvin E1 metabolome in local inactivation during inflammation-resolution. *J. Immunol.* **180**, 3512–3519.
- Krishnamoorthy, S., Recchiuti, A., Chiang, N., Yacoubian, S., Lee, C.-H., Yang, R., Petasis, N.A., and Serhan, C.N. (2010). Resolvin D1 binds human phagocytes with evidence for proresolving receptors. *Proc. Natl. Acad. Sci. USA* **107**, 1660–1665.
- Lands, W.E.M. (2005). *Fish, Omega-3 and Human Health*, Second Edition (Champaign, IL: AOCS Press).
- Lewis, R.A., Goetzl, E.J., Drazen, J.M., Soter, N.A., Austen, K.F., and Corey, E.J. (1981). Functional characterization of synthetic leukotriene B and its stereochemical isomers. *J. Exp. Med.* **154**, 1243–1248.
- Lin, J., Ziring, D., Desai, S., Kim, S., Wong, M., Korin, Y., Braun, J., Reed, E., Gjertson, D., and Singh, R.R. (2008). TNF $\alpha$  blockade in human diseases: an overview of efficacy and safety. *Clin. Immunol.* **126**, 13–30.
- Majno, G., Cotran, R.S., and Kaufman, N., eds. (1982). *Current Topics in Inflammation and Infection* (Baltimore: Williams & Wilkins).
- Marcheselli, V.L., Hong, S., Lukiw, W.J., Tian, X.H., Gronert, K., Musto, A., Hardy, M., Gimenez, J.M., Chiang, N., Serhan, C.N., and Bazan, N.G. (2003). Novel docosanoids inhibit brain ischemia-reperfusion-mediated leukocyte infiltration and pro-inflammatory gene expression. *J. Biol. Chem.* **278**, 43807–43817.
- Morris, T., Stables, M., Hobbs, A., de Souza, P., Colville-Nash, P., Warner, T., Newson, J., Bellingan, G., and Gilroy, D.W. (2009). Effects of low-dose aspirin on acute inflammatory responses in humans. *J. Immunol.* **183**, 2089–2096.
- Mukherjee, P.K., Marcheselli, V.L., Serhan, C.N., and Bazan, N.G. (2004). Neuroprotectin D1: a docosahexaenoic acid-derived docosatriene protects human retinal pigment epithelial cells from oxidative stress. *Proc. Natl. Acad. Sci. USA* **101**, 8491–8496.
- Pouliot, M., Gilbert, C., Borgeat, P., Poubelle, P.E., Bourgoin, S., Crémion, C., Maclouf, J., McColl, S.R., and Naccache, P.H. (1998). Expression and activity of prostaglandin endoperoxide synthase-2 in agonist-activated human neutrophils. *FASEB J.* **12**, 1109–1123.
- Rothwell, P.M., Wilson, M., Elwin, C.E., Norving, B., Algra, A., Warlow, C.P., and Meade, T.W. (2010). Long-term effect of aspirin on colorectal cancer incidence and mortality: 20-year follow-up of five randomised trials. *Lancet* **376**, 1741–1750.
- Rowlinson, S.W., Crews, B.C., Goodwin, D.C., Schneider, C., Gierse, J.K., and Marnett, L.J. (2000). Spatial requirements for 15-(*R*)-hydroxy-5Z,8Z,11Z, 13E-eicosatetraenoic acid synthesis within the cyclooxygenase active site of murine COX-2: why acetylated COX-1 does not synthesize 15-(*R*)-hete. *J. Biol. Chem.* **275**, 6586–6591.
- Salem, N., Jr., Litman, B., Kim, H.-Y., and Gawrisch, K. (2001). Mechanisms of action of docosahexaenoic acid in the nervous system. *Lipids* **36**, 945–959.
- Samuelsson, B. (1982). From studies of biochemical mechanisms to novel biological mediators: prostaglandin endoperoxides, thromboxanes and leukotrienes. In *Les Prix Nobel: Nobel Prizes, Presentations, Biographies and Lectures* (Stockholm: Almqvist & Wiksell), pp. 153–174.
- Samuelsson, B. (1983). Leukotrienes: mediators of immediate hypersensitivity reactions and inflammation. *Science* **220**, 568–575.
- Schwab, J.M., Chiang, N., Arita, M., and Serhan, C.N. (2007). Resolvin E1 and protectin D1 activate inflammation-resolution programmes. *Nature* **447**, 869–874.
- Serhan, C.N. (2007). Resolution phase of inflammation: novel endogenous anti-inflammatory and proresolving lipid mediators and pathways. *Annu. Rev. Immunol.* **25**, 101–137.
- Serhan, C.N., and Savill, J. (2005). Resolution of inflammation: the beginning programs the end. *Nat. Immunol.* **6**, 1191–1197.
- Serhan, C.N., Fiore, S., Brezinski, D.A., and Lynch, S. (1993). Lipoxin  $A_4$  metabolism by differentiated HL-60 cells and human monocytes: conversion to novel 15-oxo and dihydro products. *Biochemistry* **32**, 6313–6319.
- Serhan, C.N., Hong, S., Gronert, K., Colgan, S.P., Devchand, P.R., Mirick, G., and Moussignac, R.-L. (2002). Resolvins: a family of bioactive products of

- omega-3 fatty acid transformation circuits initiated by aspirin treatment that counter proinflammation signals. *J. Exp. Med.* **196**, 1025–1037.
- Serhan, C.N., Gotlinger, K., Hong, S., Lu, Y., Siegelman, J., Baer, T., Yang, R., Colgan, S.P., and Petasis, N.A. (2006). Anti-inflammatory actions of neuroprotectin D1/protectin D1 and its natural stereoisomers: assignments of dihydroxy-containing docosatrienes. *J. Immunol.* **176**, 1848–1859.
- Shimizu, T., Rådmark, O., and Samuelsson, B. (1984). Enzyme with dual lipoxygenase activities catalyzes leukotriene A<sub>4</sub> synthesis from arachidonic acid. *Proc. Natl. Acad. Sci. USA* **81**, 689–693.
- Skulas-Ray, A.C., Kris-Etherton, P.M., Harris, W.S., Vanden Heuvel, J.P., Wagner, P.R., and West, S.G. (2011). Dose-response effects of omega-3 fatty acids on triglycerides, inflammation, and endothelial function in healthy persons with moderate hypertriglyceridemia. *Am. J. Clin. Nutr.* **93**, 243–252.
- Spite, M., Norling, L.V., Summers, L., Yang, R., Cooper, D., Petasis, N.A., Flower, R.J., Perretti, M., and Serhan, C.N. (2009). Resolvin D2 is a potent regulator of leukocytes and controls microbial sepsis. *Nature* **461**, 1287–1291.
- Stark, D.T., and Bazan, N.G. (2011). Neuroprotectin D1 induces neuronal survival and downregulation of amyloidogenic processing in Alzheimer's disease cellular models. *Mol. Neurobiol.* **43**, 131–138.
- Sun, Y.P., Oh, S.F., Uddin, J., Yang, R., Gotlinger, K., Campbell, E., Colgan, S.P., Petasis, N.A., and Serhan, C.N. (2007). Resolvin D1 and its aspirin-triggered 17R epimer: stereochemical assignments, anti-inflammatory properties, and enzymatic inactivation. *J. Biol. Chem.* **282**, 9323–9334.
- Tsikitis, V.L., Morin, N.A., Harrington, E.O., Albina, J.E., and Reichner, J.S. (2004). The lectin-like domain of complement receptor 3 protects endothelial barrier function from activated neutrophils. *J. Immunol.* **173**, 1284–1291.
- Van Dyke, T.E., and Serhan, C.N. (2006). A novel approach to resolving inflammation. *Sci. Am. Oral Whole Body Health.*, 42–45.
- Vane, J.R. (1982). Adventures and excursions in bioassay: the stepping stones to prostacyclin. In *Les Prix Nobel: Nobel Prizes, Presentations, Biographies and Lectures* (Stockholm: Almqvist & Wiksell), pp. 181–206.
- Wang, Z., Ginnan, R., Abdullaev, I.F., Trebak, M., Vincent, P.A., and Singer, H.A. (2010). Calcium/Calmodulin-dependent protein kinase II delta 6 (CaMKIIdelta6) and RhoA involvement in thrombin-induced endothelial barrier dysfunction. *J. Biol. Chem.* **285**, 21303–21312.
- Wetterholm, A., Haeggström, J.Z., Samuelsson, B., Yuan, W., Munoz, B., and Wong, C.-H. (1995). Potent and selective inhibitors of leukotriene A<sub>4</sub> hydrolase: effects on purified enzyme and human polymorphonuclear leukocytes. *J. Pharmacol. Exp. Ther.* **275**, 31–37.
- Yanes, O., Clark, J., Wong, D.M., Patti, G.J., Sánchez-Ruiz, A., Benton, H.P., Trauger, S.A., Despons, C., Ding, S., and Siuzdak, G. (2010). Metabolic oxidation regulates embryonic stem cell differentiation. *Nat. Chem. Biol.* **6**, 411–417.
- Yang, R., Chiang, N., Oh, S.F., and Serhan, C.N. (2011). Metabolomics-lipidomics of eicosanoids and docosanoids generated by phagocytes. *Curr. Protoc. Immunol.*, in press.