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Resolvin D3 and Aspirin-Triggered Resolvin D3 Are Potent Immunoresolvents

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SUMMARY

Resolvins are a family of n-3 lipid mediators initially identified in resolving inflammatory exudates that temper inflammatory responses to promote catabasis. Here, temporal metabololipidomics with self-limited resolving exudates revealed that resolvin (Rv) D3 has a distinct time frame from other lipid mediators, appearing late in the resolution phase. Using synthetic materials prepared by stereocontrolled total organic synthesis and metabololipidomics, we established complete stereochemistry of RvD3 and its aspirin-triggered 17*R*-epimer (AT-RvD3). Both synthetic resolvins potently regulated neutrophils and mediators, reducing murine peritonitis and dermal inflammation. RvD3 and AT-RvD3 displayed leukocyte-directed actions, e.g., blocking human neutrophil transmigration and enhancing macrophage phagocytosis and efferocytosis. These results position RvD3 uniquely within the inflammation-resolution time frame to vantage and contribute to the beneficial actions of aspirin and essential n-3 fatty acids.

INTRODUCTION

In response to stress and/or invasion, acute inflammation is host-protective (Medzhitov, 2010) and, when uncontrolled, unresolved inflammation is linked to many widely occurring diseases (Dinarello, 2010; Majno et al., 1982; Nathan and Ding, 2010; Tauber and Chernyak, 1991). The ideal outcome for an acute inflammatory response is complete resolution. In this context, in self-limited inflammatory exudates, we identified potent local mediators within the resolution phase that possess potent anti-inflammatory and proresolving actions, coined specialized proresolving mediators, that include the resolvins, protectins, and maresins (for reviews, see Serhan, 2007 and Serhan and Petasis, 2011). Thus, within the initiation phase, chemical mediators, such as prostaglandins, leukotrienes, cytokines, and chemokines, dictate the leukocyte influx traffic (Dinarello, 2010; Samuelsson, 2012) that is actively counter-regulated by proresolving mediators, which orchestrate the resolution phase, catabasis, and the return to homeostasis (Serhan, 2007).

The resolvins are biosynthesized from essential omega-3 fatty acids (Serhan, 2007; Serhan et al., 2002; Serhan and Petasis, 2011). The E-series resolvins, i.e., resolvin E1, resolvin E2, and resolvin E3 (Isobe et al., 2012), are produced from eicosapentaenoic acid (EPA) (reviewed in Serhan, 2007 and Serhan and Petasis, 2011). D-series resolvins, including resolvin (Rv)D1 (Sun et al., 2007), RvD2 (Spite et al., 2009), RvD5 (Chiang et al., 2012), and their aspirin-triggered versions are biosynthesized from DHA (Serhan et al., 2002). Each resolvin possesses potent proresolving actions that include limiting neutrophil tissue infiltration, counterregulation of chemokines and cytokines, reduction in pain, and stimulation of macrophage-mediated actions (i.e., efferocytosis and bacterial and debris clearance). Given their unique mechanism of proresolving actions, resolvins demonstrate potent actions in animal disease models (reviewed in Serhan and Petasis, 2011). Like many other autacoids, the actions of resolvins are stereochemically selective, reflecting their routes of biosynthesis. The classic eicosanoids, for example, each carry well-established stereoselectivity in their actions (Samuelsson, 2012; Shimizu, 2009). Hence, establishing the complete stereochemical assignment for each of the separate resolvin structures is of considerable interest.

The original identification of the D-series resolvins reported the structural elucidation of several distinct bioactive structures, denoted resolvin D1 through resolvin D6, in resolving murine exudates, their biosynthesis by isolated human leukocytes, and potent actions in vivo in murine as well as human acute inflammation (Serhan et al., 2002). From the basic structures of the D-series resolvins, it was deemed important to establish each of their complete stereochemical assignments to permit further mass spectral quantitative methods for in vivo studies as well as confirmation and extension of their roles in inflammation and active resolution. Along these lines, we determined the complete stereochemical assignments of RvD1 as 7*S*,8*R*,17*S*-trihydroxydocosa-4*Z*,9*E*,11*E*,13*Z*,15*E*,19*Z*-hexaenoic acid and its aspirin-triggered AT-(17*R* epimer)-RvD1 as 7*S*,8*R*,17*R*-trihydroxydocosa-4*Z*,9*E*,11*E*,13*Z*,15*E*,19*Z*-hexaenoic acid, employing a stereocontrolled total organic synthesis approach that permitted confirmation of RvD1 potent anti-inflammatory and proresolving actions (Serhan and Petasis, 2011; Sun et al., 2007) as well as documented RvD1 production by human neutrophils, blood, trout hematopoietic organs, and in brain

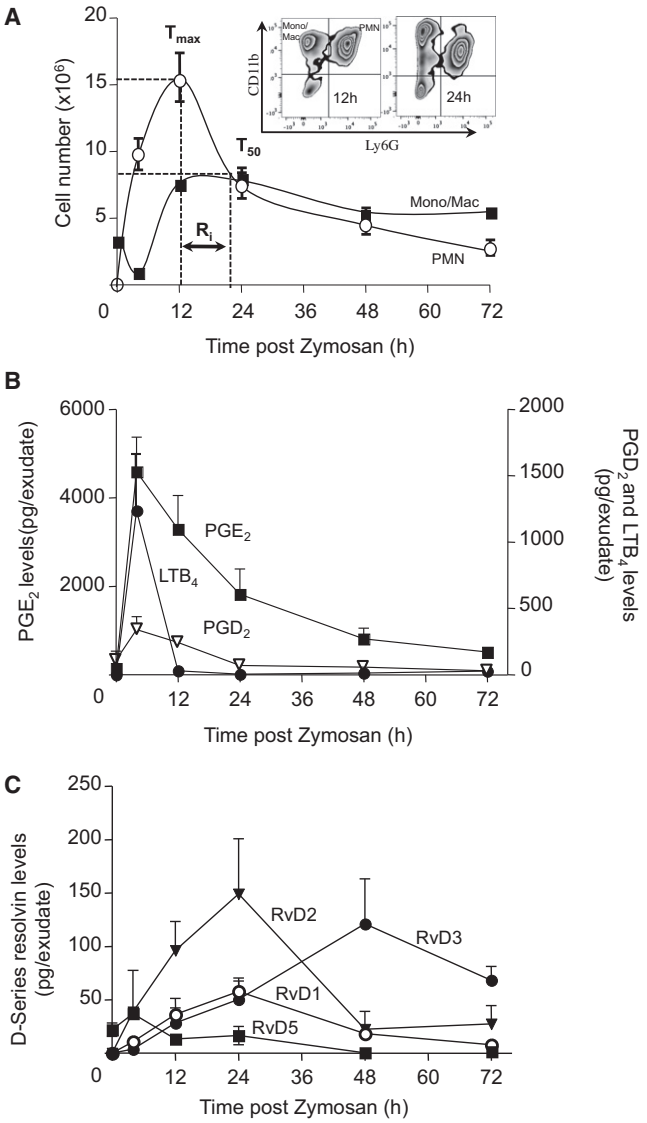

Figure 1. Endogenous Biosynthesis of RvD3 and Its Relation to Other Lipid Mediators in Inflammation Resolution

Mice were injected i.p. with zymosan (1 mg/mouse) and lavages collected at the indicated intervals.

(A) Total cell counts in the peritoneal exudates were determined by light microscopy and the number of mononuclear cells and PMN determined by flow cytometry. Lipid mediators in exudates were assessed using LC-MS-MS metabololipidomics following solid phase extraction (see Experimental Procedures).

(B and C) Exudate levels for (B) prostaglandins and leukotrienes and (C) D-series resolvins. Results are mean ± SEM n = 3–4 mice per time point.

injury (Hong et al., 2007). Hence, RvD1 actions were confirmed and extended to controlling pain (Ji et al., 2011; Lima-Garcia et al., 2011; Xu et al., 2010), reducing airway inflammation (Rogerio et al., 2012) murine colitis (Bento et al., 2011) and stimulating resolution mechanisms in vivo in mice and with human macrophages in vitro (Recchiuti et al., 2011) as well as RvD1 identification in human blood (Mas et al., 2012; Psychogios et al., 2011). For resolvin D2, establishing its complete stereochemistry as 7*S*,16*R*,17*S*-trihydroxydocosa-4*Z*,8*E*,10*Z*,12*E*,14*E*,19*Z*-hexaenoic acid permitted the identification of resolvin's ability to enhance the innate immune response without rendering immune suppression of the host (Spite et al., 2009). This also enabled the elucidation of the potent protective and proresolving actions of RvD2 in colitis (Bento et al., 2011), in reducing pain (Ji et al., 2011), and its presence in both human tissue (Mas et al., 2012) and salmon (Raatz et al., 2011).

The basic structure 4*S*,11,17*S*-trihydroxydocosa-5*Z*,7,9,13,15*E*,19*Z*-hexaenoic acid, identified in murine exudates and with human leukocytes, was denoted resolvin D3 (RvD3) (Serhan et al., 2002). By definition, resolvin D3 actions include potent reduction of neutrophil infiltration in vivo in both murine peritonitis and dorsal skin pouches. RvD3 also reduced human neutrophil transendothelial migration (Serhan et al., 2002), each cardinal actions of a proresolving mediator. Of interest, RvD3 from endogenous sources of DHA is elevated in colitis in *fat-1* transgenic mice that overexpress the enzyme that increases tissue levels of n-3 essential fatty acids without feeding DHA or EPA (Hudert et al., 2006). Also, RvD3 produced from endogenous DHA is elevated in ischemic injury of the kidney (Duffield et al., 2006). Given these properties, we have focused on establishing RvD3's complete stereochemistry and temporal positioning within inflammation resolution. Here, we report the stereochemical assignments for both RvD3 and aspirin-triggered (AT)-RvD3 and their potent anti-inflammatory and proresolving actions using synthetic materials, establishing the potent actions of these members of the D-series resolvins.

RESULTS

RvD3 Is Uniquely Positioned within the Resolution Frame of Inflammation

Self-limited inflammation results in a rapid increase in infiltration of neutrophils and their eventual loss from the tissue (Figure 1A). This is accompanied by a concomitant nonphlogistic increase in mononuclear cells. In this context, the resolution interval (Bannenberg et al., 2005) was ~11 hr. Each cell population was identified using flow cytometry and light microscopy (see inset for 12 and 24 hr). This system is an ideal example of the temporal relationship from initiation to resolution (Bannenberg et al., 2005). In this context, prostaglandins are rapidly produced (Figure 1B) and, concomitant with neutrophil infiltration into the tissue, leukotriene B_4 and prostaglandin E_2 (PGE_2) levels reach maximum within 4 hr. Leukotriene B_4 (LTB_4) levels rapidly drop and return to essentially baseline within 12 hr, whereas the levels of both PGE_2 and prostaglandin D_2 (PGD_2) persist into the resolution phase, where they were shown earlier to stimulate the production of proresolving mediators by stimulating the transcriptional regulation of a key enzyme in this process, human 15-lipoxygenase type 1 (Levy et al., 2001).

Using this setting, which amply qualifies the initiation and resolution phase, we next determined the profile of D-series resolvins within the DHA metabolome in order to temporally stage each (Figure 1C). As anticipated, endogenous production of resolvins D1, D2, and D5 lagged behind appearance of the leukotrienes and was coincident with the resolution phase as determined from a maximal PMN time point (T_{max}) to the 50% reduction in

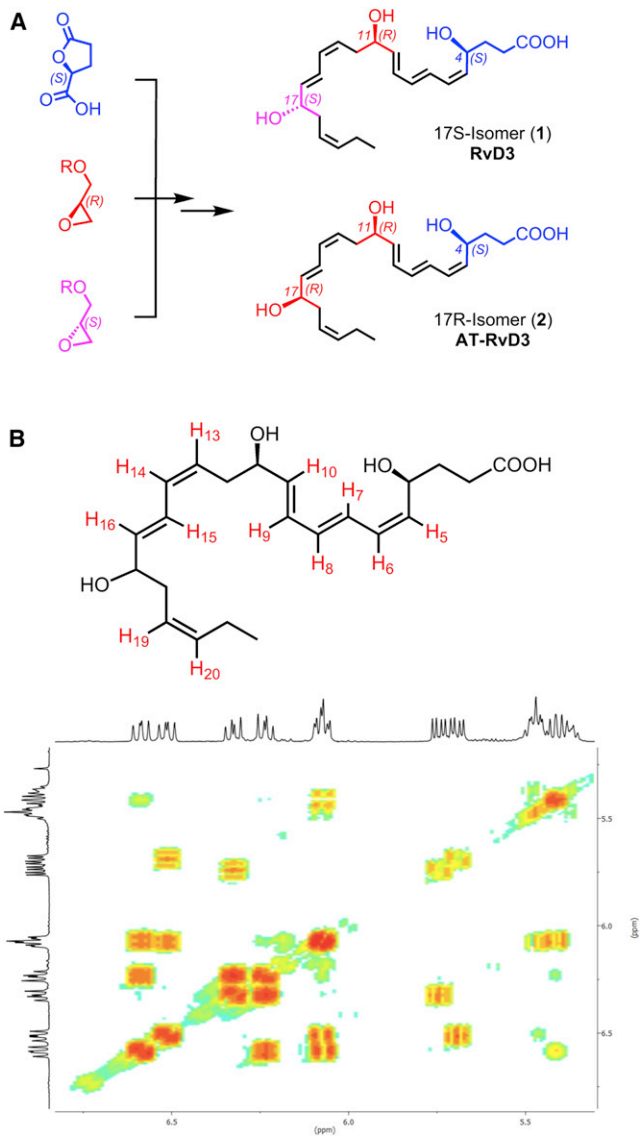

Figure 2. Synthetic RvD3/AT-RvD3 Stereoisomers

These isomers were prepared in enantiomerically pure form via stereocontrolled total synthesis and were fully characterized by NMR spectroscopy.

(A) Structures of the isomers of RvD3/AT-RvD3 and color-coded illustration depicting the origin of R or S stereochemistry of each chiral alcohol group from stereochemically pure precursors (J.W.W., C.N.S., and N.A.P., unpublished data).

(B) Assignment of the Z or E stereochemistry for each C = C bond using two-dimensional NMR spectroscopy. The shown ^{1}H-^{1}H gCOSY spectrum of a solution of RvD3 in CD_3OD [9.6 × 10^{-3}M] was acquired using a Varian VNMRS 600 MHz NMR spectrometer at 25°C on a 5 mm Triple Resonance PFG ^{1}H and referenced to the CD_3OD and an internal standard. This spectrum depicts all of the connectivities between adjacent alkenyl hydrogens (H_5-H_{10}, H_{13}-H_{16}, H_{19}-H_{20}). The colors denote a bitmap plotting method using a rainbow palette that gives depth to the positive and negative contours. The complete identification of each H-atom using this in combination with its corresponding constants (J values) permitted the unambiguous E/Z assignment of all alkenyl hydrogens (see Results).

PMN (T_{50}), defining the resolution interval (R_i). Unexpectedly, the levels of resolvin D3 increased at 24 hr after initiation and persisted to 72 hr, well into the resolution timeframe of the local inflammatory response. Thus, endogenous production of D-series resolvins gave a distinct separation between resolvins D1 and D2 accumulation early in the resolution phase and the persistence of resolvin D3, which gives a unique position within the resolution phase. Hence, we focused our attention on resolvin D3.

Synthetic RvD3 and AT-RvD3

During the initial isolation and structural characterization of RvD3 and AT-RvD3 (Serhan et al., 2002), the E/Z stereochemistry of all C = C bonds and the R/S chirality of the hydroxyl groups at C4 and C11 could not be determined, since these structures were deduced and proposed using results from liquid chromatography-tandem mass spectrometry (LC-MS-MS) and gas chromatography-mass spectrometry (GC-MS), which does not permit direct determination of the stereochemical nature of these molecules. In order to fully elucidate the stereochemical assignments of these mediators, we devised a stereocontrolled synthetic approach to obtain several potential stereoisomers of these molecules (J.W.W., C.N.S., and N.A.P., unpublished data). The complete structures of two synthetic RvD3 isomers, the 17S-isomer 1 and the epimeric 17R-isomer 2, which were obtained in stereochemically pure form, are shown in Figure 2A. The complete structure and stereochemistry for both isomers were unambiguously established through the stereocontrolled synthetic approach and were confirmed via spectroscopic analysis. The R/S chirality for each of the three OH groups (Figure 2A) was ensured by starting with enantiomerically pure starting materials with the same chirality and by employing synthetic transformations that retain this chirality throughout the synthesis. The Z/E geometry for each of the C = C bonds was secured by employing highly stereocontrolled processes that produce the desired geometry in each position. Further structural confirmation and unambiguous assignment of the Z/E geometry of each C = C bond for both isomer 1 and isomer 2 were established via two-dimensional correlated spectroscopy (COSY) nuclear magnetic resonance (NMR) spectroscopy (Figure 2B; see Experimental Procedures).

Matching of Endogenous RvD3 and AT-RvD3 with Synthetic Materials

Since both RvD3 and its aspirin-triggered epimer are produced in murine inflammatory exudates (Duffield et al., 2006; Serhan et al., 2002), it was important to establish the complete stereochemistry as well as confirm its assigned structure and actions with synthetic material prepared by stereocontrolled total organic synthesis. To this end, authentic RvD3 and its aspirin-triggered form (AT-RvD3) were obtained from endogenous DHA in murine-resolving exudates treated with or without aspirin and chromatographed using LC-MS-MS-based metabololipidomics (Figures 3A and 3B) for direct comparison with synthetic material (Figure 3C). Authentic RvD3 from both murine exudates (Figures 3A and 3B) and human macrophages (Figure S1A available online) gave a sharp peak in liquid chromatography, retention time (T_R) = 7.4 min, clearly separating from AT-RvD3, which produced a sharp peak at T_R = 7.2 min.

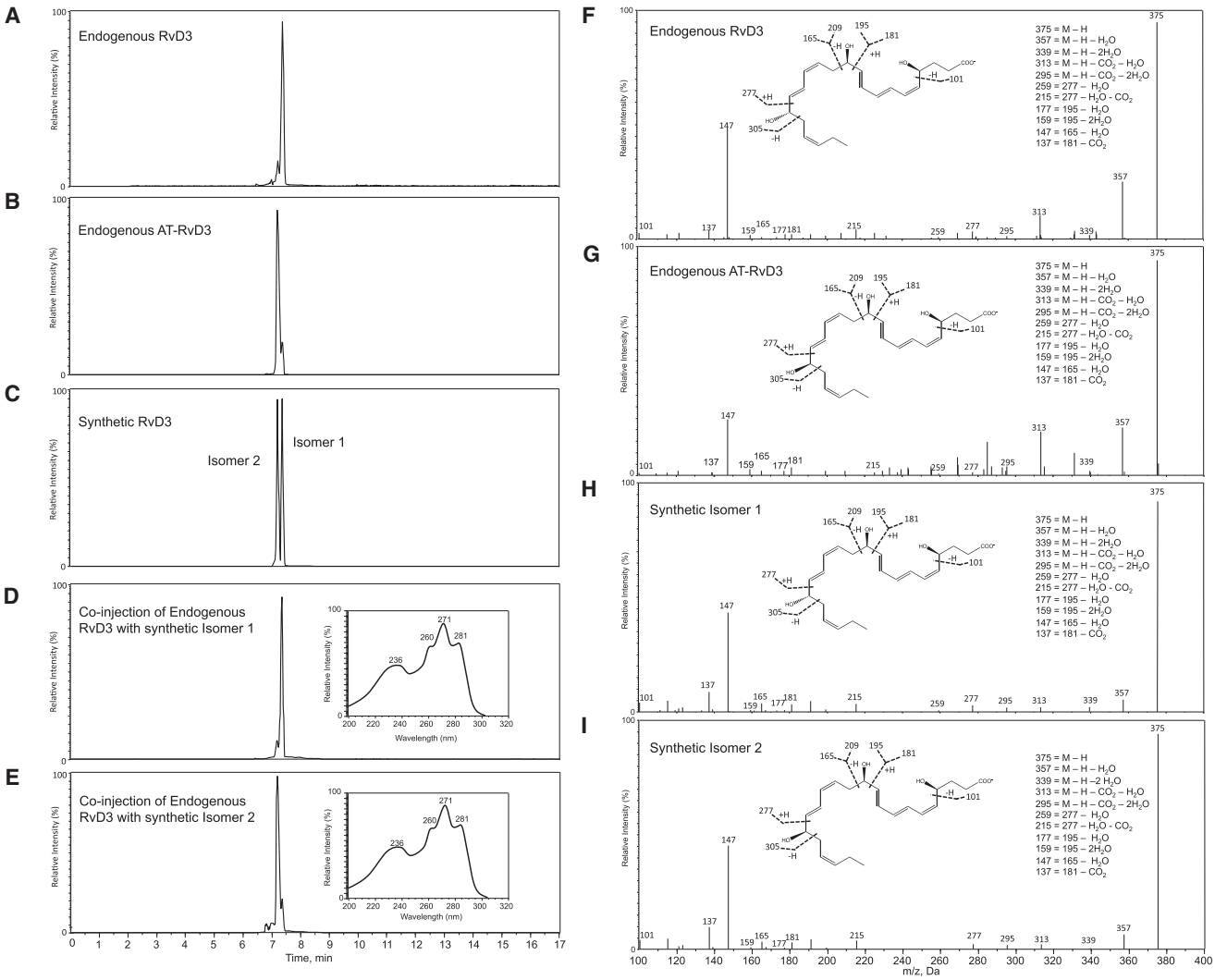

Figure 3. Endogenous RvD3 and AT-RvD3 from Resolving Inflammatory Exudates Match Synthetic Compound

(A) Endogenous RvD3 was obtained from mice injected with zymosan (1 mg/mouse) and exudates collected at 4 hr. These were subjected to lipid mediator metabololipidomics. Selected ion chromatograms (m/z 375–147) depict murine-resolving exudate-derived RvD3.

(B) Endogenous AT-RvD3 obtained from mice administered aspirin (500 μg) and zymosan (1 mg).

(C) Synthetic isomers 1 and 2.

(D) Coinjection of resolving exudate endogenous RvD3 with synthetic isomer 1 (inset: characteristic UV-absorption spectrum) (see also Figures S2 and S3 and Table S1).

(E) Coinjection of resolving exudate endogenous AT-RvD3 with synthetic isomer 2 (inset: characteristic UV-absorption spectrum).

(F–I) MS-MS spectrum for (F) endogenous RvD3 (T_R = 7.4 min); (G) endogenous AT-RvD3 (T_R = 7.2 min); (H) synthetic RvD3 (T_R = 7.4 min); (I) synthetic AT-RvD3 (T_R = 7.2min) (see also Figure S1). Representative MRM chromatograms and MS-MS spectra (n = 4).

LC-MS-MS of the synthetic isomers demonstrated that isomer 1 gave a sharp peak [T_R = 7.4 min] and isomer 2 eluted at T_R = 7.2 min (Figure 3C). Coinjection with material prepared by total organic synthesis and authentic RvD3 demonstrated coelution with isomer 1 at T_R = 7.4 min (Figure 3D). Similarly, coinjection of synthetic isomer 2 with authentic AT-RvD3 obtained from aspirin-treated inflammation demonstrated coelution T_R = 7.2 min (Figure 3E). Moreover, the two synthetic compounds contained combined characteristic conjugated triene and diene chromophores with triplet absorbance bands at λ_{max}^{MeOH} ~260, 271, and 281 nm for the triene and ~236 nm band for the diene component (Figures 3D and 3E, insets).

Of interest, although the two RvD3 epimers displayed distinct chromatographic behavior, they both demonstrated essentially identical fragmentation patterns (Figures 3F–3I). Each diastereomer gave *m/z* 375 = M-H, *m/z* 357 = M-H-H_2O, *m/z* 339 = M-H-2H_2O, *m/z* 313 = M-H-CO_2-H_2O, *m/z* 295 = M-H-CO_2-2H_2O, *m/z* 259 = 277-H_2O, *m/z* 215 = 249-CO_2, *m/z* 177 = 195-H_2O, *m/z* 159 = 195-2H_2O, *m/z* 147 = 165-H_2O, and *m/z* 137 = 181-CO_2.

To obtain further evidence for matching of RvD3, GC-MS was performed as in the original identification and basic structural elucidation (Serhan et al., 2002). RvD3 was treated with diazomethane and subsequently converted to its corresponding

trimethylsilyl derivative (Figure S2A) and subjected to GC-MS. The retention time in GC-MS for synthetic RvD3 corresponded to a C value of 27.9 (Figure S2B), while the mass spectrum (Figure S2C; Table S1) demonstrated fragmentation and ions consistent with the proposed structure of RvD3 (Serhan et al., 2002) and matched those of the derivatized synthetic materials (see Experimental Procedures). AT-RvD3 demonstrated properties similar to RvD3 (Figure S3) with a retention time of 20.5 min (Figure S3B), corresponding to a C-value of 27.9 and displaying essentially an identical fragmentation pattern to RvD3 in the GC-MS (Figure S3C). Therefore, the complete stereochemistry of RvD3 was proved to be 4*S*, 11*R*, 17*S*-trihydroxydocosa-5*Z*, 7*E*, 9*E*, 13*Z*, 15*E,* 19*Z*-hexaenoic acid (isomer 1, Figures 3A and 3F), and that for AT-RvD3 was 4*S*, 11*R*, 17*R*-trihydroxydocosa-5*Z*, 7*E*, 9*E*, 13*Z*, 15*E,* 19*Z*-hexanoic acid (isomer 2, Figures 3B and 3G).

RvD3 and AT-RvD3 Exert Potent Antineutrophil Actions

After matching the synthetic materials with endogenously produced mediators and establishing the stereochemistry of RvD3 and AT-RvD3, we required confirmation of their potent anti-inflammatory actions. Systemic administration of RvD3 and its aspirin-triggered epimer in vivo immediately prior to zymosan (1 mg) challenge at doses as low as 10 ng/mouse significantly reduced the number of transmigrated neutrophils into the peritoneal cavity (~43%; Figure 4A). Assessment of exudate cytokine and chemokine levels demonstrated a significant downregulation of the proinflammatory cytokine interleukin (IL)-6 (Figure 4B) and an increase in levels of the proresolving cytokine IL-10 (Figure 4C).

The biosynthesis and action of specialized proresolving mediators is regulated both temporally and spatially (Serhan and Savill, 2005); therefore, we next assessed the actions of the synthetic RvD3 diastereomers in murine dorsal air pouches. Local administration of RvD3 significantly reduced neutrophil transmigration in response to tumor necrosis factor alpha (TNF-α)-initiated acute inflammation (~65% reduction; Figure 4D). Assessment of inflammatory exudate cytokine and chemokine levels demonstrated a significant reduction in monocyte chemoattractant protein-1 (MCP-1) (~70%), IL-6 (~90%), and murine keratinocyte-derived cytokine/chemokine (C-X-C motif) ligand 1 (KC) (~50%) (Figures 4E–4G). Similarly, AT-RvD3 potently reduced PMN recruitment into the pouch (~75%; Figure 4D), while also reducing exudate proinflammatory cytokine and chemokine levels (Figures 4E–4G). Together, these results demonstrate the potent local and systemic anti-inflammatory actions of RvD3 and AT-RvD3, counterregulating local proinflammatory mediator production and dampening leukocyte recruitment.

RvD3 and AT-RVD3 Counterregulate Eicosanoids

Given that proresolving lipid mediators also regulate eicosanoid biosynthesis (Spite et al., 2009), we next assessed the ability of systemically delivered RvD3 as well as AT-RvD3 to regulate local eicosanoid production in vivo. We performed lipid mediator metabololipidomics using exudates obtained at 4 hr after zymosan administration to identify potential mechanism(s) underlying the actions of systemically administered RvD3 and AT-RvD3. In these experiments, we targeted and identified the following eicosanoids: prostaglandin (PG)D_2, PGE_2, and thromboxane (Tx)B_2 from the cyclooxygenase pathways and LTB_4 from the 5-lipoxygenase (LOX) pathway (Figure 5A). Identifications were validated using published criteria (Dalli and Serhan, 2012), as illustrated for TxB_2 and PGE_2 (Figure 5B). Using multiple reaction monitoring (MRM), we quantified individual mediator levels and found that systemically delivered RvD3 significantly reduced LTB_4 (~80%), PGD_2 (~67%), and TxB_2 (~50%), while stimulating the production of PGE_2 (Figure 5C). AT-RvD3 reduced local LTB_4, PGD_2, and TxB_2 levels, while increasing PGE_2 (Figure 5D). Together, these results demonstrate that both synthetic RvD3 and AT-RvD3 possess potent anti-inflammatory actions, regulating local eicosanoid levels, characteristic of specialized proresolving mediators.

RvD3 and AT-RvD3 Exert Potent Proresolving Actions

Having found that both RvD3 and AT-RvD3 exert potent anti-inflammatory and proresolving actions in murine systems in vivo, we next investigated their actions with human leukocytes. Exposure of human PMN to RvD3 or AT-RvD3 at concentrations as low at 10^{-11} M reduced transmigration ~25% (Figure 6A). AT-RvD3 displayed greater antimigratory properties at 1 and 10 pM ($p < 0.05$). We next assessed the ability of both RvD3 and its aspirin-triggered form to stimulate macrophage phagocytosis, a key step in the resolution of inflammation (Serhan and Savill, 2005). Incubation of murine naive peritoneal macrophages with RvD3 or AT-RvD3 dose-dependently increased macrophage phagocytosis of zymosan particles (Figure 6B). At 10 and 100 pM, AT-RvD3 was more potent than RvD3. Together, these results demonstrate that both RvD3 epimers exert potent anti-inflammatory and proresolving actions with leukocytes.

Since removal of apoptotic cells and cellular debris, a process termed efferocytosis, is a cellular hallmark of tissue resolution (Serhan and Savill, 2005), we next assessed whether RvD3 and its aspirin-triggered epimer stimulated murine macrophage efferocytosis, a cardinal proresolving action essential for complete resolution. Synthetic RvD3 and AT-RvD3 were each found to potently stimulate macrophage efferocytosis of apoptotic human PMN (Figures 6C and S4A). Next, we determined the rank order potencies of specialized proresolving mediators (SPM) and found that RvD3 and PD1 at 1 nM were the most potent in stimulating efferocytosis with both mouse bone marrow and resident macrophages, followed by MaR1, RvD2, and RvD1 (Figures 6D and S4B). These results demonstrate and confirm the potent anti-inflammatory and proresolving actions defining RvD3 and AT-RvD3 with synthetic materials and human and mouse leukocytes, as well as reducing levels of proinflammatory cytokines, i.e., MCP-1, IL-6, and KC (Figure 4).

Activation of Human GPR32

We next tested whether RvD3 activates the human RvD1 receptor (DRV1) GPR32, since we identified a specific G protein-coupled receptor (GPCR), denoted GPR32, that is activated by RvD1 (Krishnamoorthy et al., 2010, 2012) and RvD5 (Chiang et al., 2012). We employed electrical cell substrate impedance sensing (ECIS), which monitors impedance changes upon ligand binding to receptors (Krishnamoorthy et al., 2012). In this system, RvD3 ($-25.8 \pm 5.0\ \Omega$) and AT-RvD3 ($-20.7 \pm 3.6\ \Omega$)

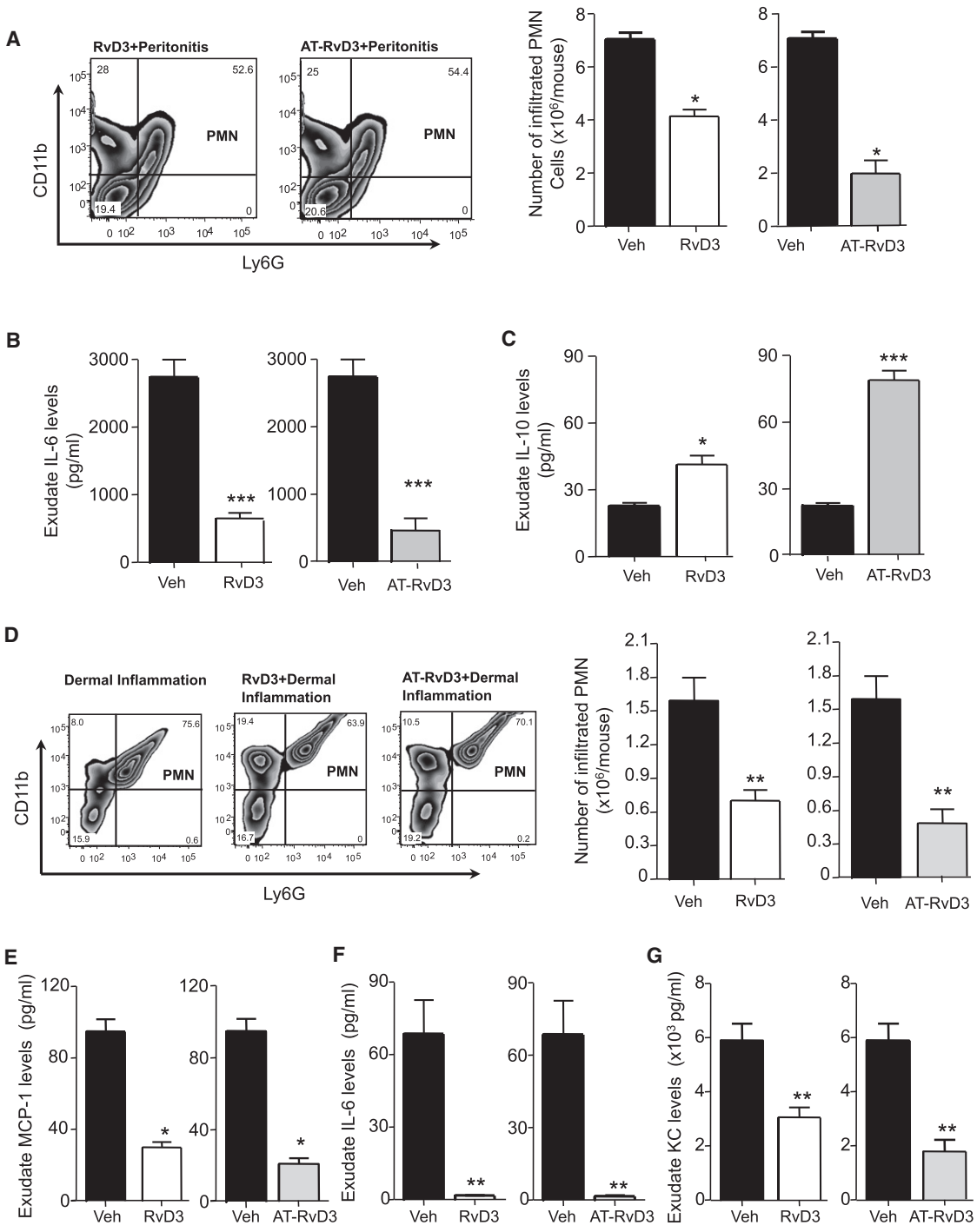

Figure 4. RvD3 Is a Potent Antineutrophil and Cytokine Regulator

RvD3, AT-RvD3 (10 ng/mouse), or vehicle (saline containing 0.1% EtOH) was administered i.v. 10 min prior to i.p. injection of zymosan (1 mg/mouse). Exudates were collected 4 hr later.

(A) Cells were enumerated and PMN identified using flow cytometry.

(B and C) IL-6 (B) and IL-10 (C) levels were measured in peritoneal exudates. Results are mean ± SEM, n = 4 mice per group. *$p < 0.05$ and ***$p < 0.001$ versus zymosan plus vehicle.

Dorsal skin inflammation (see Experimental Procedures): mice were injected on day six with murine recombinant TNF-α (100 ng/mouse) following administration of RvD3, AT-RvD3 (10 ng), or vehicle (saline containing 0.1% EtOH) by intrapouch injection and at 4 hr lavages obtained.

(D) PMN by flow cytometry.

(E–G) Levels of (E) MCP-1, (F) IL-6, and (G) KC. Results are mean ± SEM, n = 4 mice per group. *$p < 0.05$; **$p < 0.01$ versus TNF-α plus vehicle.

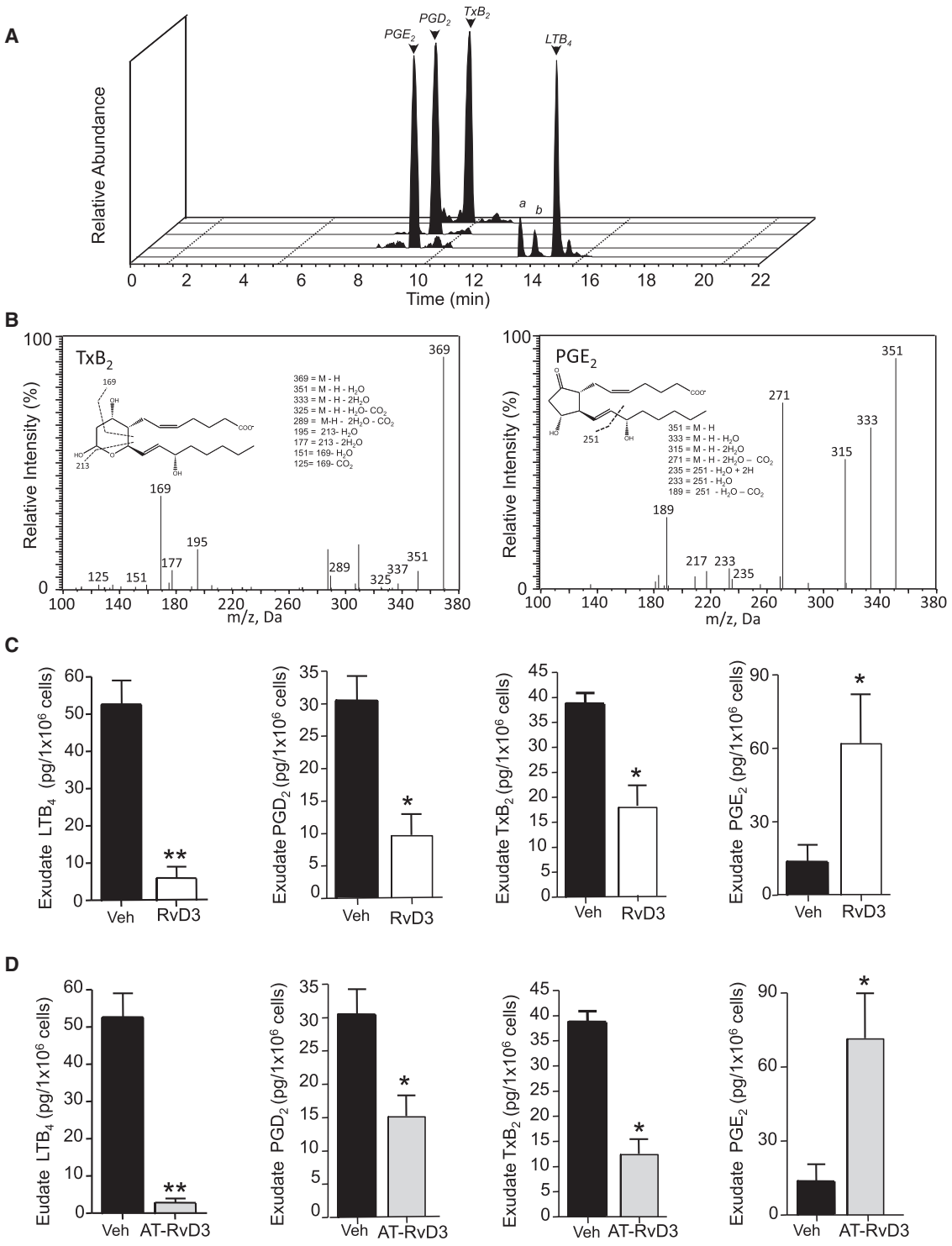

Figure 5. RvD3 and AT-RvD3 Reduce Local Prostanoids and Leukotrienes in Acute Inflammation

Lipid mediators in peritoneal exudates collected 4 hr after zymosan administration were assessed using LC-MS-MS metabololipidomics following solid phase extraction.

(A) Representative multiple reaction monitoring chromatograms (MRM) of selected ion pairs for arachidonic acid-derived eicosanoids. *a* = 6-trans-LTB_4 and *b* = 6-trans, 12-epi-LTB_4.

(B) Representative MS-MS spectra with diagnostic ions employed for the identification of TxB_2 and PGE_2 (see Experimental Procedures).

(C and D) Quantification of exudate lipid mediators following (C) RvD3 and (D) AT-RvD3 administration. Results are mean ± SEM, n = 4 mice per group. *$p < 0.05$, **$p < 0.01$ versus zymosan plus vehicle.

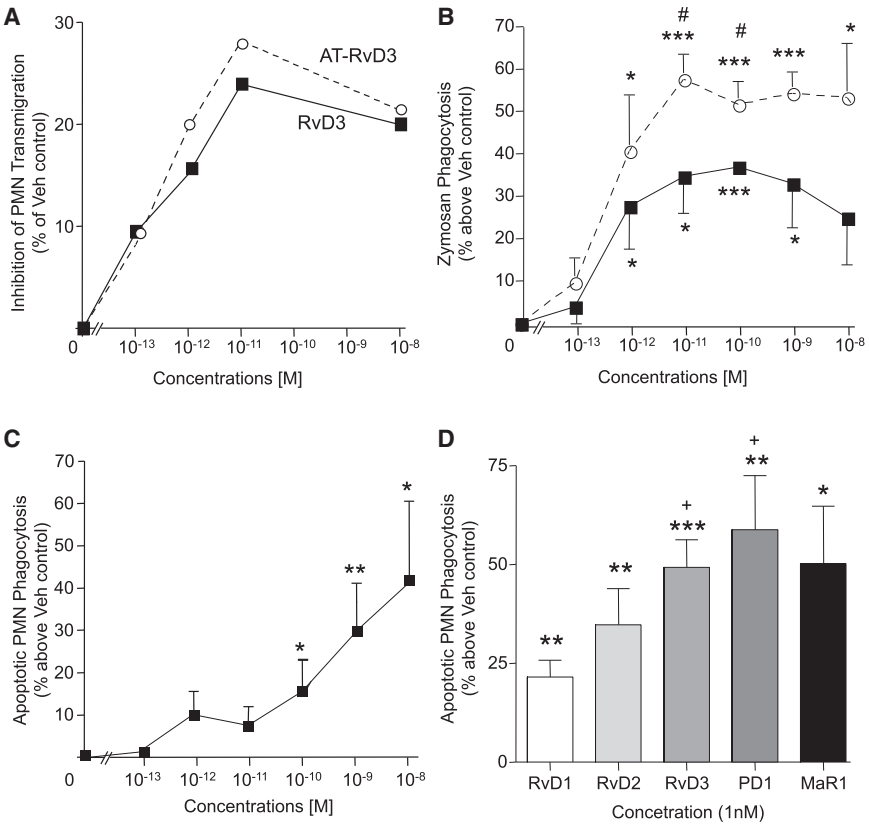

Figure 6. RvD3 and AT-RvD3 Proresolving Actions

(A) Human neutrophils were labeled with CFDA and incubated with RvD3, AT-RvD3, or vehicle (DPBS containing 0.1% EtOH) for 15 min (37°C) prior to assessing their transmigration across human umbilical vein endothelial cells exposed to TNF-α (10 ng/ml). Results are representative of n = 4 distinct PMN preparations.

(B–D) Increased macrophage phagocytosis and efferocytosis. Murine peritoneal resident macrophages were incubated with (B and C) RvD3 (black square), AT-RvD3 (open circle) (0.1 pM–10 nM) or (D) 1 nM of select SPM (15 min, 37°C) followed by addition of (B) FITC-zymosan or (C and D) CFDA-labeled apoptotic PMN for 1 hr (see also Figure S4). Results are mean ± SEM of n = 4, d = 3–4. *p < 0.05, **p < 0.01, ***p < 0.001 versus vehicle; # p < 0.05, RvD3 versus AT-RvD3; + p < 0.05 versus RvD1.

at 100 nM each elicited rapid changes in impedance with Chinese hamster ovary (CHO) cells expressing recombinant human GPR32 (Figure 7A), to a similar magnitude as RvD1 (−19.1 ± 1.8 Ω) (Krishnamoorthy et al., 2012). These changes in impedance were reduced when cells were incubated with anti-human GPR32 antibody (30 min, 37°C) prior to the addition of RvD1, RvD3, or AT-RvD3 (Figures 7B–7D).

To further examine ligand-receptor relationships, we used a beta-arrestin reporter system (Krishnamoorthy et al., 2012), which confirmed that both RvD3 and AT-RvD3 directly activated this receptor (0.1 pM–10 nM; Figure S5A). In addition, phagocytic activity with human macrophages increased by RvD3 and AT-RvD3 proved to be dependent on GPR32, since phagocytosis was significantly enhanced with GPR32 overexpression compared to mock transfected cells (Figures 7E, 7F, S5B, and S5C). Together, these results demonstrated that RvD3 and AT-RvD3 each activated human GPR32, which contributes to their proresolving actions in stimulating macrophage uptake of microbial particles (Figures 7E and 7F).

DISCUSSION

Mounting evidence indicates that resolution of acute inflammation is an active process, where lipid mediators play a pivotal role (Serhan, 2011). In this regard, the resolvins have emerged as uniquely positioned within the resolution phase of acute inflammation to actively counterregulate the initiation signals as well as stimulate specific proresolving responses. Initiation of an inflammatory response involves the orchestrated trafficking of leukocytes from peripheral blood into the tissue. The chemical mediators involved are lipid mediators, such as the eicosanoids, prostaglandins, and leukotrienes (Samuelsson, 2012; Shimizu, 2009) and the proinflammatory cytokines and chemokines (Dinarello, 2010). The stereoselective actions are a hallmark of the lipid-derived mediators, since they evoke potent and cell type-specific actions (Coulthard et al., 2012; Samuelsson, 2012). Hence, given the potent actions of the D-series resolvins, including resolvins D1 and D2, it was deemed important to establish the when and where, i.e., temporal relationships and actions of resolvin (Rv) D3, within inflammation resolution.

In the present report, we establish the complete stereochemistry of RvD3 and its aspirin-triggered 17*R* epimer, AT-RvD3. Within the time course of self-limited local inflammatory response, RvD3 stood apart from the other D-series resolvins in that it remained elevated well into the late resolution phase. The complete stereochemistry of RvD3 derived from mouse exudates as well as human leukocytes was matched to synthetic materials defined by stereocontrolled organic synthesis and NMR. The complete structure of RvD3 proved to be 4*S*,11*R*,17*S*-trihydroxydocosa-5*Z*,7*E*,9*E*,13*Z*,15*E*,19*Z*-hexaenoic acid and its aspirin-triggered epimer 4*S*,11*R*,17*R*-trihydroxydocosa-5*Z*,7*E*,9*E*,13-*Z*,15*E*,19*Z*-hexaenoic acid (Figures 1, 2, and 3). Both RvD3 and its aspirin-triggered epimer potently counterregulate proinflammatory mediators as well as limit neutrophil infiltration in vivo. Translating to human cells, both RvD3 and AT-RvD3 blocked human neutrophil transmigration across endothelial cells (Figure 6), a response pivotal to regulating the size of the inflammatory exudate in situ and potential collateral tissue damage (Majno et al., 1982). Importantly, RvD3 proved to be a potent enhancer of the uptake of apoptotic neutrophils by macrophages as well as a stimulator of IL-10. Together, these results establish the structural assignments and the elucidation of the isolated bioactive RvD3 and AT-RvD3 (Serhan et al., 2002).

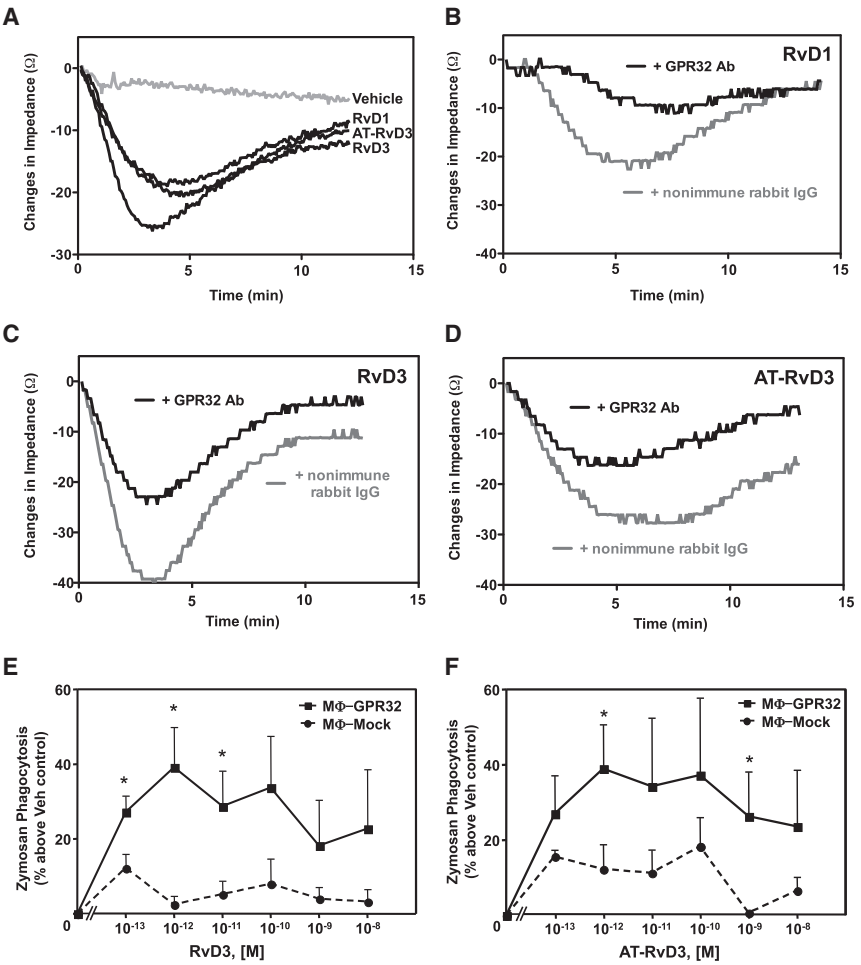

Figure 7. RvD3 and AT-RvD3 Activate Human GPR32

(A–D) Ligand–receptor-dependent changes in impedance with CHO cells overexpressing human GPR32. Impedance was continuously recorded with real-time monitoring across cell monolayers using an ECIS system. Results are tracings obtained from incubations of RvD1, RvD3, or AT-RvD3 (100 nM each) with CHO-GPR32 cells (A) or in the presence of anti-GPR32 Ab or nonimmune rabbit serum IgG (B–D). Results are expressed as (A) means from four separate tracings with each compound or (B–D) representative of three separate experiments. IgG, immunoglobulin G.

(E and F) Human GPR32 or mock-transfected human macrophages were incubated with RvD3 or AT-RvD3 (0.1 pM–10 nM) for 15 min, followed by addition of FITC-zymosan (60 min, 37°C). Results are expressed as mean ± SEM; n = 4 macrophage preparations. *p < 0.05, macrophages (MΦ) plus GPR32 versus MΦ plus mock (see also Figure S5).

These actions of RvD3 are the hallmark responses defining a proresolving mediator and immunoresolvent (Serhan, 2007, 2011). Resolvin D3 fulfilled these defining criteria as well as proved to be among the most potent of the resolvins identified to date within inflammatory exudates. It is now widely acknowledged that proinflammatory mediators, when produced in excess, can disrupt normal resolution that can lead to chronic inflammation (Serhan and Savill, 2005) and even to an eicosanoid storm via rapid activation of the inflammasome (von Moltke et al., 2012). Active containment via enhanced phagocytosis of microbes as well as the efferocytosis of apoptotic PMN leads to the clearance of invaders and cellular debris to return to homeostasis (Chiang et al., 2012). Having established the proresolving bioaction, complete stereochemistry, and synthesis of RvE1, RvD1, and RvD2 as well as PD1 (reviewed in Serhan and Petasis, 2011) permitted demonstration of their potent actions by others in many diverse systems, including colitis (Bento et al., 2011) and controlling pain (Ji et al., 2011), as well as regulating important processes, such as macrophage polarization and promoting resolution of adipose tissue inflammation in obesity (Titos et al., 2011).

Resolvin D3 is distinguished from resolvins D1 and D2 by its late appearance in vivo, promoting proresolving actions with human leukocytes as well as in vivo (Figures 4, 5, 6, 7, and 8). Given the strategic "late appearance" of RvD3 during resolution of inflammation (Figure 1), it is very likely that RvD3 is produced by macrophage subtypes known to appear late within the resolution phase of self-limited response to challenge (Stables et al., 2011). Figure 8 depicts the DHA resolution metabolome and the position of RvD3 within the resolvin biosynthesis pathway and its relation to other families of DHA-derived bioactive mediators, i.e., protectins, maresins, and resolvin family members. These late resolution-phase macrophages display high levels of 15-LOX and, hence, the capacity to produce proresolving mediators (Dalli and Serhan, 2012; Stables et al., 2011).

Using metabolomic approaches, as in the present study (Figure 1), has led to identification of previously unexplored biochemical pathways in inflammation and its resolution as well as in many other disease processes (Levy et al., 2001; Patti et al., 2012; Serhan et al., 2000). We have used this approach to profile the mediators and cell types within the initiation and resolution response (Figure 1), demonstrating that there is a clear temporal dissociation between the initiation and resolution phase of self-limited acute inflammatory response, self-limited in that it resolves on its own. We also devised quantitative indices in order to evaluate substances that regulate the magnitude and duration of the inflammatory response and its subsequent resolution (Bannenberg et al., 2005). Thus, if an acute inflammatory response does not resolve, the potential for it to progress to chronic inflammation and/or abscess formation may lie in the extent of the presence of proinflammatory mediators or, consequently, the potential loss and/or delay in the accumulation of proresolving mediators.

In murine peritonitis, the production of both PGE_2 and LTB_4 anteceded the production of D-series resolvins. Importantly, PGD_2 and PGE_2 levels linger during the time course of resolution

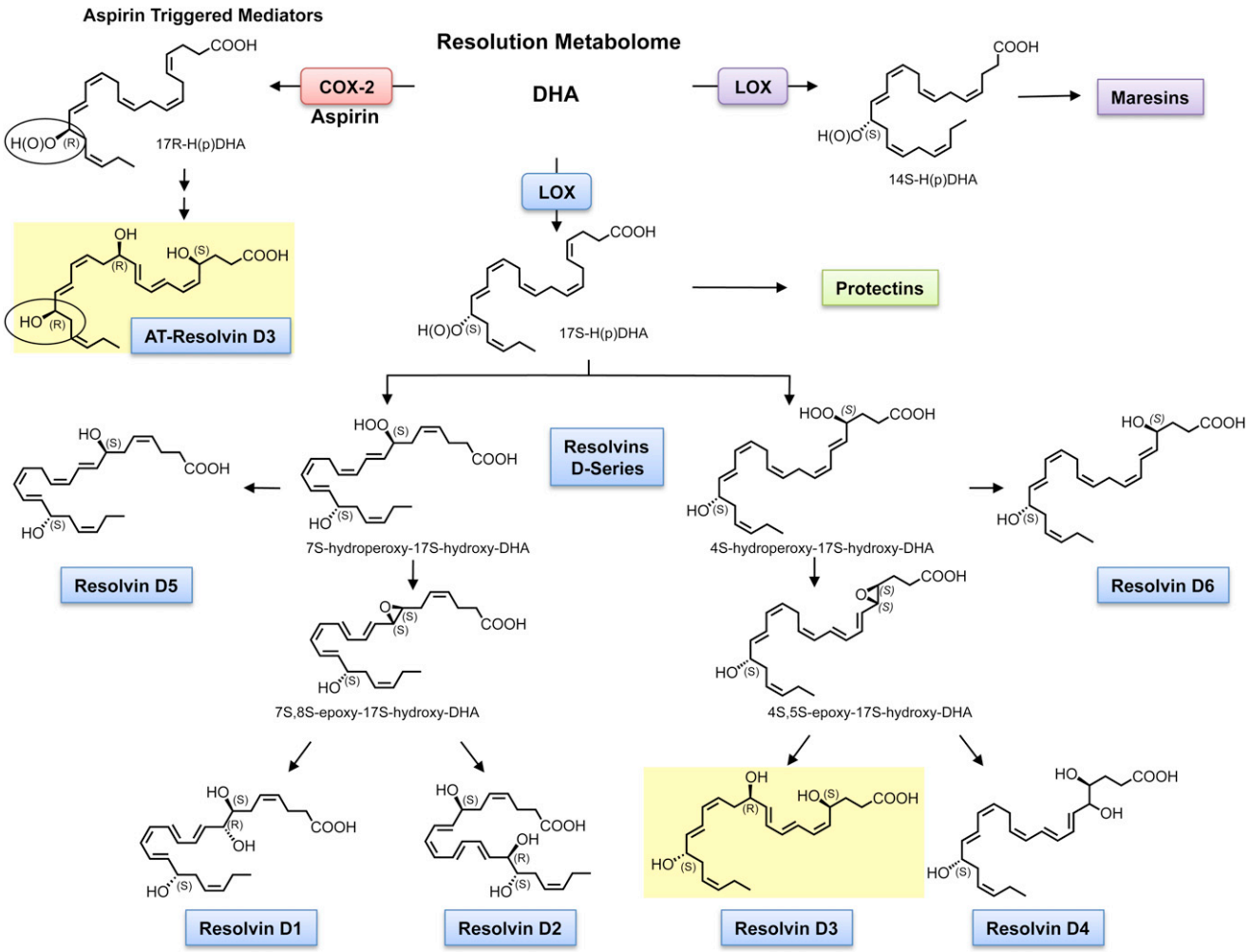

Figure 8. DHA Resolution Metabolome

Biosynthetic scheme for D-series resolvins and their relation to protectins and maresins. The position of RvD3 is depicted and that of the aspirin-triggered RvD3 within the D-series resolvins. Note that the complete stereochemistry of RvD1, RvD2, and RvD3 are established as shown. See text for further details.

(Figure 1B), and as demonstrated earlier, PGE_2 and PGD_2 in isolated human leukocytes activate the translation of messenger RNA to 15-LOX type I, a pivotal enzyme in production of lipoxins (reviewed in Romano, 2010) as well as D-series resolvins (Figure 1C). The finding that RvD3 persists well into the resolution phase and the return to homeostasis suggests that, in addition to counterregulating proinflammatory-initiating chemical mediators, such as the prostaglandins and cytokines, and regulating leukocyte traffic and functions, as demonstrated herein, RvD3 can also play a role in other metabolic processes relevant to homeostasis.

Resolvins regulate target cell expression of proinflammatory cytokines at transcriptional as well as at translational levels by interacting with specific receptors on the surface of leukocytes (Chiang et al., 2012). Proresolving mediators, including resolvins, also regulate adhesion molecules (i.e., CD11b/CD18) on leukocytes that downregulate cell-cell interactions (Dona et al., 2008; El Kebir et al., 2012). Although not formally addressed here in the context of RvD3, the reduction in proinflammatory cytokines and eicosanoids (i.e., LT) by this mediator along with its aspirin-triggered form are likely to also reflect the regulation of cell-cell interactions, as is the case with lipoxins, which reduce cell adhesion molecules, cell-cell interactions, and transcellular biosynthesis of lipid mediators (LM) (Brady and Serhan, 1992).

Aspirin, in addition to having an antithrombotic role and ability to block platelet thromboxane production (Samuelsson, 2012), was the lead agent used to produce nonsteroidal anti-inflammatories that could also block prostaglandin production (Vane, 1982). The well-appreciated antithrombotic properties of aspirin were revealed via the structural elucidation of thromboxane A_2 (Samuelsson, 1982). However, the anti-inflammatory actions of low-dose aspirin (75–81 mg doses) and its anti-inflammatory capacity with antineutrophil activities were not appreciated until the aspirin-triggered pathways for novel anti-inflammatory mediators were uncovered (Chiang and Serhan, 2004; Morris et al., 2009). It is now apparent from evidence obtained by many investigators (for example,

Clària and Planagumà, 2005 and references within Maderna and Godson, 2005 and Wu et al., 2012) that aspirin can jump-start resolution by not only blocking thromboxane and certain prostaglandins, but also by triggering aspirin-triggered lipid mediators, such as the aspirin-triggered lipoxins and the aspirin-triggered resolvins (Serhan et al., 2002).

Along these lines, aspirin-triggered lipoxin A_4 (LXA_4) is produced on dermal challenge (Morris et al., 2009), and a stable aspirin-triggered LXA_4 analog 15R/S-methyl-LXA_4 was found effective in treating infantile eczema in a double-blind two-center clinical trial (Wu et al., 2012). Hence, the complete structural elucidation of the aspirin-triggered lipid mediators (Chiang and Serhan, 2004; Serhan et al., 2002) can have far-reaching implications for clinical utility. In the present experiments, the aspirin-triggered 17*R* form of resolvin D3 proved to be highly potent (Figures 4, 5, 6, and 7), as is the case for other aspirin-triggered lipid mediators. The acetylation of cyclooxygenase-2 (COX-2) by aspirin imposes restrictions in size in the catalytic pocket, which blocks prostaglandin production, but still leads to the oxidation of polyunsaturated fats, such as arachidonic acid and DHA in vascular endothelial cells and mucosal epithelial cells. In the case of DHA, 17*R*-hydroxy-docosahexaenoic acid (17-HDHA) is produced. Albeit at low enzyme catalytic turnover levels, the 17*R*-HDHA precursor is produced by endothelial cells (Serhan et al., 2000, 2002) that have an extensive biomass in humans, in that the endothelium lines vessels from head to toe in all organs, making this a relevant site to produce 17*R*-HDHA in vivo (Serhan et al., 2002), as is the case for COX-2 in mucosal epithelia that also has an extensive biomass and surface area lining the barrier function of many organs (Louis et al., 2005; Serhan et al., 2002).

In the clinical scenario in the USA, aspirin is commonly administered at one of three doses, 81, 325, or 650 mg daily (Chiang et al., 2004; Furst and Hillson, 2001). The lower dose of 81 mg daily is the antithrombotic dose and gives both reduction in peripheral blood TXB_2 levels and a concomitant increase in AT-15-epi-LXA_4 in healthy volunteers (Chiang et al., 2004). Biosynthesis of AT-15-epi-LXA_4 following low-dose aspirin administration is activated on dermal challenge in human subjects and regulates neutrophil influx, demonstrating the anti-inflammatory actions of low-dose aspirin (Morris et al., 2009). This protective action of low-dose aspirin is a function of both age and gender, in that men older than 50 display reduced capacity to produce AT-lipid mediators (Chiang et al., 2006; Morris et al., 2010). Since RvD3 appears late in the resolution phase in murine exudates (Figure 1), the biosynthesis of its aspirin-triggered epimer would also be expected to occur late into the resolution phase at the site of inflammation within tissues. This, along with the findings that the aspirin-triggered epimer 17*R*-resolvin D1 is characteristically more resistant to enzymatic inactivation (Sun et al., 2007), likely leads to elevated local levels of RvD3 and AT-RvD3. Hence, AT-RvD3 may also provide an attractive biomarker of aspirin therapy in humans.

Studies addressing structure activity relationships of proresolving mediators demonstrate that R and S chirality at carbons 7 and 8 from the carboxylic acid end of DHA, i.e., RvD1, and carbons 5 and 6 in arachidonic acid, i.e., LXA_4, are critical in dictating the ability of these resolvins and lipoxins to engage their respective receptors (Serhan and Petasis, 2011), for example, 7*S*,8*R* in RvD1. These receptors appear to be more flexible with respect to the chirality of the hydroxyl group at carbon 17 for the resolvins and carbon 15 for the lipoxins, whereby mediators with either R or S stereochemistry at these positions are able to bind and activate their cognate GPCR to a similar extent (Chiang et al., 2000; Krishnamoorthy et al., 2012). This is also the case for RvD3 and its aspirin-triggered form, which we find here to bind and activate DRV1/GPR32 to a similar extent to each other as well as RvD1 (Figure 7).

It is noteworthy that 17-HDHA is present in the blood of healthy individuals (Mas et al., 2012; Psychogios et al., 2011) and is bioactive in animal disease models (Bento et al., 2011; Lima-Garcia et al., 2011), likely as a result of its local transformation by leukocytes to D-series resolvins (Serhan et al., 2002). In addition, the 17*R* aspirin-triggered lipid mediators, such as aspirin-triggered RvD1, remain elevated in tissues longer because they are less susceptible to local inactivation via dehydrogenation (Serhan, 2007). In this regard, aspirin-triggered RvD3 also proved to be a potent mediator in blocking neutrophil transmigration as well as enhancing proresolving responses (Figure 6), such as other aspirin-triggered members of the DHA metabolome (Serhan and Petasis, 2011). Regulating neutrophil responses can prevent collateral tissue damage and, unlike other agents that are anti-inflammatory, the resolvins and particularly D-series resolvins do this without immune suppression, as D-series resolvins, i.e., resolvin D2 and resolvin D5, enhance bacterial clearance in sepsis (Spite et al., 2009) and infections (Chiang et al., 2012).

Recently, lymph nodes were found to produce 17-HDHA, where it enhances antibody production, and the D-series resolvins and metabolome are also present in other lymphoid tissues of the mouse, such as spleen (Ramon et al., 2012). Hence, it is likely that, in addition, to the role of specialized proresolving mediators in bringing acute inflammation to homeostasis, preventing a potential for chronicity, these mediators may also play a role in acquired immunity. Although the levels in mice of n-3 essential fatty acids (e.g., EPA and DHA) and n-6 (e.g., arachidonic acid) (Weldon and Whelan, 2011) are different from those in human tissues (De Caterina, 2011), there is still a prevailing notion that n-3 fatty acids, such as DHA, have important actions in maintaining human health and preventing disease (Calder, 2012), including cardiovascular diseases (De Caterina, 2011). With the complete stereochemistry of RvD3 assigned in the present studies, namely its double-bond geometry, chirality of its alcohols, as well as anti-inflammatory and proresolving actions, they now permit gauging RvD3's role in the DHA metabolome of bioactive molecules.

SIGNIFICANCE

The results of the present report establish the stereochemistry of RvD3 and its aspirin-triggered form produced by human macrophages and in murine inflammatory exudates during inflammation resolution. These results also place the endogenous accumulation of RvD3 within the late phase of the acute inflammatory response. RvD3 and its aspirin-triggered form both displayed potent anti-inflammatory (i.e., regulating polymorphonuclear leukocyte tissue infiltration and proinflammatory mediator production) and

proresolving actions (i.e., stimulating macrophage efferocytosis and phagocytosis). The present findings provide opportunities to evaluate the role of the docosahexaenoic acid resolution metabolome (Serhan, 2007) that can also be evoked with aspirin treatment. These mediators facilitate the transition from acute inflammation to homeostasis without immunosuppression.

EXPERIMENTAL PROCEDURES

NMR of Synthetic RvD3

The following chemical shifts and coupling constants were recorded (for more details, see Figure 2): H_5 (5.42 ppm), H_6 (6.08 ppm, J = 4.4, 11.2 Hz), H_7 (6.59 ppm, J = 11.4, 14.7 Hz), H_8 (6.24 ppm, J = 10.7, 14.7 Hz), H_9 (6.33 ppm, J = 10.7, 15.1 Hz), H_{10} (5.75 ppm, J = 15.1, 6.6 Hz), H_{13} (5.47 ppm), H_{14} (6.08 ppm, J = 4.4, 11.2 Hz), H_{15} (6.52 ppm, J = 11.1, 15.3 Hz), H_{16} (5.70 ppm, J = 6.5, 15.1 Hz), H_{19} (5.44 ppm), and H_{20} (5.37 ppm).

Acute Inflammation

Male FVB mice (6 to 8 weeks old) purchased from Charles River Laboratories were fed ad libitum Laboratory Rodent Diet 20-5058 (Lab Diet, Purina Mills). All animal experimental procedures were approved by the Standing Committee on Animals of Harvard Medical School (protocol no. 02570) and complied with institutional and US National Institutes of Health (NIH) guidelines. Peritonitis: Zymosan (1 mg/ml; Sigma-Aldrich) was injected intraperitoneally (i.p.) 10 min after intravenous (i.v.) administration of RvD3, AT-RvD3 (10 ng), or vehicle (0.1% EtOH in 100 μl saline). Peritoneal lavages were collected 4–72 hr after zymosan administration. Leukocyte numbers and differential counts were assessed using Turks solution and flow cytometry analysis as detailed below. Cytokine and chemokine levels were assessed in cell-free supernatants by multiplex ELISA. Lavages were also placed in two volumes of methanol and subjected to lipid mediator metabololipidomics. Dorsal skin pouch: 2.5 ml of sterile air were injected subcutaneously on days 0 and 3. On day 6, mouse recombinant TNF-α (100 ng/mouse; R&D Systems) was administered into the airpouch in combination with either RvD3 or AT-RvD3 (10 ng/mouse). Four hours later, pouches were lavaged with Dulbecco's phosphate-buffered saline $(DPBS)^{-/-}$, leukocytes were enumerated, and a differential count was conducted; cytokine/chemokine and lipid mediator levels were assessed as above.

Flow Cytometry

Murine peritoneal and airpouch exudate cells were suspended in fluorescence-activated cell sorting (FACS) buffer (5% BSA in DPBS) and incubated with Fc block (15 min, 4°C; BD PharMingen) and then rat antimouse (from eBioscience) CD11b-PerCP/Cy5.5 (Clone:M1/70) and Ly6G- fluorescein isothiocyanate (FITC) (Clone 1A8) (30 min, 4°C) or appropriate isotype controls. Staining was assessed using FACSDiva CantoII (BD Biosciences) and analyzed using FlowJo (Tree Star).

Leukocyte Functional Responses

Macrophage Phagocytosis and Efferocytosis

To obtain apoptotic PMN, human PMN were isolated by density-gradient Ficoll-Histopaque from human peripheral blood. Blood was obtained from healthy human volunteers giving informed consent under protocol # 1999-P-001297 approved by the Partners Human Research Committee. PMN were labeled with carboxyfluorescein diacetate-succinimidyl ester (CFDA; 10 μM, 30 min, 37°C) and cultured overnight (5×10^6 cells/ml in $DPBS^{+/+}$). Mouse resident peritoneal macrophages (MΦ) were plated onto 96-well plates (Costar) at 5×10^4 cells/well and incubated with SPM (15 min, 37°C) followed by phagocytosis of fluorescent-labeled apoptotic PMN or FITC zymosan, as described (Dalli and Serhan, 2012). Fluorescent-labeled PMN were then added at a 3:1 ratio (PMN to macrophages).

Human macrophages were prepared from peripheral blood mononuclear cells (Dalli and Serhan, 2012). Macrophages (3×10^6 cells in a 10 cm petri dish) were transfected with pcDNA3 or with expression vector for human GPR32 (5 μg) using Jet-Pei transfection reagent following the manufacturer's instructions (Polyplus-transfection SA). After transfection (48 hr), cells were plated onto 96-well plates (50,000 cells/well), and phagocytosis was carried out 24 hr after replating. Expression of GPR32 was verified by flow cytometry using a polyclonal rabbit antihuman GPR32 antibody (GeneTex, GTX108119). Phagocytosis was then assessed vide supra. PMN transendothelial migration was performed as in Serhan et al. (2002).

Lipid Mediator Metabololipidomics

All samples for LC-MS-MS-based lipidomics were subject to solid-phase extraction as in Dalli and Serhan (2012). Prior to sample extraction, d_4-LTB_4 and d_4-PGE_2, representing each region in the chromatographic analysis (500 pg each), were added to facilitate quantification. Extracted samples were analyzed by a liquid chromatography-ultraviolet-tandem mass spectrometry system, QTrap 5500 (ABSciex) equipped with an Agilent HP1100 binary pump and diode-array detector. An Agilent Eclipse Plus C18 column (50 mm × 4.6 mm × 1.8 μm) was used with a gradient of methanol/water/acetic acid of 55:45:0.01 (v/v/v) to 100:0:0.01 at 0.5 ml/min flow rate. To monitor and quantify the levels of targeted LMs, we used multiple reaction monitoring (MRM) with signature ion fragments for each molecule (six diagnostic ions and calibration curves) (Dalli and Serhan, 2012). GC-MS was carried out as in Serhan et al. (2002).

GPCR-Beta-Arrestin System and Ligand-Receptor Interactions

Ligand-receptor interactions were monitored using the Beta Arrestin PathHunter system (Discoverx) and carried out essentially as in Krishnamoorthy et al. (2012), with CHO cells stably overexpressing recombinant human GPR32 (hGPR32: DRV1) receptors tagged with a prolink label of beta-galactosidase and beta-arrestin linked to the enzyme acceptor fragment of beta-galactosidase. Briefly, cells were plated in 96-well plates (20,000 cells/well) 24 hr prior to initiating experiments. Test compounds were incubated with cells (60 min, 37°C), and receptor activation was determined by measuring chemiluminescence using the PathHunter detection kit (Discoverx).

Ligand Selectivity Using ECIS

Ligand-receptor interactions were monitored by measuring impedance across cultured CHO-hGPR32 cell monolayers using an ECIS (Applied Biophysics) and carried out as in Krishnamoorthy et al. (2012). For antibody incubations, anti-GPR32 antibody (Ab) or rabbit serum was incubated with cells in the ECIS chambers at 1:50 dilutions for 30 min before addition of compounds.

Statistics

All data were expressed as means ± SEM. Differences between groups were compared using Student's t test (two groups) or one-way ANOVA (multiple groups) followed by a post hoc Bonferroni test. The criterion for statistical significance was $p < 0.05$.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and one table and can be found with this article online at http://dx.doi.org/10.1016/j.chembiol.2012.11.010.

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