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Vagus nerve controls resolution and pro-resolving mediators of inflammation

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Resolution of inflammation is now recognized as a biosynthetically active process involving pro-resolving mediators. Here, we show in zymosan-initiated peritoneal inflammation that the vagus nerve regulates local expression of netrin-1, an axonal guidance molecule that activates resolution, and that vagotomy reduced local pro-resolving mediators, thereby delaying resolution. In netrin-1−/− mice, resolin D1 (RvD1) was less effective in reducing neutrophil influx promoting resolution of peritonitis compared with Ntn1+/+. Netrin-1 shortened the resolution interval, decreasing exudate neutrophils, reducing proinflammatory mediators, and stimulating the production of resolvins, protectins, and lipoxins. Human monocytes incubated with netrin-1 produced proresolving mediators, including resolvins and lipoxins. Netrin-1 and RvD1 displayed bidirectional activation in that they stimulated each other’s expression and enhanced efferocytosis. These results indicate that the vagus nerve regulates both netrin-1 and pro-resolving lipid mediators, which act in a bidirectional fashion to stimulate resolution, and provide evidence for a novel mechanism for local neuronal control of resolution.

Acute inflammation is part of the complex response to tissue injury and infection by invading microorganisms. In the initial phase of the acute inflammatory response, immune cells extravasate and migrate to the site of inflammation guided by chemokines and eicosanoid mediators (Serhan et al., 2008; Tabas and Glass, 2013). In the resolution phase, specialized lipid mediators (SPM) endowed with novel pro-resolving properties are biosynthesized, which stimulates active resolution of tissue insult (Serhan and Savill, 2005). The resolution phase limits excessive neutrophil infiltration into affected organs, namely cessation of PMN influx and the clearance of apoptotic PMN and cellular debris by macrophages (Mφ; Serhan and Savill, 2005). Excessive PMN within tissues can give rise to sustained local inflammation via further tissue destruction that often leads to the eventual loss of organ function (Asfaha et al., 2001; Serhan and Savill, 2005; Charo and Ransohoff, 2006; Tabas and Glass, 2013).

Neuronal guidance proteins present in peripheral tissues contribute to the local control of leukocyte migration and inflammation (Wu et al., 2001; Rao et al., 2002; Mirakaj et al., 2011a). Netrin-1 is one of these neuronal proteins, which acts as a chemoattractant in axonal migration (Comamarino and Tessier-Lavigne, 1995; van Gils et al., 2012). Recently, netrin-1 was found to direct leukocyte traffic during acute inflammation in peripheral organs (Ly et al., 2005; Rosenberger et al., 2009; Mirakaj et al., 2010, 2011b; Aherne et al., 2012). With an acute inflammatory response, complete resolution of tissue inflammation is the ideal outcome for affected tissues to regain function (Bannenberg et al., 2005; Serhan and Savill, 2005; Serhan and Petsas, 2011). Although netrin-1 clearly exerts antiinflammatory and protective properties (Ly et al., 2005; Rosenberger et al., 2009; Mirakaj et al., 2010, 2011b; Aherne et al., 2012), whether netrin-1 serves to stimulate endogenous resolution...
programs remained to be explored. In this regard, resolution and eventual homeostasis are achieved via limiting further infiltration of neutrophils (PMN), as well as promoting local clearance via Mφ phagocytosis of cellular debris, apoptotic cells, and/or invading organisms (Serhan and Savill, 2005) orchestrated by novel local chemical mediators, i.e., resolvins, that possess this defining action (Serhan et al., 2002; Serhan and Petasis, 2011). Tracey (2002) uncovered neuronal reflex circuits that sense peripheral inflammation and provide regulatory feedback via specific nervous signals that regulate immune responses (Olofsson et al., 2012).

Because it is now clear that endogenous antiinflammation and pro-resolution are not equivalent processes in that pro-resolving mediators both stop/limit PMN entry and enhance nonspecific phagocytosis by Mφ (Serhan and Savill, 2005; Serhan and Petasis, 2011), we questioned whether the vagus nerve, netrin–1 expression, and local endogenous resolution programs are linked and impact the resolution of acute inflammation. Here, we report a new circuit involving the vagus nerve, netrin–1, and novel lipid mediators that control the resolution of inflammation.

RESULTS

Vagotomy regulates netrin and resolution of inflammation

Because vagotomy regulates the magnitude of the inflammatory response (Tracey, 2002), we assessed the impact of vagotomy on netrin–1 expression and in peritonitis. To this end, C57BL/6 mice were subjected to unilateral vagotomy (or a comparable sham surgical procedure) 7 d before initiating inflammation. Using Western blot analysis, we determined that in vagotomized mice, netrin–1 mRNA expression was significantly reduced in organs innervated by the vagus nerve, such as the lung and the peritoneum (Fig. 1), and protein expression was reduced as well. Of interest, during peritonitis, total exudate leukocyte numbers, PMN, and the marker myeloperoxidase were significantly increased in vagotomized mice following challenge compared with sham-operated mice (data not shown). Determination of the resolution indices (Bannenberg et al., 2005) in vagotomized animals demonstrated a clear delay in resolution, with an increase in the resolution interval from R0 = 24 h to R1 = 37 h (Fig. 1 C); also, proinflammatory cytokine and chemokine levels were elevated in inflammatory exudates from vagotomized mice compared with sham mice (Fig. 1 D).

Acetylcholine, the principal neurotransmitter involved in the inflammatory reflex, and netrin–1 were both significantly attenuated in exudates from vagotomized animals (Fig. 1, E and F). Together, these findings suggest that vagotomy regulates the resolution interval, leukocyte exudate cell numbers, chemokines, and cytokines and that netrin–1 is involved in this neuronal–inflammatory–resolution circuit.

Vagotomy impacts resolution programs: rescue with RvD1

Vagotomy shifted the profile of lipid mediators to increase the proinflammatory mediators, i.e., leukotriene B4 (Fig. 2), and decrease the pro-resolving mediators such as RvD1 in exudates. Each mediator was identified using LC–MS–MS–based metabololipidomics and signature diagnostic ions (Fig. 2). Within exudates, pro-resolving mediators are present, e.g., RvD1, LXA4, and PD1 (Fig. 2 B). These were sharply reduced in exudates from vagotomized mice throughout the time course (Fig. 2 C and Table 1). These exudates normally resolve by 24 h, as observed in sham-operated control mice (Fig. 2 C inset and Table S1).

Because vagotomy reduced RvD1 levels, we tested whether RvD1 could rescue the hyperinflammatory response obtained with vagotomy. Indeed, results in Fig. 3 indicate that administration of RvD1 rescued the excessive increased PMN infiltration that resulted from vagotomy (caused by vagus nerve cutting). RvD1 stopped PMN influx in the vagotomized animals and regulated the exudate monocyte recruitment (Fig. 3 A). In these exudates, RvD1 also reduced TNF, IL–6, KC (IL–8 in humans), MIP–1α, and HMGB–1 (Fig. 3 B). Also, netrin–1 reduced R1, when given at either T0 (Fig. 4 A) or at the maximum PMN infiltration (Fig. 4 B). In both cases, netrin–1 shortened the time required to resolve inflammation.

RvD1 pro-resolving capacity is reduced in Ntn1+/− mice

To examine potential interactions between netrin–1 and RvD1, we used Ntn1+/+ and Ntn1+/− mice in a peritonitis model. First, we determine the endogenous impact of netrin–1 and quantified the process in the resolution of acute inflammation by performing the resolution indices up to 48 h. The results from this demonstrated a delay in resolution with an increase of the resolution interval (R1) by up to 2 h in Ntn1+/− mice compared with Ntn1+/+. The administration of RvD1 shortened the R1 in WT mice, whereas this action was not found with netrin–1–deficient mice, suggesting that the action of RvD1 in vivo in this system is mediated at least in part via endogenous netrin–1 (Fig. 4, C and D).

Vagotomy reduces netrin–1 expression in pulmonary nerves

As we considered that the netrin–1 expression is likely regulated neurally, we performed immunohistochemistry staining of lungs from vagotomized or sham–operated mice to determine netrin–1 expression. We identified reduced expression of netrin–1 within lungs from vagotomized mice, especially on neurofilament structures (Serhan and Petasis, 2005; Serhan and Savill, 2005).

Netrin–1 is expressed in immunocompetent cells

To clarify the source of netrin–1 during inflammation, we determined the netrin–1 expression in immunocompetent cells. We found robust expression of netrin–1 in leukocyte subsets including CD15+ granulocytes, CD14+ monocytes, CD68+ Mφ, some CD3+ T lymphocytes, and CD19+ B lymphocytes (Fig. 5 B).

Netrin–1 enhances pro-resolving lipid mediator biosynthesis

Because SPM play pivotal roles activating resolution circuits (Serhan and Savill, 2005; Serhan and Petasis, 2011), to determine whether netrin–1 impacts SPM biosynthesis during inflammation–resolution, we performed LC–MS/MS–based profiling with inflammatory exudates. In these, netrin–1 increased protectin D1 (PD1) and resolvin D5 (RvD5; Fig. 6). Netrin–1 also significantly increased 15–HETE and 12–HETE.
RvD1 increases expression of the adenosine 2B receptor (A2BAR)

To ensure that leukocytes express receptors for netrin-1, we focused on the expression of A2BAR, Unc5B, neogenin, and the DCC. We further addressed whether RvD1 influences the expression of these receptors. The collected results showed that these receptors were expressed on human leukocytes; however, only the A2BAR is significantly induced by RvD1 (unpublished data).

Netrin-1 reduces human PMN chemotaxis and increases Mø efferocytosis: synergy with RvD1

Assessment of the antiinflammatory and pro-resolving properties displayed by netrin-1 with human peripheral blood cells from arachidonic acid as well as docosahexaenoic acid-derived lipoxygenase products (Fig. 6 C). Levels of the chemoattractant LTB4 were significantly reduced (at 4 h) in mice given netrin-1 compared with exudates from mice challenged with ZyA alone (Fig. 6 C). In contrast, at 4 h, PGD2 and PGE2 levels were significantly elevated by netrin-1, whereas at 12 h netrin-1 significantly decreased both (Fig. 6 C). To translate this to human cells, we examined whether netrin-1 has an impact on human monocyte production of lipid mediators and performed LC-MS/MS-based LM-profiling in human monocyte incubations. Netrin-1 increased the pro-resolving mediators LXA4, LXB4, RvD1, and RvE2, and decreased TNF-stimulated production of PGD2, PGE2, PGF2α, and TXB2 (Fig. S1 and Table S2).
Figure 2. Vagotomy shifts exudate lipid mediator profile to proinflammatory. Mice were subjected to right-sided cervical vagotomy or sham surgery and then challenged with 1 mg zymosan. Peritoneal exudates were collected at the indicated intervals and lipid mediator profiles determined by lipid mediator metabololipidomics (see Materials and methods). (A) MRM chromatograms for the identified lipid mediators in murine exudates. a and b denote LTB₄ isomers: a = Δ6-trans-LTB₄; b = 12-epi-Δ6-trans-LTB₄. (B) Accompanying MS/MS spectra used for identification. (C) Levels of D-series resolvins, protectins, and maresins, lipoxins, and leukotriene B₄, prostaglandin, and thromboxane measured at the indicated time points. Pie charts show the relative contribution of...
PMN revealed that netrin-1 reversed fMLP-induced CD62L shedding and CD11b neo-epitope exposure (Fig. 7 A). At concentrations as low as 1 nM, netrin-1 inhibited PMN migration (Fig. 7 B) and did not display chemotactic activities on its own in this concentration range (n = 4). RvD1 also stops PMN migration (Kasuga et al., 2008). Because resolution of lipid mediators. Blue shading denotes proresolving and red shading denotes pro-inflammatory Results are expressed as mean ± SEM; n = 4 mice per time point for two independent experiments. *, P < 0.05; **, P < 0.01 versus inflammation mice at the respective interval.

Table 1. Vagotomy shifts to proinflammatory lipid mediators-metabololipidomics

<table>
<thead>
<tr>
<th></th>
<th>Q1</th>
<th>Q3</th>
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<td></td>
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<td>SEM</td>
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<td></td>
<td>pg/exudate</td>
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<td>pg/exudate</td>
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<td>141</td>
<td>*</td>
<td>*</td>
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<td>RvD3</td>
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<td>147</td>
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<td>*</td>
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<td><strong>EPA bioactive metabolome</strong></td>
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<td>RvE1</td>
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<td>195</td>
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<td>3.5 ± 2.5</td>
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Individual lipid mediators and precursor/pathway markers at: Q1, M-H (parent ion); and Q3, diagnostic ion in the MS/MS (daughter ion) along with mean ± SEM values for each of the mediators (pg/exudate) identified in the 4-h exudates. Detection limit, ~1 pg. *, below limits.
endothelial cells (HMEC) and epithelial A549 cells exposed to netrin-1. With both cell types, netrin-1 significantly increased the expression of mRNA for human GPR32 and ALX/FPR2 and increased protein expression for both receptors (unpublished data).

**DISCUSSION**

The present results demonstrate a novel vagus-resolution circuit that involves pro-resolving lipid mediators and netrin-1. Netrin-1 reduces local injury and inflammatory responses (Wu et al., 2001; Mirakaj et al., 2010; 2011a,b). Here, we find that netrin-1 stimulates resolution mechanisms and production of resolvins (Figs. 3 and 7) and, thus, by definition (Serhan, 2011) is an innate immune system resolvent. Netrin-1 decreased PMN recruitment in vitro and in vivo, and the resolution interval was significantly shortened as a quantitative measure of resolution. Netrin-1 also increased nonphlogistic mononuclear cell recruitment and MΦ uptake of apoptotic PMNs. Together, these findings indicate that local netrin-1 is actively involved in the resolution mechanisms of acute inflammation in both lung and abdominal tissues, stimulating production of pro-resolution lipid mediators, defining a novel neuronal-resolvin circuit regulated by the vagus nerve.

**Figure 3.** RvD1 rescues hyperinflammation in vagotomized mice. Vagotomized or sham-operated control mice were injected i.p. with zymosan A (ZyA) and subsequently with RvD1 (RvD1). Peritoneal lavages were collected at indicated time points. (A) Total leukocytes were enumerated by light microscopy. PMN, classical monocytes, and nonclassical monocytes were determined by flow cytometry. (B) TNF, IL-6, KC, MIP-1α, and HMGB1 were measured in exudates by ELISA. Results are mean ± SEM, n = 6 per group for two independent experiments. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
During the acute inflammatory response, leukocytes (Nathan, 2002) diapedese and migrate to the site of inflammation guided by gradients of chemical signals, including mediators such as chemokines (Serhan et al., 2008; Tabas and Glass, 2013) and lipid mediators (Serhan and Savill, 2005; Serhan and Petasis, 2011; Samuelsson, 2012). These are critical components and steps in the initial response and host defense. If in excess or persistent, the initial inflammatory response that does not terminate or resolve can lead to collateral tissue destruction with chronicity and eventual loss of functional organ integrity (Serhan and Savill, 2005; Serhan et al., 2008). Because excessive leukocyte migration into peripheral tissues can have serious consequences, checkpoints exist at several levels to regulate local leukocyte migration and tissue infiltration (Nathan, 2002; Serhan, 2007). The circulation of leukocytes and the integrity of the vascular endothelial barrier are known impediments to aberrant infiltration (Muller, 2002). On initiation of inflammation, the resolution phase is concomitantly activated, which is a highly coordinated and biochemically active process that was once thought to be passive at the tissue level, i.e., simple dilution of the initiating chemical mediators (Bannenberg et al., 2005; Serhan and Savill, 2005; Serhan and Petasis, 2011). The process of resolution is controlled in part by the biosynthesis of endogenous chemical lipid mediators that are local autacoids stimulating, as novel agonists, pro-resolving mechanisms (Serhan and Petasis, 2011; Dalli and Serhan, 2012).
Netrin-1 activates resolution programs | Mirakaj et al.

In the present experiments, we questioned whether vagotomy impacts resolution processes and found that netrin-1 is an endogenous pro-resolution signal. Netrin-1, from the results herein, fulfills the definition of an immunoresolvent (Serhan, 2011) via its interactions with leukocytes and pro-resolving lipid mediators in the context of acute inflammation and tissue injury. Our results also reveal that mice with partial netrin-1 deficiency (Ntn1+/−) displayed increased leukocyte infiltration, and RvD1 was less effective at limiting PMN entry compared with WT littermates, illustrating the bidirectional relationship between netrin and resolvins. This finding is consistent with the ability of netrin-1 to dampen the inflammatory response in the initial phase of an acute inflammation. Using murine peritonitis and resolution indices (Bannenberg et al., 2005), we found that netrin-1 decreased PMN recruitment and protein levels in exudates during the course of inflammation. Netrin-1 also reduced proinflammatory cytokines including TNF and up-regulation of antiinflammatory cytokine IL-10 (Fig. 1). Importantly, netrin-1 administered at the peak of PMN infiltration during inflammation significantly decreased PMN numbers at later time intervals (Fig. 4). This role of netrin-1 is embedded into a neuronal reflex circuit via the vagus nerve that enables it to influence the resolution of inflammation.

During the initial events in inflammation, classic eicosanoids including prostaglandins and leukotrienes play pivotal roles as local mediators (Serhan and Petasis, 2011; Samuelsson, 2012). The prostaglandins PGE2 and PGD2 switch the mediators produced, an event called mediator class switching, which involves the biosynthesis of SPM, which actively limits inflammation by cessation of PMN influx and enhances Mφ-mediated clearance to promote resolution (Levy et al., 2001; Serhan, 2007). Accordingly, in the early inflammatory events we found that netrin-1 lowered LTB4, a primary chemoattractant, and temporally regulated both PGD2 and PGE2, which are key in mediator class switching in resolution (Levy et al., 2001). For the pro-resolving mediators, both RvD5 and PD1 exudate levels were increased by netrin-1. We also found a significant decrease in endogenous netrin-1 at 4 h in inflammatory exudates that were enhanced in early resolution (Fig. 3). These results likely reflect the local activation of pro-resolving signals by netrin-1 because both RvD5 and PD1 were dramatically increased in vivo.

In addition to limiting PMN migration, resolution of inflammation is defined by an increase in the local Mφ uptake of apoptotic PMNs and their removal (Bannenberg et al., 2005; Serhan and Savill, 2005). Netrin-1 decreased PMN migration (Fig. 4 C) and increased the uptake of apoptotic PMN. Both RvD1 and netrin-1 enhanced efferocytosis, giving substantial synergy. Recently, Liu et al. (2012) demonstrated that in neuronal degenerative diseases such as amyotrophic lateral sclerosis, with human Mφ from these patients, RvD1 potently reduced the production of inflammatory cytokines from these patients. In this context with human cells, RvD1 activates both the GPCR lipoxin receptor (ALX/FPR2) and the human GPR32 RvD1 receptor denoted DRV1 (Serhan and Petasis, 2011), and its leukocyte actions are abolished in receptor-deficient mice (Norling et al., 2012). In the present experiments, netrin-1 significantly increased the mRNA and protein expression of both ALX/FPR2 and GPR32 receptors in human endothelial and epithelial cells. Collectively, these results demonstrate novel interactions between netrin-1 and resolvins, each controlled by the vagus nerve, that are essential mechanisms in the resolution of acute inflammation.
be relevant in other inflammation-associated diseases and organ fibrosis.

Along these lines, netrin-1 promotes atherosclerosis by inhibiting the emigration of MΦ from plaques (van Gils et al., 2012), supporting a potential failure of local vascular resolution mechanisms contributing to atherosclerosis (Merched et al., 2008). Because netrin-1 evokes both positive and negative axonal migration, as well as actions with PMN, monocytes, and MΦ, netrin-1 may have divergent roles in regulating the extent of inflammation and its resolution that depend on the organ. Given the ability of netrin-1 to stop and temporally limit PMN recruitment, stimulate resolution, and SPM production (e.g., resolvins; Fig. 2), netrin-1 may also be relevant in other inflammation-associated diseases and organ fibrosis.

Combining pro-resolution mediators, e.g., RvD1, together with the axonal guidance molecule netrin-1 appears to be a useful approach to activate resolution of inflammation and protection of organs from injury from within, events controlled by vagus nerve as documented herein. Moreover, our present findings demonstrate that netrin-1 is a direct agonist of resolution, i.e., shortening the resolution interval, activating pro-resolving lipid mediator production, limiting PMN influx, and increasing MΦ efferocytosis of apoptotic PMN. The finding that vagus...
MATERIALS AND METHODS

Vagotomy. C57BL/6 mice were subjected to sham surgery, unilateral vagotomy, zymosan A administration, or a combination of vagotomy and zymosan A application. To inhibit the cholinergic antiinflammatory pathway, mice were subjected to unilateral (right side) cervical vagotomy or sham surgery 7 d before induction of peritonitis. A unilateral vagotomy was chosen because bilateral vagotomy is lethal in mice. Animal protocols were approved by the Regierungspräsidium Tübingen. Ntn1+/− and Ntn1−/− animals were bred on a CD1 background (Rosenberger et al., 2009). In brief, mice were anesthetized and a ventral cervical midline incision was performed to expose the right cervical vagus trunk, which was ligated with a 4-0 silk suture and then divided. 7 d later, peritoneal lavages and organ tissues were obtained at 0, 4, 12, 24, and 48 h after initiating peritonitis.

Murine peritonitis. Mouse protocols were approved by the Harvard Medical Area Standing Committee on Animals. C57BL/6 mice were injected i.p. with 1 ml zymosan A (ZyA; 1 mg/ml, Sigma-Aldrich) and subsequently with vehicle (saline plus 0.2% BSA) or 1 µg murine recombinant netrin-1 (R&D Systems) in 150 µl total volume. Recruited leukocytes were obtained at time points of 4, 12, 24, 48, and 72 h by peritoneal lavage with PBS solution containing 10 U/ml unfractionated heparin (Mimak et al., 2011b). Collected cells were washed, suspended in Hank’s balanced salt solution (2 ml), counted and cytopsin samples prepared. All reagents used were endotoxin-free.

Proteins: transcriptional and protein analysis. For Western blot analysis, animal samples were homogenized, normalized for protein levels, and applied to SDS containing polyacrylamide gels. Antibodies used for Western blotting included polyclonal rabbit anti-netrin-1 (Santa Cruz Biotechnology, Inc.) for murine netrin-1 analysis. Actin was stained using polyclonal rabbit anti–mouse Ly6G (eBiosciences) and FITC-conjugated anti-mouse Ly6G (BioLegend) for 30 min on ice. After washing, cell pellets were suspended in FACS buffer and analyzed using flow cytometry using BD FACS Diva Software. Cytokines were measured in the peritoneal exudates obtained from mice using standard ELISA (R&D Systems and Biocompare).

Resolution indices. The key processes in the resolution of acute inflammation can be quantified using resolution indices as defined in (Bannenberg et al., 2005) and used herein as follows: magnitude (Ψmax; Tmax), the time point (Tmax) after T0 when neutrophil numbers in tissues or exudates reach maximum (Ψmax); duration (Tmax; T50), the time point (T50) when the PMNs reduce and nonphlogistic monocyte numbers intercept nerve signals proteins and pro-resolving lipid mediators involved in axonal migration that carry additional functions outside the central nervous system opens new directions in our appreciation of the resolution of inflammation within innervated organs. These may also be useful in designing new therapeutic approaches for inflammation associated with surgery and/or traumatic tissue injury.

Figure 7. Netrin-1 reduces human PMN activation and increases Mø efferocytosis: Synergy with RvD1 pathway. (A) Isolated human PMN were incubated with either fMLP or netrin-1 for 30 min and the expression of CD11b (neo-epitope) and CD62L on PMN was measured by flow cytometry. (B) Isolated human PMN were placed on chemotaxis slides and exposed for 2 h to either fMLP or netrin-1. The cell migration was monitored for a 10-min period and analyzed using Image Pro Plus 7 software. (C) Mø differentiated from peripheral blood monocytes were incubated with indicated concentrations of netrin-1. Carboxyfluorescein diacetate-labeled apoptotic PMN were added at 1:3 ratio (Mø:PMN), and the rate of efferocytosis was determined after 1 h by measurement of fluorescence. (D) Mø differentiated from peripheral blood monocytes were incubated with netrin-1 in the absence or presence of RvD1. The degree of efferocytosis was performed using a fluorescent plate reader. (E) Peritoneal Mø from WT or 12/15LO−/− mice were incubated with the indicated concentrations of netrin-1 (15 min, 37°C), the fluorescently labeled apoptotic PMN were added and the degree of efferocytosis was assessed after 1 h (37°C) using a fluorescent plate reader (see Materials and methods). Results are representative of five independent experiments.
∼50% of \( \Psi_{\text{min}} (R_{\text{max}}) \); resolution interval (\( R_{\text{b}} \)) the time interval from maximum neutrophil and monocyte infiltration intersection time point (\( \Psi_{\text{min}} \)) to 50% reduction point \( (R_{\text{b}}, i.e., T_{\text{b}} - T_{\text{max}}) \).

**Lipid mediator metabololipidomics.** Murine peritoneal exudates or human monocyte incubations were extracted using solid-phase extraction with C-18 columns (6 mL Waters), after 2 vol of cold methanol containing deuterated internal standards (0.5 mg d\(_5\)-PGd\(_2\), d\(_5\)-LTB\(_4\), and d\(_5\)-SS-HETE) were added as in (Dalli and Serhan, 2012). Lipid mediator levels were assessed by LC-MS/MS using an HPLC system (LCZQ; Shimadzu) with a linear ion trap quadrupole mass spectrometer (QTRAP 5500; Applied Biosystems) equipped with an Eclipse Plus C18 column (4.6 × 50 mm × 1.8 mm or 4.6 × 100 mm × 1.8 mm; Agilent Technologies). Data acquisition was performed in negative ionization mode and identification was conducted using published criteria that included a minimum of matching 6 diagnostic ions for MS-MS spectra and retention times to those of authentic and synthetic standards. Calibration curves were determined using synthetic lipid mediator (LM) mixture that included d\(_5\)-SS-HETE, d\(_5\)-LTB\(_4\), d\(_5\)-LXA\(_4\), d\(_5\)-PGD\(_2\), RvD1, RvD2, RvD3, PD1, MaR1, RvE1, RvE2, LXA\(_4\), LXB\(_4\), PGE\(_2\), PGD\(_2\), PGE\(_4\), LTB\(_4\), 17-HDHA, 14-HDHA, 7-HDHA, 4-HDHA, 18-HEPE, 15-HEPE, 12-HEPE, 5-HEPE, 15-HETE, 12-HETE, and 5-HETE, each at 50, 100, 200, 400, 800 pg. Linear calibration curves for each were obtained with \( r^2 \) values of 0.98–0.99. Quantification was performed using the peak area from Multiple Reaction Monitoring (MRM) transition for a specific LM and their linear calibration curve.

**PMN chemotaxis.** Isolated human PMNs were prepared by gradient centrifugation from peripheral blood of healthy volunteers (protocol #1999P001297 approved by the Partners Human Research Committee). Cells were suspended at 10 × 10\(^5\) cells per ml in PBC (PBS plus 0.2% BSA, pH 7.45). The cell chamber and the cells exposed to either fMLP or netrin-1 (see Results). The cells were then incubated at 37°C for 2 h before imaging to allow for the chemotactic gradient to generate. Cell migration was monitored for a 10-min period using a ×40 objective microscope (Nikon) equipped with a 20× phase contrast lens and Q Imaging Fast 1394 camera. Cell migration was analyzed using the image analysis software (MediaCybernetics) and ImageJ (National Institute of Mental Health, Bethesda, Maryland).

**PMN surface antigen expression.** Whole-blood aliquots were incubated with either 10 nM fMLP or 10 nM netrin-1 for 30 min in a 37°C water bath with gentle mixing every 5 min. Red blood cells were lysed using 1 × RBC lysis buffer at 25:1 ratio with blood for 15 min in ice-bath. Direct immunofluorescence labeling was performed using FITC-conjugated anti-human CD11b (eBioscience) or PE-conjugated anti-human CD62L (eBioscience) in combination with the corresponding isotype controls in a 1:100 ratio in fluorescence-activated cell sorting (FACS) buffer for 30 min on ice. After washing, cell pellets were resuspended in FACS buffer and analyzed using FACSFlow (BD) and CellQuest software (BD).

**Flow cytometry.** Blood was collected, pooled, and incubated with erythrocyte lysing solution for 5 min at room temperature. The following antibodies were used: anti-netrin-1 (Santa Cruz Biotechnology, Inc.), anti-CD15 (Santa Cruz Biotechnology, Inc.), anti-CD41 (BD, anti-D68 (Santa Cruz Biotechnology, Inc.), and anti-CD3 (BD), and anti-CD19 (BD). FITC and PE fluorescence of species-matched secondary antibodies were evaluated using BD FACS Diva Software. For the determination of the receptors for netrin-1, isolated human PMNs were exposed for 60 min at 37°C to RvD1 (10 nM) or to recombinant netrin-1 (500 ng/ml) and the expression of A2B2AR (Santa Cruz Biotechnology, Inc.), UNC5B (Santa Cruz Biotechnology, Inc.), neogenin (Santa Cruz Biotechnology, Inc.), and DCC (Santa Cruz Biotechnology, Inc.) was evaluated using FACS Diva Software (BD).

**Immunofluorescent staining.** For murine immunofluorescence, lungs were embedded in parafilm and blocked with 5% BSA (Sigma-Aldrich). Rabbit polyclonal anti-netrin-1 (Santa Cruz Biotechnology, Inc.) was used as primary antibody. Rabbit IgG Isotype (Santa Cruz Biotechnology, Inc.) was used for negative control. FITC-conjugated goat anti-rabbit (Dianova) was used as secondary antibody. DAPI (4′, 6-diamidino-2-phenylindol; Invitrogen) was used for nuclear counter staining. For neuronal staining, mouse anti-neurofilament (Abcam) was used and Alexa Fluor 647-conjugated goat anti–chicken (Abcam) was applied as secondary antibody. Fluorescence microscopy LSM 510 Meta (Carl Zeiss) was used for imaging.

**Human Mφ efferocytosis.** For differentiation of Mφ, peripheral blood monocytes were isolated from human leukapheresis collars from the Children’s Hospital Boston Blood Bank and cultured in RPMI with 10 ng/ml human recombinant GM-CSF (R&D Systems; 37°C for 7 d). To prepare apoptotic PMN, human PMNs obtained from peripheral blood were isolated and labeled with carboxyfluorescein diacetate (10 µM, 30 min at 37°C; Invitrogen) and allowed to undergo apoptosis in serum-free RPMI for 16–18 h. Mφ (1.0 × 10\(^4\) cells/well) were incubated with human recombinant netrin-1 (R&D Systems) in the presence or absence of RvD1 (1 nM) Boc2 peptide (10 µM; Santa Cruz Biotechnology, Inc.) at 50 µg/µl and 100 µg/µl. Apoptotic PMNs were added at 1:3 ratio (Mφ/PMN) with phagocytosis incubations performed at 37°C for 60 min, and fluorescence was determined by using a fluorescent plate reader (SpectraMax; Molecular Devices).

**Transcriptional and protein analysis.** Transcriptional analysis of netrin-1 was performed using sense primer 5′-GAGCGGGGGGAGCTGTCTT-3′ and 5′-TGGTTTTGATTGCAAGTCTGT-3′ antisense primer. Samples were controlled for 18S using the following primers: sense, 5′-GTAACCCGTT-GAACCCCAT-3′; antisense, 5′-CCATCACTCGGATAGCCG-3′. Semi-quantitative analysis of mRNA was performed using real-time PCR (iCycler; Bio-Rad Laboratories).

**Data analysis.** Results are expressed as mean ± SEM. Statistical analyses were performed using the Student’s t test, one-way ANOVA, or two-way ANOVA was considered statistically significant.

**Online supplemental material.** Table S1 shows the lipid mediator levels in self-resolving exudates of vagotomized and sham operated mice. Fig. S1 and Table S2 show that Netrin-1 stimulates endogenous pro-resolving lipid mediators with human monocytes. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20132103/D1C.

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**REFERENCES**


Asfaha, S., W.K. MacNaughton, C.B. Appleyard, K. Chadee, and J.L. Wallace. 2001. Persistent epithelial dysfunction and bacterial translocation after...
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Muller, W.A. Leukocyte-endothelial cell interactions in the inflammatory response. Lab. Invest. 82:521–533. http://dx.doi.org/10.1038/ lainvest.3780446

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