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, resolvin 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.

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Abbreviations used: AT-RvD1, aspirin-triggered RvD1; LTB_4, leukotriene B_4; LXA_4, lipoxin A_4; LXB_4, lipoxin B_4; PGD_2, prostaglandin D_2; PGE_2, prostaglandin E_2; LC-MS/MS, liquid chromatography tandem mass spectrometry; Mϕ, macrophage; R_i, resolution interval; RvD1, resolvin D1.

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 proresolving 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 (Colamarino 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 Petasis, 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

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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 nonphlogistic 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 local 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 R_i = 24 h to R_i = 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 B_4 (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, LXA_4, 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 R_i when given at either T_0 (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 (R_i) by up to 2 h in *Ntn1*$^{+/-}$ mice compared with *Ntn1*$^{+/+}$. The administration of RvD1 shortened the R_i 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 neuronally, we performed immunochemistry 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 (Fig. 5 A).

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

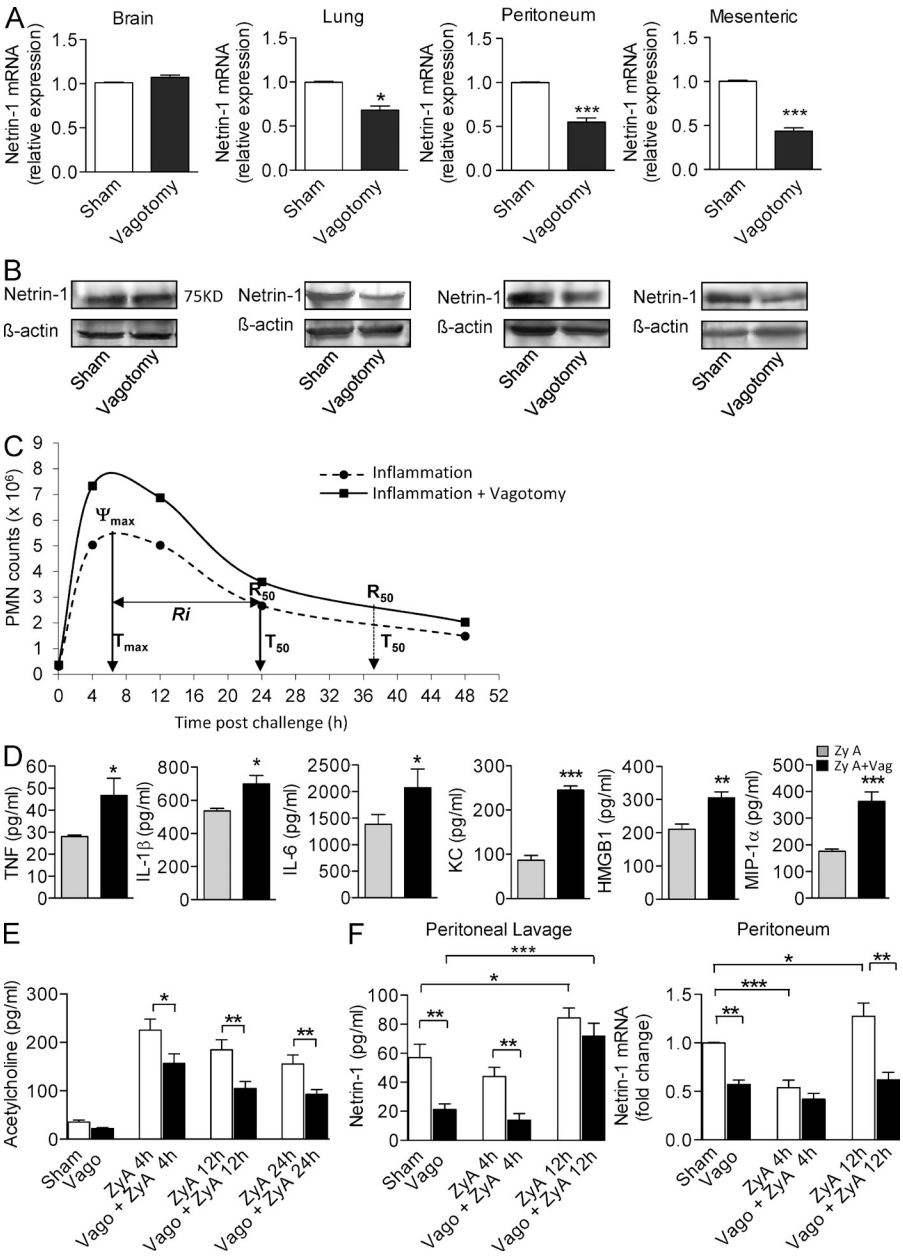

Figure 1. Vagotomy delays resolution of inflammation. Mice were subjected to right-sided cervical vagotomy or sham surgery. Netrin-1 mRNA (A) or protein (B) in the indicated tissues was quantified by quantitative real-time RT-PCR or Western blot, respectively. (C) Resolution indices as defined in (Bannenberg et al., 2005). (D and E) Mice were subjected to right-side cervical vagotomy or sham surgery and then injected i.p. with zymosan (1 mg/ml). Levels of TNF, IL-1β, IL-6, KC, MIP-1α, and HMGB1 in peritoneal lavage were measured by ELISA 4 h after zymosan injection. Acetylcholine (E) and netrin-1 levels (F, left) in peritoneal fluid at the indicated time points. Netrin-1 mRNA levels (F, right) in the peritoneal exudates were assessed by quantitative real-time RT-PCR at the indicated intervals. Results represent two independent experiments and are mean ± SEM, $n = 6$ mice per group. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

from arachidonic acid as well as docosahexaenoic acid-derived lipoxygenase products (Fig. 6 C). Levels of the chemoattractant LTB_4 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, PGD_2 and PGE_2 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 LXA_4, LXB_4, RvD1, and RvE2, and decreased TNF-stimulated production of PGD_2, PGE_2, $PGF_{2\alpha}$, and TXB_2 (Fig. S1 and Table S2).

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 Mϕ efferocytosis: synergy with RvD1

Assessment of the antiinflammatory and pro-resolving properties displayed by netrin-1 with human peripheral blood

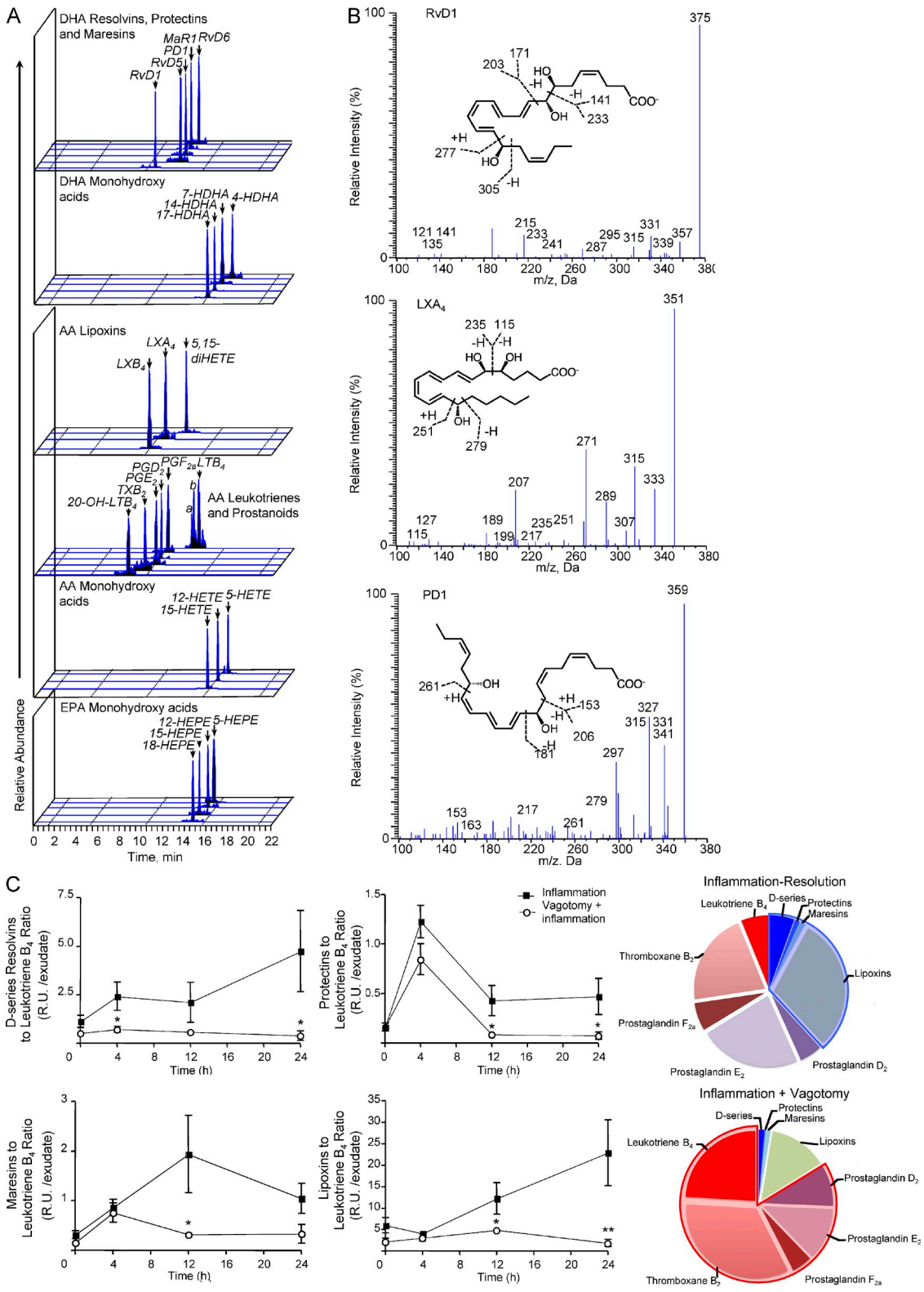

Figure 2. Vagotomy shifts exudate lipid mediator profile to proinflammatory. Mice were subjected to right-sided cervical vagotomy or sham surgery and then challenged with 1 mg zymosan. Peritoneal exudates were collected at the indicated intervals and lipid mediator profiles determined by lipid mediator metabololipidomics (see Materials and methods). (A) MRM chromatograms for the identified lipid mediators in murine exudates. a and b denote LTB_4 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 B_4, prostaglandin, and thromboxane measured at the indicated time points. Pie charts show the relative contribution of

Table 1. Vagotomy shifts to proinflammatory lipid mediators-metabololipidomics

	Q1	Q3	Inflam. time = 4 h			Vagotomy + Inflam. time = 4 h		
			Average		SEM	Average		SEM
			pg/exudate			pg/exudate		
DHA bioactive metabolome								
RvD1	375	215	27.7	±	3.8	8.5	±	2.1
RvD2	375	141		*			*	
RvD3	375	147		*			*	
RvD5	359	199	26.4	±	5.7	60.1	±	6.9
RvD6	359	159	4.8	±	1.2	5.9	±	2.2
4,14-diHDHA	359	101	10.5	±	1.9	30.1	±	3.2
MaR1	359	221	7.2	±	2.1	9.0	±	2.9
PD1	359	153	38.7	±	8.2	90.5	±	7.7
20-OH-PD1	359	153		*			*	
4-HDHA	343	101	151.8	±	54.7	104.1	±	16.9
7-HDHA	343	141	24.9	±	6.9	21.9	±	5.1
14-HDHA	343	205	10785.8	±	3675.5	7852.7	±	4296.4
17-HDHA	343	245	1249.6	±	473.2	2859.1	±	121.8
DHA	327	283	19793.0	±	7673.2	18514.9	±	689.9
AA bioactive metabolome								
LXB_4	351	115	4.7	±	2.4	4.2	±	0.7
LXB_4	351	221	43.2	±	18.5	60.6	±	13.9
5,15-diHETE	335	115	82.4	±	13.9	246.7	±	4.7
LTB_4	335	195	24.1	±	5.7	113.8	±	30.4
20-OH-LTB_4	351	195	4.4	±	1.2	6.2	±	2.7
PGD_2	351	189	79.1	±	35.4	84.6	±	7.9
PGE_2	351	233	198.3	±	19.6	295.0	±	67.4
$PGF_{2\alpha}$	353	193	86.9	±	13.5	76.2	±	19.9
TXB_2	369	169	133.3	±	9.4	266.5	±	32.9
5-HETE	319	115	368.9	±	92.9	364.1	±	95.0
12-HETE	319	179	14681.2	±	3618.1	11572.2	±	4095.4
15-HETE	319	219	857.2	±	202.8	660.4	±	212.5
AA	303	259	38.8	±	7.2	26.2	±	4.7
EPA bioactive metabolome								
RvE1	349	195		*			*	
RvE2	333	199		*			*	
LXA_5	349	115		*			*	
LXB_5	349	221		*			*	
5,15-diHEPE	333	115		*			*	
5-HEPE	317	115	20.2	±	4.5	12.8	±	1.7
12-HEPE	317	179	1176.1	±	147.1	801.9	±	116.0
18-HEPE	317	259	694.9	±	66.2	354.6	±	124.0
15-HEPE	317	219	16.3	±	4.5	17.9	±	2.4
EPA	301	257	3.5	±	2.5	8.6	±	0.7

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.

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.

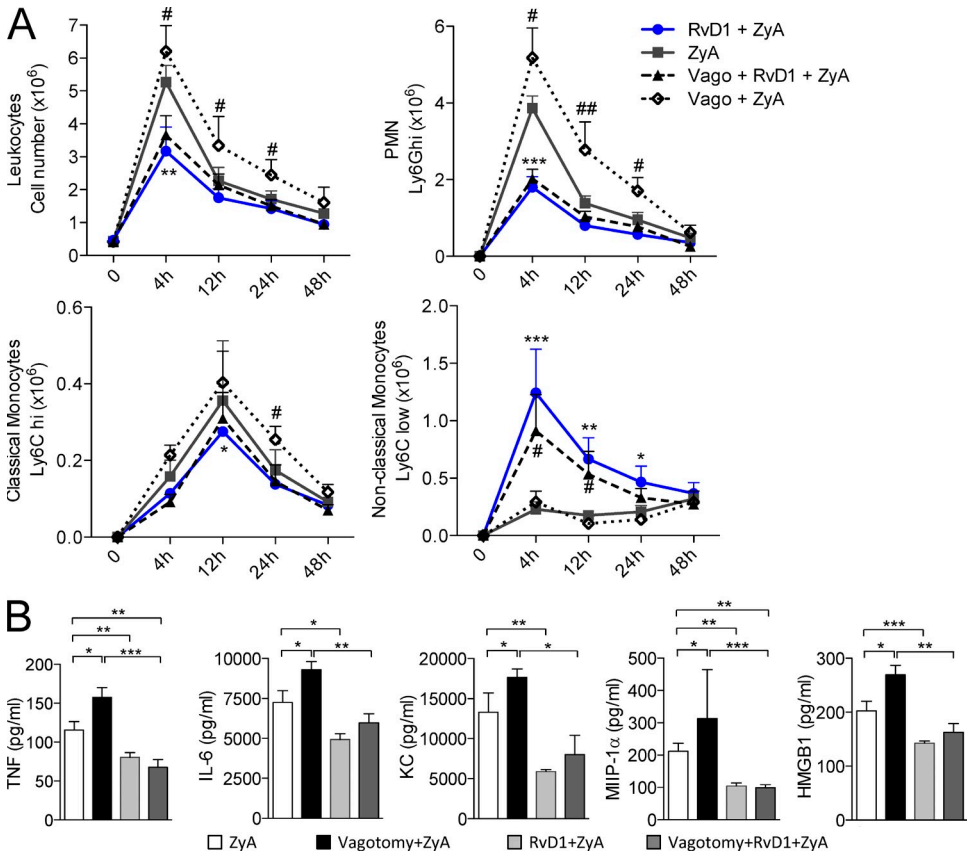

Figure 3. RvD1 rescues hyperinflammation in vagotomized mice. Vagotomized or sham-operated control mice were injected i.p. with zymosan A (ZyA) and subsequently with RvD1 (RvD1). Peritoneal lavages were collected at indicated time points. (A) Total leukocytes were enumerated by light microscopy. PMN, 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. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

inflammation is a dynamic process with one of the key steps being the clearance of apoptotic PMN by Mϕ (Serhan and Savill, 2005), we next assessed the ability of netrin-1 to regulate this pro-resolving mechanism. Netrin-1 stimulated the uptake of apoptotic PMN by human Mϕ in a dose-dependent manner (Fig. 7 C). These results were further corroborated in vivo, where netrin-1 enhanced Mϕ clearance of apoptotic PMN (unpublished data) in mice.

Co-incubations of netrin-1 and RvD1 with primary human Mϕ stimulated efferocytosis to levels above those displayed by either alone (Fig. 7 D). The actions of netrin-1 on Mϕ efferocytosis and phagocytosis were 12/15-LOX dependent because 12/15-LOX$^{-/-}$ mice displayed reduced efferocytosis (Fig. 7 E) that was also reduced >85% with phagocytosis of fluorescently labeled opsonized zymosan (not depicted). Because 12/15-LOX in mice is the enzymatic first step to RvD1 production (Serhan and Petasis, 2011), these results demonstrate the requirement of this LOX–resolvin pathway for netrin-1 actions in resolution.

Because RvD1 activates two GPCRs present on human Mϕ, the lipoxin receptor (ALX/FPR2) and DRV1/GPR32 (Krishnamoorthy et al., 2010), we next evaluated mRNA expression for these two receptors in both human microvascular 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.

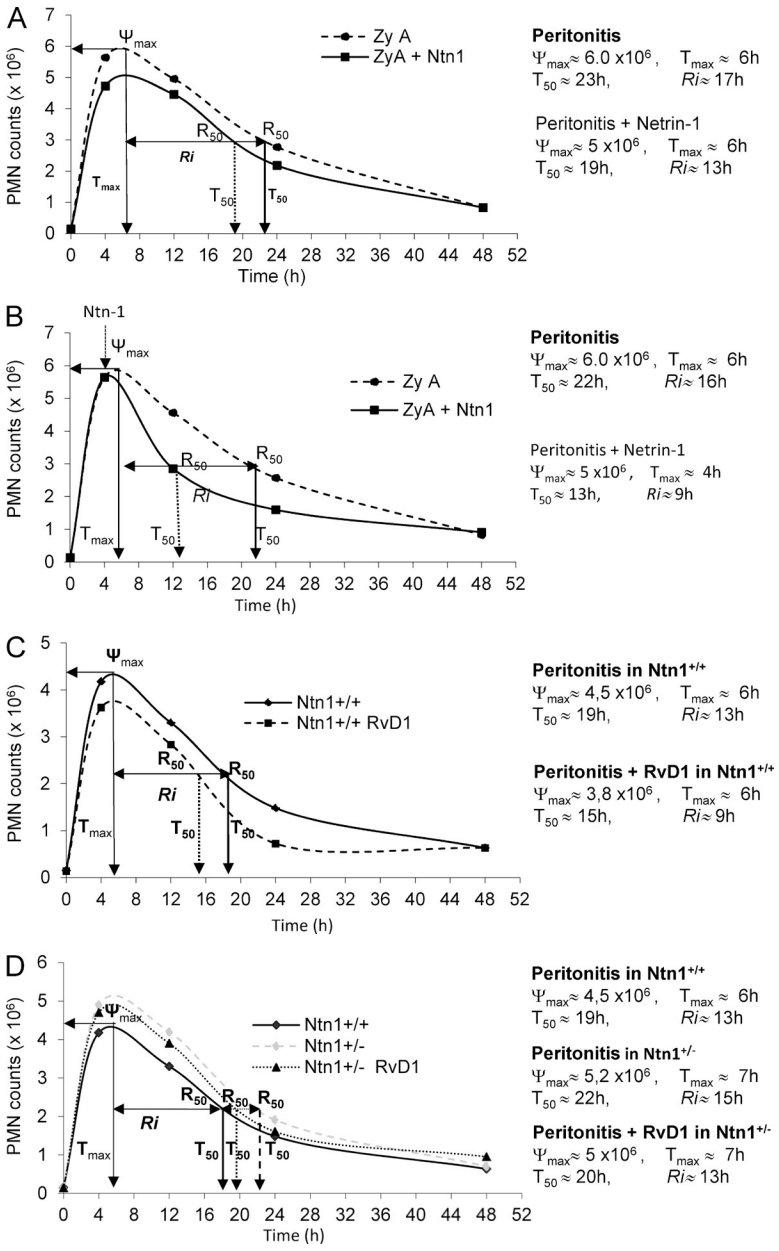

Figure 4. Netrin-1 stimulates resolution via RvD1. Resolution indices were used as previously defined (Bannenberg et al., 2005), including Ψ_{max} (maximal PMN), T_{max} (time point when PMNs reach Ψ_{max}), T_{50} (time point corresponding to 50% reduction), and R_i (resolution interval, the time interval from Ψ_{max} to 50% reduction point [i.e., T_{50}-T_{max}]). (A) Mice were injected i.p. with 1 mg of zymosan A (ZyA) and, subsequently, i.v. with either vehicle or recombinant netrin-1 (1 µg). Peritoneal exudates were collected at indicated time intervals and neutrophils (PMN) were enumerated by using light microscopy and flow cytometry. (B) Mice were treated as in panel A, and netrin-1 was given at the peak of inflammation (4h after ZyA). (C) *Ntn1*$^{+/+}$ were injected i.p. with ZyA (1 mg) and subsequently with RvD1 (1 µg/mouse). Recruited leukocytes were enumerated at indicated time points. (D) *Ntn1*$^{+/+}$ and *Ntn1*$^{+/-}$ mice were injected i.p. with ZyA (1mg) and subsequently with vehicle or RvD1 (1 µg/mouse), and peritoneal exudates were collected at indicated time intervals. Results in A–D represent 2 independent experiments with $n = 4$–6 mice per treatment per time point expressed as mean.

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).

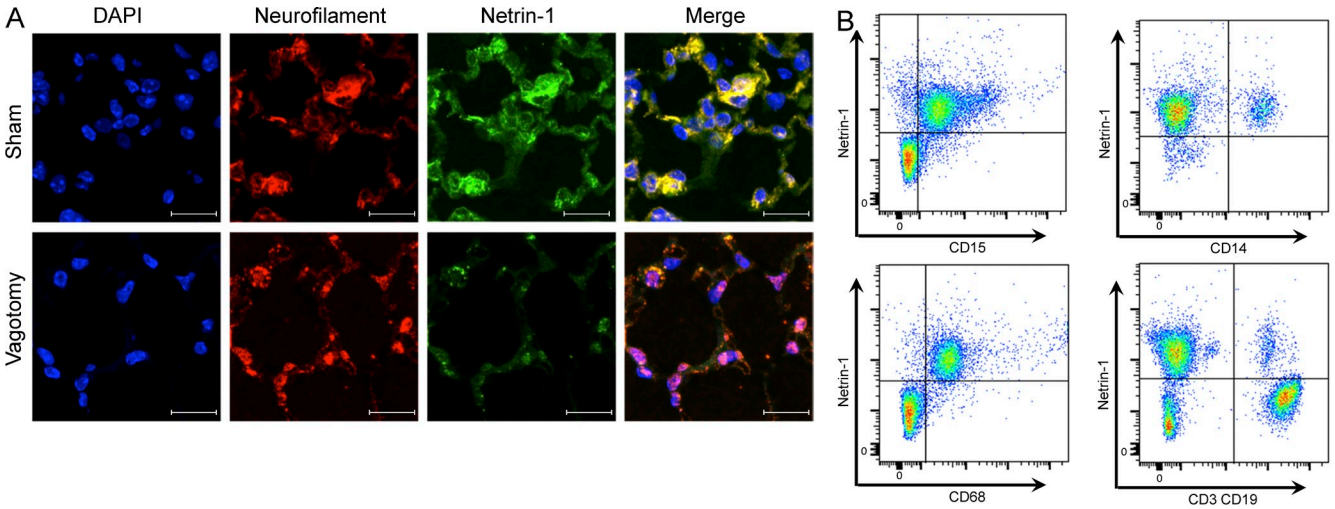

Figure 5. Vagotomy reduces netrin-1 expression. Animals were subjected to unilateral vagotomy or a comparable sham surgical procedure and netrin-1 and neurofilament expression in lungs was assessed by immunofluorescence. (A) Colocalization (green) of netrin-1 with the neuronal marker neurofilament (red) and yellow in the merged image. Bars, 100 µm. Results are representative of two separate experiments (n = 3 mice per group). (B) Leukocytes isolated from human peripheral blood were incubated with an antibody against netrin-1, CD15, CD14, CD68, CD3, CD19, and the expression of indicated marker was measured by flow cytometry. Results are representative of two separate experiments (from n = 3 healthy donors).

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 PGE_2 and PGD_2 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 LTB_4, a primary chemoattractant, and temporally regulated both PGD_2 and PGE_2, 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.

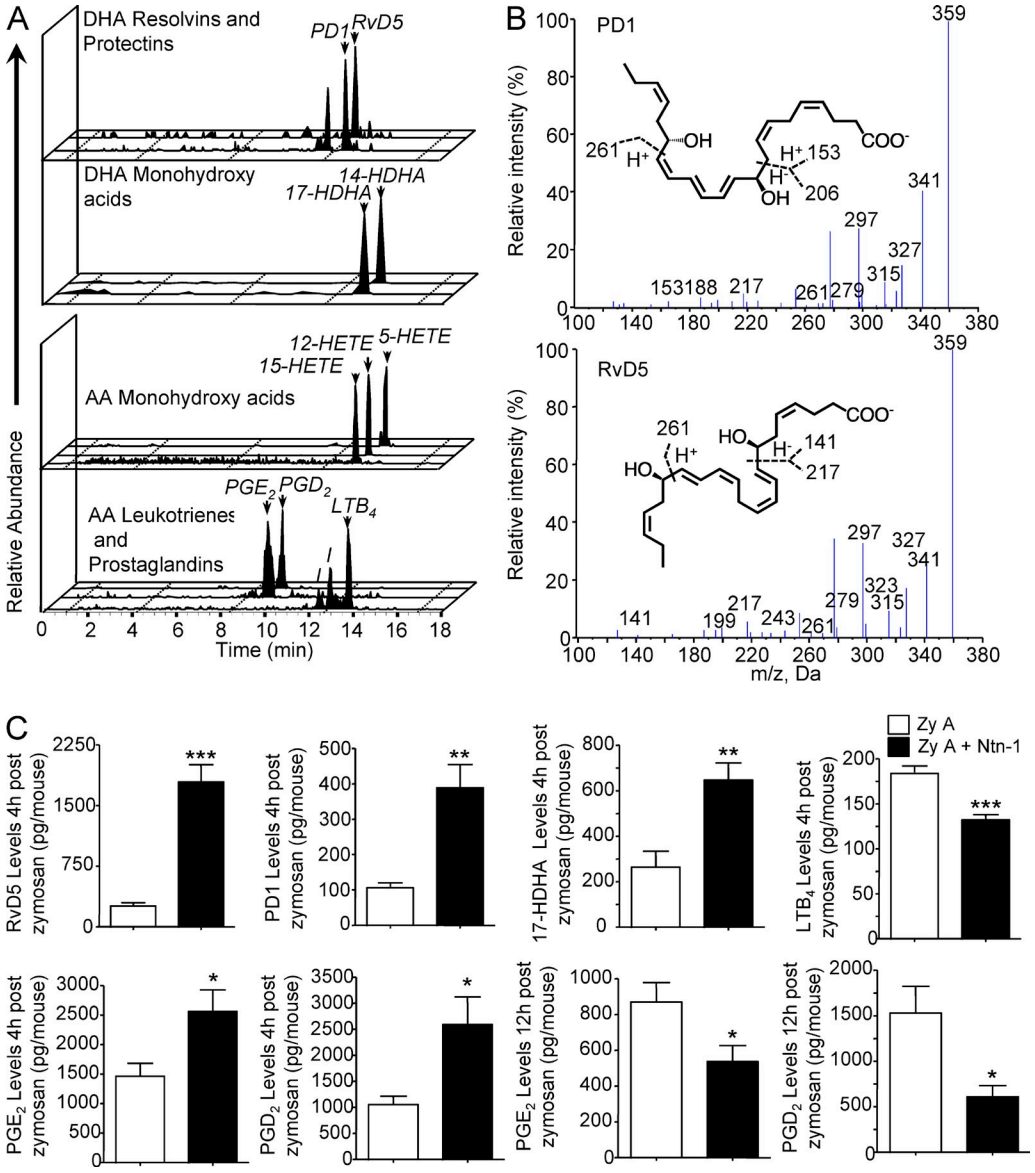

Figure 6. Netrin-1 enhances endogenous pro-resolving lipid mediators. Mouse peritonitis was initiated by i.p. injection of 1 mg of zymosan A (ZyA) and i.v. with recombinant netrin-1 (1 µg), and peritoneal exudates were collected at 4 and 12 h. Lipid mediator profiles were determined by lipid mediator metabololipidomics. (A) Representative MRM chromatograms for identified lipid mediators in murine self-resolving exudates and (B) MS/MS spectra. (C) Bioactive lipid mediators and precursor/pathway marker levels in the individual exudate at the indicated intervals as determined by MRM (see Materials and methods for details). Results are mean ± SEM and are representative of two independent experiments. *n* = 3–5 mice per group. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

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

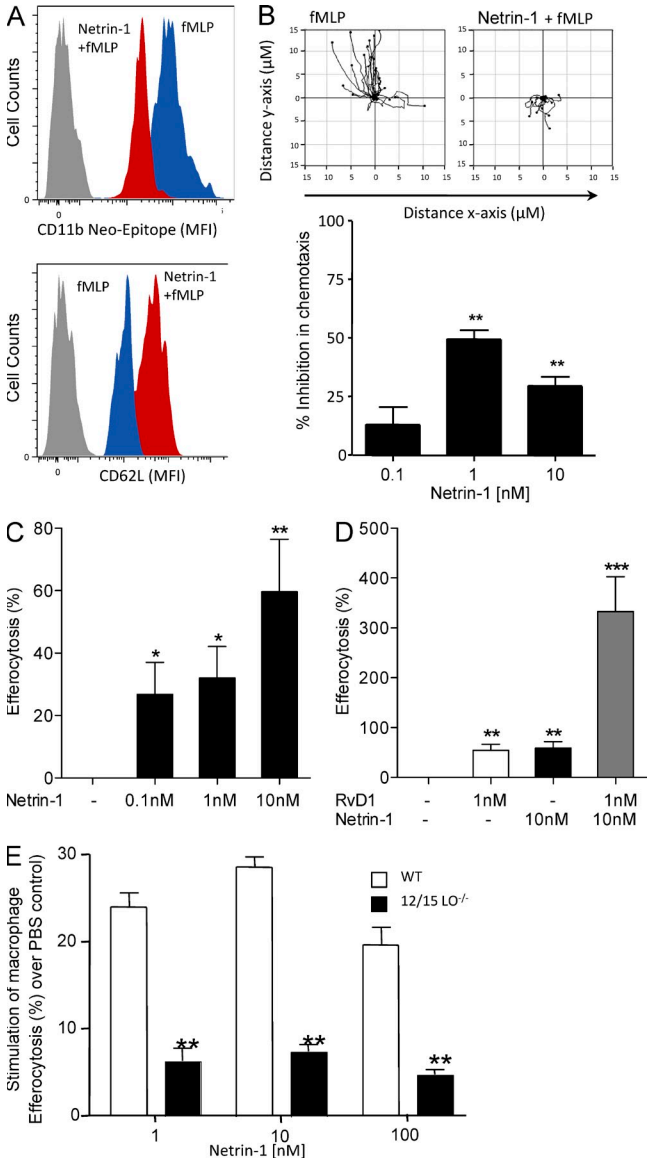

Figure 7. Netrin-1 reduces human PMN activation and increases Mϕ efferocytosis: Synergy with RvD1 pathway. (A) Isolated human PMN were incubated with either fMLP or netrin-1 for 30 min and the expression of CD11b (neo-epitope) and CD62L on PMN was measured by flow cytometry. (B) Isolated human PMN were 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) MΦ differentiated from peripheral blood monocytes were incubated with indicated concentrations of netrin-1. Carboxyfluorescein diacetate-labeled apoptotic PMN were added at 1:3 ratio (MΦ:PMN), and the rate of the efferocytosis was determined after 1 h by measurement of fluorescence. (D) MΦ 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 MΦ 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 (n = 5), mean ± SEM, in A–D. In E, results are representative of three independent experiments (n = 3 mice); *, P < 0.05; **, P < 0.01; ***, P < 0.001.

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.

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 (Mirakaj et al., 2011b). Collected cells were washed, suspended in Hanks' balanced salt solution (2 ml), counted and cytospin 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-Actin (Santa Cruz Biotechnology, Inc.). Blots were washed, and species-matched, peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Inc.) was added. Labeled bands from washed blots were detected by enhanced chemiluminescence (GE Healthcare).

Differential leukocyte counts, FACS analysis, and cytokines. Exudate cells from murine peritonitis were prepared for determining cellular composition by staining cells with APC-conjugated anti–mouse Ly6G (eBioscience) and FITC conjugated anti-mouse Ly6C (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 (Ψ_{max}, T_{max}), the time point (T_{max}) after T_0 challenge or T_0 injury when neutrophil numbers in tissues or exudates reach maximum (Ψ_{max}); duration (R_{50}, T_{50}), the time point (T_{50}) when the PMNs reduce and nonphlogistic monocyte numbers intercept

~50% of Ψ_{max} (R_{50}); resolution interval (R_i), the time interval from maximum neutrophil and monocyte infiltration intersection time point (Ψ_{max}) to 50% reduction point (R_{50}; i.e., $T_{50} - T_{max}$).

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 ng d_4-PGE_2, d_4-LTB_4, and d_8-5*S*-HETE) were added as in (Dalli and Serhan, 2012). Lipid mediator levels were assessed by LC-MS/MS using an HPLC system (LCZO; 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 µm or 4.6 × 100 mm × 1.8 µm; 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_8-5*S*-HETE, d_4-LTB_4, d_5-LXA_4, d_4-PGE_2, RvD1, RvD2, RvD5, PD1, MaR1, RvE1, RvE2, LXA_4, LXB_4, PGE_2, PGD_2, $PGF_{2\alpha}$, 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. Quantitation 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 10×10^6 cells per ml in PBC (PBS plus 0.2% BSA, pH 7.45). The cell chamber of µ-Slide chemotaxis slides (Ibidi) was coated with hr-CD62P (50 µg/ml) from R&D Systems for 20 min, 37°C. Next, the cells were placed onto the 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 YFL microscope (Nikon) equipped with a 20× phase contrast lens and QImaging Fast 1394 camera. Cell migration was analyzed using Image Pro Plus 7 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 CD 11b (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 FACSort (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-CD14 (BD), anti-CD68 (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 PMN were exposed for 60 min at 37°C to RvD1 (10 nM) or to recombinant netrin-1 (500 ng/ml), and the expression of A2BAR (Santa Cruz Biotechnology, Inc.), UNC5B (Santa Cruz Biotechnology, Inc.), neogenin (Santa Cruz Biotechnology, Inc.), and DCC (Santa Cruz Biotechnology, Inc.) was evaluated using FACSDiva Software (BD).

Immunofluorescent staining. For murine immunofluorescence, lungs were embedded in paraffin 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 microscope 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 (0.10^6 cells/well) were incubated with human recombinant netrin-1 (R&D Systems) in the presence or absence of RvD1 (1 nM) Boc2 peptide (10^{-4} M; Santa Cruz Biotechnology, Inc.; 15 min at 37°C). 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′-GAGCGGGGGAGTCTGTCT-3′ and 5′-TGGTTTGATTGCAGGTCTTG-3′ antisense primer. Samples were controlled for 18S using the following primers: sense, 5′-GTAACCCGTTGAACCCCATT-3′; antisense, 5′-CCATCCAATCGGTAGTAGCG-3′. Semiquantitative 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/DC1.

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C.N. Serhan is an inventor on patents [resolvins] assigned to Brigham and Women's Hospital and licensed to Resolvyx Pharmaceuticals. C.N. Serhan is a scientific founder of Resolvyx Pharmaceuticals and owns equity in the company. C.N. Serhan's interests were reviewed and are managed by the Brigham and Women's Hospital and Partners HealthCare in accordance with their conflict of interest policies. The other authors have no conflicting financial interests.

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