The neuroimmune guidance cue netrin-1 promotes atherosclerosis by inhibiting the emigration of macrophages from plaques

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Atherosclerotic plaque formation is fueled by the persistence of lipid-laden macrophages in the artery wall. The mechanisms by which these cells become trapped, thereby establishing chronic inflammation, remain unknown. Here we found that netrin-1, a neuroimmune guidance cue, was secreted by macrophages in human and mouse atheroma, where it inactivated the migration of macrophages toward chemokines linked to their egress from plaques. Acting via its receptor, UNC5b, netrin-1 inhibited the migration of macrophages directed by the chemokines CCL2 and CCL19, activation of the actin-remodeling GTPase Rac1 and actin polymerization. Targeted deletion of netrin-1 in macrophages resulted in much less atherosclerosis in mice deficient in the receptor for low-density lipoprotein and promoted the emigration of macrophages from plaques. Thus, netrin-1 promoted atherosclerosis by retaining macrophages in the artery wall. Our results establish a causative role for negative regulators of leukocyte migration in chronic inflammation.

Atherosclerosis is a disease of chronic inflammation that is distinguished by the persistence of cholesterol-engorged macrophages in arterial plaques. Arterial inflammation is initiated by the subendothelial retention of plasma low-density lipoproteins (LDLs) and is enhanced by oxidative modification of these lipoproteins, which triggers the influx of monocytes. Unlike other inflammatory states, atherosclerotic inflammation does not readily resolve, and cholesterol-laden macrophages persist in the arterial wall. These macrophage ‘foam cells’ cause expansion of the plaque through the recruitment of additional leukocytes and vascular smooth muscle cells (SMCs) and contribute prominently to plaque instability via the secretion of extracellular matrix–degrading proteases and cytotoxic factors. Notably, atherosclerotic plaques that cause clinical events (such as myocardial infarction and stroke) are characterized by abundant macrophage content.

Although the retention of macrophages in the artery wall has long been recognized as a fundamental step in the creation of the chronic inflammatory milieu that underlies atherosclerosis, the mechanisms that regulate this process are not well understood. The resolution of acute inflammation typically involves the emigration of monocyte-derived cells out of the inflamed site through nearby lymphatic vessels. This process seems to be impaired in atherosclerosis and has been attributed in part to the loading of cholesterol into macrophages, which shifts these cells to a more sessile phenotype. Studies of transplant-based mouse models of atherosclerosis regression have shown that decreasing plasma non–high-density lipoprotein (non-HDL) cholesterol and/or increasing HDL promotes the emigration of macrophages from lesions to regional and systemic lymph nodes.

Macrophage expression of the chemokine receptor CCR7 is essential for decreasing the macrophage content of plaques, which links the CCR7-specific ligands CCL19 and CCL21 to promotion of the egress of these cells from the artery wall. Such studies have indicated that the emigration of macrophages from plaques is actively inhibited during hypercholesterolemia, although the regulatory signals that impair this process remain largely unknown.

A paradigm for inhibitory guidance cues exists in the developing nervous system, where axonal migration relies on the integration of both chemorepulsive and chemotactive signals to steer the axonal growth cone. One such guidance molecule, netrin-1, a secreted laminin-related molecule, mediates both the chemorepulsion and chemotraction of axons navigating the spinal cord midline. This context-dependent response to netrin-1 is regulated by differences in receptor expression by the target cell. For example,
neurons that express the chemotactic netrin-1 receptors DCC or neogenin are attracted by a diffusible gradient of netrin-1 secreted at the midline. Conversely, expression of the chemorepulsive netrin-1 receptor UNC5H2 (UNC5b) together with DCC converts attraction by netrin-1 to repulsion by netrin-1, whereas expression of UNC5b alone mediates short-range repulsion and its receptors outside the nervous system in organogenesis and angiogenesis and tumorigenesis, which suggests that netrin-1 regulates cell migration in a broader context.

Netrin-1 has been identified as a leukocyte-guidance cue expressed by the endothelium that is downregulated during acute infection with Staphylococcus aureus. Those studies established that netrin-1 inhibits the migration of monocytes, neutrophils and lymphocytes via its receptor, UNC5b. Furthermore, studies of models of hypoxia and reperfusion injury have extended those findings to show that expression of netrin-1 by epithelial cells also attenuates the accumulation of leukocytes. Given its role in inhibiting leukocyte migration, we sought to determine whether netrin-1 contributes to the retention of macrophages in the chronic inflammatory milieu of the atherosclerotic plaque. We found that macrophage foam cells formed in vitro and in vivo and in atherosclerotic lesions expressed netrin-1. In functional studies, we demonstrated that netrin-1 secreted by foam cells regulated the cellular constituents of atheroma differently. Netrin-1 inactivated macrophage migration and supported the chemotraction of coronary artery SMCs. Thus, expression of netrin-1 in plaques would be predicted to simultaneously prevent the egress of inflammatory cells and induce the recruitment of SMCs into the intima, thereby promoting lesion progression. In support of that hypothesis, we demonstrated that deletion of netrin-1 in myeloid cells resulted in smaller and less-complex atherosclerotic lesions in mice deficient in the receptor for LDL (Ldlr−/−) and was associated with the emigration of macrophages from plaques.

RESULTS

Macrophage foam cells express netrin-1

Given its role in attenuating leukocyte migration, we first investigated whether netrin-1 is expressed in atherosclerotic plaques. Immunostaining of serial sections of human coronary artery atherosclerotic plaques showed expression of netrin-1 and UNC5b in lesional cells that expressed the macrophage marker HAM56 (Supplementary Fig. 1a,b). In contrast, we did not detect DCC in these plaques (data not shown). We noted a similar pattern of lesional netrin-1 staining in the Ldlr−/− mouse model of atherosclerosis (Fig. 1a). In aortic sinus plaques of Ldlr−/− mice fed a Western diet for 12 weeks, double staining for netrin-1 and the macrophage marker CD68 showed netrin-1 expression by lesional macrophages. In addition, extracellular netrin-1 staining was apparent in macrophage-rich regions of the plaque, consistent with netrin-1's being a secreted protein that can bind to extracellular matrix components. Analysis of the aortic arch, a second site of predilection toward lesions in mice, showed that Ntn1 mRNA (encoding netrin-1) was more abundant in Ldlr−/− mice than in wild-type C57BL/6 mice and that Ntn1 expression was further upregulated in mice fed a Western diet (Fig. 1b). We obtained similar results with the apolipoprotein E-deficient mouse model of atherosclerosis (Supplementary Fig. 1c), which indicated that netrin-1 expression by lesional macrophages is a common characteristic of mouse and human atheroma.

To understand the molecular mechanisms that regulate the expression of netrin-1 and UNC5b, we isolated peritoneal macrophages from Ldlr−/− mice fed either regular chow or a Western diet; the latter is a commonly used model of the in vivo formation of foam cells. Macrophages from mice fed a Western diet had much higher expression of Ntn1 and Unc5b mRNA, whereas expression of Cd68 was unchanged (Fig. 1c). We obtained similar results with hypercholesterolemic apolipoprotein E-deficient mice (Supplementary Fig. 1d), which suggested that the accumulation of cellular lipid may upregulate the expression of netrin-1 and UNC5b. To assess that,
Netrin-1 blocks the directed migration of macrophages

We next assessed the effect of netrin-1 on macrophage chemotaxis through the use of Transwell Boyden chambers and a real-time detection method. By each method, we found that recombinant netrin-1 potently inhibited chemotaxis of the mouse macrophage cell line RAW264.7 to the chemokine CCL2 (also known as monocyte chemotactrant protein-1 (MCP-1)) but had little effect on macrophage chemotaxis linked to the egress of CD68+ cells from plaques8,9.

To gain insight into the mechanisms by which netrin-1 inhibits macrophage chemotaxis, we measured its effect on the organization of the actin cytoskeleton. Stimulation of peritoneal macrophages with native LDL or LDL that had been oxidized (oxLDL), a modification that promotes the loading of cholesterol into macrophages. Consistent with our in vivo data, oxLDL resulted in macrophage expression of Ntm1 and Unc5b mRNA, but LDL did not (Fig. 1d and Supplementary Fig. 1e). Immunoblot analysis confirmed that oxLDL-treated macrophages had more cell-associated netrin-1 protein (Fig. 1e), which was paralleled by more netrin-1 in cell culture supernatants (Fig. 1f). Notably, the induction of Ntm1 mRNA (Fig. 1g) and netrin-1 protein (Supplementary Fig. 1f) by oxLDL required CD36, a scavenger receptor that has been linked to the retention of macrophages in atherosclerotic plaques22. Because the binding of oxLDL to CD36 induces activation of the transcription factor NF-κB23,24 and the Ntm1 promoter contains an NF-κB-binding site25, we investigated whether NF-κB contributes to the upregulation of Ntm1. Using a human Ntn1 promoter–luciferase reporter gene, we found that Ntn1 promoter activity was induced by oxLDL and this was diminished by the NF-κB inhibitor BAY 11-7082 (Fig. 1h). Collectively, these data demonstrated that loading of macrophages with cholesterol under physiological conditions, or by oxidized lipids via CD36 in vitro, resulted in higher expression of netrin-1 and its receptor Unc5b.

Figure 2 Netrin-1 inhibits the migration of macrophages toward CCL2 and CCL19 via UNC5b. (a) Migration of RAW264.7 cells toward CCL2 (100 ng/ml) in the presence or absence of increasing concentrations of recombinant netrin-1 placed in the lower compartment of a Boyden chamber. (b) Migration of RAW264.7 cells toward medium or toward netrin-1 (250 ng/ml) or CCL2 (100 ng/ml) alone or together. (c-e) Migration of RAW264.7 cells pretreated (PT) with netrin-1 (250 ng/ml) and exposed to CCL2 (100 ng/ml). (d,e) Migration of mouse peritoneal macrophages toward CCL2 (100 ng/ml) or CCL19 (500 ng/ml); (f) in the presence (+) or absence (−) of netrin-1 (250 ng/ml); (f) Microscopy of peritoneal macrophages treated with CCL2 (100 ng/ml) with or without (control) netrin-1 (250 ng/ml) and stained with phalloidin (to detect polymerized actin). Arrows indicate membrane ruffles in control cells and rounded morphology in netrin-1-treated cells. Scale bar, 10 μm. (g) Cell surface area of the peritoneal macrophages in f. (h) Quantification of actin polymerization by flow cytometry analysis of phallolidin staining, presented relative to results at time 0, set as 1. (i) Activated (GTP–) Rac1 in peritoneal macrophages treated for 5 min with CCL2 (100 ng/ml) with or without netrin-1 (250 ng/ml), presented relative to results of untreated cells, set as 1. (j) Immunoblot analysis of phosphorylated (p-) and total FAK in peritoneal macrophages incubated with CCL2 (100 ng/ml) with or without pretreatment with netrin-1 (250 ng/ml); results are presented relative to results at time 0, set as 1. *P < 0.05 and **P < 0.01 (Student’s t-test). Data are from one experiment representative of three independent experiments (mean ± s.d. of triplicate samples in a–e,g).
with CCL2 or CCL19 induced considerable reorganization of actin, characterized by the appearance of membrane ruffles, lamellipodia and filapodia (Fig. 2f and Supplementary Fig. 3a) and rapid cell spreading (Fig. 2g and Supplementary Fig. 3b). In contrast, peritoneal macrophages pretreated with netrin-1 before stimulation with CCL2 or CCL19 maintained a rounded morphology (Fig. 2f and Supplementary Fig. 3a) and showed no increase in mean cell area (Fig. 2g and Supplementary Fig. 3b), consistent with a nonmotile phenotype. Quantification of phalloidin-stained actin filaments by flow cytometry confirmed that netrin-1 blocked the increase in polymerized actin-1 associated with CCL2 stimulation (Fig. 2h). As the Rho GTPase Rac1 has a key role in the reorganization of actin in macrophages, we next investigated whether netrin-1 altered Rac1 activation after chemotactic stimulation with glutathione S-transferase beads conjugated to the p21-binding domain of the kinase PK-1 to detect the activated GTP-bound form of Rac1. Whereas pretreatment of macrophages with netrin-1 resulted in slightly more basal activated Rac1, it inhibited CCL2-induced activation of Rac1 (Fig. 2i) and phosphorylation of the adhesion kinase FAK (Fig. 2j), which acts together with Rac1 to link the actin cytoskeleton to the extracellular matrix during cell spreading and migration. Collectively, these data indicated that netrin-1 inhibited the directional migration of macrophages by disrupting the Rac1 signaling cascade, reorganization of the actin cytoskeleton and cell polarization.

As both netrin-1 and UNC5b were upregulated in cholesterol-loaded macrophages, we postulated that netrin-1 secreted by macrophages in atherosclerotic plaques may act in an autocrine or paracrine manner via UNC5b to immobilize these cells in the artery wall. To assess the role of UNC5b in the response to netrin-1, we preincubated macrophages with the extracellular domain of human UNC5b fused to the Fc portion of human immunoglobulin G1 (UNC5b-Fc) or an antibody that binds to the extracellular domain of UNC5b. Both UNC5b-Fc (Fig. 3a) and the antibody to UNC5b (Fig. 3b) reversed the inhibitory effect of netrin-1 on CCL19-induced migration, but control immunoglobulin G (IgG) did not. In contrast, we found no change with an inhibitor of the A2B adenosine receptor, another netrin-1 receptor linked to the attenuation of neutrophil migration (Fig. 3c).

Furthermore, consistent with the secretion of netrin-1 by macrophage foam cells, conditioned medium from oxLDL-treated macrophages inhibited the migration of cells to CCL19, but conditioned medium from LDL-treated macrophages did not (Fig. 3d), and this was reversed by UNC5b-Fc (Fig. 3e). To confirm the proposal that netrin-1 is the active component secreted by oxLDL-treated macrophages, we used peritoneal macrophages isolated from Ntn1−/− bone marrow–chimeric mice, which did not express netrin-1 in response to oxLDL (Fig. 3f). Whereas conditioned medium from oxLDL-treated Ntn1−/− peritoneal macrophages inhibited the migration of naïve macrophages toward CCL19 by 80%, conditioned medium from similarly treated Ntn1−/− macrophages resulted in only 25% less migration (Fig. 3f). Furthermore, conditioned medium from Ntn1−/− cells incubated with UNC5b-Fc inhibited migration to an extent similar to that achieved by conditioned medium from Ntn1−/− cells (−25%). In contrast, the effects of conditioned medium from Ntn1−/− cells were unchanged by the addition of recombinant UNC5b (Fig. 3f). Together these data suggest that netrin-1 secreted by cholesterol-laden macrophages would promote their accumulation in atherosclerotic plaques by inhibiting their emigration from this site of inflammation.

Studies of a mouse model of atherosclerosis regression have demonstrated that macrophages in plaques exit via nearby lymphatics after aggressive lowering of cholesterol concentrations. A similar pathway for macrophage clearance has been reported during resolving peritonitis in which inflammatory macrophages emigrate to the draining lymphatics of the omentum. To assess the effect of netrin-1 on macrophage emigration in vivo, we used the well-characterized thioglycollate-induced peritonitis model in which administration of lipopolysaccharide (LPS) induces the rapid egress of recruited leukocytes from the peritoneum. As reported before, intraperitoneal injection of LPS into control mice given no pretreatment resulted in 75% fewer leukocytes in the peritoneum (Fig. 3g), whereas mice pretreated intraperitoneally with netrin-1 45 min before this inflammatory stimulus showed no significant change in peritoneal leukocytes. Analysis of the macrophage marker F4/80 by flow
Figure 4 Netrin-1 acts as a chemoattractant for SMCs via neogenin. (a) Migration of human coronary artery SMCs in the presence of recombinant netrin-1 (a) or conditioned medium from macrophages left unstimulated (CM unstim) or treated for 0–48 h with LDL or oxLDL (50 μg/ml). b, c) Quantitative PCR analysis of mRNA for Neo1 (encoding neogenin). Unc5B and Dcc in coronary artery SMCs, presented relative to Unc5b mRNA, set as 1. d) Immunofluorescence staining for neogenin, DCC or isotype-matched control antibody (IgG) in coronary artery SMCs costained with the nuclear stain DAPI. Scale bars, 10 μm. e) Migration of coronary artery SMCs pretreated with antibody to neogenin (Anti-neogenin) or isotype-matched control antibody (Control Ab) before exposure to conditioned medium from macrophages left unstimulated or treated for 24 h with LDL or oxLDL. (f) Immunofluorescence staining of α-smooth muscle actin (SMA; green), neogenin (red) and DAPI (blue) in atherosclerotic plaques of Ldlr−/− mice fed a Western diet, showing colocalization of smooth muscle actin and neogenin (yellow in the merged image) in the media (arrows) and in SMCs that had invaded the intima (arrowheads). Scale bars, 50 μm. *P < 0.05 (Student’s t-test). Data are from one experiment representative of three independent experiments (a, c, e; mean ± s.d. of triplicate samples), three experiments (d) or two experiments with five mice (f).

Furthermore, conditioned medium from oxLDL-treated macrophages induced the migration of these cells but conditioned medium from LDL-treated macrophages did not (Fig. 4b). To understand how netrin-1 mediates the chemoattraction of these cells, we measured expression of its known receptors. Although coronary artery SMCs had low expression of DCC and UNC5b, they had abundant expression of the DCC-related receptor neogenin (Fig. 4c,d), as reported before for vascular SMCs. Notably, pretreatment of coronary artery SMCs with antibody to neogenin abrogated the chemoattractant effect of conditioned medium from oxLDL-treated macrophages (Fig. 4e), which supported the idea that foam cell–derived netrin-1 induces the migration of SMCs. Immunohistochemical staining of atherosclerotic plaques of Ldlr−/− mice showed colocalization of neogenin with smooth muscle actin (Fig. 4f) but not with the macrophage cytomtery confirmed more retention of macrophages in the peritoneum of mice pretreated with netrin-1 (1.1 × 10^6 ± 0.3 × 10^6) than in that of control mice given no pretreatment (0.4 × 10^6 ± 0.1 × 10^6). These data demonstrated that netrin-1 inhibited the emigration of macrophages from a site of active inflammation.

**Netrin-1 is a chemoattractant for SMCs**

During the progression of atherosclerosis, SMCs from the underlying medial layer are recruited to the plaque and participate in promoting plaque growth. To investigate whether netrin-1 expression in plaque affects other cellular components of the atherosclerotic lesion, we measured its effect on migration of human coronary artery SMCs. Unlike its inhibitory effect on macrophages, netrin-1 induced the migration of coronary artery SMCs in a dose-dependent way (Fig. 4a).

Figure 5 Targeted deletion of netrin-1 in cells of the immune response results in a lower atherosclerosis burden. (a) Atherosclerosis in the aorta en face of WT → Ldlr−/− and Ntn1−/− → Ldlr−/− mice (n = 10 per group). Each symbol represents an individual mouse; small horizontal lines indicate the mean (left). Scale bars (right), 5 mm. *P < 0.01 (Student’s t-test). (b,c) Quantitative PCR analysis of mRNA for Cd68 (macrophages), Cd3 (T cells), Cd11c (dendritic cells) and Elane (neutrophils; b) and of Ntn1 mRNA (c) in the aortic arches of WT → Ldlr−/− and Ntn1−/− → Ldlr−/− mice (n = 3 per group), presented relative to Gapdh (housekeeping gene), ND, not detected.

*P < 0.05 (Student’s t-test). (d,e) Lesion area of atherosclerotic plaques of the aortic arches of WT → Ldlr−/− and Ntn1−/− → Ldlr−/− mice, presented for individual mice (d) and for each genotype across the 400 μm of the aortic root (e). Each symbol represents an individual mouse; small horizontal lines indicate the mean (d). *P < 0.005 (Student’s t-test).

(f) Microscopy of hematoxylin- and eosin-stained aortic sinus lesions of WT → Ldlr−/− and Ntn1−/− → Ldlr−/− mice. Scale bars, 200 μm. Data are representative of a single experiment with ten mice per group (d, f; error bars, s.e.m.) or are from one experiment representative of two independent experiments (b, c; mean ± s.d. of triplicate samples).
marker CD68 (Supplementary Fig. 4a). Furthermore, neogenin was expressed in medial SMCs and in SMCs that had migrated into the intima, as well as some cells that were not positive for smooth muscle actin (Fig. 4f). Together these data indicated that netrin-1 modulated the migration of coronary artery SMCs and macrophages through distinct mechanisms.

Less atherosclerosis with hematopoietic deficiency in netrin-1

On the basis of the data reported above, we postulated that expression of netrin-1 by macrophage foam cells may promote atherosclerotic plaque growth by both blocking macrophage egress and eliciting the recruitment of SMCs to the intima. To test this hypothesis, we used fetal liver cells from Ntn1+/− or Ntn1−/− pups at embryonic day 14 to reconstitute the bone marrow of lethally irradiated Ldr−/− mice, thereby generating Ldr−/− mice with either Ntn1+/− macrophages (Ntn1+/−→Ldr−/−) or Ntn1−/− (wild-type) macrophages (WT→Ldr−/−). At 4 weeks after transplantation, we challenged the chimeric Ntn1+/−→Ldr−/− and WT→Ldr−/− mice with a Western diet for 12 weeks. Analysis of the expression of Ntn1 and Ldr in both circulating leukocytes and peritoneal macrophages from the recipient mice confirmed a complete change in genotype to the donor type (Supplementary Table 1 and Supplementary Fig. 4b). There was no difference in the serum concentration of total cholesterol or triglyceride or in the cholesterol distribution in very low-density lipoprotein, LDL or HDL in chimeric Ntn1+/−→Ldr−/− or WT→Ldr−/− mice (Supplementary Table 1 and Supplementary Fig. 4c).

Despite their similar serum cholesterol profiles, Ldr−/− mice lacking expression of Ntn1 in bone marrow–derived cells had much less atherosclerosis (Fig. 5). Analysis of the aortic en face showed that Ntn1−/−→Ldr−/− mice had an atherosclerotic lesion 55% smaller than that of WT→Ldr−/− mice (Fig. 5a) that was present throughout the aorta (Supplementary Fig. 4d). The aortic arch of Ntn1−/−→Ldr−/− mice had lower expression of mRNA for the macrophage marker CD68 than did that of WT→Ldr−/− mice (Fig. 5b) and, correspondingly, had lower expression of Ntn1 (Fig. 5c). The aortic arch of Ntn1−/−→Ldr−/− mice also had lower expression of markers for other leukocyte subsets, including CD3 (T cells) and CD11c (dendritic cells; Fig. 5b). Furthermore, Ntn1−/−→Ldr−/− mice had much smaller atherosclerotic plaques in a second anatomical site, the aortic root, than did WT→Ldr−/− mice (Fig. 5d–f). Quantification of lesion burden by cross-sectional analysis of the aortic root established that this smaller lesion area in Ntn1−/−→Ldr−/− mice was consistently present throughout the 400 μm of the aortic root (Fig. 5e).

Histological characterization of the aortic sinus plaques by the Stary method showed that WT→Ldr−/− mice had more advanced atherosclerosis, typified by a greater proportion of complex lesions (stages 3–4), whereas Ntn1−/−→Ldr−/− plaques tended to have undergone less progression (stages 1–3; Fig. 6a). Immunohistochemical staining of aortic sinus plaques confirmed that Ntn1−/−→Ldr−/− mice had fewer macrophages (cells positive for MOMA-2; Fig. 6b) and SMCs (α-smooth muscle actin; c). Scale bars, 100 μm. Right, quantification of staining. (d) Immunofluorescence staining (left) of apoptotic cells (green) in aortic sinus plaques; arrowheads indicate TUNEL+ nuclei. Scale bar, 50 μm. Right, quantification. (e) Quantification of necrotic areas of aortic sinus plaques. (f) In vivo analysis of the recruitment of macrophages to and retention in atherosclerotic plaques of WT→Ldr−/− and Ntn1−/−→Ldr−/− mice with a monocyte bead-tracking model, presented as bead-labeled macrophages per plaque at 3 d (baseline) and 14 d after monocyte labeling. Right, microscopy of plaques stained for CD68 (red) and cell nuclei (blue); arrows indicate the presence of the cells containing fluorescent beads (green) in the lesion. *P < 0.05 and **P = 0.07 (Student’s t test). Data are representative of one experiment each with ten mice per genotype (a,e), six to nine mice per genotype (b,c), nine mice per genotype (d) or three to four mice per group (f; mean ± s.e.m.).
at baseline and 14 d (Fig. 6f), which indicated that macrophages did not emigrate during this time. In contrast, Ntn1−/−→Ldlr−/− plaques showed a 40% decrease in beads after 14 d compared with baseline, which indicated that fewer macrophages were retained in the plaque in the absence of netrin-1. As the beads cannot be degraded, and any labeled macrophages that undergo apoptosis are phagocytosed by surrounding macrophages, which results in transfer of the bead label, the decrease in beads in Ntn1−/−→Ldlr−/− mice was indicative of the movement of macrophages out of the plaque. Collectively, our data support the proposal of the retention of macrophages in plaques by netrin-1 and have demonstrated that after the removal of this retention signal, macrophages emigrated from this site of chronic inflammation.

**DISCUSSION**

It is appreciated that plaques that cause clinical events, so-called ‘vulnerable plaques,’ have a high macrophage content. Thus, strategies targeted at diminishing macrophage accumulation and/or directing the emigration of these cells from the plaque offer promise as a complement to standard lipid-lowering therapies. However, so far the signals responsible for mediating macrophage retention in the artery wall have been poorly elucidated. Our data have established that netrin-1, a neuronal guidance molecule with immunomodulatory functions, and its receptor, UNC5b, were expressed by macrophage foam cells of human and mouse atherosclerotic plaques. Notably, recombiant netrin-1 and netrin-1 secreted by in vitro formed foam cells potently blocked the directed migration of macrophages to CCL19, a chemokine linked to the emigration of monocyte-derived cells from plaques in a transplant model of regression. Moreover, netrin-1 also blocked the migration of macrophages to CCL2, which, along with its receptor CCR2, is involved in the trafficking of myeloid cells to the lymph nodes during inflammation. Thus, netrin-1 in plaques may act to immobilize macrophage foam cells and prevent their egress to the lymphatic system. In support of this hypothesis, we found that the selective deletion of netrin-1 in bone marrow–derived cells resulted in smaller and less-complex atherosclerotic lesions in Ldlr−/− mice and was associated with the emigration of macrophages from plaques. Our data have established a causative role for netrin-1 in the persistence of inflammation in atherosclerosis and highlight the importance of such negative regulators of leukocyte migration in chronic inflammation.

Several reports have demonstrated that guidance molecules characterized in the developing nervous system can modulate leukocyte migration in inflammatory states. Such studies have shown that members of the netrin, slit, semaphorin and ephrin families of guidance cues can have both chemoattractive and chemorepulsive effects on leukocyte trafficking. Published studies have shown that netrin-1 is expressed on endothelial and epithelial cells, where it seems to function in limiting the transmigration of leukocytes into tissues. For example, endothelial expression of netrin-1 is downregulated in the lungs during acute Staphylococcus aureus–induced inflammation, coincident with the recruitment of neutrophils to the tissue. In contrast, netrin-1 is upregulated on gut epithelial cells during transient ischemia and attenuates the recruitment of neutrophils to protect the host from hypoxia-induced inflammation. Such studies showing that netrin-1 inhibits the migration of circulating leukocytes into tissues and thus is anti-inflammatory in this ability have provided insight into the homeostatic barrier functions of netrin-1. In this context, a published study has reported that intravenous viral delivery of netrin-1 to Ldlr−/− mice results in less atherosclerosis, presumably by increasing netrin-1 expression on endothelial cells, although no determination of the cell types expressing netrin-1 were made in that study. Unfortunately, that study was underpowered, and the traditional atherosclerosis measurements of cross-sectional lesion area and en face aortic lesion burden were not assessed, which limits the conclusions that can be drawn from those data. In contrast, our data have indicated that the expression of netrin-1 by macrophage foam cells in plaques was proatherosclerotic and that its inactivation of macrophage emigration from this inflamed site probably inhibited the resolution of inflammation. Thus, as in the nervous system, in which it can have both positive and negative effects on axonal migration, netrin-1 may have multifunctional roles in regulating inflammation depending on the site of its expression. Although monocytes and most tissue macrophages have low expression of netrin-1, macrophage foam cells in human and mouse atheroma have high expression of netrin-1. The mechanisms of netrin-1 upregulation in this context may be similar to those in hypoxia, during which the hypoxia-induced factor HIF1-α and NF-κB regulate Ntn1 transcription. Hypoxic stress is intimately linked to atherosclerosis, and these transcription factors are activated in lesional macrophages. The induction of netrin-1 by oxLDL involved activation of NF-κB via CD36 (refs. 23,24), a scavenger receptor linked to the retention of macrophages in atherosclerosis. Macrophages that infiltrate the arterial intima are thought to exit via lymphatic vessels or egress into the bloodstream, or undergo apoptosis and efferocytotic clearance. All of those mechanisms seem to be impaired in atherosclerosis, leading to the progressive accumulation of macrophages in plaques. However, studies of mouse models suggest that such processes can be restored with aggressive management of lipids, which provides hope that atherosclerosis regression can be achieved in humans. Genetic manipulations that decrease plasma non-HDL cholesterol and/or increase HDL cholesterol promote the emigration of macrophages from plaques to regional and systemic lymph nodes via a mechanism that involves CCR7, the receptor for CCL19 and CCL21 (refs. 5–9). Notably, as netrin-1 blocked the chemotaxis of macrophages toward CCL19 and CCL21, it would be expected to inhibit the normal migratory processes that bring about resolution of inflammation. Consistent with that, deletion of netrin-1 in lesional macrophages in Ldlr−/− mice promoted macrophage emigration and resulted in smaller plaques. Although the exact means by which netrin-1 inhibits migration remain to be elucidated, our data have indicated that netrin-1 inhibited Rac-mediated reorganization of the actin cytoskeleton, preventing cell spreading and the migration of macrophages out of plaques. In addition to its effects on macrophages, netrin-1 is a potent chemotactrant for coronary artery SMCs, a process dependent on the neogenin receptor. The recruitment of SMCs by netrin-1 represents a second mechanism by which this guidance molecule could promote atherosclerosis. During plaque progression, the internal elastic lamina can become breached and SMCs may migrate into the intima to participate in lesion growth. In plaques of Ntn1−/−→Ldlr−/− chimeric mice, we found less accumulation of SMCs, consistent with the idea that loss of netrin-1 both allowed the emigration of macrophages from the plaque and curtailed the recruitment of SMCs into it. Furthermore, Ntn1−/−→Ldlr−/− plaques had fewer apoptotic cells and a smaller necrotic area, which indicated that the mechanisms that regulate tissue homeostasis were enhanced in the absence of netrin-1. Together these data underscore the important role that negative guidance cues may have in the persistence of inflammation and indicate that local inhibition of such factors may have therapeutic value for the resolution of inflammation in atherosclerosis and other chronic inflammatory diseases.
METHODS
Methods and any associated references are available in the online version of the paper at http://www.nature.com/natureimmunology/.

Note: Supplementary information is available on the Nature Immunology website.

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AUTHOR CONTRIBUTIONS
J.M.v.G. did migration and atherosclerosis studies; M.C.D. did smooth muscle studies and fetal liver cell transplantation; K.J.R. and L.R.F. did mouse atherosclerosis studies, J.I.A.-L. and S.P. and B.R. did microscopy; T.D.R., A.J.R. and J.I.F. did biochemical assays; T.O.M. and K.D.O. did immunohistochemical studies of human atherosclerosis; E.D. and E.A.F. assisted in head-labeling experiments, L.M.S. and A.L.-H. contributed to experimental design, data analysis and provided discussions; K.J.M. designed, analyzed and interpreted the studies and wrote the manuscript with J.M.v.G.

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