Neuroimmune Guidance Cue Semaphorin 3E Is Expressed in Atherosclerotic Plaques and Regulates Macrophage Retention


Objective—The persistence of myeloid-derived cells in the artery wall is a characteristic of advanced atherosclerotic plaques. However, the mechanisms by which these cells are retained are poorly understood. Semaphorins, a class of neuronal guidance molecules, play a critical role in vascular patterning and development, and recent studies suggest that they may also have immunomodulatory functions. The present study evaluates the expression of Semaphorin 3E (Sema3E) in settings relevant to atherosclerosis and its contribution to macrophage accumulation in plaques.

Approach and Results—Immunofluorescence staining of Sema3E, and its receptor PlexinD1, demonstrated their expression in macrophages of advanced atherosclerotic lesions of Apoe–/– mice. Notably, in 2 different mouse models of atherosclerosis regression, Sema3E mRNA was highly downregulated in plaque macrophages, coincident with a reduction in plaque macrophage content and an enrichment in markers of reparative M2 macrophages. In vitro, Sema3E mRNA was highly expressed in inflammatory M1 macrophages and in macrophages treated with physiological drivers of plaque progression and inflammation, such as oxidized low-density lipoprotein and hypoxia. To explore mechanistically how Sema3E affects macrophage behavior, we treated macrophages with recombinant protein in the presence/absence of chemokines, including CCL19, a chemokine implicated in the egress of macrophages from atherosclerotic plaques. Sema3E blocked actin polymerization and macrophage migration stimulated by the chemokines, suggesting that it may immobilize these cells in the plaque.

Conclusions—Sema3E is upregulated in macrophages of advanced plaques, is dynamically regulated by multiple atherosclerosis-relevant factors, and acts as a negative regulator of macrophage migration, which may promote macrophage retention and chronic inflammation in vivo. (Arterioscler Thromb Vasc Biol. 2013;33:886-893.)

Key Words: atherosclerosis ■ macrophage ■ migration ■ regression ■ Semaphorin 3E
does not readily resolve and cholesterol-laden macrophages (foam cells) persist in the arterial wall. Macrophages in atherosclerotic plaques seem to be impaired in their ability to emigrate to draining lymph nodes.4,5 Trapped in an inflammatory milieu, some macrophages die locally, either by apoptosis or by secondary necrosis, and the retention of activated macrophages further aggravates plaque inflammation, which can lead to instability and rupture.1 Yet, the mechanisms by which macrophages are retained in the plaque are poorly understood. We have shown that emigration of macrophages from atherosclerotic lesions can occur under conditions of plaque regression induced either by reducing plasma cholesterol4,5 or by increasing levels of high-density lipoprotein (HDL).6,7 Notably, macrophage emigration was largely dependent in a transplant model of atherosclerosis regression on the expression of the chemokine receptor CCR7 implicating the CCR7-specific ligands, CCL19 and CCL21, in promoting the egress of cells from the artery wall in this model.

In addition to chemokine pathways serving to regulate the emigration of macrophages from plaques, there are also factors that are likely to actively inhibit macrophage chemotaxis. A source of potential candidates has come from the realization that the vascular and nervous systems of vertebrates share common features both in their anatomy and in the molecular factors that regulate their development.9 Indeed, the role of neuronal guidance molecules in the vasculature has been a recent focus of investigation, and a growing body of evidence demonstrates the participation of classical neuronal guidance molecules in the development of the vascular system.9 In addition, emerging evidence is revealing roles for neuronal guidance molecules in the immune system where they seem to exert diverse effects on leukocyte migration, adhesion and inflammatory responses.10–12 Notably, we recently reported the involvement of netrin-1, a neuronal guidance molecule, in promoting chronic inflammation in atherosclerosis by retaining macrophages in plaque.13

As noted above, the development of a mouse model of atherosclerosis regression involving the transplant of an atherosclerotic aortic arch into a normolipidemic donor has permitted the study of the egress of monocyte-derived cells, primarily macrophages, from aortic arch plaques.4,6,14,15 In addition, the study of gene expression changes specifically in macrophages from atherosclerotic plaques has become possible with the advent of laser capture microdissection.16 Using these tools, we recently profiled the transcriptome changes in progressing and regressing atherosclerotic plaques.15 Of the genes significantly changed during regression of atherosclerotic plaques, we were intrigued to see that Semaphorin 3E (Sema3E) was among the most highly downregulated (–6-fold) compared with that in macrophages in progressing plaque. The Semaphorins are a large family of neuronal guidance cues that have been described as having roles in vascular development and neuroimmune signaling.17–19 In particular, the class 3 Semaphorins, of which Sema3E is a member, are highly conserved secreted and matrix-associated proteins that can signal through various transmembrane receptors in the plexin or neuropilin family, mediating both repulsive and attractive signaling that seems to be cell type and context specific.20 Given our recent finding that netrin-1 expression in atheroma macrophages promotes chronic inflammation, we wanted to further investigate the expression and function of Sema3E in atherosclerosis to determine whether it had similar effects.

As will be presented, the data clearly show that Sema3E is expressed in macrophages of advanced atherosclerotic plaques in mice, and in vitro potently inhibits migration of macrophages to chemokines implicated in the recruitment of inflammatory macrophages to the draining lymph nodes (eg, CCL2 and CCL19). Sema3E expression in macrophages in vitro is upregulated by physiological drivers of plaque inflammation, such as oxidized low-density lipoprotein (oxLDL) and hypoxia, and reduced under conditions that promote cholesterol efflux. Furthermore, Sema3E is highly expressed in inflammatory M1, but not anti-inflammatory M2 macrophages, and consistent with this is the reduction in expression of Sema3E in regressing atherosclerotic plaques, in which there is also a shift in macrophage phenotype from a predominantly M1 to M2 phenotype. Together, these data suggest a role for Sema3E in the retention of macrophages in atherosclerosis and highlight the expanding functions of neuroimmune guidance cues in regulating the persistence of inflammation in atherosclerotic plaques.

Materials and Methods
Materials and Methods are available in the online-only Supplement.

Results
Sema3E Is Expressed in Macrophages of Advanced Atherosclerotic Mouse Plaques and Its Expression Decreases in Models of Plaque Regression
To investigate the expression of Sema3E at the protein level in atherosclerosis, we performed immunohistochemical staining on aortic root plaques of Apoe+/− mice fed a Western diet for 12 weeks. In these progressing atherosclerotic plaques, double-staining for Sema3E and the macrophage marker CD68 showed Sema3E protein present in lesional macrophages (Figure 1A, arrows). In addition, there seemed to be extracellular Sema3E staining in macrophage-rich regions of the plaque, consistent with Sema3E being a secreted protein that can bind to extracellular matrix. Furthermore, staining for the Sema3E receptor PlexinD1 also colocalized with CD68-positive macrophages in these advanced atherosclerotic plaques (Figure 1B, arrows), suggesting that these cells may be both the source and the target of Sema3E secreted in the plaque.

To understand the dynamics of Sema3E expression in atherosclerosis, we used an established model of atherosclerosis regression in which the aortic arch from Apoe−/− mice fed a Western diet for 16 weeks is transplanted into either a hyperlipidemic Apoe+/+ (progressive environment) or a normolipidemic wild-type C57BL6 (regressive environment) recipient mouse for 3 days.14 Similar to its expression in aortic root plaques, Sema3E was abundantly expressed in aortic arch plaques in the progressive environment (Apoe−/−→Apoe+/+) in regions that stained positive for the macrophage marker...
CD68 (Figure 2A). By contrast, staining for Sema3E was markedly reduced in plaques of aortic arch segments transplanted into the regressive environment (ApoE\(^{-/-}\)→C57BL/6), which as in previous studies\(^{21}\) also show a decrease in plaque size and macrophage content. To confirm that macrophages are a source of Sema3E expression in the plaque, we isolated mRNA from lesional CD68\(^{+}\) macrophages by laser capture microdissection and measured gene expression by quantitative reverse transcription-polymerase chain reaction. Consistent with our immunohistochemical analyses and the microarray studies,\(^{15}\) macrophages isolated from plaques in the progressive environment expressed abundant Sema3E mRNA (Figure 2B), which was reduced by 90% in macrophages from plaques transplanted into a regressive environment for only 3 days. This decrease in macrophage Sema3E mRNA also correlated with a reduction in the inflammatory M1 macrophage marker Nos2 and an increase in the anti-inflammatory M2 macrophage marker Arg1 (Figure 2B), which we have previously shown to characterize plaques undergoing regression.\(^{6,7,22}\) To investigate further whether Sema3E expression is associated with the M1 or M2 macrophage phenotype, we measured its expression in bone marrow–derived macrophages polarized in vitro. Sema3E mRNA is highly increased in M1 macrophages polarized with lipopolysaccharide and IFN-\(\gamma\), but not M2 macrophages polarized with interleukin-4, compared with untreated macrophages (Figure 2C), indicating that its expression is correlated with inflammatory macrophages.

As the atherosclerosis regression observed in the aortic arch transplant model is quite rapid, we next sought to determine whether Sema3E was also regulated in plaques under conditions simulating less aggressive lipid-lowering management. To do this, Ldlr\(^{-/-}\) mice were fed a western diet for 14 weeks and either euthanized for baseline plaque measurements or switched to chow for an additional 4 weeks. This switch to chow is associated with reductions in total plasma cholesterol and plaque lipid content.\(^{7}\) Consistent with our findings in the transplant model of regression, macrophages isolated from aortic root plaques of Ldlr\(^{-/-}\) mice fed a western diet showed a marked decrease in Sema3E mRNA compared with macrophages from baseline progressing atherosclerotic plaques (Figure 2D). Collectively, these data indicate that Sema3E expression by lesional macrophages differentially responds to progressive and regressive atherosclerotic environments.

**Macrophage Sema3E Expression Is Upregulated by Physiological Drivers of Plaque Inflammation**

To further understand the molecular mechanisms of Sema3E expression in plaque macrophages and to extend the above observations in M1 and M2 polarized macrophages, we investigated whether Sema3E is induced by proinflammatory/atherogenic stimuli in vitro. We treated macrophages with acetylated or oxLDL (modifications that promote cholesterol loading of macrophages) and measured expression of Sema3E mRNA by quantitative polymerase chain reaction. Acetylated low-density lipoprotein or oxLDL induced a 3- to 4-fold increase in Sema3E mRNA (Figure 3A and 3B), whereas native LDL did not alter expression of these genes (data not shown). Because oxLDL promotes plaque oxidative stress and induces the hypoxia-inducible factor 1\(\alpha\) transcription factor, we also treated bone marrow–derived macrophages with a chemical mimic of hypoxia, CoCl\(_2\), which stabilizes hypoxia-inducible factor 1\(\alpha\). Similar to oxLDL, CoCl\(_2\) increased macrophage Sema3E mRNA 5-fold (Figure 3C). Notably, the increase in Sema3E mRNA induced by oxLDL and CoCl\(_2\) was blocked by pretreating macrophages with an inhibitor of hypoxia-inducible factor 1\(\alpha\) (Figure 3D and 3E), implicating this transcription factor in the upregulation of Sema3E. Immunoblot analysis confirmed an increase in full-length Sema3E (89 kDa) in the supernatant of oxLDL-treated macrophages, which was paralleled by an increase in the cleaved form (69 kDa) of Sema3E (Figure 3F). Notably, treatment with HDL, which promotes cholesterol efflux from the cell and blocks the inflammatory actions of oxLDL,\(^{23}\) reduced levels of Sema3E secreted by oxLDL-stimulated cells, whereas treatment of macrophages with HDL alone did not alter levels of full-length Sema3E.
or cleaved Sema3E (Figure 3F). Similarly, macrophages treated with oxLDL, but not HDL, show increased abundance of PlexinD1 protein (250 kDa), and the effects of oxLDL on PlexinD1 are partially reversed by coincubation with HDL (Figure 3G). Collectively, these data demonstrate that Sema3E and PlexinD1 expression in macrophages is dynamically regulated by pro- and antiatherosclerotic stimuli.

**Sema3E Inhibits the Migration of Macrophages**

Sema3E has previously been reported to act as a negative guidance cue for thymocytes, and inhibits the migration of these cells to CCL25 and CCL21.24 Because recent studies have identified the importance of chemokine-mediated macrophage emigration from the plaque during atherosclerosis regression,4–6,8,13,15,21,22 we next examined the effect of Sema3E on macrophage chemotaxis using both transwell Boyden chambers and a real-time detection method (xCelligence).

Whereas Sema3E had little effect on macrophage migration in the absence of chemokine, it potently inhibited peritoneal macrophage migration toward the CCR7 ligand, CCL19, a chemokine receptor–ligand pair implicated in macrophage egress from atherosclerotic plaques5 (Figure 4A). Analogous results were obtained using real-time detection of macrophage migration, where peritoneal macrophages showed an increase in migration to CCL19 in the lower chamber within 30 minutes, whereas for up to 4 hours, macrophages treated with 250 ng/mL Sema3E and CCL19 showed no increase in migration above that in cells treated with media alone (Figure 4B). Peritoneal macrophages pretreated with Sema3E for 45 minutes, and then washed before exposure to chemokines, also showed impaired migration to CCL19 (Figure 4C), indicating that Sema3E does not require direct interaction with the chemokine to mediate its inhibitory effect. This effect was not chemokine-specific, as Sema3E also blocked migration of peritoneal macrophages to CCL2 (Figure 4D), a second chemokine implicated in the emigration of inflammatory macrophages to the lymph nodes.25,26

To gain insight into the mechanisms by which Sema3E inhibits directed chemotaxis of macrophages to multiple chemokines, we measured its effect on the organization of the actin cytoskeleton. Stimulation of pMø with CCL19 or CCL2 induced a marked reorganization of actin, characterized by the
appearance of membrane ruffles, lamellipodia, and filopodia (Figure 5A and 5B). Pretreatment with Sema3E before stimulation with CCL19 or CCL2 inhibited these effects, and cells maintained a rounded morphology consistent with their non-motile phenotype. Accordingly, whereas pMø exhibit rapid cell spreading in response to chemokine stimulation, cell pretreated with Sema3E showed no increase in mean cell area (Figure 5A and 5B).

As members of the Rho family of guanosine triphosphatases (GTPases) play key roles in the reorganization of actin in macrophages, we next investigated whether Sema3E altered activation of Rac1 and CDC42 in response to chemotactic

Figure 3. Sema3E expression is upregulated by physiological drivers of plaque inflammation. Quantitative polymerase chain reaction (qPCR) analysis of Sema3E mRNA in bone marrow–derived macrophages (BMDMs) treated with (A) 50 µg/mL of acetylated low-density lipoprotein (AcLDL) or (B) oxidized low-density lipoprotein (oxLDL) for the indicated times, or (C) CoCl2 for 24 hours. D to E, qPCR analysis of Sema3E mRNA expression in peritoneal macrophages treated with (D) oxLDL (50 µg/mL) or (E) CoCl2 (200 µmol/L) with or without hypoxia-inducible factor 1 α (HIF-1α) inhibitor (100 µmol/L). G, Western blot of full-length and cleaved Sema3E in supernatants from BMDMs stimulated with 50 µg/mL oxLDL, 10 µg/mL high-density lipoprotein (HDL), or both for 24 hours. Data in A to C are the mean of triplicate samples±SEM. Statistical analysis was performed by ANOVA. A to C, *P<0.05 compared with untreated. D to E, *P<0.05 compared with oxLDL or CoCl2 alone.

Figure 4. Sema3E inhibits the migration of macrophages. A, Transwell migration of peritoneal macrophages to CCL19 (500 ng/mL) with or without recombinant Sema3E at the concentrations indicated. B, Real-time migration (xCelligence) of peritoneal macrophages to CCL19 with or without 250 ng/mL Sema3E. C, Transwell migration of peritoneal macrophages pretreated with 250 ng/mL Sema3E for 45 minutes, washed and then exposed to 500 ng/mL CCL19. D, Transwell migration of peritoneal macrophages to 100 ng/mL CCL2 with or without recombinant Sema3E at the concentrations indicated. Data are the mean±SD of triplicate samples in a single experiment and are representative of an experimental n=3. Statistical analysis was performed by ANOVA followed by Tukey test; (A, C, and D) *P<0.01.
stimulation. Using GST beads conjugated to PAK1-p21-binding domain to immunoprecipitate the GTP-bound forms of Rac1/CDC42, we show that levels of activated Rac1 and CDC42 were rapidly increased in bone marrow–derived macrophages stimulated with CCL2 (Figure 6A and 6B). By contrast, pretreatment of macrophages with Sema3E inhibited CCL2-induced Rac1 and CDC42 activation. Thus, Sema3E blocks activation of the effectors of lamellipodia (Rac1) and filopodia (CDC42) formation, which are essential components of the migratory machinery. Collectively, these data indicate that Sema3E inhibits the directional migration of macrophages by disrupting the Rho GTPase signaling cascade, reorganization of the actin cytoskeleton and cell polarization.

**Figure 5.** Sema3E affects the organization of the actin cytoskeleton. Peritoneal macrophages stained with Phalloidin to detect polymerized actin after treatment with (A) 500 ng/mL CCL19 or (B) 100 ng/mL CCL2 in the presence or absence of recombinant Sema3E (250 ng/mL). Arrows, Membrane ruffles; scale bar, 10 μm. Mean cell surface area of macrophages is graphed at right. Statistical analysis was performed by ANOVA; *P<0.05 compared with CCL19 (A) or CCL2 (B) alone.

**Figure 6.** Sema3E impairs Rho guanosine triphosphatases (GTPase) signaling. Immunoblot of activated (A) Rac1 and (B) CDC42 in macrophages treated with 100 ng/mL CCL2 with and without 250 ng/mL Sema3E. Densitometry quantification of blots is shown graphically. Data shown are from 1 experiment and are representative of an experimental n=3.

**Discussion**

Atherosclerosis is a chronic and progressive inflammatory condition, driven by the accumulation of lipids and immune cells in plaques. Current concepts view the persistence of macrophages in plaques as a failure to resolve inflammation. Recent studies in mouse models of atherosclerosis have revived the hope that plaque macrophage lipid homeostasis and emigration can be clinically restored with aggressive lipid management, leading to the achievement of atherosclerosis regression in humans. These studies have indicated that macrophage egress from the plaque is likely to be actively inhibited during disease progression, such as with hypercholesterolemia, although the factors and their regulatory signals that impair egress remain largely unknown. In the present studies,
we have marshaled support for Sema3E to be one such factor by showing that (1) it is expressed in mouse atherosclerotic plaques, where there is significant colocalization with monocyte-derived cells; (2) its expression in vivo is significantly lower in these cells in a plaque regression versus progression environment; (3) its expression in vitro is higher in inflammatory M1 macrophages than in tissue remodeling, M2 ones, which are enriched in regressing plaques; and (4) it retards the migration of macrophages in vitro to several chemokines, including CCL19, previously shown to be a mediator of macrophage egress from regressing plaques,5 apparently by altering cytoskeletal reorganization. Thus, Sema3E is predicted to have a macrophage-retention, anti–inflammation-resolving effect in plaques similar to what we recently established for another neuronal guidance molecule, netrin-1.13

Neuronal guidance cues are increasingly recognized as important players in immune function and chronic inflammatory diseases, and recent studies have implicated members of the Semaphorin family in the pathogenesis of rheumatoid arthritis (Sema3E, Sema3C9,20,50 and atherosclerosis (Sema4D).11,32 To our knowledge, this is the first time that Sema3E has been shown to be dynamically regulated in macrophages and identified in atherosclerotic plaques. Previous studies have shown the expression of Sema3E in the outer retina of embryonic chicks, in neurons throughout the ganglion cell layer of P4 mouse retinas, in thalamostriatal projection neurons and developing somites of mice, in mouse medullary thymocytes, and in mouse calvarial osteoblasts.11,19,24,33–37 Although there is a lack of understanding of the factors that mediate Sema3E expression in most of these cell types, in addition to the factors identified herein (hyperlipidemia, oxLDL, hypoxia), 1,25-dihydroxyvitamin D3 has been reported to increase Sema3E expression in mouse osteoblasts.33 PlexinD1, the canonical receptor for Sema3E, is highly expressed in many cell types, including macrophages,38 as we also describe herein. In addition, we find that PlexinD1 is upregulated by oxLDL in vitro and is expressed by macrophages in atherosclerotic plaques.

Sema3E is unique from other Semaphorins in that it binds directly to PlexinD1 and does not require coreceptors to exert its repellent signal.33 PlexinD1 is thought to exist at the plasma membrane in an inactive folded state, and on binding with Sema3E undergoes a conformational change that activates its GTPase-activating protein domain, which may enable protein–protein interactions with other domains, such as its Rho GTPase–binding domain.19 The Sema3E activation of PlexinD1 can disrupt focal adhesions and integrin-mediated cell adhesion to the extracellular matrix, affect PI3K signaling and cause the RhoA-dependent collapse of the cytoskeleton in neurons and endothelial cells.11,31,40 Activation of PlexinD1 by Sema3E has also been shown to repress CCL25 signaling via its receptor CCR9 in thymocytes,24 and to inhibit mouse osteoblast migration in wound healing assays.34 Many of the properties of the Sema3E-PlexinD1 axis in other cell types, then, are consistent with what we observed in macrophages, namely the effects on the cytoskeleton and the inhibition of migration to several chemokines, including CCL19, previously implicated as an atherosclerosis regression factor.5 Perhaps more striking, then, was the dynamic regulation of Sema3E expression in macrophages by factors related to atherosclerosis. That Sema3E expression at the protein or mRNA level was higher in progressing than in regressing plaques, and increased in vitro by oxLDL and a hypoxia mimic (CoCl2), strongly implies that it participates in the retention of macrophages and the failure to resolve inflammation under proatherogenic conditions. This is further supported in a model of in vitro macrophage polarization, in which we found that Sema3E is highly expressed in classically activated inflammatory M1 macrophages, which are thought to be the predominant phenotype under conditions that are proatherogenic,41 but less expressed in the reparative M2 macrophages, which are considered mediators of inflammatory resolution,42 and whose markers are enriched in regressing plaques,6,7,15,22,43 coincident with decreased Sema3E mRNA expression.

Monocyte and macrophage death are well-recognized factors that determine the content of macrophages in mouse models of atherosclerosis.27 An emerging picture based on the present and prior studies4,5,17,45 is that macrophage egress is also a determinant of plaque macrophage content and that it is regulated by factors that can be broadly classified as gas pedals (emigration signals [e.g., CCR7]), and brakes (inhibitory guidance cues [e.g., Sema3E and netrin-1]), adhesion molecules [e.g, cadherins, vinculin], cellular motility factors [e.g, actin and myosin]).15 On the basis of hundreds of differences in the transcriptomes of monocyte-derived cells in regressing versus progressing plaques,15 it is likely that the list of gas pedals and brakes, as well as their modifiers, will grow. This will not only expand our fundamental understanding of atherosclerosis and other chronic inflammatory conditions but also suggest strategies to reduce the considerable residual risk of heart disease observed with the currently available therapies.

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**Disclosures**

None.

**References**


17. Significance

This study identifies semaphorin 3E as a negative regulator of macrophage migration that is expressed in progressing atherosclerotic plaques and decreased in regressing plaques. Our data suggest a role for Sema3E in the retention of macrophages in atherosclerosis and highlight the expanding functions of neuroimmune guidance cues in regulating immune cell function.
Neuroimmune Guidance Cue Semaphorin 3E Is Expressed in Atherosclerotic Plaques and Regulates Macrophage Retention


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MATERIALS AND METHODS

Experimental Animals and Diet: C57BL/6J, Ldlr−/− and Apoe−/− mice were from Charles River or Jackson Laboratories. All mice were maintained in a pathogen-free facility. Experimental procedures were performed in accordance with the New York University School of Medicine’s Subcommittees on Research Animal Care and Use. All experiments were approved by the New York University School of Medicine Institutional Animal Care and Use Committee. Aortic arch transplantation studies were conducted as previously described. Briefly, Apoe−/− mice were weaned at 4 weeks and placed on a Western diet (WD; 21% [wt/wt] fat, 0.3% cholesterol; Research Dyets) for 16 weeks. The mice were then divided into three groups (i) a pre-transplant group (n=5) for baseline analysis (ii) aortic transplants into Apoe−/− recipient mice (n=5) (iii) aortic transplants into wild type (C57/BL/6) recipient mice (n=5). Recipient mice were 20 week old males kept on a standard chow diet. The donor arch was interpositioned with the abdominal aorta in the recipient mouse and blood flow was directed through the graft. Recipient mice were maintained on a standard chow diet and sacrificed at 3 days after transplantation. In a second model of regression, 4 week old Ldlr−/− mice were placed on a WD diet for 14 weeks and were either sacrificed (baseline) or switched to chow diet for 4 weeks as previously described.

Histology and Immunostaining: Serial frozen sections of formalin fixed aortic arch or aortic sinus plaques were prepared from OCT embedded tissue using a cryostat. Sections were blocked with 10% normal serum and then incubated for 2 h at 22 °C or overnight at 4 °C with the following primary antibodies: rabbit antibody to Sema3E (10 µg/ml; AF3239; R&D), unconjugated or Alexa Fluor 488–conjugated rat antibody to mouse CD68 (1:250 dilution; FA-11; AbD Serotec), rabbit antibody to plexinD1 (gift from Jonathan Duke-Cohan, Dana-Farber Cancer Institute, Boston, MA) and goat antibody to smooth muscle actin (2.5 µg/ml; ab21027; Abcam). Sections were washed and, where necessary, incubated for 30 min with biotinylated antibody to rabbit IgG (BA-1000; Vector Laboratories), followed by incubation for 15 min with strepavadin-conjugated Texas Red (Sa-5006; Vector Laboratories) or fluorescein (Sa-5001, Vector Laboratories), or Alexa Fluor 488–conjugated donkey antibody to goat IgG (A11055; Invitrogen) or Alexa Fluor 568–conjugated goat antibody to rat IgG (A11077; Invitrogen). Sections without primary antibody served as negative controls. Sections were mounted with Vectashield medium with DAPI and were visualized with either a TCS-SP confocal laser-scanning microscope (Leica) or an Axioscope 2 Plus fluorescence microscope (Carl Zeiss).

Laser Capture Microdissection (LCM) and RNA Extraction: Isolation and capture of CD68+ cells from aortic arch or root sections was performed as previously described. Briefly, a guide slide was prepared by staining sections for CD68 as described above. Cells corresponding to CD68+ area in serial sections were collected using a PixCell II instrument (Arcturus Bioscience), and RNA was extracted using the Arcturus Picopure RNA Isolation Kit. Total RNA was amplified using the Ovation WT PicoAmp Kit (NuGen), purified using Qiagen PCR Purification Kit and used for quantitative PCR.

Quantitative RT-PCR: Total RNA was isolated from cells using TRIzol Reagent (Invitrogen). RNA (1µg) was reverse-transcribed with an iScript cDNA Synthesis kit according to the manufacturer’s instructions (Bio-Rad). KAPA SYBR FAST qPCR Master Mix was used for RT-PCR analysis. Primers used are listed in supplemental Table I. The change in mRNA expression was calculated by the comparative change in cycle method (ΔΔCT). All data were normalized to GAPDH or 28S and were expressed as fold change over the control.

Cell Culture: Peritoneal macrophages (pMø) were collected from mice by peritoneal lavage 4 d after intraperitoneal injection of 3% (wt/vol) thioglycollate as previously described. Bone marrow derived macrophages (BMDMs) were prepared from mice as previously described. For gene expression experiments, macrophages were plated at 1 x10⁶ cells/well in 6 well plates. CoCl₂ (0.1M solution) was
obtained from Sigma and used at the concentrations indicated to mimic hypoxia. Oxidation or acetylation of LDL (50 µg/mL; Biomedical Technologies) was performed as described⁴. For HIF-1α inhibitor experiments BMDMs were stimulated with 50 µg/mL of oxLDL or 100 µM CoCl2 for 24 hours. HIF-1α inhibitor [100µM] was from Calbiochem (400083, EMD Millipore). Recombinant IL-4 and IFN-γ were purchased from PeproTech. Ultrapure LPS from *E. coli* K12 was purchased from InvivoGen.

**F-actin staining:** Freshly isolated peritoneal macrophages were seeded on 8 well Lab Tek slides (Thermo Scientific) for 24 hours or used directly. The cells were pretreated with mouse recombinant Semaphorin 3E (250 ng/ml; R&D Systems) or PBS for 30 minutes and stimulated with either CCL19 (500ng/ml; R&D Systems) or CCL2 (10ng/ml; R&D Systems) for the indicated times. Macrophages were fixed (4% paraformaldehyde), permeabilized (0.1% Triton X-100) and actin filaments stained with Alexa Fluor 568 or 647 phalloidin (Molecular Probes, Invitrogen) at a dilution of 1/40. The cells on coverslips were mounted after DAPI staining and visualized with either a TCS-SP confocal laser-scanning microscope (Leica) or an Axioscope 2 Plus fluorescence microscope (Carl Zeiss). Images were background corrected followed by measurement of the average cell area of at least 10 cells per field using I-vision software.

**Migration Assays:** Chemotaxis of macrophages (pMø and BMDMs) was measured in 96-well Boyden chamber with a 5 µm (BMDMs) or 8 µm (pMø) membrane (Neuro Probe) as previously described⁵. Macrophage chemotaxis was also quantified using a Real-time Cell Invasion and Migration (RT-CIM) assay system with monitoring every 5 min (xCELLigence, Roche Applied Science). Cell migration to CCL2 (100ng/ml; R&D Systems) or CCL19 (500 ng/ml; R&D Systems) was measured in the absence or presence of recombinant Sema3E (mouse or human). In some assays cells were pretreated Sema3E for 45 min prior to migration. Following cell migration for 3 or 16 h, four random high power fields from each well were counted to determine the number of cells that had migrated through the membrane. Migration was expressed as the chemotactic index, which was calculated by dividing the number of migrating cells in the treated groups by the number of migrating cells in the lowest control well. Results of chemotaxis assays are representative of at least three independent experiments performed on triplicate samples.

**Western Blot:** Cells were lysed in ice-cold buffer containing 50 mM Tris-HCl, pH 7.5, 125 mM NaCl, 1% Nonidet P-40, 5.3 mM NaF, 1.5 mM NaP, 1 mM orthovanadate, 175 mg/ml octylglucopyr-anoside, 1 mg/mL protease inhibitor cocktail (Roche), and 0.25 mg/ml 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (AEBSF) (Roche). Cell lysates were rotated at 4 °C for 1 h before the insoluble material was removed by centrifugation at 12,000 × g for 10 min. After normalizing for equal protein concentration, cell lysates were resuspended in SDS sample buffer before separation by SDS/PAGE. After overnight transfer of the proteins onto nitrocellulose membranes, the membranes were probed with the indicated antibodies, and protein bands were visualized using the Odyssey Infrared Imaging System (LI- COR). Densitometry analysis of the gels was carried out using ImageJ software from the National Institutes of Health (http://rsbweb.nih.gov/ij/).

**Statistics:** Statistical significance was assessed by an unpaired, two-tailed Student’s t-test for single comparison, or analysis of variance for multiple comparisons. A P value of less than 0.05 was considered significant.
REFERENCES:


