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

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Abstract

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 two 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 LDL 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 up-regulated in macrophages of advanced plaques, is dynamically regulated by multiple atherosclerosis-relevant factors, and acts as a negative regulator of

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macrophage migration, which may promote macrophage retention and chronic inflammation *in vivo*.

Keywords

Atherosclerosis; Semaphorin 3E; macrophage; migration; regression

INTRODUCTION

The persistence of myeloid-derived cells in the artery wall is characteristic of advanced atherosclerotic plaques, and these inflammatory cells contribute not only to atherosclerosis progression, but also plaque instability through the secretion of extracellular matrix-degrading proteases and cytotoxic factors. Not surprisingly, then, there has been a major effort to understand macrophage dynamics in the atherosclerotic plaque. Studies examining monocyte recruitment in atherosclerosis have revealed a number of different chemokines and receptors involved in monocyte homing to the arterial wall¹. Perturbations in blood flow at areas of arterial branching, as well as both foreign and endogenous insults (including the retention of atherogenic lipoproteins) to the endothelium, are thought to be responsible for the upregulation of adhesion molecules and chemokines leading to monocyte recruitment and influx into the subendothelial layer of the aortic wall. As atherosclerosis is a chronic, progressive disease, it is thought that the accumulation of monocyte-derived macrophages compounds over time and is a major contributor to the persistence of the disease state².

As part of the resolution phase of acute inflammation, activated macrophages normally emigrate from the site of localized inflammation to the draining lymphatics³. However, unlike other acute inflammatory states, atherosclerotic inflammation does not readily resolve and cholesterol-laden macrophages (foam cells) persist in the arterial wall. Macrophages in atherosclerotic plaques appear 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 secondary necrosis, and the retention of activated macrophages further aggravates plaque inflammation, which can lead to instability and rupture¹. Yet, the mechanisms by which macrophages are retained in the plaque are poorly understood. It has been shown that emigration of macrophages from atherosclerotic lesions can occur under conditions of plaque regression induced either by reducing plasma cholesterol^{4,5} 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^{5,8} 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 development⁹. 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 system⁹. In addition, emerging evidence is revealing roles for neuronal guidance molecules in the immune system where they appear to exert diverse effects on leukocyte migration, adhesion and inflammatory responses¹⁰⁻¹². Notably, we recently reported the involvement of netrin-1, a neuronal guidance molecule, in promoting chronic inflammation in atherosclerosis by retaining macrophages in plaque¹³.

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 microdissection¹⁶. Using these tools, we recently profiled the transcriptome changes in progressing and regressing atherosclerotic plaques¹⁵. 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 to 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 appears to be cell type and context specific²⁰. 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 (e.g., CCL2 and CCL19). Sema3E expression in macrophages *in vitro* is up-regulated 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.

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 (Fig. 1a, arrows). In addition, there appeared 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 co-localized with CD68-positive macrophages in these advanced atherosclerotic plaques (Fig. 1b, arrows), suggesting that these cells may be both the source and 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 (WD) for 16 weeks is transplanted into either a hyperlipidemic *ApoE*^{–/–} (progressive environment) or normolipidemic wild type C57BL6 (regressive environment) recipient mouse for 3 days¹⁴. 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 (Fig. 2a). By contrast, staining for *Sema3E* was markedly reduced in plaques of aortic arch segments transplanted into the regressive environment (*ApoE*^{-/-} → C57BL6), 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 RT-PCR (qPCR). Consistent with our immunohistochemical analyses and the microarray studies¹⁵, macrophages isolated from plaques in the progressive environment expressed abundant *Sema3e* mRNA (Fig. 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* (Fig. 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 (BMDM) polarized *in vitro*. *Sema3e* mRNA is highly increased in M1 macrophages polarized with LPS and IFN γ , but not M2 macrophages polarized with IL-4, compared to untreated macrophages (Fig. 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 WD for 14 weeks and either sacrificed for baseline plaque measurements or switched to a chow diet for an additional 4 weeks. This switch to chow is associated with reductions in total plasma cholesterol and plaque lipid content⁷. Consistent with our findings in the transplant model of regression, macrophages isolated from aortic root plaques of *Ldlr*^{-/-} mice switched to chow diet showed a marked decrease in *Sema3e* mRNA compared to macrophages from baseline progressing atherosclerotic plaques (Fig. 2d). Collectively, these data indicate that *Sema3e* expression by lesional macrophages differentially responds to progressive and regressive atherosclerotic environments.

Macrophage *Sema3E* Expression is Up-Regulated 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 pro-inflammatory/atherogenic stimuli *in vitro*. We treated macrophages with acetylated or oxidized LDL (modifications that promote cholesterol loading of macrophages) and measured expression of *Sema3e* mRNA by qPCR. AcLDL or oxLDL induced a 3-4-fold increase in *Sema3e* mRNA (Fig. 3a-b), whereas native LDL did not alter expression of these genes (data not shown). As oxLDL promotes plaque oxidative stress and induces the hypoxia-inducible factor 1 α (HIF1 α) transcription factor, we also treated BMDM with a chemical mimic of hypoxia, CoCl₂, which stabilizes HIF1 α . Similar to oxLDL, CoCl₂ increased macrophage *Sema3e* mRNA 5-fold (Fig. 3c). Notably, the increase in *Sema3e* mRNA induced by oxLDL and CoCl₂ was blocked by pretreating macrophages with an inhibitor of HIF-1 α (Fig. 3d-e), 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* (Fig. 3f). Notably, treatment with HDL, which promotes cholesterol efflux from the cell and blocks the inflammatory actions of oxLDL²³, reduced levels of *Sema3E* secreted by oxLDL-stimulated cells, whereas

treatment of macrophages with HDL alone did not alter levels of full-length or cleaved Sema3E (Fig. 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 co-incubation with HDL (Fig. 3g). Collectively, these data demonstrate that Sema3E and PlexinD1 expression in macrophages is dynamically regulated by pro- and anti-atherosclerotic 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 CCL21²⁴. As 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 towards the CCR7 ligand, CCL19, a chemokine receptor-ligand pair implicated in macrophage egress from atherosclerotic plaques⁵ (Fig. 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 min, whereas for up to 4 h, macrophages treated with 250 ng/ml Sema3E and CCL19 showed no increase migration above that in cells treated with media alone (Fig. 4b). Peritoneal macrophages pre-treated with Sema3E for 45 min, and then washed before exposure to chemokines, also showed impaired migration to CCL19 (Fig. 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 (Fig. 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 pM ϕ with CCL19 or CCL2 induced a marked reorganization of actin, characterized by the appearance of membrane ruffles, lamellipodia and filopodia (Fig. 5a-b). Pre-treatment with Sema3E prior to stimulation with CCL19 or CCL2 inhibited these effects, and cells maintained a rounded morphology consistent with their non-motile phenotype. Accordingly, whereas pM ϕ exhibit rapid cell spreading in response to chemokine stimulation, cell pre-treated with Sema3E showed no increase in mean cell area (Fig. 5a-b).

As members of the Rho family of 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 stimulation. Using GST beads conjugated to PAK1-PBD to immunoprecipitate the GTP-bound forms of Rac1/CDC42, we show that levels of activated Rac1 and CDC42 were rapidly increased in BMDM stimulated with CCL2 (Fig. 6a-b). 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, re-organization of the actin cytoskeleton and cell polarization.

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 inflammation²⁷. 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 humans²⁸. 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 co-localization with monocyte-derived cells; 2) Its expression *in vivo* is significantly lower in these cells in a plaque regression vs. 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 a number of chemokines, including CCL19, previously shown to be a mediator of macrophage egress from regressing plaques⁵, 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-1¹³.

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, Sema3C)^{29, 30} and atherosclerosis (Sema4D)^{31, 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 the majority 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 osteoblasts³³. PlexinD1, the canonical receptor for Sema3E, is highly expressed in many cell types, including macrophages³⁸, 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 signal³⁶. PlexinD1 is thought to exist at the plasma membrane in an inactive folded state, and upon binding with Sema3E undergoes a conformational change that activates its Guanosine triphosphate (GTP-ase)-Activating-Protein (GAP) domain, which may enable protein-protein interactions with other domains, such as its Rho GTPase-Binding Domain (RBD)³⁹. 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 dependant collapse of the cytoskeleton in neurons and endothelial cells^{10, 39, 40}. Activation of PlexinD1 by Sema3E has also been shown to repress CCL25 signaling via its receptor CCR9 in thymocytes²⁴, and to inhibit mouse osteoblast migration in wound healing assays³³.

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 a number of chemokines, including CCL19, previously implicated as an atherosclerosis regression factor⁵. 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 pro-atherogenic 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 pro-atherogenic⁴¹, but less expressed in the reparative “M2” macrophages, which are considered mediators of inflammatory resolution⁴², 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 atherosclerosis²⁷. An emerging picture (recently reviewed in⁴⁴) based on the present and prior studies^{4, 5, 13, 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. Based on the hundreds of differences in the transcriptomes of monocyte-derived cells in regressing vs. progressing plaques¹⁵, 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 will also suggest strategies to reduce the considerable residual risk of heart disease observed with the currently available therapies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

Sema3E	Semaphorin 3E
oxLDL	oxidized LDL
AcLDL	acetylated LDL
BMDM	bone marrow derived macrophage
HIF-1a	hypoxia inducible factor 1 alpha

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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.

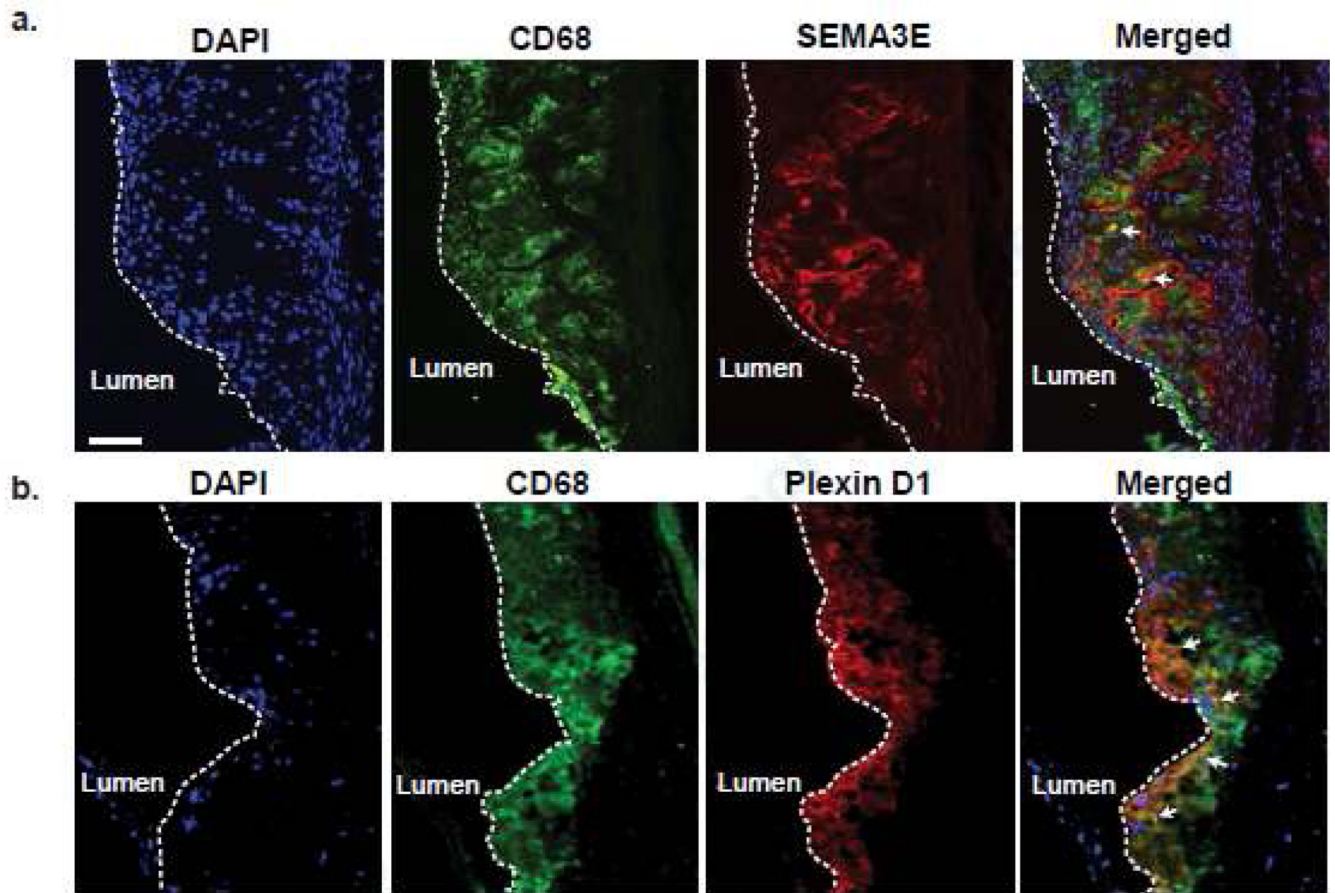


Figure 1. Sema3E and its Receptor PlexinD1 are Expressed by Macrophages in Advanced Atherosclerotic Plaques

Immunofluorescent staining of CD68 (green), DNA (DAPI, blue) and (a) Sema3E (red) or (b)^{4, 5}, PlexinD1 (red) in aortic root atherosclerotic plaques of *ApoE*^{-/-} mice fed a Western diet for 12 weeks. Areas of co-localization are shown in yellow in the merged image (arrows). Scale bar, 50 μ m. Images are representative of n = 3 mice.

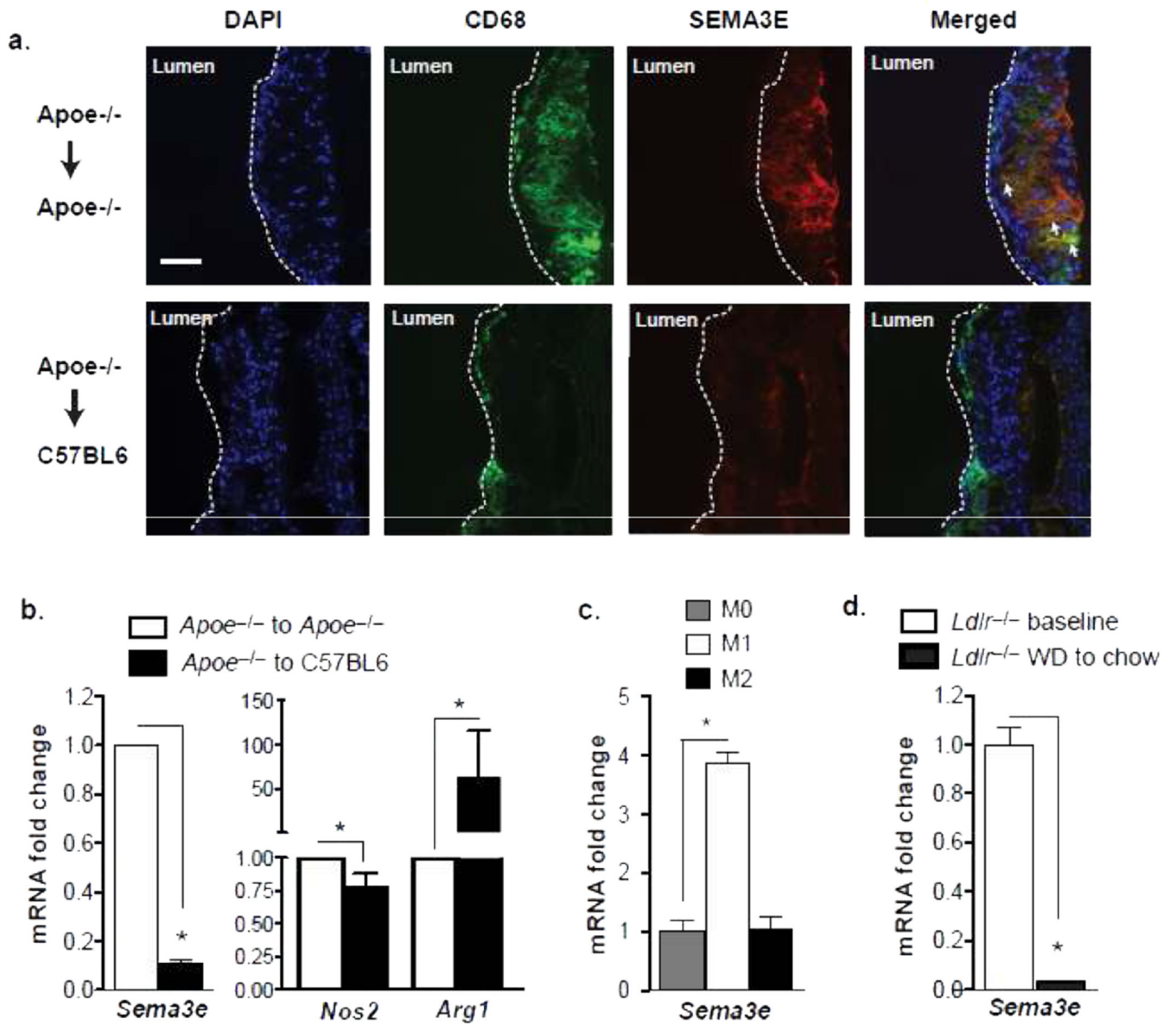


Figure 2. Sema3E Expression by Lesional Macrophages is Downregulated during Atherosclerosis Regression

(a) Immunofluorescent staining of CD68 (green) and Sema3E (red) in aortic arch plaques transplanted into a progressive (*Apoe*^{-/-} recipient) or regressive (C57BL/6 recipient) environment. Areas of co-localization (yellow) are shown in the merged image. Images are representative of n = 3 mice. (b) qPCR analysis of mRNA from laser captured CD68⁺ cells in aortic arch plaques from mice in (a) (n=4 mice/group). (c) qPCR analysis of *Sema3e* mRNA in BMDMs polarized towards M1, M2 or unpolarized (M0). (d) qPCR analysis of *Sema3e* mRNA from laser captured CD68⁺ cells in aortic root plaques from *Ldlr*^{-/-} mice fed a western diet for 14 weeks (baseline) and then switched to a chow diet for 4 weeks (regression conditions) (n=5 mice/group). Data in b-d are the mean of triplicate samples ± SEM. Statistical analyses were performed by Student's t-test (b,d) or ANOVA (c). *P0003C;0.05

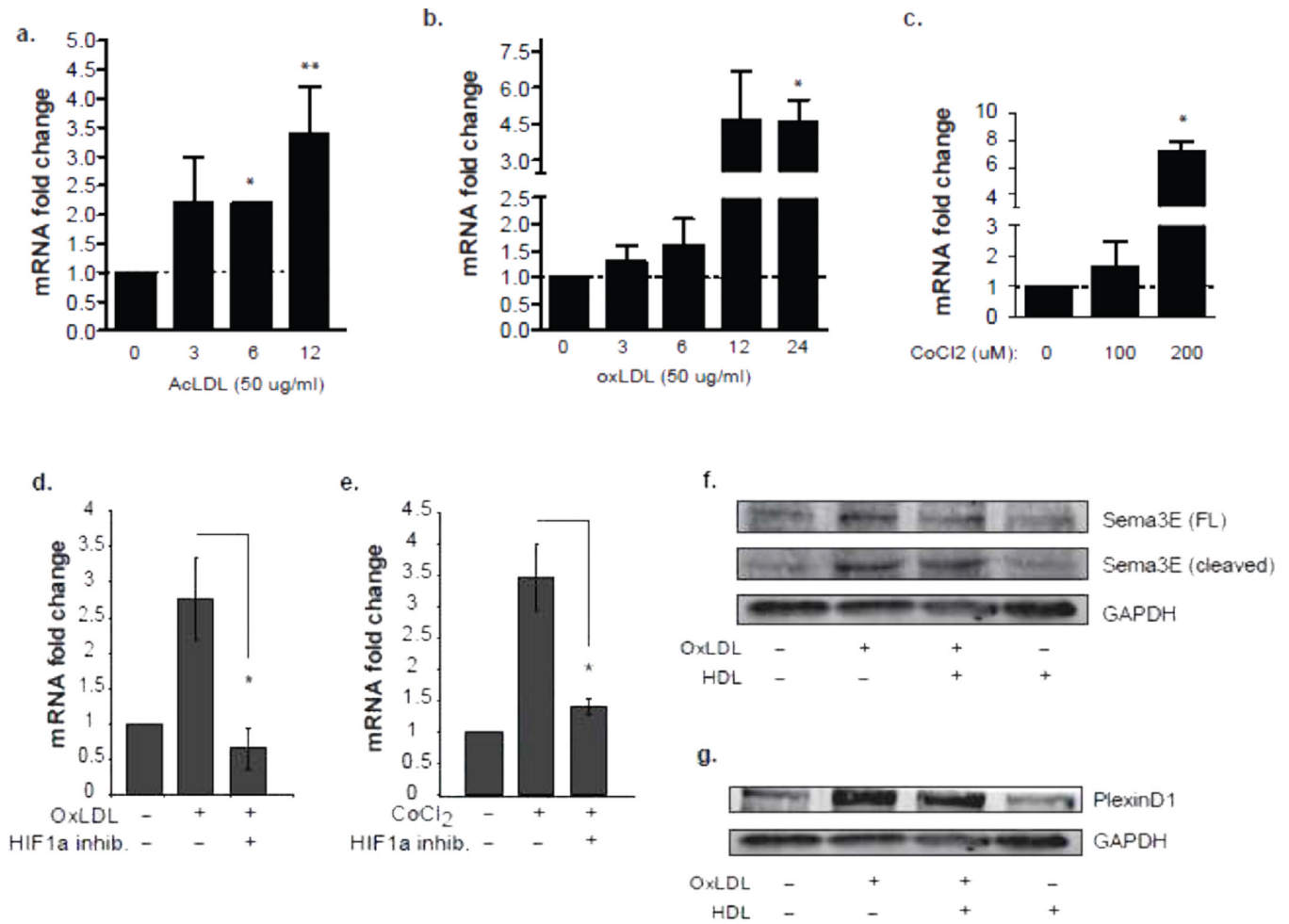


Figure 3. Sema3E Expression is Up-regulated by Physiological Drivers of Plaque Inflammation

qPCR analysis of *Sema3e* mRNA in BMDMs treated with (a) 50 μ g/ml of AcLDL or (b) oxLDL for the indicated times, or (c) CoCl₂ for 24 h. (d-e) qPCR analysis of *Sema3e* mRNA expression in peritoneal macrophages treated with (d) oxLDL (50 μ g/ml) or (e) CoCl₂ (200 μ M) with or without HIF-1 α inhibitor (100 μ mol/L). (f) Western blot of full-length and cleaved Sema 3E in supernatants from BMDMs stimulated with 50 μ g/ml oxLDL, 10 μ g/ml HDL, or both for 24 h. (g) Western blot of PlexinD1 in cell lysates of BMDMs stimulated with 50 μ g/ml oxLDL, 10 μ g/ml HDL, or both for 24 h. Data in a-c are the mean of triplicate samples \pm SEM. Statistical analysis was performed by ANOVA; (a-c) * P<0.05 compared to untreated, (d-e) * P<0.05 compared to OxLDL or CoCl₂ alone.

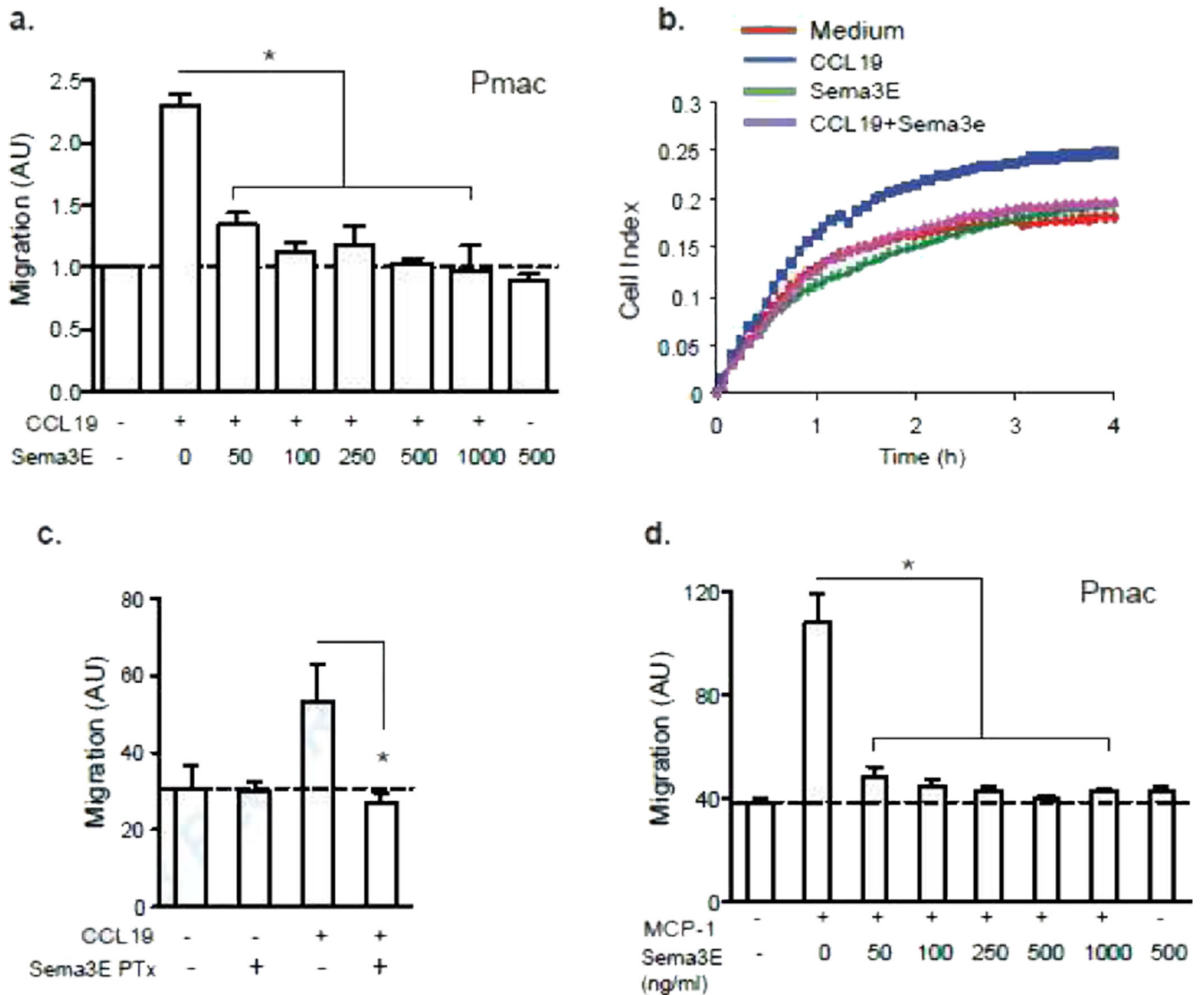


Figure 4. Sema3E Inhibits the Migration of Macrophages

(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 pre-treated 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 \pm s.d. 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,d) * $P<0.01$.

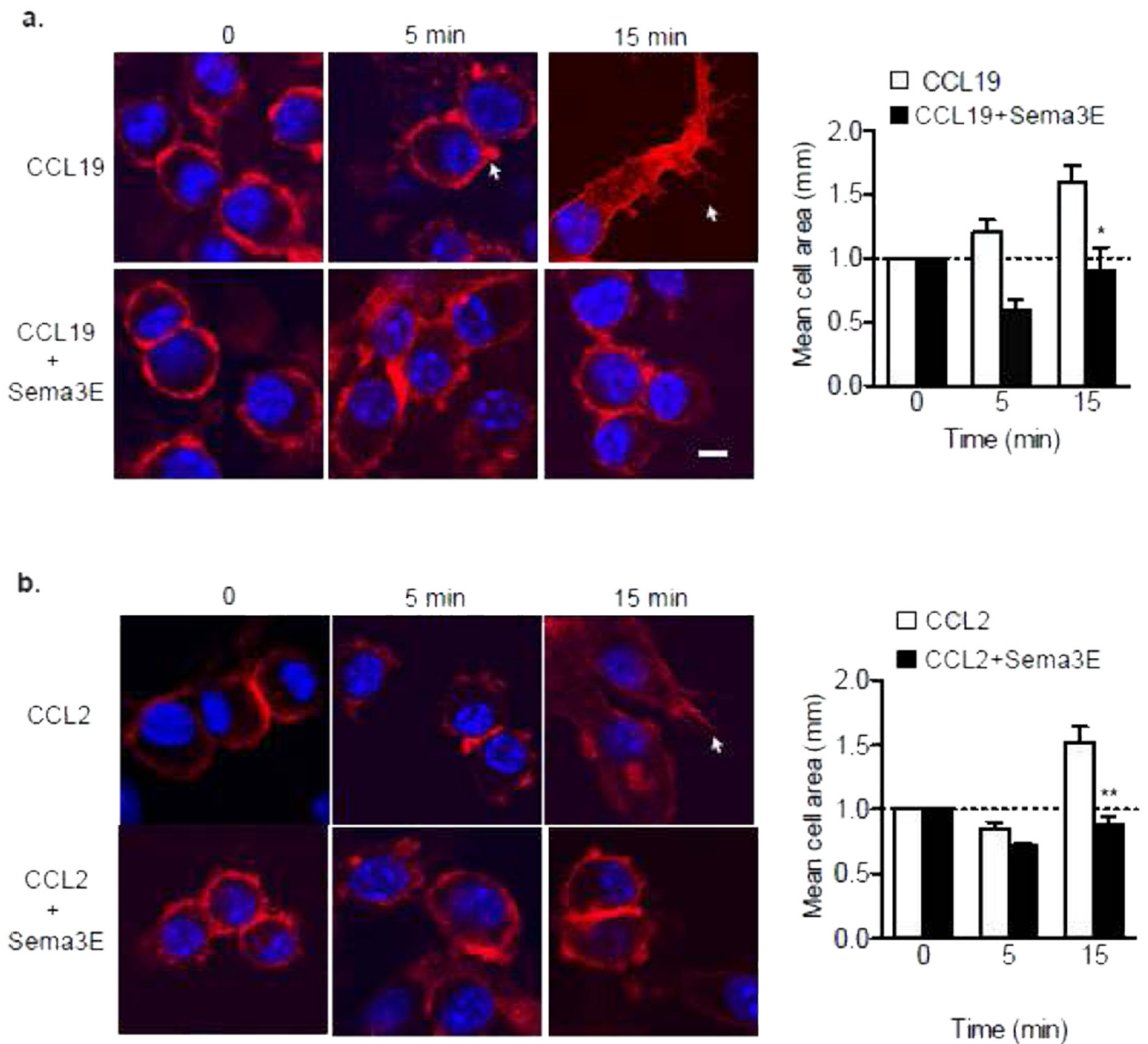


Figure 5. Sema3E Affects the Organization of the Actin Cytoskeleton

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 indicate membrane ruffles, scale bar 10 μ m.

Mean cell surface area of macrophages is graphed at right. Statistical analysis was performed by ANOVA; * $P < 0.05$ compared to CCL19 (a) or CCL2 (b) alone.

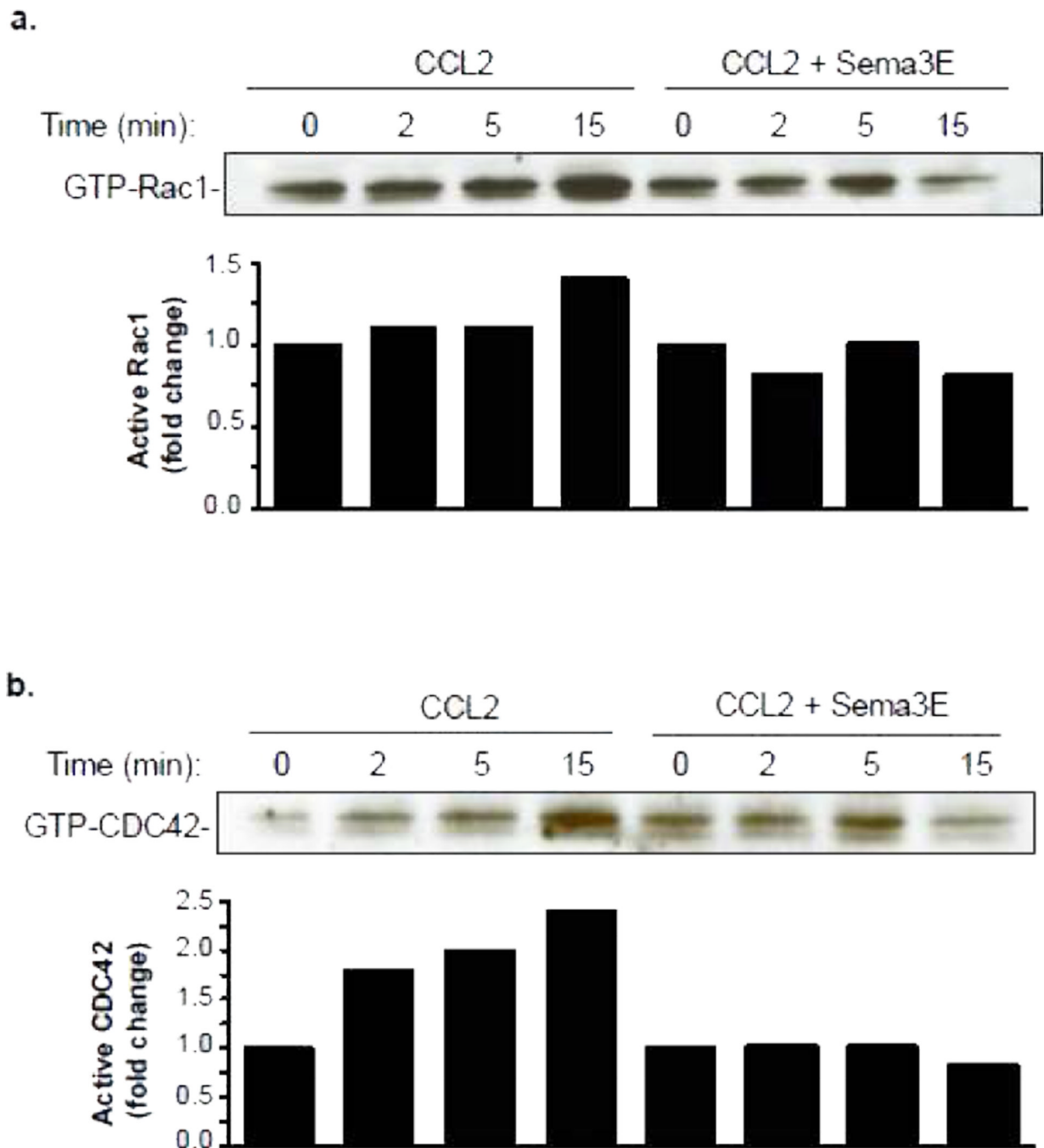


Figure 6. Sema3E impairs Rho GTPase signaling

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 is from one experiment, and is representative of an experimental n=3.