Endothelial Expression of Guidance Cues in Vessel Wall Homeostasis

Dysregulation Under Proatherosclerotic Conditions

Janine M. van Gils,* Bhama Ramkhelawon,* Luciana Fernandes, Merran C. Stewart, Liang Guo, Tara Seibert, Gustavo B. Menezes, Denise C. Cara, Camille Chow, T. Bernard Kinane, Edward A. Fisher, Mercedes Balcells, Jacqueline Alvarez-Leite, Adam Lacy-Hulbert, Kathryn J. Moore

Objective—Emerging evidence suggests that neuronal guidance cues, typically expressed during development, are involved in both physiological and pathological immune responses. We hypothesized that endothelial expression of such guidance cues may regulate leukocyte trafficking into the vascular wall during atherogenesis.

Approach and Results—We demonstrate that members of the netrin, semaphorin, and ephrin family of guidance molecules are differentially regulated under conditions that promote or protect from atherosclerosis. Netrin-1 and semaphorin3A are expressed by coronary artery endothelial cells and potently inhibit chemokine-directed migration of human monocytes. Endothelial expression of these negative guidance cues is downregulated by proatherogenic factors, including oscillatory shear stress and proinflammatory cytokines associated with monocyte entry into the vessel wall. Furthermore, we show using intravital microscopy that inhibition of netrin-1 or semaphorin3A using blocking peptides increases leukocyte adhesion to the endothelium. Unlike netrin-1 and semaphorin3A, the guidance cue ephrinB2 is upregulated under proatherosclerotic flow conditions and functions as a chemoattractant, increasing leukocyte migration in the absence of additional chemokines.

Conclusions—The concurrent regulation of negative and positive guidance cues may facilitate leukocyte infiltration of the endothelium through a balance between chemoattraction and chemorepulsion. These data indicate a previously unappreciated role for axonal guidance cues in maintaining the endothelial barrier and regulating leukocyte trafficking during atherogenesis. **(*Arterioscler Thromb Vasc Biol*. 2013;33:911-919.)**

Key Words: atherosclerosis ■ axonal guidance ■ endothelial–leukocyte interaction ■ migration

Atherosclerosis is a chronic inflammatory disease of the large arteries, in which monocyte recruitment into the vessel wall plays an essential role in both the initiation and progression of disease. Atherosclerotic plaques form predominantly at sites of disturbed laminar flow, notably, arterial branch points and bifurcations, and are initiated by the subendothelial accumulation of apolipoprotein B-containing lipoproteins.[1,2] The key early inflammatory response to these atherogenic lipoproteins is activation of overlying endothelial cells in a manner that leads to recruitment of blood-borne monocytes.[3] Research in the last decades has provided a rich description of the molecules involved in the recruitment of leukocytes into the artery wall, and highlighted the importance of the coordinated action of chemokines, integrins, and other adhesion molecules in directing this pathological process.[4] The chemoattractant and adhesive forces that recruit leukocytes to the vessel wall have been well studied in this context.[5] However, our understanding of other aspects of immune cell migration remains incomplete, particularly the mechanisms by which these cells are excluded from the artery in the absence of inflammation. In addition to the well-known positive migration cues that promote atherosclerotic plaque formation, it is reasonable to assume that chemorepulsive forces, or negative guidance cues, also exist to inhibit leukocyte–endothelial interactions under homeostatic conditions that may become dysregulated during disease.

Received on: August 21, 2012; final version accepted on: February 4, 2013.

From the Marc and Ruti Bell Vascular Biology and Disease Program, Leon H. Charney Division of Cardiology, Department of Medicine, New York University School of Medicine, New York, NY (J.M.V.G., B.R., M.C.S., L.G., E.A.F., K.J.M.); Department of Biochemistry and Immunology, Federal University of Minas Gerais, Belo Horizonte, MG, Brazil (L.F., G.B.M., D.C.C., J.A.-L.); University of Ottawa Heart Institute, Ottawa, Ontario, Canada (T.S.); Program of Developmental Immunology, Massachusetts General Hospital, Harvard Medical School, Boston, MA (C.C., B.K., A.L.-H.); Massachusetts Institute of Technology, Division of Health Sciences and Technology, Cambridge, MA (M.B.); and Institut Quimic de Sarria, IQS School of Engineering, Ramon Llull University, Barcelona, Spain (M.B.).

*These authors contributed equally to this study.

The online-only Data Supplement is available with this article at http://atvb.ahajournals.org/lookup/suppl/doi:10.1161/ATVBAHA.112.301155/-/DC1.

Correspondence to Kathryn J Moore, New York University School of Medicine, 522 First Ave, Smilow 705, New York, NY 10016. E-mail Kathryn.moore@nyumc.org

The integration of chemoattractive and chemorepulsive signals is essential for controlling neuronal migration during development, and this paradigm is evolutionarily conserved from *Caenorhabditis elegans* to mammals.[6] Netrins, slits, semaphorins, and ephrins compose the 4 major families of conserved neuronal guidance cues that control neuronal migration and vascular patterning through a complex interplay of signals. However, accumulating evidence points to additional functions for these guidance molecules in regulating cell migration outside of the nervous system, including roles in the physiological and pathological regulation of the immune response.[7–9] Such studies have shown that members of the netrin, slit, semaphorin, and ephrin families of guidance cues can regulate immune cell activation or differentiation, and have both chemoattractive and chemorepulsive effects on leukocyte migration.[9–12] For example, recent work from our group identified netrin-1 as a leukocyte guidance cue expressed by the endothelium, where its expression was modulated during acute inflammation because of *Staphylococcus aureus* infection.[11] In this model, netrin-1 was found to be expressed on the luminal surface of lung endothelial cells, where it acted to block the migration of monocytes to such bacterial factors as the N-formylated peptide N-formylmethionine-leucine-phenylalanine, suggesting that it may play a role in endothelial barrier function. At the onset of *S. aureus* infection or on treatment with tumor necrosis factor-α (TNF-α) in vitro, endothelial expression of netrin-1 was rapidly downregulated consistent with a lowering of this barrier to leukocyte infiltration of the tissues.[11]

Given the emerging roles for neuronal guidance cues in regulating inflammation, we hypothesized that these molecules may also have roles in early atherosclerosis, when, as alluded to above, endothelial dysfunction attributable to injury or lipoprotein retention precedes the development of atherosclerotic plaques. To perform a systematic evaluation of the 4 major classes of evolutionarily conserved neuronal guidance molecules, we used a microarray approach using RNA samples harvested from atherosclerosis-prone and -resistant aortic sites in a mouse model of early atherogenesis. We identified key members of the netrin, semaphorin, and ephrin families that are regulated in the initial stages of plaque formation, and confirmed these changes in arterial endothelial cells exposed to atherogenic factors in vitro. Notably, we identified guidance cues that both block (netrin-1 and semaphorin3A) or promote (ephrinB2) monocyte migration, and showed that their expression by endothelium is concurrently regulated by proatherosclerotic conditions in a manner that would facilitate leukocyte recruitment. Furthermore, using intravital microscopy, we demonstrated that blocking peptides targeting these molecules alters leukocyte adhesion to the endothelium in vivo, as would have been predicted by the array and in vitro results. Together, these data suggest an emerging paradigm for the coordination of leukocyte–endothelial interactions in vessel wall homeostasis by neuroimmune guidance cues.

Materials and Methods

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

Results

Neuronal Guidance Molecules Are Differentially Regulated by Flow

It is well established that atherosclerotic lesions develop in areas of branching or high curvature that are associated with oscillatory or turbulent flow, so-called atheroprone regions.[13] We therefore investigated the expression of neuronal guidance molecules in 2 different regions of the vessel wall: (1) the inner curvature (atheroprone region) and (2) the outer curvature (atheroprotected or homeostatic region) of the aortic arch (Figure 1A). We placed *Ldlr*$^{-/-}$ mice on a Western diet for 2 weeks to model conditions for the early initiation of atherosclerosis, and isolated the inner and outer curvature from their aortic arches. Using custom mRNA arrays that covered all 4 families of neuronal guidance molecules, we compared the expression profile between these 2 vascular sites. In the aortic arch, we found that 31 of the 36 neuronal guidance molecules we profiled were expressed (Figure 1B and 1C). Comparing the expression of neuronal guidance molecules from the inner curvature relative to the outer curvature, we found that 10 were >25% downregulated in the inner compared with the outer curvature, and only 2 were >25% upregulated (Figure 1C). From these, we selected candidate molecules from the netrin, semaphorin, slit, and ephrin families and confirmed the array findings by independent quantitative real-time PCR (Figure 1D). Expression of netrin-1 (*Ntn1*), which we previously showed can limit the migration of monocytes, granulocytes, and lymphocytes,[11] was reduced by 48% in the inner curvature (atheroprone) compared with the outer curvature. Like *Ntn1*, another reported repulsive cue of the semaphorin family, semaphorin3A (*Sema3a*), was downregulated in the inner curvature compared with the outer curvature (–53%), consistent with roles in endothelial barrier function for these 2 guidance molecules. From the slit family, *Slit1* was not detectable, whereas *Slit2* was expressed at very low levels. However, *Slit3* was confirmed to be expressed at similar levels between the inner and outer curvature. From the ephrin family, the ephrinB2 gene (*Efnb2*) was selected for validation, as ephrinB2 has been demonstrated to be expressed in vascular endothelial cells and may be sufficient to initiate monocyte–endothelial interactions.[12,14] However, we did not detect differential expression of *Efnb2* mRNA between the inner curvature and outer curvature of the aorta (Figure 1D).

To directly investigate whether it was the endothelial cells of the aorta that contributed to the observed differences in *Ntn1*, *Sema3a*, and *Efnb2* gene expression, and to extend those differences to the protein level, we performed immunostaining of longitudinal sections of the aortic arch of the *Ldlr*$^{-/-}$ mice fed a Western diet for 2 weeks. At this time point, changes in endothelial cell morphology are observed in the disturbed flow regions of the lesser curvature of the aorta, before the subendothelial accumulation of monocytes.[15] Netrin-1 immunostaining was detected in endothelial cells (positive for CD31) of the outer curvature, but was highly downregulated on endothelial cells of the inner curvature of the aortic arch (Figure 2). Similarly, semaphorin3A was expressed by endothelial cells in the atheroprotected outer curvature, whereas there was little to no semaphorin3A expression by endothelial cells of the inner aortic curvature. In contrast,

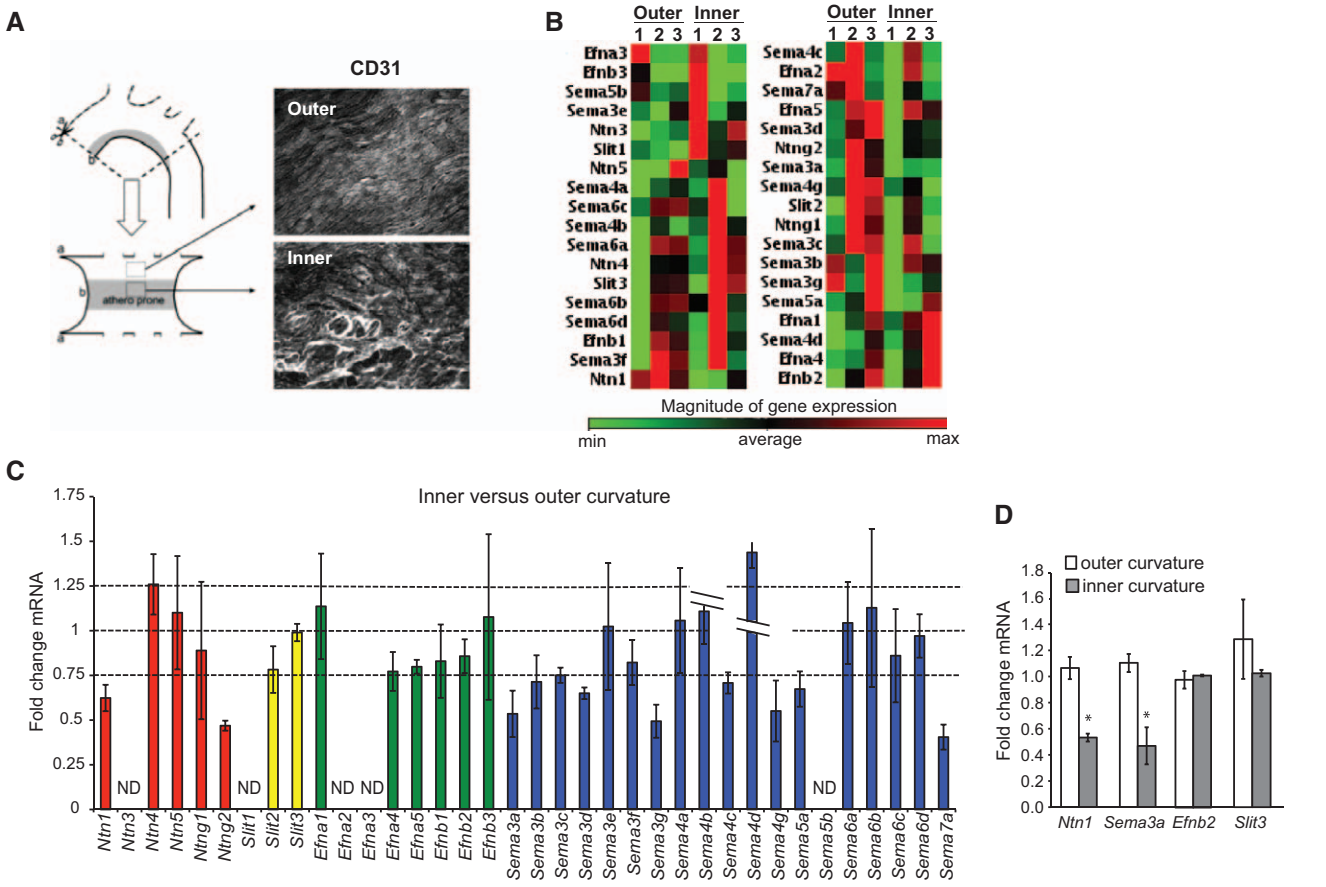

Figure 1. Differential expression of neuronal guidance molecules in the inner vs outer curvature of the aortic arch. **A**, *Ldlr*$^{-/-}$ mice were fed a Western diet for 2 weeks, after which their aortas were harvested and the atheroprotected outer curvature or atheroprone inner curvature isolated. CD31 staining confirmed disruption of endothelial integrity suggestive of early atherosclerosis in the inner curvature compared with outer curvature. **B** and **C**, mRNA expression profiling of these aortic regions for netrin, slit, ephrin, and semaphorin family members using custom quantitative real-time PCR arrays (n=3 mice); (**B**) heat-map; (**C**) relative fold change in mRNA expression in the inner curvature compared with outer curvature. **D**, Validation of candidate guidance cue expression in the inner and outer curvature by quantitative real-time PCR. Data are mean±SEM. *P<0.05.

endothelial cells of the inner aortic curvature expressed ephrinB2; however, staining for ephrinB2 was not detected in the atheroprotected outer curvature.

Endothelial Expression of Netrin-1, Semaphorin3A, and EphrinB2 Are Regulated by Proatherosclerotic Conditions In Vitro

Given the difference in netrin-1, semaphorin3A, and ephrinB2 mRNA, and protein levels in atheroprotected versus atheroprone regions of the aorta, we postulated that hemodynamic proatherosclerotic forces regulate the expression of these guidance cues on endothelial cells. We therefore seeded human coronary artery endothelial cells (HCAECs) onto fibronectin-coated gas-permeable laboratory tubes and exposed them to arterial (atheroprotective), oscillatory (atheroprone) flow, or no flow (static) for 6 hours. As a positive control, we measured endothelial nitric oxide (*Nos3*), an atheroprotective factor known to be expressed under arterial laminar flow conditions, and found that its mRNA abundance was upregulated 3-fold compared with static control but was not significantly changed by oscillatory flow conditions compared with static control (Figure 3A). Similarly, *Ntn1* mRNA was almost 5-fold upregulated by arterial flow, but unchanged in oscillatory flow conditions when compared with static control (Figure 3A). A different pattern emerged for *Sema3a*; expression of this gene was unchanged between static and arterial flow conditions, however it was highly downregulated by oscillatory flow (≈50%; Figure 3B). Notably, *Efnb2* gene expression was also regulated by oscillatory flow, but unlike *Sema3a*, it was significantly upregulated under these proatherosclerotic flow conditions (1.7-fold; Figure 3B). *Slit3* mRNA and protein were not affected by either atheroprotective or atheroprone flow conditions, and, therefore, was not investigated further (Figure IA in the online-only Data Supplement).

The observed changes in the expression of *Ntn1*, *Sema3a*, and *Efnb2* under differential flow conditions led us to consider whether other proatherogenic factors could modulate the expression of these molecules. To examine this, we stimulated HCAECs with chemokines that have been implicated in the recruitment of monocytes during early atherosclerosis, including CCL2 (monocyte chemotactic protein-1 [MCP-1]), CX3CL1 (Fractalkine; FKN), or interleukin (IL)-8.[5] Analysis of HCAEC mRNA expression revealed that MCP-1, FKN, and IL-8 all significantly reduced *Ntn1* after 6 hours of treatment (Figure 4A). Similarly, MCP-1 and FKN, but not IL-8, reduced Semaphorin3A expression under these conditions

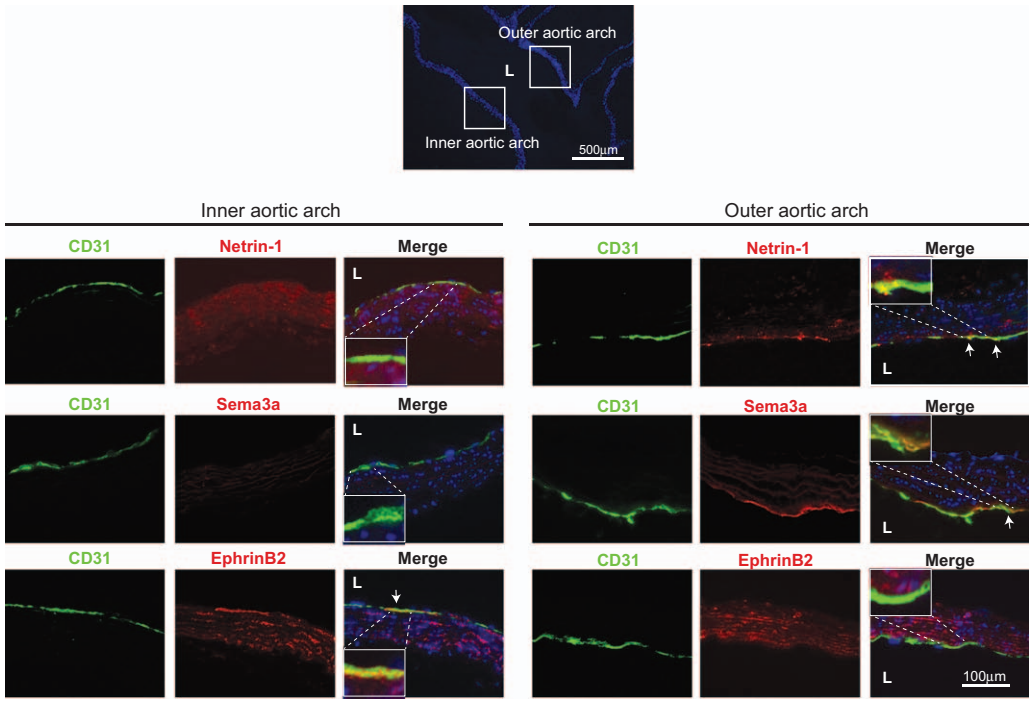

Figure 2. Immunofluorescent staining of netrin-1, semaphorin3A, and ephrinB2 in atheroprotected and atheroprone regions of the aorta. Longitudinal sections of the aortic arch of the *Ldlr*$^{-/-}$ mice fed a Western diet for 2 weeks were stained for CD31 (green), netrin-1, semaphorin3A, or ephrinB2 (red), and DAPI (blue) in the atheroprone inner curvature (**left**) or atheroprotected outer curvature (**right**) of the aortic arch. Areas of colocalization (yellow) are shown in the merged image. Images are representative of 5 mice (L=lumen).

(Figure 4A). In contrast, ephrinB2, which we found to be induced by oscillatory flow conditions, was not affected by atherosclerotic cytokines/chemokines (Figure 4A). Similar effects on *Ntn1* and *Sema3a* mRNA were seen with other classical proatherogenic activators of the endothelium, such as IL-1β and TNF-α, as well as with bacterial lipopolysaccharide (Figure 4B). Notably, these proinflammatory stimuli increased *Efnb2* mRNA in HCAEC (Figure 4B). To ascertain whether these changes were reflected at the protein level, we treated HCAECs with MCP-1 or TNF-α for 24 hours, and performed Western blotting for netrin-1, semaphorin3A, and ephrinB2. Expression of netrin-1 and semaphorin3A by HCAECs was decreased after treatment with MCP-1 and TNF-α, whereas ephrinB2 was increased under similar conditions (Figure 4C).

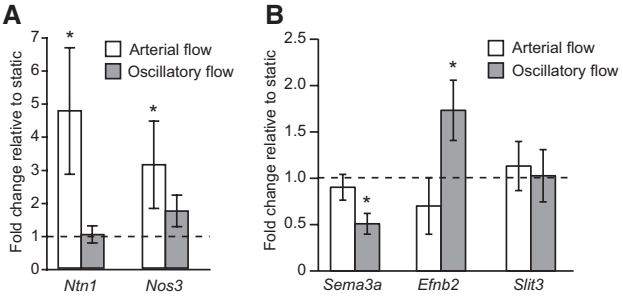

Figure 3. Regulation of netrin-1, semaphorin3A, and ephrinB2 expression by arterial flow conditions. (**A**) *Ntn1*, *Nos3* (endothelial nitric oxide) and (**B**) *Sema3a*, *Efnb2*, and *Slit3* mRNA in human coronary artery endothelial cells (HCAECs) subjected to arterial (17±1 dynes/cm^2 continuous flow in one direction) or oscillatory (17±1 dynes/cm^2 biphasic flow in opposing directions) for 6 hours. Data are expressed as the fold change in mRNA in each condition compared with static flow. Data are the mean±SD of quadruplicate samples in a single experiment and are representative of an experimental n of 4. *P<0.05.

Netrin-1, Semaphorin3A, and EphrinB2 Regulate Leukocyte Migration

Because we observed lower expression of netrin-1 and semaphorin3A in regions of the aorta subject to proatherosclerotic/oscillatory flow and in endothelial cells exposed to oscillatory flow conditions or proatherosclerotic cytokines, we hypothesized that endothelial expression of netrin-1 and semaphorin3A may act as a barrier to prevent monocyte migration into the arterial intima under basal conditions. To test this hypothesis, we investigated the effects of netrin-1 and semaphorin3A on monocyte migration to 2 chemokines implicated in monocyte recruitment during early atherogenesis; FKN (CX3CL1) and MCP-1 (CCL2). Cultured THP-1 monocytes or freshly isolated human peripheral blood mononuclear cells were used in modified Boyden chamber assays to examine monocyte migration to FKN or MCP-1 by adding recombinant netrin-1 to the lower chamber in the absence or presence of chemoattractants. As we previously reported,[11] recombinant netrin-1 did not alter monocyte migration in the absence of chemokine (data not shown). However, addition of recombinant netrin-1 inhibited peripheral blood mononuclear cell migration to FKN and MCP-1 by up to 50% (Figure 5A and 5B). This inhibitory effect of netrin-1 was reversed by preincubation of peripheral blood mononuclear cells with an antibody that binds to the extracellular domain of Unc5b, a receptor for netrin-1 expressed by monocytes[11] (Figure 5C). Similarly, when semaphorin3A was added in combination with FKN or MCP-1, it was found to be a potent inhibitor of peripheral

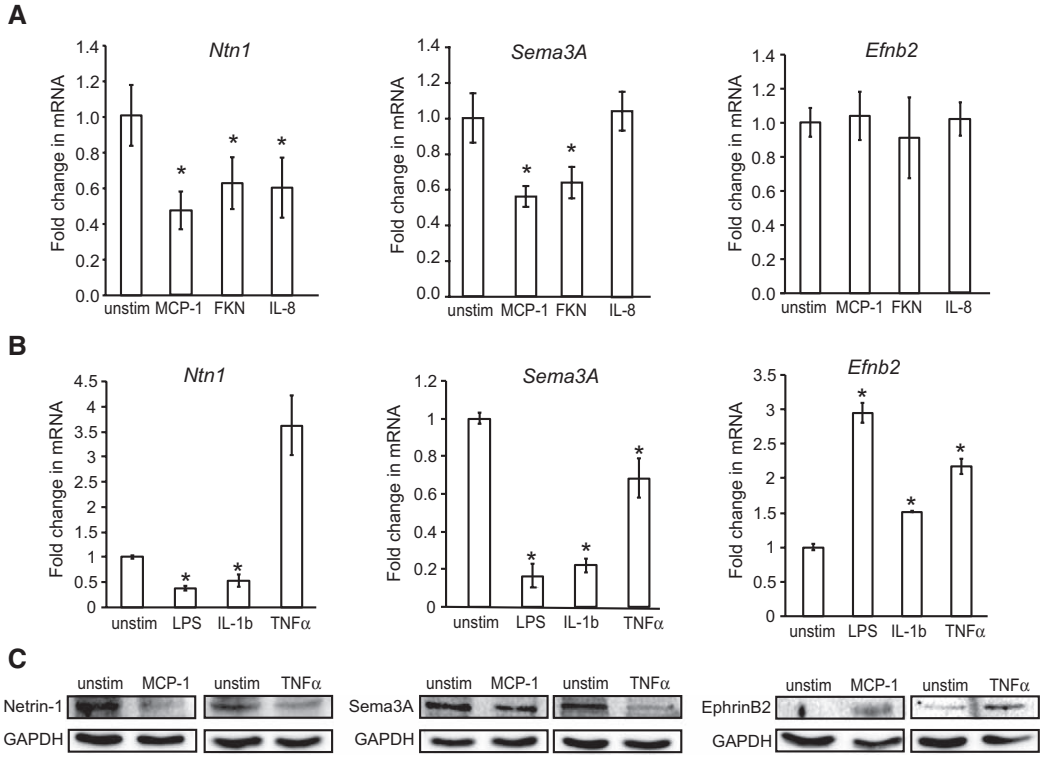

Figure 4. Proatherosclerotic chemokines regulate netrin-1, semaphorin3A, and ephrinB2 expression in human coronary artery endothelial cells (HCAECs). **A** and **B**, *Ntn1*, *Sema3a*, and *Efnb2* mRNA in HCAECs treated with proatherosclerotic chemokines/cytokines (**A**; MCP-1, 5 nmol/L; Fractalkine, 5 nmol/L; and IL-8, 5 nmol/L) or (**B**) lipopolysaccharide (LPS; 1μg/mL), IL-1b (20ng/mL), or tumor necrosis factor-α (TNF-α) (10ng/mL). Data are the mean±SD of triplicate samples in a single experiment and are representative of an experimental n of 3. **P*<0.05. **C**, Western blot analysis of netrin-1, sema3A, ephrinB2, or GAPDH (internal control) in HCAECs stimulated with MCP-1 or TNF-α for 24 hours.

blood mononuclear cell chemotaxis to both FKN (Figure 5D) and MCP-1 (Figure 5E). In dose–response experiments, semaphorin3A-dependent effects on migration produces a bell-shaped curve (also typically seen with chemokines), with 60% to 90% inhibition observed at 250 ng/mL and reduced effects observed at the highest doses (1000 ng/mL; Figure 5D and E).

It has been previously demonstrated that class III secreted semaphorins can signal through neuropilins and plexins to mediate their effects on target cells.[16,17] We therefore investigated whether neuropilin-1 was required for semaphorin3A-mediated inhibition of monocyte chemotaxis. THP-1 monocytes were preincubated with a blocking antibody to neuropilin-1 or an IgG isotype control antibody. Preincubation with the control antibody did not restore monocyte chemotaxis to MCP-1 (Figure 5F). However, preincubation with the neuropilin-1–blocking antibody restored the ability of THP-1 monocytes to respond to MCP-1 (Figure 5F).

In contrast to netrin-1 and semaphorin3A, ephrinB2 expression was observed to be upregulated in endothelial cells exposed to oscillatory flow conditions. Thus, we hypothesized that the upregulation of ephrinB2 may act to recruit monocytes into the arterial intima. To test this hypothesis, we examined the ability of ephrinB2 to act as a monocyte chemoattractant. Indeed, we found that ephrinB2 was a potent monocyte chemoattractant when added to the lower chamber of Boyden migration assays (Figure 5G). Migration was induced in a dose-dependent manner, with maximal chemotaxis observed at 250 ng/mL of ephrinB2. When ephrinB2 was added with FKN or MCP-1 to the lower well, chemotaxis was similar to that seen with ephrinB2 alone (data not shown), indicating that the effects on chemotaxis were not additive.

Netrin-1 and Semaphorin3A Inhibit Leukocyte Adhesion

We next investigated whether netrin-1 and semaphorin3A could alter leukocyte adhesion. Treatment with netrin-1 or semaphorin3A, both at 250 ng/mL, reduced the adhesion of RAW264.7 cells to surfaces coated with BSA by 50% to 60% (Figure 6A). As netrin-1 and semaphorin3A inhibit both leukocyte migration and adhesion in vitro, we next determined whether enhancing or blocking these 2 negative guidance molecules would alter leukocyte–endothelial dynamics. We find that netrin-1 and semaphorin3A actively inhibit THP-1 monocyte binding to HCAEC treated with TNF-α (Figure 6B and 6C) or lipopolysaccharide (Figure IB in the online-only Data Supplement). Conversely, blocking peptides to netrin-1 and semaphorin3A increased THP-1 adhesion to quiescent HCAEC (Figure 6D). Hence, the expression of netrin and semaphorin3A by the endothelium would be expected to actively prevent leukocyte arrest and tissue entry.

To examine this we used intravital microscopy, a technique that allows the observation of leukocyte–endothelial cell interactions in vivo.[18] Similar to areas of disturbed blood flow in the conduit artery, the venous system is exposed to low shear stress ranging from 1 to 6 dynes/cm^2. Thus, intravital microscopy

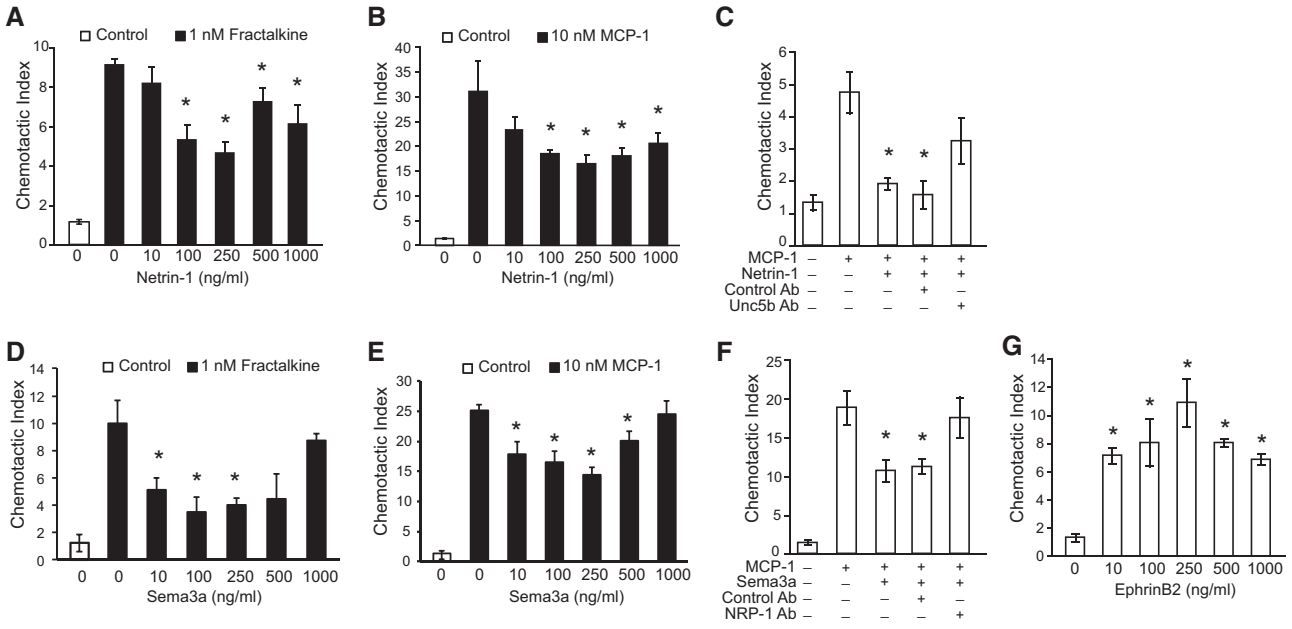

Figure 5. Netrin-1, semaphorin3A, and ephrinB2 alter leukocyte migration. Migration of peripheral blood mononuclear cells (PBMCs) to (**A**) fractalkine (FKN; 1 nmol/L) or (**B**) MCP-1 (10 nmol/L) with or without recombinant netrin-1 at the concentrations indicated. **C**, Preincubation of PBMCs with an Unc5b-blocking antibody (5 μg/mL) blocks the inhibitory effect of netrin-1 (250 ng/mL) on migration to MCP-1 (10 nmol/L). **D** and **E**, Migration of THP-1 monocytes to fractalkine (1 nmol/L; **D**) or MCP-1 (10 nmol/L; **E**) with or without recombinant semaphorin3A at the concentrations indicated. **F**, Preincubation of THP-1 monocytes with a blocking neuropilin-1 antibody (5 μg/mL), but not a control antibody (5 μg/mL), reverses the inhibitory effect of semaphorin3A on migration to 10 nmol/L MCP-1. **G**, Migration of PBMCs to ephrinB2 added at the indicated concentrations in the absence of a chemotactic stimulus. Data are the mean±SD of triplicate samples in a single experiment and are representative of an experimental n=3. **P*<0.05.

of the cremaster vein has proven to be a useful in vivo model for assessing leukocyte–endothelial cell interactions relevant to atherosclerosis.[19–21] Intravenous infusion of netrin-1– or semaphorin3A–blocking peptides to C57BL/6J mice did not alter leukocyte rolling but significantly increased leukocyte adhesion to the endothelium compared with control peptides (Figure 7A and 7B). The number of leukocytes adherent to the endothelium increased approximately 2-fold after the addition of netrin-1– or semaphorin3A–blocking peptides (Figure 7B), further supporting our hypothesis that these guidance cues act to impair this earliest interaction with endothelial cells in vivo.

Discussion

The pioneering studies from the Gimbrone laboratory showed that the properties of atheroprone and susceptible regions of the aorta varied in a number of significant ways that could be attributed to the different flow characteristics at specific sites.[13] For example, endothelial cells in the lesser (inner) curvature of the aortic arch or at branch points, where the laminar flow tended to be lower, were relatively activated. These observations were subsequently extended by a number of investigators to show that before there was evidence of monocyte infiltration, a host of molecules, such as P-selectin, vascular cell adhesion molecule-1, intercellular adhesion molecule-1, and endothelial nitric oxide, were already adversely affected in the susceptible areas.[3,13] Based on the results of the present studies, we can now add certain neuronal guidance molecules as early-disease stage, atheroregulatory, factors that also operate at the level of the endothelium.

Based on the microarray gene expression results (Figure 1), we focused on netrin-1 and semaphorin3A, because of their decreased expression in the atheroprone, lesser curvature in the aortae of prelesional, but diet-stimulated, *Ldlr*$^{-/-}$ mice. We also included analyses of eprinB2 and slit3 as members of their respective classes of neuronal guidance molecules. In our previously published studies, we reported that netrin-1 inhibits monocyte migration in vitro in response to bacterial N-formyl-methionine-leucine-phenylalanine.[11] In that same study, we found that netrin-1 was expressed in the vascular endothelium of the lung, as well as in human umbilical vein endothelial cells, and that its expression was rapidly downregulated during acute inflammation. Based on these findings, we hypothesized that the higher expression of netrin-1 in the endothelium of the atheroresistant greater curvature reflected an important and novel mechanism by which leukocyte interactions with endothelial cells are limited in homeostatic conditions. Furthermore, we considered that there could also be regulation of leukocyte entry into susceptible arterial regions by other neuronal guidance molecules, given some overlap in properties among the family members.

Importantly, we found that the expression of netrin-1 and semaphorin3A was increased under conditions that recapitulated the steady flow found in healthy arterial segments relative to the oscillatory pattern found at arterial regions prone to development of atherosclerotic plaques, as would be expected if these molecules were atheroprotective at the endothelial cell level. Endothelial expression of netrin-1 and semaphorin3A was also decreased by treatment with proinflammatory cytokines or chemokines implicated

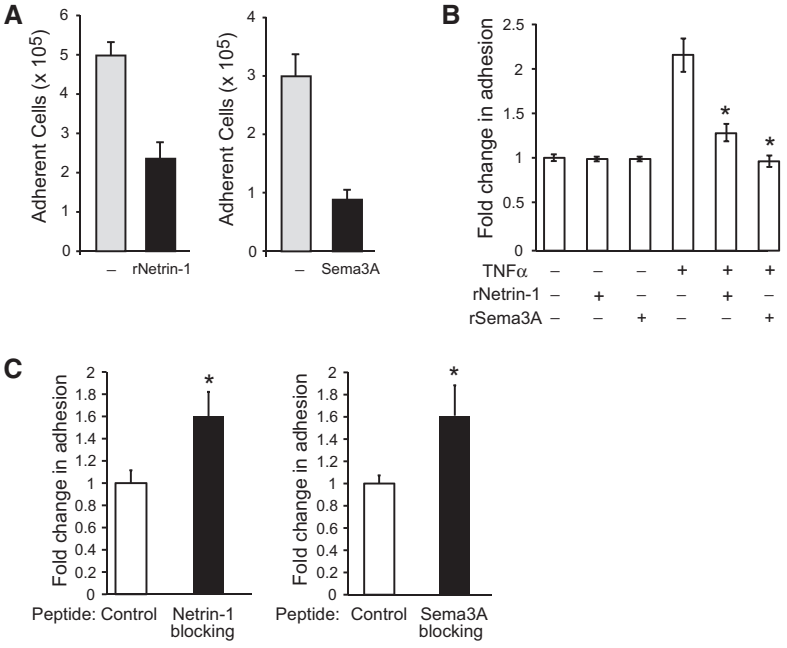

Figure 6. Netrin-1 and semaphorin3A inhibits leukocyte adhesion. **A**, Adhesion of RAW264.7 cells, treated without or with netrin-1 (250 ng/mL) and sema3A (250 ng/mL), to BSA-coated tissue culture plates. **B**, Adhesion of THP-1 monocytes to untreated or tumor necrosis factor-α (TNF-α)–(10ng/mL) treated human coronary artery endothelial cells (HCAECs) in the presence/absence of recombinant netrin-1 or sema3A (250 ng/mL). Data are the mean±SEM of 4 experiments. **C**, Adhesion of THP-1 monocytes to HCAECs treated with either netrin-1– or semaphorin3A-blocking peptide or control peptides. Data are the mean±SEM of 6 experiments. *$P<0.05$.

in leukocyte recruitment to atheroprone regions. We had previously demonstrated that most leukocyte subclasses, including monocytes/macrophages and neutrophils, express the chemorepulsive receptor for netrin1, Unc5b.[11,22] This suggests that when circulating monocytes encounter an endothelial cell expressing relatively more netrin-1, a barrier mechanism would be triggered either because secreted netrin-1 inhibited monocyte chemotaxis by creating a diffusible netrin-1 gradient across endothelial cell layers (similar to that created by endothelial cell-secreted MCP-1), or through presentation of netrin-1 on the surface of endothelial cells. In this regard, there is evidence of netrin-1 binding to α6β4 and α3β1 integrins on pancreatic epithelial cells,[23] and this is one mechanism by which netrin-1 could be presented on arterial endothelial cells.

Consistent with either scenario, our results show that (1) netrin-1 inhibits leukocyte migration to the proatherogenic chemokines, MCP-1 and FKN, in vitro, in a manner that involves the Unc5b receptor; (2) netrin-1 inhibits monocyte binding to endothelial cells; and (3) peptides that block netrin-1 increase leukocyte adhesion to endothelium in vitro. In addition to supporting an atheroprotective role for netrin-1 at the endothelial level, the current study also suggests a similar role for semaphorin3A. For example, semaphorin3A inhibited monocyte migration to MCP-1 in vitro in a process dependent on its receptor neuropilin, and a semaphorin3A-blocking peptide also reduced leukocyte adhesion in vitro. Notably, using intravital microscopy of the cremaster vein, we show that administration of netrin-1 and semaphorin3A-blocking peptides similarly increase leukocyte adhesion in vivo.

Turning to ephrinB2, in contrast to netrin-1 and semaphorin 3A, it was upregulated in HCAECs in vitro by proatherogenic (oscillatory) flow (Figure 3), and at the protein level, it was more highly expressed in the inner curvature endothelium (Figure 2). Another contrast was that it acted to stimulate leukocyte recruitment. These findings are supported by Goettsch et al,[24] who found that ephrinB2 expression was downregulated by arterial, but not venous, laminar shear stress in HUVECs,[24] suggesting that this downregulation may keep endothelial cells in a nonactivated state. Other supporting data for a role of ephrinB2 in vivo are contained in a report that showed that monocytes express *EphB2*, one of the possible receptors for ephrinB2, and that the expression of *EphB2* in monocytes is increased on their adhesion.[14] In agreement with the present results, in that report, there was also greater expression of ephrinB2 in the endothelium of the lesser curvature, which correlated with an increased abundance of macrophages in the underlying neointima.[14]

Although we have shown that there is concurrent regulation of netrin-1, semaphorin3A, and ephrinB2 in the endothelium, resulting in concerted atheroprotective effects, whether this represents a common coordinating regulatory mechanisms or a more complex process remains to be determined. In contrast to *Ntn1* and *Sema3a*, we did not detect a difference in *EfnB2* mRNA expression in the atheroprone and atheroresistant sites of the aorta, which implies that a combination of transcriptional and posttranscriptional mechanisms may regulate the expression of these factors. Similarly, whereas TNF-α treatment of HCAECs markedly downregulated *Sema3a* mRNA, as well as netrin-1 and semaphorin3A protein levels, this cytokine increased *Ntn1* mRNA in vitro. There are scant data about the regulation of endothelial expression for any of the factors we studied. There is some evidence for netrin-1 that NF-κB and hypoxia-inducible factor-1α may be involved in its transcriptional upregulation,[25,26] but on the surface, this would be counter to the present results in that (1) treatment with lipopolysaccharide or TNF-α (activators of NF-κB) or MCP-1 (a classic target of NF-κB) was associated with decreased netrin-1 expression (Figure 4); and (2) in areas of

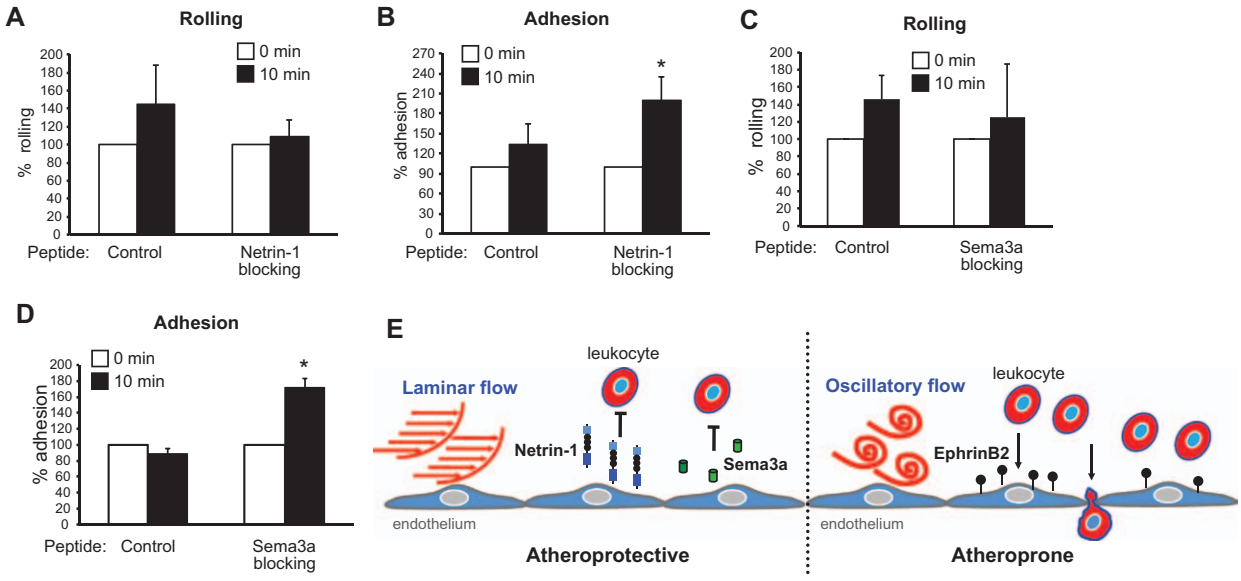

Figure 7. Blocking netrin-1 or semaphorin3A increases leukocyte adhesion to endothelium in vivo. Leukocyte rolling and adhesion before and after administration of a (**A** and **B**) netrin-1– or (**C** and **D**) semaphorin3A–blocking peptide or control peptide measured by intravital microscopy in C57BL/6 mice. Data are the mean±SEM, n=5 to 7 mice/group. *P<0.05. **E**, Schematic diagram of the regulated expression of neuroimmune guidance cues under atheroprotective and atheroprone (**right**) conditions. Under conditions of laminar flow (**left**), netrin-1 and semaphorin3A are expressed on endothelial cells and inhibit leukocyte recruitment, maintaining an atheroprotective state. In contrast, oscillatory flow characteristic of atheroprone regions of the vasculature downregulates expression of netrin-1 and semaphorin3A, while increasing levels of ephrinB2, thereby facilitating leukocyte recruitment.

disrupted laminar flow (as in the lesser curvature of the aortic arch), hypoxia-inducible factor-1α is induced independent of hypoxia. Thus, although the phenomenon of intersecting protective changes in the 3 guidance molecules is clear, a dissection of the underlying regulatory mechanisms awaits detailed analyses beyond the scope of the present report.

Our data suggest that netrin-1 and semaphorin3A may reduce leukocyte migration and recruitment to atherosclerotic plaque by inhibiting firm adhesion to the vessel wall. In vivo, leukocyte recruitment to inflamed sites is mediated by a combination of adhesion and signaling molecules. Selectins, expressed at inflamed sites, slow leukocytes and initiate rolling; next, chemokine signaling to leukocytes activates integrins to mediate firm adhesion and subsequent extravasation. Our observations that netrin-1 and semaphorin3A inhibit the initiation of firm adhesion are consistent with known roles for neuronal guidance molecules. For example, semaphorin3A inhibits the activation and adhesive function of β1 and β3 integrins in endothelial cells,[27] and similar effects might be expected to regulate activity of β2 integrins on leukocytes, which are primarily responsible for firm adhesion of leukocytes to the vessel wall. Netrin-1 has also been shown to bind to integrins (α3 and α6) and to activate integrin ligand binding through focal adhesion kinase and small GTPases.[23]

In summary, our data support a model (Figure 7) in which the inhibitory guidance molecules netrin-1 and semaphorin3A are more highly expressed by endothelial cells in atheroresistant aortic regions, where they help to maintain tissue homeostasis by preventing leukocyte influx. In atheroprone regions, however, they become downregulated by disturbed laminar flow and local inflammation, which lowers the endothelial barrier to leukocyte entry. Consistent with this model, systemic delivery of netrin-1 to *Ldlr*$^{-/-}$ mice using adenovirus-associated virus reduced atherosclerosis,[28] presumably by increasing its expression on endothelial cells. Adding to the increased predilection for leukocyte recruitment in the prone regions is the upregulation of another guidance molecule, eprinB2, which increases monocyte migration in vitro. In addition to these guidance cues, which we selected for in-depth study, our microarray profiling data suggest that additional family members, including *Ntng2*, *Sema3g*, *Sema4d*, and *Sema7a*, may also be relevant to atherosclerotic processes. During development, the various families of guidance molecules have been shown to have overlapping effects and to also coregulate each other positively or negatively. Further exploration of the expression of neuronal guidance molecules and their receptors under resting and inflammatory conditions, and their functional interactions, will likely identify new regulatory mechanisms and therapeutic targets in atherosclerosis and other inflammatory disorders.

Sources of Funding

Support for this work came from the American Heart Association (Grant-in-aid 0655840T to K.J.M.; 09POST2080250 to J.M.V.G.), Fondation de la recherche Medicale to B.R., National Institutes of Health (RC1HL100815 to K.J.M.; R01 HL084312 to E.A.F.; R01/GM049039 to M.B.), Canadian Institute of Health Research (CGS-MSFSS to T.S.), Conselho Nacional de Desenvolvimento Científico e Tecnológico (01558/2007-6 to L.R.F. and J.I.A.-L.), Ministerio de Innovación Plan Nacional BFU2009-09804 (M.B.), Fundació Empreses IQS (M.B.), and POSIMAT (M.B.).

Disclosures

None.

References

1. Tabas I, Williams KJ, Borén J. Subendothelial lipoprotein retention as the initiating process in atherosclerosis: update and therapeutic implications. *Circulation*. 2007;116:1832–1844.
2. Williams KJ, Tabas I. The response-to-retention hypothesis of early atherogenesis. *Arterioscler Thromb Vasc Biol*. 1995;15:551–561.
3. Mestas J, Ley K. Monocyte-endothelial cell interactions in the development of atherosclerosis. *Trends Cardiovasc Med*. 2008;18:228–232.
4. Moore KJ, Tabas I. Macrophage in the pathogenesis of atherosclerosis. *Cell*. 2011;doi:10.1016/j.cell2011.04.005
5. Galkina E, Ley K. Leukocyte influx in atherosclerosis. *Curr Drug Targets*. 2007;8:1239–1248.
6. Kolodkin AL, Tessier-Lavigne M. Mechanisms and molecules of neuronal wiring: a primer. *Cold Spring Harb Perspect Biol*. 2011;3:.
7. Suzuki K, Kumanogoh A, Kikutani H. Semaphorins and their receptors in immune cell interactions. *Nat Immunol*. 2008;9:17–23.
8. Cirulli V, Yebra M. Netrins: beyond the brain. *Nat Rev Mol Cell Biol*. 2007;8:296–306.
9. Guan H, Zu G, Xie Y, Tang H, Johnson M, Xu X, Kevil C, Xiong WC, Elmets C, Rao Y, Wu JY, Xu H. Neuronal repellent Slit2 inhibits dendritic cell migration and the development of immune responses. *J Immunol*. 2003;171:6519–6526.
10. Mirakaj V, Brown S, Laucher S, Steinl C, Klein G, Köhler D, Skutella T, Meisel C, Brommer B, Rosenberger P, Schwab JM. Repulsive guidance molecule-A (RGM-A) inhibits leukocyte migration and mitigates inflammation. *Proc Natl Acad Sci USA*. 2011;108:6555–6560.
11. Ly NP, Komatsuzaki K, Fraser IP, Tseng AA, Prodhan P, Moore KJ, Kinane TB. Netrin-1 inhibits leukocyte migration *in vitro* and in vivo. *Proc Natl Acad Sci USA*. 2005;102:14729–14734.
12. Korff T, Braun J, Pfaff D, Augustin HG, Hecker M. Role of ephrinB2 expression in endothelial cells during arteriogenesis: impact on smooth muscle cell migration and monocyte recruitment. *Blood*. 2008; 112:73–81.
13. Gimbrone MA Jr, Topper JN, Nagel T, Anderson KR, Garcia-Cardeña G. Endothelial dysfunction, hemodynamic forces, and atherogenesis. *Ann N Y Acad Sci*. 2000;902:230–9; discussion 239.
14. Braun J, Hoffmann SC, Feldner A, Ludwig T, Henning R, Hecker M, Korff T. Endothelial cell ephrinB2-dependent activation of monocytes in arteriosclerosis. *Arterioscler Thromb Vasc Biol*. 2011;31:297–305.
15. Mullick AE, Soldau K, Kiosses WB, Bell TA III, Tobias PS, Curtiss LK. Increased endothelial expression of Toll-like receptor 2 at sites of disturbed blood flow exacerbates early atherogenic events. *J Exp Med*. 2008;205:373–383.
16. Horie T, Ono K, Horiguchi M, Nishi H, Nakamura T, Nagao K, Kinoshita M, Kuwabara Y, Marusawa H, Iwanaga Y, Hasegawa K, Yokode M, Kimura T, Kita T. MicroRNA-33 encoded by an intron of sterol regulatory element-binding protein 2 (Srebp2) regulates HDL in vivo. *Proc Natl Acad Sci USA*. 2010;107:17321–17326.
17. Lwigale PY, Bronner-Fraser M. Semaphorin3A/neuropilin-1 signaling acts as a molecular switch regulating neural crest migration during cornea development. *Dev Biol*. 2009;336:257–265.
18. Gavins FN, Chatterjee BE. Intravital microscopy for the study of mouse microcirculation in anti-inflammatory drug research: focus on the mesentery and cremaster preparations. *J Pharmacol Toxicol Methods*. 2004;49:1–14.
19. Jin SY, Tohyama J, Bauer RC, Cao NN, Rader DJ, Zheng XL. Genetic ablation of Adamts13 gene dramatically accelerates the formation of early atherosclerosis in a murine model. *Arterioscler Thromb Vasc Biol*. 2012;32:1817–1823.
20. Eriksson EE. Intravital microscopy on atherosclerosis in apolipoprotein e-deficient mice establishes microvessels as major entry pathways for leukocytes to advanced lesions. *Circulation*. 2011;124:2129–2138.
21. Missiou A, Köstlin N, Varo N, et al. Tumor necrosis factor receptor-associated factor 1 (TRAF1) deficiency attenuates atherosclerosis in mice by impairing monocyte recruitment to the vessel wall. *Circulation*. 2010;121:2033–2044.
22. van Gils JM, Derby MC, Fernandes LR, et al. The neuroimmune guidance cue netrin-1 promotes atherosclerosis by inhibiting the emigration of macrophages from plaques. *Nat Immunol*. 2012;13:136–143.
23. Nikolopoulos SN, Giancotti FG. Netrin-integrin signaling in epithelial morphogenesis, axon guidance and vascular patterning. *Cell Cycle*. 2005;4:e131–e135.
24. Goettsch W, Augustin HG, Morawietz H. Down-regulation of endothelial ephrinB2 expression by laminar shear stress. *Endothelium*. 2004;11:259–265.
25. Paradisi A, Maisse C, Bernet A, Coissieux MM, Maccarrone M, Scoazec JY, Mehlen P. NF-kappaB regulates netrin-1 expression and affects the conditional tumor suppressive activity of the netrin-1 receptors. *Gastroenterology*. 2008;135:1248–1257.
26. Rosenberger P, Schwab JM, Mirakaj V, Masekowsky E, Mager A, Morote-Garcia JC, Unertl K, Eltzschig HK. Hypoxia-inducible factor-dependent induction of netrin-1 dampens inflammation caused by hypoxia. *Nat Immunol*. 2009;10:195–202.
27. Serini G, Valdembri D, Zanivan S, Morterra G, Burkhardt C, Caccavari F, Zammataro L, Primo L, Tamagnone L, Logan M, Tessier-Lavigne M, Taniguchi M, Püschel AW, Bussolino F. Class 3 semaphorins control vascular morphogenesis by inhibiting integrin function. *Nature*. 2003;424:391–397.
28. Khan JA, Cao M, Kang BY, Liu Y, Mehta JL, Hermonat PL. Systemic human Netrin-1 gene delivery by adeno-associated virus type 8 alters leukocyte accumulation and atherogenesis in vivo. *Gene Ther*. 2011;18:437–444.

Significance

We identified key members of the netrin, semaphorin, and ephrin families that are regulated on the endothelium in the initial stages of atherosclerotic plaque formation. We identified guidance cues that both block (netrin-1 and semaphorin3A) or promote (ephrinB2) monocyte migration, and showed that their expression by endothelium is concurrently regulated by proatherosclerotic conditions in a manner that would facilitate leukocyte recruitment. Furthermore, blocking peptides targeting these molecules alter leukocyte adhesion to the endothelium in vivo, as would have been predicted by the array and in vitro results. Together, these data suggest an emerging paradigm for the coordination of leukocyte–endothelial interactions in vessel wall homeostasis by neuroimmune guidance cues.