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Semaphorin 4A Exerts a Proangiogenic Effect by Enhancing Vascular Endothelial Growth Factor-A Expression in Macrophages

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The axon guidance cues semaphorins (Semas) and their receptors plexins have been shown to regulate both physiological and pathological angiogenesis. SemA4 plays an important role in the immune system by inducing T cell activation, but to date, the role of SemA4A in regulating the function of macrophages during the angiogenic and inflammatory processes remains unclear. In this study, we show that macrophage activation by TLR ligands LPS and polyinosinic-polycytidylic acid induced a time-dependent increase of SemA4A and its receptors PlexinB2 and PlexinD1. Moreover, in a thioglycollate-induced peritonitis mouse model, SemA4A was detected in circulating Ly6Chigh inflammatory monocytes and peritoneal macrophages. Acting via PlexinD1, exogenous SemA4A strongly increased macrophage migration. Of note, SemA4A-activated PlexinD1 enhanced the expression of vascular endothelial growth factor-A, but not of inflammatory chemokines. SemA4A-stimulated macrophages were able to activate vascular endothelial growth factor receptor-2 and the PI3Kserine/threonine kinase Akt pathway in endothelial cells and to sustain their migration and in vivo angiogenesis. Remarkably, in an in vivo cardiac ischemia/reperfusion mouse model, SemA4A was highly expressed in macrophages recruited at the injured area. We conclude that SemA4A activates a specialized and restricted genetic program in macrophages able to sustain angiogenesis and participates in their recruitment and activation in inflammatory injuries.

PlexinB1-B2 and B3 induces COS7 cell contraction through the R-Ras GTPase-activating protein enzymatic activity that characterizes all Plexin cytodomains studied so far (21). Besides acting as a chemorepulsive cue via B-typeplexins (21, 22), Sema4A has been shown to be constitutively expressed by dendritic cells, where it stimulates T cell activation through Tim-2 receptor, a member of the T cell Ig and mucine domain proteins expressed on activated T cells (19). Moreover, T cells require Sema4A to allow Th cell differentiation. Sema4A-deficient mice displayed defective Th1 responses, indicating that the induction of Sema4A in Th1 cells is necessary for their differentiation either by cognate cellular interactions among Th1 cells or by following an autocrine pathway (23). Recently, Sema4A has been shown to be upregulated by allergen or VEGF in bronchial epithelial cells expressing both PlexinD1 and PlexinB1, thus pointing to Sema4A as a potential player for the allergic airway inflammatory diseases (24).

This evidence highlights the important role played by Sema4A in regulating angiogenesis and the immune system function; however, its specific involvement in controlling both angiogenic and inflammatory response in macrophages is still poorly understood.

In this work, we analyzed the expression and the function of Sema4A in macrophages in vitro and in vivo experimental models, including chicken chorioallantoic membrane (CAM) angiogenic assay, thioglycollate-induced peritonitis, and a model of cardiac ischemia/reperfusion (I/R) injury, characterized by increased angiogenesis, tissue remodeling, and recruitment of inflammatory cells (25–28). In this study, we identify a new function for Sema4A as modulator of macrophage functions in the context of both angiogenic and inflammatory processes.

Materials and Methods

Cell culture

Human monocytes were isolated from buffy coats of healthy donors obtained through the courtesy of Transfusion CenterAVIS (Torino, Italy). Blood was washed once with PBS 1× at 400 × g to remove plasma and platelets and then centrifuged at 600 × g for 30 min at room temperature in a Ficoll gradient (GE Healthcare). Cells at the interface were collected, washed twice with PBS 1×, and monocytes were isolated in a Percoll gradient (Sigma-Aldrich) at 700 × g for 30 min at room temperature. Cells at the interface were collected, washed twice with PBS 1×, and plated at a density of 1.5 × 10^6 cells/cm^2 in RPMI 1640 (Lonza). Monocytes were allowed to adhere for 1 h, and then the medium was replaced by RPMI 1640 supplemented with 10% FCS. HUVECs were isolated from umbilical cord veins, characterized, and grown in M199 (Lonza) containing 20% FCS (Invitrogen), bovine brain extract, heparin (50 μg/ml; Sigma-Aldrich), and penicillin-streptomycin (200 U/ml; Lonza) on gelatin-coated tissue-culture dishes, as previously described (29).

Human macrophage cell culture conditions, differentiation, and polarization

To obtain differentiated macrophages, human monocytes from buffy coats were cultured for 7 d in RPMI 1640 supplemented with 10% FCS and 100 ng/ml M-CSF (PeproTech) at normoxic oxygen levels (21% O_2) in a humidified incubator at 37°C. For hypoxic experiments, differentiated macrophages were incubated for a further 18 h at an atmosphere of 1% O_2. One week-differentiated macrophages were polarized by replacing the medium for additional 18 h with RPMI 1640 supplemented with 5% FCS. To obtain polarization, 100 ng/ml LPS (Sigma-Aldrich) with 20 ng/ml IFN-γ (PeproTech; M1 polarization) or 20 ng/ml IL-4 (PeproTech; M2 polarization) was added to the medium for 18 h. Differentiated macrophages were exposed to PAM3Cys (2 μg/ml; Calbiochem), polyinosinic-polyribocytidylic acid (poly I:C, 10 μg/ml; Invivogen), flagellin (5 μg/ml; Invivogen), R848 (3 μg/ml; Alexis Biochemicals), and CpG ODN 2006 (1 μg/ml; MWG Biotech) for 18 h.

Real-time RT-PCR analysis

RT-PCR analysis was performed as previously described (15). cDNA from I/R tissues and Sema4A-treated macrophages were analyzed by TaqMan Low Density Array based on Applied Biosystems Microfluidic Card (Applied Biosystems) containing 48 probes for I/R tissues and 96 probes for macrophages. cDNA from cells were analyzed by real-time RT-PCR and performed in triplicate using a TaqMan Gene Expression Assay Mix (Applied Biosystems) specific for Sema4A, PlexinD1, PlexinB1, and PlexinB2. cDNAs were run on an ABI Prism 7900HT Fast Real-Time PCR System (Applied Biosystems), and the data were analyzed by SDS and RQ Manager Software to obtain a relative quantification based on the arithmetic equation 2^-ΔΔCt, in which ΔΔCt is the normalized signal level in a sample relative to the normalized signal level in the corresponding calibrator sample. mRNA from I/R tissues were normalized to the housekeeping 18S gene, whereas the GAPDH gene was used to normalize mRNA from human macrophages. Normalized relative quantification (RQ) fold changes were calculated compared with ΔCt of nontreated mice or control cells.

Western blot analysis

Western blot analysis was performed as previously described (30). Briefly, human macrophages, HUVECs, and mice peritoneal cell proteins were extracted with lysis buffer. For Sema4A detection, coating was performed using 2 μg/ml protein lysates or 50 μl supernatants dispensed into 96-well polystyrene microtiter plates (Nunc). The plate included duplicates of each sample. After incubation at 4°C overnight, plates were washed once with PBS and blocked with 3% BSA in PBS 1× for 1 h at 37°C. Primary anti-Sema4A (PeproTech) was diluted 1:200 in 5% skim milk, membranes were incubated with anti-VEGF and anti-Sema4A (PeproTech), anti-Sema3E (Everest), anti-VEGF receptor (VEGFR)-2, anti-phospho-VEGFR-2, anti- phospho-Akt, anti-phospho-ERK, anti-Akt, anti-ERK (Cell Signaling Technology), anti-phospho-VEGFR-1 (R&D Systems), and anti-β-tubulin (Santa Cruz Biotechnology) primary Abs at 4°C overnight. Membranes were incubated with HRP-conjugated secondary Abs, and immunoreactive proteins were visualized by ECL system (GE Healthcare). The intensity of the signal was quantified by means of the Chemidoc Bio-Rad program (Bio-Rad), and data are reported as ratios of Sema4A/β-actin or β-tubulin densitometry.

ELISA

ELISA has been performed as previously described (15). Briefly, macrophage supernatants were collected and concentrated by the use of centrifugal filter devices (Centricon YM-10; Millipore), whereas macrophage proteins were extracted using lysis buffer. For Sema4A detection, coating was performed using 2 μg/ml protein lysates or 50 μl supernatants dispensed into 96-well polystyrene microtiter plates (Nunc). The plate included duplicates of each sample. After incubation at 4°C overnight, plates were washed once with PBS and blocked with 5% BSA in PBS 1× for 1 h at 37°C. Primary Ab anti-Sema4A (PeproTech) was diluted 1:200 in 3% BSA in PBS 1× and added to the plate. After incubation at 37°C for 2 h, plates were rinsed three times, and the secondary Ab peroxidase-conjugated AffiniPure goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories) was diluted 1:2000 in wash solution and added to the plate. The plate was incubated at 37°C for 1 h and washed again. The 1-Step Turbo TMB-Elisa peroxidase substrate (Pierce) was added and incubated at room temperature in the dark. The reaction was stopped by adding 2 M H_2SO_4. The OD was measured at a wavelength of 450 nm using a Synergy HT automated microplate reader (Bio-Tek). For VEGF-A detection, supernatants from macrophages were treated with human macrophage supernatants with Sema4A (10 and 100 mmol/l) for 18 h were collected, concentrated, and loaded on a Quantikine 96-well plate for VEGF-A level determination (R&D Systems). To generate a standard curve, we measured the absorbance of increasing concentration of recombinant Sema4A or VEGF-A (10, 25, 50, and 100 ng).

Migration assay

The migration of macrophages was measured with a 48-well chemotaxis Boyden chamber (Neuro Probe) using polyvinylpyrrolidone filter membranes with a pore diameter of 5 μm. Upon 18 h exposure to LPS (100 ng/ml) and IFN-γ (20 ng/ml), human macrophages were harvested with Accutase (PAA Laboratories) and resuspended in RPMI 1640 supplemented with 1% FCS at a concentration of 4 × 10^5 cells/ml. Lower wells of the chamber were loaded with 29 μl RPMI 1640 plus 1% FCS alone or supplemented with 50 mmol/l human recombinant Sema4A or Sema3E (R&D Systems). Upper wells were loaded with 50 μl cells alone or in the presence of 50 mmol/l recombinant Sema4A, 20 μg/ml rabbit anti-PlexinD1 Ab (H-70, sc-67145; Santa Cruz Biotechnology), 20 μg/ml mouse anti-PlexinB2 Ab (MAB5329; R&D Systems), and 20 μg/ml IgG or IgG2A controls, respectively (DakoCytomation). After incubation for 4 h at 37°C, the top side of the insert membrane was scrubbed free of cells.
and the bottom side was fixed with methanol and stained with hematoxylin and cosin. Cells that migrated to the bottom side of the membrane were counted under an Olympus BX60F-3 microscope (Olympus) using a 10X original magnification. For ECs migration assay, cells resuspended in M199 medium were seeded on the upper surface of a polyvinylpyrolidone filter membrane with a pore diameter of 8 μm coated with either 50 μg/ml fibronectin or 0.1% gelatin. Lower wells of the chamber were loaded with M199 medium alone or supplemented with 10 or 50 nmol/l human recombinant Sema4A in the presence or not of human VEGF-A (30 ng/ml). For EC and macrophage coculture migration assay, a monolayer of human macrophages was incubated for 18 h with Sema4A (10 and 100 nmol/l) with or without bevacizumab (1 μg/ml; Avastin; Roche), anti-IL-8 (1 μg/ml; MAB208; R&D Systems), anti-PlexinD1 (20 μg/ml; Santa Cruz Biotechnology), or anti-PlexinB2 (20 μg/ml; R&D Systems) blocking Abs or for 18 h with human recombinant Sema4A (100 nmol/l) in the presence or not of bevacizumab (34 μg/CAM). After 48 h of incubation, CAM vessels were isolated, fixed, and photographed in ovo with a stereomicroscope connected to a camera by the use of the Transwell membrane (BD Falcon) were allowed to migrate toward the human macrophage monolayer. After 5 h of incubation, ECs on the upper side of the filters were then mechanically removed. ECs of the filter’s lower side were then fixed in 2.5% glutaraldehyde for 30 min and stained with 0.1% crystal violet (15, 30).

**Chicken embryo chorioallantoic membrane assay**

Fertilized chicken eggs were incubated at 37°C in a humidified incubator, as previously described (31). On day 10 of incubation, CAM were added with sterilized paper disks. The disks were saturated with 100 ng VEGF-A in the presence or not of human recombinant Sema4A (100 nmol/l) or bevacizumab (34 μg/CAM). In addition, the disks were loaded with 10 μl conditioned medium (CM) from human macrophages or 10 μl CM from human recombinant Sema4A in the presence or not of human VEGF-A (30 nmol/l). For EC and macrophage coculture migration assay, the filter disk was suspended in RPMI 1640 medium supplemented with 1% FCS and seeded on the upper surface of a polycarbonate 8-μm porous Transwell membrane (BD Falcon) were allowed to migrate toward the human macrophage monolayer. After 5 h of incubation, ECs on the upper side of the filters were then mechanically removed. ECs of the filter’s lower side were then fixed in 2.5% glutaraldehyde for 30 min and stained with 0.1% crystal violet (15, 30).

**Thiglycollate-induced peritonitis**

Peritonitis was induced by instillation of thioglycollate as previously described (32). Healthy C57/B16 mice were subjected to i.p. injection of a sterile 2.95% thioglycollate solution. After 24 and 48 h, mice were anesthetized with isoflurane 1.5% and euthanized, 5 ml sterile PBS 1× and 1.5 ml air were injected in the peritoneal cavity, and peritoneal exudates were collected. For each experimental condition, pools of eight animals were used. Peritoneal cells were pelleted, washed with PBS 1×, and used for immunofluorescence, FACS analysis, and RNA and protein extraction.

**Flow cytometry analysis**

Mouse peritoneal cells were recovered washed twice with PBS 1× and stained for flow cytometry. A total of 2.5 × 10^5 cells was incubated with anti–Sema4A-FTTC (MBL), anti–F4/80–PE (AbD Serotec), anti–Tim-2–647 (BioLegend), and anti–PlexinD1 and PlexinB2 (Santa Cruz Biotechnology) conjugated with allophycocyanin in PBS/1% FCS for 30 min in the dark. Plexins were revealed by incubation with allophycocyanin- secondary Ab (Jackson & Jackson). Samples were washed, resuspended in PBS 1×, and acquired and analyzed on a flow cytometer (CyAn ADP; DakoCytabation). Blood and blood cells were washed twice with PBS 1×, and stained for flow cytometry. A total of 2.5 × 10^5 cells was incubated with anti–Sema4A-FTTC (MBL), anti–F4/80–PE (AbD Serotec), anti–Tim-2–647 (BioLegend), and anti–PlexinD1 and PlexinB2 (Santa Cruz Biotechnology) conjugated with allophycocyanin in PBS/1% FCS for 30 min in the dark. Plexins were revealed by incubation with allophycocyanin-secondary Ab (Jackson & Jackson). Samples were washed, resuspended in PBS 1×, and acquired and analyzed on a flow cytometer (CyAn ADP; DakoCytabation).

**Statistical analysis**

The results of all experiments are expressed as mean ± SD. For all statistical analyses, a two-tailed, unpaired Mann–Whitney U test was used. A p value <0.05 was considered significant.

**Results**

**Sema4A is expressed in activated human macrophages**

Sema4A enhances T cell activation (19), but its role in the innate immune system and, in particular, on the activation of macrophages, is still unknown. Therefore, we first analyzed Sema4A expression in human macrophages obtained by in vitro differentiation of PBMCs. Significant amount of Sema4A transcript was present in nonstimulated macrophages and significantly increased by LPS, but not by IL-4 (data not shown). IFN-γ alone did not modulate the gene expression profile and slightly decreased the induction by LPS (Fig. 1A). LPS, but not IFN-γ or IFN-γ plus LPS, significantly increased the expression levels of PlexinD1 and PlexinB2 (Supplemental Fig. 1B, 1C and data not shown), whereas PlexinB1 was poorly expressed both in resting and activated cells (data not shown). LPS activates cellular response mainly by activating TLR4, a member of the TLR family (33) that recognizes pathogen-specific molecular patterns, thus playing a crucial role in both innate and adaptive immunity (34). Therefore, we investigated the expression of Sema4A and its receptors after macrophage activation by other TLR agonists (PAM3Cys, a TLR1-TLR2 agonist; poly I:C, a TLR3 agonist; flagellin, a TLR5 agonist; R848, a TLR7/8 agonist; and CpG ODN 2006, a TLR9 agonist). Transcript levels of Sema4A were strongly increased by long-term exposure to LPS, poly I:C, and, to a lesser extent, CpG ODN 2006 (Supplemental Fig. 1A), whereas no
significant modulation was observed for the other TLR agonists flagellin, R848, and PAM3Cys (data not shown). Interestingly, poly I:C and LPS treatment also resulted in the upregulation of PlexinD1 and PlexinB2 transcripts, whereas the other TLR agonists did not modulate their expression (Supplemental Fig. 1B, 1C and data not shown). PlexinB1 was not significantly regulated by any TLR agonist (data not shown). All together, these data indicate that Sema4A expression is induced during selective macrophage activation, and indeed, it may play an important role in the regulation of their inflammatory properties.

**Sema4A stimulates the chemotactic activity of macrophages via PlexinD1**

To better understand the role of Sema4A in human macrophages, we evaluated its activity in eliciting their migration. In a chemotactic Boyden’s chamber assay, human recombinant Sema4A induced a bell-shaped dose-dependent chemotactic response (Fig. 1B). Notably, the increased macrophage migration induced by Sema4A was comparable to that mediated by MCP-1 (data not shown). To identify what plexin receptor was involved in the Sema4A-dependent chemotactic activity, macrophages were incubated with blocking Abs for PlexinD1 or PlexinB2 prior to testing their chemotactic migration. Although the PlexinB2 Ab was unable to block the Sema4A-induced migration, PlexinD1 neutralization strongly inhibited the effect (Fig. 1C). Interestingly, the PlexinD1 inhibition also decreased the basal migration, possibly indicating the involvement of endogenous Sema4A in the basal motility. Because PlexinD1 binds also Sema3E (35), we investigated whether this molecule was involved in the regulation of macrophage motility. By performing migration assay, we observed that exogenous Sema3E, differently from Sema4A, did not increase macrophage basal migration (Fig. 1D). Although PlexinD1 neutralization efficiently inhibited the basal migration of macrophages (Fig. 1C, 1D), exogenous Sema3E counteracted the...

**FIGURE 1.** Sema4A expression is increased in activated human macrophages. (A) Real-time RT-PCR analysis using RNA from human macrophages exposed to IFN-γ, LPS alone, or combined with IFN-γ for 18 h. RQ values are compared with control untreated macrophages and are mean ± SD of n = 3 donors for each condition. Sema4A enhanced macrophage migration via PlexinD1. (B) Human recombinant Sema4A (from 0 up to 100 nmol/l) increased the migration in Boyden’s chamber of human macrophages treated for 18 h with LPS and IFN-γ in a dose-response curve with a peak at 50 nmol/l of Sema4A. Values are mean ± SD of four independent experiments. (C) Human macrophages exposed for 18 h to LPS and IFN-γ were incubated in the presence or not of 20 μg/ml of PlexinD1 or PlexinB2-blocking Abs or appropriate control Ig and allowed to migrate toward 50 nmol/l of recombinant Sema4A for 4 h. Values are mean ± SD (n = 4 donors) of four independent experiments. (D) Human macrophages exposed for 18 h to LPS and IFN-γ were incubated in the presence or not of 20 μg/ml of PlexinD1-blocking Ab or IgG and stimulated with 50 nmol/l of recombinant Sema3E and Sema4A for 4 h. Values are mean ± SD (n = 4 donors) of four independent experiments. (E and F) Sema4A and Sema3E protein levels were analyzed by Western blot analysis of lysates of control or LPS- and IFN-γ–treated human macrophages. The data shown are representative (n = 4 donors) of four independent experiments. F) Relative levels of Sema4A and Sema3E protein expression normalized to β-tubulin. (G) Protein levels of Sema4A were measured by ELISA both in the supernatants and cell lysates of human macrophages exposed or not for 18 h to LPS and IFN-γ (see Materials and Methods). Values are mean ± SD (n = 4 donors) of four independent experiments. Ctrl, Control. **p < 0.01, ***p < 0.001.
inhibitory effect on cell motility induced by PlexinD1-blocking Ab. Of note, PlexinD1 neutralization in macrophages triggered by Sema4A associated with Sema3E was not effective in reducing the migration of macrophages under basal levels (Fig. 1D), as observed in the presence of Sema4A and PlexinD1-blocking Ab (Fig. 1C). Based on these results, we can hypothesize that this effect could be mostly due to a competition between Sema3E and Sema4A for the PlexinD1 receptor. By Western blot analysis, we observed that Sema4A, but not Sema3E, expression increased in activated cells compared with control macrophages (Fig. 1E, 1F). Remarkably, we detected significant amounts of both cell-associated and released forms of Sema4A in macrophages (Fig. 1G), as detected by ELISA. The amount of Sema4A protein significantly increased in both supernatants and lysates of activated macrophages, highlighting that Sema4A acts as a soluble factor regulating macrophage functions both in a paracrine and autocrine manner.

**Sema4A is upregulated during peritoneal inflammation**

Next, we extended the above in vitro observations to a murine model of peritonitis induced by thioglycollate and looked at the expression of Sema4A in peritoneal recruited cells. CD11c, CD4, CD8, and Ly6G+ cells did not express Sema4A, neither in controls nor in thioglycollate-treated animals (data not shown). In contrast, whereas CD11b+ and CD68+ cells isolated from control animals did not express significant amounts of Sema4A, thioglycollate treatment induced a dramatic upregulation of the protein in these cells (Fig. 2). Interestingly, after 24 h, Sema4A was mainly expressed by CD11b+ cells with few positive CD68+ macrophages (Fig. 2C, 2D, 2F). Remarkably, 48 h after the treatment, the majority of CD11b+ cells were negative (data not shown), whereas Sema4A accumulated in CD68+ cells (Fig. 2E, 2F).

FACS analysis revealed that peritoneal cells positive for the macrophage F4/80 marker expressed increasing levels of Sema4A upon thioglycollate treatment (Fig. 3A). In fact, the number of Sema4A-expressing peritoneal cells rose from 8% in resting conditions to 48% and 58% 24 and 48 h after thioglycollate injection, respectively. This increased expression of Sema4A in peritoneal cells 24 and 48 h after thioglycollate treatment was confirmed by Western blot analysis (Fig. 3D, 3E). Next, we investigated the expression of Sema4A receptors in these peritoneal cells. Interestingly, both PlexinD1 and PlexinB2, significantly expressed in control cells, were strongly upregulated in Sema4A+ macrophages upon 24 and 48 h of thioglycollate treatment. It has been shown that under inflammatory conditions, various inflammatory cell types including macrophages increased Tim-2 expression, which is one of the Sema4A receptors (2, 24). Therefore, we evaluated the expression of Tim-2 in peritoneal cells upon

![FIGURE 2. Sema4A is upregulated in thioglycollate-induced peritonitis in mice.](https://www.jimmunol.org/)
thioglycollate treatment. Of note, whereas we observed a significant Tim-2 expression in both control and elicited Sema4A + macrophages, differently from the other two receptors, we did not detect a significant increase of Tim-2 after the inflammatory stimulus compared with untreated cells (Fig. 3B, 3C).

Circulating monocytes can be grouped in inflammatory and resident subsets depending on the level of expression of the Ly6C marker (36). When cell-surface expression of Sema4A was analyzed by flow cytometry on Ly6C<sub>high</sub> and Ly6C<sub>low</sub> peripheral blood monocyte subsets, it was higher on Ly6C<sub>high</sub> than on Ly6C<sub>low</sub> monocytes, both in control and in thioglycollate-treated mice. Furthermore, thioglycollate significantly upregulated Sema4A expression on Ly6C<sub>high</sub> monocytes but not in Ly6C<sub>low</sub> cells (Fig. 3F). These data suggest that Sema4A belongs to a specific signature for circulating inflammatory monocytes that could migrate at site of tissue damage.

Sema4A increases VEGF-A expression in human macrophages through PlexinD1

To further study the involvement of Sema4A in macrophage response, we analyzed its effect on the expression of genes encoding for chemokines and angiogenic inducers (Supplemental Tables I, II). Sema4A selectively upregulated VEGF-A and, to a lesser extent, IL-8, whereas it did not modify the expression of other angiogenic factors and chemokines compared with controls (Fig. 4, Supplemental Tables I, II). Consistent with the gene-expression analysis, we detected increased VEGF-A protein expression in lysates and supernatants of macrophages treated with Sema4A (Fig. 4B–D). Hypoxia regulates angiogenesis and is a strong inducer of VEGF-A production (37, 38). Accordingly to previous data (39–41), hypoxia positively regulated the basal and LPS-stimulated expression of VEGF-A, but it did not display any synergistic effect in upregulating the amount of VEGF-A produced in normoxic conditions by Sema4A. In addition, hypoxia did not affect Sema4A expression in macrophages (Supplemental Fig. 1D).

To evaluate the cellular mechanism of the increased expression of VEGF-A induced by Sema4A, we investigated whether this effect was mediated by PlexinD1 or PlexinB2. As detected by ELISA, we observed that PlexinD1, but not PlexinB2-blocking, Abs strongly reduced the enhanced VEGF-A levels induced by both endogenous and exogenous Sema4A in macrophage supernatants (Fig. 4E) and cell lysates (Fig. 4F). Therefore, these data indicate that Sema4A induces VEGF-A expression through a plexin-mediated pathway and that PlexinD1 is critical in mediating the effects of Sema4A.
Because VEGF-A enhances monocyte/macrophage activity and triggers their motility (42–44), we assessed whether Sema4A was able to increase macrophage function through VEGFR-1 or VEGFR-2 activation. Western blot analysis of lysates of macrophages exposed or not to Sema4A revealed barely detectable levels of phospho–VEGFR-2 in both control and Sema4A-stimulated cells. Remarkably, Sema4A induced an increase of VEGFR-1 phosphorylation (Fig. 4G). These data suggest that the increased activation of VEGFR-1 could be involved, in part, in the Sema4A promigratory effect on macrophages.

Sema4A-treated macrophages increase in vitro and in vivo angiogenesis through VEGF-A/VEGFR-2 signal pathway

To study the proangiogenic effect of macrophages mediated by Sema4A, we first assessed whether the VEGF-A expressed by Sema4A-treated macrophages was able to stimulate EC functions. To this aim, we stimulated HUVECs with supernatants of control and Sema4A-treated macrophages, and we checked both VEGFR-2 phosphorylation and the VEGF-induced signal pathway. VEGFR-2 phosphorylation was increased in ECs exposed to supernatants of Sema4A-stimulated macrophages, but not in ECs incubated with CM of control cells (Fig. 5B, 5D). These data suggest that Sema4A-treated macrophages exert a proangiogenic effect by activating specifically the PI3K/Akt pathway in ECs.

Next, we assessed the chemotactic activity of Sema4A-treated macrophages on ECs. Notably, although Sema4A directly inhibited EC motility induced by VEGF-A (Supplemental Fig. 1E, 1F) (20), we observed a significant increase of EC migration in the presence of Sema4A-treated macrophages compared with non-treated macrophages (Fig. 5E). Remarkably, the EC migration induced by Sema4A-treated macrophages was completely abrogated by the VEGF-A–blocking Ab bevacizumab (Fig. 5E), whereas anti–IL-8–specific or control Abs were ineffective. Of note, treatment of macrophages with PlexinD1, but not PlexinB2, Abs significantly reduced the EC migration triggered by Sema4A-treated macrophages (Fig. 5E), further confirming that the increased VEGF-A expression observed in Sema4A-treated macrophages is mediated by PlexinD1 receptor (Fig. 5E).

To investigate whether macrophages activated by Sema4A were able to promote in vivo angiogenesis, we performed CAM experiments. Interestingly, although recombinant Sema4A inhibited the VEGF-A–induced formation of new blood vessels (Fig. 6C, 6G), supernatants derived from macrophages elicited with Sema4A strongly enhanced vessel branching and number (Fig. 6E, 6G). As described for the migration assay, this increased angiogenesis could be blocked by the VEGF-A–blocking Ab bevacizumab (Fig. 6E, 6G).
genesis induced by Sema4A-elicited macrophages was completely blocked by bevacizumab (Fig. 6F, 6G), indicating that VEGF-A specifically mediates the proangiogenic effect of Sema4A on macrophages in vivo (Fig. 6F, 6G). Taken together, these data demonstrate that Sema4A activates a proangiogenic phenotype in macrophages by specifically inducing the expression of VEGF-A, which, in turn, enhances EC migration and in vivo angiogenesis.

**Sema4A is upregulated in the heart after I/R**

Based on the data describing the role played by Sema4A during peritoneal inflammation and on its ability to induce a proangiogenic phenotype in macrophages, we next investigated its expression in the cardiac I/R experimental mouse model, which is characterized by activated angiogenesis and inflammatory response. It has been shown that blood reperfusion by eliciting the recruitment of inflammatory cells such as monocytes at the injury site, accentuates the inflammatory cardiac response to ischemia (26–28). To this aim, mice were sacrificed at different time points after heart I/R, and the expression of Sema4A in the infarcted and control sham-operated tissues was evaluated. Sema4A expression was evident after 6 h, peaked at 24 h, and persisted up to 48 h (Fig. 7A). Of note, Sema4D, which was previously implicated in angiogenesis and inflammation (16, 17), was not significantly modulated during cardiac ischemia (Fig. 7A). Moreover, the increased expression of Sema3A, Sema3E, Sema3F, Sema6A, and Sema7A transcripts at 24 h I/R (Supplemental Fig. 2A, 2B) agrees well with their role in regulating angiogenesis, thus suggesting a potential role of these Semas in the early stages of the ischemic process (15, 45, 46). By Western blot analysis, we detected a very similar expression pattern of Sema4A protein at 24 and 48 h after I/R (Fig. 7C, 7D). Next, we focused our analysis on the differential expression in these experimental conditions of Sema4A receptors, showing that whereas PlexinD1 expression was unchanged, PlexinB1 was significantly downregulated, and PlexinB2 was upregulated (Fig. 7B). These data suggest the involvement of Sema4A and its receptors in the cellular processes occurring after an ischemic event in the heart.

**Sema4A is specifically expressed by myeloid precursors and mature macrophages in the infarcted heart**

To better understand the involvement of Sema4A in the cardiac ischemia after I/R, we investigated Sema4A protein localization and expression at the different time points after reperfusion by confocal microscope analysis (Fig. 8). Sema4A was not expressed in the noninfarcted heart (i.e., interventricular septum and right ventricle), but its expression was readily detected in the infarcted and necrotic free wall of the left ventricle (Fig. 8A) (47). Notably, Sema4A was not detectable in the heart of sham-operated control mice (Fig. 8B). Next, we examined Sema4A expression pattern in
different cell types that can be found within the infarcted cardiac tissue and noticed that this protein did not colocalize with either α-actinin+ cardiomyocytes or CD31+ vascular ECs (Supplemental Fig. 2C, 2D). Based on the scattered staining of Sema4A, we then evaluated its expression in the different subsets of recruited leukocytes. At 24 h post–I/R, Sema4A did not colocalize with Ly6G+, CD4+, CD8+, or CD11c+ cells (data not shown). Conversely, Sema4A was expressed by CD11b+ and a subset of CD68+ cells infiltrating the tissue at 24 h post-I/R (Fig. 8C, 8D, 8F). At 48 h post-I/R, when most of the CD11b+ cells were replaced by CD68+ mature macrophages, Sema4A was mainly localized in these cells (Fig. 8E, 8F). Moreover, at 48 h, Sema4A colocalized with CD11b+ cells not yet differentiated into macrophages and, to a lesser extent, with Ly6G+ cells. Similar results were obtained using anti-F4/80 Ab at 24 and 48 h post-I/R (data not shown). Taken together, these data indicate that Sema4A is produced by mature macrophages recruited at injury sites and suggest it may play an important role in regulating their functions during cardiac I/R.

Discussion

In the last years, a growing body of evidence demonstrated an important role of Semas and their receptors plexins in regulating cellular functions related to angiogenesis and immunity (2, 3, 15, 46). Among these, Sema4A regulates the function of dendritic cells and T lymphocytes and inhibits EC migration and experimental angiogenesis (19, 20), but its role in mediating the interplay between inflammatory and angiogenic processes is still unclear. By using in vitro approaches and in vivo models of peritoneal inflammation and cardiac ischemia, we uncovered in this study a new role of Sema4A in regulating macrophage functions in angiogenesis and inflammation.

In this study, we showed that exogenous Sema4A stimulated macrophage migration in a dose-dependent manner. Moreover, we found that PlexinD1 is the most upregulated plexin in human macrophages stimulated with LPS and poly I:C, suggesting its potential involvement in the response of macrophages to Sema4A during the inflammatory process. Consistently, blocking Ab anti-PlexinD1 but not anti-PlexinB2 abrogated for the migratory effect of Sema4A. The observation that Sema4A slightly increased the expression of IL-8, but did not significantly change the expression levels of any inflammatory chemokine tested (Supple-
Sema4A expression was negligible in the noninfarcted myocardium (NI), as shown in the phase-contrast image. (B) Sema4A was not expressed in CD11b+ or Cd68+ cells in the hearts of sham-operated mice. Twenty-four hours after I/R, Sema4A was expressed mainly by precursors of monocytes/macrophages (C) and to a lesser extent by tissue macrophages (D) as revealed by colocalization of Sema4A (red) with CD11b (green) and CD68 (green) Abs, respectively. (E) Most of Sema4A was observed in infiltrated macrophages (CD68+ cells) 48 h after I/R. No signal was detected using CD11b blocking Ab directed against this receptor did not inhibit the Sema4A-stimulated chemotaxis of macrophages. It is intriguing to speculate that this receptor may mediate other functions or act as a decoy receptor.

Among the several angiogenic factors analyzed, Sema4A specifically activates the transcription of VEGF-A gene in macrophages. VEGF-A is a marker of alternatively activated (M2) macrophages with a well-recognized role in tissue remodeling, angiogenesis, and tumor progression (51). It has been widely described that tumor-associated macrophages share properties with M2-activated cells, having a propelling role in tumor angiogenesis, and induce cancer progression (52). For instance, it has been shown that tumor-associated macrophages expressing matrix metalloproteinase-9 trigger the angiogenic switch and tumor progression by releasing VEGF-A from the extracellular matrix in transgenic mouse models of skin and uterine cervix carcinogenesis (53, 54). Matrix metalloproteinase-9 was highly expressed, but not significantly modulated, in both Sema4A-stimulated and non-stimulated macrophages, suggesting that other proteases may be involved in regulating VEGF-A–induced angiogenesis. Conversely, Sema4A did not significantly modulate the inflammatory signature of macrophages, with the remarkable exception of IL-8, which also plays a relevant role in angiogenic processes (55).

It has been widely shown that hypoxia induced expression of VEGF-A in macrophages (39, 41, 56). However, our data show that hypoxia is not involved in the enhanced VEGF-A expression in macrophages induced by Sema4A. Moreover, we demonstrated that Sema4A induces VEGF-A expression specifically through the PlexinD1 pathway and the consequent binding with VEGF-A produced by macrophages or other cell types could be part of the mechanism by which Sema4A exerts its promigratory effect on macrophages. Recently, Sema4A has been reported to bind to all three B-type plexins (PlexinB1, B2, and B3) and plexins found on macrophages. Recently, Sema4A has been reported to bind to all three B-type plexins (PlexinB1, B2, and B3) and plexins found on macrophages. Recently, Sema4A has been reported to bind to all three B-type plexins (PlexinB1, B2, and B3) and plexins found on macrophages. Recently, Sema4A has been reported to bind to all three B-type plexins (PlexinB1, B2, and B3) and plexins found on macrophages. Recently, Sema4A has been reported to bind to all three B-type plexins (PlexinB1, B2, and B3) and plexins found on macrophages.
pathways, leading to overexpression of VEGF-A by NF-κB activation. TGF-β1 has been recently involved in the upregulation of VEGFRs and VEGF-A expression in macrophages and dendritic cells through Smad3/4 (58). Based on our findings describing the activation of VEGFR-1 in macrophages upon stimulation with exogenous Sem4A, we can hypothesize that the activation of the TGF-β1/Smad signaling pathway may represent an alternative mechanism by which Sem4A upregulates VEGF-A expression in these cells.

The increased ECs migration and vessel formation in vivo induced by macrophages treated with recombinant Sem4A is consistent with the induction by Sem4A of a proangiogenic phenotype in macrophages. It has been recently described that Sem4A inhibits ECs functions in experimental angiogenesis (20). In this study, we described that Sem4A can exert an indirect proangiogenic effect mediated by macrophages that trigger EC migration. Interestingly, a similar opposite effect of Semas has been previously described. For instance, it has been shown that Sem3B, indicated as a putative tumor suppressor that inhibits tumor growth and angiogenesis (45), induced the recruitment of tumor-associated macrophages and a consequent activation of a prometastatic program (46). Based on our data describing that bevacizumab completely abrogated EC migration and vessel formation in vivo induced by Sem4A-treated macrophages, we can speculate that during pathological angiogenesis, Sem4A may promote neovascularization by recruiting VEGF-A–expressing macrophages to activate ECs.

In this article, we show that VEGF-A produced by Sem4A–elicited macrophages activates a specific signal pathway in ECs by increasing the levels of phospho-Akt. It has been widely described that PI3K/Akt pathways can regulate endothelial migration, proliferation, and survival through the effect of its downstream targets such as endothelial NO synthase, p70 ribosomal protein S6 kinase 1, and forkhead box factor O1 to regulate tumor angiogenesis (59–61). In addition, endothelial Akt activation causes the appearance of an enlarged and highly permeable vasculature that recapitulates the abnormal structural and functional phenotype of tumor blood vessels (62). Based on our data, it is conceivable to hypothesize that the induction of a proangiogenic phenotype in macrophages by Sem4A causes the activation of the endothelial PI3K/Akt pathway leading to an increased EC migration and in vivo vessel formation that could prove crucial for angiogenesis in cancers and other pathological conditions. Remarkably, we detected a significant amount of Sem4A in the culture medium of differentiated human macrophages, corroborating previous observations demonstrating that a soluble form of Sem4A is cleared from membrane-bound Sem4A overexpressed in human embryonic kidney 293 (20). These data indicate therefore that Sem4A can be expressed as soluble mediator and exerts its promigratory function on macrophages as locally released protein. Therefore, the final effects of Sem4A on angiogenesis result from a homeostatic balance between its anti- and proangiogenic activities, respectively, mediated by a direct effect on ECs and macrophages.

It has been widely shown that during cardiac I/R, monocytic/macrophages play a crucial role in the phagocytosis of the necrotic myocardium and the inflammatory response necessary for tissue healing (26). We show in this study for the first time, to our knowledge, that Sem4A is specifically expressed by macrophages recruited in the infarcted area and significantly enhanced macrophage migration, suggesting that Sem4A may be involved in recruitment and activation of a subset of macrophages that contribute early to tissue remodeling after I/R.

Interestingly, by performing a wider gene expression analysis of axon guidance cues at the early stages of cardiac ischemia, we revealed a significant modulation of several Semas along the injury development. Interestingly, at the early stages, members of class 3, such as Sem3A, Sem3E, and Sem3F, known to be relevant for pathological angiogenesis (45), were upregulated. This is reminiscent of the observations done in tumor mouse models describing that the angiogenic inhibitors Sem3A and Sem3F were upregulated to balance the prominent production of angiogenic inducers (15). Moreover, Sem6A, another angiogenesis inhibitor (63), and Sem7A, a mediator of T cell-mediated inflammatory response (64), were upregulated as well. Altogether, these results suggest a complex role of Semas in tissue repair of ischemic injury. This concept is further supported by the persistent expression of a high level of Sem4A early on during the I/R process. Further studies will be performed to better understand this new role of Sem4A on angiogenesis in a pathological condition.

In conclusion, our work unveils a new role of Sem4A in inducing a restricted genetic program in macrophages able to sustain angiogenesis. Based on our in vivo and in vitro observations, it is conceivable to hypothesize that at the site of inflammatory injuries or during pathological angiogenesis such as I/R, Sem4A could recruit macrophages to the ischemic/tumor tissue able to regulate the angiogenic response necessary for tissue healing and remodeling. The increased expression of Sem4A may therefore contribute to the activation of the onset of the angiogenesis process by specifically enhancing VEGF-A expression in infiltrating macrophages.

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Disclosures

The authors have no financial conflicts of interest.

References
