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

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Semaphorins (Semas) are a large family of extracellular proteins present from viruses to primates and divided into eight classes. Classes 1, 4, 5, 6, and 7 are membrane-bound, whereas classes 2, 3, and V are secreted (1–3), though some membrane-associated Semas become soluble after proteolytic cleavage (4). Originally discovered as steering molecules for developing axons, Semas have subsequently been shown to be involved in neuronal function, vascular patterning, heart development, and the immune response (1). The biological effects of Semas impinge on neurones that, alone or in association with other coreceptors endowed with modulatory functions, signal inside cells (5, 6). However, most secreted Semas, including Sema3A and Sema3F, do not bind directly to plexins and use neuropilins as coreceptor ligand-binding subunits (7–9). As reported in axon navigation (10–12), Semas may be both chemorepellent and chemomagnetic on endothelial cells (ECs) (1). Class 3 Semas have also been implicated in experimental models of tumor angiogenesis (13). For instance, Sema3E regulates cancer growth, tumor dissemination, and angiogenesis (14). Moreover, recent studies indicated that Sema3A is an endogenous angiogenic inhibitor that regulates the angiogenic switch, inhibits tumor growth, and normalizes tumor vasculature by reducing tumor hypoxia (15).

Besides class 3 Semas, class 4 Semas also regulate angiogenesis as well. Both in vitro and in vivo Sema4D promotes angiogenesis through PlexinB1 (16). More recently, these observations have been extended to tumor angiogenesis, and strong evidence indicated that macrophages recruited in tumors are a major source of Sema4D (17). Another membrane-bound class 4 Semaphorin, namely Sema4A, progressively increases its expression in developing mouse embryos (18) and becomes prominent in the adult brain, lung, kidney, spleen, testis, and mammary gland (19). In contrast to Sema4D, upon binding to PlexinD1, Sema4A inhibits EC migration and in vivo angiogenesis by suppressing the vascular endothelial growth factor (VEGF)-mediated activation of Rac and integrin-dependent cell adhesion (20). Moreover, in the presence of the Rho family GTPase Rnd1, the binding of Sema4A to
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-type plexins (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 fromuffy coats of healthy donors obtained through the courtesy of Transfusion Center VIS (Torino, Italy). Blood was washed once with PBS 1× 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 × 10⁶ cells/ml 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 fromuffy 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% O2) in a humidified incubator at 37°C. For hypoxic experiments, differentiated macrophages and Sema4A-treated macrophages were analyzed by 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 arithmetical 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 (50 mmol/l Tris-HCl, 150 mmol/l NaCl, 5 mmol/l Na2VO4, 1 mmol/l PMSF, 0.01 mmol/l ZnCl2, protease inhibitors, and 1% Triton X-100). Proteins were quantified using the BCA assay reagent (Thermo Scientific). Equal amounts of proteins (50 µg) were resolved by SDS-PAGE on a 12% or 8% polyacrylamide gel and transferred to nitrocellulose membranes (Amersham Biosciences). After blocking with 5% nonfat milk in PBS or 5% skim milk, membranes were incubated with anti-VEGF and anti-Sema4A (PeproTech), anti-Sema3E (Everest), anti-VEGF receptor (VEGFR)-2, anti-VEGF-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 3% 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 H2SO4. 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 transfected 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 (PAAB Laboratoraries) and resuspended in RPMI 1640 supplemented with 1% FCS at a concentration of 4 × 10⁶ 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 eosin. Cells that migrated to the bottom side of the membrane were counted under an Olympus BX60F-3 microscope (Olympus) using a 10× original magnification. For ECs migration assay, cells resuspended in M199 medium were 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).

**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 sterile 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/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 g/ml; Santa Cruz Biotechnology), or anti–PlexinB2 (20 μg/ml; R&D Systems) blocking Abs or IgG1 and IgG2A control Abs (1 and 20 g/ml; DakoCytomation). ECs 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).

**Thiglycocolate-induced peritonitis**

Peritonitis was induced by instillation of thiglycocolate as previously described (32). Healthy C57/B6 mice were subjected to i.p. injection of a sterile 2.95% thiglycocolate 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 × 10⁶ cells was incubated with anti–Sema4A-ITTC (MBL), anti–F4/80-PE (AbD Serotec), anti–Tim-2-Alexa Fluor 488 fluorochromes (Invitrogen). Cell nuclei were DAPI stained for flow cytometry. A total of 2.5 × 10⁵ cells was incubated with Abs anti–CD11b (BioLegend), -Ly6G (Abcam), -CD45 (Sigma-Aldrich), -F4/80, -CD68, -CD4, -CD8, and -CD11c (AbD Serotec). Secondary Abs were conjugated to Alexa Fluor 555, Alexa Fluor 647, and Alexa Fluor 488 fluorochromes (Invitrogen). Cell nuclei were DAPI counterstained (1:5000; Invitrogen). Immunofluorescence images were captured and analyzed with a Leica TCS SP2 AOBS confocal laser-scanning microscope (Leica Microsystems), as previously shown (15). All immune-localization experiments were repeated three times on multiple tissue sections and included negative and IgG controls for determination of background staining, which was negligible. To quantify the extent of colocalization, we measured the mean fluorescence intensity of red and green channels by means of the Leica Confocal Software Histogram Quantification Tool (Leica Microsystems). In each analyzed picture, we considered five random regions of interest of the same size, and then we calculated the ratio between red (Sema4A) and green (CD11b or CD68) channel mean fluorescence intensity.

**In vivo induction of I/R**

Twelve-week-old C57BL/6dJ mice (The Jackson Laboratory) were used and maintained in specific pathogen-free organism facilities. All procedures were conducted in conformity with the institutional guidelines in compliance with national and international laws and policies and approved by the Ethical Commission of the University of Turin and by the Italian Ministry of Health. Animals were anesthetized with isoflurane 1.5%, ventilated with a mechanical ventilator (0.2 ml, 120 acts/min; Ugo Basile 28026 mouse ventilator; Ugo Basile) through an endotracheal cannula, and kept at a body temperature of 37°C. The left anterior descending coronary artery was ligated with a 7–0 silk (Ethicon) suture after exteriorization of the heart through a 15-mm opening at the fourth intercostal space. An overall knot was tied with two pieces of suture to arrest blood flow and then removed after 45 min. Ischemia was confirmed by the appearance of ventricular ectopy and blanching of the myocardium. The chest was closed under negative pressure, and mice were weaned from mechanical ventilation. Posturgical analgesia was achieved by buprenorphine (0.1 mg/kg s.c. every 12 h for 1 d). Mice were sacrificed after 1, 6, 12, 24, and 48 h and 6 d of IR, and hearts were excised. The right ventricle and the septum and left ventricular free wall (i.e., infarcted myocardium) and sham-operated hearts were separately snap-frozen and used for RNA extraction. Whole hearts were embedded in OCT and stored at −80°C for tissue analysis.

**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 immune macropages**

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 enhanced macrophage migration via PlexinD1 (Fig. 1B). 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. 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. 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.

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. 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) 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. (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% in 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...
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 Ly6Chigh and Ly6C low peripheral blood monocyte subsets, it was higher on Ly6C high than on Ly6C low monocytes, both in control and in thioglycollate-treated mice. Furthermore, thioglycollate significantly upregulated Sema4A expression on Ly6C high monocytes but not in Ly6C low cells (Fig. 3F). These data suggest that Sema4A belongs to a specific signature for circulating inflammatory monocytes that could migrate to sites 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-treated 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 angio-

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**FIGURE 4.** Sema4A induces a proangiogenic activity on human macrophages. (A) Real-time RT-PCR analysis using RNA from human macrophages treated for 18 h with human recombinant Sema4A (10, 50, and 100 nmol/l). RQ values are compared with control macrophages and are mean ± SD of n = 4 donors for each condition. (B and C) VEGF-A protein levels were analyzed by Western blot analysis of lysates of human macrophages upon 18-h treatment with different concentrations of recombinant Sema4A (10, 50, and 100 nmol/l). The data shown are representative (n = 4 donors) of four independent experiments. (C) Relative levels of VEGF-A protein expression normalized to β-tubulin. (D) Protein levels of VEGF-A were measured by ELISA in the supernatants and lysates of human macrophages treated with 50 nmol/l of human recombinant Sema4A in the presence or not of PlexinD1 and PlexinB2-blocking Abs or LPS. Values are mean ± SD (n = 4 donors) of four independent experiments. (G) Phospho–VEGFR-2 and phospho–VEGFR-1 protein levels were analyzed by Western blot analysis of lysates of human macrophages upon 18-h treatment with 50 nmol/l of recombinant Sema4A. The data shown are representative (n = 4 donors) of four independent experiments. ***p < 0.001.

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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 is expressed by myeloid precursors and mature macrophages after heart I/R. Fluorescent confocal microscopy analysis of Sema4A expression in heart I/R tissues. (A) Sema4A (red) was specifically detected in the infarcted myocardium (I) 24 h after I/R, whereas its 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 a control goat IgG Ab (not shown). Confocal analysis has been performed on tissue sections from n = 6 mice per time point, and images are representative of five fields observed per mouse. Scale bars, 25 \( \mu \text{m} \). (F) Bar graph shows the percentage of Sema4A colocalization with CD11b+ and CD68+ cells. **p < 0.01.

Sema3E did not enhance macrophage migration and that Sema3E expression was not enhanced in activated cells. Furthermore, our data of the simultaneous treatment of macrophages with Sema4A and Sema3E in presence of PlexinD1-blocking Ab suggest a competition between the two Semas for PlexinD1 receptor. Even though we did not observe a significant increase of Sema3E expression in activated macrophages, we can hypothesize that the low levels of Sema3E produced by macrophages may be sufficient to counteract the effects of Sema4A on basal migration. Our findings unveil a crucial and specific role of Sema4A in enhancing the motility of activated macrophages and suggest that the competition with Sema3E may regulate the activity of macrophages during inflammation or pathological angiogenesis.

While looking for additional mechanisms by which Sema4A could regulate macrophages, we noticed that the stimulation of these cells with Sema4A induced a significant phosphorylation of VEGFR-1, but not of VEGFR-2. Notably, it has been shown that VEGFR-1 is the principal receptor regulating the VEGF-A–triggered motility of monocytes/macrophages. Moreover, VEGF-1 mediates the activation and recruitment of these cells during chronic inflammation and pathological angiogenesis. Based on these findings and our data, we can assume that the increased activation of VEGFR-1 induced by the Sema4A/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 to mediate Sema4A-induced growth cone collapse in mouse hippocampal neurons. In this study, we showed that, among the different PlexinBs, macrophages stimulated with poly I:C and LPS displayed increased levels of PlexinB2; however, a function-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. It has been widely described that tumor-associated macrophages share properties with M2-activated cells, have a propelling role in tumor angiogenesis, and induce cancer progression. 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. 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.

It has been widely shown that hypoxia induced expression of VEGF-A in macrophages. 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 PlexinD1. It has been described that one of the main mechanisms of induction of VEGF-A production in macrophages involves an NF-κB–dependent pathway. Therefore, we can speculate that Sema4A, through PlexinD1, may activate hypoxia-independent
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 Sema4A, we can hypothesize that the activation of the TGF-β1/Smad signaling pathway may represent an alternative mechanism by which Sema4A upregulates VEGF-A expression in these cells.

The increased ECs migration and vessel formation in vivo induced by macrophages treated with recombinant Sema4A is consistent with the induction by Sema4A of a proangiogenic phenotype in macrophages. It has been recently described that Sema4A inhibits ECs functions in experimental angiogenesis (20). In this study, we described that Sema4A 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 Sema3B, 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 Sema4A-treated macrophages, we can speculate that during pathological angiogenesis, Sema4A may promote neovascularization by recruiting VEGF-A–expressing macrophages to activate ECs.

In this article, we show that VEGF-A produced by Sema4A–elicited macrophages activates a specific signal pathway in ECs by increasing the levels of phospho-Akt. It has been widely described that P13K/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 Sema4A causes the activation of the endothelial P13K/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 Sema4A in the culture medium of differentiated human macrophages, corroborating previous observations demonstrating that a soluble form of Sema4A is cleaved from membrane-bound Sema4A overexpressed in human embryonic kidney 293 (20). These data indicate therefore that Sema4A can be expressed as soluble mediator and exerts its promigratory function on macrophages as locally released protein. Therefore, the final effects of Sema4A 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, monocytes/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 Sema4A is specifically expressed by macrophages recruited in the infarcted area and significantly enhanced macrophage migration, suggesting that Sema4A 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 Sema3A, Sema3E, and Sema3F, 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 Sema3A and Sema3F were upregulated to balance the prominent production of angiogenic inducers (15). Moreover, Sema6A, another angiogenesis inhibitor (63), and Sema7A, 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 Sema4A early on during the I/R process. Further studies will be performed to better understand this new role of Sema4A on angiogenesis in a pathological condition.

In conclusion, our work unveils a new role of Sema4A 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, Sema4A could recruit macrophages to the ischemic/tumor tissue able to regulate the angiogenic response necessary for tissue healing and remodeling. The increased expression of Sema4A 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

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