Inflammation, Vol. 42, No. 4, August 2019 (© 2019)
DOI: 10.1007/s10753-019-00985-4

ORIGINAL ARTICLE

Semaphorin 3F Promotes Transendothelial Migration of Leukocytes in the Inflammatory Response After Survived Cardiac Arrest

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Abstract—Leukocyte transmigration through the blood vessel wall is a fundamental step of the inflammatory response and requires expression of adhesion molecule PECAM-1. Accumulating evidence implicates that semaphorin (Sema) 3F and its receptor neuropilin (NRP) 2 are central regulators in vascular biology. Herein, we assess the role of Sema3F in leukocyte migration *in vitro* and *in vivo*. To determine the impact of Sema3F on leukocyte recruitment *in vivo*, we used the thioglycollate-induced peritonitis model. After the induction of peritonitis, C57BL/6 mice were intraperitoneally (i.p.) injected daily with recombinant Sema3F or solvent for 3 days. Compared with solvent-treated controls, leukocyte count was increased in the peritoneal lavage of Sema3F-treated mice indicating that Sema3F promotes leukocyte extravasation into the peritoneal cavity. In line with this observation, stimulation of human endothelial cells with Sema3F enhanced the passage of peripheral blood mononuclear cells (PBMCs) through the endothelial monolayer in the transwell migration assays. Conversely, silencing of endothelial Sema3F by siRNA transfection dampened diapedesis of PBMCs through the endothelium *in vitro*. xMechanistically, Sema3F induced upregulation of adhesion molecule PECAM-1 in endothelial cells and in murine heart tissue shown by immunofluorescence and western blotting. The inhibition of PECAM-1 by blocking antibody HEC7 blunted Sema3F-induced leukocyte migration in transwell assays. SiRNA-based NRP2 knockdown reduced PECAM-1 expression and migration of PBMCs in Sema3F-treated endothelial cells, indicating that PECAM-1 expression and leukocyte migration in response to Sema3F depend on endothelial NRP2. To assess the regulation of Sema3F in human inflammatory disease, we collected serum samples of patients from day 0 to day 7 after survived out-of-hospital cardiac arrest (OHCA, $n = 41$). First, we demonstrated enhanced migration of PBMCs through endothelial cells exposed to the serum of

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patients after OHCA in comparison to the serum of patients with stable coronary artery disease or healthy volunteers. Remarkably, serum samples of OHCA patients contained significantly higher Sema3F protein levels compared with CAD patients (CAD, $n = 37$) and healthy volunteers ($n = 11$), suggesting a role of Sema3F in the pathophysiology of the inflammatory response after OHCA. Subgroup analysis revealed that elevated serum Sema3F levels after ROSC are associated with decreased survival, myocardial dysfunction, and prolonged vasopressor therapy, clinical findings that determine the outcome of post-resuscitation period after OHCA. The present study provides novel evidence that endothelial Sema3F controls leukocyte recruitment through a NRP2/PECAM-1-dependent mechanism. Sema3F serum concentrations are elevated following successful resuscitation suggesting that Sema3F might be involved in the inflammatory response after survived OHCA. Targeting the Sema3F/NRP2/PECAM-1 pathway could provide a novel approach to abolish overwhelming inflammation after resuscitation.

KEY WORDS: inflammation; leukocyte transmigration; semaphorin 3F; neuropilin-2; PECAM-1.

INTRODUCTION

Tissue damage of any kind causes an inflammatory reaction of the immune system characterized by localized emigration of leukocytes from post-capillary venules into inflamed tissue [1, 2]. During this process, leukocytes move from the blood stream through the tightly apposed endothelial borders (paracellular) or through the endothelial cell itself (transcellular). Intercellular junction molecules such as PECAM-1 (platelet endothelial cell adhesion molecule-1, CD31) are involved in transendothelial migration of leukocytes [1, 3–5]. PECAM-1 is a member of the immunoglobulin family and expressed on platelets, neutrophils, monocytes, lymphocyte subsets, and endothelial cells [1, 4, 5]. Homophilic PECAM-1/PECAM-1 interactions between leukocytes and endothelial cells facilitate passage of leukocytes through the endothelium. The inhibition of PECAM-1 by genetic deletion or blocking antibodies dramatically impaired leukocyte transmigration [5, 6]. *Vice versa* endothelial overexpression of PECAM-1 cDNA promoted transendothelial migration of leukocytes [7]. Therefore, PECAM-1 has important functions in the process of leukocyte extravasation and is a key player in the inflammatory response following tissue injury.

Semaphorin (Sema) 3F is a secreted member of the class 3 semaphorin family that was originally identified as neuronal guidance molecule in the developing brain [8, 9]. Outside the nervous system, Sema3F plays important roles in tumor and vascular biology [8, 10, 11]. In the last years, Sema3F has been identified as an effective inhibitor of angiogenesis, tumor progression, and metastasis [12–16]. Sema3F signals in endothelial cells mostly through a receptor complex consisting of plexin A1 and neuropilin (NRP) 2. NRP2 is a transmembrane glycoprotein essential for proper formation of lymphatic vessels and capillaries [16–18]. Despite the acknowledged importance of Sema3F in vascular biology, studies assessing the role of Sema3F in endothelial inflammation and leukocyte transmigration have not been reported. Herein, we provide novel evidence that Sema3F is an essential regulator of leukocytes diapedesis contributing to the pathogenesis of systemic inflammation after survived cardiac arrest.

MATERIALS AND METHODS

Reagents

Recombinant human tumor necrosis factor (TNF)α, monocyte chemoattractant protein (MCP)-1, and Sema3F were purchased from R&D Systems (Minneapolis, USA). Sema3F ELISAs were obtained from MYBioSource (San Diego, CA, USA) and thioglycollate from Merck (T9032; Darmstadt, Germany). Primary antibodies were used as follows: monoclonal rabbit anti-human Sema3F (Novus Biologicals, Littleton, USA), monoclonal mouse anti-human α-tubulin (R&D Systems, Minneapolis, USA), monoclonal rabbit anti-human PECAM-1 (R&D Systems, Minneapolis, USA), monoclonal mouse anti-human PECAM-1 HEC7 (Abcam, Cambridge, UK), monoclonal rabbit anti-human GAPDH (Cell Signaling, Danvers, USA), PE-conjugated monoclonal anti-mouse PECAM-1 (Thermo Scientific™, Darmstadt, Germany), and monoclonal goat anti-rabbit Cy2 (Koma Biotech, Seoul, Korea).

Cell Culture

Human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical cords as previously

described [19]. HUVECs from passages 4 to 6 were used for experiments and were grown to full confluency at 37 °C and 5% CO2 in endothelial cell growth medium (PELO-biotech GmbH, Planegg, Germany) supplemented with 10% fetal calf serum (FCS).

RNA Interference

Non-specific scrambled siRNA oligos (scrsiRNA) and three different human Sema3F-directed siRNA oligos were purchased from Thermo Scientific (Darmstadt, Germany). Human NRP2-directed siRNA oligos were acquired from Dharmacon (Lafayette, USA). HUVECs were plated 1 day before transfection in endothelial cell medium supplemented with 10% FCS. On the day of transfection, scrsiRNA or specific siRNAs (100 nM) were transfected with Lipofectamine RNAiMAX (Thermo Scientific™, Darmstadt, Germany) according to the manufacturer's protocol. Forty-eight-hour post transfection endothelial cells were used for transwell migration assays or were harvested for RNA or protein analyses.

Western Blot Analysis

HUVECs were transfected with indicated siRNA and cultured for 48 h. In another set of experiments, HUVECs were cultured in the presence of recombinant Sema3F for indicated times. Cell proteins were extracted in RIPA buffer (0.1% SDS, 0.5% sodium deoxycholate, 1% NP40, Tris-HCl, pH 7.4) supplemented with a protease inhibitor and phosphatase inhibitor cocktail (Roche). Extracted proteins were subjected to western blot analysis as previously described [19].

Immunofluorescence

HUVECs were seeded on glass coverslips and stimulated for 72 h with solvent or recombinant human Sema3F (100 ng/ml). Cells were fixed in ice-cold methanol/acetone at − 20 °C for 10 min and blocked with 5% goat serum at room temperature for 30 min. Subsequently, HUVECs were incubated with a monoclonal rabbit anti-human PECAM-1 antibody overnight. A goat anti-rabbit Cy2 was used as a secondary antibody. Nuclei were visualized using 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI; 1:30000; Sigma-Aldrich, Munich, Germany).

Transwell Migration Assay

The migration of human peripheral blood mononuclear cells (PBMCs) through the endothelial cell monolayer was analyzed in transwell chambers with 5-μm pore-size inserts (Costar Transwell, Corning Inc., New York, USA). HUVECs were cultured to full confluence on fibronectin-coated transwell inserts. After 24 h, the medium was replaced with endothelial cell medium containing TNFα (20 ng/ml), Sema3F (100 ng/ml), or solvent daily. After 48 h, PBMCs were added on top of HUVECs in the upper compartment. Chemotaxis of PBMCs was induced by monocyte chemoattractant protein (MCP)-1 (50 ng/ml) in the bottom chamber. After 2 h, PBMCs were collected in the bottom chamber. Cells were spun down and counted by blinded investigators. The results are expressed as the percentage of migrated PBMCs in the presence of solvent.

Chemotaxis Assay

Chemotaxis assays were performed in 12-well transwell chambers (Costar Transwell, Corning Inc., New York, USA). Human PBMCs were resuspended in 100 μl RPMI medium containing 1% bovine serum albumin (BSA) and placed on top of the transwell insert (pore size, 5 μm) to migrate towards recombinant Sema3F (100 ng/ml) and/or MCP-1 (50 ng/ml). After 2 h, PBMCs in the lower chamber were collected, spun down, and counted by blinded investigators. Results are expressed as percentage of migrated PBMCs in the presence of solvent.

Animal Studies

Mice studies were approved by the local ethics committee (Regierungspräsidium Freiburg) and were performed according to the respective guidelines. To induce sterile peritonitis, 8–12-week-old male C57BL/6 mice were administered with 500 μl of 16% thioglycollate i.p. (Sigma-Aldrich, Munich, Germany) [20]. Additionally, mice were injected with Sema3F (50 ng/g BW, 500 μl) or solvent i.p. daily for 72 h. Afterwards, the mice were euthanized and peritoneal cells were recovered by peritoneal lavage with 6 ml of RPMI medium. Peritoneal and blood cells were counted by a hemocytometer.

Patient Recruitment and Sample Collection

The study was approved by the ethics committee of the University of Freiburg and confirms to the tenets of the declaration of Helsinki. Forty-one patients following successful resuscitation after OHCA from January 2017 to February 2018 were included after admission to the intensive care unit at the University Hospital of Freiburg (Germany). Informed consent was obtained from resuscitated patients that survived to discharge with a good neurological outcome or from the next of kin. Patients younger than

18 years or resuscitated following traumatic injury were excluded. First blood sampling drawn from a venous catheter was performed after hospital admission within the first 3 h after ROSC (day 0). After containing informed consent, blood samples from patients with stable coronary artery disease (CAD, $n = 37$) or healthy volunteers ($n = 11$) were studied as control groups. All samples were centrifuged immediately at 1000×*g* at 4 °C for 10 min. The supernatants were frozen at − 20 °C.

ELISA

Human serum Sema3F protein concentrations were measured using the Sema3F ELISA kit from MYBioSource (San Diego, CA, USA) according to the manufacturer's protocol.

Statistical Analysis

Densitometric analysis of western blots was performed by Imagelab™ software (Bio-Rad Laboratories, Hercules, USA). Statistical differences between more than two groups were analyzed by ANOVA followed by the Bonferroni *post hoc* analysis. The two-tailed *t* test was used for two-group comparisons of normally distributed data with GraphPad PRISM 4.0 (GraphPad Software, Inc., La Jolla, CA, USA). Differences were considered significant when the *p* value was less than 0.05. After linear transformation of our ELISA results, differences of serum Sema3F concentrations were analyzed by ANOVA to assess the effects of time or different groups. A Bonferroni *post hoc* test was used to identify specific differences between indicated groups. Data are expressed as mean ± SEM.

RESULTS

Sema3F Causes Leukocyte Accumulation in the Peritoneal Cavity during Peritonitis

To assess the impact of Sema3F on leukocyte recruitment during inflammation, C57BL/6 mice were administered at first with thioglycollate i.p. and recombinant Sema3F i.p. daily. Thioglycollate elicits a sterile peritonitis leading to extravasation of leukocytes into the peritoneal cavity [20]. Control mice were injected with appropriate solvent. After 3 days, cells were counted in the peritoneal cavity of Sema3F- or solvent-injected mice. Remarkably, increased accumulation of inflammatory cells was observed in the peritoneal cavity of mice injected with Sema3F (Fig. 1a). Blood analysis did not reveal significant differences in total leukocyte blood count between these groups. This result suggests that different peritoneal cell numbers cannot be explained by distinct white blood counts and are most likely a consequence of heightened leukocyte extravasation (Fig. 1b). Take together, Sema3F increased leukocyte diapedesis through the vessel wall in a murine inflammation model.

Sema3F Promotes Transendothelial Migration of Leukocytes *in vitro*

To minimize environmental variables present in mice, we analyzed the impact of Sema3F on transendothelial leukocyte migration in transwell assays with cultured human endothelial cells. HUVECs were plated on transwell inserts with porous filters and co-stimulated with Sema3F alone or solvent. Peripheral blood mononuclear cells (PBMCs) were added to the upper chamber and migrated into the bottom chamber towards a monocyte chemoattractant protein (MCP)-1 gradient (Fig. 1c). Compared with solvent-treated cells, migration of PBMCs was significantly promoted by Sema3F. Next, we performed loss-of-function studies and quantified the migration of PBMCs through HUVECs transfected with non-silencing scrambled (scr) siRNA or siSema3F. Knockdown of endothelial Sema3F was confirmed by qRT-PCR and significantly reduced Sema3F expression compared with control cells transfected with scrsiRNA (Fig. 1d). Notably, Sema3F knockdown significantly reduced the number of PBMCs crossing the endothelial monolayer, indicating that loss of endothelial Sema3F expression attenuates leukocyte extravasation (Fig. 1e). These findings identify Sema3F as a positive regulator of leukocyte recruitment in acute inflammation *in vitro* and *in vivo*.

To assess endothelial-independent Sema3F effects on leukocyte migration, we determined chemotaxis of PBMCs in the absence of endothelial cells in chemotaxis assays (Fig. 1f). PBMCs were placed in the upper transwell chamber and migrated into the lower transwell chamber loaded with Sema3F and/or MCP-1-containing medium. In the absence of endothelial cells, Sema3F was ineffective as a chemoattractant and caused no significant alteration in PBMC migration compared with the control medium (Fig. 1f). This data demonstrates that recruitment of leukocytes in response to Sema3F requires the presence of endothelial cells.

Sema3F Regulates Intercellular Junction Protein PECAM-1 in Endothelial Cells

Extravasation of leukocytes through the endothelium requires adhesion molecule PECAM-1 [2, 4]. In order to assess whether Sema3F regulates PECAM-1, we performed

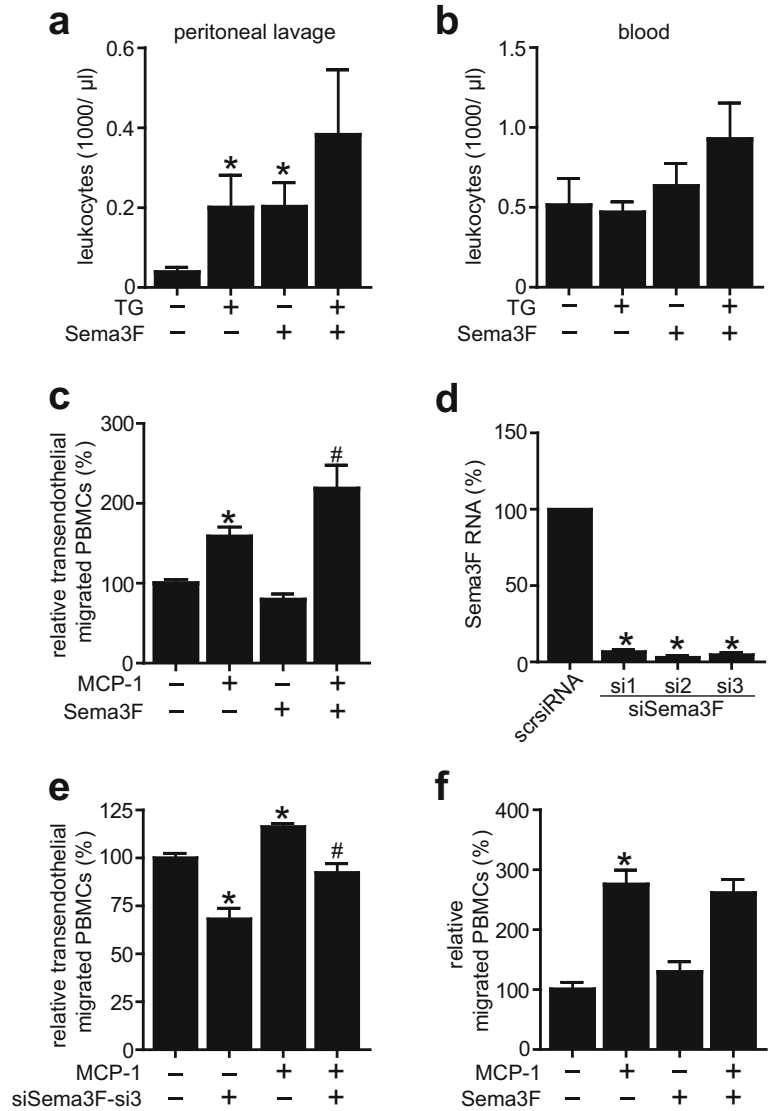

Fig. 1. Sema3F promotes leukocyte migration *in vitro* and *in vivo*. **a**, **b** Sema3F promotes leukocyte diapedesis in a murine peritonitis model. To induce peritonitis, 8–12-week-old male C57BL/6 mice were challenged once with 16% thioglycollate (TG) i.p. Mice were administered daily with recombinant human Sema3F (50 ng/g mouse BW) or an appropriate solvent. After 72 h, cell counts were analyzed in peritoneal lavage (a) or blood (b) of Sema3F- or solvent-treated mice. Results are displayed as means ± SEM ($n = 6$/group; *$p < 0.05$ *versus* TG−/ Sema3F–mice). **c** Sema3F augments transendothelial migration of leukocytes in transwell migration assays. Human umbilical venous endothelial cells (HUVECs) were grown to confluence on transwell inserts. Endothelial cells were exposed to Sema3F (100 ng/ml) alone or to solvent. After 3 days, peripheral blood mononuclear cells (PBMCs) were added on top of the endothelial monolayer. PBMCs migrate through the endothelium towards an MCP-1 gradient (50 ng/ml). After 2 h, PBMCs collected in the bottom chamber were counted by blinded investigators. Results are expressed as the percentage of PBMCs migrated in the presence of control (MCP-1–/ Sema3F–). The graph shows combined data obtained from 3 independent experiments; bars denote means ± SEM ($n = 6$ wells for each condition;*$p < 0.05$ *versus* Sema3F−/ MCP-1–; #$p < 0.05$ *versus* MCP-1+/ Sema3F–). **d** siRNA-mediated Sema3F knockdown repressed Sema3F RNA expression in endothelial cells ($n = 3$/condition; *$p < 0.05$ *versus* scrsiRNA). **e** Sema3F knockdown significantly impeded migration of PBMCs through the endothelial monolayer *in vitro*. HUVECs transfected with Sema3F siRNA3 (siSema3F-si3+) or scrambled siRNA (siSema3F-si3–) were seeded on transwell inserts. After transfection, MCP-1-mediated migration of PBMCs through the endothelial monolayer was assessed by blinded investigators ($n = 6$ wells for each condition; *$p < 0.05$ *versus* MCP-1−/ siSema3F-si3–; #$p < 0.05$ *versus* MCP-1+/ siSema3F-si3–). **f** Sema3F had no chemotactic effect on PBMCs in chemotaxis assays *in vitro.* PBMCs in the upper chamber of the transwell were allowed to migrate through the pores into the lower chamber filled with Sema3F (100 ng/ml)– and/or MCP-1 (50 ng/ml)–containing RPMI medium. Cell migration was quantified by blinded investigators after 2 h ($n = 6$ wells for each condition;*$p < 0.05$ *versus* MCP-1–/Sema3F–).

loss-of-function studies and quantified PECAM-1 expression in HUVECs transfected with scrsiRNA or siSema3F (Fig. 2a). Silencing of Sema3F in HUVECs diminished endothelial PECAM-1 RNA expression demonstrated by qRT-PCR. Next, we performed gain-of-function experiments and analyzed PECAM-1 expression in HUVECs cultured in the presence of recombinant Sema3F. Interestingly, Sema3F augmented endothelial PECAM-1 protein expression in a concentration- and time-dependent manner shown by western blotting and immunocytochemistry (Fig. 2b–d). Consistently, an increase of PECAM-1 protein was observed in the hearts derived from Sema3F-injected mice (Fig. 2e). Taken together, these data identify Sema3F as a novel regulator of endothelial PECAM-1.

Sema3F Promotes Transendothelial Migration of Leukocytes *via* NRP2 and PECAM-1

To further substantiate PECAM's role in the transduction of Sema3F effects, we performed transwell migration assays and inhibited function of PECAM-1 by the administration of HEC7, a blocking anti-human PECAM-1 antibody [3]. Inhibition of endothelial PECAM-1 by HEC7 repressed passage of PBMCs across Sema3F-treated endothelial cells (Fig. 3a). To assess whether Sema3F receptor NRP2 participates in Sema3F-dependent recruitment of leukocytes *in vitro*, HUVECs were transfected with siNRP2, seeded on transwell inserts, and cultured in the presence of Sema3F or solvent (Fig. 3c). NRP2 knockdown diminished NRP2 protein expression in HUVECs (Fig. 3b) and prevented passage of PBMCs across HUVECs in response to Sema3F (Fig. 3c). To substantiate the importance of NRP2 in Sema3F-mediated effects, we quantified PECAM-1 in the endothelial cells after NRP2 knockdown by western blotting (Fig. 3d). Notably, loss of endothelial NRP2 prevented upregulation of PECAM-1 protein in the endothelial cells stimulated with Sema3F. Collectively, these studies identify a novel Sema3F/NPR2/PECAM-1 pathway that positively regulates leukocyte extravasation *in vitro*.

Serum from Resuscitated Patients Induces Transendothelial Leukocyte Recruitment and Contains Elevated Levels of Sema3F Protein

Inflammation is a central finding in resuscitated patients after return of spontaneous circulation (ROSC) [21]. To assess the regulation of Sema3F in the inflammatory response after ROSC, we collected serum samples of patients from day 0 to day 7 after survived OHCA. To confirm the pro-inflammatory effects of the serum from OHCA patients, we performed transwell migration assays and analyzed passage of PBMCs through endothelial cells in response to serum from resuscitated patients (Fig. 4a). TNFα served as positive control and induced transendothelial leukocyte migration through the endothelium. Increased transendothelial leukocyte migration was observed in response to serum of resuscitated patients compared with control serum. Interestingly, the pro-migratory effect of the serum from cardiac arrest patients increased over time suggesting that concentrations of pro-migratory factors underlie changes in the post-resuscitation period (Fig. 4a).

Next, we assessed whether Sema3F is regulated in the post-resuscitation period in OHCA patients by ELISA. Circulating serum Sema3F levels were analyzed in 41 patients after survived cardiac arrest (Fig. 4b). Thirty-seven patients with stable coronary artery disease (CAD) and 11 healthy volunteers were also included. Clinical characteristics of all patients are presented in Table 1. All resuscitated patients were intubated and mechanically ventilated. Five patients (12.2%) received extracorporeal life support (ECLS) during cardiopulmonary resuscitation. Resuscitated patients were followed for 1 month to assess neurological outcome by modified Rankin scale. Approximately half of OHCA patients showed evidence of cardiac origin (56.1%). The majority of survivors (71.4%) presented initially with a shockable rhythm. Two-thirds of resuscitated patients (n = 27, 65.9%) died during hospital stay within the first month after ROSC. Non-survivors were older (66.9 ± 15.2 *versus* 53.7 ± 16.6 years) and had more frequent (74.1%) non-shockable rhythm (asystole and pulseless electrical activity (PEA)) in the first ECG. Compared with the serum of CAD patients or healthy donors, Sema3F protein was elevated in the serum of resuscitated patients and increased over time with a maximum at day 2 after ROSC (Fig. 4a).

Serum Sema3F Levels Are Associated with Prolonged Vasopressor Support and Reduction of Left Ventricular Function and Survival in Post-Cardiac Arrest Patients

Elevated serum levels of inflammatory factors after ROSC predict worse outcome after OHCA [22, 23]. To assess whether Sema3F concentrations are associated with survival, our post-cardiac arrest patients were divided into a survivor (n = 14) and non-survivor (n = 27) cohort (Fig. 5a). Notably, Sema3F serum levels were significantly higher in non-survivors at day 3 after ROSC (Fig. 5a). Next, we compared Sema3F levels in OHCA patients with normal, mild (LV-EF 50–40%), and severely impaired left ventricular ejection fraction (LV-EF < 40%) (Fig. 5b). Sema3F levels were significantly elevated in patients with

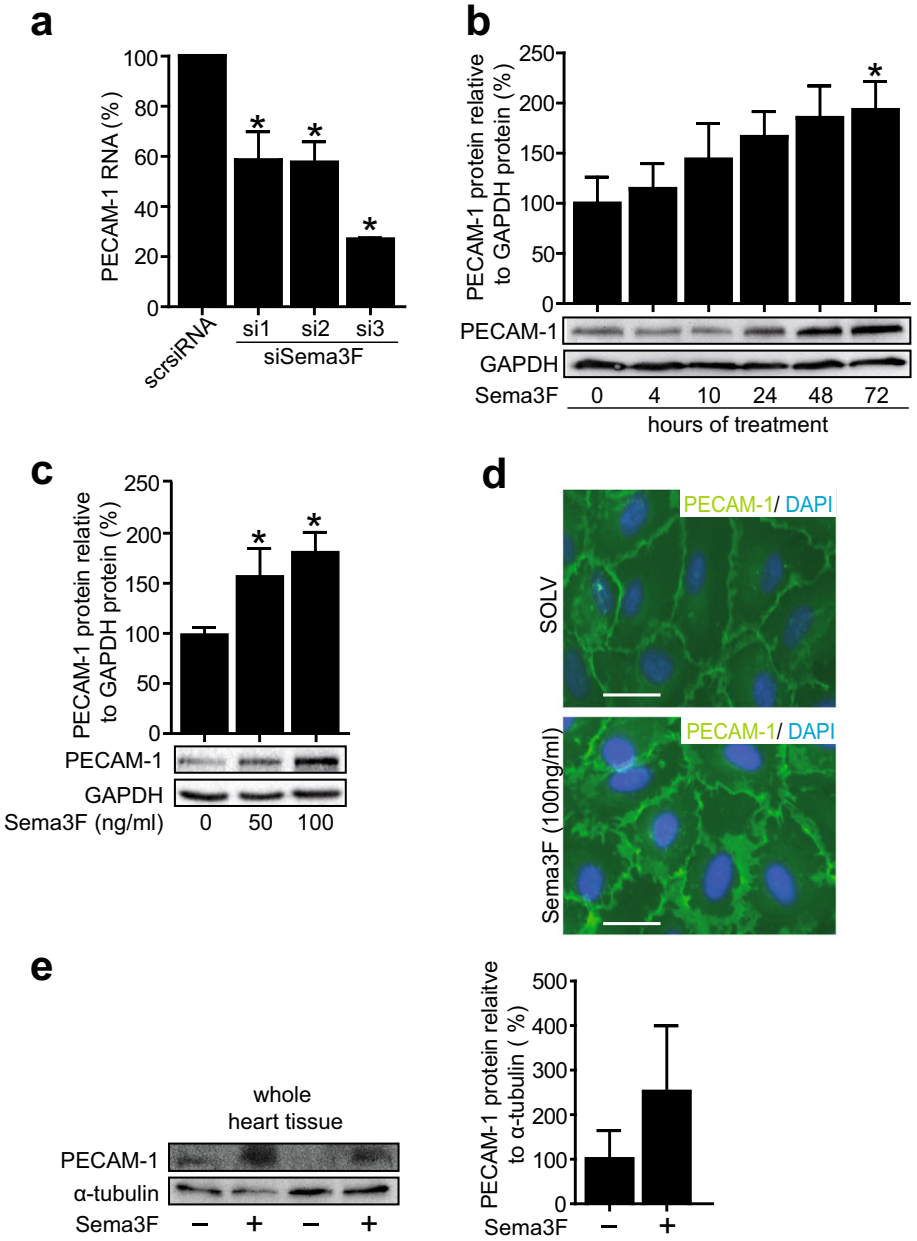

Fig. 2. Sema3F regulates intercellular junction protein PECAM-1 in endothelial cells. **a** siRNA-mediated Sema3F knockdown reduced Sema3F PECAM-1 RNA in endothelial cells ($n = 3$/condition; $^*p < 0.05$ *versus* scrsiRNA). HUVECs were transfected with three different Sema3F siRNAs (siSema3F-si1-3) or scrambled siRNA (scrsiRNA). RNA was isolated after 48 h and reverse transcribed. PECAM-1 cDNA expression was assessed by qRT-PCR ($n = 3$, $^*p < 0.05$ *versus* scrsiRNA). PECAM-1 protein expression is upregulated by Sema3F in endothelial cells. **b** HUVECs were exposed to solvent or recombinant Sema3F (100 ng/ml) and cell lysates were collected at indicated time points. PECAM-1 expression was analyzed by western blotting with an anti-PECAM-1 antibody. GAPDH served as loading control. Results are presented as mean ± SEM ($n = 4$, $^*p < 0.05$ *versus* solvent). **c** Endothelial cells were stimulated with indicated concentrations of Sema3F for 72 h and cell proteins were used for western blot analysis. Representative blots of five independent experiments are shown ($^*p < 0.05$ *versus* solvent). **d** Sema3F augmented endothelial PECAM-1 expression demonstrated by immunocytochemistry of endothelial cells treated with recombinant Sema3F (100 ng/ml) for 72 h. DAPI is used for nuclear staining. Scale bar, 10 μm. **e** C57BL/6 mice were administered daily with recombinant Sema3F (50 ng/g mouse BW) or solvent i.p. After 72 h, hearts were removed and total heart proteins were extracted and subjected to western blot analysis with antibodies against PECAM-1 or α-tubulin.

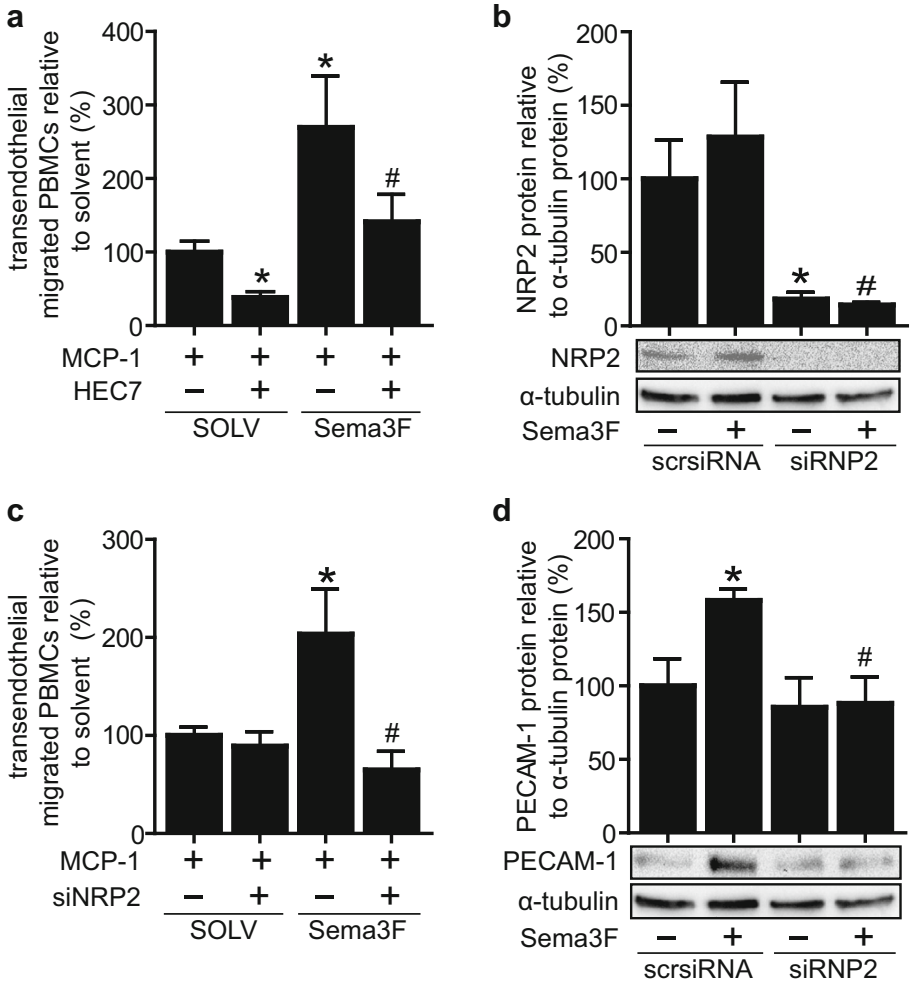

Fig. 3. Sema3F promotes transendothelial migration of leukocytes *via* NRP2 and PECAM-1. **a** Recruitment of leukocytes in response to Sema3F is inhibited by blocking anti-PECAM-1 antibody HEC7. HUVECs seeded on transwell inserts were cultured in the presence of Sema3F or solvent. After 3 days, endothelial cells were exposed to HEC7 or control mouse antibody. After 2 h, PBMCs were added to upper compartment and transendothelial leukocyte migration was assessed by blinded investigators ($n = 6$/ for each condition, *$p < 0.05$ *versus* MCP-1+/ Sema3F−/ HEC7–; #$p < 0.05$ *versus* MCP-1+/ Sema3F+/ HEC7–). **b** NRP2 knockdown significantly reduced NRP2 protein expression. Endothelial cells were transfected with specific siNRP2 or scrambled siRNA and cultured in the presence of Sema3F (100 ng/ml) or control medium. Three days post transfection, endothelial cell lysates were used for western blot analysis with anti-NRP2 and anti-α-tubulin antibody. **c** NRP2 knockdown in endothelial cells markedly reduced transendothelial migration of PBMCs in response to Sema3F. After transfection of siNRP2 or scrsiRNA, we performed transwell migration assays. ($n = 6$ wells for each condition; *$p < 0.05$ *versus* MCP-1+/ Sema3F−/ siNRP2–; #$p < 0.05$ *versus* MCP-1+/Sema3F+/siNRP2–). **d** Loss of endothelial NRP2 diminished endothelial PECAM-1 protein expression.

severe myocardial dysfunction compared with patients with normal or mild reduced ejection fraction (Fig. 5b). In OHCA patients, the levels of inflammatory mediators are associated with hemodynamic instability and increased need for vasopressor support [23]. To explore a relation between Sema3F levels and hemodynamic stability, we analyzed Sema3F levels in OHCA patients with short- and long-term catecholamine therapy (days 0–4 *versus* day ≥ 5). Serum Sema3F levels at days 2 and 5 after ROSC were significantly higher in patients with prolonged catecholamine therapy (day ≥ 5) compared with OHCA patients that required short-term vasopressor support (days 0–4, Fig. 5c). Collectively, this data suggests that increased Sema3F levels are associated with higher mortality after ROSC, impaired left ventricular function, and hemodynamic instability in post-cardiac arrest patients.

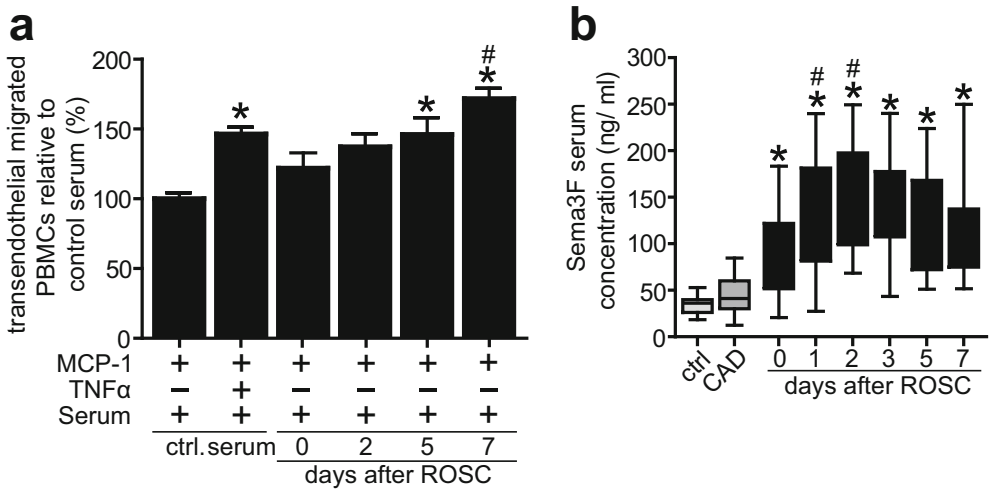

Fig. 4. Serum from resuscitated patients induces transendothelial leukocyte recruitment and contains elevated levels of Sema3F protein. **a** Endothelial cells were grown on gelatin-coated transwell inserts (pore size, 5 μm) to full confluency. Cells were cultured for additional 24 h in the presence of control serum (fetal calf serum, FCS) or serum from patients at different time points of the post-resuscitation period (days 0, 2, 5 or day 7 after ROSC). After 24 h, PBMCs were added to the upper chambers on top of the endothelial monolayer and were allowed to migrate into the bottom chambers containing chemoattractant MCP-1 (50 ng/ml). Results are expressed as the percentage of PBMCs migrated in the presence of control serum. The graph shows combined data obtained from 3 independent experiments; bars denote means ± SEM ($n = 9$ wells for each condition;*$p < 0.05$ *versus* control serum+/TNFα–/MCP-1+, #$p < 0.05$ *versus* serum day 0 post ROSC serum+/TNFα–/MCP-1+). **b** Kinetics of serum Sema3F levels on admission and over a 7-day period in resuscitated patients. Serum concentrations of Sema3F were measured by ELISA in healthy volunteers ($n = 11$), patients with stable coronary artery disease (CAD, $n = 37$), or after successful resuscitation following OHCA ($n = 41$). Boxplot whiskers indicate min and max. Error bars indicate ± SEM (*$p < 0.05$ *versus* CAD, #$p < 0.05$ *versus* day 0 after ROSC).

DISCUSSION

The present study discloses the so far unknown role of Sema3F signaling during endothelial inflammation and reveals that Sema3F promotes leukocyte recruitment and is upregulated in human serum of successfully resuscitated patients. We demonstrate that recombinant Sema3F led to increased leukocyte accumulation at the site of inflammation in a murine peritonitis model (Fig. 1). Consistent with this observation, Sema3F promoted the passage of leukocytes through cultured endothelial cells *in vitro* (Fig. 1c). In Sema3F-treated endothelial cells, we observed upregulation of endothelial PECAM-1 protein, a key molecule that is required for leukocyte transmigration during inflammation (Fig. 2). Blocking PECAM-1 antibody abolished leukocyte transmigration in response to Sema3F (Fig. 3). Moreover, RNA interference of Sema3F receptor NRP2 diminished Sema3F-induced PECAM-1 expression and leukocyte recruitment in cultured endothelial cells, indicating that Sema3F-induced leukocyte migration and PECAM-1 expression require endothelial NRP2 (Fig. 3). Finally, we identify that Sema3F protein concentrations are elevated in the serum of resuscitated patients after survived cardiac arrest (Fig. 4). Subgroup analyses revealed that Sema3F serum levels are associated with increased mortality, myocardial dysfunction, and hemodynamic instability in the post-resuscitation period (Fig. 5). Collectively, this data strongly suggest a role of the Sema3F during the systemic inflammatory response after survived cardiac arrest.

Studies of the last years led to the appreciation that members of the semaphorin family are the central regulators of the immune system and differentially control leukocyte migration during inflammation [24, 25]. Supporting this notion, Sema7A increased transmigration of neutrophils and aggravated lung inflammation [26, 27]. In the same line, Sema3A retarded monocyte migration and reduced inflammation after myocardial infarction [28]. Despite its importance in vascular biology regulation, function and mechanisms of Sema3F/NRP2 signaling during endothelial inflammation are incompletely understood [12, 14–16, 29–31]. The central finding of the present work is that Sema3F/NRP2 signaling exerted pro-inflammatory effects and increased migration of leukocytes across mesenteric venules *in vivo* and cultured endothelial cells *in vitro* (Figs. 1 and 2). Two studies have related Sema3F or respectively NRP2 signaling to leukocyte migration in different settings [32, 33]. In the first study, Sema3F/NRP2 signaling diminished migration of thymocytes and

Table 1. Patients Characteristics and Resuscitation Variables

	CPR cohort ($n = 41$)	CAD group ($n = 37$)	Healthy volunteers ($n = 11$)
Median age (years)	62 ± 16.7	69.1 ± 10.2	29.6 ± 9
Male sex, *n* (%)	28 (68.3)	26 (70.3)	7 (63.6)
Duration of CPR (min)	21.3 ± 16.7	–	–
Etiology of arrest	23 (56.1)	–	–
• Cardiac, *n* (%)	15 (36.6)	–	
• Non- cardiac, *n* (%)	3 (7.3)	–	
• Unknown, *n* (%)			
Initial rhythm	17 (41.5)	–	–
• Shockable, *n* (%)	24 (58.5)		
• Non-shockable, *n* (%)			
pH at admission 0 (pH)	7.16 ± 0.16	–	–
Lactate day 0 (mmol/l)	7.5 ± 4.2	–	–
Performed coronary angiography, *n* (%)	28 (68.3)	37 (100)	–
LVEF (%)	35.9 ± 19.1	57 ± 0.1	–
• Day 0	40.2 ± 18.3		–
• Hospital stay			
Catecholamine req. (days)	3.8 ± 1.9	–	–
ECLS, *n* (%)	5 (12.2)	–	–
ICU length of stay (days)	8.3 ± 7.7	–	–
Survival to discharge, *n* (%)	14 (34.1)	–	–
Neurological outcome	10 (24.4)	–	–
• Rankin ≤ 3, *n* (%)	31 (75.6)	–	–
• Rankin > 3, *n* (%)			

Data are expressed as mean ± SD. *CAD*, coronary artery disease; *CPR*, cardiopulmonary resuscitation; *LVEF*, left ventricular ejection fraction; *ECLS*, extracorporeal life support; *ICU*, intensive care unit; *Rankin*, modified Rankin scale for neurological disability

malignant T cells [32]. Another study reported that myeloid-specific ablation of NRP2 altered the composition of leukocyte subsets in the bronchoalveolar lavage following LPS inhalation indicating that NRP2 in myeloid cells is involved in the pathophysiology of airway inflammation [33]. These studies together with our observations substantiate the idea that Sema3F/NRP2 signaling is an important pathway during inflammation. Future experiments with conditional NRP2 and Sema3F knockout mice are needed to contrast the cell-type specific effects in endothelium and myeloid cells during inflammation.

Leukocyte transmigration requires the expression of PECAM-1 in endothelial cells [1, 4, 5, 20]. Despite the pivotal role of PECAM-1 in inflammation biology, little is known about the regulation of PECAM-1 expression in endothelial cells. In the present study, we provide novel insights into the regulation of PECAM-1 expression and identify PECAM-1 as a downstream target of Sema3F/NRP2 signaling in endothelial cells (Figs. 3 and 4). Previous studies have shown that endothelial PECAM-1 expression is regulated by inflammatory cytokines [34, 35] and nitric oxide [36]. Binding of transcription factor GATA-2 is essential for PECAM-1 promoter activity [37] and mediated PECAM-1 transcription in endothelial cells exposed to leukocyte-specific protein (LSP)-1 [38]. In addition, GATA -2 directly binds to the NPR2 promoter and activated NRP2 expression in endothelial cells [39]. To understand the regulation of PECAM-1 by Sema3F on the transcriptional level, further experiments should clarify whether GATA-2 is required for the upregulation of endothelial PECAM-1 in response to Sema3F.

OHCA is one of the leading causes of death in Europe and in the USA [40–42]. After achieving ROSC, whole body ischemia/reperfusion injury causes the activation of the endothelium and the immune system leading to the post-cardiac arrest syndrome (PCAS) [43]. In addition to brain injury, PCAS comprises myocardial dysfunction, hemodynamic instability, and a sepsis-like inflammatory syndrome that strongly predicts poor outcome after OHCA [22, 44, 45]. A growing body of evidence implicates that the serum of OHCA patients contains a variety of inflammatory cytokines including TNFα, interleukin (IL)-1, IL-6, IL-8, and IL-10 that cause endothelial inflammation and contribute to the pathophysiology of PCAS [22, 23, 46–49]. The present study confirms the pro-inflammatory activity of OHCA serum and demonstrates that endothelial cells exposed to serum of OHCA patients promote transendothelial migration of leukocytes (Fig. 4a). In our

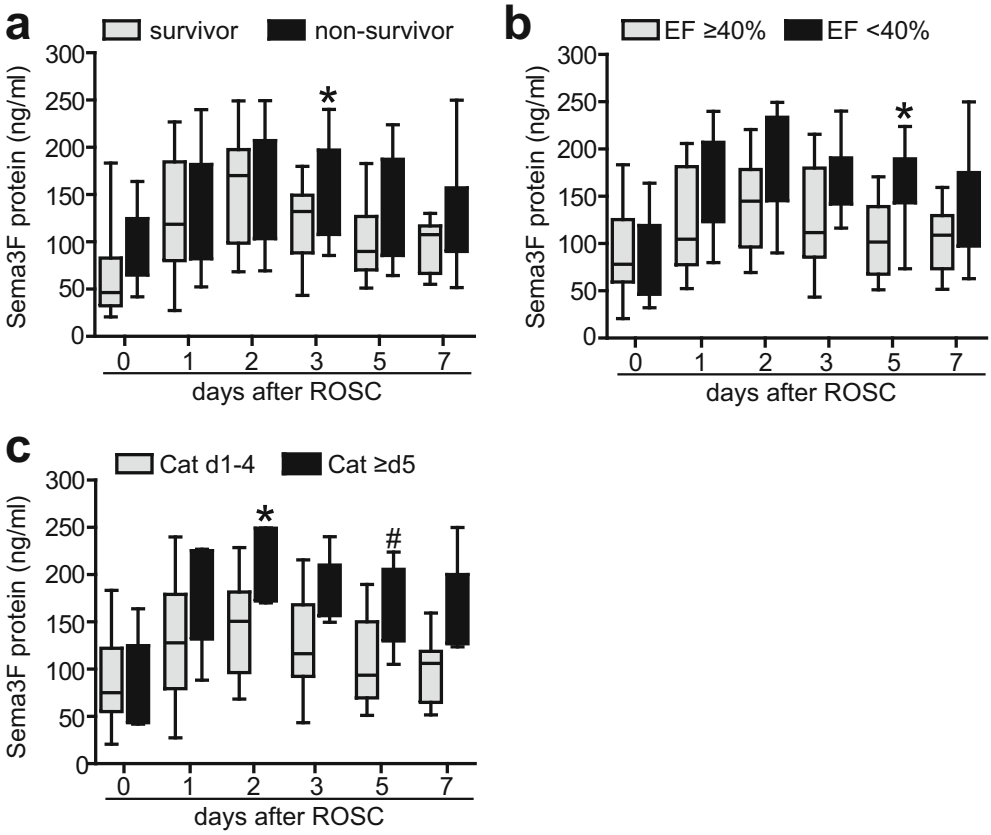

Fig. 5. Serum levels of Sema3F in subgroup analysis for survival, left ventricular myocardial dysfunction, and prolonged catecholamine therapy after resuscitation. **a** Kinetics of Sema3F serum levels on admission and over a 7-day period in survivors ($n = 14$) and non-survivors ($n = 27$) after successful resuscitation (*$p < 0.05$ *versus* survivor at day 3). **b** Variations of serum Sema3F concentrations over a 1-week period after ROSC in resuscitated patients with severe myocardial dysfunction in comparison with patients with mild or normal left ventricular function (LV-EF ≥ 40%) following ROSC (*$p < 0.05$ *versus* EF ≥ 40% at day 3). **c** Serum Sema3F concentrations in OHCA patients with prolonged need for catecholamine (day ≥ 5) compared with OHCA patients with short-term catecholamine therapy (days 0–4). Box and whisker plots compare serum Sema3F levels at indicated time points. Boxplot whiskers indicate min and max. Error bars indicate ± SEM (*$p < 0.05$ *versus* CAD, #$p < 0.05$ *versus* day 0 after ROSC).

experiments, the pro-inflammatory endothelial phenotype was accompanied by increased Sema3F serum levels in patients after OHCA (Fig. 4b), suggesting that Sema3F is induced after systemic ischemia/reperfusion injury and may contribute to systemic inflammation of PCAS (Fig. 4). This observation is consistent with previous studies that semaphorins are known regulators of ischemia/reperfusion injury. Sema3A was upregulated after ischemia/reperfusion injury of the kidney and inhibition of Sema3A protected from acute kidney injury [50]. Another study demonstrated that Sema7A reduced ischemia/reperfusion injury of the liver by repression of neutrophil recruitment [51]. Our results indicate that Sema3F is a novel player in response to systemic ischemia/reperfusion injury after cardiac arrest. Furthermore, we provide novel evidence that increased serum Sema3F levels were associated with reduced survival, left ventricular dysfunction, and hemodynamic instability after survived cardiac arrest, clinical findings that characterize PCAS (Fig. 5). To assess the direct effects of Sema3F on the severity of PCAS *in vivo*, experiments in a cardiac arrest/resuscitation model are needed that address outcome, survival, myocardial function, and hemodynamic stability in mice with Sema3F deletion.

Collectively, studies of the last decade implicate that semaphorins are key regulators during inflammation and control migration of inflammatory cells [24]. The present data expand our understanding of Sema3F in endothelial biology and demonstrate that the Sema3F plays a key role during endothelial inflammation and may serve as a

potential drug target in ischemia/reperfusion injury. Future experiments have to determine whether modulation of the Sema3F/NRP2/PECAM-1 pathway has the impact to diminish inflammation and protect from ischemia/reperfusion injury following OHCA.

ACKNOWLEDGEMENTS

We are indebted to Jessica Beckert for her outstanding technical assistance.

FUNDING

This work was supported by the Deutsche Forschungsgemeinschaft to T. H. (HE 7432/1-1) and to M. M. (973/8-1).

COMPLIANCE WITH ETHICAL STANDARDS

All procedures performed in studies involving murine or human tissues were in accordance with the ethical standards of the institution and/or national research.

Conflict of Interest. The authors declare that they have no conflict of interest.

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