ENDOTHELIAL DYSFUNCTION DURING INFLAMMATION AND ALLOIMMUNITY

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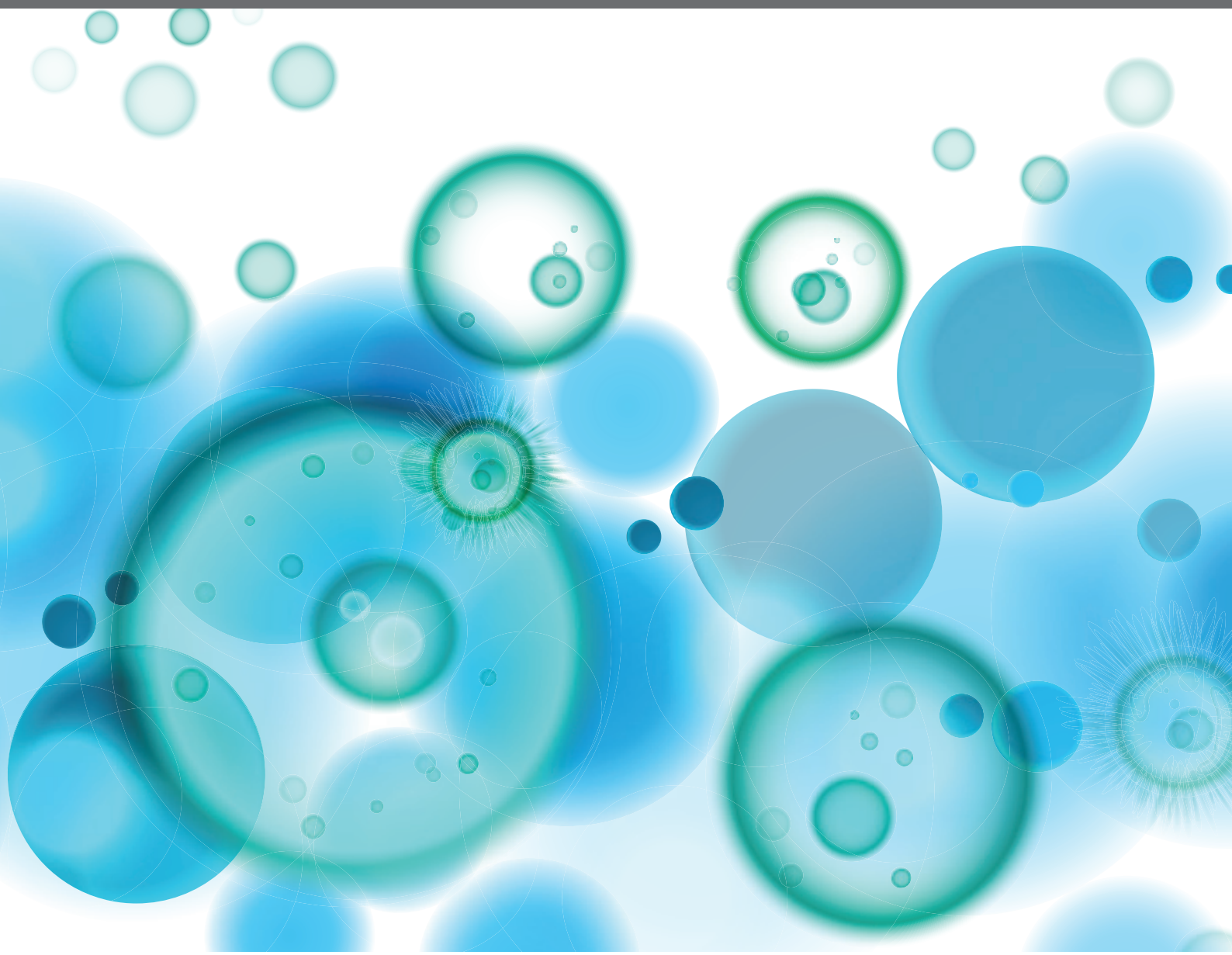

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ENDOTHELIAL DYSFUNCTION DURING INFLAMMATION AND ALLOIMMUNITY

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EDITORIAL
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Editorial: Endothelial Dysfunction During Inflammation and Alloimmunity

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Keywords: endothelial, dysfunction, alloimmune, transplantation, inflammation

Editorial on the Research Topic

Endothelial Dysfunction During Inflammation and Alloimmunity

Endothelial cells form the inner lining of blood and lymphatic vessels and they have frequent interactions with immune cells as well as foreign agents (1, 2). Endothelial function is crucially involved in physiologic immunity at different stages including recruitment of leukocytes, angiogenesis, and tissue repair (3–5). The term endothelial dysfunction is widely used to describe the non-physiologic activity of endothelial cells. Endothelial dysfunction plays a role in a variety of human diseases, such as arteriosclerosis, cancer, autoimmunity, and sepsis (6). More recently, a role of endothelial dysfunction during inflammatory diseases and transplantation has been demonstrated (7, 8). This research topic addresses the role of endothelial dysfunction during allo-immune reactions as well as during inflammatory diseases.

The first section deals with endothelial dysfunction and allo-antibody responses in organ transplantation. A mini review article by Cardinal et al. presents the evidence for endothelial injury, its causes and long-term consequences on graft outcomes in kidney transplantation. The authors describe the current view on the involvement of the endothelium on graft rejection including apoptotic pathways, autoantibody production, complement deposition, and microvascular rarefaction. Another concise review article by Cross et al. further deepens our understanding on the role of the endothelium during antibody-mediated rejection of solid organ transplants. The authors picture endothelial cells as "victims" of immune reactions, such as allo-reactive lymphocytes and donor-specific antibodies. Subsequently, they discuss the potential role of endothelial cells as "accomplice" of graft rejection facilitating pro-inflammatory allo-responses by upregulation of MHCII molecules and antigen presentation. The original research article by Morales et al. shows that pre-transplantation levels of anti-beta-2-glycoprotein-I antibodies are useful as a biomarker to predict renal graft loss. The authors prospectively followed a large cohort of 740 renal transplant recipients and found a higher rate of vessel thrombosis and subsequent graft loss in the subgroup of patients with high antibody levels before transplant. This finding implicates that an interventional study on prophylactic anticoagulation in renal transplant recipients with elevated anti-beta-2-glycoprotein-I antibody levels is warranted.

The second section of the research topic addresses new findings on the role of endothelial cells in maintenance of vascular barrier function and potential implications for therapeutically targeting of vascular integrity. Disruption of vascular integrity and increased permeability are associated with inflammation and adverse outcome of organ transplantation. A review article by Rahimi highlights recent advances that have provided new insights into endothelial barrier function and mechanisms involved. He describes that endothelial barrier function and integrity are regulated by highly specialized transmembrane receptors through various distinct mechanisms

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and signaling cascades. Major pathways acting in destabilization of endothelial barrier function as well as potential implications for therapeutic interventions are explained. The role of the Angiopoietin 2/Tie2 Axis, which regulates vascular leakiness during sepsis (9), is addressed in the review article by Leligdowicz et al. The manuscript summarizes new findings on the mechanisms of Ang/Tie2-mediated regulation of vascular leakiness. Furthermore, the authors explain how the Ang/Tie2 pathway can serve clinically as prognostic biomarker and as therapeutic target using small molecules or monoclonal antibodies. Wang et al. present original research results on the mechanisms of sepsis-related injury of the pulmonary endothelium. In previous work in murine models, the authors found a role of Neutrophils in the damage of pulmonary microvasculature during sepsis (10, 11). In the current manuscript, they demonstrate *in vitro* that human pulmonary endothelial barrier dysfunction under septic conditions is caspase-dependent. Pan-caspase inhibition attenuated septic neutrophil-dependent pulmonary microvascular barrier dysfunction.

The third section contains four articles on different mechanistic aspects of endothelial cells dysfunction during inflammation and how endothelial dysfunction and inflammation are interlinked. Le Gallo et al. commence with a review of the CD95/Fas axis in modulating immune functions via induction of mainly non-apoptotic signaling pathways in endothelial cells. Zhang et al. demonstrate in an original research paper that the endogenous sulfide pathway is involved in endothelial cell inflammation. Cho et al. present novel knowledge on how inflammation and endothelial dysfunction are linked by a process that is termed "endothelial to mesenchymal transition." Vascular endothelial cells undergo dynamic phenotypic switching during inflammation. Endothelial to mesenchymal transition is a complex biological process in which endothelial cells lose their endothelial characteristics, acquire mesenchymal phenotypes, and express mesenchymal cell markers. This process contributes to vascular dysfunction and is involved in tissue fibrosis, pulmonary arterial hypertension and atherosclerosis. The research topic terminates with a review manuscript of Sedding et al. presenting current knowledge on the role of vasa vasorum angiogenesis in endothelial dysfunction during inflammatory arteriosclerotic plaque formation.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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ORIGINAL RESEARCH
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Differential Mechanisms of Septic Human Pulmonary Microvascular Endothelial Cell Barrier Dysfunction Depending on the Presence of Neutrophils

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Sepsis is characterized by injury of pulmonary microvascular endothelial cells (PMVEC) leading to barrier dysfunction. Multiple mechanisms promote septic PMVEC barrier dysfunction, including interaction with circulating leukocytes and PMVEC apoptotic death. Our previous work demonstrated a strong correlation between septic neutrophil (PMN)-dependent PMVEC apoptosis and pulmonary microvascular albumin leak in septic mice *in vivo*; however, this remains uncertain in human PMVEC. Thus, we hypothesize that human PMVEC apoptosis is required for loss of PMVEC barrier function under septic conditions *in vitro*. To assess this hypothesis, human PMVECs cultured alone or in coculture with PMN were stimulated with PBS or cytomix (equimolar interferon γ, tumor necrosis factor α, and interleukin 1β) in the absence or presence of a pan-caspase inhibitor, Q-VD, or specific caspase inhibitors. PMVEC barrier function was assessed by transendothelial electrical resistance (TEER), as well as fluoroisothiocyanate-labeled dextran and Evans blue-labeled albumin flux across PMVEC monolayers. PMVEC apoptosis was identified by (1) loss of cell membrane polarity (Annexin V), (2) caspase activation (FLICA), and (3) DNA fragmentation [terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)]. Septic stimulation of human PMVECs cultured alone resulted in loss of barrier function (decreased TEER and increased macromolecular flux) associated with increased apoptosis (increased Annexin V, FLICA, and TUNEL staining). In addition, treatment of septic PMVEC cultured alone with Q-VD decreased PMVEC apoptosis and prevented septic PMVEC barrier dysfunction. In septic PMN–PMVEC cocultures, there was greater trans-PMVEC macromolecular flux (both dextran and albumin) vs. PMVEC cultured alone. PMN presence also augmented septic PMVEC caspase activation (FLICA staining) vs. PMVEC cultured alone but did not affect septic PMVEC apoptosis. Importantly, pan-caspase inhibition (Q-VD treatment) completely attenuated septic PMN-dependent PMVEC barrier dysfunction. Moreover, inhibition of caspase 3, 8, or 9 in PMN–PMVEC cocultures also reduced septic PMVEC barrier dysfunction whereas inhibition of caspase 1 had no effect. Our data demonstrate that human PMVEC barrier

dysfunction under septic conditions *in vitro* (cytomix stimulation) is clearly caspase-dependent, but the mechanism differs depending on the presence of PMN. In isolated PMVEC, apoptosis contributes to septic barrier dysfunction, whereas PMN presence enhances caspase-dependent septic PMVEC barrier dysfunction independently of PMVEC apoptosis.

Keywords: human sepsis, endothelial barrier dysfunction, neutrophil, caspase activity, cell co-culture

INTRODUCTION

Sepsis, defined as organ dysfunction due to a dysregulated host response to infection, is the most common cause of death in Intensive Care Units (ICU), and is one of the leading healthcare expenses for patients in the hospital consuming up to 45% of total ICU costs (1–3). Mortality in sepsis is due to multiple organ dysfunction, most commonly the lung leading to acute respiratory distress syndrome (ARDS), but also systemic organs such as heart, brain, and kidneys (1, 2, 4). Septic organ dysfunction is due to an overwhelming systemic inflammatory process, characterized by the enhanced production and release of a plethora of soluble inflammatory mediators, including bacterial-derived lipopolysaccharide (LPS) and various host-derived cytokines [e.g., tumor necrosis factor (TNF) α, interleukin (IL) 1β, interferon (IFN) γ], as well as the activation of both circulating [e.g., polymorphonuclear leukocytes (PMN)] and tissue-resident (e.g., macrophages) inflammatory cells (4–6).

Septic organ dysfunction is also recognized to be due, in large part, to significant activation and dysfunction of the microvasculature of individual organs. Microvascular dysfunction is characterized by impaired barrier function (increased permeability leading to extra-vascular leak of protein-rich edema) and PMN influx into organs (7–10), microvascular thrombosis (11), and impaired distribution of blood flow in microvascular beds (12). Moreover, microvascular dysfunction is clinically important, as it has been documented early in the course of sepsis in humans, and is associated with increased mortality (13, 14), especially if it persists over time (15).

The mechanism of microvascular dysfunction in sepsis is primarily thought to be activation and dysfunction of microvascular endothelial cells (MVEC). This septic activation and dysfunction of MVEC leads to severe tissue edema, inflammation, and ultimately, organ dysfunction and failure. Multiple factors promote septic MVEC activation and dysfunction, including cytokine signaling, mechanical interaction with activated leukocytes, and exposure to harmful leukocyte-derived molecules, such as proteases and oxidants [e.g., nitric oxide (NO)] (4, 8, 16–21). Moreover, PMN interaction with pulmonary MVEC (PMVEC) has been shown to exacerbate the observed barrier dysfunction in response to stimulation with a mixture of pro-inflammatory cytokines (TNFα, IL1β, and IFNγ) and this is thought to be due, at least in part, to PMN-derived NO (8, 22). Further evidence supporting the critical function for PMN in driving septic pulmonary microvascular permeability was provided by *in vivo* murine studies in which PMN were removed (9). Specifically, septic pulmonary microvascular permeability was found to be completely abrogated in septic mice *in vivo* in the absence of PMN or following antibody-mediated blocking of the CD18-β2 integrin adhesion pathway (9).

Apoptosis is a tightly regulated form of cell death known to be important both in tissue homeostasis and under pathological conditions (i.e., sepsis). One of the hallmarks of apoptosis is activation of a family of cysteine proteases known as caspases (23, 24). In addition to caspase activation, apoptotic cell death is also associated with a loss of cell membrane polarization leading to phosphatidyl serine appearing on the outer leaflet of the cell membrane, and fragmentation of the DNA leading to condensed nuclei (23, 24).

Our previous *in vivo* studies in mice revealed both a temporal association and a strong correlation between septic PMVEC apoptosis and PMVEC barrier dysfunction, and importantly, this PMVEC apoptosis appeared to be dependent on PMN–PMVEC interaction (9, 25). However, further assessment of the role of apoptosis in septic murine PMVEC barrier dysfunction *in vitro* demonstrated that early septic barrier dysfunction in PMVEC cultured alone did not appear to be associated with PMVEC apoptosis (26). This finding was supported by multiple other studies that suggested that the connection between MVEC apoptosis and septic barrier dysfunction is unclear and appears to depend on MVEC type, as well as the specific stimulation and time course (26–31). Collectively, these studies highlight the importance of further studies examining the mechanisms responsible for septic MVEC barrier dysfunction, including more clinically relevant direct examination of human MVEC dysfunction and the specific contribution of PMN.

MATERIALS AND METHODS

Reagents

Calcein-AM or calcein-red: Thermo Fisher Scientific (Burlington, ON, Canada); Caspase 1 inhibitor (Ac-YVAD-CMK): Sigma (Oakville, ON, Canada); Caspase 3 inhibitor (Z-DEVD-FMK): APExBIO (Boston, MA, USA); Caspase 8 inhibitor (Z-IETD-FMK): MBL International Corporation (Woburn, MA, USA); Caspase 9 inhibitor (Z-LEHD-FMK): MBL International Corporation (Woburn, MA, USA); CD31 Dynabeads: Life Technologies AS (Oslo, MN, USA); Endothelial Cell Growth Medium-2 (EGM-2): Lonza (Walkersville, MD, USA); Evans blue (EB)-labeled bovine serum albumin (BSA): Sigma (Oakville, ON, Canada); fluoroisothiocyanate (FITC) or Alexa Fluor 647-labeled Annexin V: BioLegend (San Diego, CA, USA); FITC-labeled dextran: Sigma (Oakville, ON, Canada); Human TNFα, IL1β, IFNγ: PeproTech

(Rocky Hill, NJ, USA); Lymphocyte Separation Medium (LSM): MP Biomedical (Canada); Pan-caspase inhibitor (Q-VD-OPh hydrate): APExBIO (Boston, MA, USA); Sulforhodamine (SR) FLICA Poly Caspase Assay Kit: Immunohistochemistry Technologies (Bloomington, MN, USA); terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) *In Situ* Cell Death Detection kit: Roche (Laval, QC, Canada); Type II collagenase: Worthington Biochemical Corporation (Lakewood, NJ, USA).

Isolation and Culture of Human PMVEC

PMVEC were isolated from human lung as previously reported (22). Briefly, human peripheral lung tissue isolated from a grossly normal-appearing region obtained during resectional surgery for localized lung cancer was rinsed in PBS, finely minced, and digested in 0.3% type II collagenase at 37°C with occasional agitation. The digested suspension was filtered, centrifuged at 200 *g*, and washed in PBS. The cell pellet was then resuspended in binding buffer (2 μM Na-citrate, 1.2 μM $NaH_2PO_4.H_2O$, 5.6 μM Na_2HPO_4, 138.6 μM NaCl, and 0.1% BSA) and incubated at 4°C with magnetic Dynabeads coated with anti-human CD31 antibody. Bound cells were magnetically isolated and washed with binding buffer. Isolated cells were resuspended in 10% EGM-2 and placed at 37°C in 5% CO_2 until 50% confluent. PMVEC were monitored daily and quickly growing fibroblasts were removed mechanically under direct microscopy. Once PMVEC reached 50% confluence, they were harvested and re-purified using anti-CD31-coated magnetic microbeads as above. PMVEC were used for experiments at passages 4–10.

Isolation and Labeling of Human Blood Polymorphonuclear Leukocytes (PMN)

PMN were isolated from healthy human blood donors using LSM as described previously (22, 32). In some experiments, PMN were labeled with calcein-AM or calcein-red according to the manufacturer's instructions.

Experimental Conditions

PMVEC medium was changed to fresh 10% EGM-2 at 16 h before PMVEC stimulation. For PMVEC cultured alone, cytomix (equimolar human TNFα, IL1β, and IFNγ in PBS) was added to PMVEC at a final concentration of 30 ng/mL (vs. an equal volume of PBS control). For PMN–PMVEC coculture experiments, PMN and PMVEC were individually stimulated with cytomix at 0.3 and 3 ng/mL vs. PBS control (Figure S1 in Supplementary Material). After 3 h, PMN were added to the corresponding PMVEC monolayer (PMN:PMVEC ratio = 10:1), and cocultured together with or without cytomix stimulation for another 2 h (Figure S1 in Supplementary Material). Isolated PMVEC were treated with caspase inhibitors (Q-VD, Ac-YVAD-CMK, Z-DEVD-FMK, Z-IETD-FMK, and Z-LEHD-FMK) for the entire duration (5 h) of the study, but for PMVEC–PMN coculture studies, caspase inhibitors were applied to PMVEC for the 3 h prior to coculture and then for the 2 h of coculture.

Assessment of PMVEC Barrier Function

PMVEC were seeded at a concentration of 10^4 cells/well on gelatin-coated 24-well cell-culture inserts (3.0 μm pore, VWR Scientific) in 10% EGM-2 as we have done previously (33). Once confluent, PMVEC monolayer permeability was assessed by (1) transendothelial electrical resistance (TEER; EVOM2 Endothelial Voltohmmeter; World Precision Instruments, Sarasota, FL, USA); (2) FITC-labeled dextran flux (FITC-dextran, 4 kDa); and (3) EB-labeled BSA flux (EB-BSA, 67 kDa). Trans-PMVEC macromolecular flux from the upper chamber into the lower chamber of the cell-culture inserts was measured over exactly 1 h (Figure S1 in Supplementary Material). Briefly, 50 μL of EB-BSA (containing 33.5 μg of EB) and FITC-dextran (125 μg) was added gently to the upper chamber (final volume 250 μL) of the cell-culture insert at 1 h before each indicated time point of assessment. At the same time, 150 μL of the same concentration of unlabeled BSA was added to the lower chamber. After exactly 1 h, inserts were removed and the media from the lower chamber collected. EB-BSA flux was determined by measuring absorbance (620 nm) and FITC-dextran flux was determined by measuring fluorescence (excitation 488 nm and emission 525 nm; Victor3 microplate reader, PerkinElmer, Woodbridge, ON, Canada). All leak measures (TEER, EB-BSA, and dextran) were normalized to a blank well containing only an insert with no PMVEC present.

Assessment of PMVEC Apoptosis

PMVEC apoptosis was detected by measuring caspase activation, loss of cell membrane polarization, and DNA fragmentation as we previously described (26). To detect caspase activation, PMVEC were stained with the FLICA Poly Caspase Assay Kit as per the manufacturer's instructions. For PMN–PMVEC coculture experiments, PMN were pre-labeled with calcein-AM to identify them during fluorescence microscopy. PMVEC were then fixed with methanol and Hoechst stain was used to identify nuclei. Cells were then imaged using fluorescent microscopy (FLICA excitation/emission: 550/590–600 nm; Hoechst excitation/emission: 361/486 nm). The number of FLICA and Hoechst positive cells per field of view was assessed through manual counting (two blinded reviewers) and automated counting using ImageJ (National Institutes of Health).

Loss of cell membrane polarization (as indicated by presence of cell surface phosphatidylserine) was assessed by staining PMVEC with FITC-conjugated Annexin V and propidium iodide (PI). Following stimulation with PBS or cytomix, the medium containing detached cells was collected, and the remaining attached cells lifted by Accutase. Both detached and attached PMVEC populations were pooled and stained with Annexin V and PI in binding buffer (0.1 M HEPES pH 7.4; 140 mM NaCl; 25 mM $CaCl_2$). PMVEC were then analyzed by flowcytometry (easyCyte Guava 12HT). Annexin V+/PI− cells were considered early-phase apoptotic cells, whereas Annexin V+/PI+ cells were considered dead cells and Annexin V−/PI− cells were considered live cells.

Late-stage apoptotic DNA fragmentation in PMVEC was examined by analysis of terminal deoxynucleotidyl TUNEL using the *In Situ* Cell Death Detection kit. For these studies, PMVEC were fixed in methanol following stimulation and then

permeabilized with a 0.1% Triton X-100 solution. Following permeabilization, TUNEL staining was used to identify PMVEC with DNA fragmentation and Hoechst stain was used to label all PMVEC nuclei. Cells were imaged and the number of TUNEL and Hoechst positive cells per field of view was determined as above. In addition, for all FLICA and TUNEL experiments, all detached cells were collected and stained as above, cytospun onto a slide, and images captured.

Statistics

Data are reported as mean ± SEM and were analyzed using GraphPad Prism 5. Differences between groups were assessed by *t*-tests (one measured variable) or by a two-way ANOVA with Bonferroni *post hoc* testing (two independent variables). Significance threshold was set at $\alpha = 0.05$ and experiments were replicated at least three times using three different primary human PMVEC. The correlation of septic PMVEC permeability (EB-BSA flux) with caspase activation (FLICA+ EC) was assessed by Pearson's method.

RESULTS

Effects of Septic (Cytomix) Treatment on Human PMVEC Barrier Function and Apoptosis

To examine the relationship between human PMVEC barrier dysfunction and apoptosis under septic conditions, we initially characterized the time course of septic (cytomix 30 ng/mL) stimulation-induced barrier dysfunction of human PMVEC monolayers cultured alone over 6 h. In our previous dose-response studies, this was the maximally effective cytomix dose that did not induce massive loss of PMVEC viability (22). PMVEC permeability was assessed by measuring trans-PMVEC ion movement using TEER. TEER was significantly reduced following cytomix stimulation of PMVEC compared to PBS-treated PMVEC, with the greatest decrease in TEER occurring by 5–6 h post-stimulation, indicating peak permeability (**Figure 1A**).

Based on the time course of septic changes in TEER, we subsequently assessed specifically at 5 h post-cytomix treatment changes in PMVEC permeability to macromolecules, of greater relevance to human sepsis and ARDS, including EB-BSA (67 kDa) and FITC-dextran (4 kDa). Septic PMVEC barrier dysfunction was confirmed by significant increases in both BSA and dextran flux at 5 h post-cytomix stimulation vs. PBS treatment (**Figure 1B**). Basal trans-PMVEC flux of the smaller dextran molecule was significantly greater than the flux of the larger BSA (1.92 ± 0.10% of total dextran loaded vs. 0.76 ± 0.04% of total BSA loaded, respectively, $p < 0.05$). Moreover, this higher dextran flux vs. BSA flux under basal conditions resulted in the relative cytomix-induced increase being greater for BSA than for dextran (777 ± 116% of PBS-treated vs. 454 ± 47% of PBS-treated for BSA and dextran, respectively, $p < 0.05$).

We next assessed the effects of septic treatment of human PMVEC on markers of PMVEC apoptosis. Caspase activation, one of the molecular features of apoptosis, was assessed using the fluorescent marker FLICA. We observed a significant increase in the number of FLICA+ PMVEC by 4 h post-cytomix vs. PBS, that remained similarly increased at 6 h (**Figures 2A,B**). Based on the time course of changes in caspase activation, we also assessed two other markers of PMVEC apoptosis specifically at 5 h post-cytomix treatment, including changes in PMVEC membrane polarization (Annexin V staining) and DNA fragmentation (TUNEL). We observed significant increases in both Annexin V+ (**Figures 2C,D**) and TUNEL+ (**Figures 2E,F**) PMVEC following cytomix stimulation vs. PBS.

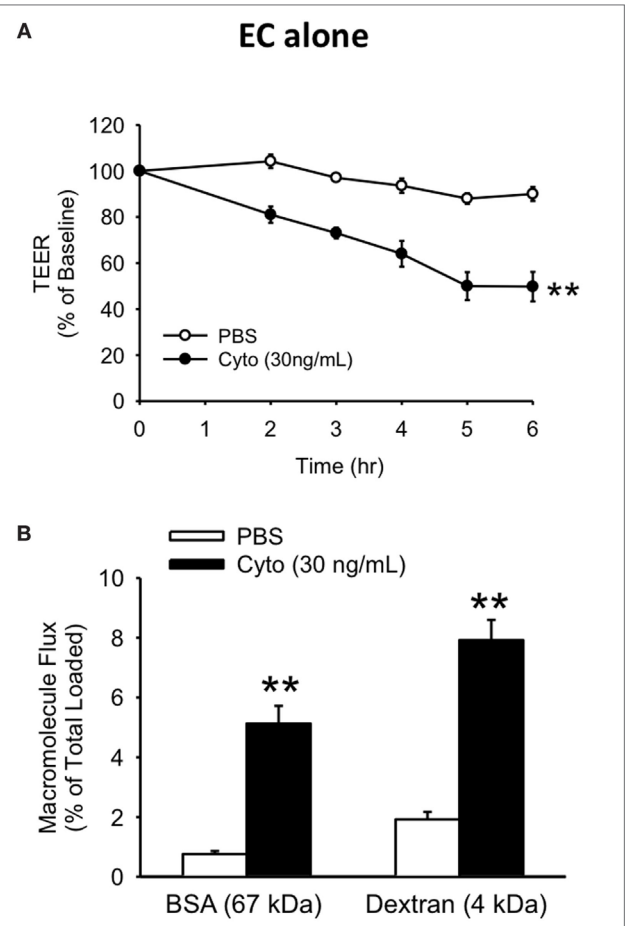

FIGURE 1 | Cytomix induces significant loss of human PMVEC barrier function. Cytomix-stimulated PMVEC cultured alone had significantly increased permeability that peaked by 5 h post-stimulation and persisted at 6 h post-stimulation vs. PBS by three assays: lower transendothelial electrical resistance (TEER) **(A)** and higher macromolecular flux including Evans blue-bovine serum albumin (BSA) and fluoroisothiocyanate-dextran **(B)**. $^{**}p < 0.01$ compared with respective PBS (repeated measures two-way ANOVA or *t*-test). $n = 6$. Note: for the sake of simplicity, the term EC refers specifically to PMVEC within all figures.

Effect of Caspase Inhibition on Cytomix-Induced Permeability and Apoptosis in Human PMVEC Cultured Alone

To determine the contribution of PMVEC apoptosis to septic PMVEC barrier dysfunction, human PMVEC were cultured in the presence or absence of Q-VD, a pan-caspase inhibitor

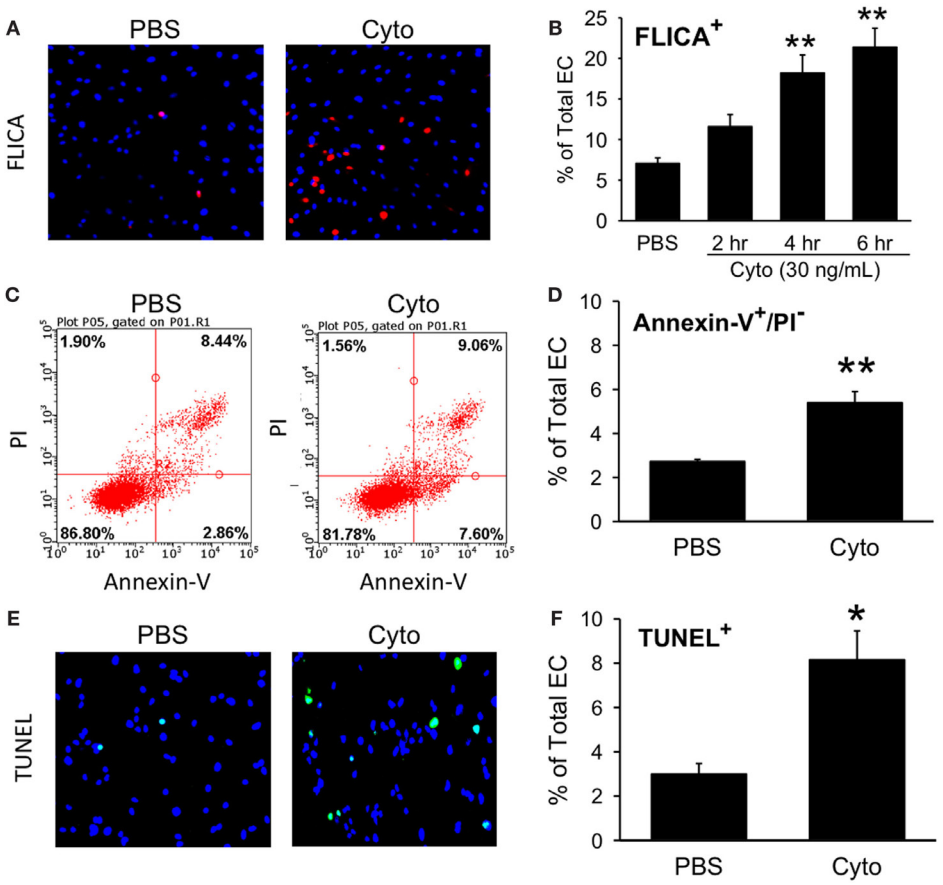

FIGURE 2 | Cytomix induces significant human PMVEC apoptosis. Cytomix stimulation of PMVEC cultured alone leads to an increased number of cells stained positive for **(A)** active caspases [fluorescent inhibitor of caspases (FLICA); red]; **(C)** loss of cell membrane polarization (Annexin V); and **(E)** DNA fragmentation [terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL); green]. Quantification revealed significant increases in FLICA+ cells **(B)** by 4 h post-cytomix that persisted at 6 h post-cytomix. The number of Annexin-V+/propidium iodide (PI)– cells **(D)** and TUNEL+ cells **(F)** were also significantly increased by 5 h post-cytomix. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with respective PBS-treated group (two-way ANOVA or *t*-test). $n = 4–7$.

previously found to inhibit PMVEC apoptosis (26). Treatment of cytomix-stimulated PMVEC with Q-VD led to a significant restoration of PMVEC barrier function. Specifically, Q-VD treatment slightly attenuated the septic fall in PMVEC TEER (**Figure 3A**), but dramatically blunted the septic increases in trans-PMVEC macromolecule flux of both EB-BSA and FITC-dextran, compared to vehicle-treated septic PMVEC (**Figures 3B,C**). Furthermore, treatment of PMVEC with Q-VD completely abrogated the cytomix-induced increases in Annexin V+ and TUNEL+ PMVEC vs. vehicle treatment (**Figures 3D,E**).

Effects of Septic (Cytomix) Treatment on Human PMVEC Barrier Function and Apoptosis in the Presence of PMN

We have previously shown that PMN contribute to septic PMVEC barrier dysfunction and apoptosis in murine sepsis *in vivo* (9). To assess a possible contribution of human PMN to septic human PMVEC barrier dysfunction and apoptosis, PMVEC were cocultured with PMN under basal (PBS) and septic (cytomix) conditions. Based on our previous studies, 30 ng/mL cytomix is an excessive stimulus for PMN–PMVEC cocultures, leading to massive loss of cell viability (22). As such, two lower cytomix doses were used for septic PMN–PMVEC coculture studies, low (0.3 ng/mL) and medium-dose (3 ng/mL) cytomix treatment. The presence of PMN slightly decreased PMVEC barrier function under basal conditions compared to PMVEC cultured alone, as indicated by significantly lower TEER (**Figure 4A**) and significantly higher EB-BSA and FITC-dextran flux (**Figures 4B,C**).

Although basal PMVEC TEER was lower in PMN–PMVEC coculture than in PMVEC alone, the presence of PMN had no effect on the septic decrease in PMVEC TEER vs. PMVEC cultured alone, at both low and medium cytomix doses (**Figure 4A**). By contrast, coculture of PMVEC with PMN significantly

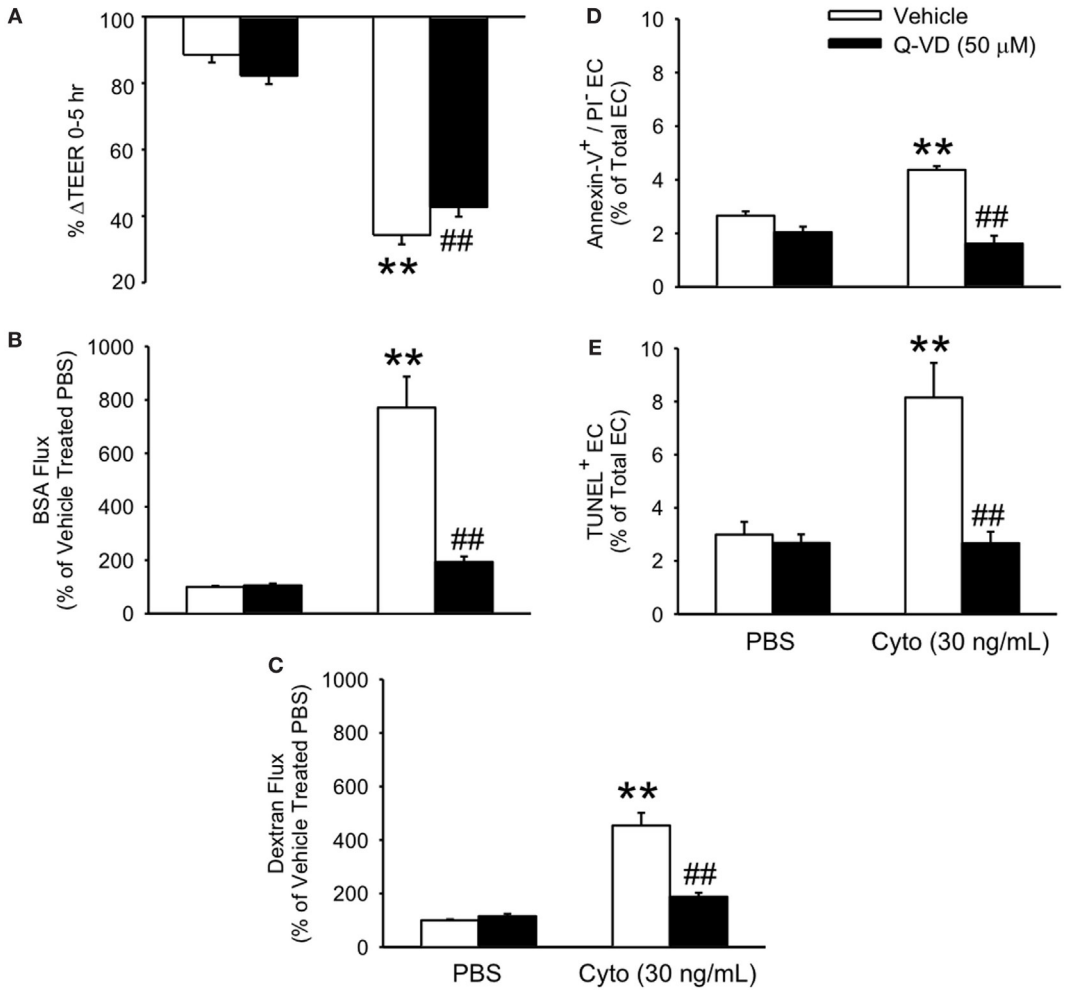

FIGURE 3 | Inhibition of caspase activity reduces septic human PMVEC hyper-permeability and the associated apoptosis. Inhibition of caspase activity (Q-VD, 50 μM) following cytomix stimulation of human PMVEC cultured alone modestly attenuated the septic decrease in transendothelial electrical resistance (TEER) **(A)** but more markedly blunted the septic increases in macromolecular flux, including Evans blue-albumin **(B)** and fluoroisothiocyanate-dextran **(C)** vs. vehicle treatment (dimethyl sulfoxide). Furthermore, treatment of human PMVEC cultured alone with Q-VD also completely prevented the septic increase in the number of Annexin-V+/propidium iodide (PI)– **(D)** and TUNEL+ **(E)** cells. ** or $^{\#\#}p < 0.01$ compared with cytomix or vehicle group, respectively (two-way ANOVA). $n = 4–6$.

enhanced septic PMVEC barrier dysfunction (increases in both EB-BSA and FITC-dextran flux) vs. PMVEC cultured alone, at both low and medium-dose cytomix (**Figures 4B,C**).

To assess the contribution of PMVEC apoptosis to PMN-enhanced septic PMVEC barrier dysfunction, markers of apoptosis were examined in PMVEC cocultured with PMN under basal and septic conditions. Under basal conditions, the presence of PMN had no effect on PMVEC apoptosis vs. PMVEC cultured alone, as evidenced by all three measures of apoptosis (FLICA, Annexin V, and TUNEL; **Figure 5**). In PMVEC cultured alone, medium-dose cytomix stimulation (3 ng/mL) induced septic PMVEC apoptosis, but not low-dose cytomix stimulation (0.3 ng/mL; **Figure 5**). Compared to cytomix-treated PMVEC alone, the presence of PMN-enhanced septic PMVEC FLICA staining (this achieved significance at a medium cytomix dose, 3 ng/mL; **Figure 5A**), but PMN presence had no effect on the septic increases in other markers of PMVEC apoptosis, including both Annexin V and TUNEL staining (**Figures 5B,C**).

For both PMVEC cultured alone and PMVEC cocultured with PMN, septic PMVEC barrier dysfunction, as reflected by increased EB-BSA flux, was strongly correlated with PMVEC caspase activation, as indicated by an increased number of FLICA+ PMVEC (**Figures 6A,B**). Although the percentage increase in trans-PMVEC flux of dextran was smaller, because of higher basal flux of the smaller dextran molecule, compressing the septic signal, there were also significant correlations between PMVEC

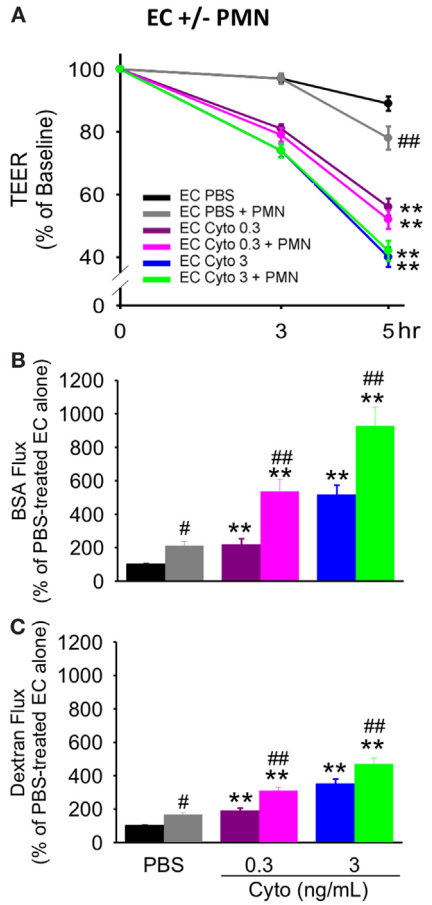

FIGURE 4 | PMN coculture with PMVEC significantly augments septic trans-PMVEC macromolecular flux. Compared to human PMVEC alone, coculture of PMVEC with PMN led to significantly increased leak under basal (PBS) conditions, as assessed by three measures: transendothelial electrical resistance (TEER) **(A)**, Evans blue (EB)-bovine serum albumin (BSA) **(B)**, and fluoroisothiocyanate (FITC)-dextran **(C)**. Cytomix treatment (0.3 and 3 ng/mL) of PMVEC cultured alone or in coculture with PMN increased permeability, including decreased TEER and increased EB-BSA and FITC-dextran flux. However, under septic conditions, PMN–PMVEC coculture only significantly augmented macromolecular flux **(B,C)** compared to PMVEC alone. $^{**}p < 0.01$ compared with respective PBS-treated group; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ for PMN + PMVEC compared with PMVEC alone (two-way ANOVA). $n = 6$.

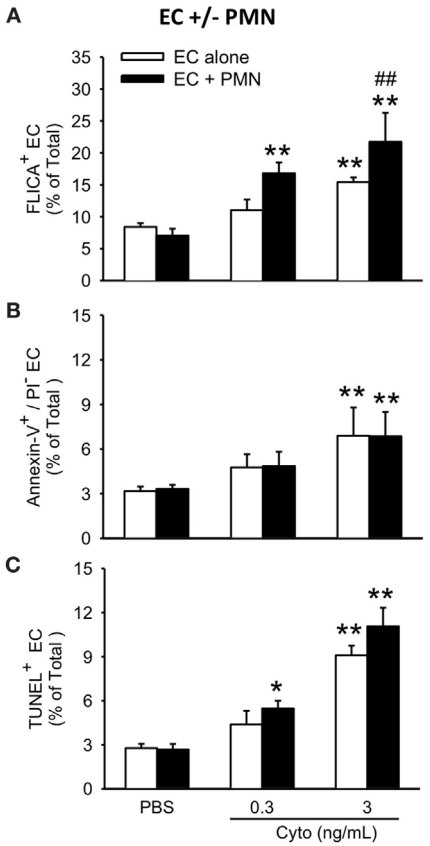

FIGURE 5 | PMN coculture with PMVEC increases septic caspase activation but does not augment septic PMVEC apoptosis. In isolated PMVEC, only moderate-dose cytomix (3 ng/mL), but not low-dose (0.3 ng/mL), increased the number of apoptotic PMVEC as indicated by three complementary measures: **(A)** FLICA+ cells, **(B)** Annexin-V+/propidium iodide (PI)– cells, and **(C)** TUNEL+ cells. Cytomix stimulation of PMN–PMVEC cocultures increased PMVEC caspase activation (FLICA+) at both low- and moderate-doses but had negligible effects on other markers of apoptosis (Annexin-V+/PI– and TUNEL+). $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with respective PBS-treated group; $^{\#\#}p < 0.01$ for PMN + PMVEC compared with PMVEC alone (two-way ANOVA). $n = 3$–4.

FLICA staining and trans-PMVEC dextran flux in both isolated PMVEC and in PMN–PMVEC coculture (data not shown).

Effect of Caspase Inhibition on Cytomix-Induced Permeability and Apoptosis in Human PMN–PMVEC-Coculture

To determine the contribution of caspase activation to PMN-enhanced septic PMVEC barrier dysfunction, human PMN–PMVEC cocultures were treated with Q-VD vs. dimethyl sulfoxide vehicle. Q-VD treatment of PMVEC cocultured with PMN led to a partial, but significant restoration of PMVEC barrier function (**Figure 7**). Specifically, treatment with Q-VD significantly attenuated the septic decrease in PMVEC TEER compared to vehicle-treated PMVEC (**Figure 7A**). Moreover, Q-VD treatment also significantly blunted the increased trans-PMVEC macromolecular flux across PMVEC cocultured with PMN under septic conditions, including both EB-BSA (**Figure 7B**) and FITC-dextran (**Figure 7C**). Furthermore, the cytomix-induced increase in trans-PMVEC macromolecular flux across PMVEC cultured alone or in coculture with PMN was associated with the formation of PMVEC intercellular gaps and treatment with Q-VD significantly blunted this gap formation (Figure S2 and Materials and Methods of Data Sheet 1 in Supplementary Material).

In addition to blunting cytomix-induced macromolecular flux, Q-VD treatment of PMVEC cocultured with PMN also significantly reduced the sepsis-induced increases in Annexin V+ and TUNEL+ PMVEC vs. vehicle-treated (**Figures 7D,E**). To assess the potential contribution of other cell death sub-types, such as necroptosis and necrosis, further analysis of Annexin V

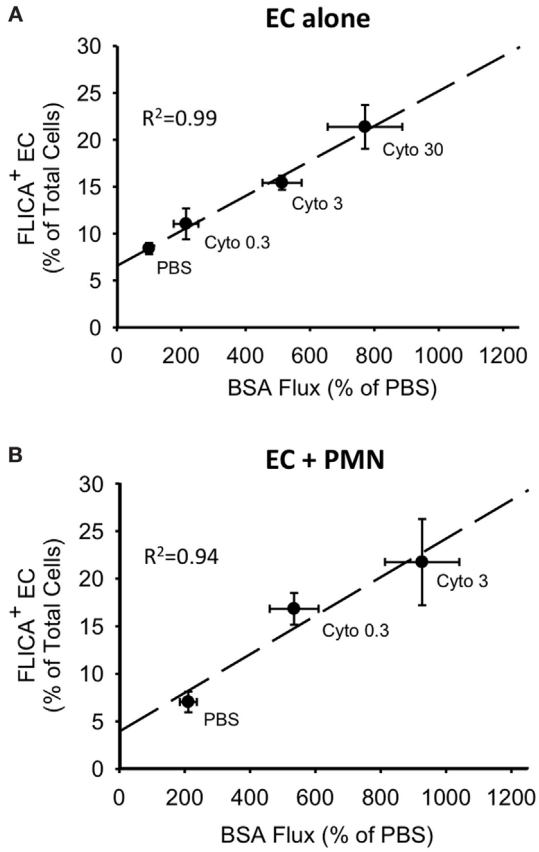

FIGURE 6 | Correlations between cytomix-induced increases in PMVEC caspase activity and trans-PMVEC macromolecular flux in isolated PMVEC and in PMN–PMVEC cocultures. Cytomix dose-dependently increased PMVEC macromolecular permeability [Evans blue-bovine serum albumin (BSA); *x*-axis], which was highly correlated with caspase activation (FLICA+ cells; *y*-axis) in PMVEC cultured alone **(A)** and in coculture with PMN **(B)**. Note: given the greater sensitivity of PMN–PMVEC cocultures **(B)** to cytomix stimulation, only low (0.3 ng/mL) and medium (3 ng/mL) doses of cytomix were used, compared to high dose (30 ng/mL) cytomix in PMVEC alone **(A)**. $n = 4$–6 for each dose.

and PI staining of PMVEC cultured alone or in coculture with PMN was performed. Cytomix stimulation or treatment with Q-VD had no effect on PI staining, including the percentage of Annexin V−/PI+ or Annexin V+/PI+ PMVEC (**Figure 8**; **Table 1**). Importantly, while the percentage of Annexin V−/PI+ PMVEC appeared to increase following Q-VD treatment of PMVEC stimulated with cytomix in coculture with PMN, these differences did not reach significance (**Figure 8**; **Table 1**).

To further define the role of caspase activity in septic PMVEC barrier dysfunction, human PMVEC cultured alone or in coculture with PMVEC were treated with inhibitors against specific caspases, including caspase 1, 3, 8, and 9. In PMVEC cultured alone, inhibition of caspase 3 and 8 significantly attenuated cytomix-induced trans-PMVEC EB-BSA flux while inhibition of caspase 1 and 9 had no significant impact (**Figure 9A**). Similarly, treatment with caspase 3 or 8 inhibitors of PMN–PMVEC cocultures significantly reduced cytomix-stimulated EB-BSA flux while inhibition of caspase 1 had no effect (**Figure 9B**). However, unlike PMVEC cultured alone (**Figure 9A**), treatment of PMVEC cocultured with PMN with a caspase 9 inhibitor significantly reduced cytomix-stimulated EB-BSA flux (**Figure 9B**).

DISCUSSION

In the present report, isolated human PMVEC were cultured either alone or in the presence of human PMN, under septic conditions induced by exposure to multiple sepsis-relevant pro-inflammatory cytokines, as an *in vitro* model of human sepsis. Septic stimulation of isolated human PMVEC resulted in significant barrier dysfunction associated with increased PMVEC presence of three markers of apoptosis, including FLICA (caspase activation), Annexin V (cell surface phosphatidyl serine), and TUNEL (DNA fragmentation) staining. Pan-caspase inhibition with the most potent chemical inhibitor available, Q-VD, in isolated PMVEC expectedly prevented septic PMVEC apoptosis (as evidenced by reduced Annexin V and TUNEL staining), and markedly blunted the septic increase in trans-PMVEC macromolecule flux (both BSA and dextran), but only slightly attenuated the septic decrease in TEER. When human PMN were cocultured with human PMVEC, septic PMVEC barrier dysfunction was exacerbated, and this was associated with greater septic PMVEC caspase activation (FLICA staining). By contrast, PMN-dependent septic PMVEC barrier dysfunction was not associated with any greater degree of septic PMVEC apoptosis (as assessed by both Annexin V and TUNEL staining). Pan-caspase inhibition in PMN–PMVEC cocultures using Q-VD markedly attenuated PMN-dependent septic increases in trans-PMVEC macromolecule flux (both BSA and dextran) but had no effect on septic decreases in TEER. Moreover, inhibition of caspase 3, 8, or 9 in PMN–PMVEC cocultures significantly reduced septic increases in trans-PMVEC EB-BSA flux whereas inhibition of caspase 1 had no effect.

The new definition of human sepsis incorporates the critical idea of septic organ dysfunction (34, 35), which is a primary determinant of the severity of sepsis, and especially of clinical outcomes such as mortality (4). Of all the organs, septic injury of the lung, termed ARDS, is the most common and the most serious in terms of adverse clinical outcomes (36). Organ dysfunction in human sepsis can be related to the actual infection and its dissemination, to the resulting systemic inflammation, and importantly, to organ-specific microvascular dysfunction and injury (11). Septic microvascular dysfunction is clinically relevant, as it is associated with more severe sepsis, greater organ dysfunction, and increased mortality in human sepsis (13, 14, 37, 38). Septic microvascular dysfunction is largely driven by activation and injury specifically of MVEC. For example, increased circulating numbers of EC and higher levels of soluble markers of EC activation/damage [e.g., intercellular adhesion molecule 1, von Willebrand factor, vascular endothelial (VE) growth factor receptor 1] correlate with more severe sepsis and higher mortality (39–45).

Multiple initiating mechanisms for MVEC activation and injury leading to loss of MVEC barrier function in sepsis have been postulated. These include the actions of cytokines

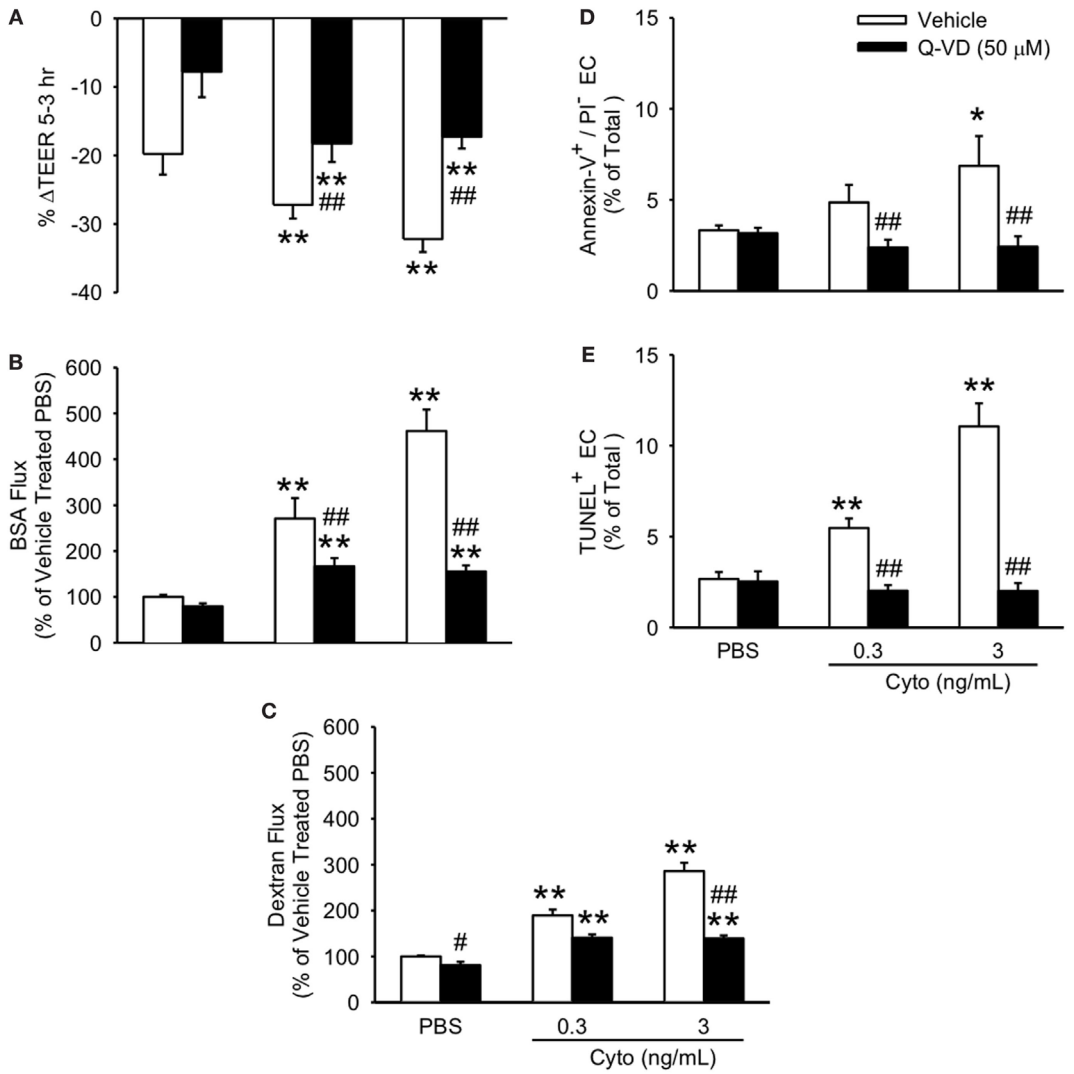

FIGURE 7 | Inhibition of caspase activity in human PMVEC cocultured with PMN reduces septic hyper-permeability and apoptosis. Treatment of PMN–PMVEC cocultures with Q-VD (50 μM) significantly attenuated the septic decreases in transendothelial electrical resistance (TEER) **(A)** as well as the septic increases in macromolecular flux, including Evans blue-bovine serum albumin (BSA) **(B)** and fluoroisothiocyanate-dextran **(C)** vs. vehicle treatment (dimethyl sulfoxide). Furthermore, Q-VD treatment of PMVEC cultured with PMN also significantly blunted the septic increase in the number of Annexin-V+/propidium iodide (PI)– **(D)** and TUNEL+ **(E)** cells. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with respective PBS-treated group; $^{\#\#}p < 0.01$ compared with respective vehicle-treated group (two-way ANOVA). $n = 3$–6.

and other soluble circulating molecules on MVEC, mechanical interaction of activated leukocytes and platelets with MVEC, and paracrine exposure to injurious molecules released by these circulating cells (8, 16–19, 46). Furthermore, septic MVEC barrier dysfunction and increased paracellular protein-fluid leak can also result from actin cytoskeleton-driven MVEC retraction, signaling-activated cleavage, and/or internalization of cell–cell junctional proteins (e.g., VE-cadherin), which may be associated with altered expression/function of cytosolic adaptor proteins (e.g., β-catenin), or MVEC loss due to cell death and/or disruption of MVEC–matrix interactions (47, 48). The predominant mechanisms of septic PMVEC injury and dysfunction, of greatest direct relevance to human sepsis and ARDS, remain uncertain.

In the current study, we examined septic responses in human PMVEC, because of the clinically important and prognostic role of septic lung dysfunction leading to ARDS in septic humans, the central role of the pulmonary microvasculature

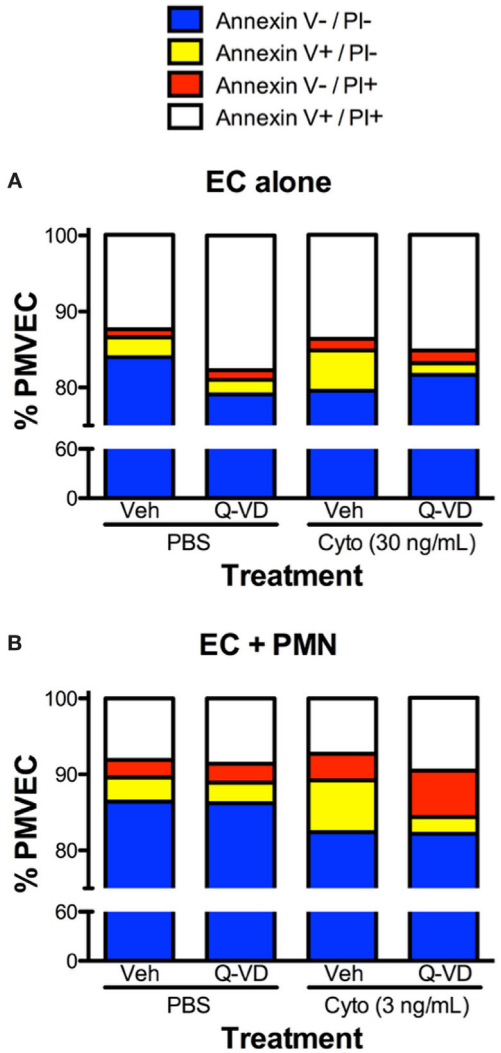

FIGURE 8 | Effects of cytomix treatment of human PMVEC on different types of cell death. **(A)** Cytomix (30 ng/mL) treatment increased Annexin V+/propidium iodide (PI)– PMVEC (yellow bar; suggestive of early apoptosis), but had no effect on PI staining, including specifically both Annexin V–/PI+ cells (red bar; indicative of pyroptosis) and Annexin V+/PI+ cells (white bar; later stage apoptosis and necrosis/necroptosis). The cytomix-induced increase in Annexin V staining was completely abrogated by Q-VD treatment. **(B)** Similarly, in PMN–PMVEC cocultures, cytomix treatment (3 ng/mL) increased Annexin V+/PI– PMVEC (yellow bar) and was not associated with any significant change in Annexin V–/PI+ (red bar) and Annexin V+/PI+ (white bar) PMVEC vs. PBS. The cytomix-induced increase in Annexin V+/PI– PMVEC was completely inhibited by Q-VD treatment. Complete data and variance are provided in **Table 1**.

TABLE 1 | Effects of cytomix and Q-VD treatment on different types of cell death in isolated human PMVEC and in PMN–PMVEC cocultures.

Group/treatment	Annexin V–/ propidium iodide (PI)– (%)	Annexin V+/PI– (%)	Annexin V–/PI+ (%)	Annexin V+/ PI+ (%)
EC alone				
PBS	84.0 ± 2.3	2.6 ± 0.1	1.1 ± 0.3	12.4 ± 2.2
PBS + Q-VD	79.1 ± 1.6	1.9 ± 0.2	1.3 ± 0.2	17.7 ± 1.8
Cytomix (30 ng/mL)	79.6 ± 1.6	5.3 ± 0.5**	1.5 ± 0.3	13.7 ± 1.8
Cytomix + Q-VD	81.7 ± 1.7	1.5 ± 0.2##	1.7 ± 0.2	15.2 ± 1.5
EC + PMN				
PBS	86.4 ± 0.4	3.2 ± 0.2	2.3 ± 0.2	8.1 ± 0.5
PBS + Q-VD	86.2 ± 1.0	2.7 ± 0.1	2.5 ± 0.6	8.6 ± 1.0
Cytomix (3 ng/mL)	82.4 ± 0.5	6.8 ± 1.4*	3.5 ± 0.2	7.3 ± 1.8
Cytomix + Q-VD	82.2 ± 1.9	2.2 ± 0.6##	6.1 ± 1.8	9.6 ± 1.1

One-way ANOVA with Tukey HSD post hoc test for each column.
**p < 0.05 and **p < 0.01 for cytomix vs. respective PBS.*
##p < 0.01 for Q-VD-treated vs. cytomix.
n = 3–6.

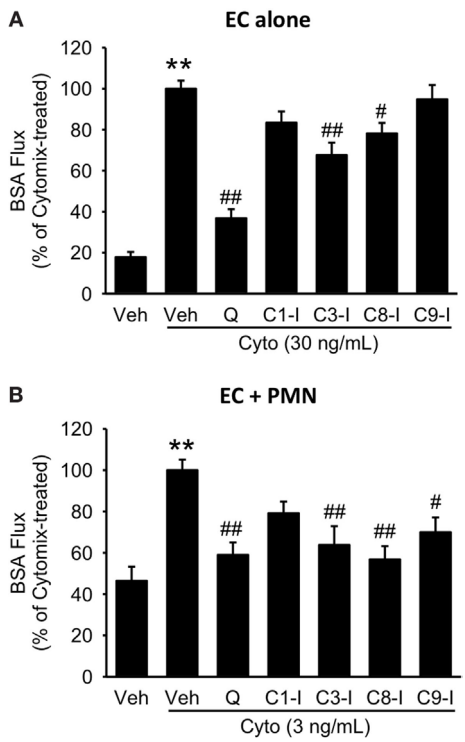

FIGURE 9 | Effect of pan-caspase vs. selective caspase inhibition on septic hyper-permeability in human PMVEC alone or cocultured with PMN. **(A)** In isolated PMVEC, pan-caspase inhibition with Q-VD treatment markedly inhibited cytomix-induced trans-PMVEC bovine serum albumin (BSA) flux. Inhibition of caspase3 (C3-I) and caspase8 (C8-I) had a similar but smaller inhibitory effect on septic PMVEC hyper-permeability, but inhibition of caspase1 (C1-I) or caspase9 (C9-I) had no effect. **(B)** In PMN–PMVEC cocultures, pan-caspase inhibition with Q-VD almost completely inhibited septic PMVEC hyper-permeability, with similar effects of individual caspase3, caspase8, and caspase9 inhibition, and no effect on septic PMVEC hyper-permeability of inhibition of caspase1. $^{**}p < 0.01$ for cytomix vs. non-cytomix treatment; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ for caspase inhibitor treatments vs. cytomix alone (one-way ANOVA). $n = 7–8$.

in sepsis-associated ARDS, and the direct human relevance of such studies. Specifically, we assessed mechanisms driving septic PMVEC barrier dysfunction under two conditions, the presence or absence of PMN. In isolated PMVEC alone, the loss of PMVEC barrier function is strongly associated with increased PMVEC apoptosis and is caspase-dependent as inhibition of caspases through Q-VD treatment prevented

septic PMVEC apoptosis and restored PMVEC barrier function. By contrast, the presence of PMN greatly enhanced septic human PMVEC barrier dysfunction/leak and increased septic PMVEC caspase activation but did not induce any greater degree of septic PMVEC apoptosis compared to isolated PMVEC alone. To date, there have been no studies specifically using human PMVEC in the presence of PMN to assess the connection between septic PMVEC apoptosis and barrier dysfunction. Thus, our comprehensive examination of human septic PMVEC barrier dysfunction in isolated PMVEC and in PMVEC cultured in the presence of PMN clearly establishes for the first time the contributions of apoptosis vs. caspase activation. Collectively, our study suggests that early septic cytomix-induced human PMVEC barrier dysfunction in the presence of PMN is caspase-dependent but is not mediated through PMVEC apoptosis.

The contribution of apoptosis to septic injury and dysfunction of isolated PMVEC *in vitro* has been uncertain. Specifically, there are conflicting data, based on studies examining barrier function in EC from many species and often EC types that are genotypically and phenotypically very distinct from PMVEC and thus of questionable direct relevance to septic lung biology; these include macrovascular pulmonary artery endothelial cells (PAEC) and EC from systemic vascular beds, including macrovascular (e.g., HUVEC) and microvascular (e.g., brain MVEC) (27–31). Most relevant to the present study, EC from different vascular beds exhibit markedly heterogeneous responses to inflammatory environments, especially with respect to the association of apoptosis with increased trans-EC permeability (26–31). Moreover, many studies of EC apoptosis inadequately characterize apoptosis using only one method (e.g., caspase activation or TUNEL) (23, 24, 26).

Death of PMVEC, possibly *via* apoptosis, has been presumed to play a central role in human ARDS, both in the setting of systemic disease like sepsis, but also in direct lung injury (e.g., pneumonia, acid aspiration) (49). There is more evidence to support PMVEC death and specifically apoptosis in various animal models of sepsis and resulting lung injury. We recently reported that PMVEC apoptosis appears to contribute to PMVEC injury and dysfunction in murine cecal ligation/perforation (CLP)-induced sepsis *in vivo* (9, 25). For example, septic pulmonary microvascular barrier dysfunction *in vivo* was temporally associated with and highly correlated with enhanced PMVEC apoptosis, and moreover, inhibition of apoptosis *in vivo* following treatment with Q-VD (a synthetic pan-caspase inhibitor), significantly reduced septic pulmonary microvascular permeability (9, 25). Studies in murine CLP-sepsis by other groups have also demonstrated that inhibition of apoptosis through treatment with siRNA against caspases or FAS-associated death domain rescues septic EC dysfunction, including reducing septic hyper-permeability (46, 50, 51).

Other sub-types of cell death, such as pyroptosis or necrosis, may also be involved in the septic PMVEC barrier dysfunction. Importantly, each cell death sub-type is associated with a specific set of molecular markers (23, 24). For example, both necrosis and necroptosis are thought to be caspase-independent and associated with a loss of cell membrane integrity (i.e., necrotic cells stain positive with PI) (23, 24). Our observation that the increased in PMVEC barrier dysfunction under septic conditions is dependent on caspase activity as well as the apparent lack of any changes in the percentage of PI+ PMVEC under septic conditions or following treatment with Q-VD suggests that necrosis and necroptosis are likely not involved in septic PMVEC barrier dysfunction (23, 24). Pyroptosis is known to be dependent on caspase 1 activity and is also thought to be associated with the formation of pores in the cell membrane (23, 24). Our data found a lack of any significant effect of caspase 1 inhibition on cytomix-stimulated PMVEC barrier dysfunction as well as a lack of changes in the percentage of Annexin V−/PI+ PMVEC under any conditions. Together, this suggests that PMVEC are not undergoing pyroptosis in our model of septic PMVEC barrier dysfunction.

Measurements of TEER and macromolecular permeability are known to reflect different aspects of EC barrier function, and not surprisingly, may be differentially regulated under various inflammatory conditions resulting in barrier dysfunction (52). Our data suggests that while apoptosis occurs in isolated human PMVEC under septic conditions *in vitro* and may be associated with cytomix-induced trans-PMVEC macromolecular flux, it does not appear to be associated with changes in TEER. For example, acute cytomix-induced PMVEC barrier dysfunction was consistent between TEER and both FITC-dextran and EB-albumin techniques. However, this acute septic cytokine-induced PMVEC barrier dysfunction was differentially affected by Q-VD pan-caspase inhibition, which rescued the increased septic macromolecular flux but not septic decreases in TEER. Thus, acute septic trans-PMVEC macromolecular flux appears to be caspase-dependent but septic changes in TEER are not, which is supported by our previous studies using mouse PMVEC (26). Other groups have also reported divergent TEER and macromolecule-leak measurements in studies with PAEC and corneal EC, and have suggested that TEER is inadequately sensitive to biologically important changes in EC barrier function (30, 31). Moreover, the most important feature of PMVEC barrier dysfunction clinically is the increased pulmonary leak of plasma macromolecules, especially albumin accompanied by fluid and resulting in the typical lung edema that characterizes the severe often refractory hypoxemic respiratory failure of septic ARDS (53, 54). The measurement of EB-BSA flux specifically assesses trans-PMVEC permeability to albumin, and is a marker of both paracellular and transcellular permeability pathways (55).

Caspases are multi-functional proteases and while most studies focus on their function in apoptosis, there is clear literature evidence supporting their function in several fundamental cellular processes other than apoptosis (56). Of relevance to this study, multiple caspases have been linked to cleavage and subsequent degradation of the adherens junction adaptor protein β-catenin, including caspases 3, 6, and 8 (57). Furthermore, caspase 3 has also been found to cleave γ-catenin (58). Consequently, it is possible that caspases may regulate PMVEC barrier function through controlling the formation and stability of cell–cell junctions *via* cleavage and processing of critical adapter proteins, such as β-catenin.

PMN have previously been linked to septic PMVEC permeability and this function for PMN was found to be dependent on inducible nitric oxide synthase (iNOS) as the removal of PMN or iNOS deficiency/inhibition restored PMVEC barrier function (9, 32). Thus, it is likely that the mechanism through which PMN mediate PMVEC barrier function is dependent on oxidant/nitrosative stress. Oxidant stress has previously been linked to the activation of caspases, although this function has generally been assumed to lead to apoptosis. Specifically, oxidant stress leads to serial activation of BAX/BAK, mitochondria, and subsequently caspase 9 (59). Moreover, our current data that caspase 9 inhibition in PMVEC cocultured with PMN significantly attenuated cytomix-stimulated permeability but had no effect in PMVEC cultured alone supports this potential function for PMN-induced oxidant stress. Oxidant stress also activates calpains, a family of Ca^{2+}-dependent, cytoplasmic cysteine proteases (60). Among several calpain isoforms, the most important and ubiquitously expressed in murine and human cells is calpain1 (μ-calpain). Calpains can cleave many peptide targets, including caspase 3 (60). Thus, it is very plausible that PMN-dependent oxidant stress may result in increased caspase activation and loss of PMVEC barrier function.

Another putative mechanism of microvascular barrier dysfunction is through disruption of MVEC-–extracellular matrix (ECM) interactions resulting in increased MVEC detachment (61). For example, LPS-induced PAEC detachment has been found to be associated with caspase-dependent cleavage of α- and β-catenin as well as focal adhesion kinase, highlighting the ability of caspases to regulate not just inter-EC junctions, but also EC–ECM interactions (62, 63). Interestingly, while caspase inhibition prevented degradation of proteins involved in EC–ECM interactions and rescued EC detachment, it did not prevent LPS-induced disruption of inter-EC junctions or rescue LPS-induced leak (62).

It is likely that there are multiple mechanisms mediating septic EC barrier dysfunction, depending on EC species and type, septic conditions, and timing. For example, stimulation of macrovascular PAEC with a single cytokine (TNFα) resulted in apoptosis as early as 4 h post-stimulation that persisted at 20 h (30). While this apoptosis was also associated with increased permeability across the PAEC monolayer, treatment with Z-VAD did not rescue the enhanced permeability at any time point (30). Furthermore, TNFα has been found to drive loss of corneal EC barrier function through activation of p38 mitogen-activated protein kinase and subsequent disassembly of microtubules, as well as adherens and tight junctions (31). In addition, examination of barrier function in mouse renal MVEC following stimulation with TNFα demonstrated that increased permeability to albumin was associated with altered actin cytoskeleton, as well as formation of gaps between previously confluent cells and a loss of tight junctions and the EC glycocalyx (64). In both of these studies, inhibition of caspase activity (i.e., treatment with caspase inhibitors) had no effect on EC barrier dysfunction (62, 64).

We recognize that our study has limitations. For example, our *in vitro* model of human septic ARDS employed PMVEC cultured alone or with PMN vs. the many different cell types normally present in the lung *in vivo*, such as pericytes and multiple types of circulating inflammatory cells, which interact with PMVEC. Moreover, PMVEC reside *in vivo* on a complete interstitial ECM, and there is an extensive glycocalyx on the surface of the PMVEC, all of which are missing or limited in the *in vitro* setting (65–69). Furthermore, our *in vitro* model employed stimulation with a mixture of three sepsis-relevant cytokines, which is still a less robust septic stimulus than EC would face *in vivo* (e.g., bacterial products such as LPS), as well as the potentially injurious effects of shear stresses associated with blood flow *in vivo*. However, use of this simplified *in vitro* model, as well as the comprehensive assessment of apoptosis (use of three different markers) and PMVEC permeability (use of three complementary measures) allowed for the examination of the function of specifically PMVEC over a comprehensive time course, and thereby the identification of potentially novel mechanisms mediating septic human PMVEC barrier dysfunction.

In addition, the coculture model means that inhibitors, e.g., Q-VD, may have a direct effect on PMN. However, while caspase inhibition has been found to inhibit neutrophil apoptosis and promote neutrophil survival (70), it should be noted that PMN were not exposed to inhibitors (e.g., Q-VD) for the first 3 h of incubation, but only for the final 2 h in cocultures. Moreover, prolonged PMN survival has been shown to increase EC injury and barrier dysfunction (71–73), which was not what we observed in Q-VD treated PMN–PMVEC cocultures. We also recognize that the small $n = 3$ we used to perform our linear correlation analysis could be considered a limitation as it challenges assumptions of normality and homoscedascity. However, we believe our correlation analysis is clearly valid, as it is solidly supported in the literature to use such small *n*'s in order to calculate "*p*" values (74).

In conclusion, our current data suggest for the first time using human PMVEC cultured in the presence or absence of PMN that septic PMVEC barrier dysfunction in the absence of PMN is dependent on apoptosis and caspase activity. However, septic PMVEC barrier dysfunction in the presence of PMN appears to be independent of apoptosis, but very much still caspase-dependent. Future work will pursue the mechanisms of caspase-dependent human PMVEC barrier dysfunction in order to identify potential new therapeutic targets to prevent and treat human septic PMVEC dysfunction in ARDS, and potentially other systemic septic organ dysfunction.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Western University Health Sciences Research Ethics Board with written informed consent from all healthy blood donors. All healthy blood volunteers gave written informed consent in accordance with the Declaration of Helsinki. Discarded lung tissue samples were obtained from the pathology department following removal of all clinical identifiers, and as such, the institutional review board waived the need for written informed consent from lung tissue donors. The protocol was approved by

the Western University Health Sciences Research Ethics Board (Protocol #10536E).

AUTHOR CONTRIBUTIONS

LW, YA, SW, and MP performed experiments; LW, SM, YA, SW, MP, and SG analyzed data, interpreted results of experiments, and approved final version of manuscript; LW and SG prepared figures; LW, SM, and SG drafted, edited, and revised manuscript; SM and SG conception and design of research.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.01743/full#supplementary-material.

FIGURE S1 | Schematic of neutrophil (PMN)–pulmonary microvascular endothelial cell (PMVEC) coculture and septic stimulation. PMN and PMVEC were individually stimulated with cytomix (equimolar human tumor necrosis factor α, interleukin 1β, and interferon γ in PBS) at 0.3 and 3 ng/mL vs. PBS control. After 3 h, PMN were added to the corresponding PMVEC monolayer (PMN:PMVEC ratio = 10:1), and cocultured together with or without cytomix stimulation for another 2 h. EC barrier function was assessed by measuring transendothelial electrical resistance (TEER) at 0, 3, and 5 h. To examine trans-PMVEC macromolecular flux, Evans blue (EB)-labeled bovine serum albumin (BSA; 67 kDa) and fluoroisothiocyanate (FITC)-labeled dextran (4 kDa) were added to the apical aspect of the PMVEC (upper chamber of the transwell) at 5 h post-cytomix. The transwell insert was removed at 6 h post-cytomix and the amount of EB-BSA and FITC-dextran in the lower chamber assessed. Note: for the sake of simplicity, the term EC refers specifically to PMVEC within all figures.

FIGURE S2 | Cytomix treatment of human PMVEC induces caspase-dependent intercellular gap formation. **(A)** Cytomix-treated isolated PMVEC had obvious intercellular gaps (arrows) at 5 h vs. PBS, and the gap area in each image was quantified as a percentage of the total PMVEC monolayer area, in both isolated PMVEC [**(B)** EC alone] and for PMN–PMVEC coculture [**(C)** EC + PMN]. The cytomix-induced increase in PMVEC intercellular gaps was completely prevented by pan-caspase inhibition in PMVEC pre-treated with Q-VD **(B,C)**. $N = 3$–4/group. $^{**}p < 0.01$ for cytomix vs. respective PBS; $^{\#\#}p < 0.01$ for Q-VD vs. cytomix alone.

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Endothelial Dysfunction in Kidney Transplantation

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Kidney transplantation entails a high likelihood of endothelial injury. The endothelium is a target of choice for injury by ischemia-reperfusion, alloantibodies, and autoantibodies. A certain degree of ischemia-reperfusion injury inevitably occurs in the immediate post-transplant setting and can manifest as delayed graft function. Acute rejection episodes, whether T-cell or antibody-mediated, can involve the graft micro- and macrovasculature, leading to endothelial injury and adverse long-term consequences on graft function and survival. In turn, caspase-3 activation in injured and dying endothelial cells favors the release of extracellular vesicles (apoptotic bodies and apoptotic exosome-like vesicles) that further enhance autoantibody production, complement deposition, and microvascular rarefaction. In this review, we present the evidence for endothelial injury, its causes and long-term consequences on graft outcomes in the field of kidney transplantation.

Keywords: kidney transplantation, endothelial injury, apoptosis, necroptosis, alloantibodies, autoantibodies

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INTRODUCTION

The endothelium plays an important role in vascular biology and regulation of renal function. Healthy endothelial cells are involved in vasodilation through nitric oxide (NO) release, which also inhibits platelet adhesion and aggregation, as well as leukocyte adhesion. Conversely, injured endothelial cells can develop a vasoconstrictive, pro-inflammatory, and procoagulant phenotype. Endothelial dysfunction is associated with traditional cardiovascular risk factors such as hypertension and diabetes, and it predicts atherosclerosis progression and cardiovascular events in the general population (1, 2). A large body of data shows that chronic kidney disease (CKD) is associated with endothelial dysfunction and/or apoptosis (3–7). Increased levels of circulating microparticles from apoptotic endothelial cells have been observed in patients with CKD (5, 6). Uremic solutes foster the production of these microparticles by endothelial cells (6), which in turn decrease NO release and impair endothelium-mediated dilation (5).

Kidney transplantation is the best mode of renal replacement therapy, improving both quality of life and life expectancy compared to dialysis (8, 9). Kidney transplantation restores renal function and improves endothelial function compared to dialysis (10, 11). Nevertheless, kidney transplantation entails a high likelihood of endothelial injury in the allograft. Given its intimate contact with the blood, the allograft endothelium is a target of choice for interactions with circulating inflammatory cells, cytokines, antibodies, and circulating pharmacological agents. First, a certain degree of ischemia-reperfusion injury (IRI) inevitably occurs in the immediate posttransplant setting and manifests as delayed graft function (DGF). IRI is associated with both tubular and endothelial damage, especially in the peritubular capillary network. Second, acute rejection episodes, whether T-cell or antibody-mediated, occur in 15–20% of kidney transplant recipients (2) and can involve the graft micro- and macrovasculature, leading to endothelial injury. This can alter renal blood flow

and impair renal function, both acutely and on the long-term, favoring renal fibrosis and loss of renal function.

Last, the most commonly used immunosuppressive agents may have divergent impact on the graft endothelium after transplantation. Mycophenolic acid may protect the endothelium, but calcineurin inhibitors have an adverse impact on endothelial function and glucocorticoids can worsen endothelial function under physiological conditions and improve it in the presence of inflammation. While these topics are reviewed elsewhere (12–14), here we present the evidence for allograft endothelial injury that is associated with IRI, alloimmunity, and autoimmunity in kidney transplantation and describe its long-term consequences on graft outcomes.

IRI INDUCES ENDOTHELIAL DAMAGE, MICROVASCULAR RAREFACTION AND ADVERSE GRAFT OUTCOMES

The kidney transplant procedure is inevitably associated with a certain degree of IRI. Donor type (deceased after cardiocirculatory arrest and neurologically deceased versus living) and length of cold and warm ischemic times are important risk factors for IRI (15). Clinically significant IRI manifests as DGF, or acute kidney injury (AKI) in the immediate posttransplant period. DGF is defined as the need for hemodialysis in the first week posttransplantation or failure of serum creatinine to decrease by more than 10% on the first three postoperative days, although other definitions have been used (16). Episodes of AKI are strong predictors of CKD in the general population (17–20). Similarly, DGF is associated with decreased long-term kidney graft survival (15, 21).

In the past decade, microvascular injury and endothelial dysfunction have emerged as pivotal elements in the pathogenesis of AKI (22, 23). In experimental models of IRI, renal perfusion in peritubular capillaries is compromised within minutes of unclamping (24). Endothelial dysfunction/injury and apoptosis compromise microcirculatory renal blood flow through decreased vasodilatory capacity, coagulation activation and the formation of microvascular thrombi, and increased rolling/adhesion of inflammatory cells (23, 25).

Because the regenerative capacity of endothelial cells in peritubular capillaries appears limited (26–28), microvascular damage occurring during an episode of AKI can lead to permanent peritubular capillary rarefaction (26–28). Loss of peritubular capillaries favors chronic hypoxia, leading to overexpression of hypoxia inducible factor 1 α (HIF-1α), favoring transcription of fibrogenic genes such as transforming growth factor β (TGF-β) and connective tissue growth factor (CTGF). It also favors accumulation of α-smooth muscle actin (α-SMA) positive myofibroblasts and production of fibrogenic mediators (22, 23, 28–31).

These phenomena eventually lead to progressive interstitial fibrosis/tubular atrophy and renal dysfunction in animal models and in human AKI (31, 32). In kidney transplant patients, peritubular capillary loss, assessed by comparing capillary density on 3-month posttransplant biopsies with capillary density on preimplantation biopsies, is strongly associated with interstitial fibrosis/tubular atrophy and graft dysfunction 1 year posttransplant (33). Recent animal studies using *in vivo* imaging and electron microscopy in murine models of AKI demonstrated a tight correlation between peritubular capillary injury, rarefaction, and renal fibrosis (34, 35). Ultrastructural changes to peritubular capillaries include focal widening of the subendothelial space, higher numbers of endothelial vacuoles, reduced numbers of fenestrations, and increased thickness of the basement membrane (35). Human kidney biopsy samples with progressive renal fibrosis showed strikingly similar ultrastructural findings. Taken together, these studies support the concept that IRI-associated AKI can lead to microvascular rarefaction which in turn plays a pivotal role in favoring interstitial fibrosis and long-term renal dysfunction in patients with native kidney disease and in kidney transplant recipients.

Kidneys from older donors are more susceptible to IRI and more likely to develop DGF (36–39). Increasing age and the presence of age-associated disorders, such as hypertension and type 2 diabetes, favor the accumulation of senescent cells within the vasculature and the kidney. Senescence is characterized by proliferative arrest, cell flattening and enlargement, and the production of an array of pro-inflammatory cytokines (IL-1α, IL-1β, IL-6, IL-8, matrix metalloproteiases, CTGF) known as senescence associated secretory phenotype (40). Senescent cells lack replicative potential and hence tissues with higher levels of senescent cells display lower repair capacity in the face of injury. Increased microvascular rarefaction and enhanced fibrosis have been observed following IRI in rodent models and in transplant patients (41, 42).

IMMUNE-MEDIATED VASCULAR AND ENDOTHELIAL INJURY IS ASSOCIATED WITH ADVERSE KIDNEY GRAFT OUTCOMES

Acute rejection episodes occur in 15–20% of kidney transplant recipients (2). T-cell mediated rejections that involve the tubulointerstitial compartment are responsive to corticosteroid therapy and are reversible in a majority of cases. However, vascular involvement by the rejection process, also termed graft endarteritis, is an important risk factor for decreased long-term graft survival (43, 44). Endarteritis has classically been regarded as a T-cell-mediated phenomenon (45), with both alloreactive CD8+ and CD4+ T-cells infiltrating the allograft small-sized arteries (46). However, mounting evidence shows that endarteritis often clusters with microvascular inflammation (glomerulitis, peritubular capillaritis) and antibody-mediated damage (47). The deleterious impact of donor-specific alloantibodies (DSA) is illustrated by recent data showing that antibody-mediated rejection with endarteritis entails a worse prognosis than cell-mediated endarteritis alone (44). DSA can target class I human leukocyte antigen (HLA) molecules, which are constitutively expressed on all nucleated cells or class II HLA molecules, whose expression is restricted to B lymphocytes, antigen-presenting cells, and activated endothelial cells. Both class I and class II DSA can injure the endothelium though complement-dependent

mechanisms and antibody-dependent cell-mediated cytotoxicity. DSA class I binding also affects the graft endothelium by inducing intracellular signaling which results in migration, proliferation, and resistance to apoptosis and complement-induced death that can have an impact on vascular remodeling and chronic allograft rejection (48). The effect of HLA class II DSA on cell signaling remains to be fully defined given constraints in experimental systems due to the restricted expression of their antigenic target. Although DSA IgG have long been recognized as deleterious to the allograft, the clinical relevance of DSA IgM remains unclear. Some studies have reported associations between IgM DSA, rejection, and decreased graft survival (49, 50).

Even when the allograft arteries are not involved, DSA can affect the graft microcirculation, which is associated with adverse outcomes. A threefold increase in the risk of graft loss was reported in DSA-positive cases of rejection affecting only the microcirculation compared to pure cell-mediated tubulointerstitial rejection (44). In another study, diffuse C4d staining in peritubular capillaries, which marks antibody-mediated complement activation through the classical pathway, was an independent adverse prognostic factor in patients with concurrent cell-mediated rejection, whether or not the graft arteries were involved (51). Hence, the presence of antibody-mediated damage to the microcirculation has prognostic implications in cases of acute rejection, whether or not graft arterial involvement is also present.

Donor-specific antibodies lead to adverse outcomes by injuring the graft endothelium. In patients with antibody-mediated rejection, elevated levels of endothelial transcripts including von Willebrand's factor, caveolin 1, platelet/endothelial cell adhesion molecule, and E selectin have been found in the allograft tissue (52). The presence of circulating DSA and elevated endothelial transcripts in the allograft were associated with poorer long-term graft survival (52), even when evidence for complement activation was lacking (53). Taken together, these studies illustrate that endothelial injury in the allograft macro- or microvascular beds, especially when antibody-mediated, reduces graft survival. DSA-mediated endothelial damage can occur through both complement-dependent and independent pathways.

The persistence of cell- or antibody-mediated vascular and endothelial injury are closely linked with the development of allograft fibrosis and demise. In a swine kidney transplantation model, persistent inflammation in peritubular capillaries was strongly associated with the presence of proliferating α-actin positive myofibroblasts around peritubular capillaries and progression of interstitial fibrosis (54). Similar results were found in human kidney graft biopsies, where microvascular injury in peritubular capillaries (angioregression or capillary drop-out, apoptotic endothelial cells and lamination of the basement membrane) was strongly correlated with interstitial fibrosis, graft dysfunction, and proteinuria (55). Glomerular capillary loss was also associated with glomerular sclerosis and proteinuria.

Recent data suggest that, in addition to DSA, autoantibodies present at the time of transplantation or produced in the posttransplant period can accentuate and aggravate microvascular injury. This concept, coined "innate autoimmunity," was put forward by Carroll and co-workers, as they identified the aggravating role of naturally occurring polyspecific IgM autoantibodies targeting non muscle myosin heavy chain and glycogen phosphorylase in models of intestinal and skeletal muscle IRI (56–58). They also showed that blockade of this autoantibody attenuated tissue damage in a model of cardiac IRI (59). Our group identified anti-perlecan/LG3 IgG autoantibodies of the IgG1 and IgG3 sub-types that target a cryptic C-terminal fragment of perlecan (LG3), as predictors of renal dysfunction in a murine model of renal IRI and in renal transplant patients (60). Elevated levels of anti-perlecan/LG3 at the time of transplantation are associated with an increased risk of vascular rejection and DGF (60, 61). In patients with DGF, anti-perlecan/LG3 autoantibodies predict reduced long-term renal function (60). Anti-perlecan/LG3 autoantibodies exhibit a specific tropism for the ischemic vasculature. In experimental models of vascular rejection and renal IRI, deposition of anti-perlecan/LG3 autoantibodies was significantly increased by ischemia (60, 61). This led to enhanced activation of the classical complement pathway, C4d deposition, peritubular capillary rarefaction, and renal fibrosis. Other autoantibodies, such as anti-angiotensin II type 1 receptors (AT1R) and anti-fibronectin antibodies, have been implicated in accentuation of renal acute vascular rejection and transplant glomerulopathy (38, 39). Anti-AT1R IgG autoantibodies also increase the risk of acute rejection and graft loss in renal transplant patients (62, 63). Ischemia was shown to increase the contractile activity of AT1R autoantibodies in isolated renal artery rings (64), suggesting the possibility of enhanced renal vasoconstriction and ischemia. Collectively, these reports add further support to the notion that renal microvascular injury, either induced by IRI, allo- or autoantibodies or through synergistic interactions between these different factors, plays a major role in long-term renal allograft dysfunction.

ENDOTHELIAL CELL DEATH CONTRIBUTES TO VASCULAR REMODELING, AUTOIMMUNITY AND INFLAMMATION

The presence of dying renal cells in association with AKI or rejection episodes has been known for decades. However, the characterization of molecular pathways controlling regulated renal cell death responses is still an evolving field. Two major types of programmed cell death, apoptosis and necroptosis, have been characterized in association with AKI (23, 26, 65–73), although various death and inflammatory pathways such as ferroptosis and pyroptosis also likely contribute (74–76). Apoptosis can be initiated by two major initiating pathways: cell surface death receptors or mitochondrial outer membrane permeabilization. Both pathways converge on an effector phase triggered by caspases-3 activation and responsible for definitive degradation of key nuclear and cytoskeletal substrates leading to morphological changes such as membrane blebbing and nuclear condensation. However, ligation of death receptors, such as tumor necrosis factor or Fas, in conditions when caspases are inhibited can also activate a regulated form of necrosis referred to as "necroptosis" [reviewed in Ref. (77, 78)]. In this context, receptor-interacting protein 1 (RIPK1) phosphorylates RIPK3

and mixed lineage kinase domain-like protein (MLKL) leading to cell swelling and rupture (77, 78). Necroptosis is associated with an important inflammatory response secondary to the release of damage-associated molecular patterns and to the activation of the inflammasome leading to caspase-1 activation and release of IL-1β, IL-18, and IL-1α. Like necroptosis, pyroptosis is a type of regulated necrotic cell death. Pyroptosis is characterized by caspase-11/gasdermin D-dependent plasma membrane rupture, is highly pro-inflammatory, and has a unique feature: the caspase-1 dependent maturation of pro-inflammatory cytokines in a multiprotein complex called the inflammasome during the cell death process (71, 75).

Apoptosis has classically been considered as an inert or anti-inflammatory type of cell death, responsible for the physiological turnover of multiple cell types. During apoptosis, caspase activation inactivates mitochondrial DNA-induced type I interferon secretion and oxidizes danger signals. This inactivates danger associated molecular patterns (DAMP) molecules and prevents the development of an innate immune response to apoptotic cells (79). Effector caspase activation also leads to the release of chemotactic factors that recruit phagocytes and enhance the clearance of apoptotic cells, preventing secondary necrosis and the release of DAMP factors (79).

Nevertheless, the impact of apoptosis may vary according to cell type and in certain conditions also favor inflammatory responses. Apoptotic endothelial cells externalize phosphatidylserine (80), which binds Factor XII to promote coagulation (81). Apoptotic endothelial cells also interact with other cell types through the release of extracellular vesicles which can in turn promote inflammation. Extracellular vesicles include microvesicles, such as apoptotic bodies, that are produced by cytoplasmic membrane blebbing and shedding, and exosomes, that are smaller and stored in multivesicular bodies or alpha-granules (82). For example, endothelial apoptotic bodies that contain the full-length precursor and processed mature form of IL-1α have pro-inflammatory effects when injected in the peritoneal cavity of mice (83). Both types of vesicles are involved in cellular crosstalk, as will be discussed later.

The relative importance of regulated death pathways in AKI or rejection-induced microvascular injury is only beginning to be unraveled. It is generally accepted that broad caspase inhibition can prevent apoptosis at the expense of increased necroptosis

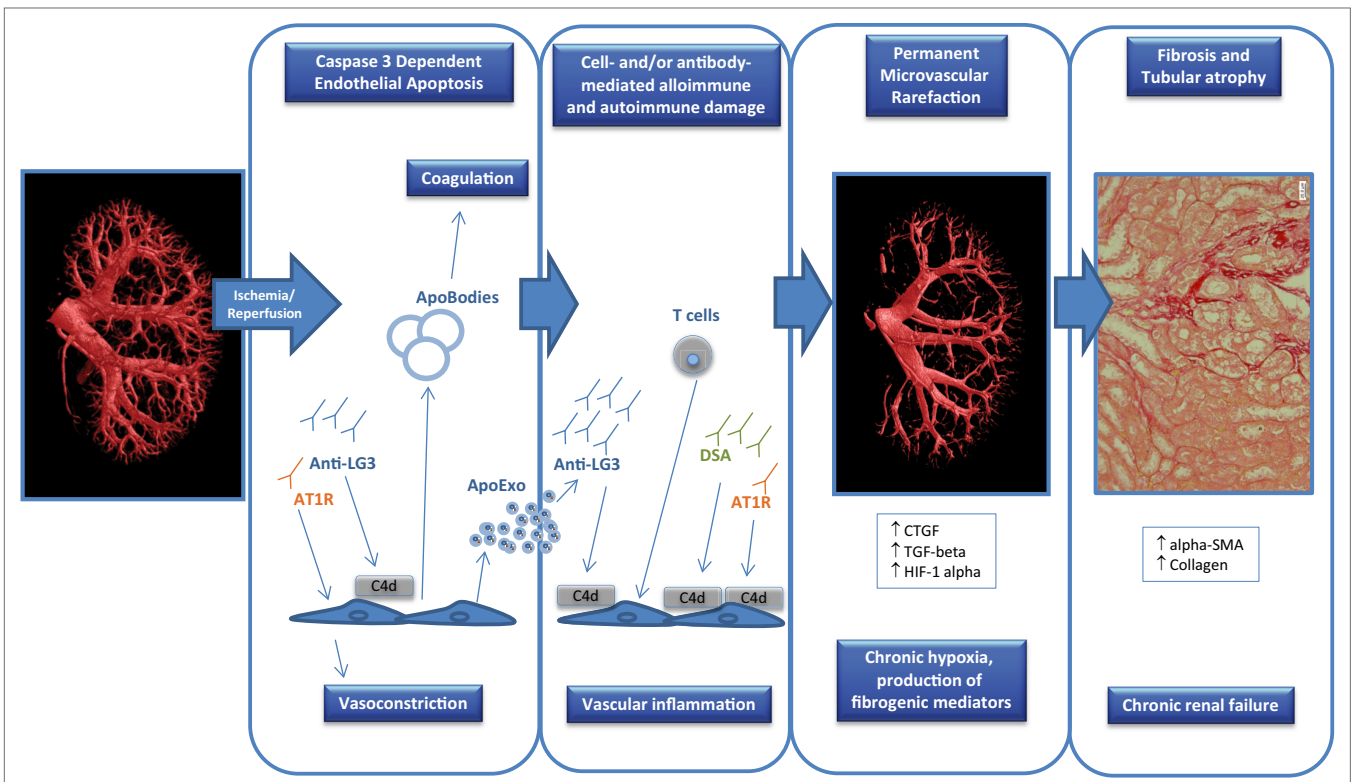

FIGURE 1 | Factors contributing to endothelial dysfunction in transplantation. Ischemia-reperfusion injury that occurs at the time of transplantation can trigger endothelial apoptosis through caspase-3 activation which can in turn release apoptotic bodies with procoagulant activity. In addition, apoptotic endothelial cells also release apoptotic exosome-like vesicles that favor the recruitment of inflammatory cells (e.g., T cells) and the production of autoantibodies such as anti-perlecan/LG3 antibodies (anti-LG3). The synergistic interactions between cellular alloimmunity and autoimmunity, donor-specific antibodies (DSA), and autoantibodies [anti-LG3, anti-angiotensin II type 1 receptors (AT1R)] amplify microvascular damage, through complement-dependent (C4d deposition) or -independent pathways, which leads to permanent peritubular capillary rarefaction. Loss of peritubular capillaries favors chronic hypoxia, leading to overexpression of hypoxia inducible factor 1 α (HIF-1α), favoring transcription of fibrogenic genes such as transforming growth factor β (TGF-β) and connective tissue growth factor (CTGF). It also favors accumulation of collagen, α-smooth muscle actin (α-SMA) positive myofibroblasts and of fibrogenic mediators. These phenomena eventually lead to progressive interstitial fibrosis/tubular atrophy and renal graft dysfunction.

and accentuated renal dysfunction (70, 78), a phenomenon well characterized in renal tubular epithelial cells (84, 85). Cardiac endothelial cells have also been shown to develop RIPK3-dependent cell death after TNF-alpha treatment *in vitro* and following transplantation *in vivo*. RIPK3−/− mice show better preservation of microvascular integrity in a model of cardiac rejection (86). Whether RIPK-dependent death also occurs in the renal microvasculature during AKI and/or rejection remains to be evaluated. However, microvascular apoptosis, evaluated by caspase-3 activation, has been documented in models of renal IRI and rejection (23, 87). Also, inhibition of caspase-3 at the time of renal IRI has generally been associated with improved long-term renal function and reduced extracellular matrix deposition (78, 88, 89). Collectively, these results suggest an important role for caspase-3 in regulating renal vascular cell death whereas the importance of RIPK-dependent death remains to be characterized. While pyroptosis has been observed in renal tubular epithelial cells in a rat model of renal IRI (90), this type of cell death has not been described in endothelial cells.

Endothelial caspase-3 activation can promote vascular dysfunction through various and non-mutually exclusive pathways (**Figure 1**). It favors the release of a number of fibroproliferative mediators, such as CTGF, LG3, and translationally controlled tumor protein, which can in turn favor neointima formation and myointimal thickening (91–94). Endothelial caspase-3 activation also leads to the release of apoptotic bodies or membrane blebs with procoagulant activity (95, 96). Recently, we showed that, in addition to apoptotic bodies, endothelial caspase-3 activation prompts the release of a novel type of extracellular vesicles whose protein content and function are dramatically different from classic apoptotic bodies (97). These apoptotic exosome-like vesicles (ApoExo) are smaller than apoptotic bodies, ranging from 30 to 100 nm and carry active 20S proteasome complexes with pro-inflammatory activity. ApoExo injection in mice favors the recruitment of T and B cells in a model of vascular allograft rejection (97). Endothelial ApoExos also favor the production of autoantibodies such as anti-perlecan/LG3 antibodies, anti-nuclear antibodies, and anti-double-stranded DNA antibodies (97), which in turn further aggravate vascular inflammation. In animal models, renal IRI favors the release of ApoExos within the bloodstream, followed by augmented levels of anti-perlecan/LG3 antibodies (97). Collectively, these results highlight an important role for vascular caspase-3 activation in triggering the release of a number of mediators and extracellular vesicles that will, both at the local and systemic levels, initiate multiple positive feedback mechanisms that favor vascular remodeling, inflammation, and autoimmunity.

CONCLUDING REMARKS

Kidney transplantation is associated with an elevated likelihood of damage to the graft macro- and microvasculature, given the IRI that occurs at the time of transplantation and the physical location of the graft endothelium that makes it a target of choice for cell- or antibody-mediated alloimmune injury. IRI, alloimmune damage, and autoantibodies can activate programmed cell death pathways in the graft endothelium, which can in turn trigger microvascular rarefaction, interstitial fibrosis, and graft dysfunction. These pathways represent potential targets for pharmacological intervention that could be delivered in preservation solutions during the period cold ischemia, with the aim of improving long-term graft outcomes.

AUTHOR CONTRIBUTIONS

HC, MD, and M-JH reviewed the literature and drafted the manuscript.

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The Increased Endogenous Sulfur Dioxide Acts as a Compensatory Mechanism for the Downregulated Endogenous Hydrogen Sulfide Pathway in the Endothelial Cell Inflammation

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Endogenous hydrogen sulfide (H_2S) and sulfur dioxide (SO_2) are regarded as important regulators to control endothelial cell function and protect endothelial cell against various injuries. In our present study, we aimed to investigate the effect of endogenous H_2S on the SO_2 generation in the endothelial cells and explore its significance in the endothelial inflammation *in vitro* and *in vivo*. The human umbilical vein endothelial cell (HUVEC) line (EA.hy926), primary HUVECs, primary rat pulmonary artery endothelial cells (RPAECs), and purified aspartate aminotransferase (AAT) protein from pig heart were used for *in vitro* experiments. A rat model of monocrotaline (MCT)-induced pulmonary vascular inflammation was used for *in vivo* experiments. We found that endogenous H_2S deficiency caused by cystathionine-γ-lyase (CSE) knockdown increased endogenous SO_2 level in endothelial cells and enhanced the enzymatic activity of AAT, a major SO_2 synthesis enzyme, without affecting the expressions of AAT1 and AAT2. While H_2S donor could reverse the CSE knockdown-induced increase in the endogenous SO_2 level and AAT activity. Moreover, H_2S donor directly inhibited the activity of purified AAT protein, which was reversed by a thiol reductant DTT. Mechanistically, H_2S donor sulfhydrated the purified AAT1/2 protein and rescued the decrease in the sulfhydration of AAT1/2 protein in the CSE knockdown endothelial cells. Furthermore, an AAT inhibitor L-aspartate-β-hydroxamate (HDX), which blocked the upregulation of endogenous SO_2/AAT generation induced by CSE knockdown, aggravated CSE knockdown-activated nuclear factor-κB pathway in the endothelial cells and its downstream inflammatory factors including ICAM-1, TNF-α, and IL-6. In *in vivo* experiment, H_2S donor restored the deficiency of endogenous H_2S production induced by MCT, and reversed the upregulation of endogenous SO_2/AAT

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pathway *via* sulfhydrating AAT1 and AAT2. In accordance with the results of the *in vitro* experiment, HDX exacerbated the pulmonary vascular inflammation induced by the broken endogenous H_2S production in MCT-treated rat. In conclusion, for the first time, the present study showed that H_2S inhibited endogenous SO_2 generation by inactivating AAT *via* the sulfhydration of AAT1/2; and the increased endogenous SO_2 generation might play a compensatory role when H_2S/CSE pathway was downregulated, thereby exerting protective effects in endothelial inflammatory responses *in vitro* and *in vivo*.

Keywords: endothelial cells, inflammation, sulfhydration, H_2S, SO_2

INTRODUCTION

Hydrogen sulfide (H_2S), a new member of gaseous signal molecule family, has been found as a metabolic end product of sulfur-containing amino acids and to be involved in various physiologic and pathophysiologic processes since the end of the last century (1, 2). Cystathionine-γ-lysase (CSE) is regarded as a predominant H_2S-generating enzyme in the cardiovascular tissues, and H_2S is generated with the substrates of cystathionine or cysteine, catalyzed by CSE (3, 4). The regulatory effect of endogenous H_2S on the endothelial cell function attracted great attention because of the importance of endothelial cells in the vascular injury and repair. H_2S was reported to stimulate the proliferation and migration of endothelial cells, promote endothelial cell angiogenesis, inhibit the endothelial cell inflammation, protect mitochondrial function, and mediate endothelial-dependent vasorelaxation, etc (5–8). Plenty of research demonstrated that H_2S protected the endothelial cells against various insults from hypoxia, high-salt, high-glucose, angiotensin II, and tumor necrosis factor-α (TNF-α), and so on (7, 9–12). Impaired endogenous H_2S production, bioavailability, and its function were involved in the pathogenesis of endothelium dysfunction-related diseases including hypertension, vascular complication of diabetes, atherosclerosis, restenosis, and aging, etc (13–15).

Recently, sulfur dioxide (SO_2), a brother of H_2S, attracted more and more concerns in the field (16, 17). SO_2 was found to be endogenously generated from the enzymatic reaction catalyzed by aspartate amino transferase (AAT) in the metabolic pathway of sulfur-containing amino acids (18). Endogenous SO_2/AAT pathway was discovered to exist in the endothelium, vascular smooth muscles, fibroblasts, cardiac myocytes, adipocyte, and alveolar epithelial cells and play an important role in the cardiovascular homeostasis (19–24). Our research group firstly put forward the hypothesis that endogenous SO_2 might be the fourth gaseous signal molecule involved in the regulation of cardiovascular system (25). Endogenous SO_2 was discovered to promote the nitric oxide production and enhance the nitric oxide-induced vasodialation (26). It could protect against acute lung injury induced by limb ischemic/reperfusion (I/R) or by lipopolysaccharide or by oleic acid in rats (27–29). Moreover, AAT1 overexpression could alleviate the lung inflammatory response caused by oleic acid in a mice model of acute lung injury (29).

Collectively, both H_2S and SO_2 are generated from the same metabolic pathway in the similar origin tissues and exert similar biological effect (24, 29–33). For instance, Xiao et al. discovered that H_2S mitigated cardiomyocyte injury caused by hypoxic-reoxygenation *via* decreasing autophagy (30), while Chen et al. demonstrated that SO_2 also alleviated myocardial hypertrophy by inhibiting Ang II-activated autophagy in mice (31). Furthermore, the two gasotransmitters sometimes share the same signaling pathway, and even the same target residue. The activation of PI3K/Akt pathway mediated the protective effect of H_2S preconditioning on the cerebral I/R injury (32). Meanwhile, it was involved in SO_2 preconditioning-induced protection against myocardial I/R injury (24). H_2S can inactivate inflammatory response by inhibiting the phosphorylation and nuclear translocation of NF-κB p65 *via* sulfhydrating NF-κB p65 cysteine 38 (33), whereas SO_2 suppresses inflammatory response by sulfenylating NF-κB p65 at the same residue (29).

So, here comes the question that what is the significance of the coexistence of H_2S and SO_2 in the biologic tissues. Li and Luo et al. found that SO_2 increased endogenous H_2S production in the development of artherosclerosis and pulmonary hypertension, and the upregulation of endogenous H_2S pathway might be one of protective mechanisms responsible for endogenous SO_2 (23, 34). However, the impact of endogenous H_2S on the endogenous SO_2 production and its significance have been unclear. In the present study, we attempted to construct an endogenous H_2S-defiency endothelial cell inflammation model by transfecting lentivirus-containing CSE shRNA using human umbilical vein endothelial cell (HUVEC) line (EA.hy926), investigate the effect of endogenous H_2S on the endothelium-derived SO_2 generation and explore its significance in the development of inflammatory response induced by the H_2S/CSE deficiency. In addition, we also used the primary HUVECs, rat pulmonary artery endothelial cells (RPAECs) and rats with pulmonary vascular inflammation in the study to verify the effect of H_2S on the endogenous SO_2 production and its implication.

MATERIALS AND METHODS

Cell Culture

The HUVEC line (EA.hy926) was purchased from China Infrastructure of Cell Line Resources Center, China. The cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% streptomycin, and 1% penicillin (Gibco, USA). Primary HUVECs were kindly provided by professor Jing Zhou, Peking University Health Science Center, Beijing, China, and RPAECs (PriCells, Wuhan, China) were cultured in the specialized endothelial cell medium (PriCells, Wuhan, China) supplemented with 10% FBS and 100

IU/mL penicillin-streptomycin. The endothelial cells were maintained in a humidified atmosphere of 5% CO_2 at 37°C.

CSE knockdown endothelial cells were obtained by infecting lentivirus containing CSE shRNA plus green fluorescent protein (GFP) cDNA (Cyagen, China). For the purpose of determining the appropriate concentration of lentivirus used for the treatment, the cells were seeded in 6-well plate, grown to 60–70% confluence, and transfected with different viral titers of lentivirus (1×10^4 to 2×10^5 TU/mL). After 12 h of the infection, freshly completed culture medium was replaced. After another 72 h, the green fluorescence of GFP was observed in the successfully transfected cells under fluorescence microscope. Moreover, the protein expression of CSE in the cells was detected by western blot and the H_2S level in cell supernatant was detected by H_2S-selective sensor. The screening results demonstrated that the appropriate concentration of lentivirus containing CSE shRNA plus GFP cDNA was 1×10^5 TU/mL (Figure S1 in Supplementary Material). The endothelial cells were seeded in T25 flasks and infected with lentiviral CSE shRNA (1×10^5 TU/mL) at 60–70% confluency. G418 antibiotics (200 μg/mL) was used for EA.hy926 cell screening for 2 week and G418 antibiotics (300 μg/mL) was used for primary HUVECs and primary RPAECs screening for 1 week. At the same time, vehicle lentivirus was used to infect the endothelial cells as the control according to the same protocol.

To explore the effect of endogenous H_2S deficiency on the SO_2/AAT pathway in the endothelial cell and its mechanism, cells were randomly divided into vehicle group, CSE shRNA group, and CSE shRNA plus H_2S group. Cells in the CSE shRNA + H_2S group were pretreated with 200 μM of H_2S donor sodium hydrosulfide hydrate (NaHS) for 24 h. Cells in the vehicle group and CSE shRNA group were incubated with equal volume of ddH_2O. NaHS was freshly dissolved in ddH_2O.

To investigate the significance of the increased endogenous SO_2 generation in the endothelial cell inflammation caused by CSE knockdown, cells were divided into vehicle group, CSE shRNA group, and CSE shRNA + L-aspartate-β-hydroxamate (HDX) group. Cells in the CSE shRNA + HDX group were pretreated with 200 μM HDX for 24 h. Cells in the control group and CSE shRNA infected group were incubated with equal volume of ddH_2O. HDX is an inhibitor of AAT and freshly prepared.

Animal Preparation and Grouping

All animal care and experimental procedures complied strictly with the Animal Management Rule of the Ministry of Health of the People's Republic of China (Documentation 55, 2001). The protocol was specifically approved by the Animal Research Ethics Committee of Peking University First Hospital (permit number 201215 and 201326).

Eighteen male Wistar rats provided by the Animal Research Committee of the First Hospital, Peking University, weighing 160 ± 20 g, were randomly divided into three groups ($n = 6$ each group): control group, monocrotaline (MCT) group, and MCT + H_2S group. On the first day, the rats of MCT and MCT + H_2S groups were administered with MCT (60 mg/kg) by intraperitoneal injection, while the rats of control group were injected with the same dose of saline (5, 35). The rats of MCT + H_2S group were injected daily with the H_2S donor, NaHS (56 μmol/kg), for 21 days, while the rats of the control and MCT groups were given the same dose of saline.

Another 21 male Wistar rats were divided into three groups ($n = 7$ each group): control group, MCT group, and MCT + HDX group. The rats in the MCT and MCT + HDX groups were administered with MCT (60 mg/kg) by intraperitoneal injection on day 1. The rats in the MCT + HDX group were given HDX orally at 25 mg/kg on days 0, 7, and 14 (36). The rats in the control group received the same dose of saline.

Rat Pulmonary Artery Pressure Measured by Right Heart Catheterization

Rats were anesthetized *via* intraperitoneal injection of 0.5% sodium pentobarbital (0.1 mL/100 g) after 21 days of MCT challenge. The pulmonary artery pressure was measured *via* right heart catheterization as previously described (22). Briefly, the right external jugular vein was exposed and a catheter was guided through the superior vena cava, right atrium, and right ventricle into the pulmonary artery. The extracorporal end of the catheter was connected to a pressure sensor (BL-410, Chengdu TME Technology, China) to record the continuous changes of pulmonary artery pressure, including systolic pulmonary artery pressure, diastolic pulmonary artery pressure, and mean pulmonary artery pressure.

Morphological Change of Pulmonary Arteries

The rat lung tissue was immersed in the 10% (wt/vol) paraformaldehyde for fixation and then embedded in paraffin. The lung tissue was sectioned at a thickness of 4 μm. The elastic fiber in the pulmonary artery was stained using the modified Weigert's elastic fiber staining kit according to the manufacturer's protocol (Leagene, Beijing, China). The internal and external elastic lamina were shown as dark-purple color under microscope.

Western Blotting

The specific protein expression in the endothelial cell and lung tissues was detected by western blotting. After treatment, the cells and rat lung tissues were lysed in lysis buffer (50 mM Tris base, 150 mM NaCl, 1 mM EDTA, 0.25% sodium deoxycholate, 1% NP-40, protease inhibitor cocktail, PH 7.4) (37). Protein concentration was determined using Bradford kit. Equal amounts of proteins were boiled and separated using 8–15% SDS-PAGE, and transferred using electrophoresis to a nitrocellulose membrane (Amersham, USA). The primary antibody dilutions were 1:200 for CSE (Sigma, USA), 1:1,000 for AAT1 and AAT2 (Sigma, USA), 1:200 for ICAM-1 (Boster, China), 1:1,000 for NF-κB p65 and IκBα (CST, USA), 1:500 for p-NF-κB p65 and p-IκBα (CST, USA), 1:2,000 for β-actin (Santa Cruz, CA, USA), and 1:2,000 for GAPDH (Kangcheng, China). Horseradish peroxidase-conjugated secondary antibodies were used at a dilution of 1:3,000–1:5,000 (Sigma, USA). The bands were visualized using a chemiluminescence detection kit on the FluorChem M MultiFluor System (Proteinsimple, USA). The densitometric analysis of the bands was performed using AlphaEaseFC (Alpha, USA). All experiments were performed independently for at least three times.

Measurement of H_2S Level by an H_2S-Selective Sensor

The H_2S level in endothelial cell supernatant and rat lung tissues was measured using the free radical analyzer TBR4100 with an H_2S-selective sensor (ISO-H_2S-100, WPI, China) as previously described (38, 39). The rat lung homogenate was prepared by grinding with cold PBS buffer (pH 7.2, 0.01 M). Firstly, an H_2S-selective sensor was polarized with PBS buffer (pH 7.2, 0.05 M) until a stable baseline current was reached, and then the calibration curve of pA–H_2S concentration began to be plotted as follows. The sensor tip was immersed by 10 mm into 20 mL of PBS buffer solution-containing Na_2S at different concentrations (0.5, 1, 4, 8, 16, and 32 μM) sequentially. Then the calibration curve was constructed by plotting the signal output (pA) against the concentration (μM) of H_2S. Secondly, the sensor tip was immersed into each sample by 10 mm to detect the H_2S content in the sample according the calibration curve of pA–H_2S concentration. All experiments were performed independently for at least three times.

Measurement of SO_2 Level by High-Performance Liquid Chromatography (HPLC) Analysis

SO_2 content in the supernatant of EA.hy926 cells, primary HUVECs and RPAECs supernatants, and rat lung tissues was examined by HPLC (Agilent 1100 series, Agilent Technologies, Palo Alto, CA, USA) as previously described (29). The rat lung homogenate was prepared by grinding with cold PBS buffer (pH 7.2, 0.01 M). In brief, the sample was mixed with 0.212 mM sodium borohydride in 0.05 M Tris–HCl (pH 8.5) and incubated at room temperature for 30 min; this mixture was subsequently combined with 70 mM monobromobimane in acetonitrile. Then, perchloric acid was added, followed by vortex mixing. After that, the mixtures were centrifuged at 12,400× *g* for 10 min, and the supernatant was neutralized by 2.0 M Tris and subsequently centrifuged again at 12,400× *g* for 10 min. Eventually, the neutralized supernatant was transferred and injected into an HPLC column. Sulfite-bimane adduct was detected by the excitation at 392 nm and the emission at 479 nm. All experiments were performed independently for at least three times.

In Situ Detection of H_2S by Fluorescent Probe

The H_2S generation in the endothelial cells was *in situ* detected by H_2S fluorescent probe kindly provided by professor Xinjing Tang, Peking University Health Science Center, Beijing, China, as described previously (5). The cells were cultured using Lab-Tek chambered coverglass (Thermo, USA), rinsed with PBS for twice before incubation with the H_2S fluorescent probe, then subsequently incubated with H_2S fluorescent probes (100 μM) for 30 min, and fixed with ice-cold 4% paraformaldehyde for 20 min. Immunofluorescent images were obtained using a confocal laser-scanning microscope (TCS SP5, Leica, Wetzlar, Germany). Green fluorescent indicates endogenous H_2S in the cells and the fluorescent signal intensity was measured using Image J software (NIH, Bethesda, MD, USA). All experiments were performed independently for at least three times.

In Situ Detection of Endogenous SO_2 by Fluorescent Probe

The SO_2 generation in endothelial cells was detected *in situ* by SO_2 fluorescent probe kindly provided by Professor Kun Li, College of Chemistry of Sichuan University, Sichuan, China. The specificity and sensitivity of this probe were previously verified (40, 41). The cells were cultured using Lab-Tek chambered coverglass (Thermo, USA) and subsequently incubated with SO_2 fluorescent probes (20 μM) for 30 min and then rinsed twice with PBS prior to fixation with ice-cold 4% paraformaldehyde for 20 min. Then, cells were rinsed twice with PBS, each for 5 min before testing. Immunofluorescent images were obtained using a confocal laser-scanning microscope (TCS SP5, Leica Microsystems, Wetzlar, Germany). Blue fluorescent indicates endogenous SO_2 in the cells. The fluorescent signal intensity was measured using Image J software (NIH, Bethesda, MD, USA). All experiments were performed independently for at least three times.

AAT Activity Detected by Colorimetry Assay

The activity of AAT in endothelial cells, purified AAT protein from pig heart, and rat lung tissues was tested by colorimetry assay (JianCheng, Nanjing, China) according to the manufacturer's instructions as described previously (18). AAT catalyzes the transfer of amino group and keto group in α-ketoglutaric acid and aspartic acid to form glutamic acid and oxaloacetic acid. Oxaloacetic acid is then decarboxylated by itself to form pyruvic acid, and the latter was reacted with 2,4-dinitrophenylhydrazine to produce the 2,4-dinitrophenylhydrazone which shows a red–brown color in alkaline solution and can be detected by colorimetric method. Pyruvic acid solution (2 mM) was used as the standard to plot the standard curve. Endothelial cells were homogenized in PBS with an ice-water bath and centrifuged at 5,000× *g* for 10 min at 4°C to get the supernatant. Equivalent AAT purified proteins (0.375 μg, Sigma, USA) were incubated at different concentrations of H_2S (100, 200, and 500 μM) or double distilled water for 2 hr in 37°C water bath. In the 200 μM H_2S plus DTT treatment, purified AAT protein was pretreated with NaHS (200 μM) for 1 h and then incubated with 1 mM DTT for a further 1 h in the continuous presence of NaHS. The rat lung homogenate was prepared by grinding with cold PBS buffer (pH 7.2, 0.01 M). AAT activity was expressed as Carmen's unit, which was calculated according to the standard curve after colorimetric determination. One unit of Carmen's is defined as follows: NADH is oxidized to NAD^+ by pyruvic acid generated from 1 mL of the sample within 1 min at 25°C in the total reaction capacity of 3 mL, which causes the absorbance deceased by 0.001 at 340 nm wavelength using a light path length of 1 cm. All experiments were performed independently for at least three times.

S-Sulfhydrtation Detected by Biotin Switch Analysis

S-sulfhydration of AAT1 and AAT2 in EA.hy926 cell line, primary HUVECs, RPAECs, and rat lung tissues was detected by biotin switch assay as described previously (33, 42). Endothelial cells or rat lung tissues were homogenized in

non-denaturing lysis buffer with protease inhibitors and centrifuged at 13,000× *g* for 20 min at 4°C. Supernatant reserved for sulfhydration analysis was incubated with blocking buffer (lysis buffer supplemented with 2.5% SDS and 20 mM S-methyl methanethiosulfonate) at 50°C for 30 min with continuous vortexing. The sample was added with acetone for removing S-methyl methanethiosulfonate at −20°C for 2 h. After acetone removal by centrifuge, the protein was resuspended in lysis buffer and incubated with EZ-link iodoacetyl-PEG2 biotin (10 mg/mL) at 4°C for 12 h. Biotinylated proteins were precipitated by UltraLink™ Immobilized Neutravidin™ for 4 h on a roller system (100 rpm) at 4°C and then washed three times with PBS. The sulfhydrated proteins were boiled with loading buffer without β-mercaptoethanol and centrifuged at 5,000× *g* for 10 min to get supernatant, and then subjected to western blot using 8% SDS-PAGE as described previously. All experiments were performed independently for at least three times.

S-Sulfhydrtation Detected by Biotin Thiol Assay

S-sulfhydrtation of AAT1 and AAT2 in EA.hy926 cell line and purified AAT protein from pig heart was detected by the biotin thiol assay as described before (43). The schematic protocol is shown in Figure S2 in Supplementary Material. Cells were homogenized in non-denaturing lysis buffer with protease inhibitors and centrifuged at 13,000× *g* for 20 min at 4°C. The protein concentrations were determined by the BCA assay. Equal amount (1 mg) of total protein was incubated with 100 μM maleimide-PEG2-biotin (Thermo, USA) for 0.5 h on a roller system (100 rpm) at room temperature. Subsequently, the mixture was added with acetone at −20°C for 20 min. After washing for three times with 70% precooled acetone, the sample was centrifuged at 12,000× *g* for 5 min. The precipitate was resuspended in the buffer (0.1% SDS, 150 mM NaCl, 1 mM EDTA and 0.5% Triton X-100, 50 mM Tris–HCl, pH 7.5) mixed with streptavidin–agarose resin (Thermo, USA) and kept rotating overnight at 4°C. The beads were washed three times with PBS containing 0.5% Triton X-100 and centrifuged at 5,000× *g* for 5 min, and then the precipitate was mixed with 50 μL of loading buffer containing or not containing DTT (20 mM) with gentle shaking for 1.0 h on a roller system (100 rpm) at room temperature. After centrifugation at 5,000× *g* for 10 min, supernatant subjected to western blot using 8% SDS-PAGE as described previously. Equal amount (3 μg) of purified AAT protein (Sigma, USA) was incubated with NaHS (200 μM) for 2 h. The sulfhydrated AAT was separated and measured using the abovementioned protocol. All experiments were performed independently for at least three times.

Expression of IκBα in Primary RPAECs Detected by Immunofluorescence

Immunofluorescent imaging was obtained using a confocal laser-scanning microscope (TCS SP5, Leica, Germany). Briefly, RPAECs were rinsed with PBS before the fixation with 4% paraformaldehyde. The RPAECs were then incubated with the anti-IκBα antibody (1:50, CST, USA) at 4°C overnight. RPAECs were subsequently incubated with the anti-mouse-FITC conjugated secondary antibody (Thermo, USA) at 37°C for 1 h. After washing, the slides were observed under confocal microscope (5). All experiments were performed independently for at least three times.

Inflammatory Cytokine Levels Detected by Enzyme-Linked Immunosorbent Assay (ELISA)

Inflammatory cytokines including TNF-α, IL-6, and ICAM-1 in the cell supernatant and rat lung tissue homogenates were measured using ELISA kits (eBioscience, CA, USA). Recombinant TNF-α, IL-6, and ICAM-1 were used as standard substances. Samples and standard substances were incubated separately with an equal volume of diluent in an microplate coated with specific primary antibody at room temperature for 2 h using a shaker. Subsequently, the supernatant was removed, and the wells were rinsed with washing solution and dried. Horseradish peroxidase-conjugated primary antibody was then added to the wells and incubated for 1 h. After rinsing with washing solution, 100 μL of substrate solution was added to each well to develop the chromogenic reaction for 15 min. Then, 50 μL of stop solution was added to each well to stop the reaction. A standard curve was made by absorbance at 450 nm as the vertical axis and standard substance concentration as the horizontal axis. The concentrations of inflammatory cytokines in the samples were then calculated (5). The protein concentration of rat lung tissue homogenate was determined with Bradford kit and used for adjusting the content of cytokines in the rat lung tissue. All experiments were performed independently for at least three times.

Statistical Analysis

Data are expressed as mean ± SEM. Comparisons among groups were analyzed by one-way ANOVA using SPSS 17.0 (SPSS Inc., USA). Means between groups with equal variance were analyzed by least-significance difference (LSD). When equal variance not assumed, means between groups were analyzed using Tamhane. $P < 0.05$ was considered statistically significant.

RESULTS

Endogenous SO_2 Production Was Increased in CSE Knockdown Endothelial Cells

For the purpose of revealing the effect of endogenous CSE/H_2S pathway on endogenous SO_2 production, EA.hy926 cell line was treated with CSE shRNA followed by H_2S donor supplement. Compared with vehicle group, H_2S level in cell supernatant was decreased in endothelial cells of CSE shRNA group, while H_2S donor reimbursed the H_2S deficiency caused by CSE knockdown (**Figure 1A**). In *in situ* fluorescent probe experiment, the data showed that CSE knockdown decreased the endogenous H_2S level but promoted endogenous SO_2 production (**Figures 1B,C**). Moreover, H_2S donor NaHS inhibited the increase in endogenous SO_2 level in the CSE knockdown EA.hy926 cells (**Figures 1B,C**). To further confirm the above result, we selected primary HUVECs and RPAECs in addition to EA.hy926 cells (**Figures 1D–I**). Interestingly, the results in both primary endothelial cells were

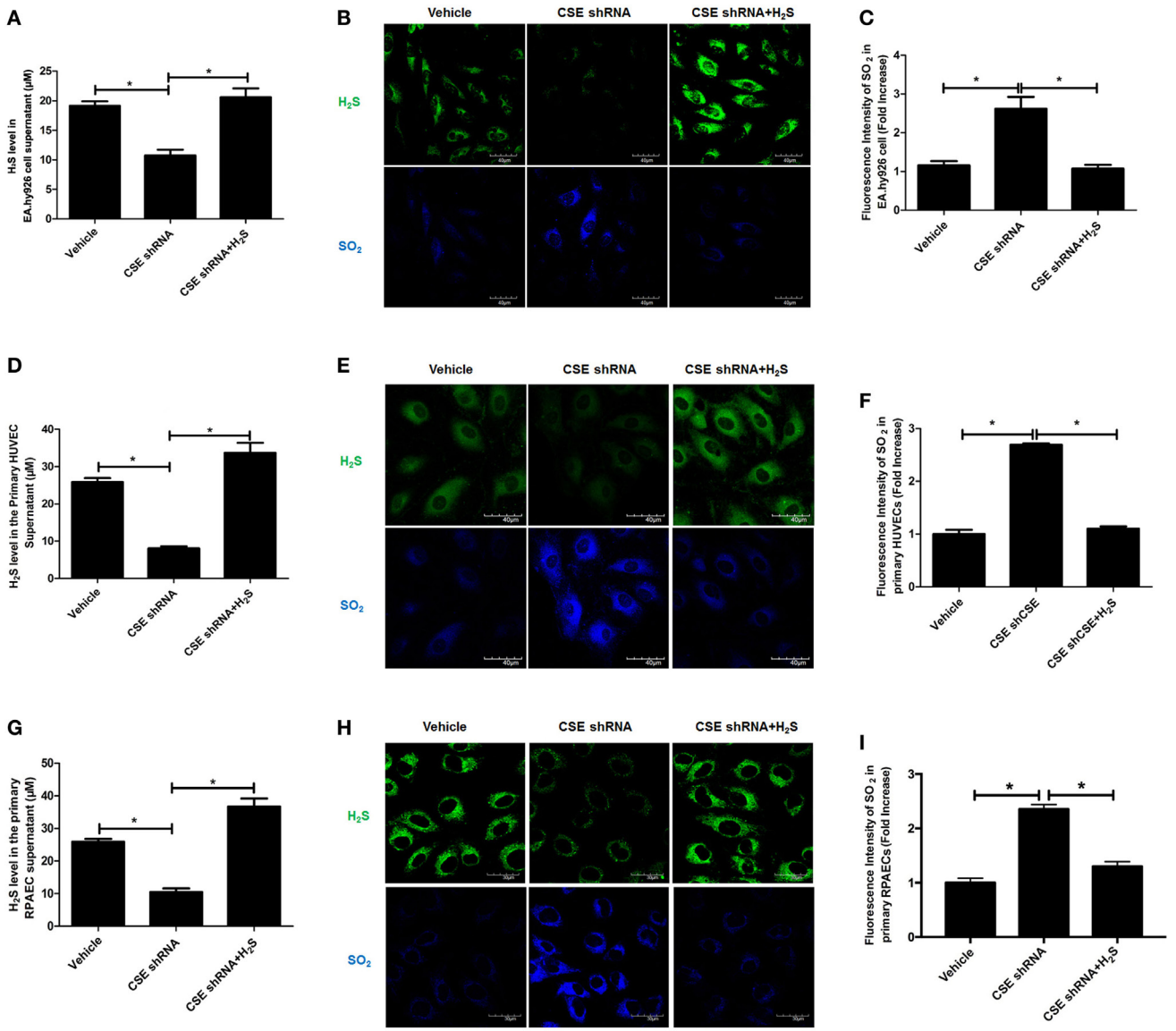

FIGURE 1 | Endogenous SO_2 production was increased in cystathionine-γ-lyase (CSE) knockdown endothelial cells. The EA.hy926 cells, primary human umbilical vein endothelial cells (HUVECs), and primary rat pulmonary artery endothelial cells (RPAECs) were transfected with vehicle lentivirus or lentivirus containing CSE shRNA, and then treated with or without H_2S donor (200 μM). **(A)** The H_2S level in cell supernatant was detected by H_2S-selective sensor in EA.hy926 cell. **(B)** H_2S generation in HUVECs was detected by *in situ* fluorescent H_2S probe (green color), while SO_2 generation in HUVECs was detected by *in situ* fluorescent SO_2 probe (blue color) in EA.hy926 cell. **(C)** The blue fluorescence intensity indicating endogenous SO_2 content was analyzed using Image J software in EA.hy926 cell. **(D)** The H_2S level in cell supernatant was detected by H_2S-selective sensor in primary HUVECs. **(E)** H_2S generation in primary HUVECs was detected by *in situ* fluorescent H_2S probe (green color), while SO_2 generation in primary HUVECs was detected by *in situ* fluorescent SO_2 probe (blue color). **(F)** The blue fluorescence intensity indicating endogenous SO_2 content was analyzed using Image J software in primary HUVECs. **(G)** The H_2S level in cell supernatant was detected by H_2S-selective sensor in primary RPAECs. **(H)** H_2S generation in primary RPAECs was detected by *in situ* fluorescent H_2S probe (green color), while SO_2 generation in primary RPAECs was detected by *in situ* fluorescent SO_2 probe (blue color). **(I)** The blue fluorescence intensity indicating endogenous SO_2 content was analyzed using Image J software in primary RPAECs. $^*P < 0.05$. Data are expressed as means ± SEM, and all experiments were performed independently for at least three times.

accordant with that in the EA.hy926 cell. Compared with the vehicle group, SO_2 level in both primary endothelial cells of CSE shRNA group was upregulated, while H_2S donor blunted the effect of CSE knockdown on the endogenous SO_2 level in both primary endothelial cells (**Figures 1E,F,H,I**). The results suggested that endogenous H_2S suppressed the SO_2 production in endothelial cells.

The Protein Expression of AAT1 and AAT2 in Endothelial Cells Was Not Affected by CSE Knockdown

In order to elucidate the target on which endogenous CSE/H_2S inhibited SO_2 production, we first detected the protein expression of AAT1 and AAT2, the two key endogenous SO_2

producing enzymes in EA.hy926 cells. Compared with vehicle group, the expression of CSE in the endothelial cells of CSE shRNA group was markedly decreased (**Figure 2A**). However, there was no difference in the expressions of AAT1 and AAT2 in the endothelial cells between vehicle group and CSE shRNA group (**Figures 2B,C**). Next, the same protocol of the experiments was done on both kinds of primary cells. Compared with the vehicle group, the expression of CSE was both downregulated in primary HUVECs and primary RPAECs (**Figures 2D,G**), while the expression of AAT1 and AAT2 was not affected by CSE knockdown (**Figures 2E,F,H,I**). The results proved that AAT1 and AAT2 protein expressions were not involved in the inhibitory effect of endogenous H_2S/CSE on the endogenous SO_2 production.

The Endogenous H_2S/CSE Inhibited the AAT Activity

Aspartate aminotransferase activity is another important element involved in the regulation of endogenous SO_2 production. Therefore, we further detected the activity of AAT in the HUVECs and purified AAT protein. The result showed that activity of AAT was significantly increased in the CSE knockdown EA.hy926 cells. While compared with the vehicle cells, the exogenous supplementation of NaHS (200 μM) reversed the increase in the AAT activity caused by CSE knockdown (**Figure 3A**). The similar results were observed in both primary endothelial cells as shown in **Figures 3B,C**. Furthermore, NaHS (100–500 μM) directly inhibited the AAT activity in a concentration-dependent manner in purified AAT protein (**Figure 3D**), which further supported the direct inhibitory effect of H_2S on the AAT activity.

H_2S S-Sulfhydrated AAT to Inhibit AAT Activity

In *in vitro* experiment, DTT, a thiol reductant, could reverse the impact of H_2S on the AAT activity (**Figure 3D**), suggesting that the thiol group at the cysteine of AAT protein might be involved in the mechanisms by which H_2S suppressed AAT activity. Considering that S-sulfhydration, a special posttranslation modification on the thiol group at the cysteine, was reported to participate in the wide biological effects of H_2S, we detected the S-sulfhydration of AAT in the EA.hy926 cell, using modified biotin switch assay. The data showed that compared with vehicle group, S-sulfhydration of AAT1 and AAT2 was sharply reduced in EA.hy926 cell of CSE shRNA group, while the supplementation of NaHS significantly reversed the decrease in the S-sulfhydration of AAT in the EA.hy926 cell caused by CSE knockdown (**Figure 4A**). Furthermore, we also used another method for detecting S-sulfhydration, known as biotin thiol assay. The results in **Figure 4B** are in accordance with those shown in **Figure 4A**, suggesting that H_2S can sulfhydrate AAT1 and AAT2.

Next, we detected S-sulfhydration of AAT by H_2S in both primary HUVECs and primary RPAECs using biotin switch assay. Compared with vehicle group, S-sulfhydration of AAT1 and AAT2 was decreased significantly in both primary endothelial cells of CSE shRNA group, while the supplementation of NaHS significantly reversed the decrease in the S-sulfhydration of AAT (**Figures 4C,D**). Moreover, NaHS-induced S-sulfhydration of AAT1 and AAT2 in the purified protein from pig heart, which was blocked by the treatment with a thiol reductant DTT (**Figure 4E**).

Collectively, the above data suggested that H_2S might inhibit the activity of AAT *via* the sulfhydration of AAT.

Upregulation of Endogenous SO_2 Production Exerted Compensatory Effects to Inhibit Inflammation Caused by Downregulated H_2S/CSE Pathway *In Vitro*

In order to explore the biological significance of elevated endogenous SO_2 levels induced by downregulation of endogenous H_2S/CSE pathway, CSE knockdown EA.hy926 cells were treated with HDX, an AAT inhibitor. The results showed that compared with vehicle group, the SO_2 level in the EA.hy926 cell supernatant was sharply increased, and the further treatment by HDX reversed the increased SO_2 caused by CSE knockdown (**Figure 5A**). Meanwhile, the phosphorylation of NF-κB p65 (pp65/p65) and the expression of ICAM-1 which denoted the inflammatory response in the EA.hy926 cell were also upregulated by CSE knockdown. However, the treatment of HDX aggravated the increase in the phosphorylation of NF-κB p65 and the expression of ICAM-1, which resulted from the deficiency of endogenous H_2S/CSE pathway (**Figures 5B,C**).

Furthermore, the same protocol of the experiment was done on primary HUVECs. The ratio of phosphorylated IκBα/IκBα (p-IκBα/IκBα), IκBα protein level, the ratio of pp65/p65, and the expression of ICAM-1 were also detected by western blot. The inflammatory cytokines IL-6 and TNF-α in primary HUVEC supernatant were detected by ELISA. Compared with the vehicle group, the SO_2 level in primary HUVECs was markedly increased by CSE knockdown, and HDX blocked the increase in SO_2 content in cell supernatant (**Figure 6A**). The ratio of p-IκBα/IκBα, the ratio of pp65/p65, and the expression of ICAM-1 were all upregulated but IκBα protein level was reduced by CSE knockdown (**Figures 6B–D**). Meanwhile, the inflammatory cytokines, IL-6 and TNF-α, in primary HUVEC supernatant were elevated by CSE knockdown (**Figures 6E,F**). However, the treatment of HDX promoted the increase in IκBα and NF-κB p65 phosphorylation and the level of inflammatory cytokines, and aggravated the decrease in IκBα protein level, which resulted from the deficiency of endogenous H_2S/CSE pathway in the primary HUVECs (**Figures 6B–F**).

The results observed in the following primary RPAECs were in accordance with those in both EA.hy926 cells and primary HUVECs (**Figure 7**). HDX inhibited the increased SO_2 content in the supernatant of primary RPAECs but aggravated the decrease in IκBα protein level and the increase in the phosphorylation of p65 and the levels of ICAM-1, IL-6, and TNF-α in cell supernatants induced by CSE knockdown.

Collectively, the above data implied that the upregulated endogenous SO_2 production might exert compensatory effects to inhibit the inflammation caused by H_2S/CSE deficiency.

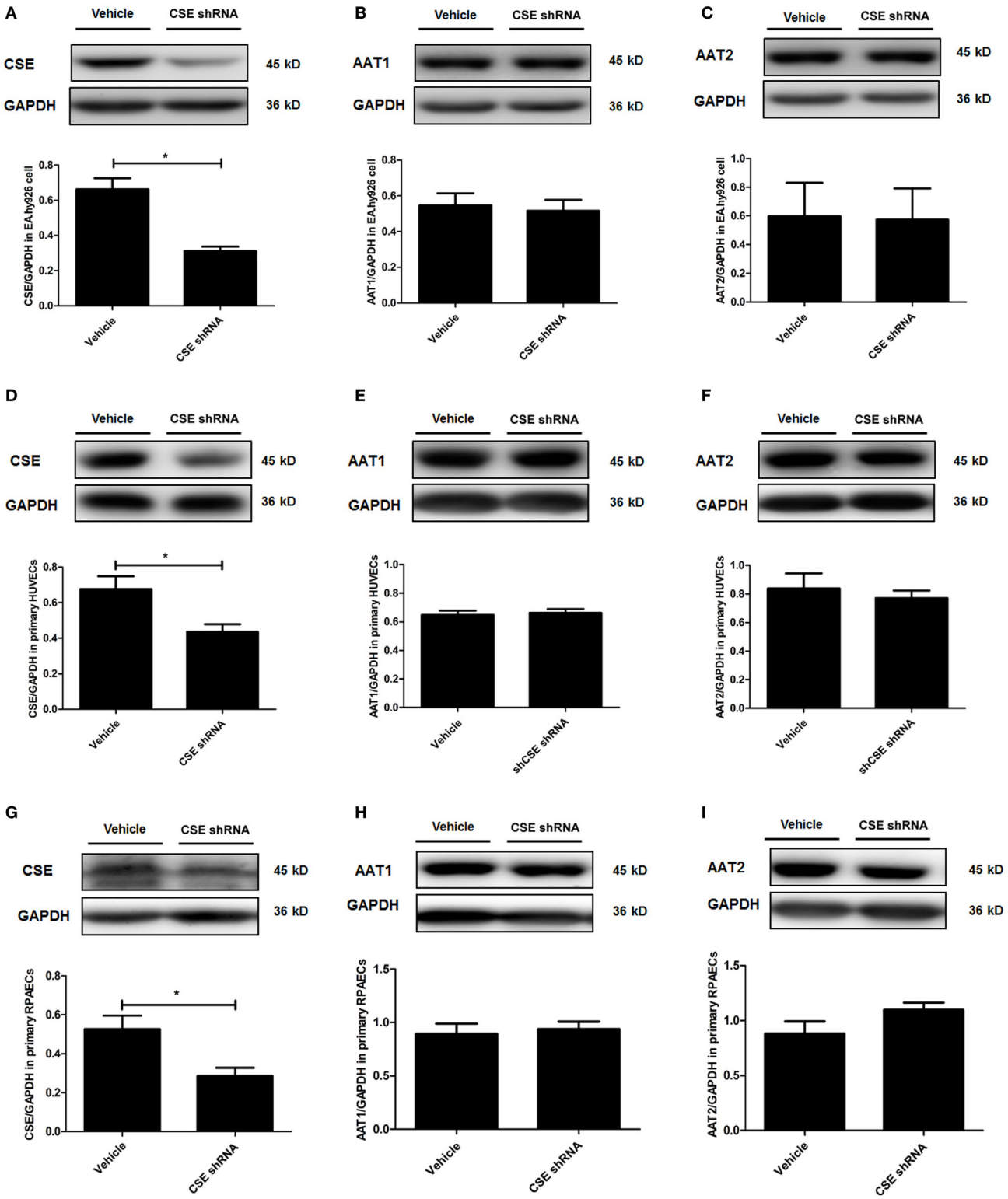

FIGURE 2 | Cystathionine-γ-lyase (CSE) knockdown did not affect the expression of AAT1 and AAT2 in the endothelial cells. The EA.hy926 cells, primary human umbilical vein endothelial cells (HUVECs), and primary rat pulmonary artery endothelial cells (RPAECs) were transfected with vehicle lentivirus or lentivirus containing CSE shRNA. The expressions of CSE **(A)**, AAT1 **(B)**, and AAT2 **(C)** in the EA.hy926 cell were detected by western blot. The expressions of CSE **(D)**, AAT1 **(E)**, and AAT2 **(F)** in the primary HUVECs were detected by western blot. The expressions of CSE **(G)**, AAT1 **(H)**, and AAT2 **(I)** in the primary RPAECs were detected by western blot. $^*P < 0.05$. Data are expressed as means ± SEM, and all experiments were performed independently for at least three times.

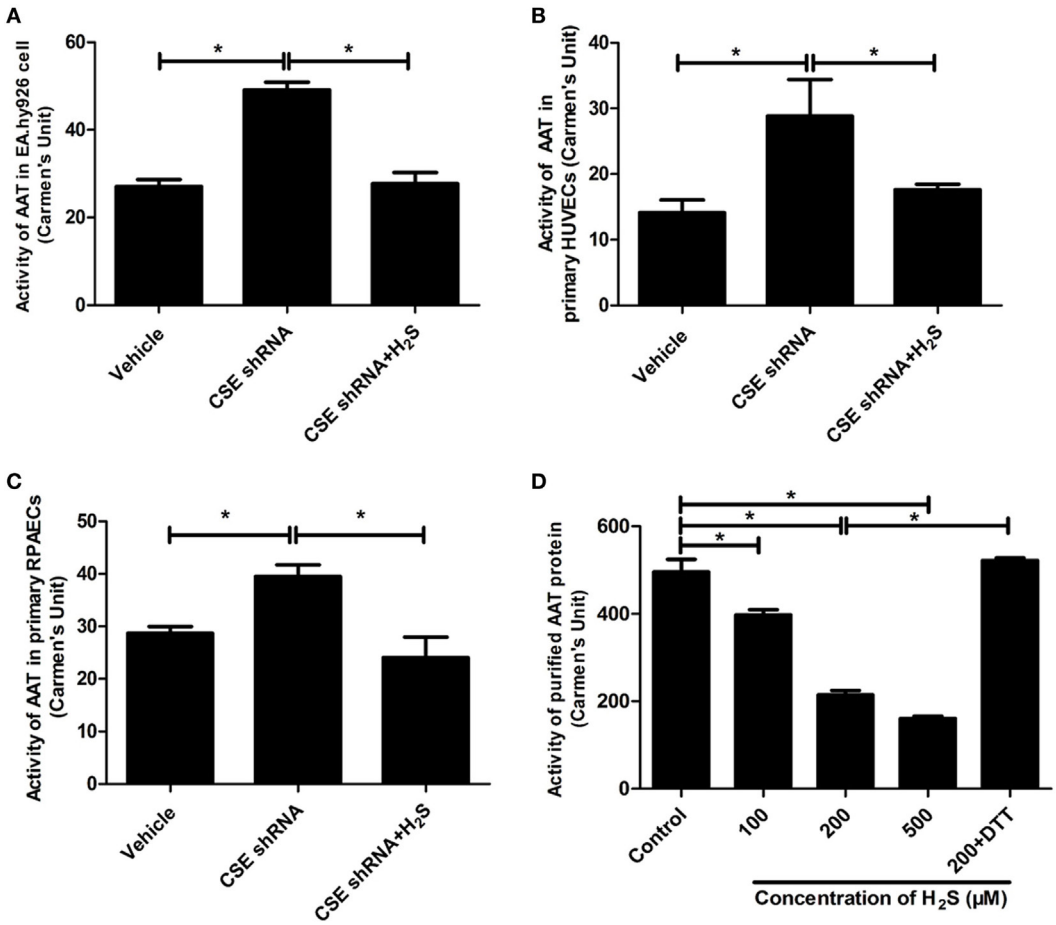

FIGURE 3 | Endogenous H_2S inhibited aspartate aminotransferase (AAT) activity in the endothelial cells and purified AAT protein. The activity of AAT in the EA.hy926 cells, primary human umbilical vein endothelial cells (HUVECs), primary rat pulmonary artery endothelial cells (RPAECs), and purified AAT protein were detected using colorimetric method. The endothelial cells were intervened by cystathionine-γ-lyase (CSE) knockdown, then pretreated with or without H_2S donor (200 μM) for 24 h. **(A)** AAT activity in the EA.hy926 cells. **(B)** AAT activity in the primary HUVECs. **(C)** AAT activity in the primary RPAECs. **(D)** AAT activity of purified AAT protein. Different concentrations of H_2S donor NaHS (100, 200, and 500 μM) were incubated with purified AAT protein from pig heart for 2 h. In the 200 μM H_2S plus DTT treatment, purified AAT protein was pretreated with NaHS (200 μM) for 1 h, and then incubated with 1 mM DTT for a further 1 h in the continuous presence of NaHS. $^*P < 0.05$. Data are expressed as means ± SEM, and all experiments were performed independently for at least three times.

Upregulation of Endogenous SO_2 Production Exerted Compensatory Effects to Inhibit Pulmonary Vascular Inflammation Caused by Downregulated H_2S/CSE Pathway *In Vivo*

In order to further elucidate the significance of upregulated endogenous SO_2 production induced by the deficiency of endogenous H_2S/CSE pathway in the development of vascular inflammation, we constructed a rat model of pulmonary hypertension in which the endogenous H_2S production was suppressed by MCT stimulation. The data showed that compared with the control group, the systolic, diastolic, and mean pulmonary arterial pressures in the rats of MCT group were increased, respectively (**Figures 8A–C**). Moreover, the thickened media of small pulmonary artery and the increased inflammatory cytokines IL-6 and TNF-α in the lung tissue in MCT-treated rat were demonstrated (**Figures 8D–F**). Simultaneously, the H_2S content in the lung tissue of rats in the MCT group was lower than that of the control group (**Figure 8G**). The supplement of H_2S donor NaHS rescued the pulmonary hypertension, pulmonary vascular remodeling and pulmonary vascular inflammation in the rats of MCT group (**Figures 8A–F**).

In the above rat model, the effect of H_2S on the endogenous SO_2/AAT pathway was examined. The results demonstrated that compared with the control group, SO_2 level, and AAT activity in the lung tissues of rats in the MCT group were increased significantly (**Figures 8H,I**). Moreover, the sulfhydration of AAT1 and AAT2 in the lung tissues of MCT rats was decreased compared with the control group (**Figures 8J,K**). Compared with MCT group, SO_2 level and AAT activity in the lung tissues of the rats in the MCT + H_2S group were reduced, while sulfhydrated AAT1 and AAT2 were increased (**Figures 8H–K**), suggesting that the supplement of H_2S donor NaHS restored the H_2S level in the lung tissue of MCT rats, and subsequently blocked the upregulation of endogenous SO_2/AAT pathway.

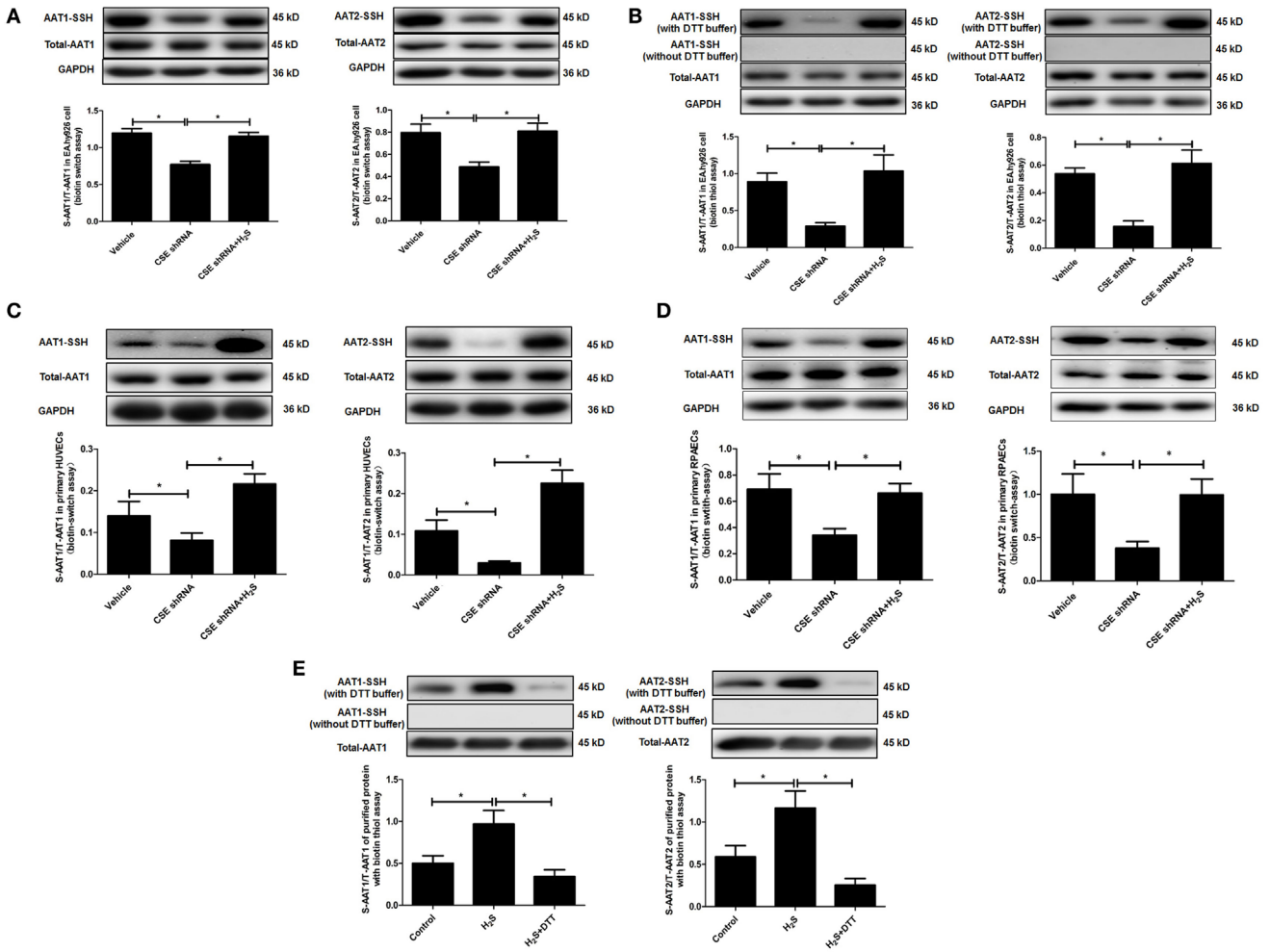

FIGURE 4 | Endogenous H_2S induced the sulfhydration of aspartate aminotransferase (AAT) in the endothelial cells and purified protein. **(A)** Sulfhydration of AAT1 and AAT2 in the EA.hy926 cell detected by biotin switch assay. **(B)** Sulfhydration of AAT1 and AAT2 in the in the EA.hy926 cell detected by biotin thiol assay. **(C)** Sulfhydration of AAT1 and AAT2 in the primary human umbilical vein endothelial cells (HUVECs) detected by biotin switch assay. **(D)** Sulfhydration of AAT1 and AAT2 in the primary rat pulmonary artery endothelial cells (RPAECs) detected by biotin switch assay. **(E)** Sulfhydration of purified AAT1 and AAT2 proteins from pig heart detected by biotin thiol assay. *$P < 0.05$. Data are expressed as means ± SEM, and all experiments were performed independently for at least three times.

Furthermore, the significance of upregulated SO_2/AAT pathway in the pulmonary vascular inflammation associated with the downregulation of endogenous H_2S production was explored in the MCT rats treated with an AAT inhibitor HDX. The data showed that compared with the MCT group, AAT activity and SO_2 level were suppressed significantly in the lung tissue of rats in the MCT + HDX group (**Figures 9A,B**). While HDX aggravated the increase in the phosphoralyation of NF-κB p65, ICAM-1 protein expression, the level of IL-6, and TNF-α in the lung tissue of MCT-treated rats (**Figures 9C–F**). In addition, the thickened media of small pulmonary artery in MCT-treated rats was exacerbated by HDX (**Figure 9G**), suggesting that the upregulation of endogenous SO_2 pathway might be an important compensatory response when the endogenous H_2S pathway collapsed in the development of pulmonary vascular inflammation and pulmonary vascular remodeling.

DISCUSSION

The impaired H_2S/CSE pathway was one of important pathogenesis of many cardiovascular diseases due to the lack of protective effect of endogenous H_2S on the heart and vessel. The facts that CSE knockout mice exhibited a series of marked cardiovascular pathological phenotypes further supported the significance of endogenous H_2S/CSE in the cardiovascular regulation and diseases. For example, Yuan et al. found that vascular endothelial growth factor (VEGF)-induced vascular solute hyperpermeability was blunted in the CSE gene deficient mice, suggesting that endothelium-derived H_2S protected the endothelial solute barrier function (44). Mani et al. discovered that CSE gene depletion promoted aortic intimal proliferation and accelerated atherosclerotic development in the ApoE knockout mice fed with atherogenic diet (45). CSE knockout mice were also found to exhibit a delayed

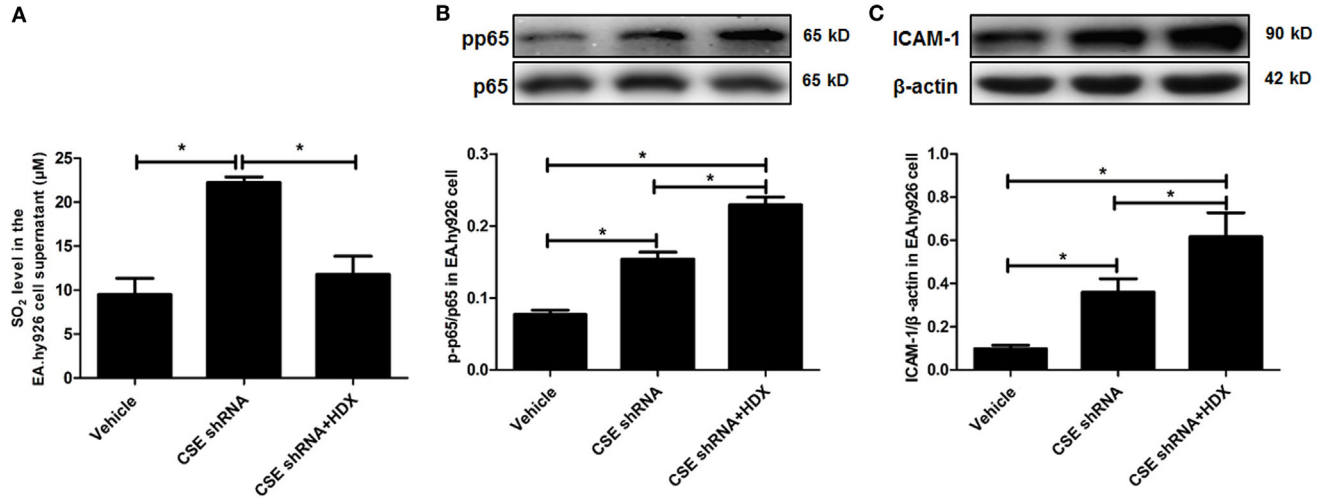

FIGURE 5 | Aspartate aminotransferase inhibitor L-aspartate-β-hydroxamate (HDX) aggravated the inflammation caused by cystathionine-γ-lyase CSE knockdown in EA.hy926 cell. **(A)** The SO_2 level in the supernatant of EA.hy926 cells detected by HPLC analysis. **(B)** The phosphorylation of NF-κB p65 in the EA.hy926 cell detected by western blot. **(C)** The expression of ICAM-1 in the EA.hy926 cell detected by western blot. *$P < 0.05$. Data are expressed as means ± SEM, and all experiments were performed independently for at least three times.

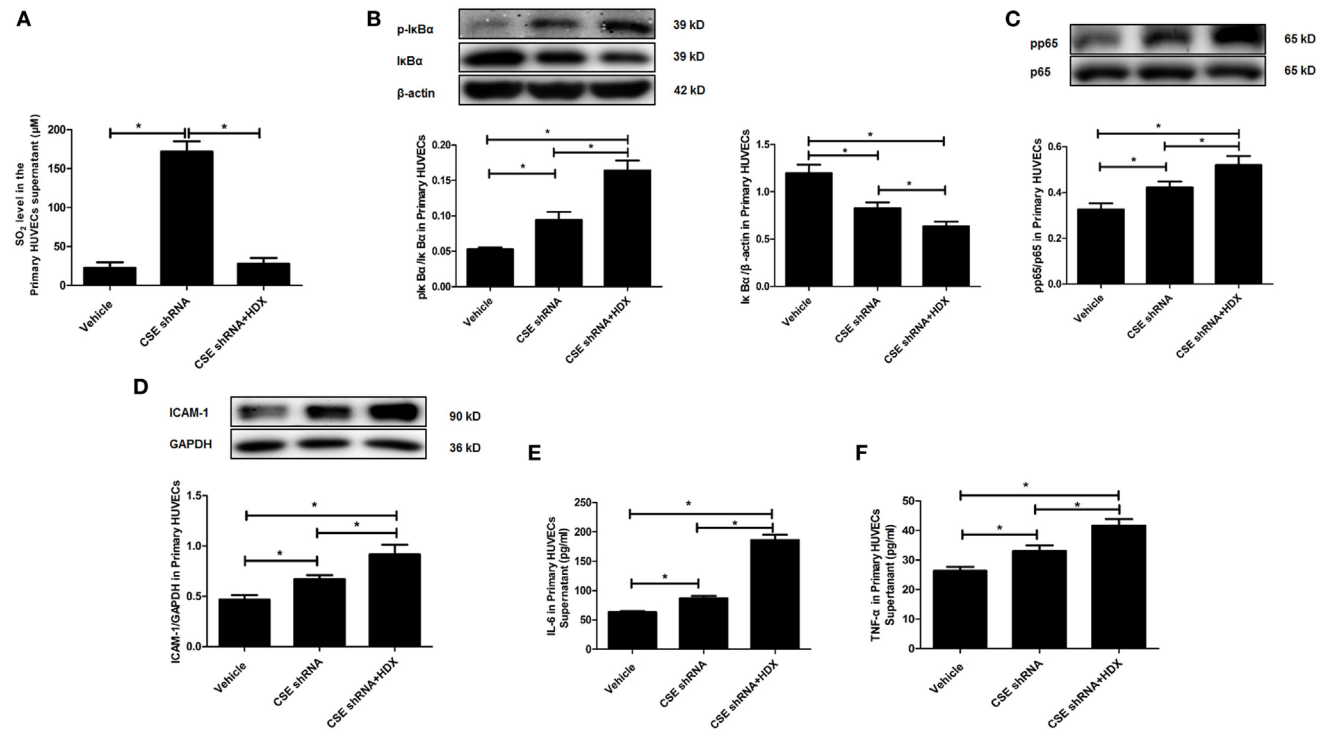

FIGURE 6 | Aspartate aminotransferase inhibitor L-aspartate-β-hydroxamate (HDX) aggravated the inflammation caused by cystathionine-γ-lyase (CSE) knockdown in primary human umbilical vein endothelial cells (HUVECs). **(A)** The SO_2 level in the supernatant of primary HUVECs detected by high-performance liquid chromatography analysis. **(B)** The phosphorylated and total IκBα in the primary HUVECs detected by western blot. **(C)** The phosphorylation of NF-κB p65 in the primary HUVECs detected by western blot. **(D)** The expression of ICAM-1 in the primary HUVECs detected by western blot. The levels of IL-6 **(E)** and TNF-α **(F)** in the supernatant of the primary HUVECs detected by ELISA. *$P < 0.05$. Data are expressed as means ± SEM, and all experiments were performed independently for at least three times.

wound healing and a markedly reduced microvessel formation in response to VEGF (46). In the present study, we observed the variation of endogenous SO_2/AAT pathway, another protector in the cardiovascular system, in a CSE knockdown endothelial cell model and further explored its pathological significance in the endothelial cell inflammation.

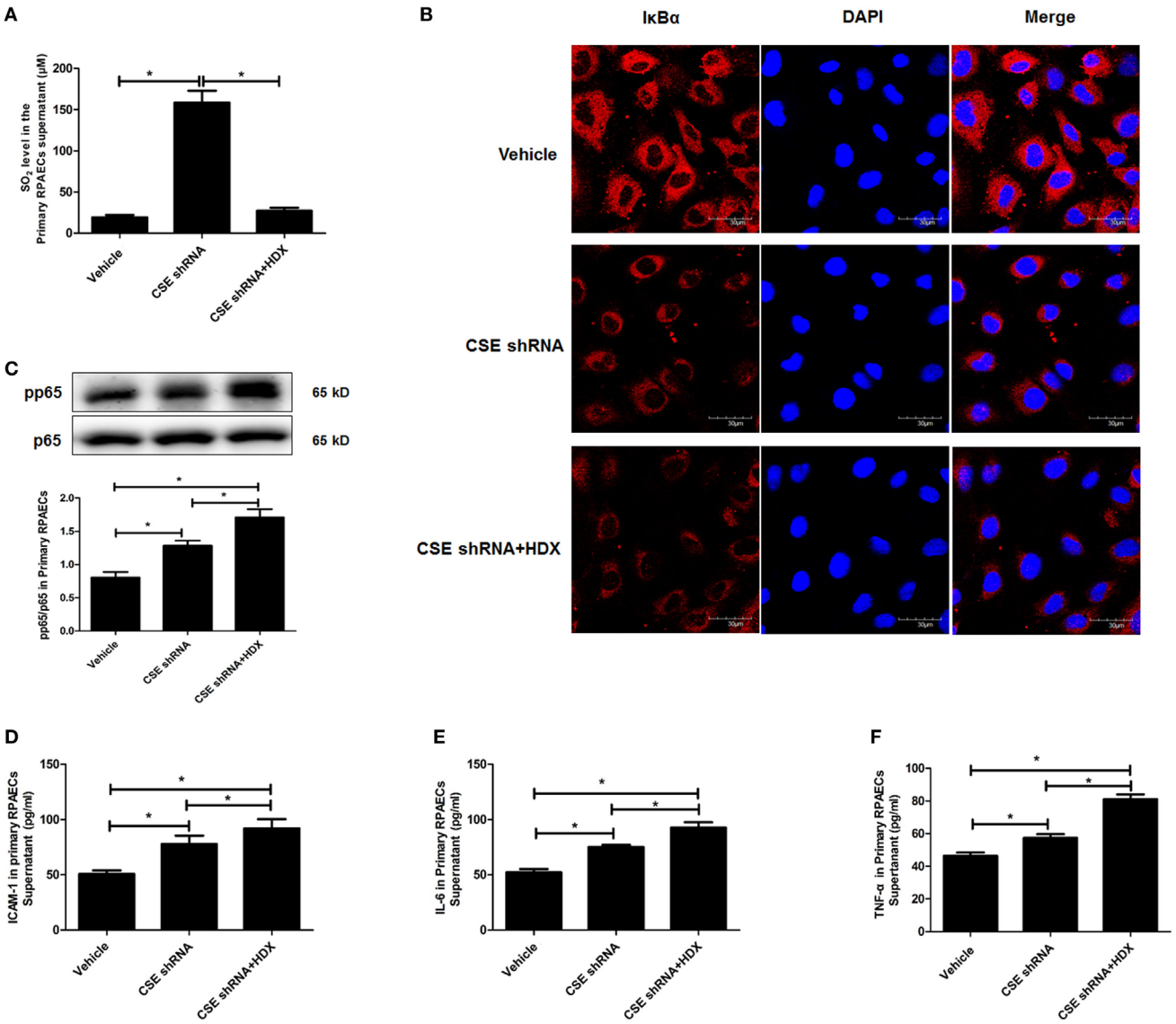

FIGURE 7 | Aspartate aminotransferase inhibitor L-aspartate-β-hydroxamate (HDX) aggravated the inflammation caused by cystathionine-γ-lyase (CSE) knockdown in primary rat pulmonary artery endothelial cells (RPAECs). **(A)** The SO_2 level in the supernatant of primary RPAECs detected by HPLC analysis. **(B)** The expression of IκBα in the primary RPAECs detected by western blot. **(C)** The phosphorylation of NF-κB p65 in the primary RPAECs detected by western blot. The level of ICAM-1 **(D)**, IL-6 **(E)**, and TNF-α **(F)** in the supernatant of the primary RPAECs detected by enzyme-linked immunosorbent assay. *$P < 0.05$. Data are expressed as means ± SEM, and all experiments were performed independently for at least three times.

Firstly, we observed the change of endogenous SO_2 generation in CSE knockdown EA.hy926 cells using SO_2 fluorescent probe. The results showed that endogenous SO_2 level in the CSE knockdown endothelial cells was markedly higher than that in the vehicle endothelial cells, while H_2S level in the culture supernatant and endothelial cells of CSE shRNA group was decreased compared with vehicle group. Moreover, H_2S donor NaHS raised the H_2S level in the supernatant and endothelial cells of CSE shRNA group, and blocked the increase in the SO_2 level caused by CSE knockdown. In accordance with the results obtained from the HUVEC line, the levels of SO_2 in the primary HUVECs and RPAECs were also increased by the impaired H_2S/CSE pathway, while the restoration of H_2S content in the primary endothelial cells abolished the increase in the endogenous SO_2 generation. The abovementioned data confirmed that the endogenous H_2S inhibited endothelium-derived SO_2 production.

Aspartate aminotransferase is regarded as the key enzyme generating endogenous SO_2 in the mammal animals. There are two kinds of AAT isoenzymes: AAT1 locates in the cytoplasm and AAT2 in the mitochondria (18, 47). Considering that the expression and activity of AAT are the major elements to control the endogenous SO_2 production (48), we measured the expression and activity of AAT in the CSE knockdown EA.hy926 cells to explore the mechanism by which endothelium-derived H_2S

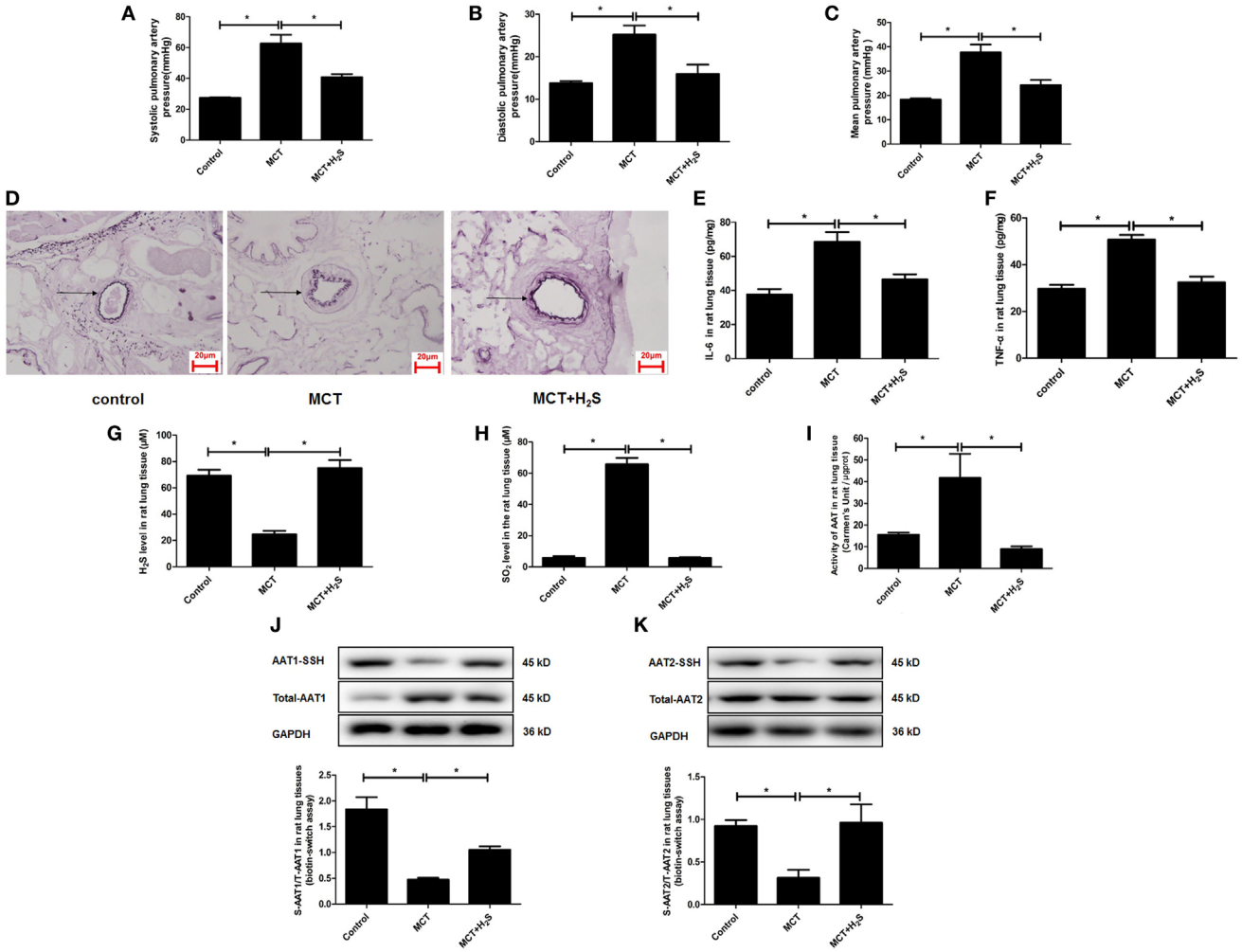

FIGURE 8 | The endogenous sulfur dioxide (SO_2)/aspartate aminotransferase (AAT) pathway was upregulated by the downregulated H_2S production in the rats with pulmonary vascular inflammation. **(A–C)** The systolic pulmonary artery pressure, diastolic pulmonary artery pressure and mean pulmonary artery pressure of rats measured by right heart catheterization. **(D)** The morphological change of small pulmonary arteries in the rats observed following the staining by the modified Weigert's elastic fiber dye. The small pulmonary artery was indicated by dark arrow. The internal and external elastic lamina were shown as dark-purple color under microscope. **(E,F)** The level of IL-6 and TNF-α in the rat lung tissue detected by enzyme-linked immunosorbent assay. **(G)** The H_2S level in the rat lung tissues detected by a an H_2S-selective sensor. **(H)** The SO_2 level in the rat lung tissues detected by HPLC assay. **(I)** AAT activity in the rat lung tissues detected by colorimetry assay. **(J,K)** Sulfhydration of AAT1 and AAT2 in the rat lung tissues detected by biotin switch assay. *$P < 0.05$. Data are expressed as means ± SEM, $n = 6$.

repressed endogenous SO_2 generation. The western blot results showed that there was no difference in the expression of AAT1 and AAT2 in the EA.hy926 cells between vehicle group and CSE shRNA group, suggesting endogenous H_2S did not affect the expression of AAT protein. We further investigated the role of endogenous H_2S in the control of AAT activity. Interestingly, the enzymatic activity of AAT in the CSE shRNA endothelial cells was higher than that in the vehicle endothelial cells, while H_2S donor supplement alleviated the enhancement of AAT activity induced by CSE knockdown. The discrete regulation of the AAT protein expression and activity by H_2S in the EA.hy926 cells was completely reproduced in both primary endothelial cells. Moreover, in *in vitro* experiment, H_2S donor was found to directly inhibit activity of purified AAT protein in a concentration-dependent manner, which further supported the speculation that endogenous H_2S suppressed endothelium-derived SO_2 generation *via* inhibiting AAT activity. In fact, the detached regulation of the AAT expression and activity was reported, although the expression and activity of AAT were identically controlled in general. For example, Barouki et al. found that the regulation of AAT1 mRNA in the Fao rat hepatoma cell line by dexamethasone correlated with the variation of the AAT1 activity, suggesting that dexamethasone acted at the transcriptional level (49). However, cortisol acetate treatment did not alter AAT1 activity but reduced AAT1 mRNA in rat muscles (50). Therefore, we supposed that the discrete effects of H_2S on the AAT1/2 protein expression, and activity might result from the fact that H_2S regulated SO_2/AAT at a posttranslational level.

Secondly, we tested how H_2S inhibited AAT activity. It is well known that endogenous H_2S regulates various cellular processes

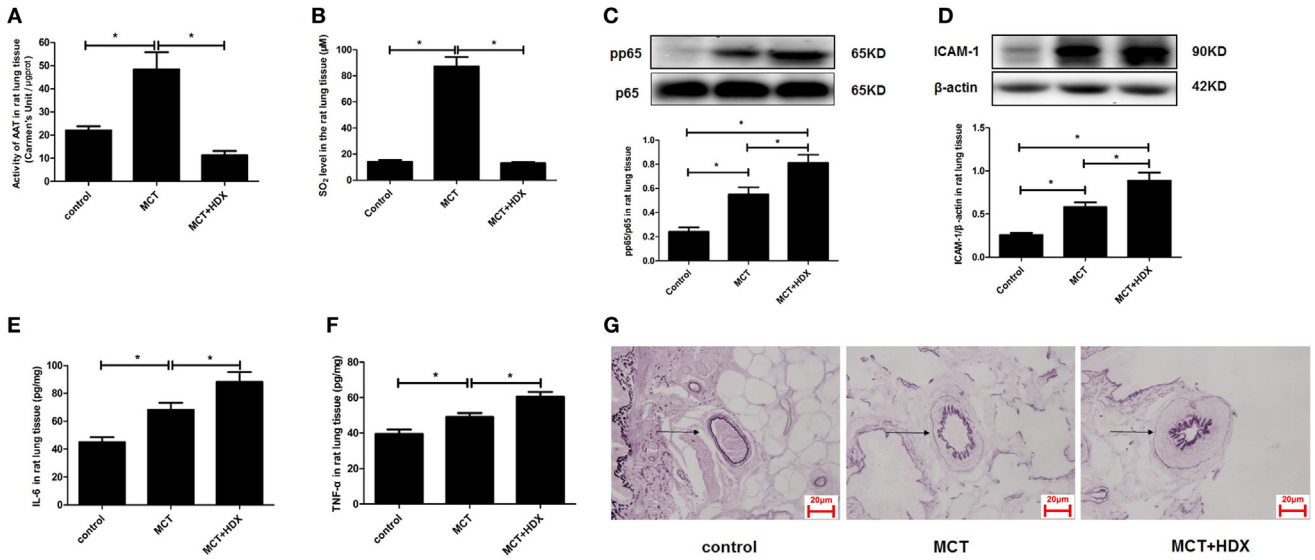

FIGURE 9 | Upregulation of endogenous SO_2 production exerted compensatory effects to inhibit pulmonary vascular inflammation caused by downregulated H_2S/cystathionine γ-lyase (CSE) pathway *in vivo*. **(A)** Aspartate aminotransferase (AAT) activity in the rat lung tissues detected by colorimetry assay. **(B)** The SO_2 level in the rat lung tissues detected by high-performance liquid chromatography assay. **(C)** The ratio of pp65/p65 in the rat lung tissues detected by western blotting. **(D)** The expression of ICAM-1 in the rat lung tissues detected by western blotting. **(E,F)** The level of IL-6 and TNF-α in the rat lung tissue detected by enzyme-linked immunosorbent assay. **(G)** The morphological change of small pulmonary arteries in the rats observed following the staining by the modified Weigert's elastic fiber dye. The small pulmonary artery was indicated by dark arrow. The internal and external elastic lamina were shown as dark-purple color under microscope. $^*P < 0.05$. Data are expressed as means ± SEM, $n = 7$.

via S-sulfhydration of target proteins, a posttranslational modification at the thiol group in the cysteine residue in the proteins such as Keap1, P66Shc, and NF-κB (1, 33, 42, 51–53), while the thiol group in the cysteine residue is also the molecular target of redox regulation. Coincidentally, AAT expression and activity were controlled in an oxygen-related manner in a rodent model of acute ischemic stroke (54). In the present study, we found that DTT, a thiol reductant, could reverse the H_2S-induced decrease in the AAT activity in the *in vitro* experiment, suggesting the thiol group might be involved in the regulation of the AAT activity by H_2S. Therefore, we detected the sulfhydration of AAT1 and AAT2 in the CSE knockdown EA.hy926 cells using the modified biotin switch assay (33). The data showed that CSE knockdown reduced the sulfhydration of AAT1 and AAT2 in the endothelial cells, while H_2S donor enhanced the sulfhydration of AAT1 and AAT2 in the EA.hy926 cells of CSE shRNA group. To confirm the fact that endogenous H_2S sulfhydrated AAT protein, we used biotin thiol assay (43), another method for detecting sulfhydration, to investigate the modification of AAT by H_2S. The change of sulfhydration of AAT detected by biotin thiol assay was similar to the results using modified biotin switch assay. Moreover, in the *in vitro* experiments we discovered that NaHS induced a marked sulfhydration of AAT1 and AAT2, which was blocked by DTT treatment. In the experiment on the primary HUVECs and RPAECs, the decrease in the sulfhydration of AAT1 and AAT2 caused by CSE knockdown was also rescued by NaHS. Those data suggested that sulfhydration of AAT might mediate the inhibitory effect of endogenous H_2S on the AAT activity.

On the basis of H_2S/CSE deficiency-induced inflammation endothelial cell model, we further investigated the pathological significance of CSE knockdown-enhanced endogenous SO_2 production. We used HDX, an AAT inhibitor, to block the increased endogenous SO_2 production in the HUVEC line, primary HUVECs and RPAECs, and observed the changes of NF-κB pathway, a pivot regulator of cellular inflammation, and its downstream target genes including inflammatory cytokine ICAM-1, IL-6, and TNF-α. NF-κB, consisting of p65 and p50 subunits, locates in the cytosol complexed with the inhibitory protein IκBα in an inactivated state. Inflammatory stimuli such as hypoxia can activate the phosphorylation of IκBα, leading to the IκBα degradation and dissociation from NF-κB. The released NF-κB is subsequently phosphorylated, translocates into the nucleus and increases the transcription of inflammatory cytokines (33). ICAM-1 is typically expressed on the surface of endothelial cells and of other inflammatory cells and mediates the binding of leukocytes to endothelial cell by coupling its ligand integrin. ICAM-1 is regarded as a classical marker of endothelial inflammation (55). Therefore, we detected the phosphorylated IκBα, total IκBα, phosphorylated NF-κB p65, and ICAM-1 protein in the endothelial cells and ICAM-1, IL-6, and TNF-α levels in the supernatant to reflect the endothelial cell inflammation. As we expected, HDX aggravated the increase in the expression of ICAM-1 and the phosphorylation of NF-κB p65 in the CSE knockdown EA.hy926 cells. Moreover, HDX was found to promote phosphorylation of IκBα, decrease IκBα protein level, and raise the phosphorylated NF-κB p65 in both primary endothelial cells. The effects of HDX on the inflammatory cytokines in the

supernatant of primary endothelial cells were in line with the regulation on the NF-κB pathway. Therefore, we supposed that endogenous SO_2/AAT pathway was upregulated as a compensatory mechanism for the downregulated endogenous H_2S pathway in the endothelial cell inflammation.

Finally, we further explored the importance of upregulated SO_2/AAT pathway following the broken H_2S/CSE pathway in the *in vivo* experiments. As previously reported, endogenous H_2S production in the rat lung tissues was downregulated by MCT treatment in a rat model of pulmonary vascular inflammation (5). Conversely, SO_2 content and AAT activity in the lung tissue of MCT-treated rats were enhanced, while the AAT1 and AAT2 sulfhydraton was reduced. More interestingly, the restoration of H_2S level reversed the upregulation of endogenous SO_2/AAT pathway, demonstrated by the facts that NaHS increased the sulfhydrated AAT1 and AAT2, inactivated the AAT activity and reduced SO_2 level in the lung tissue of rats in the MCT groups. Furthermore, as designed in the endothelial cell experiments, we used HDX and found that it inhibited the upregulation of endogenous SO_2/AAT pathway. As we expected, the pulmonary vascular inflammation reflected by the phosphorylation of NF-κB p65 and the elevated inflammatory cytokines including ICAM-1, IL-6, and TNF-α was exacerbated when the deficient H_2S-induced SO_2/AAT pathway was blocked by HDX. Moreover, in our previous studies, HDX was also found to exacerbate the MCT-induced pulmonary vascular inflammation, demonstrated by the fact that HDX enhanced NF-κB p65 and ICAM-1 expression in the pulmonary artery endothelial cells in an immunohistochemical study (56). As a result, HDX aggravated the thickened media of pulmonary artery in the MCT-treated rats in accordance with the findings previously reported (56, 57). The abovementioned results were in accordance with the data obtained from *in vitro* endothelial cell experiment. Therefore, we supposed that endogenous SO_2/AAT pathway was upregulated as a compensatory mechanism for the downregulated endogenous H_2S pathway in the endothelial cell inflammation.

In brief, we firstly demonstrated that endogenous H_2S inhibited endothelial cell-derived SO_2 generation through suppressing AAT activity *via* sulfhydration *in vitro* and *in vivo*. When injury factors impaired H_2S/CSE pathway, the endogenous SO_2 production was subsequently induced as a reserved protector to protect the endothelial cell functions such as anti-inflammatory effects. Our findings deepen the understanding of regulatory mechanism responsible for cardiovascular homeostasis, providing a new insight for the exploration of interaction among bioactive small molecules. More molecular and cellular biological studies, however, need to be done for disclosing the precise target and mechanisms by which endogenous H_2S functions.

ETHICS STATEMENT

This study was carried out in accordance with the Animal Management Rule of the Ministry of Health of the People's Republic of China. The protocol was approved by the Animal Research Ethics Committee of Peking University First Hospital.

AUTHOR CONTRIBUTIONS

DZ, XW, XT and CL carried out the experimental work. DZ wrote the paper. HJ and YH designed and supervised the experiments. HJ, KL, XY and XT revised the primary manuscript. JZ, WK, JD and CT were responsible for the quality control and analysis. DZ, LZ, GY and YT participated in the data analysis. All authors approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.00882/full#supplementary-material.

FIGURE S1 | Transfection of cystathionine-γ-lyase (CSE) shRNA lentivirus downregulated the H_2S/CSE pathway in EA.hy926 cell. The EA.hy926 human umbilical vein endothelial cells (HUVECs) were, respectively, transfected with lentivirus containing CSE shRNA plus green fluorescent protein (GFP) cDNA at different concentrations, 1×10^4, 2×10^4, 4×10^4, 1×10^5, and 2×10^5 TU/mL to determine the appropriate concentration. **(A)** The GFP green fluorescence observed in the infected EA.hy926 cells after 72 h under fluorescence microscope. The images showed that more than 90% of cells showed green fluorescence at the lentivirus concentration of 1×10^5 and 2×10^5 TU/mL compared with control group, suggesting that the lentivirus was successfully transfected. **(B)** The expression of CSE in the EA.hy926 cells detected by western blot. Compared with the control group, the expression of CSE was decreased by 60.2% and 65.1% at the lentivirus concentration of 1×10^5 and 2×10^5 TU/mL. **(C)** The H_2S level in the supernatant of EA.hy926 cells was detected by H_2S-selective sensor. Compared with control group, the H_2S level in EA.hy926 cell supernatant was significantly decreased by 63.6% and 75%, respectively, which was similar to the change of CSE expression. * $P < 0.05$. Data are expressed as means ± SEM, and all experiments were performed independently for at least three times.

FIGURE S2 | The schematic protocol of biotin thiol assay to detect the S-sulfhydration. The sample protein was incubated with maleimide-PEG2-biotin to alkylate both cysteine residue and sulfhydrated cysteine residue at the first step. At the subsequent step, the high-capacity affinity streptavidin–agarose resin was used to pull down the proteins which were alkylated by maleimide-PEG2-biotin at the first step. At the last step, the proteins were reacted with buffer with or without DTT, a thiol reductant, for 30 min followed by centrifugation. DTT was used for cleaving the mixed disulfide bond and releasing the sulfhydrated protein. Therefore, sulfhydrated protein separated from the mixture containing DTT was detected by western blot, while supernatant separated from the mixture without DTT was used as negative control.

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REVIEW
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Endothelial Activation: The Ang/Tie Axis in Sepsis

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Sepsis, a dysregulated host response to infection that causes life-threatening organ dysfunction, is a highly heterogeneous syndrome with no specific treatment. Although sepsis can be caused by a wide variety of pathogenic organisms, endothelial dysfunction leading to vascular leak is a common mechanism of injury that contributes to the morbidity and mortality associated with the syndrome. Perturbations to the angiopoietin (Ang)/Tie2 axis cause endothelial cell activation and contribute to the pathogenesis of sepsis. In this review, we summarize how the Ang/Tie2 pathway is implicated in sepsis and describe its prognostic as well as therapeutic utility in life-threatening infections.

Keywords: endothelial dysfunction, Tie2 receptor, angiopoietins, sepsis, malaria, critical care

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INTRODUCTION

Sepsis is a state of life-threatening organ dysfunction caused by a dysregulated host response to infection (1). Despite being a leading cause of global morbidity and mortality, sepsis has no known specific therapies (2). The current critical illness classification defines organ dysfunction by an increase in the Sequential [sepsis-related] Organ Failure Assessment (SOFA) score (3). However, sepsis is a heterogeneous syndrome that is not completely characterized using non-specific clinical variables. The use of generic classification models for complex, critically ill patients may impede

Abbreviations: ABIN2, A20-binding inhibitor of nuclear factor-κB-2; ABA, Ang2-blocking antibody; ABTAA, Ang2-binding and Tie2-activating antibody; ADAM-15, disintegrin and metalloproteinase domain-containing protein 15; ADMA, asymmetric dimethyl arginine; Ang, angiopoietin; ARDS, acute respiratory distress syndrome; BBB, blood brain barrier; BDNF, brain-derived neurotrophic factor; Ca^{2+}, calcium; CaM, calmodulin; CD36, cluster of differentiation 36; CDDO-EA, 2-cyano-3,12-dioxooleana- 1,9(11)-dien-28-oic acid ethyl amide; CM, cerebral malaria; COMP-Ang1, cartilage oligomatrix protein Ang1; EC, endothelial cell; ECM, experimental cerebral malaria; EPCR, endothelial protein C receptor; ER, endoplasmic reticulum; ET-1, endothelin-1; FOXO1, forkhead box protein O1; GPCR, G-protein-coupled receptor; PAMP, pathogen-associated molecular pattern; ICAM-1, intracellular adhesion molecule-1; ICU, intensive care unit; IP_3R, inositol triphosphate receptor; IQGAP, IQ motif containing GTPase activating protein; KLF2, Krüppel-like factor-2; iRBC, infected red blood cell; LPS, lipopolysaccharide; MAT, matrilin; mDia, mammalian diaphanous; MEF2, myocyte enhancer factor-2; miR-30, micro ribonucleic acid-30-5p; MLCK, myosin light-chain kinase; NF-κB, nuclear factor-κB; NO, nitric oxide; NOS, nitric oxide synthase; eNOS, endothelial nitric oxide; NFR2, nuclear factor erythroid 2-related factor 2; p38 MAPK, p38 mitogen-activated protein kinase; p190RhoGAP, p190 Rho GTPase-activating protein; PfEMP-1, *Plasmodium falciparum* erythrocyte membrane protein-1; PI3K, phosphoinositide triphosphate kinase; Rac1, RAS-related C3 botulinum toxin substrate 1; rh-Ang1, recombinant Ang1; Rap1, Ras-related protein 1; RBC, red blood cell; rh, recombinant human; RhoA, Ras homolog gene family, member A; RhoGEF, Rho guanine nucleotide exchange factor; SOFA, sequential organ failure assessment; Src, proto-oncogene tyrosine-protein kinase; TRPC1, transient receptor potential channel-1; VE-cadherin, vascular endothelial-cadherin; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor; VE-PTP, vascular endothelial protein tyrosine phosphatase; WPB, Weibel-Palade bodies; p38 MAPK, p38 mitogen-activated protein kinase; PPAR, peroxisome proliferator-activated receptor.

appropriate triage and management and limit the application of personalized treatment strategies (4). A more comprehensive characterization of sepsis pathophysiology may reveal new opportunities for precision medicine-based therapies with either novel or repurposed agents that target specific pathways contributing to disease (4, 5).

Microvascular dysfunction is the endpoint of many life-threatening infections (6), and a well-established relationship exists between endothelial injury and sepsis (7–9). Determining the severity of microbial infections is challenging early in the course of disease when clinical scoring systems have limited prognostic utility. Many classic markers of end-organ compromise (such as serum lactate, bilirubin, and creatinine clearance) are not informative until significant clinical deterioration has occurred. However, the timely recognition of sepsis is critical as early aggressive management can considerably reduce morbidity and mortality (10). Early prognostic indicators of critical illness severity are needed to improve early recognition, appropriate triage, management, and outcomes, as well as to enable rational health resource allocation. This review examines the role of the angiopoietin (Ang)/Tie2 axis in sepsis and summarizes its potential applications in the early recognition of sepsis and as a therapeutic target to improve clinical outcomes.

THE Ang/Tie AXIS IN SEPSIS

Vascular function and permeability are regulated by endothelial-specific receptor tyrosine kinases and their ligands, including the vascular endothelial growth factor (VEGF)-VEGF-receptors (VEGFRs), and the Ang-Tie receptors. The Tie1 and Tie2 receptors constitute the Tie receptor family and are almost exclusively expressed in the endothelium (11, 12). Tie2 functions as a receptor for the Ang family of proteins (Ang1, Ang2, and Ang4), while Tie1 is an orphan receptor that can be activated by Angs *via* its interaction with Tie2 (13). Binding of Angs to Tie2 in the stable vasculature promotes the formation of a Tie1/Tie2 heterodimer in a β_1 integrin-dependent manner, resulting in Tie2 trafficking to cell–cell junctions (14, 15).

During vascular quiescence, mesenchymal cells secrete Ang1, a strong Tie2 agonist, to support endothelial survival and vascular stability (16). Under these conditions, oligomerized Ang1 promotes the *trans*-association of Tie2 at cell–cell contacts and can also anchor Tie2 to the extracellular matrix (ECM) through binding fibronectin, collagen, and vitronectin with high affinity (17). In addition to forming adhesive structures between cell–cell and cell–substratum contacts, Tie2 activation by Ang1 induces a number of downstream signaling cascades as shown in **Figure 1**. Notably, the serine kinase, Akt, is activated and results in the phosphorylation of the Forkhead box protein O1 (FOXO1) transcription factor, leading to the nuclear exclusion of FOXO1 and decreased expression of its target genes (18–20). The inhibition of *Foxo1* transcriptional activity in endothelial cells (ECs) induces expression of genes involved in vessel stability and the repression of genes involved in vascular destabilization, including Ang2. Consequently, during quiescence, Ang2 is constitutively expressed at low levels and co-localizes with von Willebrand factor (vWF) within the Weibel Palade bodies (WPBs) of ECs (21).

Upon stimulation of ECs by inflammatory cytokines or VEGF, Ang2 expression and secretion from WPB are increased, creating an autocrine regulatory mechanism of Tie2 signaling (36, 37). However, in contrast to Ang1, the action of Ang2 on Tie2 signaling has an additional level of complexity that is dependent on the microenvironment of ECs (38–41). While the Tie1/Tie2 heterodimeric complex enables both Ang1 and Ang2 to function as Tie2 agonists (14, 15), in the presence of an infection or inflammation ECs shed the Tie1 ectodomain, and Ang2 binding results in Tie2 antagonism (14). Similarly, Tie1 shedding decreases Ang1 agonistic activity (reduced Tie2 phosphorylation), demonstrating that Tie1 is required for the full activation of Tie2 (14, 15). Taken together, infection increases Ang2 expression and its release from WPBs, tipping the luminal Ang balance in favor of Ang2. Consequently, the increase in Ang2/Tie2 binding, particularly under conditions of enhanced Tie1 shedding, blocks Tie2 activation and contributes to the destabilization of the endothelium.

In addition, binding of Ang1 to Tie2 can also stimulate the association of vascular endothelial (VE)-protein tyrosine phosphatase (PTP) with the Tie receptor complex (42). Under conditions of hypoxia, such as that resulting from infection-induced reduction in laminar flow, VE-PTP expression is up-regulated (43), and a negative feedback loop is triggered to limit Tie2 activation (42, 44). As outlined in **Figure 2**, there are a number of mechanisms employed by ECs to modulate Ang/Tie2 signaling during infection-induced endothelial activation. Findings from our group (45–53) and many others (54–60) have shown that disruption of any of these components related to the Ang/Tie axis may result in endothelial dysregulation and microvascular leak, regardless of the microbial etiology.

Although beyond the scope of this review it should be noted that both coagulation and complement activation contribute to the course and outcome of sepsis. A connection between the Ang/Tie2 pathway and coagulation in sepsis was revealed in a proteomic analysis of septic patients with disseminated coagulation (DIC). Findings from this study demonstrated that changes in Tie2 signaling was an initiating event in septic DIC and, at least in a mouse model, restoring Tie2 activation was sufficient to mitigate thrombosis (78). Additionally, the anticoagulant, activated protein C (APC) has been shown to bind and activate Tie2, leading to improved endothelial barrier integrity (79). Still, little remains known about the interplay between complement activation and Ang/Tie2 pathways in sepsis; this is an area that requires further investigation.

THE Ang/Tie PATHWAY IN SEVERE BACTERIAL INFECTIONS

The incidence of severe sepsis in the United States is estimated at 3 cases per 1,000 population (80, 81). Mortality due to sepsis is high at approximately 20–55% (80–85), estimates that are relatively stable over nearly the past decade (86). Despite a high burden of disease and improved application of management strategies (87), there is a lack of effective treatments specific for sepsis (5, 88).

Gram-negative and Gram-positive infections occur with similar frequencies in hospitalized patients (89), and both can trigger

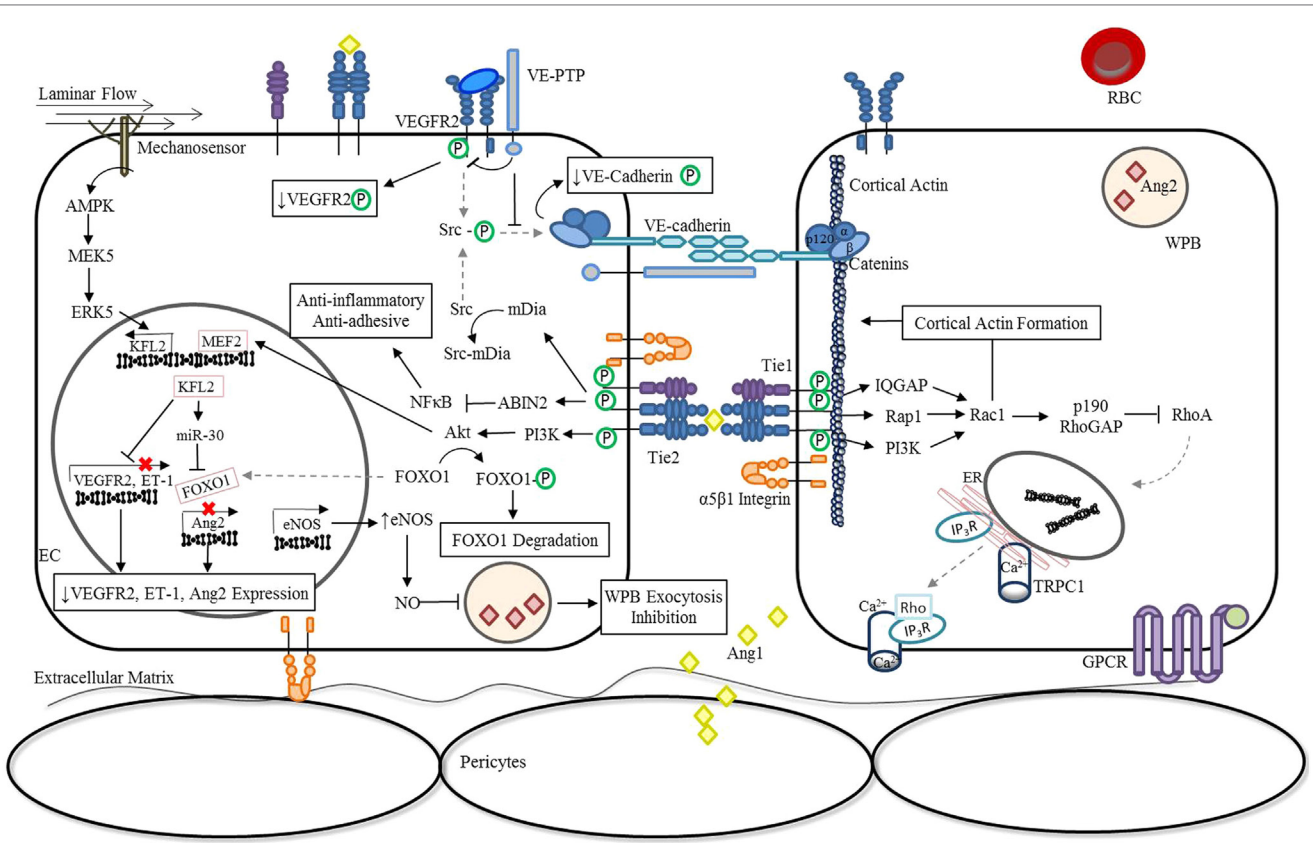

FIGURE 1 | Ang/Tie pathway regulation of vascular stability. In the absence of inflammation or infection (stable vasculature), Ang1 is secreted by mesenchymal cells (22, 23) and low levels of autocrine Ang2 are constitutively secreted (24). Binding of Angs to Tie2 promotes the interaction of Tie1 and Tie2 in a β_1 integrin-dependent manner and regulates Ang-induced Tie2 trafficking to cell–cell junctions (14, 15). Under these conditions, endothelial Tie1 enhances the activity of Ang1 and is essential for Ang2 agonistic activity (14, 15). Oligomerized Ang1 also induces the translocation of Tie2 to cell–cell contacts and induces an Ang1-bridged Tie2 *trans*-association (17). The resulting phosphorylation of Tie2 leads to the activation a number of downstream signaling pathways that are involved in vessel stability and endothelial barrier function. Activation of the downstream serine kinase, AKT, leads to the phosphorylation and nuclear exclusion of FOXO1, and repression of its target genes which include Ang2 (18–20). Ang1 inhibition of NF-κB reporter gene activity *via* activation of ABIN2 dampens the expression of adhesion molecules and pro-inflammatory cytokines (25, 26), preventing further activation of the endothelium through localized inflammatory mediators. In parallel, Ang/Tie2 signaling stimulates the transcriptional activity of MEF2 through the PI3K/AKT pathway to induce the expression of a second transcription factor KFL2 to ultimately counteract VEGF-mediated vascular permeability (↑eNOS expression; ↓VEGFR2 and ET-1 expression) (27). The increase in NO generated by eNOS combined with the negative regulation of Ang2 expression during quiescence significantly reduces luminal concentrations of Ang2 (28). In addition, KFL2 induces miR-30 expression, further blocking the transcription of Ang2 (29). The phosphorylation of Src, which generally culminates in the phosphodependent internalization of VE-cadherin, is also inhibited by Ang1/Tie2. Signaling through Ang1 leads to the activation of mDia, resulting in the sequestration of Src, and prevention of subsequent phosphorylation by VEGFR2 (30). At cell–cell junctions, Ang1/Tie2 also blocks VEGF signaling by promoting the interaction of VEGFR2 with VE-PTP (31). Lastly, activation of Tie2 can lead to the activation of the GTPase, Rac1, *via* IQGAP (32), Rap1 (33), or PI3K/Akt (34)-dependent pathways to stabilize the cortical actin cytoskeleton and maintain adherens and tight junctions between cells (35). In the presence of LPS, activation of the RhoA-specific GTPase activating protein, p190RhoGAP, by Rac1, is essential for shifting the balance away from RhoA rearrangement of the actin cytoskeleton and preventing vascular permeability (32, 35). Abbreviations: ABIN2, A20-binding inhibitor of nuclear factor-κB-2; Ang1, angiopoietin-1; Ang2, angiopoietin-2; EC, endothelial cell; eNOS, endothelial nitric oxide synthase; ER, endoplasmic reticulum; ET-1, endothelin-1; FOXO1, forkhead box protein O1; GPCR, G-protein-coupled receptor; IP_3R, inositol triphosphate receptor; IQGAP, IQ motif containing GTPase activating protein; KLF2, Krüppel-like factor-2; LPS, lipopolysaccharide; mDia, mammalian diaphanous; MEF2, myocyte enhancer factor-2; miR-30, microRNA-30-5p; NF-κB, nuclear factor-κB; NO, nitric oxide; PI3K, phosphoinositide triphosphate kinase; Rac1, RAS-related C3 botulinum toxin substrate 1; Rap1, Ras-related protein 1; RBC, red blood cell; RhoA, Ras homolog gene family, member A; Src, proto-oncogene tyrosine-protein kinase; TRPC1, transient receptor potential channel-1; VE-cadherin, vascular endothelial-cadherin; VEGF, vascular endothelial growth factor; VEGFR2, vascular endothelial growth factor receptor 2; VE-PTP, vascular endothelial protein tyrosine phosphatase; WPB, Weibel-Palade Body; P, phosphorylation.

sepsis mediated in part by the production of exotoxins or the release of bacterial cell wall components into the systemic circulation (32). These microbial products modulate many host response pathways, including in the Ang/Tie axis. The suppression of Ang1/Tie2 signaling and the associated microvascular leak are common features of many severe infections including bacterial sepsis (54). Reduced Tie2 activation during sepsis is the result of several perturbations to the pathway, including decreased Tie2 and Ang1 expression (24, 54, 57, 90–92), generation of soluble Tie receptors (14, 15, 40, 41, 66, 93, 94), and the antagonistic activity of Ang2 (14, 15).

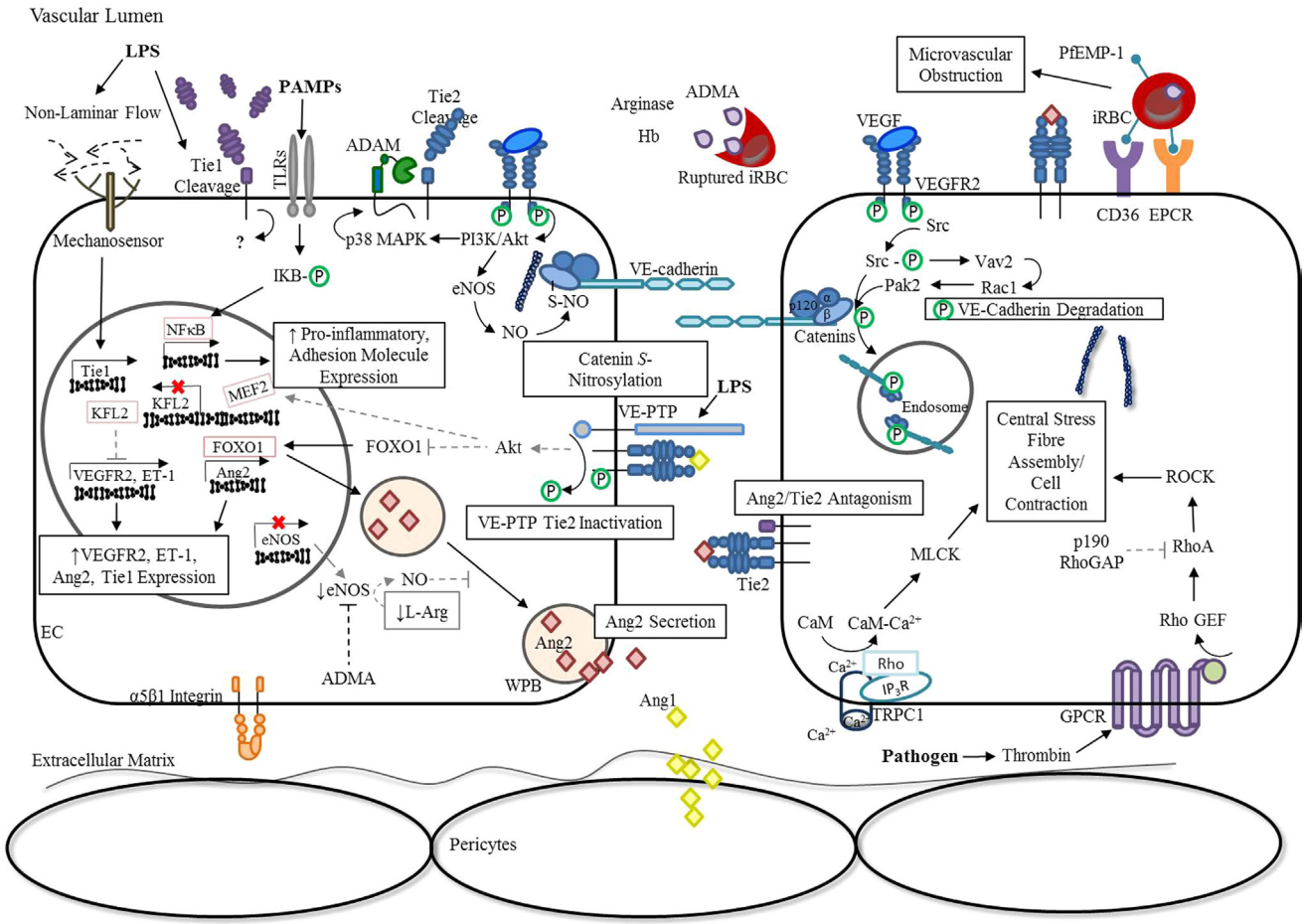

FIGURE 2 | Endothelial activation: dysregulation of Ang/Tie signaling during severe infection. In response to inflammation, ECs release Ang2 from WPB into the vascular lumen, tipping Ang2:Ang1 ratios in favor of Ang2 (21). The release of ADMA, arginase, and hemoglobin from ruptured *Plasmodium falciparum* infected erythrocytes additionally decrease the availability of NO, enhancing WPB exocytosis (61, 62). Simultaneously, the presence of infection or TNF-α leads to Tie1 inactivation by ectodomain cleavage, thereby reducing the agnostic activity of Ang1 and promoting the antagonistic action of Ang2 (14, 15). Under conditions of hypoxia, such as an infection-induced reduction in laminar flow, VE-PTP expression is up-regulated (43), and a negative feedback loop is triggered that limits Tie2 activation by enhancing its association with VE-PTP (42). Interestingly, the transcription of Tie1 is also up-regulated in response to reduced laminar flow, despite ectodomain cleavage, suggesting an unknown role for the intracellular tyrosine kinase domain in signaling under these conditions (63). The resulting inactivation of Tie2 during infection and/or inflammation promotes FOXO1 transcriptional activity, thereby increasing Ang2 expression (15, 18, 20). The reduction in KFL2 expression associated with Tie2 inactivation stimulates VEGF-induced monocyte adhesion and vascular permeability (27, 64, 65). Furthermore, increased VEGFR2-signaling during infection leads to the activation of downstream pathways, such as PI3K/Akt and Src. Consequently, p38 MAPK-dependent activation of the protease, ADAM-15, induces Tie2 shedding and prevents its downstream signaling (66). In parallel, VEGFR2 activation of eNOS and Src further disrupt adherens junction complexes through *S*-nitrosylation of β-catenin (67) and phosphorylation of VE-cadherin (68, 69), respectively. Pathogens can indirectly lead to the activation of RhoA through GPCRs (70), for example, thrombin binding of protease-activated receptors (71, 72), to promote the formation of actin stress fibers that increase centripetal tension throughout the cytoskeleton (73). Furthermore, limited Tie2 activation during infection prevents p190RhoGAP inhibition of RhoA (35), and subsequent coupling of IP_3R and TRPC1 to form a Ca^{2+} channel in the plasma membrane (74). This rise in intracellular Ca^{2+} leads to CaM-dependent activation of MLCK to further support EC contraction (35, 75). Lastly, TLR activation by PAMPs triggers the downstream activation of NF-κB to induce the expression of pro-inflammatory cytokines and adhesion molecules (76). This generates a positive feedback mechanism for endothelial destabilization, until infection and the associated inflammatory response are resolved (77). Abbreviations: ADAM-15, disintegrin and metalloproteinase domain-containing protein 15; ADMA, asymmetric dimethylarginine; Ang1, angiopoietin-1; Ang2, angiopoietin-2; Ca^{2+}, calcium; CaM, calmodulin; CD36, cluster differentiation 36; EC, endothelial cell; eNOS, endothelial nitric oxide synthase; EPCR, endothelial protein C receptor ET-1, endothelin-1; FOXO1, forkhead box protein O1; GPCR, G-protein-coupled receptor; iRBC, *Plasmodium*-infected red blood cell; IP_3R, inositol triphosphate receptor; IQGAP, IQ motif containing GTPase activating protein; KLF2, Krüppel-like factor-2; LPS, lipopolysaccharide; mDia, mammalian diaphanous; MEF2, myocyte enhancer factor-2; miR-30, microRNA-30-5p; MLCK, myosin light-chain kinase; NF-κB, nuclear factor-κB; NO, nitric oxide; p190RhoGAP, p190Rho GTPase-activating protein; p38 MAPK, p38 mitogen-activated protein kinase; PAMPs, pathogen-associated molecular patterns; PI3K, phosphoinositide triphosphate kinase; Rac1, RAS-related C3 botulinum toxin substrate 1; Rap1, Ras-related protein 1; RhoA, Ras homolog gene family, member A; RhoGEF: Rho guanine nucleotide exchange factor; S-NO, *S*-nitrosylation; Src, proto-oncogene tyrosine-protein kinase; TRPC1, transient receptor potential channel-1; VE-cadherin, vascular endothelial-cadherin; VEGF, vascular endothelial growth factor; VEGFR2, vascular endothelial growth factor receptor 2; VE-PTP, vascular endothelial protein tyrosine phosphatase; WPB, Weibel-Palade Body; P, phosphorylation.

Preclinical models of sepsis that have been used to explore the mechanisms and consequences of Tie2 signaling have shown that Tie2 expression and phosphorylation are greatly reduced during systemic infection. Inhibition of Tie2 activation leads to the nuclear localization of FOXO1 and transcription of its target genes, resulting in increased microvascular permeability (14, 15, 57, 91, 92). Tie2 mRNA levels have been shown to decline in response to decreases in endothelial shear stress associated with severe infection in a nuclear factor (NF)-κB dependent manner (92, 95). In contrast, the downregulation of Tie2 protein on the EC surface is not mediated by NF-κB but rather by the proteolytic cleavage of the Tie2 extracellular domain (92). *In vitro* studies have demonstrated both constitutive and VEGF-stimulated Tie2 receptor cleavage result in the release of a 75-kDa soluble Tie2 (sTie2) protein (66, 94, 96). The cleavage of the Tie2 ectodomain prevents Ang/Tie2 signaling and the circulating sTie2 which is generated may then function as a ligand trap, binding, and further inhibiting Ang activity. Indeed, the intravenous administration of adenoviral vectors expressing sTie2 blocked Ang/Tie2 signaling in *Mycoplasma pulmonis*-infected mice (97). Notably, the presence of sTie2 has been documented *in vivo* (93, 98, 99) and its levels are significantly increased in septic versus non-septic Intensive Care Units (ICU) patients (100). However, recent evidence incorporating mathematical and *in vitro* experimental modeling suggests that the molar ratio of sTie2:Ang1 levels found in patients with severe sepsis would have little influence on Ang/Tie2 activation *in vivo* (101).

In addition to reducing Tie2 expression, bacterial infections may also alter the functional activation state of this receptor, contributing to a leaky microvascular phenotype. Murine models of sepsis demonstrate a significant decline in the phosphorylated Tie2:total Tie2 ratio following LPS administration (91). This may be due, at least partly, to the influence of Tie1 on Tie2 activation. As already discussed, under baseline conditions, Ang1 or Ang2 stimulation of Tie2 promotes its interaction with Tie1 to form a heteromeric complex that is translocated to areas of cell–cell contacts (14, 17, 102). Oligomerized Ang1 bridges Tie2 at cell junctions resulting in the formation of *trans*-associations with Tie2 that preferentially activate Akt and its downstream signaling pathways, maintaining vascular quiescence (17). However in the mouse model, both LPS challenge and *M. pulmonis* infection induce cleavage of the Tie1 ectodomain responsible for its interaction with Tie2, thereby reducing Tie2 activation (14, 15). During endotoxemia, Tie1 cleavage also promotes antagonistic Ang2 activity resulting in the suppression of Tie2 signaling. This restores FOXO1 activity and establishes a positive feedback loop whereby FOXO1-driven Ang2 expression promotes microvascular leak during infection (14, 15).

Interestingly in murine models, Gram-negative bacteria may increase Ang2 expression by a different mechanism than that of Gram-positive bacteria. LPS has been shown to increase Ang2 expression *via* nicotinamide adenine dinucleotide phosphate (NADPH) oxidase 2 signaling through the inhibition of NFκ-B kinase subunit β/NF-κB and mitogen-activated protein kinase (MAPK)/activator protein 1 pathways (103), as well as the histone/protein deacetylase, Sirtuins 3 (24). Although mice treated with Gram-positive cell wall components (peptidoglycan and lipoteichoic acid) also exhibit elevated levels of Ang2 (24, 104), a comparison of bacterial etiologies among septic ICU patients revealed that Ang2/Ang1 ratios were significantly higher among patients with Gram-negative than those with Gram-positive infections (105). Recent evidence suggests that increased circulating Ang2 in Gram-positive infections is not the result of *de novo* biosynthesis, but rather the stimulated secretion of intracellular storage pools in response to the binding of cell wall components to TLR2 (24). While mechanistic differences may account for the more robust Ang2 increase observed in Gram-negative bacterial infections, these findings require *in vivo* confirmation.

During sepsis, an increase in Ang1 or decrease in Ang2 levels can enhance survival in murine bacterial sepsis models (56, 90, 106, 107). The overexpression of Ang1 using an adenoviral construct expressing human Ang1 (rh-Ang1) was found to attenuate LPS-induced expression of endothelial adhesion molecules in mouse lungs and kidneys, resulting in decreased leukocyte infiltration into interstitial spaces and minimizing hemodynamic instability (106, 107). In these mice, endothelial nitric oxide synthase (eNOS) expression was preserved and inducible nitric oxide (NO) synthase activity was decreased, contributing to reduced microvascular permeability in major organs (106, 107). The corresponding anti-inflammatory and anti-permeability effects of overexpressing Ang1 ultimately resulted in reduced organ injury as well as enhanced survival in endotoxemic mice compared to their Fc-controls. Despite the observed protective effect, little remains known as to how elevated levels of Ang1 impact the innate immune response. However, a recent study demonstrated that the improved survival conferred by the administration of rh-Ang1 in a mouse model of cerebral malaria (CM) was independent of its direct effects on parasitemia as both mice receiving rh-Ang1 and the Fc control had comparable parasite burdens (45). These findings suggest that increasing Ang1 levels during severe infection does not impair the host's ability to resolve infection.

The protective effect observed with Ang1 has also been described in a cecal ligation and perforation (CLP) model of mice with one functional Ang2 allele ($Ang2^{\pm}$), suggesting Ang2 contributes to multi-organ dysfunction and death in sepsis (56). In contrast, compared to their wild-type littermates, $Ang2^{-/-}$ knockout mice developed acute kidney injury following LPS exposure (90). Interestingly, the complete loss of Ang2 was previously observed to result in developmental abnormalities in mouse vasculature (108) likely rendering $Ang2^{-/-}$ mice susceptible to LPS-induced kidney injury. Nevertheless, these pre-clinical studies suggest that increasing circulating Ang1 and reducing Ang2 are associated with improved endothelial function during bacterial sepsis.

Bacterial pathogens are an important cause of sepsis, at least in part, due to their ability to induce systemic microvascular dysfunction through their interactions with the Ang/Tie system. A detailed understanding of mechanisms that regulate this pathway, and the ways in which bacteria modulate Tie2 activity, may suggest intervention strategies to maintain endothelial quiescence and microvascular integrity during severe bacterial infections. Preclinical studies of Tie2 directed therapies are reviewed in a later section.

ENDOTHELIAL DYSFUNCTION IN MALARIA INFECTION

Malaria is an acute infectious disease caused by parasitic protozoa of the genus *Plasmodium*. Severe malaria is a sepsis syndrome that was responsible for an estimated 429,000 deaths in 2016 (109). It is typically caused by *Plasmodium falciparum* and is a complex multisystem disease with cerebral involvement (CM) being the most severe manifestation. Despite treatment with intravenous artesunate, CM has a reported case fatality rate of 30% in adults and 18% in children, with approximately one-third of survivors left with long-term neurological and neurocognitive deficits (110–114). While the pathophysiology of severe malaria is incompletely understood, it is characterized by marked inflammation, oxidative stress, and endothelial dysfunction and microvascular leak associated with disruption of the Ang/Tie2 axis.

The interaction between parasitized red blood cells (RBCs) and the host endothelium is central to the pathobiology of malaria infection and the development of severe disease. *P. falciparum*-infected RBCs (iRBCs) express the *P. falciparum* erythrocyte membrane protein 1 (PfEMP-1) on their cell surface, which mediates binding of the iRBCs to host EC receptors, including intracellular adhesion molecule-1 (ICAM-1), cluster of differentiation 36 (CD36), endothelial protein C receptor (EPCR), and gC1qR (115–118). This cytoadhesion leads to organ-specific sequestration of iRBCs, resulting in microvascular obstruction, impaired perfusion, hypoxia and metabolic derangements (119), all of which can contribute to endothelial activation (37, 63, 120, 121).

Endothelial stability is mediated in large part by two distinct but inter-related pathways, the Ang/Tie2 axis and the NO biosynthetic pathway (discussed below). Similar to bacterial sepsis, Ang1 has been shown to play a protective role in the severe malaria models (45), while several human studies have demonstrated a positive correlation between increased circulating Ang2 levels and poor clinical outcomes in pediatric and adult malaria infections (49, 52, 61, 122–124). Increased levels of Ang2 have also been implicated in the pathogenesis of placental malaria and its associated adverse birth outcomes, including stillbirth and fetal growth restriction (125–127).

The murine model of experimental CM (ECM) recapitulates several features of human severe and CM (128–132), including Ang dysregulation (45, 133). Deletion of the Ang1 locus increased susceptibility to ECM, while restoring circulating Ang1 levels maintained blood–brain barrier (BBB) integrity and enhanced survival, demonstrating that Ang1 is required to stabilize the microvasculature and improve outcome (45). The pathophysiology of human CM is debated, but likely results from both parasite and host determinants (134–138). Similar to ECM, recent human MRI data has provided evidence that BBB dysfunction and microvascular leak may contribute to BBB breakdown and cerebral edema in both adult and pediatric patients with CM (139, 140). In both pre-clinical and clinical studies, higher levels of Ang1 are associated with better outcomes, whereas higher levels of Ang2 correlate with disease severity and mortality. Thus, interventions that enhance Ang1 and/or Tie2 expression and activation may be beneficial in reducing malaria-associated adverse outcomes.

Nitric oxide is produced by a family of NO synthases (NOS) that use L-arginine as a substrate and the cofactor tetrahydrobiopterin. The rupture of iRBCs releases free hemoglobin, arginase, and contributes to the generation of asymmetric dimethylarginine (ADMA) in the circulation (**Figure 2**). These compounds collectively limit NO bioavailability *via* several mechanisms. Hemoglobin does so by reacting with NO, converting it to a biologically inactive nitrate. Proteolysis of erythrocyte proteins releases ADMA (an endogenous inhibitor of NOS) as well as arginase and converts arginine to ornithine, limiting the pool of arginine available for NO production (62, 141). Reduced levels of bioavailable NO lower the threshold for cytokine-induced EC activation and exocytosis of vWF and Ang2 from WPBs (21). Therefore, malaria infection fosters microenvironment conditions that facilitate the release of Ang2 and promote the switch from a quiescent to an activated endothelial phenotype. Interventions that improve NO bioavailability may be promising candidates for the treatment of severe malaria [discussed in Ref. (142–144)].

Less is known about the role of the Tie2 component of the pathway in malaria infection but reduced expression of Tie2 is observed in the lungs of malaria-infected mice (54) and increased levels of circulating sTie2 are observed in human severe malaria (48). These observations support the hypothesis that therapeutics that increase Tie2 expression may restore endothelial quiescence and reduce the risk of ALI and acute respiratory distress syndrome (ARDS) in human malaria infection (145). Ang/Tie2 targeted therapeutics in the treatment of malaria are discussed later in this review.

BENCH TO BEDSIDE: THE Ang/Tie PATHWAY COMPONENTS AS BIOMARKERS OF LIFE-THREATENING INFECTIONS

Septic shock is the quintessential state of systemic endothelial dysfunction and microvascular leak. Over the last two decades, multiple studies of adult and pediatric populations have consistently shown that sepsis is marked by decreased levels of circulating Ang1 and increased levels of Ang2 [reviewed in Ref. (146–148)] and that the Ang2/Ang1 ratio can risk-stratify patients with critical illness. Notably, increased levels of Ang2 or a higher Ang2/Ang1 ratio predict mortality in septic patients (50, 53, 56, 100, 149–163). Among critically ill patients admitted to ICUs, plasma levels of Ang2 or the Ang2/Ang1 ratio increased across the spectrum of patients with sepsis and septic shock independent of the infecting pathogen (53, 56, 60, 150, 153, 155, 156, 158, 160, 161, 163–168). Ang2 levels correlated with surrogates of disease severity, including markers of tissue hypoperfusion, such as serum lactate (56, 60, 151, 155, 161), kidney injury (151, 154, 161), hepatic dysfunction (151), coagulopathy (151, 152), and markers of systemic inflammation (56, 60, 167). Ang2 levels were also associated with other clinical correlates of disease severity including Acute Physiology and Chronic Health Evaluation II (APACHE II) scores (56, 155, 161, 167), ICU length of stay (60), bacteremia (159), positive fluid balance (151, 162), need

for corticosteroid support (151), and measures of organ failure (50, 53, 56, 60, 151–155, 160, 161, 164, 167, 169). Importantly, Ang2 levels measured early in the course of sepsis, including within 24 h of symptoms onset (158, 163, 166, 168), presentation to an Emergency Department (56, 150), and admission to ICU (50, 53, 60, 155, 157, 159, 160, 162, 164, 165), were associated with disease severity and predicted hospital mortality.

The circulating mediators of Tie2 signaling are particularly valuable in predicting outcomes of lung injury, likely owing to the high level of Tie2 expression in pulmonary vascular endothelium (147). In the lungs, Ang2 contributes to microvascular leak leading to pulmonary edema, ALI, and ARDS (55, 163, 168–173) [reviewed in Ref. (174)]. Elevated levels of circulating Ang2 not only correlate with disease severity, but also predict the degree of pulmonary microvascular leak (100, 163), duration of mechanical ventilation (163, 175), the partial pressure arterial oxygen to fraction of inspired oxygen (PaO_2/FiO_2) ratio (161–163, 168), and mortality (163, 169, 172, 173, 176, 177). Ang2 levels were a strong predictor of death in infection-mediated ARDS (149), an association that holds regardless of the inflammatory trigger.

Ang1 and Ang2 proteins have been evaluated as potential biomarkers of malaria disease severity and mortality, with Ang2 levels predicting not only in-hospital but also post-discharge mortality in children with severe malaria (46). Increased circulating levels of circulating Ang2 and decreased levels of Ang1 are associated with both severe *P. falciparum* (48, 49, 52, 122–124, 141, 178–183) and severe *Plasmodium vivax* infections (184–186) [reviewed in Ref. (187)]. Furthermore, the degree of Ang derangement correlates with the severity and outcome of *P. falciparum* infection (48, 49, 61, 122, 141, 181–184, 188), including anemia, jaundice, hypoglycemia (180), kidney injury (178, 180, 182), respiratory distress (181), CM (48, 52, 122, 178, 180, 189), and death (48, 49, 52, 61, 141, 180, 182, 189–191). Incorporating Ang2 concentrations into clinical scoring tools significantly improved the prediction accuracy of the models for mortality (48). Moreover, circulating Ang2 levels were informative in monitoring response to therapy and were predictive of short and long-term mortality (61, 122, 192).

THERAPEUTIC INTERVENTIONS TARGETING THE Ang/Tie AXIS

The observation that the Ang/Tie2 pathway contributes to disease pathobiology and that circulating ligands of Tie2, Ang1, and Ang2 can risk-stratify critically ill patients suggests that this pathway is a therapeutic target to prevent microvascular leak associated with sepsis. Off target effects of corticosteroids and HMG-CoA reductase inhibitors, both of which can reduce the severity of critical illness (193–198), have been shown to modulate Ang1 and Ang2 levels (199, 200) [reviewed in Ref. (36, 201, 202)]. Ang/Tie2-directed anti-angiogenic pharmacotherapies are in preclinical and clinical trials for the treatment of several malignancies and neovascular eye diseases [reviewed in Ref. (203)]; however, the development of adjunctive therapies for the management of sepsis and other critical illnesses associated with microvascular dysfunction have lagged behind. To date, pre-clinical studies with interventions that have stabilized the Tie2 receptor provide evidence that targeting this pathway may enable precision medicine approaches to improve outcomes of severe infections in humans [**Table 1**; reviewed in Ref. (146, 148)].

Studies using murine models of sepsis have demonstrated that therapeutic compounds that augment Ang1 expression can attenuate many of the adverse outcomes associated with endotoxemia. Increased Ang1, driven by adenovirus-mediated rh gene delivery prior to an LPS challenge, preserved eNOS activity in lung tissue and reduced lung injury, prevented up-regulation of cellular adhesion molecules, improved hemodynamics, and reduced mortality (106, 204). A more potent and stable Tie2 phosphorylating molecule, cartilage oligomatrix protein (COMP)-Ang1 (215) similarly prevented adhesion molecule expression and conferred renal protection in the sepsis model (107). Although these findings establish the protective benefit of sustaining Tie2 phosphorylation through Ang1 treatment in sepsis, the use of an adenoviral delivery vector is problematic for translation to human therapy. Subsequent studies demonstrated that rh Ang1 (rh-Ang1) delivered systemically to mice undergoing cecal ligation and perforation (CLP) stabilized endothelial barrier function, preventing pulmonary capillary leak, and decreased leukocyte infiltration into both lungs and kidneys by suppressing ICAM-1 expression (35). Despite the short half-life of rh-Ang1 (216), treatment was able to avert multi-organ dysfunction and increased survival following CLP (59). Matrilin-1-Ang1 (MAT.Ang1) was developed as a stable Ang1 variant amenable to direct intravenous administration; it too stabilized the endothelium in the setting of LPS-induced endotoxemia (58).

Ang1 therapy has also been shown to mitigate the adverse sequelae of severe malaria infection. Using BowAng1, a rh Ang1 protein capable of phosphorylating Tie2 (217), it was demonstrated that its addition to artesunate therapy preserved the integrity of the BBB and improved survival in a murine model of CM, even when administered during the late stage of infection (45). Other therapeutic agents that increase Ang1 expression also prevent BBB leak and improve survival in ECM. Mice treated with the ethyl amide of a synthetic oleanane triterpenoid, 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid (CDDO-EA), had increased Ang1, reduced Ang2 and Ang2:Ang1 ratio, and this was associated with improved BBB integrity (208). Similarly, treatment of malaria infected mice with the PPAR-γ agonist rosiglitazone in combination with artesunate at the onset of neurological symptoms achieved higher plasma and brain levels of Ang1 and a lower Ang2:Ang1 ratio compared to mice treated with artesunate alone. Furthermore, these mice had enhanced BBB integrity, improved survival and better cognitive and motor outcomes than mice treated with anti-malarials alone (133). Rosiglitazone has entered human clinical trials. To date, a randomized clinical trial of rosiglitazone in young adults with uncomplicated malaria showed reduced levels of pro-inflammatory mediators, a lower Ang2:Ang1 ratio, and higher levels of brain-derived neurotrophic factor, a protein involved in neuronal survival and proliferation (133, 218). A phase IIa study has shown rosiglitazone to be safe and well tolerated in pediatric patients with uncomplicated malaria and is currently being tested in a phase IIb trial in children with severe malaria (219).

TABLE 1 | Ang/Tie2-targeted therapies in pre-clinical studies of sepsis.

Compound(s)	Description	Pre-clinical studies/model
AdhAng1/rAAV.ANG1/AdAng1	Adenovirus construct expressing rh Ang1	Mouse—endotoxemia (106, 204) Mouse—ECM (45)
rh-Ang1	Commercial rh Ang1 protein (R&D Systems)	Mouse—endotoxemia (35) Mouse—Gram-negative sepsis (205)
COMP-Ang1	Adenovirus expressing rh-Ang1 variant: N-terminal is replaced with short coiled-coil domain of COMP for increased stability, solubility and Tie2 activating potency over rh-Ang1	Mouse—endotoxemia (107)
MAT.Ang1	rh-Ang1 variant: central coiled-coil N-terminal of Ang1 is replaced with short coiled-coil domain of matrilin for increased stability and solubility over rh-Ang1	Mouse—sepsis (58)
BOWAng1	rhAng1 variant: C-terminal fibrinogen-like domain of Ang1 protein fused to human IgG Fc fragment, engineered to tetramer conformation for optimal Tie2 phosphorylation	Mouse—ECM (45)
ANGPT1	Human Ang1 gene plasmid transfected into syngeneic MSCs for engraftment into injured pulmonary vasculature	Mouse—endotoxemia (206, 207)
CDDO-EA	Synthetic oleanane triterpenoid, activator of Nrf2. Increased Ang1 and decreased Ang2 levels in plasma, and reduced cerebrovascular leak in ECM model	Mouse—ECM (208)
Rosiglitazone	PPAR-γ agonist increased Ang1 levels in brains of ECM models	Mouse—ECM (133)
LC10, LOC06, ABA	Selective anti-Ang2 antibodies inhibit Ang2 binding to Tie2	Mouse—polymicrobial sepsis (24, 204)
ABTAA	Ang2 clustering converts antibody into Tie2 activating ligand	Mouse—endotoxemia, Gram-positive bacteremia, polymicrobial sepsis (24)
Angpt-2 siRNA	Ang2 siRNA highly specific for pulmonary endothelium, reduced Ang2 expression in murine lung tissue and resulted in increased Tie2 phosphorylation	Mouse—endotoxemia, polymicrobial sepsis (209)
rh-Ang2	Commercial rh Ang2 protein (R&D Systems)	Mouse—Gram-negative sepsis (205) Rabbit—Gram-negative sepsis (210)
AKB-9778	Small molecule inhibitor of VE-PTP; promotes Tie2 activation	Mouse—endotoxemia (33) Mouse—stroke/BBB permeability (211) Mouse—choroidal neovascularization and ischemic retinopathy (43, 212)
Vasculotide	Synthetic tetrameric polyethylene glycol-clustered Tie2 agonist	Mouse—polymicrobial sepsis (213) Mouse—influenza infection (214)

Therapeutic agents that target Tie2 by augmenting Ang1 levels, inhibiting Ang2, or promoting Tie2.
Abbreviations: ABA, Ang2-blocking antibody; ABTAA, Ang2-binding and Tie2 agonist antibody; Angiopoietin (Ang), CDDO-EA, 2-cyano-3,12-dioxooleana- 1,9(11)-dien-28-oic acid ethyl amide; COMP, cartilage oligomeric matrix protein; ECM, experimental cerebral malaria; Fc, IgG, immunoglobulin; MAT, matrilin; nrf2, nuclear factor-like 2; siRNA, small interfering RNA; rh, recombinant human; VE-PTP, vascular endothelial protein tyrosine phosphatase.

Other novel modes of augmenting Ang1 expression are currently in preclinical trials. Preliminary work utilizing cell-based therapy has demonstrated that mesenchymal stem cells (MSCs) transfected with Ang1 are able to engraft the pulmonary endothelium damaged during sepsis, preserve pulmonary endothelial integrity, and ameliorate ALI/ARDS (206, 207).

In contrast to the constitutive expression of Ang1, Ang2 is released in response to infectious triggers with considerable dynamic range (56, 168), making this molecule an appealing target for pharmacologic inhibition in sepsis. In preclinical trials, lung-targeted small interfering RNA (siRNA) against Ang2 delivered both pre- and post-sepsis induction reduced pulmonary inflammatory cytokine levels, ICAM-1 expression, neutrophil organ infiltration, and overall disease severity while improving survival (209). Functional inhibition of Ang2/Tie2 binding using anti-Ang2 antibodies decreased rates of hemodynamic shock and mortality in murine sepsis and ARDS models (24, 204).

Furthermore, the novel Ang2-binding and Tie2-activating antibody (ABTAA) utilizes a mechanism through which Ang2 clustering converts the antibody-antigen cluster into a Tie2 activating ligand, thus allowing simultaneous Ang2 inhibition and Tie2 activation (24). When compared to the conventional anti-Ang2 antibody, ABTAA conferred increased protection against microvascular dysfunction, end-organ damage, and mortality in CLP, endotoxemia, and *Staphylococcus aureus* models of sepsis (24). In combination with broad-spectrum antibiotics (imipenem/cilastatin), ABTAA improved survival to 70%, compared to 20% survival in animals treated with antibiotics alone in the CLP model (24). Seemingly paradoxically, studies in murine and rabbit models of pyelonephritis and sepsis found that rh-Ang2 administration prolonged survival in Gram-negative sepsis (205, 210). Notably, these studies did not assess Tie2 phosphorylation status, leaving the mechanism of Ang2-mediated survival in these models unclear.

In light of these findings, further analyses have been performed examining the role of simultaneous Ang2 inhibition and Tie2 activation in vascular protection during sepsis (24). When treated with the antibody ABTAA, mice with high-grade CLP had significantly improved survival rates (40%) compared to the conventional Ang2-blocking antibody (ABA; 13%). These findings were further extended to two other sepsis models used in this study: endotoxemia (rate of survival increase: 63% ABTAA vs 33% ABA) and *S. aureus* bacteremia (rate of survival increase: 55% ABTAA vs. 9% ABA). In these models, it was observed that ABTAA ameliorated endotoxemic and CLP-induced sepsis by preserving endothelial glycocalyx and microvascular integrity of major organs (24). Taken together, these studies underline the importance of Tie2 activation in ameliorating the progression of sepsis and demonstrate that solely blocking Ang2 is insufficient for preserving endothelial integrity during severe bacterial sepsis.

In addition to targeting the Tie2 receptor through Ang1 and Ang2, several other agents have been used to maintain Tie2 phosphorylation in animal models of sepsis. For example, pharmacologic inhibition of the Tie2 phosphatase, VE-PTP, with AKB-9778 stabilized the pulmonary endothelium following LPS administration in mice (33), offering another potential mechanism to modulate the activity of Tie2 in sepsis. This compound has already been used in human trials to treat diabetic macular edema and ocular neovascularization (43, 212). Alternatively, Vasculotide, a synthetic polyethylene glycol-clustered Tie2 agonist, has been shown to sustain Tie2 activation *in vivo*. Its administration both pre- and post-CLP reduced end-organ dysfunction and mortality in the murine abdominal sepsis model (155). Vasculotide administration also preserved pulmonary endothelial barrier function and survival following murine infection with several strains of influenza. Importantly, the protective effect was realized even with therapy delayed up to 72 h after infection, conditions similar to typical septic patient presentations (214). Unlike interventions that manipulate Ang1 or Ang2 expression, Vasculotide is highly specific for the Tie2 receptor and does not displace Ang1 or Ang2. As such, Vasculotide may avoid interfering with off-target effects of Ang1 during the dynamic host sepsis response.

Although the pathophysiology Tie2-mediated vascular dysfunction in sepsis remains incompletely understood, the effects of these therapies in preclinical sepsis models warrant further investigation to develop human Tie2-directed therapies.

CONCLUSION

The Ang/Tie2 axis plays an essential role in maintaining endothelial barrier stability and its disruption during systemic infection contributes to the pathologic cascade that culminates in end-organ failure and death. In addition to its mechanistic role in the pathobiology of sepsis, components of the Ang/Tie2 system can function as prognostic biomarkers of disease severity and outcomes, and potentially serve as important therapeutic targets in the management of sepsis.

The dysregulation of Ang/Tie2 signaling is "pathogen agnostic" and appears to represent a final common pathway in many different types of microbial infections, including bacterial and parasitic processes described in this review. As such, therapeutic interventions to restore Tie2 activity may be useful in the early management of serious infections where there is a high degree of diagnostic uncertainty. Furthermore, use of Ang/Tie2 adjunctive therapy in sepsis may confer protection against the collateral systemic damage that results in significant morbidity and mortality. The pathophysiology Tie2-mediated microvascular dysfunction in sepsis remains incompletely understood, but the findings from preclinical sepsis models warrant further investigation with the aim of developing human Tie2-directed therapies to improve outcomes of life-threatening infections.

AUTHOR CONTRIBUTIONS

AL, MR-G, JW, VC, and KK conceived the ideas for preparing this review. AL, MR-G, JW, VC, and KK contributed to the writing, editing, and approval of the final review.

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REVIEW
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Vasa Vasorum Angiogenesis: Key Player in the Initiation and Progression of Atherosclerosis and Potential Target for the Treatment of Cardiovascular Disease

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Plaque microvascularization and increased endothelial permeability are key players in the development of atherosclerosis, from the initial stages of plaque formation to the occurrence of acute cardiovascular events. First, endothelial dysfunction and increased permeability facilitate the entry of diverse inflammation-triggering molecules and particles such as low-density lipoproteins into the artery wall from the arterial lumen and vasa vasorum (VV). Recognition of entering particles by resident phagocytes in the vessel wall triggers a maladaptive inflammatory response that initiates the process of local plaque formation. The recruitment and accumulation of inflammatory cells and the subsequent release of several cytokines, especially from resident macrophages, stimulate the expansion of existing VV and the formation of new highly permeable microvessels. This, in turn, exacerbates the deposition of pro-inflammatory particles and results in the recruitment of even more inflammatory cells. The progressive accumulation of leukocytes in the intima, which trigger proliferation of smooth muscle cells in the media, results in vessel wall thickening and hypoxia, which further stimulates neoangiogenesis of VV. Ultimately, this highly inflammatory environment damages the fragile plaque microvasculature leading to intraplaque hemorrhage, plaque instability, and eventually, acute cardiovascular events. This review will focus on the pivotal roles of endothelial permeability, neoangiogenesis, and plaque microvascularization by VV during plaque initiation, progression, and rupture. Special emphasis will be given to the underlying molecular mechanisms and potential therapeutic strategies to selectively target these processes.

Keywords: atherosclerosis, inflammation, vasa vasorum, plaque angiogenesis, unstable plaque

Abbreviations: ApoE, apolipoprotein E; BET, bromodomain and extra terminal domain; BRD4, bromodomain-containing protein 4; CD40, cluster of differentiation 40; DIT, diffuse intimal thickening; Fbn, fibrillin; FGF2, fibroblast growth factor-2; HIF, hypoxia-inducible transcription factors; ICAM, intercellular adhesion molecule; LDL, low-density lipoprotein; LDLR, low-density lipoprotein receptor; PFKFB3, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3; PlGF, placental growth factor; qPCR, quantitative polymerase chain reaction; ROS, reactive oxygen species; rPAI-1, recombinant plasminogen activator inhibitor-1; TGF, transforming growth factor; TNFα, tumor necrosis factor α; VCAM, vascular cell adhesion molecule; VEGF, vascular endothelial growth factor; VSMCs, vascular smooth muscle cells; VV, vasa vasorum.

INTRODUCTION

As atherosclerotic lesions progress, they can become unstable, and plaque rupture or erosion followed by luminal thrombosis is the primary cause of clinical complications such as myocardial infarction, stroke, and sudden death (1–4). Despite sophisticated interventional and surgical treatment options, the morbidity and mortality from acute cardiovascular events remain unacceptably high. While cholesterol-lowering (5), anti-inflammatory (6), and anti-platelet therapies offer benefits in survival when used in primary or secondary prevention, the benefits of such treatments are still limited and not sufficient in the prevention of acute complications in all treated patients. Specifically, 61% of primary major cardiovascular adverse events are not prevented with current statin treatment regimens in patients as recently shown in the WOSCOPS trial (5). Thus, there is a clear need for novel strategies to both prevent atherosclerotic plaque development as well as to stabilize existing atherosclerotic plaques. Flow-limiting lesions have long been the focus of therapeutic approaches; however, attention has now shifted to the importance of cellular plaque composition rather than the stenotic features alone. Indeed, the cellular features and composition of atherosclerotic plaques have emerged as the most robust predictors of future cardiovascular events (7). Microvessel expansion within the arterial wall and their impact on plaque progression is an area of increasing interest, albeit the precise mechanisms still remain to be determined (8–13). Therefore, the present review will focus on the role of vasa vasorum (VV) neovascularization in atherosclerotic plaque progression and its impact on plaque stability. Furthermore, resulting treatment options focusing on VV neovascularization are discussed.

PHYSIOLOGICAL ADAPTION OF VV TO THE GROWTH OF THE ARTERIAL WALL

It was recognized in the early twentieth century that the vessel wall architecture is structurally dynamic and changes with growth and aging (14). At birth, the innermost layer of the vessels is comprised solely of endothelial cells attached to an underlying matrix and surrounded by an internal elastic lamina, while the medial layer has lamellar units consisting of vascular smooth muscle cells (VSMCs), connective tissue, and elastic fibers. The collagen-rich adventitia comprises fibroblasts, perivascular nerves, pericytes, adipocytes, as well as resident leukocyte populations. Due to a pressure gradient, the diffusion of solutes through a permeable medium like the vessel wall is facilitated by the high intra-arterial pressure and is dependent on the permeability of the endothelial layer. With growth, the thickness of the intimal layer increases and the intimal layer gains a higher level of cellular complexity. This process is referred to as diffuse intimal thickening and is now considered a developmental process associated with the growth of the arteries rather than being linked to atherosclerosis itself (15).

While diffusion is responsible for the exchange of nutrients of thin-walled blood vessels, at a critical thickness of more than 0.5 mm, diffusion alone is insufficient (16–18). Hypoxic conditions that arise in the vessel wall of larger blood vessels give rise to VV, defined as arterial microvessels that supply nutrients and oxygen to the adventitia and outer media of the parent vessel.

CORRELATION BETWEEN ARTERIAL WALL NEOVASCULARIZATION AND ATHEROSCLEROSIS PROGRESSION

While the thickness of the blood vessel wall is an important parameter governing the neovascularization of VV, other stimuli such as inflammation can trigger neovascularization. For example, even though the murine arterial wall does not exceed the 0.5 mm diffusion limit, VV are seen in atherosclerotic mouse arteries (19–21). In atherosclerotic pigs, vessel wall thickening and plaque development follow the growth of VV in atherosclerotic models (22, 23). The structure of VV is different in non-diseased versus diseased arteries. Early low-resolution X-ray images failed to detect VV in non-diseased human coronary arteries, but in diseased vessels, the presence of a dense microvascular plexus was observed (24). This pattern was also seen in coronary arteries from hypercholesterolemic pigs, where the longitudinal VV externa (defined as first-order VV) originate from the coronary artery as seen in healthy pigs or human arteries. These longitudinal VV further branch to form circumferential arches around the vessel wall, which are defined as second-order VV. Non-diseased porcine coronary arteries display a significantly higher density of first-order VV than the second-order VV. By contrast, the second-order vessel density is twofold greater than the first-order vessel density in hypercholesterolemic pigs (22). Interestingly, the branching patterns of VV reflect the dichotomous tree structure with a hierarchical branching pattern, as seen in the physiological systemic circulation structure. In further findings, Gõssl et al. demonstrated that VV are not connected by a plexus but rather are end arteries (25). Using *ex vivo* micro-CT scans, we could demonstrate that this pathological sprouting pattern can also be observed in VV of small animal models of atherosclerosis, namely, apolipoprotein $E^{-/-}$ (ApoE)/$LDLR^{-/-}$ mice, and can be prevented by an antiangiogenic therapeutic approach (26, 27). Moreover, structural hierarchy in adventitial VV was also later demonstrated *in vivo* in diseased $LDLR^{-/-}$ApoB 100/100 mice by using high-resolution confocal microscopy (28). During VV neoangiogenesis, the branched vessels further branch, occupying the space between two larger vessels. However, in the presence of angiogenesis inhibitors, the newly formed neovessels collapse whereas the larger vessels remain intact. Taken together, data from different animal models demonstrate the presence of some rare, stable, larger VV in healthy vessels. By contrast, under pathological conditions, before and during atherosclerotic plaque progression, neovessels branch out, significantly expand, and exert a disarrayed structure (28).

Thus, microvessels are rarely present in the healthy intima of the vessel wall but are usually observed in pathological conditions such as atherosclerosis. Indeed, a link between atherosclerosis and intraplaque neovascularization was first observed by Koester (29) and Winternitz et al. (30) while the first insights into the mechanism behind the association between atherosclerosis and

intraplaque neovascularization was presented by Paterson (31), who was able to identify the rupture of capillaries accompanied by erythrocyte and platelet leakage into the plaque as the cause of plaque progression, rupture, and coronary thrombosis (intraplaque hemorrhage). Further research in the past three decades has largely focused on the role of intraplaque neovascularization in plaque progression and rupture (3, 32–34), confirming the presence of an expansive network of intraplaque neovessels in human stenotic lesions in close proximity to inflammatory infiltration and the necrotic core. Intraplaque hemorrhages are an important trigger for plaque progression, instability, and rupture (3, 35). However, intraplaque neovascularization is also associated with plaque vulnerability and plaque erosion, even in the absence of intraplaque hemorrhage. Moreover, microvessels in the plaque express high levels of cell adhesion molecules (CAM) like intercellular adhesion molecule (ICAM), vascular cell adhesion molecule (VCAM), E-selectin, and cluster of differentiation 40, which facilitate the further recruitment of inflammatory cells into the plaque (36, 37).

One practical example of the importance of VV for vessel integrity and patency can be seen in saphenous veins used for coronary artery bypass graft surgery (38, 39). During the process of saphenous vein harvesting, the connective tissue containing VV is stripped from the vein (38, 40, 41). This often manifests in venospasm (42), which can progress into vein-graft disease and even vein-graft failure, a process analogous to atherosclerosis (40, 43). In addition, venous VV play an important role in vein relaxation, and any damage to the venous VV during saphenous vein harvesting severely impairs flow-induced vasodilation of the graft (43–45).

VV EXPANSION AND PLAQUE ANGIOGENESIS

It has been proposed that VV formation occurs as response to the nutritional needs of the artery's outer medial layer, as the metabolic needs exceed the diffusion levels of oxygen from the luminal blood (17, 18). Under hypoxic conditions, hypoxia-inducible transcription factors (HIF)-1 and HIF-2 induce the transcription of proangiogenic genes like vascular endothelial growth factor (VEGF) (46, 47). Hypoxic conditions in the blood vessel wall also upregulate the expression of important enzymes required for the synthesis of heparan sulfate in microvascular endothelial cells, providing binding sites for fibroblast growth factor-2 (FGF2) (48), which is a known potent stabilizing agent for VV and a promoter of endothelial cell growth (28). In hypercholesterolemic LDLR$^{-/-}$ ApoB100/100 mice, FGF2 is the primary angiogenic growth factor expressed in the adventitial VV, and quantitative polymerase chain reaction measurements have shown an eightfold increase in FGF2 mRNA in hypercholesterolemic mice compared with age- and sex-matched chow-fed mice (21). In hypercholesterolemic mice, FGF2 stimulates the formation of complex VV networks not seen in healthy mice. Tanaka and colleagues delivered FGF2 to the adventitia of ApoE$^{-/-}$ mice demonstrating its role in the expansion of VV and acceleration of plaque progression (49). Other studies investigating the role of placental growth factor (PlGF), a member of the VEGF family of proteins, have shown that the delivery of PlGF into the periadventitial space of the carotid artery significantly increases adventitial neovascularization and macrophage accumulation in hypercholesterolemic rabbits (50). In ApoE$^{-/-}$PlGF$^{-/-}$ mice, the absence of PlGF significantly reduces macrophage accumulation and plaque size (51).

However, and in contrast to the above described "nutritional demand" theory, observations in hypercholesterolemic pigs revealed that VV begin to sprout even before aortic wall thickening and this sprouting is in turn preceded by the infiltration of inflammatory cells into the adventitia (22, 23). Several factors could contribute to the above phenomena, including the secretion of angiogenic growth factors by inflammatory cells infiltrating from the adventitia or by periadventitial fat cells (52, 53). Taken together, it remains to be determined whether hypoxia and nutritional needs, or rather inflammatory stimuli and angiogenic growth factors, induced by the accumulation of pathological particles in the subintimal space, are the initial triggers responsible for VV expansion.

While we have gained tremendous insight from the above studies regarding the sequence of pathophysiological events (**Figure 1**), there are only limited and correlative studies in humans available, suggesting that the infiltration of inflammatory cells into the plaque can be limited by the inhibition of VV angiogenesis and vice versa, resulting in a plaque stabilizing effect (54). Studies in cancer patients revealed a regression of angiogenic blood vessels upon antiangiogenic therapy and, importantly, their reversal to a normal, stable and mature phenotype (55). This normalization of the tumor vasculature is accompanied by a decrease in microvessel density and blood vessel diameter, and at the same time, by an increase in the perivascular cell coverage. As a result, vascular permeability and the interstitial fluid pressure are decreased, resulting in an improved oxygenation of the tumor cells. Hypoxia is a hallmark of solid tumors and drives the production of angiogenic factors. Therefore, by improving tumor oxygenation, normalization of the tumor microvasculature inhibits tumor growth (56). This concept is also valid for VV, where a truncated mutant of plasminogen activator inhibitor-1 (rPAI-1_{23}), a potent antiangiogenic protein, has been shown to limit plaque area and plaque volume, and decrease inflammatory cell accumulation and necrotic area, resulting in a reduction in blood vessel stenosis (21, 56). Interestingly, the treatment with rPAI-1_{23} only affects the microvasculature, leaving the larger VV unaffected (28).

EFFECTS OF CARDIOVASCULAR RISK FACTORS ON VV EXPANSION

Studies in experimental animal models on the role of cardiovascular risk factors on VV formation and adventitial remodeling processes are scarce and have yielded varying results (57–59). High cholesterol levels have been associated with an increase in the density of the VV while increased adventitial matrix deposition was observed in hypertensive animals (59). Interestingly, animal models of diabetes mellitus show attenuated growth of VV (60), whereas patients with diabetes mellitus display increased microvessel density, an increased number of

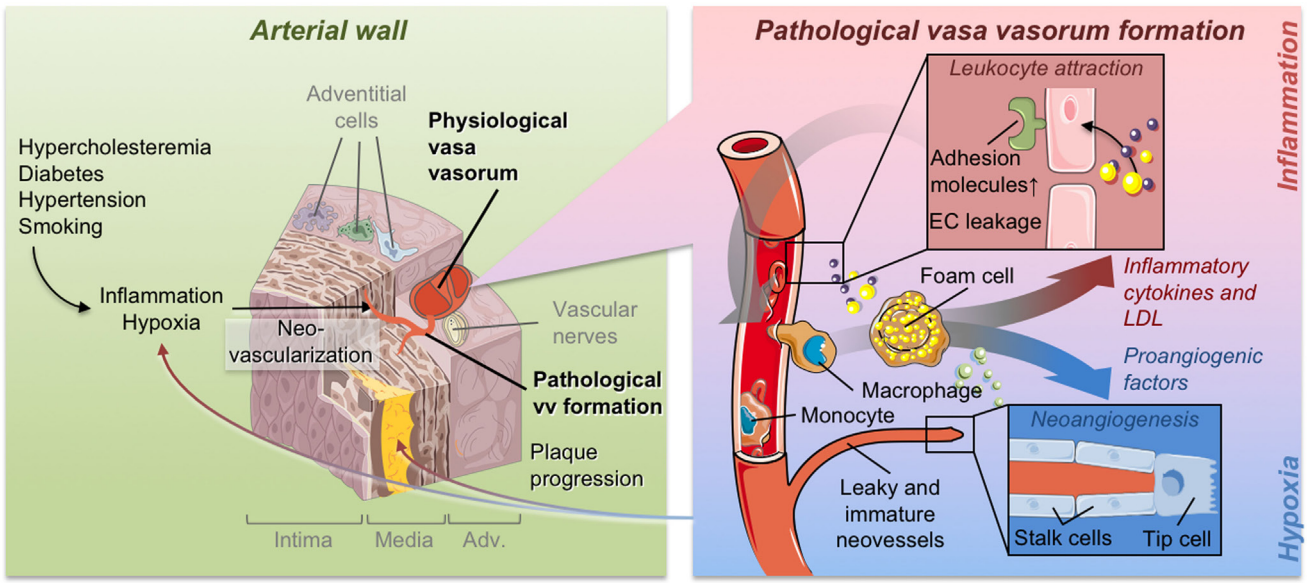

FIGURE 1 | Pathological vasa vasorum formation and its contribution to plaque formation.

inflammatory cells, and more intraplaque hemorrhage (61). Intraplaque hemorrhage, in turn, aggravates atherosclerosis progression due to the increased hemoglobin–haptoglobin complex deposition, which results in oxidative stress-mediated endothelial dysfunction (62). Thus, taken together, there are only limited and partially contradictory data regarding the direct impact of classical cardiovascular risk factors on the development of VV.

By contrast, there is good evidence that chronic inflammation drives angiogenesis, plaque progression, and the occurrence of cardiovascular events. Elevated levels of inflammatory markers are associated with an increased cardiovascular risk (4), and reduced inflammatory levels were shown to yield an equivalent benefit to cardiovascular outcome as the reduction of lipid levels, the classical therapeutic target (63). In humans, a clear correlation between adventitial inflammation, chronic infiltration of $CD68^+$ macrophages, and VV expansion was demonstrated (64). Furthermore, perivascular inflammation in autoimmune rheumatoid arthritis, which is associated with an increase in cardiovascular risk, goes along with enhanced VV formation (65).

As a proof of principle, the randomized placebo-controlled multicenter CANTOS trial clinically showed that targeting inflammation potently reduces cardiovascular events. Patients with previous myocardial infarction receiving canakinumab, a monoclonal neutralizing antibody targeting IL-1β, had a significantly lower rate of recurrent cardiovascular events (66). These effects were particularly pronounced in patients with effective reduction of inflammation as evidenced by low C-reactive protein levels during treatment (66). As inflammation is a key trigger of VV expansion, one consequence of dampening inflammation would be to reduce VV neoangiogenesis. Thus, targeting inflammation is a promising strategy to prevent VV growth, plaque progression, and subsequent cardiovascular events.

VASCULAR INFLAMMATION AS A TRIGGER FOR VV FORMATION—"FROM OUTSIDE IN"

Inflammatory mediators are well-known potent triggers of neovascularization in different settings like tumor development or acute ischemic damage [for review see Ref. (67, 68)]. Inflammation and neovascularization tend to feed each other in a vicious cycle in that inflammatory cells in the plaque increase oxygen demand, thereby triggering further neovascularization. Furthermore, as neovascularization progresses, the inherent leakiness of the neovessels, together with the increased expression of adhesion molecules, results in the recruitment of more inflammatory cells into the plaque. Increased expression of angiogenic chemokines like IL-1β have been detected in human atherosclerotic plaques (69) and further increase endothelial cell permeability, allowing the infiltration of leukocytes into the plaque (70). Moreover, continuous inflammatory stimulation causes an irreversible change in endothelial cells to a phenotype consistent with a migratory and proangiogenic state (71).

There was a long-held belief that the infiltration of inflammatory cells, mainly macrophages, occurred through the luminal side of the artery during atherosclerosis progression. By contrast, it has more recently been proposed that vascular inflammation is initiated in the adventitia and progresses toward the media and intima (72). The detection of resident macrophages and T cells in the adventitia further fueled this hypothesis (73). Furthermore, the adventitia is the main site for the acute inflammatory response following vascular injury induced by balloon angioplasty in porcine coronary arteries (74). Adhesion molecules attracting circulating inflammatory cells (VCAM-1 and P-selectin) are expressed most prominently in VV endothelial cells following injury (74). In agreement with these findings, we

recently showed that adventitial inflammation is mandatory for the activation of medial smooth muscle cells and subsequent neointima formation (75). Data from a rat model of aortic transplantation between histocompatible strains further supported this hypothesis. In this model, the VV in the adventitia of the aortic allografts triggered a robust angiogenic response in the allograft. Moreover, infiltrating leukocytes were detected in the adventitial VV of the graft, suggesting a role for VV as conduits for the entry of inflammatory cells into the graft (76). Further studies in mice documented the presence of adventitial immune cells already in young wild-type mice, but the number of these adventitial immune cells, especially T-cells, was dramatically increased in hypercholesterolemic ApoE$^{-/-}$ mice (77). Immune cells were further found to organize into tertiary lymphoid structures in the mouse adventitia (73) and are predominantly found in regions next to the external elastic lamina and the atherosclerotic plaque (78).

Studies in humans are consistent with the abovementioned observations in animal experiments. The presence of resident immune cells (T cells, B cells, macrophages, and dendritic cells) in the adventitia was documented in human atherosclerotic arteries (79), and an infiltration of inflammatory cells is observed from the adventitia of the plaque along with the formation of adventitial lymph follicles (80, 81). O'Brien et al. have confirmed the expression of VCAM-1, ICAM-1, and E-selectin, which mediate the recruitment of inflammatory cells, primarily on the intimal and medial VV in human coronary artery segments from patients with atherosclerotic plaques (36, 37). Moreover, leukocytes are present near the adventitial VV even in aortas from healthy children (82). Taken together, there is accumulating evidence that adventitial immune cells play a pivotal role in atherosclerotic disease development and progression and are associated with and probably trigger the growth of VV. However, future research is required to clarify the relevance of this association in human atherosclerosis.

INCREASED PERMEABILITY OF VV FUELS ATHEROSCLEROTIC PLAQUE PROGRESSION

Endothelial cell junctions are responsible for maintaining vessel wall integrity and preventing the leakage of intravascular components to the extravascular space. Consequently, increased permeability across the endothelial cell layer is an early indicator of vascular dysfunction or the induction of endothelial cell sprouting. As angiogenic processes are initiated, vessel permeability increases, enabling the deposition of serum proteins that form a provisional matrix, triggering and facilitating proangiogenic inflammatory cell adhesion and (trans-) migration. Both local as well as systemic inflammation results in increased permeability of endothelial cell junctions. This process is similar for the early VV expansion phase, where increased endothelial permeability facilitates the infiltration of lipoproteins, inflammatory cells, and red blood cells before and during VV angiogenesis (32). Examination of atherosclerotic coronary arteries in humans showed the lack of mural cells which stabilize endothelial cells and vessel structure and prevent leakage in adventitial VV microvessels invading the medial layer (34). Thus, the sprouting and expanding immature plaque VV are highly fragile and permeable, and thus susceptible to hemorrhage.

Extravasated erythrocytes undergo hemolysis upon exposure to plaque lipids. The released hemoglobin undergoes oxidation and free heme or iron is released, triggering a cycle where hemoglobin interacts with plaque lipids resulting in further oxidation of plaque lipids. Oxidized lipids, in turn, trigger an upregulation of the heme oxygenase-1 in endothelial cells, which catalyzes the formation of active iron, thus further increasing lipid toxicity and endothelial cell damage (83). These events contribute to a thinning of the fibrous cap, making it more prone to rupture (84). This is supported by rabbit data where exogenous erythrocyte injection enhanced plaque progression (85). Moreover, magnetic resonance imaging studies in humans show a direct correlation between intraplaque hemorrhage, plaque growth, and an increase in the volume of the lipid-rich necrotic core (86).

Erythrocytes leaking out into the plaque also act as a source of free cholesterol, increasing the risk of plaque rupture by triggering an influx of macrophages to remove the cell debris (87). Furthermore, the mechanisms regulating the influx and efflux of phagocytic macrophages and clearance of cholesterol, erythrocytic debris, and apoptotic macrophages from the blood vessel walls are largely impaired in advanced atherosclerotic plaques, resulting in the accumulation of free cholesterol from membranes of extravasated erythrocytes in the necrotic core. This, in turn, triggers an inflammatory response and further infiltration of macrophages, resulting in the damage of neighboring cells due to the action of proteases and reactive oxygen species.

To further exploit therapeutic strategies to target pathological VV expansion, reliable preclinical models have to be established which allow an adequate translation of results into the clinical scenario in humans. Unfortunately, most of the available models, especially in small animals, exhibit profound differences in plaque characteristics and VV formation as compared with human arteries and thus suffer from severe limitations regarding their translational relevance. As we will discuss in the following section, only recently have new small animal models been established that more closely resemble the correlation of expanding VV with spontaneous plaque rupture as seen in humans.

ANIMAL MODELS TO STUDY VV AND PLAQUE RUPTURE IN ATHEROSCLEROSIS

Current knowledge on the significance of neovascularization in atherosclerosis is mainly based on studies with human atheroma tissue. A direct association between plaque rupture and intraplaque neovascularization has not been confirmed, as there was a lack of suitable animal models of atherosclerosis. Wild-type rats are particularly resistant to developing atherosclerosis, even on high-fat diets (88) and are therefore not suitable to study plaque neovascularization. As intraplaque microvessels

are rare or absent in wild-type pigs, pigs are not often used to study intraplaque neovascularization. However, plaque neovascularization in pigs can be induced with a combination of vascular injury and high cholesterol diets (89). More recently, further pig models have been developed to study intra-plaque neovascularization (90–92). Moreover, a very promisig model of a transgenic Yucatan mini pig overexpressing pro-protein convertase subtilisin/kexin type 9 (PCSK9) showed a more humanized plaque phenotype upon high cholesterol diet. Plaques show intra-plaque and adventitial angiogenesis and micro vessels, although their frequency and microvascular density remains to be determined and quantified (93). More often, intraplaque neovascularization has been investigated in small animal models such as genetically modified mice and rats (94) as well as in rabbits (95, 96).

Atherosclerotic plaques in rabbits are induced by feeding them with a high cholesterol diet in addition to repeated endothelial denudations (97). ApoE$^{-/-}$ or LDLR$^{-/-}$ mice have been extensively used as standard models in atherosclerosis research (98). Moreover, ApoE$^{-/-}$/LDLR$^{-/-}$ double knockout mice kept on a high-fat diet develop plaques rich in lipid content, which until recently, was considered to be the best model of human plaque composition containing foam cells, necrotic cores, and VSMC-rich fibrous caps (99). Moreover, these mice exert an extensive angiogenic activity in the adventitia and develop prominent adventitial and intimal plaque VV (19, 100). Since these mice most closely mimicked atherosclerotic plaques in humans, we and others have used this model extensively during the last years to study VV formation in mice (26, 27, 101). It is commonly supported by cardiovascular pathologists trained in human and experimental pathology (102, 103) that murine plaques most closely resemble human plaque morphology. However, morphology of the recent model of PCSK9-overexpressing mini pig (93) certainly bears strong humanlike features too. Possibly, this is explained by very severe hypercholesterolemia in both PCKS9-mini pigs and apoE/LDLRko models, compared to non-transgenic pigs. However, despite the similarities to human plaque architecture, these models do not develop plaque rupture or thrombosis and are thus unsuitable to investigate correlations of VV formation and plaque instability/rupture.

In a further model utilizing vein grafts in ApoE*3-Leiden mice, the developing atherosclerotic lesions were shown to be even more similar to human plaques, including intraplaque neovascularization together with intimal dissections and intramural thrombus formation (104, 105). Immunostaining studies of histological sections have demonstrated an insufficient pericyte coverage, indicative of leaky and immature microvessels. Importantly, these microvessels were surrounded by extravasated erythrocytes suggesting micro-hemorrhages similar to those found in human plaques (105). Therefore, this model very closely resembles plaque phenotypes in which VV neovascularization is accompanied by intraplaque hemorrhages and plaque ruptures. On the other hand, this model is difficult to establish as it requires a complex vein-graft transplantation surgery and the results are prone to high variability. In a recently established mouse model, a heterozygous mutation (C1039G$^{+/-}$) in the fibrillin 1 gene (*fbn1*) was created, resulting in the fragmentation of the elastic fibers in the medial layer. When back crossed to ApoE$^{-/-}$ mice, the resulting ApoE$^{-/-}$ Fbn1C1039G$^{+/-}$ on a Western-type diet develop spontaneous plaque ruptures (106). Furthermore, these mice spontaneously develop plaques that closely resemble unstable lesions in human plaques, while displaying intraplaque neovascularization and hemorrhages as well as sporadic spontaneous plaque ruptures manifesting into myocardial infarctions (107). Overall, this novel mouse model will facilitate the design of further studies shedding light on the complex interaction of VV formation and plaque progression up to plaque ruptures and the resulting vascular complications. Moreover, this model will be helpful to study novel therapeutic approaches and will allow a better translation efficacy of the gained results into the human/clinical situation.

ANTIANGIOGENIC STRATEGIES IN THE PREVENTION AND TREATMENT OF ATHEROSCLEROSIS

As VV and their dysfunction are associated with the initiation and progression of the atherosclerotic process and are later implicated in plaque destabilization, several preventive therapeutic opportunities can be envisioned. One logical consequence would be to target the inflammatory- and angiogenic factors or the endothelial cell response to these factors by targeting cell-specific and cell state-specific signaling events which regulate endothelial cell differentiation, integrity, metabolism, inflammatory- or angiogenic response (**Figure 2**).

INHIBITING VASCULAR GROWTH FACTORS

Investigations in animal models have shown that inhibiting vascular growth factors can dampen the expansion of VV. We and others could show that thalidomide (27, 108), endostatin (109), angiostatin (20), angiopoietin-2 (Ang-2) blocking antibodies (110), and rPAI-1_{23} (21, 111) all block VV neovessel formation and slow the progression of atherosclerotic lesions. However, from the cancer field, it is also established that antiangiogenic therapies often have transient effects as there are multiple compensatory mechanisms that take over (112). Therefore, a future strategy could be to combine antiangiogenic factors with anti-inflammatory treatment regimens for the long-term treatment of atherosclerosis.

PRESERVING VASCULAR INTEGRITY

Microvessel quality is determined by its ultrastructural composition: the morphology and integrity of endothelial cells and their junctions, and the extent of pericyte coverage. Defective microvessels are a major source of intraplaque hemorrhage in humans and as already discussed, intraplaque hemorrhage is thought to originate from microvessel hyperpermeability. Restoring microvessel integrity might therefore reduce intraplaque hemorrhage risk and prevent subsequent plaque aggravation. Interesting targets to restore integrity are the main orchestrators

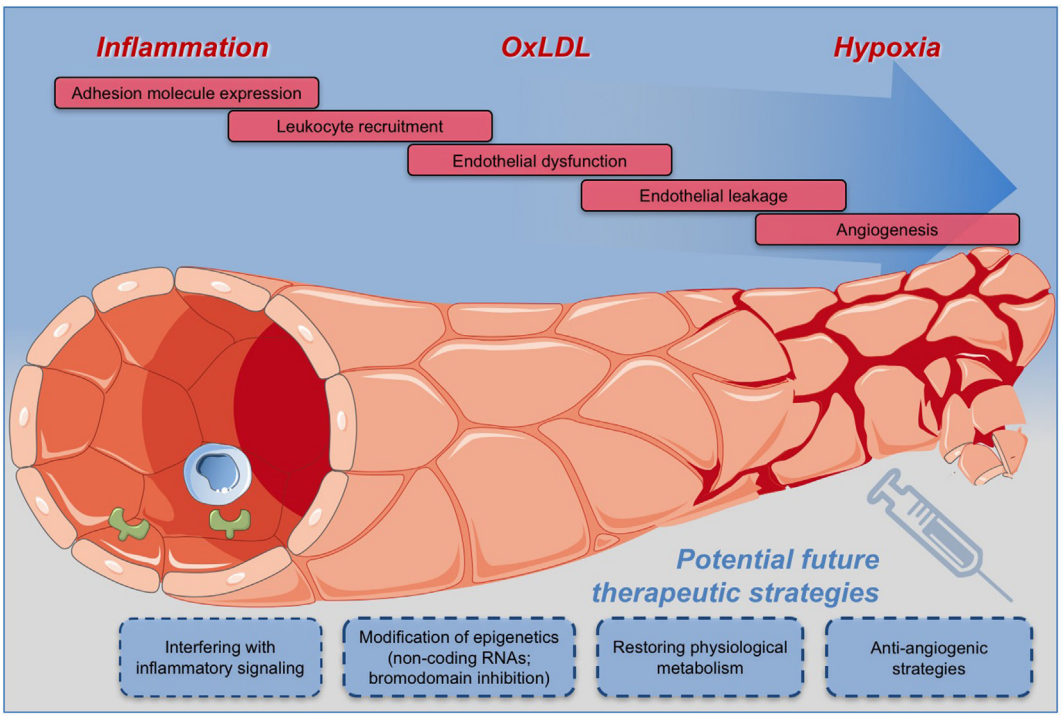

FIGURE 2 | Potential therapeutic strategies for effective and safe targeting of vasa vasorum for the prevention and treatment of atherosclerosis and the related cardiovascular diseases.

of angiogenesis and vascular maturation: VEGF, its receptors, and the angiopoietin family. VEGFs loosen endothelial junctions to allow angiogenic sprouting, which inherently causes vessel leakage in parallel. Several VEGF subtypes, with high affinity for the VEGFR-2 receptor (i.e., VEGF-A and VEGF-F), are able to induce vascular hyperpermeability in a time-dependent and tissue-specific manner (113). VEGF (preferably subtype-specific) inhibition or normalization could be a promising approach to reduce plaque microvessel dysfunction, in addition to its antiangiogenic properties. However, given the extensive physiologically important functions of VEGF, it would be desirable to interfere with downstream players of the VEGF pathway as identified by Laakkonen et al., e.g., SNAI2, RCAN1, MYCN, and NR4A1. Moreover, despite promising effects of VEGF therapy on tumor angiogenesis, serious adverse effects on cardiovascular events (angina pectoris, arterial thrombosis, cerebral- or myocardial ischemia and infarction) have been shown for the VEGF-A inhibitor bevacizumab (114). This is of particular relevance for its potential use as therapy for atherosclerosis, as a history of atherosclerosis greatly enhances the risk of cardiovascular events. The so-called "Janus" face of VEGF is explained by its positive effect on maintenance and regeneration of arterial endothelium, as opposed to its destabilizing effect on microvascular endothelium. Similar concerns for the increased risk of stroke have been described for the intraocular use of the VEGF-A inhibitor ranibizumab (115), suggesting that VEGF inhibition in general should be used with caution.

In addition, Ang-2 increases vascular permeability and decreases pericyte recruitment. Despite the beneficial effects of Ang-2 inhibition in several types of cancer (116, 117), a study examining effects of Ang-2 inhibition in atherosclerosis did not affect murine atherosclerosis and importantly found no impact on adventitial microvessel density (110). Interestingly, angiopoietin-1 (Ang-1), the counterpart of Ang-2, increases the stability of the junctions between the endothelial cells, hence promoting vessel maturity, stability, and reducing leakiness (118). Moreover, it was shown that the balance between Ang-1 and -2 correlates with intraplaque microvessel density in human atherosclerotic plaques, in which the relative abundance of Ang-2 increases microvessel quantity (119).

In addition to endothelial malformations, surrounding pericytes were found to be absent in a majority of microvessels in ruptured plaques (34), clearly linking these cells to plaque destabilization. Unfortunately, little is known about the role of pericytes in endothelial dysfunction and research regarding their relative contribution to plaque development is lacking. From other fields we know that platelet derived growth factor beta (PDGF-B) plays a role in pericyte recruitment at the blood–brain barrier (120, 121). Mice deficient in the PDGF-B retention motif (PDGF-$B^{ret/ret}$) have diminished pericyte coverage leading to permeability of the blood–brain barrier (122). Moreover, the recombinant humanized monoclonal antibody against VEGF-A, bevacuzimab, was shown to reduce vascular leakage by restoring pericyte function through induction of PDGF-B expression *in vivo* in a hindlimb ischemia mouse model (123).

Endothelial barrier integrity is also typically hampered in numerous types of cancer. Firstly, tumor cells constitute an important source of the aforementioned VEGFs. Secondly, pro-inflammatory cytokines like IL-8, often overexpressed by

cancerous cells, evoke enhanced endothelial permeability *via* both VEGF-dependent and -independent mechanisms (124, 125). Also, transforming growth factor-β1 (TGF-β1), produced by many cancer type cells in humans (126), is implicated in vascular leakage. *In vitro* data show TGF-β1 inhibits Ang-1 production (127) and stimulates VEGF release (128) in multiple cell lines thereby driving angiogenesis and vascular leakage. Interestingly, in the light of cardiovascular event prevention, the extensively used HMGCoA-reductase inhibitors ("statins") have shown to increase apoptotic cell death of pericytes both *in vitro* and *in vivo*, possibly counteracting their antiatherogenic features by destabilizing plaques (129). Although pericyte presence in coronary arteries and large blood vessels has been shown (130), their function there remains elusive. However, it is suggested they may play a role in the impairment of adequate microvascular reperfusion after myocardial infarction treatment (131).

In summary, there are several pathways and mechanisms involved in endothelial barrier destabilization, and thus multiple plausible targets to prevent this. VEGF subtypes appear especially interesting as these keep emerging as important players in different pathways. Unfortunately, VEGFs are involved in important physiological processes and therefore perhaps it is not surprising that multiple trials with VEGF inhibiting compounds show also harmful effects (e.g., hypertension, arterial thromboembolic events, and cardiotoxicity). Moreover, as previously addressed, current mouse models are not sufficient for reliably studying the contribution of intraplaque microvessels to plaque aggravation. Hence, advances in animal models to study intraplaque microvessels are needed to gain more insight in this important but underexposed contributor in atherosclerotic plaque development.

MODULATING ENDOTHELIAL CELL METABOLISM TO PREVENT VV DYSFUNCTION

Endothelial cells have the ability to switch between a mature quiescent state and an angiogenic state. Angiogenesis is an energy-intensive process and requires increased endothelial cell metabolism to support sprouting, migration, and proliferation. Restricting endothelial cell metabolism is a recently recognized strategy that can be used for the inhibition of angiogenesis. Several important recent reviews focus on the strategies that could be used to exploit endothelial metabolism for the development of antiangiogenesis therapy (132–135). As angiogenic endothelial cells rely heavily on glycolysis for ATP generation (136), inhibitors of the key glycolysis enzyme 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3) have been shown to maintain endothelial cells in a quiescent state, reducing injury- and inflammation-induced pathological angiogenesis *in vivo* (135, 137).

Upregulation of CAM has been observed in VV endothelial cells, facilitating the recruitment of circulating leukocytes. In tumor models, inhibition of PFKFB3 impairs nuclear factor kappa B (NF-κB) transcriptional activity in endothelial cells by targeting the phosphorylation of p65 and IκBα, which in turn decreases CAM expression (138). As VV play an important role in the recruitment of inflammatory cells into atherosclerotic plaques, specific reduction of CAMs by a PFKFB3 inhibitor could impact plaque initiation and progression.

We suspect that modulating endothelial cell metabolism could be used to develop strategies to stabilize VV neovessels and therefore might be of help in controlling the initiation and progression of atherosclerosis. Pericytes near the parental endothelial layer are known to surround the VV microvessel endothelium, stabilizing it, and thus establishing a mature, non-leaky vessel phenotype (139). Studies in cancer models have shown that PFKFB3 inhibitors can decrease pericyte glycolysis and impair their migration and proliferation. PFKFB3 inhibitors also promote cell quiescence and tighter cell–cell junctions (138), resulting in a tighter pericyte layer covering the endothelial cell layer and leading to the maturation and normalization of the tumor vasculature. Thus, targeting pericyte cell metabolism could be advantageous in stabilizing VV structure (13, 140, 141).

Experimentally used glycolysis inhibitors result in transient and incomplete inhibition of glycolysis. To maintain cell homeostasis, glycolysis flux is required to avoid detrimental systemic side effects (137, 142). And in fact, it can be assumed, that blocking VV neoangiogenesis could create an environment that is highly hypoxic and could worsen cell necrosis and promote plaque development. Therefore, incomplete or partial inhibition of glycolysis might very likely be advantageous in this setting.

EPIGENETIC MODIFICATION TO RENDER ENDOTHELIAL CELLS LESS SENSITIVE TO INFLAMMATORY STIMULI

Epigenetic regulation of gene expression *via* DNA methylation and histone posttranslational modifications can modulate gene expression by affecting the binding of specific transcription factors. Recent research has revealed the role of several epigenetic modifications in the pathology of atherosclerosis. These modifications are specific to particular cell types and affect specific stages of the disease (143, 144). The following section elaborates the involvement of two specific inflammatory triggers that result in epigenetic modifications in atherosclerosis and the mechanisms that could be specifically targeted to reduce the sensitivity of the VV endothelial cells to inflammatory triggers. Exposure of endothelial cells to oxidized low-density lipoproteins (oxLDL) upregulates expression of DNA methyltransferase (DNMT) 1. DNMT1 then methylates the promoter encoding the anti-inflammatory transcription factor Krüppel-like factor 2 (KLF2), resulting in its repression. Inhibition of DNMT1 by 5-aza-2′-deoxycytidine prevents methylation of the KLF2 promoter (145), and therefore this strategy could suppress the response of the endothelium to oxLDL by blocking the inflammatory and angiogenic signaling mechanisms.

Brown et al. recently uncovered the signaling mechanisms involved in inflammatory cytokine-mediated epigenetic changes that occur in endothelial cells. These epigenetic changes further drive the inflammatory processes (143). When endothelial cells are exposed to tumor necrosis factor α (TNFα), the transcription factor NF-κB locates to enhancers and promoters genome-wide,

where it recruits bromodomain-containing protein 4 (BRD4). Through the recruitment of BRD4, TNFα rapidly induces new super enhancers (inflammatory super enhancers) that drive NF-κB-mediated pro-inflammatory gene expression. Inhibiting BRD4 in endothelial cells decreases the expression of pro-inflammatory cytokines and CAM and attenuates leukocyte extravasation and plaque burden in a mouse model of atherosclerosis. Similarly, inhibition of bromodomain and extra terminal domain (BET) proteins, as occurring in BRD4, by the use of specific inhibitors like, i.e., I-BET and JQ1, have also shown positive results by dampening endothelial inflammation (146).

Epigenetic changes also regulate endothelial cell responses to hypoxia, as has been shown in cancer biology. BET inhibitors impair endothelial cell response to hypoxia thereby reducing hypoxia-induced angiogenesis (147). Investigations adapting these mechanisms to atherosclerosis research would elucidate the role of BET inhibitors in modulating the response of VV to hypoxic conditions.

Investigation of the specific responses that occur in VV under hypoxic and inflammatory conditions would help identify the best target molecules for therapeutic modalities. Similarly, specific targeting of epigenetic regulators of the neovasculature could provide a method for site-specific targeting to maintain the VV endothelial cells in a quiescent and mature state.

THERAPEUTIC POSSIBILITIES OF MICRORNAs (miRNAs) TO TACKLE VV DYSFUNCTION

MicroRNAs are small non-coding RNAs that can target multiple genes and inhibit gene expression. miRNA dysfunction is associated with atherosclerosis (148), and multiple different miRNAs, as well as target genes, can affect the progression of atherosclerosis. The therapeutic use of miRNAs is complicated by the fact that each miRNA can target mRNA of several different, completely unrelated genes and these effects may further be influenced by the cell type, its activation and/or differentiation state, and its microenvironment (149). miR-126 is thought to have atheroprotective effects as it reduces the inflammatory response by decreasing the expression of leukocyte adhesion molecules and inhibits angiogenesis in mature endothelial cells (150). However, under hypoxic conditions, or in the case of a injured vessel wall, miR-126 stimulates the formation of neovessels, thereby assuming a proangiogenic function (151–153). Therefore, given the hypoxic conditions in the VV, upregulation of miR-126 may have adverse effects assuming a proatherogenic response, resulting in a localized proliferation of unstable neovessels. Other miRNAs identified with potential therapeutic uses include miR-221 and miR-222 (154). While miR-221/222 support endothelial quiescence, they also downregulate endothelial nitric oxide synthase (155), causing endothelial dysfunction. miRNA-221/222 also simulate VSMC proliferation and accelerate neointima formation (156), which is a contributing factor for plaque progression.

Inhibition of endothelial cell-specific miRNAs like miR-92a augments angiogenesis during cardiac regeneration (157) and has a favorable effect on re-endothelialization and neointima formation after vascular injury (158). Special features of miRNAs such as cell-specific expression and cell- and activation state dependent regulation make them attractive targets for precise therapeutic approaches. While miRNA-targeting strategies hold a valid therapeutic potential, their feasibility, safety, and effectiveness in the prevention and treatment of atherosclerosis will have to be determined.

CONCLUSION AND FUTURE PERSPECTIVES

Substantial scientific evidence documents a clear association between the expansion of VV and plaque neovascularization with atherosclerotic plaque growth and progression toward an inflammatory and unstable plaque phenotype leading to plaque rupture and related clinical events. In physiological conditions, VV enable the access of oxygen and nutrients to the vessel wall. However, when expanding due to pathological stimuli, VV set the milieu for plaque growth and function as carriers of cholesterol, inflammatory cells, erythrocytes, provisional extracellular matrix, or other atherogenic molecules into the growing plaque (**Figure 1**). Conversely, prevention of new VV growth or stabilization of existing physiological VV was documented to be followed by a reduction in plaque growth and increased plaque stability. Despite these clear associations, there is a gap in the knowledge regarding the precise mechanisms regulating pathological VV expansion.

Consequently, therapeutic approaches specifically targeting the expanding microvessels in developing plaques will have to be established and evaluated. In particular, advances in the understanding of the metabolic and epigenetic players involved in the regulation of disease-specific functions of endothelial cells will enable the development of new treatment modalities for an effective and safe targeting of VV and thus for the prevention and treatment of atherosclerosis and the related cardiovascular diseases (**Figure 2**).

AUTHOR CONTRIBUTIONS

DS, EB, JAFD, and JS performed literature search and analysis and wrote the content of the manuscript. AH and JB contributed by editing and proofreading. JD designed the figures and edited the manuscript.

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Pretransplant IgA-Anti-Beta 2 Glycoprotein I Antibodies As a Predictor of Early Graft Thrombosis after Renal Transplantation in the Clinical Practice: A Multicenter and Prospective Study

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Background: Graft thrombosis is a devastating complication after renal transplantation. We recently described the association of anti-beta-2-glycoprotein-I (IgA-ab2GP1) antibodies with early graft loss mainly caused by thrombosis in a monocenter study.

Methods: Multicenter prospective observational cohort study.

Setting and participants: Seven hundred forty patients from five hospitals of the Spanish Forum Renal Group transplanted from 2000 to 2002 were prospectively followed-up for 10 years.

Outcomes: Early graft loss and graft loss by thrombosis.

Measurements: The presence of IgA anti-B2GP1 antibodies in pretransplant serum was examined using the same methodology in all the patients.

Results: At transplantation, 288 patients were positive for IgA-B2GP1 (39%, Group-1) and the remaining were negative (Group-2). Graft loss at 6 months was higher in Group-1 (12.5 vs. 4.2% $p < 0.001$), vessel thrombosis being the most frequent cause of early graft loss, especially in Group-1 (6.9 vs. 0.4% $p < 0.001$). IgA-aB2GP1 was the most important independent risk factor for graft thrombosis (hazard ratio: 13.83; 95% CI: 3.17–60.27, $p < 0.001$). Furthermore, the, presence of IgA-aB2GP1 was associated with early graft loss and delayed graft function. At 10 years, survival figures were also lower in Group-1: graft survival was lower compared with Group-2 (60.4 vs. 76.8%, $p < 0.001$). Mortality was significantly higher in Group-1 (19.8 vs. 12.2%, $p = 0.005$).

Limitations: Patients were obtained during a 3-year period (1 January 2000–31 December 2002) and kidneys were only transplanted from brain-dead donors. Nowadays, the patients are older and the percentage of sensitized and retransplants is high.

Conclusion: In a prospective observational multicenter study, we were able to corroborate that pretransplant presence of IgA-aB2GP1 was the main risk factor for graft thrombosis and early graft loss. Therefore, a prospective study is needed to evaluate the efficacy and safety of prophylactic anticoagulation to avoid this severe complication.

Keywords: graft thrombosis, kidney transplant, autoimmunity, autoantibodies, antiphospholipid syndrome, antiphospholipid antibodies, B2GP1, IgA

INTRODUCTION

The introduction of modern immunosuppression, advances in the control of infections, better methodologies, and improvement in histocompatibility tests in recent decades have substantially increased short- and long-term results after renal transplantation. However, the percentage of patients who suffer graft loss in the first months posttransplant (mainly by thrombosis) has remained unchanged (5–8%) (1).

Humoral immune response to the allograft after kidney transplantation is one of the main factors responsible for the deterioration of graft function, or even graft loss. The main target of this immune attack is the donor major histocompatibility complex (alloreactivity) (2). It has been recently described that antibody-based autoimmune responses may also affect the outcome of renal transplantation (3). The main antigens associated with an autoimmune-mediated allograft response are angiotensin 1 receptor (4), endotelin 1 receptor (5), LG3 fragment of perlecan (6), and β_2 glycoprotein I (B2GP1) (7).

Antiphospholipid antibodies (aPL) are a group of autoantibodies directed against phospholipid-binding plasma proteins, including both those circulating in the blood and/or located in the plasma membrane of blood vessel cells. Antiphospholipid syndrome (APS) is an autoimmune disorder characterized by the presence of circulating aPL associated with vessels thrombosis or adverse pregnancy outcome (8).

B2GP1 is a protein that is mainly synthesized not only in the liver but also in kidney and heart. It is localized in plasma and in the membrane of endothelial cells, the platelets being the most frequent antigen recognized by pathogenic aPL (9, 10). The prevalence of anti-B2GP1 antibodies of IgA isotype (IgA-aB2GP1) is higher in patients with chronic kidney disease than in the general population (30 vs. 1.5%) (11), and an association between these antibodies in patients undergoing hemodialysis and thrombotic events and mortality has been found (12, 13).

We recently described the association of a variety of IgA anti-beta-2 glycoprotein-I (aB2GP1) antibodies with early loss of kidney grafts, mainly from thrombosis (7). This observation was confirmed in a large historical cohort of patients transplanted over a 12-year period in a single hospital (14).

Abbreviations: OR, odds ratio; HR, hazard ratio; BMI, body mass index; PRA, panel reactive antibody score; aB2GP1, anti-beta-2 glycoprotein-I; aCL, anti-cardiolipin.

Our work has aimed to corroborate the association of IgA aB2GP1 with early graft thrombosis after renal transplantation in a prospective multicenter study.

MATERIALS AND METHODS

Study Design

We performed an observational, "non-interventional" follow-up study that included patients transplanted in five hospitals of the Spanish Forum Renal Group cohort. The original series ("Forum Renal") included all transplanted patients, without exclusions, during 2000–2002 in 14 renal transplant units in Spain ($N = 2,600$).

In our study, only patients from the five units with stored pretransplant serum samples were included, it not being possible to include the patients from the remaining centers because pretransplant serum samples were not available. Patients with hemolytic uremic syndrome, factor V Leiden, or primary APS were excluded.

A total of 740 patients who had received a kidney transplant in a 3-year period (from 01/01/2000 to 12/31/2002) were evaluated in a 10-year prospective follow-up study. Presence of aPL was examined in pretransplant serum samples used for donor–recipient crossmatch (disposition algorithm and flow diagram, **Figure 1**).

The centers and number of patients in each center included in the study were as follows: Unit-1 ($N = 269$): "Hospital 12 de Octubre" (Madrid); Unit-2 ($N = 86$): "Hospital Ramon y Cajal" (Madrid); Unit-3 ($N = 63$): "Hospital Marques de Valdecilla" (Santander); Unit-4 ($N = 282$): "Hospital de Cruces" (Bilbao); and Unit-5 ($N = 20$): "Hospital la Paz" (Madrid).

Primary Aim

The primary aim is to confirm the prevalence of pretransplant IgA-aB2GP1 antibodies and its influence on outcomes after kidney transplantation in a multicenter study. Main endpoints: (A) early graft loss and (B) graft loss by thrombosis. Secondary endpoint: delayed graft function (DGF).

Secondary Aims

The secondary aims are to perform a Replication Study comparing the results from the hospitals 2 to 5 with the results from hospital 1 that had previously described such a prevalence. Secondary endpoints: prevalence of IgA-aB2GP1 antibodies, graft and patient survival, and causes of graft loss and death.

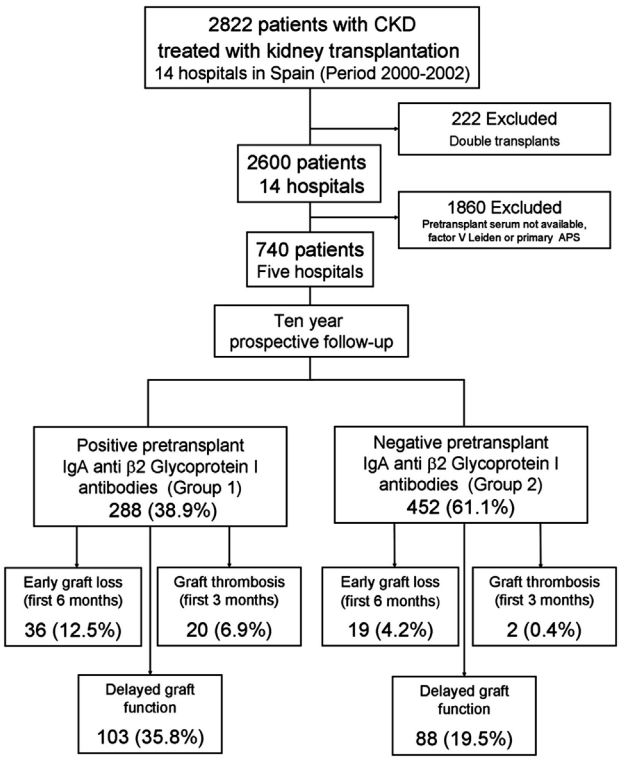

FIGURE 1 | Disposition of study and early outcomes. This cohort included 740 patients from five renal transplant units that were followed-up for 10 years.

Ethical Issues

The "Forum renal" study (prospective observational and non-interventional) was approved by all the Ethics Committees and Nephrology Departments of the 14 hospitals, assuring data confidentiality (15, 16). The patients were not asked to sign an informed consent because the Spanish legislation does not require it for observational studies without intervention. This study was approved by the ethics committee of the five hospitals, assuring data confidentiality. To control the center effect, each participating hospital was assigned a blinded code.

In addition, prior to centralizing the laboratory and database work, the study was submitted for approval to the Hospital 12 de Octubre Ethics Committee for Clinical Research and it received a favorable report (Reference Number CEIC-14/021).

Patients

Seven hundred forty patients (99.1% Caucasian) received a kidney transplant from heart-beating (brain dead) donors. Although an exhaustive coagulation study was not performed, all patients were negative for Factor V Leiden (disposition algorithm and outcomes, **Figure 1**). No patient was diagnosed of hemolytic uremic syndrome as an original disease. All of them were complement-dependent cytotoxicity crossmatch negative. All 740 patients were followed-up for a period of up to 10 years or until graft failure or death.

Database

The donor and recipient characteristics were stored in an anonymized database. The pretransplant variables included were gender, age, original disease, serology, immunological data, time on dialysis, and pretransplant conditions. In this way, hypertension, hyperlipidemia, diabetes, body mass index (BMI), smoking, and pretransplant cardiovascular disease were recorded. Immunosuppressive drug treatment was also included.

After surgery, incidence of thrombotic events, cardiovascular events, neoplasia, DGF, and acute rejection (AR) episodes were recorded. Patient and graft survival (GS), and causes of mortality and graft loss were also recorded.

Immunosuppressive Treatment

The most frequently used immunosuppressive protocol (88.5% of patients) was based on triple therapy with calcineurin inhibitors, mainly tacrolimus, associated with steroids and MMF with or without induction. In patients older than 60 years who received kidneys from donors older than 60 years, immunosuppression regimen was based on Cyclosporine (CsA), steroids, and MMF with or without induction. Immunosuppressive treatments in all the patients are described in Table S1 in Supplementary Material.

Definitions

Thrombotic Events

Thrombotic events were defined as venous thrombosis, arterial thrombosis, graft thrombosis, pulmonary thromboembolism acute stroke, or transient ischemic attack. Thrombotic episodes were diagnosed by imaging techniques or by histological study (9).

Graft Thrombosis

Graft thrombosis was diagnosed with imaging techniques. Furthermore, most of the patients who suffered graft thrombosis underwent a transplactectomy. Immediately after the surgery, the kidneys were studied in the Pathology Department, the results of macroscopic and histopathology analysis confirmed the presence of graft thrombosis.

Graft Loss

Death of the patient or loss of kidney function that requires the beginning of permanent renal replacement therapy. It is considered early graft loss if it occurs in the first 6 months after the transplant. Censored graft loss is graft loss excluding death of the patient.

Delayed Graft Function

Delayed graft function is a form of posttransplantation acute renal failure defined as a temporary graft non-function that requires hemodialysis during the first week after surgery. DFG was diagnosed once discarded hyperacute rejection, vascular complications, and urinary tract obstruction.

Arterial Hypertension

Arterial hypertension was defined as blood pressure greater than 140/90 mm Hg.

Primary Non-Function

Primary non-function was considered to exist in grafts with good perfusion that never functioned in which a biopsy study had excluded other causes of graft dysfunction as AR.

Cardiovascular Event

Cardiovascular event was considered if at least one of the following was present: heart failure, angina, coronary revascularization, myocardial infarction, stroke, or peripheral bypass.

Acute Rejection

Acute rejection was applied to acute deterioration in allograft function associated with specific histopathologic changes in the graft.

Clinically Suspected AR

Clinically suspected AR is defined as patients with AR clinical criteria lacking histological data that confirmed the diagnostic.

Panel Reactive Antibody Score (PRA)

Panel Reactive Antibody Score was defined as the percentage of the general population to which the patient reacts by preformed antibodies. PRA was studied by complement-mediated cytotoxicity using pooled lymphocyte panel with at least 35 unrelated genotypes. Patients were considered as "sensitized" with PRA values ≥50%.

Hyperlipidemia

Hyperlipidemia was defined as when hypertriglyceridemia (>150 mg/dL) or hypercholesterolemia (>200 mg/dL) were seen.

Diabetes Mellitus

Diabetes mellitus was diagnosed in patients with fasting plasma glucose greater than 126 mg/dL (7.0 mmol/L).

Normal Weight

Normal weight was defined by a BMI range from 18.5 to 25 kg/m^2. Overweight was defined when BMI is >30 kg/m^2.

Laboratory Determinations

Autoantibodies were measured in pretransplant serum used for crossmatch or in a serum sample obtained up to 15 days before transplantation. All the aPL determinations were performed in center 1.

IgA-aB2GP1 antibodies were quantified in all the centers by enzyme-linked immunosorbent assay (ELISA) using the QUANTA Lite B2 GPI IgA (INOVA Diagnostics Inc., San Diego, CA, USA). A unique assay lot was used for the analysis of the samples from centers 2, 3, 4, and 5.

The anti-cardiolipin (aCL) and aBGPI antibodies of IgG and IgM isotypes in patients from center 1 were measured with QUANTA Lite aCL IgG, QUANTA Lite B2 GPI IgG, QUANTA Lite aCL IgM, and QUANTA Lite B2 GPI IgM (INOVA Diagnostics Inc.). In patients from centers 2, 3, 4 and 5, these were measured using BioPLex 2200 multiplex immunoassay system APLS IgG and IgM (Bio-Rad, Hercules CA, USA).

Antibody levels higher than 18 U/mL were considered positive for aPL of IgG and IgM isotypes and higher than 20 U/mL were considered positive for IgA-aB2GP1. The cutoff values were those recommended by the manufacturer, which coincided with those determined in the healthy population in our country (17, 18).

Statistical Methods

Results were expressed as mean ± SE, or absolute frequency and percentage. Association between qualitative variables was determined with Pearson's Chi-square test or Fisher's exact test, when appropriate. Comparisons were performed using the Mann–Whitney *U* test in scaled variables with two categories. Probabilities less than 0.05 were considered significant.

Survival was calculated using the Kaplan–Meier Method and differences between the distributions of survival were assessed with the log-rank test.

Multivariate analyzes of graft loss and graft thrombosis-associated variables were performed using Cox regression (proportional hazards model). The relative measure of an effect was expressed as hazard ratio (HR).

Multivariate analysis of DGF (dichotomous outcome concentrated in a very short period of time) was performed using logistic regression model (19). Probabilities less than 0.05 were considered significant.

The policy regarding donor–recipient selection was based on trying to match recipients and donors with similar ages. Therefore, donor age is a recipient age-dependent variable. Donor age was not included as a statistical analysis variable except when studying DGF because it is more associated with donor age than recipient age in the literature (20).

Data were processed and analyzed using Medcalc for Windows version 16.1 (MedCalc Software, Ostend, Belgium).

RESULTS

Antiphospholipid Antibodies

The average pretransplant levels of aCL antibodies were IgM 5.4 U/mL ± 0.7 and IgG 4.0 U/mL ± 0.4. Mean levels of aB2GP1 antibodies were as follows: IgM 4.3 U/mL ± 0.8, IgG were 4.1 U/mL ± 0.5, and IgA were 32.4 U/mL ± 1.8 (Table S2 in Supplementary Material). Patients whose antibody levels were above the cutoff were considered positive. Prevalence of aCL positive patients was 1.1% for IgM and 1.2% for IgG. Prevalence of aB2GP1 antibodies patients was 1.6% for IgM and 1.2% for IgG.

Patients with IgA-aB2GP1 Antibodies

Two hundred eighty-eight (38.9%) patients were positive for IgA-aB2GP1 antibodies (Group-1) and 452 were negative (Group-2). Patients in Group-1, were immunologically less complex and there were fewer retransplanted patients (10.8 vs. 17.5%; $p = 0.017$) and less hyperimmunized patients (6.6 vs. 11.9%; $p = 0.024$). The prevalence of dyslipidemia and hypertension was slightly higher in Group-1. The remaining pretransplant characteristics did not differ between both groups (**Table 1**). The correlation between recipient age and IgA-aB2GP1 levels was very weak (Correlation coefficient $r = 0.184$, 95% CI: 0.114–0.253).

Clinical Events and Course in the Early Posttransplant Period (6 Months)

Thirty-six patients in Group-1 (12.5%) lost their graft during the first semester after transplantation vs. 19 in the Group-2 (4.2%, $p < 0.001$). At 3 months, the percentage of patients with graft

TABLE 1 | Pretransplant condition of patients in Group-1 (positive for IgA-aB2GP1 antibodies) and in Group-2 (negative patients).

Condition	Group-1 (*N* = 288)		Group-2 (*N* = 452)		*P*-value
	Patients/ mean	%/SE	Patents/ mean	%/SE	
Sex (women)	107	37.2%	198	43.8%	N.S.
Age (years)[a]	51.9	±0.8	47.4	±0.6	<0.001
Donor age (years)[a]	47.9	±1	44.2	±0.8	N.S.
Body mass index[a]	25.5	±0.3	24.9	±0.2	N.S.
Time on dialysis (months)[a]	36.5	±2.2	28.8	±2.0	N.S.
Type of dialysis					
Hemodialysis	217	75.3%	342	75.7%	N.S.
Peritoneal dialysis	58	20.1%	100	22.1%	N.S.
Both	12	4.2%	8	1.8%	N.S.
Undialyzed	1	0.3%	2	0.4%	N.S.
Panel reactive antibody score (PRA) at transplantation >50%	5	1.7%	19	4.2%	N.S.
Historical PRA >50%	19	6.6%	54	11.9%	0.024
Previous kidney transplant	31	10.8%	79	17.5%	0.017
Cold ischemia (h)[a]	19.5	±0.3	19.8	±0.3	N.S.
Associated conditions					
Diabetes mellitus	36	12.5%	41	9.1%	N.S.
Type 1 diabetes	14	4.9%	17	3.8%	N.S.
Type 2 diabetes	22	7.6%	24	5.3%	N.S.
Dyslipidemia	90	31.2%	98	21.7%	0.004
Hypertension	230	79.9%	311	68.8%	0.001
Causes CKD					
Chronic glomerulonephritis	73	25.3	137	30.3%	N.S.
Interstitial kidney disease	41	14.2%	59	13.1%	N.S.
Nephroangiosclerosis	20	6.9%	40	8.8%	N.S.
Polycystic kidney disease	47	16.3%	71	15.7%	N.S.
Diabetes mellitus	27	9.4%	29	6.4%	N.S.
Unknown	45	15.6%	67	14.8%	N.S.
Others	35	12.2%	49	10.8%	N.S.

N.S., non-significant.

[a]*Mann–Whitney test was used because variable is not normally distributed.*

TABLE 2 | Posttransplant events of patients in Group-1 (positive for IgA-aB2GP1 antibodies) and in Group-2 (negative patients).

Condition	Group-1 (*N* = 288)		Group-2 (*N* = 452)		*P*-value
	Patients/ mean	%/SE	Patents/ mean	%/SE	
Delayed graft function (DGF)	103	35.8%	88	19.5%	<0.001
Graft loss on the complete follow-up (global 29.6%)	114	39.6%	105	23.2%	<0.001
First-month (global 3.9%)	20	6.9%	9	2%	0.001
First-trimester (global 5.9%)	31	10.8%	13	2.9%	<0.001
First semester (global 7.4%)	36	12.5%	19	4.2%	<0.001
First year (global 8.5%)	38	13.2%	25	5.5%	<0.001
Causes graft loss first semester					
Acute rejection (AR)	7	2.4%	1	0.2%	0.014
Non-functioning graft	1	0.3%	6	1.3%	N.S.
Death (with a functioning kidney)	4	1.4%	4	0.9%	N.S.
Cardiovascular diseases (CVDs)	1		0		N.S.
Infections	3		0		N.S.
Sudden death	0		2		N.S.
Others	0		2		N.S.
Graft thrombosis	20	6.9%	2	0.4%	<0.001
Others	4	1.4%	6	1.3%	N.S.
Graft loss from month 7 to end of follow-up	78	27.1%	86	19%	<0.001
AR	5	1.7%	0	0%	0.019
Death (with a functioning kidney)	41	14.2%	47	10.4%	N.S.
CVDs	9		11		N.S.
Infections	8		7		N.S.
Cancer	6		10		N.S.
Sudden death	3		3		N.S.
Others	15		16		N.S.
Chronic allograft nephropathy	26	9%	32	7.1%	N.S.
Others	6	2.1%	7	1.5%	N.S.
Cardiovascular events					
Myocardial infarction	7	2.4%	18	4%	N.S.
Stroke	18	6.3%	9	2%	0.005
Angina pectoris	9	3.1%	7	1.5%	N.S.
Patients with AR episodes	28	9.7%	43	9.5%	N.S.
Death in follow-up	57	19.8%	55	12.2%	0.005
Death first semester	11	3.8%	8	1.8%	N.S.
Death from months 7 to 24	46	16%	47	10.4%	0.034

N.S., non-significant.

loss in the Group-1 was also significantly higher than in Group-2 (10.8 vs. 2.9%, $p < 0.001$) (**Table 2**). Differences between patients with early graft loss (<6 months) and remaining patients were age (55.7 ± 1.7 vs. 48.6 ± 0.5 years, $p < 0.001$), presence of DGF (50.9 vs. 23.8%, $p < 0.001$), positivity for IgA-aB2GP1 antibodies (65.5 vs. 36.8%, $p < 0.001$), and a higher proportion of patients with nephroangiosclerosis as cause of end-stage renal disease (ESRD) (20 vs. 7.2%, $p = 0.001$) (**Table 3**). As the risk of graft loss and graft thrombosis is partially dependent on the donor factors, we performed an analysis of same-donor paired kidneys (21) showing the same results (data not shown).

A Kaplan–Meier survival analysis showed significantly lower 6-month GS rates in Group-1 (HR: 3.10, 95% CI: 180–5.35, $p < 0.001$, **Figure 2A**). Graft thrombosis was the most common cause of graft loss (22 patients, 61% of losses), this occurring more frequently in Group-1 (6.9 vs. 0.4%, $p < 0.001$, **Figure 2B**).

Delayed graft function was also significantly higher in Group-1 (35.8 vs. 19.5%; $p < 0.001$). There were no differences regarding AR episodes in both groups (**Table 2**).

IgA anti-B2GP1 Antibodies Are an Independent Risk Factor for Early Graft Loss

Early graft loss-associated factors that were significant in the univariate analysis (**Table 3**) were included in a multivariate analysis [**Table 4** (A)].

Presence of IgA-aB2GP1 antibodies continued to be an independent and significant risk factor for graft loss after adjusting for other risk factors (HR: 2.49; 95% CI: 1.40–4.43, $p = 0.002$). Recipient age, presence of DGF, and nephroangiosclerosis as cause of ESRD were also independent risk factors for early graft loss [**Table 4** (A)].

TABLE 3 | Clinical characteristics of patients with early graft loss vs. patients with functioning graft at 6 months posttransplant.

Condition	Early graft loss (*N* = 55)		Functioning graft (*N* = 685)		
	N/ mean	%/SE	*N*/ mean	%/SE	*P*
Sex (women)	17	30.9%	288	42%	N.S.
Age (years)[a]	55.7	±1.7	48.6	±0.5	**<0.001**
Donor age (years)[a]	55.2	±2.2	44.9	±0.7	<0.001
Body mass index[a]	25.6	±0.6	25.1	±0.2	N.S.
Time on dialysis (months)[a]	44.9	±7.1	32.6	±1.6	N.S.
Pretransplant clinical characteristics					
Diabetes mellitus	5	9.1%	72	10.5%	N.S.
Type 1 diabetes	2	3.6%	29	4.2%	N.S.
Type 2 diabetes	3	5.5%	43	6.3%	N.S.
Dyslipidemia	18	32.7%	170	24.8%	N.S.
Hypertension	44	80%	497	72.6%	N.S.
Patients IgA-aB2GP1 positive	36	65.5%	252	36.8%	**<0.001**
Causes CKD					
Chronic glomerulonephritis	15	27.3%	195	28.5%	N.S.
Interstitial kidney disease	9	16.4%	91	13.3%	N.S.
Nephroangiosclerosis	**11**	**20%**	**49**	**7.2%**	**0.002**
Polycystic kidney disease	6	10.9%	112	16.4%	N.S.
Diabetes mellitus	3		53		N.S.
Unknown	4		108		N.S.
Others	7		77		N.S.
Transplant-associated factors					
Previous kidney transplant	13		97		N.S.
Panel reactive antibody score (PRA) at time of transplant >50%	4	7.3%	20	2.9%	N.S.
Historical PRA >50%	7		66		N.S.
Cold ischemia (h)[a]	20.7	±0.6	19.6	±0.2	N.S.
DGF	**28**	**50.9%**	**163**		**<0.001**

N.S., non-significant.

The variables that were selected for the multivariate analysis are marked in bold.

[a]*Mann–Whitney test was used because variable is not normally distributed.*

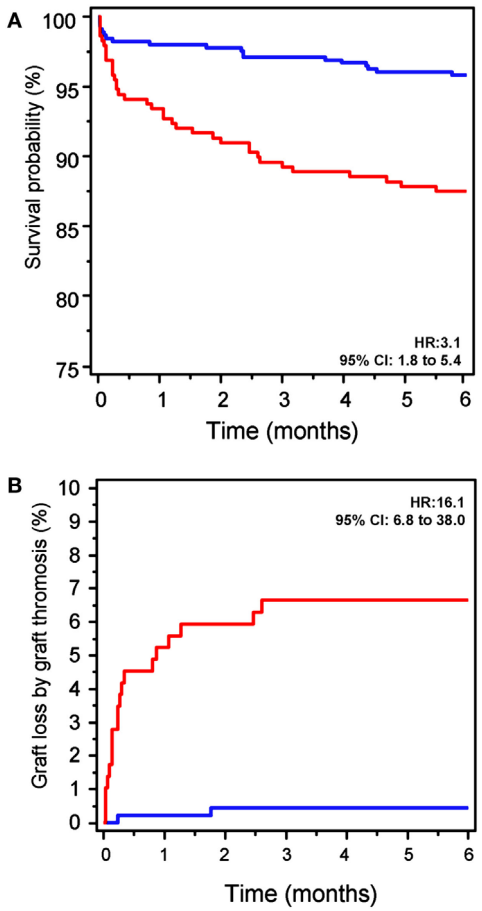

FIGURE 2 | Early graft loss. **(A)** Evolution of graft survival (GS) at the first 6 months of the follow-up. GS in patients in Group-1 (positive for IgA-aB2GP1 antibodies, red line) was significantly lower than that observed in Group-2 (blue line). HR, hazard ratio (Kaplan–Meier analysis). **(B)** Graft loss by thrombosis in the 6 months after transplantation was significantly higher in patients in Group-1 (red) than in patients in Group-2 (blue).

IgA-aB2GP1 Antibodies Are an Independent Risk Factor for Graft Thrombosis

IgA-aB2GP1, cold ischemia time, and age were identified in univariate analysis as significant and associated factors for graft thrombosis (Table S3 in Supplementary Material). In a Cox proportional regression multivariate analysis, cold ischemia time, DGF, and especially IgA-aB2GP1-ab (HR: 13.83; 95% CI: 3.17–60.27; $p < 0.001$) were identified as independent risk factors for graft thrombosis [**Table 4** (B)].

No significant differences were observed between the group I patients who suffered graft loss due to thrombosis and the remaining patients of this same group who had cardiovascular risk factors: dyslipidemia (5.6 vs. 7.6%; $p = 0.533$), hypertension (6.1% vs. 10.3%; $p = 0.255$), type 2 diabetes (9.1 vs. 6.8%; $p = 0.681$), and BMI (23.9 ± 0.8 vs. 25.6 0.3; $p = 0.128$).

IgA-aB2GP1 Antibodies Are an Independent Risk Factor for DGF

Variables previously significantly associated to DGF (Table S4 in Supplementary Material) were analyzed in a logistic regression univariate analysis. Those that continued to be significant were studied in a multivariate analysis: donor age, BMI, hypertension, and especially IgA-aB2GP1 antibodies [odds ratio (OR): 2.08, 95% CI: 1.47–2.95; $p < 0.001$] were identified as independent risk factors for DGF [**Table 4** (C)].

Late Posttransplant Period (from 7 Months to 10 Years)

Graft survival (**Figure 3**) was significantly lower (Kaplan–Meier analysis) in Group-1, both non-censored GS (HR: 1.63; 95% CI: 1.19–2.25; $p = 0.002$) and death-censored GS (HR: 1.80; 95% CI: 1.12–2.89; $p = 0.009$).

Causes of graft loss in this period were similar in both groups except for death with a functioning kidney that was more frequent in Group-1 patients (OR: 1.60; 95% CI: 1.02–2.51; $p = 0.042$).

Complete Follow-up (0–120 Months)

Non-censored GS was 70.4% at 10 years when the total group was considered. GS analysis (Kaplan–Meier) showed that graft loss

TABLE 4 | Multivariate analysis.

Factors	Univariate			Multivariate		
	Hazard ratio (HR)	**95% CI**	***P*-value**	**HR**	**95% CI**	***P*-value**
A. Early graft loss						
Patients IgA-aB2GP1 positive	3.11	1.78–5.41	<0.001	**2.49**	**1.40–4.43**	**0.002**
Recipient age (years)	1.04	1.02–1.06	<0.001	**1.02**	**1.00–1.05**	**0.042**
Nephroangiosclerosis	3.01	1.55–5.82	0.001	**2.61**	**1.32–5.17**	**0.006**
Delayed graft function (DGF)	3.14	1.85–5.33	<0.001	**2.35**	**1.37–4.04**	**0.002**
B. Early graft loss by thrombosis						
IgA-aB2GP1 positive	16.09	3.76–68.85	<0.001	**13.83**	**3.17–60.27**	**<0.001**
DGF	4.29	1.83–10.03	<0.001	2.42	1.01–5.81	0.047
Cold ischemia time (h)	1.09	1.01–1.17	0.024	**1.09**	**1.056–3.233**	**0.041**
	Odds ratio (OR)	**95% CI**	***P*-value**	**OR**	**95% CI**	***P*-value**
C. DGF						
IgA-aB2GP1 positive	2.30	1.65–3.22	<0.001	**2.08**	**1.47–2.95**	**<0.001**
Donor age (years)	1.02	1.01–1.03	<0.001	**1.02**	**1.01–1.03**	**0.003**
Cold ischemia (h)	1.04	1.01–1.07	0.011	1.03	1.00–1.07	0.050
Body index mass	1.07	1.04–1.11	<0.001	**1.06**	**1.02–1.10**	**0.002**
Hypertension	1.67	1.12–2.49	0.012	**1.56**	**1.03–2.37**	**0.039**
Time on dialysis (months)	1.00	1.00–1.01	0.307	–	–	–

(A) Cox proportional regression multivariate analysis (p < 0.001) of graft loss-associated variables significant in univariate analysis. (B) Cox proportional regression multivariate analysis (p < 0.001) of variables associated with graft loss by thrombosis. (C) Logistic regression multivariate analysis (p < 0.001) of DGF-associated variables significant in univariate analysis. The variables that were selected for the multivariate analysis are marked in bold.

was higher in Group-1 vs. Group-2: survival was 60.4 vs. 76.8%; HR: 1.91; 95% CI: 1.45–2.52; $p < 0.001$ (**Figure 3B**, dotted lines).

Death-censored GS (Kaplan–Meier analysis) was lower in Group-1 than Group-2: 76.1 vs. 86.5%; HR: 2.34; 95% CI: 1.61–3.39; $p < 0.001$ (**Figure 3B**, solid lines).

Global mortality in the follow-up was 15.1%, this being significantly higher in Group-1 (19.8 vs. 12.2%, $p = 0.005$; **Table 2**). Therefore, survival probability was lower in Group-1 (HR: 1.53; 95% CI: 1.07–2.18; $p = 0.015$, **Figure 4A**).

GS and Outcomes Excluding Center 1

When patients from centers 2, 3, 4, and 5 were considered alone with the exclusion of center 1, GS (**Figure 3C**) was also significantly lower in Group-1 vs. Group-2 patients, at 6 months (86.9 vs. 94.1%; HR: 2.30; 95% CI: 1.24–4.24; $p = 0.007$) and at 10 years (71.6 vs. 86.5%; HR 2.07 95% CI: 1.47–2.91; $p < 0.001$).

Notably, graft thrombosis (6 vs. 0.7%; OR: 8.66; 95% CI: 1.92–39.16; $p = 0.005$; **Figure 4B**) and DGF (39.7 vs. 22.4%; OR: 2.28; 95% CI: 1.52–3.40; $p < 0.001$) were significantly more frequent in patients in Group-1 than observed in Group-2 patients.

Remarkably, in a Cox proportional regression multivariate analysis (Table S5 in Supplementary Material), IgA-aB2GP1 antibodies continued to be an independent and significant risk factor associated with early graft-loss (HR: 1.90; 95% CI: 1.00–3.61; $p = 0.049$) and graft thrombosis (HR: 8.28; 95% CI: 1.75–39.07; $p = 0.008$). Likewise, IgA-aB2GP1 continued to be an independent risk factor for DGF in a multivariate logistic regression analysis (OR: 2.19; 95% CI: 1.43–3.37; $p < 0.001$).

Mortality was also higher on Group-1 (21.6 vs. 12.5%; $p = 0.008$; HR: 1.81; 95% CI 1.15–2.85; $p = 0.009$).

DISCUSSION

We previously described that the presence of pretransplant IgA-aB2GP1 is an independent risk factor for early graft loss in two unicenter studies (7, 14) and graft thrombosis (14) after renal transplantation. For the first time, herein, we have been able to demonstrate in an observational, multicenter, and prospective study including 740 renal transplant patients from five hospitals of Spain that preformed IgA-aB2GP1 is an independent risk factor for early graft thrombosis. Therefore, the presence of pretransplant IgA-aB2GP1 may be considered as a new tool to predict early graft loss by thrombosis after renal transplantation. In addition, these important results can be the rationale to investigate if prophylactic treatment of pretransplant IgA-aB2GP1 positive patients could improve this catastrophic early complication.

In this study we found that 55 (7.4%) of renal transplant patients suffered early loss in the first 6 posttransplant months and 22 of them (40%) due to graft thrombosis. Therefore, graft thrombosis was the main cause of early graft loss, representing 3% of the total group, a percentage almost similar to the other series (22). In addition, it is important to consider that these findings were obtained from a database representing the clinical practice in our country, including all transplanted patients from brain-death donors during 2000–2002, without exclusions. Notably, 90% of patients with graft thrombosis exhibited pretransplant IgA-aB2GP1 and consequently this parameter was the most important significant risk factor for graft thrombosis. This interesting finding was also corroborated when only centers 2, 3, 4, and 5 were analyzed. This partial analysis that excluded center 1 was done in order to demonstrate that the data from center 1 were not conditioning the final results. Therefore, the relationship between renal graft thrombosis and the presence of pretransplant IgA-aB2GP1 seems to be very clearly significant.

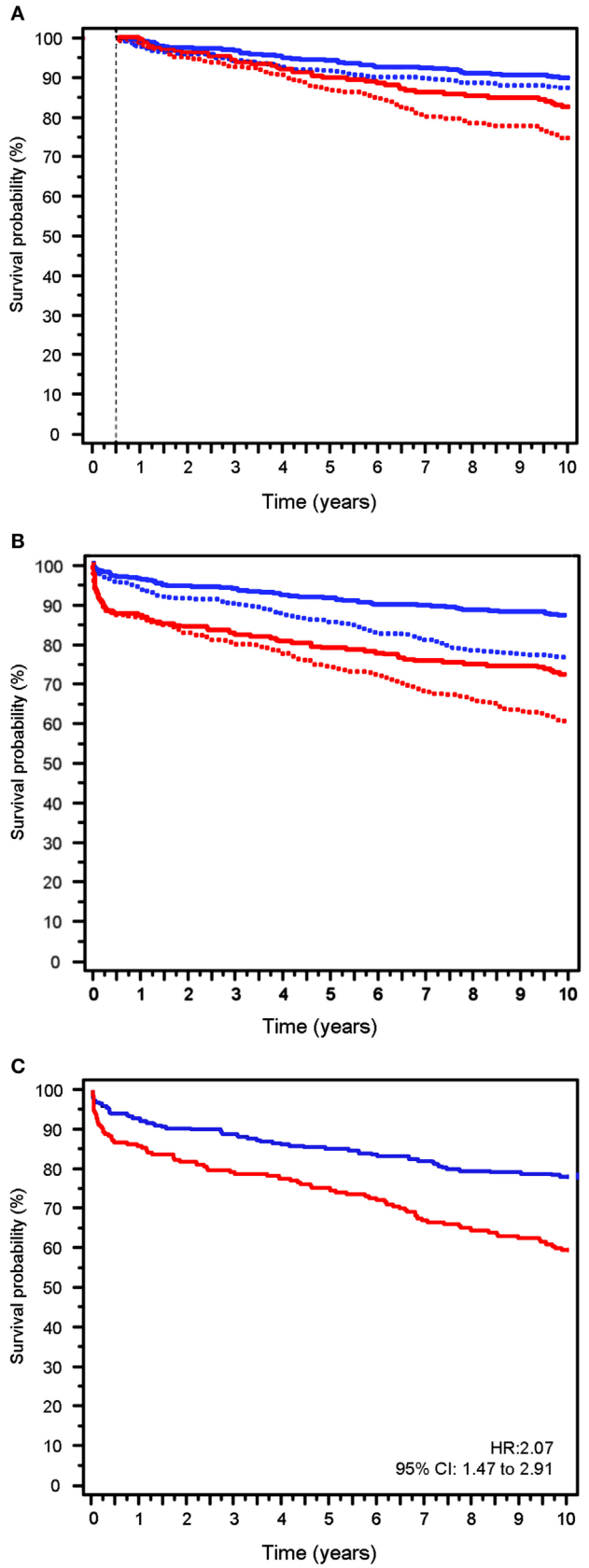

FIGURE 3 | Continued

FIGURE 3 | Graft survival (GS) on the complete follow-up. **(A)** GS from the end of the sixth month to the end of follow-up. Death-censored GS (solid line) vs. uncensored GS (dotted line). **(B)** GS during the 10 years of follow-up. Death-censored GS (solid line) vs. uncensored GS (dotted line). **(C)** Graft loss in the follow-up excluding Center 1 patients (uncensored data). Red lines patients in Group-1. Blue lines patients in Group-2.

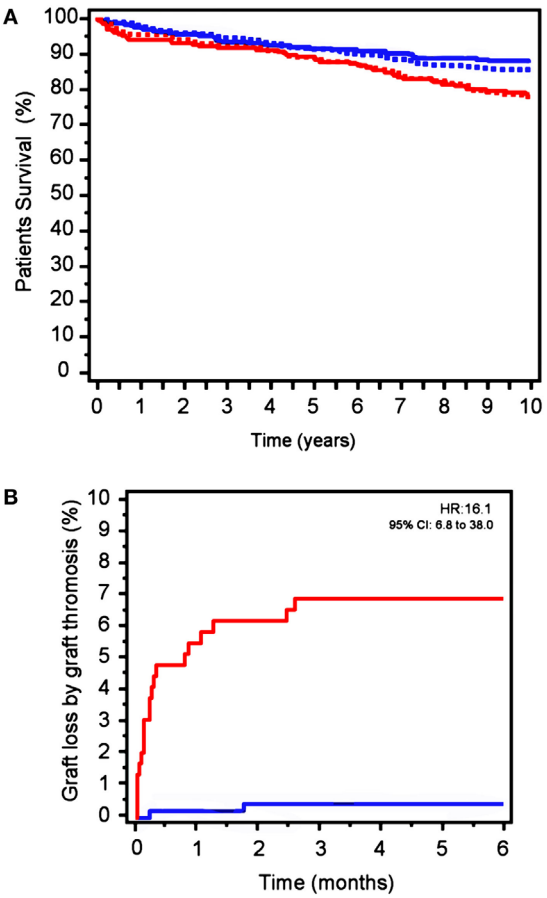

FIGURE 4 | **(A)** Mortality in patients in Group-1 (red) vs. Group-2 (blue) in the follow-up. Solid lines: excluding center 1. Dotted lines: complete study. **(B)** Graft-loss by graft thrombosis excluding center 1. Observation: the survival curves from the study that excludes center 1 and the survival curves from the complete study are so similar that they practically overlap and impede a correct visualization.

The prevalence of anti-B2GP1 IgA was significantly lower in retransplanted patients than in those receiving a first transplant. This finding confirms that observed in previous studies, that is, that the prevalence of these autoantibodies is lower in patients who have undergone immunosuppressant treatment prior to transplantation, either due to an autoimmune illness or a previous transplant (11, 14).

The prevalence of hyperlipidemia and hypertension in Group-1 patients is higher than in those in Group-2. We could hypothesize that this higher prevalence could be related to their older age; however, there is no known explanation for this finding. The presence of these factors could affect the development of vascular conditions such as arteriosclerosis. However, as the

prevalence of hyperlipidemia and hypertension does not show significant differences in the group I patients who have lost the graft due to thrombosis and the remaining group I patients who did not suffer graft loss, these factors do not seem to affect the vascular thrombosis of the graft.

Although it is known that the binding of antibodies with B2GP1 is critical to the development of events APS, the physiological functions of are unknown. Thus, the specific mechanism by which the antibodies act remains elusive (23).

Studies with electron microscopy suggest that the tridimensional structure of B2GP1 is not limited to a single conformation and it has been suggested that the geometry of the B2GP1 can alter their potential to interact with autoantibodies (24, 25).

Membrane-bound B2GP1 acquires a J-shaped structure and binding of anti-B2GP1 antibodies stabilizes the interaction of the protein with membrane phospholipids that is hypothesized to potentiate signaling through several receptors associated with prothrombotic cellular actions (26). Patients receiving a graft should undergo surgery, a known "second hit" to trigger the event. The risk of thrombotic events in carriers of IgA-aB2GP1 is higher for carriers of other thrombosis-associated risk factors such as smoking, infections, prolonged immobilization, use of estrogens, or surgical procedures (27). However, the contribution of these factors as second hits in association with IgA-aB2GP1 needs to be established in subsequent studies.

Since not all IgA-aB2GP1 positive patients develop thrombotic complications (18), the next step should be to find a marker that would identify the patients with a higher risk of thrombosis among those are IgA-aB2GP1 positive (28). We recently described that the presence of circulating immune complexes (CIC) of IgA bounded to B2GP1 was associated with occurrence of recent thrombotic events (29) and are a predictor of acute thrombotic events, including graft thrombosis after renal transplantation (30). However, we have not been able to detect the presence of CIC as, unfortunately, although the conditions of preservation of serum samples were adequate for the determination of IgA-aB2GP1, these conditions were not consistent with the maintenance of stable CIC and they had not been reliably determined in the present group of patients in some centers.

On the other hand, global results of this population at 10 years may be considered to be in agreement with other series of renal transplantation with deceased donors: patient survival 84.9%, DCGS and NCGS of 81 and 70.4%, respectively. It is important to note that our patients were closely followed-up in the renal transplant offices under the umbrella of a national health service with universal and lifelong support. These findings are in agreement with previous results in Spain (31). As we noted previously, IgAB2GP1 positive patients show lower survival figures than negative patients. Notably, the immunosuppressive protocol was based on calcineurin inhibitors and MMF. Only six patients did not receive calcineurin inhibitors as an initial immunosuppressive protocol, and therefore, we cannot discuss if m-TOR inhibitors can be useful in the prevention of vascular lesions associated with aPL (32). Furthermore, prior results have demonstrated a superior capacity of CNI over m-TOR inhibitors to inhibit alloantibodies production in renal transplantation (33, 34).

One of the main limitations is that these results were obtained during the first 3 years of the twenty-first century. Currently, donors and patients are older, show a high percentage of sensitized and retransplants as well as new forms of renal transplantation and with non-heart-beating donors. At present, and with these demographic changes, short- and long-term results could be different. At this point, such a limitation makes it necessary to design a long-term study.

Determination of IgA-aB2GPI antibodies in patients from all the centers was performed with the same diagnostic system (Quanta Lite ELISA). However, a different diagnostic system was used to quantify aPL of IgG and IgM isotype for center 1 (ELISA) than in rest of the centers (multiplex immunoassay). This change of methodology is irrelevant because the efficiency in determination of aPL of IgG and IgM isotypes using ELISA and multiplex diagnostic systems is very similar (35, 36). Furthermore, the average amount of antibody levels and the proportion of aPL of IgG and IgM isotype-positive patients was similar to those described in previous studies (7, 11).

The need to include the determination of aB2GP1 IgA in diagnostic protocols has been suggested recently. Currently, the IgA isotype is not included among the APS classification criteria of the APS, so that very few centers perform the IgA determination aB2GP1. For this reason, cases of IgA-mediated thrombosis are clearly underdiagnosed (37).

In summary, for the first time, we have been able to corroborate in a large cohort of patients from five hospitals that the presence of pretransplant IgA-aB2GP1 is an independent risk factor for graft thrombosis after renal transplantation, a devastating condition without available prevention and treatment. This finding can be the rationale for a prospective study to demonstrate if prophylactic anticoagulation can be useful to improve this early complication.

ETHICS STATEMENT

The study was submitted to the Ethics Committee for Clinical Research (ECCR) of Hospital "12 de Octubre" and received a favorable report (Reference Number CEIC-14/021).

AUTHOR CONTRIBUTIONS

AS and JM conceived the project, designed the research, discussed the results, and wrote the manuscript. AS, MS, and JM-F performed the antiphospholipid determinations and were responsible for the database and the statistical analysis. JM, JG, MA, RM, FE, AA, NM, and EA were responsible for the patients' care and clinical data collection. AS, MS, JM-F, JC, and ML-H were responsible for coordination of the Organ Transplant Waiting List Serum Bank. MM evaluated the histopathology. All authors contributed to the data interpretation, reviewed the manuscript, and agreed with the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.00468/full#supplementary-material.

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MINI REVIEW
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Endothelial to Mesenchymal Transition Represents a Key Link in the Interaction between Inflammation and Endothelial Dysfunction

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Endothelial cells that line the inner walls of blood vessels are in direct contact with blood and display remarkable heterogeneity in their response to exogenous stimuli. These ECs have unique location-dependent properties determined by the corresponding vascular beds and play an important role in regulating the homeostasis of the vascular system. Evidence suggests that vascular endothelial cells exposed to various environments undergo dynamic phenotypic switching, a key biological program in the context of endothelial heterogeneity, but that might result in EC dysfunction and, in turn, cause a variety of human diseases. Emerging studies show the importance of endothelial to mesenchymal transition (EndMT) in endothelial dysfunction during inflammation. EndMT is a complex biological process in which ECs lose their endothelial characteristics, acquire mesenchymal phenotypes, and express mesenchymal cell markers, such as alpha smooth muscle actin and fibroblast-specific protein 1. EndMT is induced by inflammatory responses, leading to pathological states, including tissue fibrosis, pulmonary arterial hypertension, and atherosclerosis, *via* dysfunction of the vascular system. Although the mechanisms associated with inflammation-induced EndMT have been identified, unraveling the specific role of this phenotypic switching in vascular dysfunction remains a challenge. Here, we review the current understanding on the interactions between inflammatory processes, EndMT, and endothelial dysfunction, with a focus on the mechanisms that regulate essential signaling pathways. Identification of such mechanisms will guide future research and could provide novel therapeutic targets for the treatment of vascular diseases.

Keywords: endothelial dysfunction, inflammatory process, endothelial to mesenchymal transition, endothelial heterogeneity, vascular disease

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INTRODUCTION

Endothelial cells (ECs) play a key role in maintaining vascular homeostasis in response to various stimuli. They can regulate vascular tone, permeability, coagulation, and inflammation through the regulation of numerous mediators, such as endothelium-derived relaxing and contracting factor, cell-adhesion molecules, cytokines, and chemokines (1, 2). However, vascular injuries resulting from procedures and conditions, such as angioplasty, stenting, diabetes, hypertension, and immune-mediated damage, can lead to endothelial dysfunction, resulting in disturbance or loss of normal endothelial functions (1, 3–5). Many studies have revealed an association between

endothelial dysfunction and inflammatory stress in vascular biology. Under conditions of chronic inflammation, sustained activation of ECs by inflammatory stimuli, such as interleukin (IL)-6, tumor necrosis factor-α (TNF-α), IL-1β, and pathogens, cause alterations in normal endothelial function, resulting in impaired endothelial-dependent immune response, which is the hallmark of endothelial dysfunction (6–9). Indeed, endothelial dysfunction due to inflammatory stress contributes to the pathogenesis of many diseases, including fibrosis, atherosclerosis, pulmonary arterial hypertension (PAH), and pathological angiogenesis (10–17). In addition, emerging evidence shows that the nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3 (NLRP3) inflammasome not only has a role as a critical sensor in immune response, but also has a critical role in endothelial dysfunction and the pathogenesis of vascular diseases, such as atherosclerosis and metabolic syndrome (18–20). Assembly and activation of the NLRP3 inflammasome results in the conversion of the inactive procaspase-1 into active caspase-1, with subsequent secretion of mature IL-1β and IL-18 in such diseases (21–25).

Accumulating evidence suggests that endothelial to mesenchymal transition (EndMT) represents a key link in the complex interactions between inflammatory stress and endothelial dysfunction. EndMT is a phenotypic switching process by which ECs lose their characteristics and acquire mesenchymal traits (26, 27). EndMT exhibits features similar to those of epithelial to mesenchymal transition (EMT) and is often considered a specific form of EMT (26, 28). Although both processes use the same signaling pathways and result in cells with a mesenchymal phenotype, studies of the differences between EMT and EndMT are needed due to differences in the origin, fundamental function, and microenvironment of ECs and epithelial cells (29). EndMT was first discovered and has been studied in heart development and emerging studies show that EndMT can occur in postnatal pathologies associated with several diseases, such as fibrosis, cancer, neointima formation, cerebral cavernous malformations, atherosclerosis, and PAH (26, 28–30). Indeed, it has been reported that EndMT contributes to endothelial dysfunction during inflammatory conditions, and that some inflammatory mediators, such as IL-1β, TNF-α, nuclear factor kappa B (NF-κB) transcription factor, and endotoxins, can activate ECs and convert them to mesenchymal-like cells through the EndMT process (6, 7, 31). However, how EndMT contributes to disease progression remains unclear (32), and the specific role of EndMT in inflammatory stimulus-induced endothelial dysfunction has not been fully elucidated due to the dynamic nature of the EndMT process, which consists of multiple steps.

A single layer of ECs lining blood vessels displays heterogeneity in function, morphology, gene expression, and antigen composition depending on location (2) and behaves differently based on its exposure to different microenvironments (33). Therefore, it is also important to understand the molecular basis of inflammation-induced EndMT in the context of endothelial heterogeneity, because this can be critical for developing personalized vascular therapies for patients with vascular bed specific diseases (33).

In this review, we summarize the knowledge currently available regarding the role of EndMT in inflammatory processes and discuss endothelial heterogeneity in the context of inflammation.

ENDMT MEDIATORS AND SIGNALING PATHWAYS DURING INFLAMMATION

Endothelial cells play an important role in the maintenance of homeostasis across the entire vascular system (10, 34). ECs actively participate in the regulation of immune responses to various stimuli. To this end, the inflammation-mediated signaling pathway has been extensively studied (6, 17). However, cell signaling associated with inflammation-induced EndMT remains poorly understood. Nevertheless, the molecular mechanisms underlying inflammation-induced EndMT have been gradually identified based on observations of EMT processes that are relatively well studied on inflammatory responses (6). Current evidence suggests that inflammation-induced EndMT, similar to that of EMT, is largely governed by two signaling pathways: the transforming growth factor beta (TGFβ) pathway and the non-TGFβ pathway (35). TGFβ is the most well-known EndMT inducer and upregulates the expression of transcription factors, such as snail, slug, and zinc finger E-box-binding homeobox 1 (ZEB1). These transcription factors then upregulate the expression of mesenchymal markers, such as alpha smooth muscle actin (α-SMA), smooth muscle protein 22 alpha (SM22α), calponin, vimentin, type I collagen, fibronectin, fibroblast-specific protein 1 (FSP-1), N-cadherin, matrix metalloprotein (MMP)-2, and MMP-9 (6, 36, 37).

It has been identified that EndMT related to direct immune responses is triggered in response to pro-inflammatory cytokines, such as TNF-α, IL-1β, and their combinations. Similarly, inflammation-induced EndMT is characterized by the loss of endothelial phenotypes and gain of mesenchymal-like characteristics, and endothelial/mesenchymal markers are tightly controlled by EndMT mediators, such as ZEB1, β-catenin, Akt/NF-κB, snail, slug, Notch1, bone morphogenetic protein (BMP)-4, Sp1, phosphoinositide 3-kinase (PI3K), and enhancer of zeste homolog 2 (EZH2) (**Figure 1**).

Tumor necrosis factor-α, a pro-inflammatory cytokine, plays an important role in the regulation of various cellular activities (38). In ECs, TNF-α responses are initiated by the binding of one of two receptors, TNF receptor type 1 and TNF receptor type 2, allowing these receptors to activate transcription factors, such as NF-κB, which leads to the induction of transcription of multiple genes such as vascular cell adhesion molecule 1 (*VCAM-1*) and intercellular adhesion molecule 1 (31, 39–42). TNF-α also induces EndMT through activation of multiple signaling pathways in various ECs types (39, 40, 43, 44). However, future studies will be needed to clarify what type of TNF-α receptor is involved in EndMT. In lymphatic endothelium, TNF-α-induced EndMT occurs through inhibition of vascular endothelial (VE)-cadherin expression while increasing the expression of β-catenin, N-cadherin, and ZEB1, key molecules involved in the EndMT processes (44). A previous study (40) showed that TNF-α drives

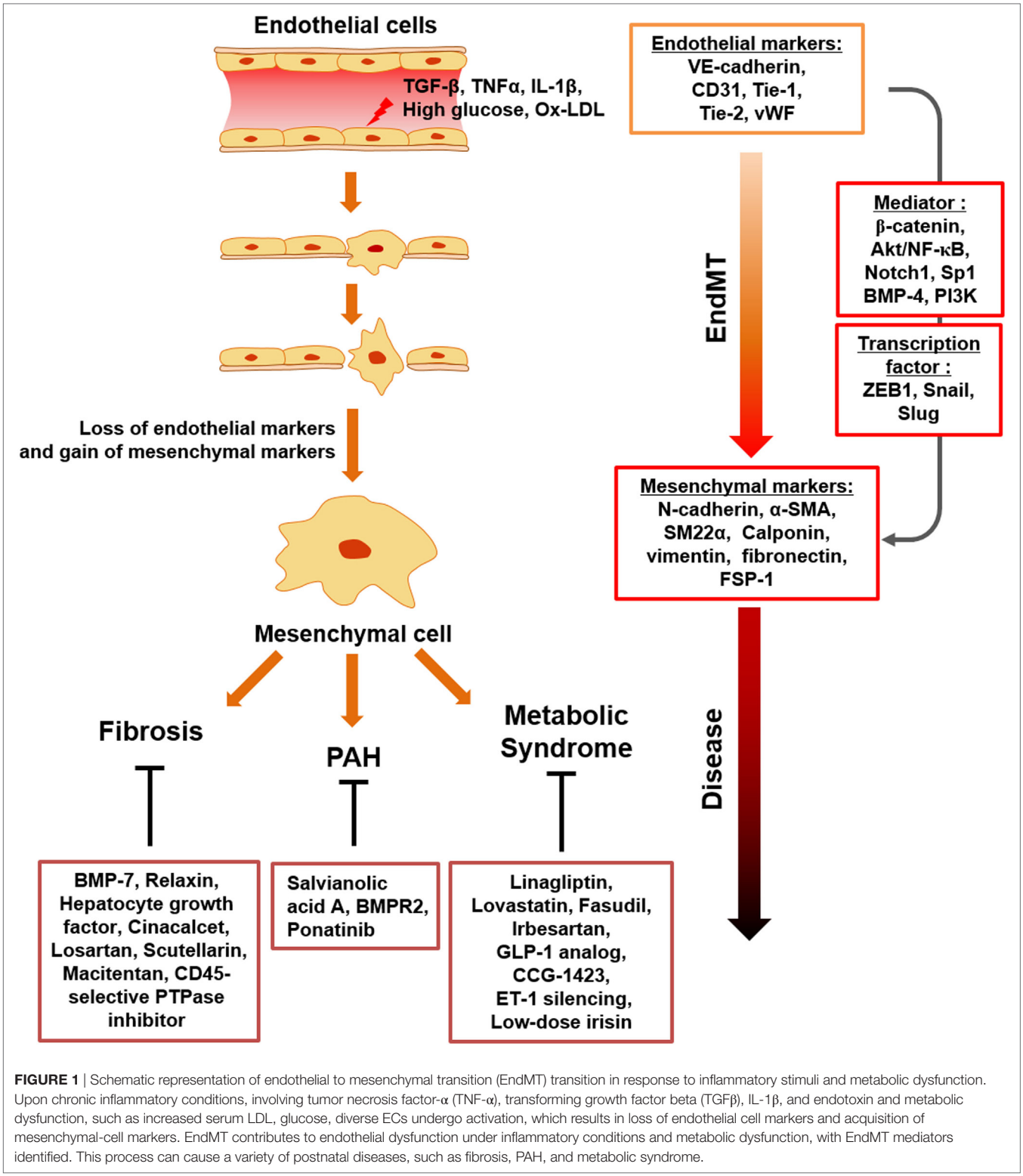

FIGURE 1 | Schematic representation of endothelial to mesenchymal transition (EndMT) transition in response to inflammatory stimuli and metabolic dysfunction. Upon chronic inflammatory conditions, involving tumor necrosis factor-α (TNF-α), transforming growth factor beta (TGFβ), IL-1β, and endotoxin and metabolic dysfunction, such as increased serum LDL, glucose, diverse ECs undergo activation, which results in loss of endothelial cell markers and acquisition of mesenchymal-cell markers. EndMT contributes to endothelial dysfunction under inflammatory conditions and metabolic dysfunction, with EndMT mediators identified. This process can cause a variety of postnatal diseases, such as fibrosis, PAH, and metabolic syndrome.

EndMT through Akt/NF-κB activity in both embryonic and adult-valve endothelium, finding that EndMT-related protein expression involving α-SMA and snail was significantly upregulated, whereas VE-cadherin was significantly downregulated in response to TNF-α in porcine aortic valve ECs (PAVECs), but not porcine aortic ECs (40); suggesting the importance of determining the molecular mechanism of EndMT in the context of endothelial heterogeneity during inflammation. The same group also demonstrated heterogeneous susceptibility to EndMT in PAVECs in response to TNF-α. Under TNF-α stimulation, non-transforming

cells that maintain endothelial-cell marker expression and transforming cells that acquire mesenchymal-marker expression were isolated using membrane-based three-dimensional culture systems. Transforming cells decreased endothelial marker expression, such as VE-cadherin and endothelial nitric oxide synthase and acquired mesenchymal markers, such as α-SMA, Notch1, MMP-9, BMP-4, and TGFβ in PAVECs (**Figure 2**) (39).

Interleukin-1β is a proinflammatory cytokine (45) involved in endothelial dysfunction (46) and a key inducer of EndMT. IL-1β-induced phenotypic changes in ECs were first demonstrated in IL-1β-treated human dermal microvascular ECs undergoing morphological changes and cytoskeletal reorganization, in addition to decreased expression of typical endothelial markers, such as von Willebrand Factor (vWF) and CD31 (47). In addition, long-term exposure of human dermal microvascular ECs to IL-1β induces the expression of mesenchymal markers such as α-SMA, type I collagen, and calponin and inhibits the expression of vWF (48). Maleszewska et al. (49) reported that the molecular mechanism underlying IL-1β-induced EndMT involves increased expression of SM22α, which is encoded by *TAGLN*. Their results demonstrated the epigenetic regulation of *TAGLN via* EZH2, which acts as a key negative regulator in IL-1β-induced EndMT (49). Moreover, in corneal ECs, IL-1β induced an EndMT phenotype by increasing fibroblast growth factor (FGF) expression through the PI3K-signaling pathway (50, 51) in accordance with changes in the actin cytoskeleton and cellular morphology (**Figure 2**). The most recent study has shown that the NLRP3 inflammasome, closely associated with mature IL-1β secretion, is involved in mechanical stretch-induced EndMT in lung fibrosis and NLRP3 inactivation could inhibit EndMT, suggesting novel therapeutic options against mechanical ventilation-induced pulmonary fibrosis (52).

Several studies reported that a combination of cytokines, including TNF-α, IL-1β, and TGFβ, is more powerful than a single cytokine at inducing EndMT. The combination of TGFβ1, IL-1β, and TNF-α induces EndMT *via* the Sp1 transcription factor, which is a key transcriptional regulator of EndMT-related genes in human intestinal microvascular endothelial cells (31). TGFβ2 and IL-1β synergistically induce EndMT through increased expression of mesenchymal markers while decreasing the

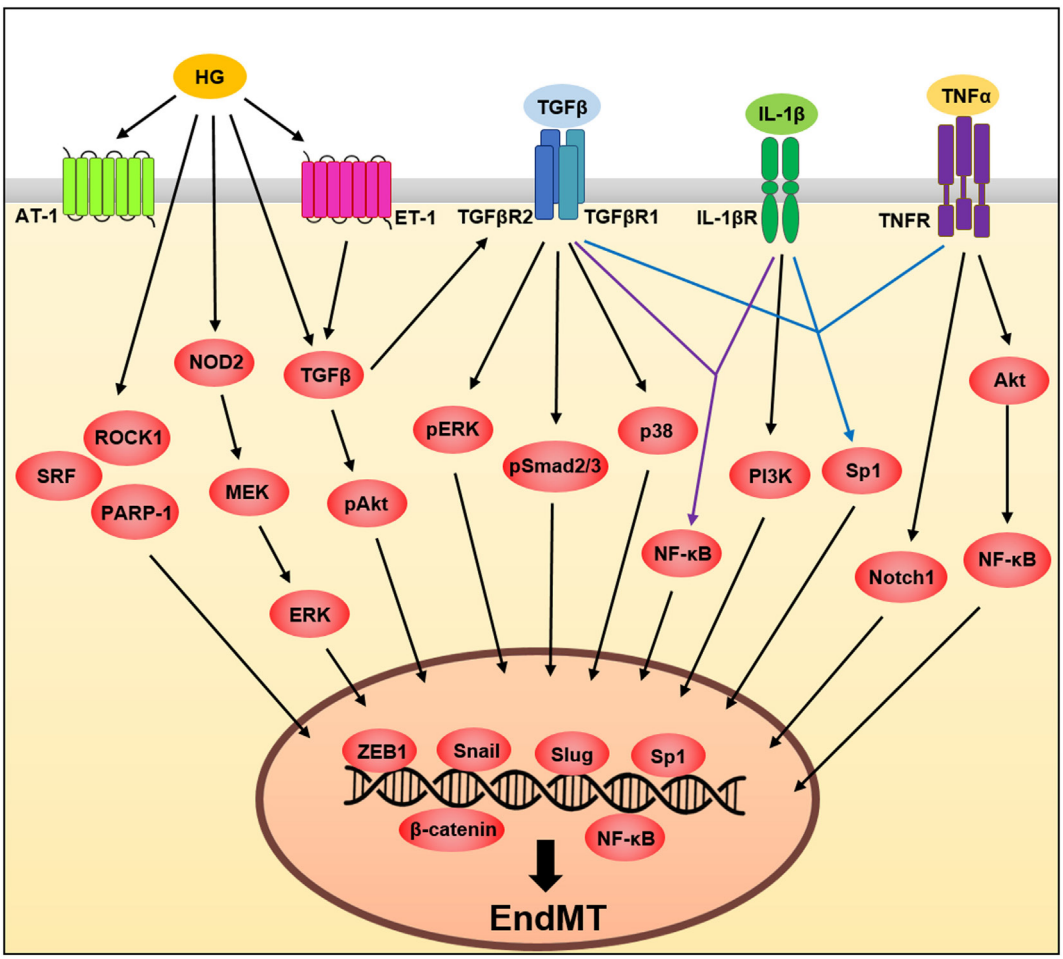

FIGURE 2 | A schematic illustration of the signaling pathways governing endothelial to mesenchymal transition (EndMT). Tumor necrosis factor-α (TNF-α), transforming growth factor beta (TGFβ), interleukin (IL)-1β, and high glucose influences EndMT by regulating signaling pathways. These pathways converge and induce the expression of transcription factors involving Slug, Snail and zinc finger E-box-binding homeobox 1 (ZEB1) (see text for details).

expression of endothelial markers in human esophageal microvascular endothelial cells and human umbilical vein ECs (HUVECs) (53, 54). The combination of TNF-α, IL-1β, and TGFβ1 also induces EndMT in pulmonary artery ECs, with EndMT cells exhibiting morphological changes, as well as changes in endothelial and mesenchymal markers (7).

Emerging evidence has shown that endothelial dysfunction induced by metabolic disorders such as obesity, hyperglycemia, and dyslipidemia is critically associated with induction of EndMT. Several studies have demonstrated that high glucose induces EndMT, which leads to increased expression of mesenchymal markers, such as α-SMA, FSP-1, type I collagen, fibronectin, vimentin, and MMP-2 along with decreased expression of endothelial markers CD31 and VE-cadherin in various EC types (55–57). It was shown that high-glucose-induced EndMT occurs through positive regulators, such as Smad2/3, Snail, Rho-associated kinase 1 (ROCK1), serum response factor (SRF), nucleotide-binding oligomerization domain-containing protein 2 (NOD2), and ERK in glomerular ECs (57–60). In human aortic ECs (HAECs) and HUVECs, high glucose also induces EndMT through positive regulators, such as angiotensin II, poly (ADP-ribose) polymerase 1 (PARP-1), endothelin 1 (ET-1), Smad, Akt, p38, and ERK, contributing to diabetic cardiomyopathy (55, 56, 61–63). It has also been shown that oxidized low-density lipoprotein (ox-LDL) accelerates radiation-induced EndMT in HAECs and contributes to radiation-induced atherosclerosis (64), whereas high-density lipoprotein (HDL) inhibits TGFβ1-induced EndMT in HAECs suggesting anti-fibrotic effects of HDL (**Figure 2**) (65).

Although much attention has recently been directed to EndMT because of its importance in many diseases, most studies have been limited to the identification of endothelial and mesenchymal markers in response to inducers of EndMT. Therefore, elucidation of the potential molecular mechanisms regulating pathological EndMT induced by inflammatory stimuli will be important in the future. **Table 1** shows a summary of the main studies exploring EndMT under specific inflammatory stimuli and metabolic dysfunction, including EndMT mediators, endothelial and mesenchymal markers, and EC types.

EC HETEROGENEITY DURING INFLAMMATION

Endothelial cells line the inner wall of blood vessels and exhibit diverse subtypes (2, 8, 33). Different ECs have different structural and functional characteristics based on their exposure to distinct microenvironments (2, 8, 9, 33, 66). They are activated in response to inflammatory stimuli, with this activation resulting in the expression of adhesion molecules necessary for leukocyte binding (33).

TABLE 1 | Summary of the key studies exploring EndMT under specific inflammatory stimuli and metabolic dysfunction.

Stimuli	Endothelial markers	Mesenchymal markers	Endothelial cell types	Positive regulator of EndMT	Reference
TNF-α	VE-cadherin	N-cadherin	LEC	ZEB1 and β-catenin	(44)
TNF-α	VE-cadherin, CD31, eNOS	α-SMA and MMP-9	PAVEC and eQEE	Akt/NF-κB, Snail, Slug, TGFβ, Notch1, and BMP-4	(39, 40)
IL-1β	vWF	α-SMA, collagen I, and calponin	HDMEC	Non determined	(47, 48)
IL-1β	Non determined	SM22α	HUVEC	pSmad2 and TGFβ2	(49)
IL-1β	Non determined	Cell shape change and actin cytoskeleton	CECs	PI3K	(50, 51)
TNF-α, IL-1β and TGFβ1	VE-cadherin, CD31, and vWF	α-SMA, FSP-1, vimentin, N-cadherin, and fibronectin	HIMEC	Sp1	(31)
TGFβ2 and IL-1β	CD31, vWF, and VE-cadherin	SM22α, FSP-1, collagen 1 A2, vimentin, and α-SMA	HEMEC	Snail	(53)
TGFβ2 and IL-1β	eNOS and vWF	SM22α, calponin	HUVEC	NF-κB	(54)
TNF-α, IL-1β and TGFβ1	vWF, CD31, VE-cadherin, and Occludin	Calponin, α-SMA, and collagen I	PAECs	Non determined	(7)
High glucose	CD31 and VE-cadherin	α-SMA, α-SMA, FSP-1, and fibronectin	GEnC	TGFβ, pSmad2/3, Snail, ROCK1, NOD1, MEK/ERK, SRF, and Snail	(57–60)
High glucose	CD31 and VE-cadherin	α-SMA, FSP-1, collagen I, collagen III, and MMP-2/9	HAEC	Angiotensin II, Snail, and PARP-1	(55, 61)
High glucose	VE-cadherin and CD31	α-SMA, collagen I, FSP-1, vimentin, and MMP-2/9	HUVEC	TGFβ1, ERK, pSmad2/3, and MAPK (p38 and ERK)	(56, 63)
High glucose	VE-cadherin	FSP-1 and collagen I	HUVEC and HAEC	ET-1, TGFβ1, pSmad3, pAKT, and Snail	(62)
ox-LDL + Radiation	VE-cadherin and CD31	α-SMA, FSP-1, and vimentin	HAEC	Non determined	(64)

TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β; TGFβ, transforming growth factor-β; VE-cadherin, vascular endothelial cadherin; eNOS, endothelial nitric oxide synthase; vWF, von Willebrand Factor; α-SMA, α-smooth muscle actin; SM22α, smooth muscle protein 22-α; FSP-1, fibroblast-specific protein 1; LEC, lymphatic endothelial cell; PAVEC, porcine aortic valve endothelial cell; eQEE, embryonic quail endocardial explant; HDMEC, human epithelioid dermal microvascular endothelial cell; HUVEC, human umbilical vein endothelial cell; CEC, corneal endothelial cell; HIMEC, human intestinal microvascular endothelial cell; HEMEC, human esophageal microvascular endothelial cell; PAEC, pulmonary artery endothelial cell; ZEB1, zinc finger E-box-binding homeobox 1; NF-κB, nuclear factor kappa B; BMP-4, bone morphogenetic protein 4; EZH2, enhancer of zeste homolog 2; FGF-2, fibroblast growth factor 2; PI3K, phosphatidylinositol 3-kinase. GEnC, glomerular endothelial cell; ROCK1, Rho-associated kinase 1; HAEC, human aortic endothelial cell; GLP-1, glucagon-like peptide-1; PARP-1, Poly (ADP-ribose) polymerase 1; SRF, Serum response factor; ET-1, endothelin-1; ox-LDL, oxidized low-density lipoprotein; NOD2, Nucleotide-binding oligomerization domain-containing protein 2.

Many studies showed that each EC subtype responds differently to different inflammatory stimuli *in vitro* (33). Viemann et al. (67) suggested that genes differentially regulated upon TNF-α stimulation between human microvascular ECs and HUVECs exhibit functional differences, and that genes whose expression was altered only in the human microvascular ECs group were associated with signaling and transcription factors, apoptosis, cell proliferation, immune response, and cell structure. However, genes showing altered expression only in HUVECs were associated with chemokines, cytokines, cell-surface molecules, and signaling and transcription factors (67). VCAM-1 expression was only increased in response to TNF-α in HUVECs and glomerular ECs, but not in dermal microvascular ECs (68). In addition, Scott et al. (69) reported changes in heterogeneous gene expression in response to TNF-α, lipopolysaccharide (LPS), and IL-1β in HUVECs, human pulmonary microvascular ECs, HAECs, carotid artery ECs, coronary artery ECs, subclavian artery ECs, and brachiocephalic artery ECs. TNF-α, and IL-1β-stimulated organ-specific endothelial heterogeneity has also been reported (70).

These findings suggest that each EC subtype might respond differently to different inflammatory stimuli in the context of EndMT. Indeed, Pinto et al. (71) compared responses to TGFβ1 or TGFβ2 between human coronary artery ECs and microvascular pulmonary artery ECs, finding that in human coronary artery ECs, both TGFβ1 and TGFβ2 upregulated the expression of the mesenchymal markers α-SMA and SM22α, but only TGFβ1 had an effect on α-SMA expression in human pulmonary microvascular ECs. In addition, TNF-α increased the expression of the mesenchymal markers in PAVECs, although this response was not observed in porcine aortic ECs (40).

Importantly, other inflammation-associated endothelial activators, such as shear stress or protein kinase C (PKC), have also been studied in this context (33). Methe et al. (72) showed that venous and coronary artery specific flows differentially regulate the expression of endothelial adhesion molecules as well as KLF2/KLF4 transcription factors in human saphenous vein ECs and human coronary artery ECs. Two ECs have also been reported to show heterogeneity in adhesion molecule expression in response to PKC. Here, PKC activation induces E-selectin and VCAM-1 expression in HUVECs, but not in human dermal microvascular ECs (73).

Differences in the behavior of various EC subtypes in response to inflammatory stimuli have also been reported *in vivo* (33). Tamaru et al. (74) showed that induction of adhesion molecule expression in response to IL-1β stimulation is both tissue- and cell-type specific. However, no changes were observed in VCAM-1 expression in brain and liver microvascular ECs in response to LPS stimulation (75, 76). Furthermore, van Meurs et al. (77) showed that E-selectin and VCAM expression in human glomerular ECs differs from that in other ECs. In CD31-deficient mice, apoptosis of peritubular-capillary ECs occurs upon LPS administration, although other microvessel ECs were unaffected (78). Given the extent of endothelial heterogeneity found both *in vitro* and *in vivo*, studying the molecular mechanisms and functions associated with the EndMT process in inflammation in the context of endothelial heterogeneity will eventually enable us to better understand vascular diseases and develop more sophisticated and effective therapeutic drugs.

TARGETING ENDMT FOR THERAPEUTIC AND CLINICAL APPLICATIONS IN VASCULAR DISEASES

Endothelial to mesenchymal transition is recognized to not only occur during development but also it is now clear that EndMT underlies pathological processes associated with multiple diseases (6, 26, 79, 80). EndMT is also controlled by a variety of stimuli, including inflammation, growth factors, and hypoxia (81–83). Particularly, inflammation-induced EndMT aggravates inflammation and destroys vascular homeostasis, leading to pathogenesis of several diseases, such as cardiac fibrosis, PAH, and atherosclerosis (31, 84, 85). Given the involvement of EndMT in multiple inflammatory diseases, preventing EndMT may represent a useful approach to treat inflammatory diseases.

Several factors have been identified as the negative regulators of EndMT signaling pathways (86–88). Vascular endothelial growth factor-A reverses TGFβ2-induced EndMT (89), and HDL and the extracellular-matrix protein fibulin 1 and kallistatin also exert inhibitory effects on TGFβ-induced EndMT (65, 90, 91). Furthermore, the most common aldosterone receptor antagonist, spironolactone has a protective role against TGFβ-induced EndMT in HUVECs (92) and, rapamycin suppresses mechanistic target of mTOR signaling, leading to the inhibition of EndMT (93). Although emerging studies report multiple EndMT mediators that play critical roles in EndMT induction, the targeting of EndMT mediators requires careful evaluation due to the modulation of EndMT exhibiting differential effects in different ECs based on endothelial heterogeneity. For example, IL-1β upregulates FGF2 expression through PI3K activation, which leads to EndMT of corneal ECs (51). Lee et al. (50) suggested that blocking the IL-1β and FGF2 pathways would prevent inflammation-induced EndMT in corneal ECs; however, FGF2 exerts an inhibitory effect on TGFβ-mediated EndMT *via* miR-20a in HUVECs (81, 94). Moreover, another study showed that FGF receptor-1 is a key inhibitor of TGFβ-driven EndMT in HUVECs (95), and the endogenous antifibrotic peptide N-acetyl-seryl-aspartyl-lysyl-proline restores FGF receptor levels and upregulates levels of the let-7, resulting in the inhibition of EndMT in human dermal microvascular ECs (96). Therefore, further studies are needed to completely elucidate the mechanisms associated with FGF2, as well as many mediators involved in EndMT (81).

In the context of diseases, studies on the inhibitory effect of EndMT have mainly focused on fibrosis. The common feature of many fibro-proliferative diseases is inflammation (97). Fibrosis results from chronic inflammation, possibly owing to infection, autoimmune reactions, or allergic reactions, which results in the release of inflammatory mediators, abnormal cell proliferation, and deposition of extracellular-matrix components (81, 82, 84, 97). BMP7 has been shown to exhibit anti-EndMT effects and reduce cardiac fibrosis; however, most other BMPs are positive regulators of EndMT (81, 85, 98, 99). Hepatocyte growth factor

reduces cardiac fibrosis by suppressing TGFβ1-mediated EndMT (100). Cinacalcet, a calcimimetic agent, reduces serum levels of parathyroid hormone and suppresses EndMT, leading to attenuation of cardiac fibrosis (101). Similarly, losartan and irbesartan, two angiotensin II-receptor type 1 blockers, ameliorate cardiac fibrosis by inhibiting EndMT (61, 102). Scutellarin and relaxin are also EndMT inhibitors and prevent cardiac fibrosis by regulating Notch1 and Jagged-1 (86, 103). Furthermore, inhibition of CD45 protein tyrosine phosphatase leads to reduced EndMT in TGFβ1-treated mitral valve ECs (104). The anti-fibrotic effects of linagliptin, which blocks EndMT, have also been reported *in vitro* and in diabetic kidneys (105). Similarly, macitentan inhibits endothelin-1 or TGFβ1-induced EndMT in systemic sclerosis (106).

Cytokine-induced inflammation is widely considered a major cause of PAH development (107). Moreover, remodeling of the pulmonary artery under inflammatory conditions is a major feature of PAH (12–15). Recent evidence suggests that inflammation-induced EndMT is a key contributor to pathological pulmonary vascular remodeling associated with transition of ECs to α-SMA-expressing mesenchymal-like cells in obstructive vascular lesions of PAH (7, 29). Clinical data also indicate that the serum levels of IL-1, -6, -8, -10, and TNF-α are elevated in PAH patients (107). In this context, salvianolic acid A, a polyphenol compound, inhibits EndMT in PAH, thereby attenuating inflammation associated with monocrotaline-induced PAH (108). Another study showed that the delivery of BMP receptor-2 resulted in less right-ventricle hypertrophy, pulmonary vascular resistance, and improved cardiac function through attenuation of EndMT (109). Kang et al. (87) suggested that ponatinib, a multi-target tyrosine-kinase inhibitor, delays TGFβ1-mediated EndMT and has therapeutic potential for use in PAH therapy, where it could act by regulating Wnt signaling.

Atherosclerosis is a vascular disease mediated by a typical inflammatory response. Inflammatory stimuli continuously lead to calcified plaque formation (110). Atherosclerosis lesions mostly comprise EndMT-derived fibroblast-like cells, which are regulated by various EndMT mediators, such as snail, slug, and β-catenin (84, 111, 112). Other pathways might also lead to atherosclerosis by inducing EndMT *via* TGFβ, oxidative stress, hypoxia, Wnt/β-catenin signaling, and BMP signaling (81, 84, 113). These data suggest that EndMT is a major source of neointimal hyperplasia and plays a role in the progression of arteriosclerosis through inflammation. In particular, excessive BMP activity promotes the calcification of atherosclerotic lesions through EndMT and serine-protease inhibitors also reduce EndMT and vascular calcification (88, 114). Consistent with this report, vascular calcification was found to be reduced in response to a BMP inhibitor in matrix-gla-protein-deficient mice (115).

It has been shown that metabolic syndrome, which is associated with metabolic dysfunction such as obesity, hyperglycemia, insulin resistance, and dyslipidemia has a central role in the

TABLE 2 | Summary of the key studies exploring endothelial to mesenchymal transition (EndMT) as a therapeutic target in various diseases.

Model of Study	Negative regulator of EndMT	Clinical relevance	Reference
Isoproterenol-induced myocardial fibrosis rat model	Relaxin	Cardiac fibrosis	(86)
Bleomycin-induced PAH model	Ponatinib (multi-targeted tyrosine-kinase inhibitor)	Pulmonary arterial hypertension (PAH)	(87)
TGFβ1-induced EndMT	HDL	Non-determined	(65)
TGFβ-induced EndMT	Spironolactone (aldosterone receptor antagonist)	Non-determined	(92)
Mouse models of pressure overload and chronic allograft rejection	BMP-7	Cardiac fibrosis	(85)
Heterotopic heart transplantation model	BMP-7	Endocardial fibroelastosis	(99)
Pressure-overload mouse model	HGF	Cardiac fibrosis	(100)
Rat model of uremia and secondary hyperparathyroidism	Cinacalcet (calcimimetic agent)	Cardiac fibrosis	(101)
TGFβ1-induced EndMT	Losartan (angiotensin II receptor type 1 blocker)	Non determined	(102)
Isoproterenol -induced myocardial fibrosis rat model	Scutellarin	Cardiac fibrosis	(103)
Ovine inferior myocardial infarction model	CD45-selective PTPase inhibitor	Myocardial infarction	(104)
STZ-induced diabetic mice	Linagliptin (DPP-4 inhibitor)	Diabetic kidney fibrosis	(105)
TGFβ and ET-1-induced EndMT	Macitentan (ET-1 receptor antagonist)	Systemic sclerosis	(106)
MCT-induced PAH model	Salvianolic acid A	Pulmonary arterial hypertension	(108)
Hypoxia, MCT-induced PAH model	Delivery of *BMPR2*	Pulmonary arterial hypertension	(109)
STZ-induced diabetic rats	Lovastatin	Diabetic nephropathy	(58)
db/db diabetic mice	Fasudil (ROCK1 inhibitor)	Diabetic nephropathy	(59)
STZ-induced SHR diabetic rats	Irbesartan (angiotensin II receptor type 1 blocker)	Diabetic cardiomyopathy	(61)
STZ-induced diabetic mice	GLP-1 analog	Diabetic cardiomyopathy	(55)
STZ-induced diabetic rats	CCG-1423 (SRF inhibitor)	Diabetic nephropathy	(57)
STZ-induced diabetic ET-1$^{f/f}$; Tie2-Cre(+) mice	ET-1 silencing	Diabetic cardiomyopathy	(62)
STZ-induced diabetic mice	Low-dose irisin	Diabetic cardiomyopathy	(63)
TGFβ1-induced EndMT	HDL	Non determined	(65)

TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β; TGFβ, transforming growth factor-β; VE-cadherin, vascular endothelial cadherin; eNOS, endothelial nitric oxide synthase; vWF, von Willebrand factor; α-SMA, α-smooth muscle actin; SM22α, smooth muscle protein 22-α; FSP-1, fibroblast-specific protein 1; LEC, lymphatic endothelial cell; PAVEC, porcine aortic valve endothelial cell; eQEE, embryonic quail endocardial explant; HDMEC, human epithelioid dermal microvascular endothelial cell; HUVEC, human umbilical vein endothelial cell; CEC, corneal endothelial cell; HIMEC, human intestinal microvascular endothelial cell; HEMEC, human esophageal microvascular endothelial cell; PAEC, pulmonary artery endothelial cell; ZEB1, zinc finger E-box-binding homeobox 1; NF-κB, nuclear factor kappa B; BMP-4, bone morphogenetic protein 4; EZH2, enhancer of zeste homolog 2; FGF-2, fibroblast growth factor 2; PI3K, phosphatidylinositol 3-kinase. STZ, streptozotocin; SHR, spontaneously hypertensive rats; HDL, High-Density Lipoproteins; HGF, hepatocyte growth factor; MCT, monocrotaline.

pathogenesis of cardiovascular disease, diabetes mellitus type 2, and tissue remodeling (116). Abnormalities associated with chronic inflammation are key risk factors of metabolic dysfunction, leading to the endothelial dysfunction that is critically involved in the development of such diseases (64, 116–119). In the context of metabolic syndrome, studies on EndMT induced by metabolic dysfunction have mainly focused on diabetic nephropathy and cardiomyopathy. Several studies have shown that EndMT contributes to diabetic nephropathy, while inhibition of EndMT by lovastatin, fasudil, and CCG-1423 could ameliorate diabetic nephropathy in streptozotocin (STZ)-induced diabetic animal models (57–59). In addition, inhibition of EndMT by Irbesartan, glucagon-like peptide-1 analog, ET-1 inhibition and low-dose irisin could prevent diabetic cardiomyopathy in diabetic animal models (55, 61–63).

Taken together, the currently available data indicate that EndMT plays a key role in various fibrosis-related and cardiovascular diseases (**Figure 1**; **Table 2**). Considering the large number of studies that suggest targeting EndMT as a novel therapeutic approach for many diseases, clarifying the underlying signaling mechanisms associated with EndMT and establishing strategies to regulate EndMT are urgently needed.

CONCLUSION

Endothelial to mesenchymal transition plays an important role not only during the development process but also in adults under physiological and pathological conditions. A central role for EndMT emerges from the complex network of interactions that underlie inflammation-induced endothelial dysfunction. There is accumulating evidence indicating that EndMT is a key feature in inflammation-related endothelial dysfunction. It is through this phenotypic switch that EndMT causes diverse vascular diseases, such as atherosclerosis, PAH, and fibrosis. Therefore, the modulation of EndMT might yield new therapeutic strategies for the treatment of diverse diseases. Although our current understanding of the molecular mechanisms underlying EndMT in the context of inflammation is advancing, further studies are needed in the future to completely understand the molecular mechanism associated with EndMT in inflammation-related diseases. Given that heterogeneity is apparent in ECs of different organs in response to different inflammatory stimuli, it will also be important to determine the molecular mechanisms associated with EndMT in the context of endothelial heterogeneity during inflammation in future studies. In conclusion, the study of EndMT will provide valuable insights into the molecular mechanisms leading to various human diseases and will help develop more sophisticated and effective therapeutic drugs for patients suffering from these diseases.

AUTHOR CONTRIBUTIONS

JK, JC, and AL wrote the manuscript. JK, WC, and MS-L were critically involved in the design of the work and the discussion of the content. All the authors approved the final manuscript.

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The Role of the Endothelium during Antibody-Mediated Rejection: From Victim to Accomplice

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Antibody-mediated rejection (AMR) of solid organ transplants is characterized by the activation and injury of the allograft endothelium. Histological and transcriptomic studies have associated microvascular inflammation and endothelial lesions with the severity of rejection and poor graft outcomes. The allograft endothelium forms the physical barrier between the donor organ and the recipient; this position directly exposes the endothelium to alloimmune responses. However, endothelial cells are not just victims and can actively participate in the pathogenesis of rejection. In healthy tissues, the endothelium plays a major role in vascular and immune homeostasis. Organ transplantation, however, subjects the endothelium to an environment of inflammation, alloreactive lymphocytes, donor-specific antibodies, and potentially complement activation. As a result, endothelial cells become activated and have modified interactions with the cellular effectors of allograft damage: lymphocytes, natural killer, and myeloid cells. Activated endothelial cells participate in leukocyte adhesion and recruitment, lymphocyte activation and differentiation, as well as the secretion of cytokines and chemokines. Ultimately, highly activated endothelial cells promote pro-inflammatory alloresponses and become accomplices to AMR.

Keywords: endothelial cells, antibody-mediated rejection, donor-specific antibodies, transplantation immunology, inflammation

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ANTIBODY-MEDIATED REJECTION (AMR)

With the decline in the incidence of acute rejection, chronic rejection has become the major cause of allograft loss in solid organ transplantation (1). Moreover, AMR represents the predominant mechanism of chronic rejection (2, 3). In the case of renal transplantation, AMR is identified by the presence of circulating donor-specific antibodies (DSAs), morphologic evidence for tissue injury such as transplant glomerulopathy (characterized by reduplication of glomerular basement membranes), and evidence for antibody interaction with the vascular endothelium such as C4d deposition and/or microvascular inflammation (4, 5). Similar criteria for AMR diagnosis are used in cardiac (6), pulmonary (7), and pancreatic (8) transplantation and establish the involvement of the endothelium in the processes of AMR.

Both DSA existing prior to transplantation and DSA produced *de novo* post transplantation are harmful, although a recent study reported that AMR patients with preexisting DSA had superior graft survival to patients with *de novo* DSA (9). In the subset of patients with *de novo* DSA, the detection of endothelial lesions was associated with the poorest survival rates. While the correlation between DSA and microvascular endothelial lesions is well recognized, recent data have

revealed that DSAs also accelerate arteriosclerosis (10) and that they have a key role in premature and accelerated fibrosis of the allograft (11).

In AMR, the vast majority of known DSA are directed against the highly polymorphic HLA antigens. DSAs directed against either class of HLA antigen are deleterious; however, DSAs directed against HLA class II antigens have been more strongly associated with late-onset AMR, *de novo* antibody production, and reduced graft survival (12). Patients who develop HLA class II DSA have a higher risk of developing transplant glomerulopathy and microvascular endothelial damage associated with reduced graft survival (13). Of note, DSAs do not exclusively target HLA antigens; several non-HLA DSAs have been identified in proteomic and transcriptomic studies following allograft rejection (14). Such non-HLA DSAs often target endothelial-expressed antigens and accelerate vascular injury, such as vimentin, MICA, collagen, laminin-like globular domains of perlecan, and the angiotensin receptor type 1 (15, 16).

Endothelium damage in the presence of DSA was first considered to be a consequence of complement activation on the basis of the detection of a product of the activated complement cascade, C4d, in the microvasculature of AMR graft biopsies. Following studies on animal and human tissues, it became clear that complement activation is not a prerequisite for allograft vasculopathy (17, 18). Nonetheless, complement-binding anti-HLA DSAs have been associated with an increased rate of AMR, worse tissue damage, and more extensive microvascular inflammation than non-complement-binding anti-HLA DSAs (19).

These data demonstrate the clear involvement of the endothelium in AMR. Furthermore, they associate DSA and even complement with increased endothelial cell inflammation and vascular damage.

ENDOTHELIUM ACTIVATION IN AMR

The allograft microvasculature is the initial site of contact between the recipient's circulating immune system and donor antigens. As such, the endothelium takes on the role of a primary target of alloresponses. However, the endothelium is not an inert structure and actively participates in vascular and immune homeostasis. AMR-associated inflammation, alloantibodies, and activation of the complement cascade have been shown in recent studies to produce distinct endothelial phenotypes and impact their capacity to regulate and activate the alloimmune response (20–22).

Comparison of gene transcripts isolated from DSA-positive and -negative patient biopsies has confirmed endothelium activation as a characteristic of AMR (23). Hidalgo et al. reported a set of 132 DSA-specific transcripts that were functionally associated with HLA, interferon gamma effects, macrophages, natural killer (NK) cells, endothelial cells, inflammation, and immunoglobulins. Furthermore, 23 transcripts were selectively expressed during rejection from DSA-positive patients and they were chiefly expressed by the endothelium (8/23). This evidence supports the notion that DSAs contribute to a distinctive activation of endothelial cells during rejection.

The allograft endothelium is capable of expressing HLA molecules, which in the context of allotransplantation exposes the endothelium to recognition by the recipient's humoral and cellular immune system. The expression of HLA class I antigens is readily detected in the renal microvascular endothelium, whereas that of HLA class II antigens is modest in the steady state and significantly increased in rejection (24, 25). HLA class II antigen expression is regulated by pro-inflammatory factors and displays a hierarchical expression of its isotypes (DR > DP > DQ) (26) and a non-identical time course of expression with HLA-DR and −DP being more readily induced than HLA-DQ. HLA-DR and HLA-DQ are frequently the target of *de novo* DSA and are significantly upregulated by the microvasculature post transplantation, as well as in AMR (27, 28).

Signaling mediated by ligand binding to HLA molecules in different cell types has been extensively reviewed elsewhere (29–31). In the endothelial cell, the alloantibody binding of endothelial HLA class I and II molecules has been shown to mediate signal transduction, independent of complement activation ((20, 21) and reviewed in ref. (32)). The relevance of these signaling pathways has been confirmed by the detection of phosphorylated-signaling proteins, such as S6RP, in biopsies from patients with cardiac allograft vasculopathy and their quantitative correlation with AMR, but not other forms of rejection (33). Non-HLA DSAs directed against the angiotensin receptor type 1 DSA have been reported to be biologically active in vascular cells through the activation of Erk, AP-1, and NF-kB activation (34).

Complement activation, as a result of DSA binding, can synergize with the direct signal transduction induced by DSA-HLA ligation on the endothelium to further modify endothelial activation. This synergy has been observed to increase exocytosis of pro-thrombotic and pro-inflammatory molecules. While DSA-activated exocytosis was independent of complement activation, it could be increased by the addition of the C5a fragment of complement (35). Also, panel-reactive antibodies and the complement membrane attack complex combine to amplify a set of pro-inflammatory genes and the molecular expression of VCAM-1 (21).

In this review, we will use recent studies to explore endothelial cell participation in the physiopathology of AMR. We will primarily address atypical endothelial activation in the allograft and how these conditions may dictate alternative interactions with the cellular effectors of AMR, such as lymphocytes, NK cells, and myeloid cells.

ACTIVATED ENDOTHELIAL CELLS MODULATE ALLOGENEIC LYMPHOCYTES

As antigen-presenting cells, albeit non-professional, endothelial cells are in a unique position to promote the recognition of "non-self" allograft antigens, to recruit leukocytes and to modulate alloimmunity. Leukocyte infiltration is typical during allograft rejection. Interestingly, the composition of the infiltrates has been observed to contain equivalent numbers of intravascular CD3+ lymphocytes and CD68+ myeloid cells in the biopsies from cardiac-transplant patients with AMR (36).

Under inflammatory conditions, endothelial HLA molecules and adhesion molecules are upregulated and are sufficient to activate the alloproliferation of CD4+ T lymphocytes and concomitant increases in IL-2 and IFNγ secretion (37, 38). In an *in vitro* experimental model, allogeneic human ECs expressing HLA-DR were capable of selectively activating CD4+ T cell differentiation toward the pro-inflammatory Th17 and the anti-inflammatory Treg subsets, by mechanisms involving IL-6 secretion and CD54 expression, respectively (38). Allograft infiltration of Th17 cells has been associated with shorter allograft survival (39), while the proportion of intragraft Treg cells has been positively correlated with graft survival (40, 41). The importance of HLA class II was also underlined by a study wherein the blocking of expression of endothelial HLA class II antigens by antibodies or siRNA was able to prevent CD4+ T activation and the IL-2 production required for a CD8+ alloresponse (42). These data led to the proposal that the extent of endothelial expression of HLA class II has functional consequences on lymphocyte-associated alloresponses.

In our most recent study, HLA class II antibody binding to activated endothelial cells, in an inflammatory context, induced the phosphorylation of Akt, MEK, and ERK. The downstream effect of HLA class II ligation was modified by endothelial immunogenicity. The activation of Akt by anti-HLA-DR antibody was implicated in the increased IL-6 secretion by the endothelial cells in an allogeneic setting and therefore enhanced endothelial cell-mediated differentiation of pro-inflammatory Th17 (20). Jane-Wit et al. showed that panel-reactive antibodies activated an inflammatory gene expression profile in endothelial cells through the activation of the non-canonical pathway of NF-kB. This signal transduction increased endothelial cell immunogenicity and promoted CD4+ -T cell differentiation toward the pro-inflammatory Th1 subset. Antibody-mediated increases in endothelial cell immunogenicity were significantly enhanced by the activation of the classical complement cascade (21) (see **Figure 1**).

Regarding costimulatory molecules, ICOSL is strongly expressed by human endothelial cells in the presence of TNFα and IL-1β and played a functional role in CD4+ T activation (43). There are discrepancies in the literature regarding PD-L1; vascular endothelial expression was required for the generation of allogeneic Treg in a mouse-transplant model (44), whereas the lack of endothelial PD-L1 expression resulted in a greater generation of effector T cells and cardiac-transplant rejection in a chimeric mouse model (45). PD-L1 did not appear to be implicated in Treg expansion in an experimental model of human microvascular EC (38).

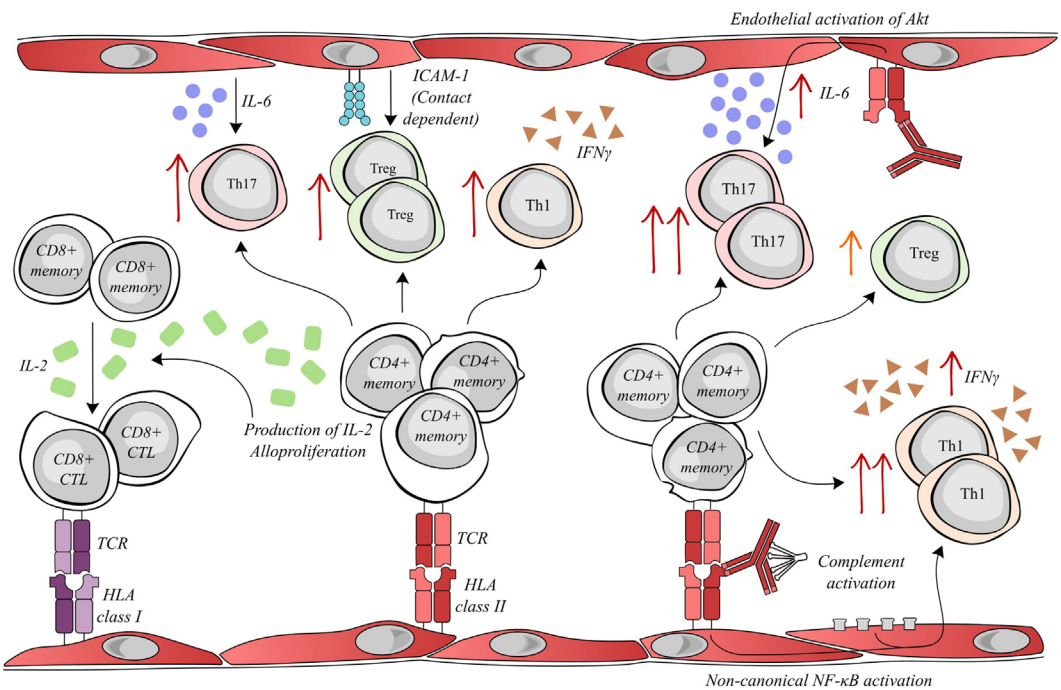

FIGURE 1 | Activated endothelial cells modulate allogenic T lymphocyte activity. Allogenic endothelial cells expressing HLA class II molecules are capable of inducing the secretion of IL-2 and alloproliferation by CD4+ memory lymphocytes. The T helper cell production of IL-2 facilitates the differentiation of memory CD8+ T cells into cytotoxic lymphocytes (CTLs), which can mediate rejection by targeting donor HLA class I molecules. Meanwhile, alloproliferation in CD4+ lymphocytes is associated with the selective expansion of pro-inflammatory Th17 (endothelial IL-6-dependent), anti-inflammatory Treg (endothelial ICAM-1 dependent) and pro-inflammatory Th1 subsets. Interestingly, donor-specific antibodies (DSAs) binding to HLA class II molecules alter endothelial cell immunogenicity. DSA binding can activate the Akt/PI3K-signaling pathway, consequently increasing endothelial IL-6 secretion and increasing the expansion of the pro-inflammatory Th17 subset. In addition, the amplification of the Treg population was decreased after DSA binding. Both the binding of DSA and the sub-lytic activation of the complement cascade can synergize to activate non-canonical NF-κB signaling in endothelial cells, ultimately resulting in an increased expansion of the Th1 subset and a greater secretion of IFNγ.

THE NK CELL CONTRIBUTION TO ENDOTHELIUM DAMAGE

Strong support for the implication of NK cells in AMR came from a mouse model of cardiac transplant in which the adoptive transfer of DSA led to chronic allograft vasculopathy and in which the depletion of NK cells limited cardiac allograft vasculopathy (46). Further support for the implication of NK cells in AMR in patients also came from the initial study by Hidalgo et al., which revealed that 6/23 transcripts selectively associated with the presence of DSA were highly expressed in NK cells (23). The data from transcript studies were supported by the detection of CD56 + cells (alongside CD68 + cells) in peritubular capillaries of biopsies from patients with AMR. Further study indicated that high NK transcript expression was associated with late-stage AMR, with microvascular inflammation, and with DSA (3). The notion of IFNγ release by the NK cell, following triggering by DSA fixation to both the EC and the NK cell, heightening local inflammation, which in turn would increase both HLA class I and II molecular expression by the endothelium and thus enrich the density of targets for DSA binding, was raised. While high NK transcript expression is associated with AMR, they can also be detected in biopsies from patients with T cell-mediated rejection.

The role of NK cell interactions with the graft endothelium in AMR has also been postulated as a mechanism of allograft damage. Such interactions were proposed to take place after DSA binding to the endothelium and the simultaneous binding of the constant antibody fragment (Fc) to circulating NK cells. Several outcomes of antibody binding to NK cells through activating receptors are possible, including antibody-dependent cell-mediated cytotoxicity (ADCC) with target cell lysis or the release of pro-inflammatory cytokines. The latter is a more likely consequence in AMR as lytic damage to the endothelium is infrequently observed (see **Figure 2A**).

A recent study set out to model the DSA interaction with the NK cells *in vitro* by stimulating human NK cells from healthy donors with an immobilized antibody directed against CD16a (47). Interestingly, the detection of both chemokines and inflammatory cytokines supported the idea that NK cytokine production may be more relevant than cell lysis to AMR. Although the six NK-associated transcripts initially described by Hidalgo et al. (23) were expressed, they were not increased by antibody binding to NK cells.

A novel functional assay of NK activity was recently tested using DSA and NK cells from ABMR patients to indirectly determine NK lytic function and the cytotoxic potential of patient alloantibodies, the NK Cellular Humoral Activation Test (NK-CHAT) (48). Results from the study showed that ADCC activity was lower in transplant recipients with preserved graft function and that scoring the outcome of the NK-CHAT test allowed the prediction of ABMR.

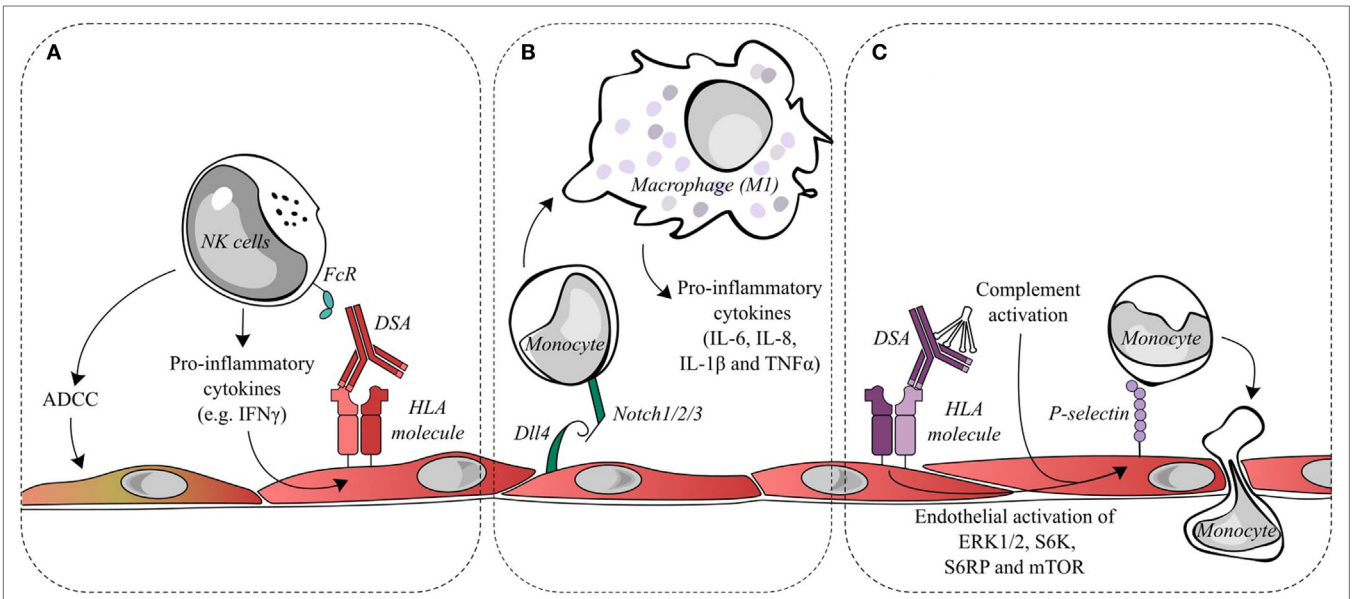

FIGURE 2 | Endothelial cell activation and recruitment of natural killer (NK) cells and monocytes. **(A)** Donor-specific antibodies (DSAs) binding to endothelial cells may lead to the ligation of Fc receptors on NK cells and contribute to antibody-dependent cell-mediated cytotoxicity (ADCC) directed against the allograft or to the release of pro-inflammatory cytokines, such as interferon gamma (IFNγ). IFNγ is a key factor in the quantitative expression of HLA class II molecules. Changes in IFNγ could conceivably increase the targets for DSA and thus increase antibody-mediated damage. **(B)** During antibody-mediated rejection, Dll4 is upregulated on endothelial cells and is capable of modulating the differentiation of monocytes into macrophages displaying a pro-inflammatory M1 phenotype. **(C)** DSA binding to endothelial HLA class I molecules initiates intracellular signaling, such as the activation of ERK1/2, S6 kinase, S6 ribosomal protein, and mTOR. This signal transduction leads to the exocytosis of Weibel–Palade bodies and increases cell surface P-selection, which can augment monocyte recruitment and adhesion to the endothelial layer. Classical complement activation, resulting from DSA binding, is capable of synergizing with DSA signaling to further upregulate endothelial cell surface P-selectin and increase monocyte recruitment.

ENDOTHELIAL CELL RECRUITMENT AND POLARIZATION OF MONOCYTES/ MACROPHAGES

Immunohistochemical studies of rejecting biopsies have observed significant leukocyte infiltration associated with the severity of allograft damage. A majority of these cells are intravascular CD68+ monocytes/macrophages (49, 50). In biopsies from cardiac-transplant patients with AMR, an equivalent number of intravascular CD3+ lymphocytes and CD68+ myeloid cells were observed (36). Transcriptomic studies of biopsies have identified macrophage transcripts as the selective genes set that allow the discrimination of AMR and non-AMR patients (51). The myeloid gene set correlates with both the severity of AMR and the MFI of anti-HLA DSA. Furthermore, the changes in macrophage-associated genes suggest Fcγ receptor-mediated phagocytosis as an active pathway in AMR (51).

Monocyte and macrophages may be important mediators of rejection through different mechanisms including antigen processing and presentation, co-stimulation, cytokine production, and tissue remodeling (52). Endothelial cells interact with monocytes through cytokine crosstalk as well as contact-dependent mechanisms, and recent research has elucidated specific mechanisms by which AMR-activated endothelial cells may act upon monocyte responses.

Reed et al. found that HLA class I antibodies are capable of activating endothelial cells by an increase in intracellular calcium, leading to the exocytosis of Weibel–Palade bodies and a concomitant increase in cell surface P-selectin (53). This increase in P-selectin is sufficient to modulate and increase monocyte adhesion to the endothelial cells, and a mouse model of anti-HLA class I DSA-induced AMR found that blocking P-selectin significantly reduced the macrophage load in the graft (54). Furthermore, following the increase in surface P-selectin, Fc receptors binding to the HLA class I-bound DSA contributed to the stability of monocyte adhesion (55). Additionally, the activation of the classical complement cascade by the Fc portion of DSA that releases components such as C5a and C3a has been shown to further enhance endothelial surface P-selectin increases mediated by HLA class I DSA and thereby increase monocyte recruitment to the endothelium (56) (see **Figure 2C**).

Notch ligands and receptors are important regulators of immune cells. Within endomyocardial biopsies, the Notch4 receptor is exclusively expressed on the apical side of endothelial cells and is downregulated during AMR. Conversely, the Notch ligand Dll4 is detected and upregulated on both intravascular macrophages and endothelial cells during AMR. Pabois et al. found that the pro-inflammatory stimulation of endothelial cells *in vitro* could recreate the changes in Notch4 and Dll4 observed in AMR (22). Endothelial Dll4 regulates Notch signaling in monocytes and encourages the differentiation of monocytes into macrophages with a predominantly pro-inflammatory M1 phenotype, which corresponds to the phenotype of intravascular macrophages observed in the endomyocardial biopsies during AMR. The M1 subset is considered to be phagocytic and pro-inflammatory, while the M2 subset is attributed to anti-inflammatory and pro-fibrotic abilities (see **Figure 2B**).

FROM VICTIM TO ACCOMPLICE

The endothelium has been long recognized as a victim in AMR. Endothelial lesions, endothelial cell swelling, and reduplication of the capillary basement membranes are strongly associated with the severity of AMR and poor graft outcomes.

However, studies have only recently begun to reveal the active involvement of endothelial cells in the physiopathology of rejection. Allotransplantation and AMR expose the endothelium to inflammation, HLA/non-HLA DSA, and often complement activation. As a consequence of such stimulation, endothelial cells become atypically activated with an altered immunogenicity and an altered capacity to modulate alloimmune responses. DSA-bound endothelial cells act as a scaffold for Fc-dependent NK cell activation. Activated endothelial cells can induce the alloproliferation of CD4+ T lymphocytes, alter T cell polarization, and indirectly aid anti-graft CD8+ T cell responses. Activated endothelial cells possess a greater capacity for monocyte recruitment and are implicated in pro-inflammatory monocyte differentiation.

Is endothelial cell activation a consequence or a cause of AMR? Endothelial cell activation is associated with episodes of AMR, but this does not exclude the possibility that the activation precedes the clinical diagnosis. For example, HLA-DQ expression is upregulated post transplantation in stable as well as in deteriorating allografts, despite the molecule being regulated by inflammation (27, 28). The idea of a subclinical activation of the endothelium could contribute to the chronicity of AMR through long-standing low-level activation of T cells, NK cells, and myeloid cells despite the systemic therapeutic immunosuppression. Few studies have assessed the impact of such therapies on the immunomodulatory properties of the allograft endothelium (57) and fewer still have tried to directly manipulate the endothelium. Yet, with the growing understanding of endothelial involvement in the mechanisms of AMR, this avenue for treatment remains to be explored.

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AC, DG, and NM contributed to the writing of this review.

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REVIEW
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Defenders and Challengers of Endothelial Barrier Function

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Regulated vascular permeability is an essential feature of normal physiology and its dysfunction is associated with major human diseases ranging from cancer to inflammation and ischemic heart diseases. Integrity of endothelial cells also play a prominent role in the outcome of surgical procedures and organ transplant. Endothelial barrier function and integrity are regulated by a plethora of highly specialized transmembrane receptors, including claudin family proteins, occludin, junctional adhesion molecules (JAMs), vascular endothelial (VE)-cadherin, and the newly identified immunoglobulin (Ig) and proline-rich receptor-1 (IGPR-1) through various distinct mechanisms and signaling. On the other hand, vascular endothelial growth factor (VEGF) and its tyrosine kinase receptor, VEGF receptor-2, play a central role in the destabilization of endothelial barrier function. While claudins and occludin regulate cell–cell junction *via* recruitment of zonula occludens (ZO), cadherins *via* catenin proteins, and JAMs *via* ZO and afadin, IGPR-1 recruits bullous pemphigoid antigen 1 [also called dystonin (DST) and SH3 protein interacting with Nck90/WISH (SH3 protein interacting with Nck)]. Endothelial barrier function is moderated by the function of transmembrane receptors and signaling events that act to defend or destabilize it. Here, I highlight recent advances that have provided new insights into endothelial barrier function and mechanisms involved. Further investigation of these mechanisms could lead to the discovery of novel therapeutic targets for human diseases associated with endothelial dysfunction.

Keywords: cell adhesion molecules, vascular permeability, endothelial dysfunction, vascular endothelial growth factor A, adherens junctions, gap junctions

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INTRODUCTION

To live and reproduce, all vertebrate animals are evolved to have a circulatory system (i.e., heart, veins, and arteries) that safeguards an uninterrupted supply of blood and oxygen to all tissues, followed by the return of the deoxygenated blood to the lungs for re-oxygenation. In addition to its emissary function, the vascular system also plays an indispensable role in hemostasis, immune surveillance, angiogenesis, and vascular permeability (1). Although they differ in function and morphologies, endothelial cells are the main constituents of blood vessels. In some organs such as the brain, endothelial cells form a strong and highly selective blood–brain barrier, but in other organs such as the kidney and pancreas, endothelial cells display selective permeability by forming highly specialized holes on the plasma membrane called fenestrae, which allows rapid exchange of solute and molecules such as hormones.

To maintain the structural and functional integrity that retains the highly dynamic barrier function of blood vessels, which permits continuous leakage of solutes and small molecules but limits extravasation of larger molecules and cells, metazoan cells are evolved to form highly specialized

cell–cell junctions such as desmosomes, adherent junctions, and gap junctions. Not only do these junctions glue cells together, they also generate intracellular signaling and permit junctional remodeling in response to various external and internal cues (2). Curiously, certain viruses employ cell adhesion receptors for their entry into human cells. For example, hepatitis C virus (HCV) uses occludin and claudins to enter liver cells. Coxsackievirus and adenovirus use junctional adhesion molecule (JAM)/coxsackie and adenovirus receptor (CAR) and reoviruses uses JAM-A for their entry into cells [for review see Ref. (3)]. In many human diseases, such as cancer, diabetes, age-related macular degeneration, and chronic inflammatory conditions, this core barrier function of endothelial cells breaks down, leading to the leakage of larger molecules and blood with serious life-threatening consequences. Blood vessel leakiness also is associated with tumor-induced angiogenesis and represents a significant challenge for an effective delivery of anti-cancer drugs to the site of tumors as tumor-associated blood vessels are structurally fragile and hyperpermeable (4).

In addition to their pivotal roles in angiogenesis and inflammation, endothelial cells also play important functions in various other conditions such as surgical trauma, ischemia–reperfusion, alloimmune responses, and chemotherapy and immunosuppressant treatments (5, 6). Activated endothelial cells often upregulate expression of various growth factors, cytokines and chemokines that stimulate endothelial cell proliferation, permeability, and migration (7, 8). Furthermore, they upregulate thrombogenic molecules and specific adhesion molecules that promote thrombosis and immune cell activation (**Figure 1**). Endothelial cells also respond to immunosuppressant and chemotherapeutic drugs. Although the cardiotoxic effects of conventional chemotherapeutic agents are well-documented, the targeted therapeutic drugs such as the antiangiogenic are also associated with endothelial dysfunction, such as hypertension, thromboembolism, myocardial infarction, and proteinuria (9, 10). In organ transplantation, the host immune system is brought into direct contact with the endothelial cell lining of graft vessels, where the graft endothelial cells play a major role in allograft vasculopathy (i.e., allograft rejection) and in the overall long-term survival after any organ transplantation (11, 12).

REGULATION OF ENDOTHELIAL CELL–CELL JUNCTIONS BY CELL ADHESION MOLECULES (CAMs)

Adherens junctions, gap junctions, and desmosomes are principal cell–cell junctions that provide structural integrity and create highly polarized barriers with selective paracellular permeability to solutes, macromolecules, and other cells, which is an essential element of homeostatic maintenance in endothelial and epithelial cells. Tight junctions, in particular, control monolayer permeability and play a significant role in endothelial cells that maintain rigorous barriers, whereas

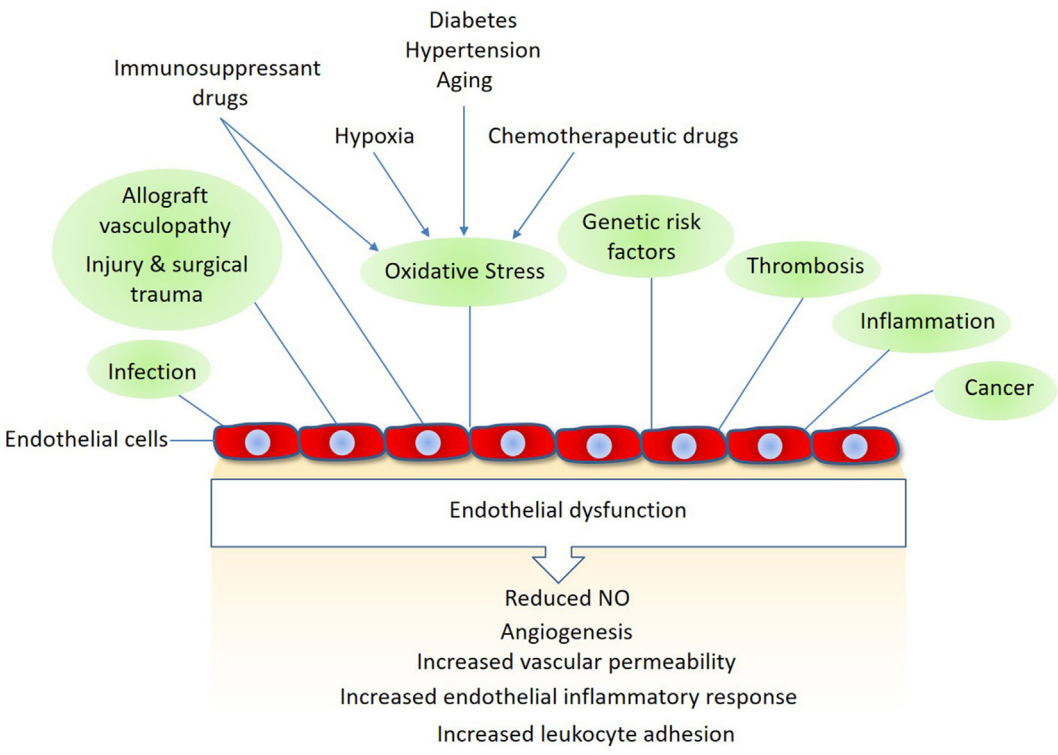

FIGURE 1 | Mechanisms linked to endothelial dysfunction: several key mechanisms that promote endothelial dysfunction and vascular damage are shown. Also, shown are the major endothelial responses triggered by these factors Nitric oxide (NO).

adherens junctions partake in multiple roles, such as establishment and maintenance of cell–cell adhesion, actin cytoskeleton remodeling, signal transduction, and transcriptional regulation. However, unlike epithelial cells, adherens and tight junctions in endothelial cells are highly interconnected. In addition to their cardinal role in the regulation of homeostatic maintenance and barrier function, proteins involved in the regulation of cell–cell junctions play major role in cellular differentiation, proliferation, migration, signal transduction, and gene expression (13, 14). Altered cell–cell junctions are also associated with the pathogenesis of various diseases, including cancers, diabetic retinopathy, and inflammation (15, 16). A plethora of cell surface receptors including claudins family proteins, occludin, JAMs, vascular endothelial (VE)-cadherin, and the recently identified immunoglobulin (Ig) and proline-rich receptor-1 (IGPR-1) are involved in cell–cell junction signaling through various means and mechanisms. While occludin through its cytoplasmic coiled-coil (CC) domain interacts ZO proteins, claudins family proteins and JAMs through their PDZ-binding motif interact with PDZ-containing proteins such as ZO. JAMs also interact with PAR3, PAR6 and AF6, which are also PDZ-containing proteins. On the other hand, VE-cadherin through its armadillo-binding domain recruits p120, catenin proteins, whereas IGPR-1 through its proline-rich motif interacts with (BPAG1 or BP230), also called dystonin (DST) and SH3 protein interacting with Nck90 (SPIN90)/WISH (SH3 protein interacting with Nck), also called NCK-interacting protein with SH3 domain (NCKIPSD) (**Figure 2**). Regardless of their mechanisms of recruitment of cytoplasmic-binding partners, it is clear that these receptor-interacting proteins transduce signals that are required for cell–cell junction assembly, cell morphology, and barrier function. In a way, these transmembrane receptors along with their intracellular-binding partners are the defenders of endothelial integrity and barrier function.

Claudins

Claudin family proteins are four-transmembrane type proteins and there are at least 24 claudins present in human genome (17, 18), which represents the largest family of tight junction proteins. By forming homophilic- and heterophilic-*trans*/*cis* dimerization, claudins determine the barrier properties and cell–cell interactions (19, 20). Claudins, with the exception of claudin-12, contain a PDZ-binding domain at C-terminal tail that allows them to interact with PDZ-containing scaffold proteins such as zonula occludens (ZO) (19). It appears that unlike cadherins, claudins mediate cell–cell adhesion through a calcium-independent manner (20). Claudins are expressed in both endothelial and epithelial cells, though with some degree of cell type specificity. Claudin-3, claudin-5, and claudin-12 are predominantly expressed in brain endothelial cells (21, 22), whereas renal endothelial cells express claudin-5 and claudin-15 (21, 23). Some claudins such as claudin-2 and claudin-16 specifically control paracellular ionic selectivity by forming ion channels (24, 25), while others such as claudin-8 is proposed to control paracellular Na+ permeability (26). In addition to their canonical function, some claudins also interact with other proteins. For example, claudin-1 acts as a receptor for HCV (27) and for dengue virus (28).

Occludin

Similar to claudins, occludin is a four-transmembrane protein and one of the key components of tight junctions that plays a critical role in the regulation of trans-epithelial/endothelial electrical resistance (29, 30) and actin assembly (31). While the N-terminal extracellular domain is involved in the adhesive function of occludin, its C-terminal is subject to phosphorylation at several tyrosine and serine/threonine residues through multiple kinases and is also involved in the recruitment of SH3 and PDZ-containing zonula occluden (ZO) proteins, which anchor occludin to the actin fibril assembly (**Figure 3**). In endothelial cells, it regulate tight junction barriers in response to IFNγ and vascular endothelial growth factor (VEGF) (32–34). The barrier function of occludin is regulated by the phosphorylation of key residues at the cytoplasmic domain (19, 35). For example, phosphorylation of Ser490 was proposed to promote ubiquitination of occludin, which promotes its downregulation (36), whereas

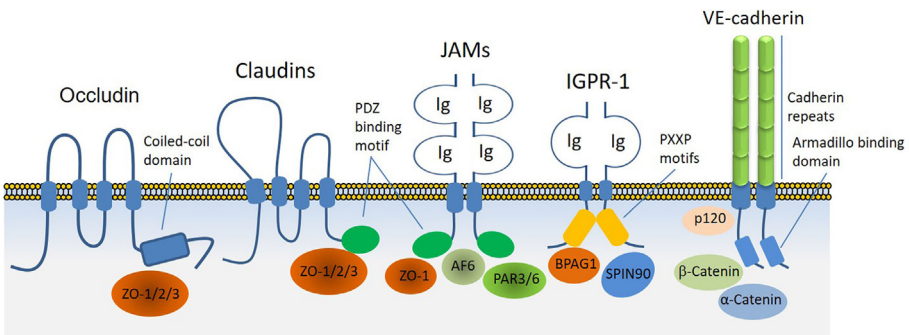

FIGURE 2 | Transmembrane receptors involved in the endothelial cell–cell junction and their key cytoplasmic-binding partners. Claudin family proteins, occludin, junctional adhesion molecules (JAMs), vascular endothelial (VE)-cadherin an immunoglobulin (Ig) and proline-rich receptor-1 (IGPR-1) are major receptors in endothelial cells that regulate endothelial cell–cell junctions and barrier. The core mechanism associated with the function of these receptors involves with their ability to recruit specific signaling proteins that signal to strength the cell–cell junctions. Zonula occludens (ZO 1–3), catenin proteins (α-catenin, β-catenin, and γ-catenin), polarity protein-3 and 6 (PAR3/6), afadin (AF6), bullous pemphigoid antigen 1 [BPAG1 also called dystonin and SH3 protein interacting with Nck90 (SPIN90)/WISH (SH3 protein interacting with Nck)] are key substrates involved with these receptors.

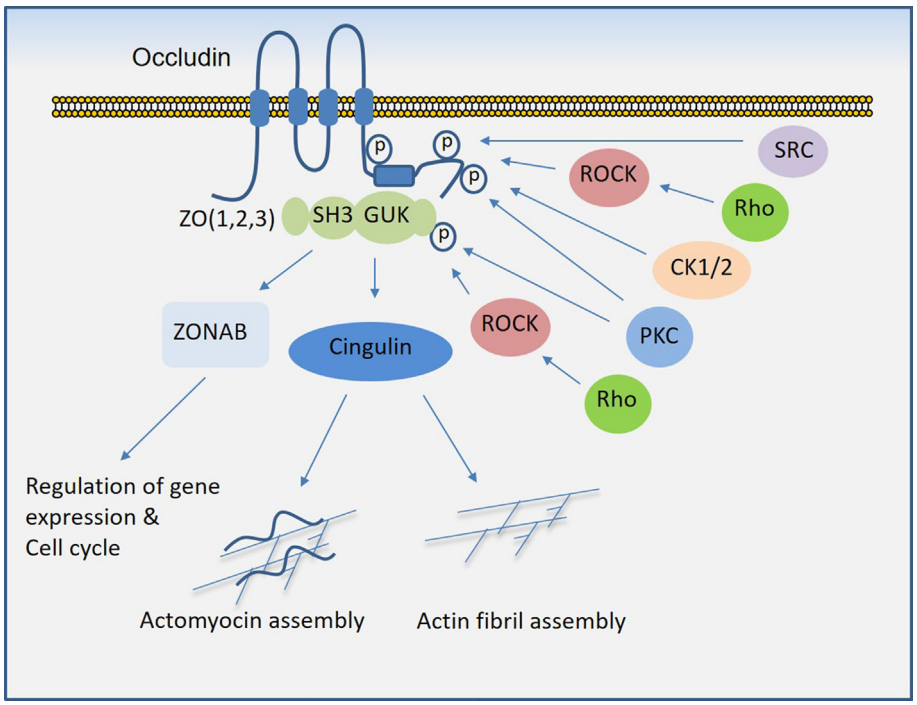

FIGURE 3 | Regulation of occludin mediated cell–cell junction assembly and organization. Occludin *via* its coiled-coil domain recognizes guanylate kinase (GUK) domain, PDZ, and SH3 domain (43), that further recruits Cingulin and ZONAB that participate in the formation and regulation of the tight junction and paracellular permeability barrier. C-terminal of occludin is subject to phosphorylation at serine/threonine and tyrosine residues by multiple serine/threonine kinases and tyrosine kinases, which in part regulate occludin binding with ZO proteins and its tight junctional function (see the text).

phosphorylation of occludin at different sites is associated with its barrier function (37–39). The key kinases involved in the phosphorylation of occludin are shown (**Figure 3**). The interaction of occludin with tight junction proteins such as ZO family proteins is also affected by phosphorylation at its CC domain (40, 41). Overall, phosphorylation of the C-terminal of occludin at serine/threonine and tyrosine sites by various kinases (**Figure 3**) and dimerization (not shown) of occludin appear to be key mechanisms that govern occludin function (42).

Junctional Adhesion Molecules

Junctional adhesion molecules are distinct and important cell surface proteins that are involved in the regulation of cell–cell adhesion and barrier. JAMs belong to the Ig superfamily proteins and contain two extracellular Ig-like domains, a single transmembrane domain and a C-terminal cytoplasmic domain (44). The cytoplasmic domain of JAMs contains a PDZ domain, which recruits PDZ-binding proteins such ZO and afadin that connects JAM proteins to actin assembly and regulation of epithelial and endothelial barrier function (44–46). JAM-A regulates the barrier function of tight junctions in both endothelial and epithelial cells (47) and is involved in the migration of endothelial cells (48). JAM-C was proposed to be involved in tumor angiogenesis (49). Furthermore, other JAMs such as CAR and endothelial cell-selective adhesion molecule are also expressed in endothelial cells and are involved in the regulation of permeability, angiogenesis, and cell migration (50, 51).

MECHANISMS OF DESTABILIZATION OF ENDOTHELIAL BARRIER FUNCTION

The control of the endothelial barrier function is largely mediated by cell-to-cell junctions, which include adherens and tight junctions. CAMs are the key mediators of endothelial barrier function. CAMs mediate cell–cell and cell–matrix adhesion and transmit signals across the plasma membrane to process information from the extracellular environment involved in tissue morphogenesis, angiogenesis, and tumor progression (52, 53).

Various proteins and molecules could destabilize endothelial barrier function and stimulate vascular permeability. Proteins and molecules such as Ang2, chemokines, and IL-8 (interleukin-8), bradykinin, histamine, thrombin, fibrinogen, tumor necrosis factor-α (TNF-α), and endotoxins such LPS could destabilize endothelial barrier. However, VEGF, also called vascular permeability factor, is perhaps the most potent factor involved in the disruption of endothelial barrier function and induction of vascular permeability in pathological conditions (16, 54–56). In tumorigenesis, not only does VEGF induce angiogenesis but also mediates disruption of the vascular barrier, resulting in the leaky vessels leading to an increase in tumor cell extravasation and reduced drug delivery to tumor site which is associated with the development of drug resistance and inefficacy (57). Similarly, VEGF causes vascular permeability and edema in various other diseases such as diabetic retinopathy, age-related macular degeneration, and inflammation (58–61). One of

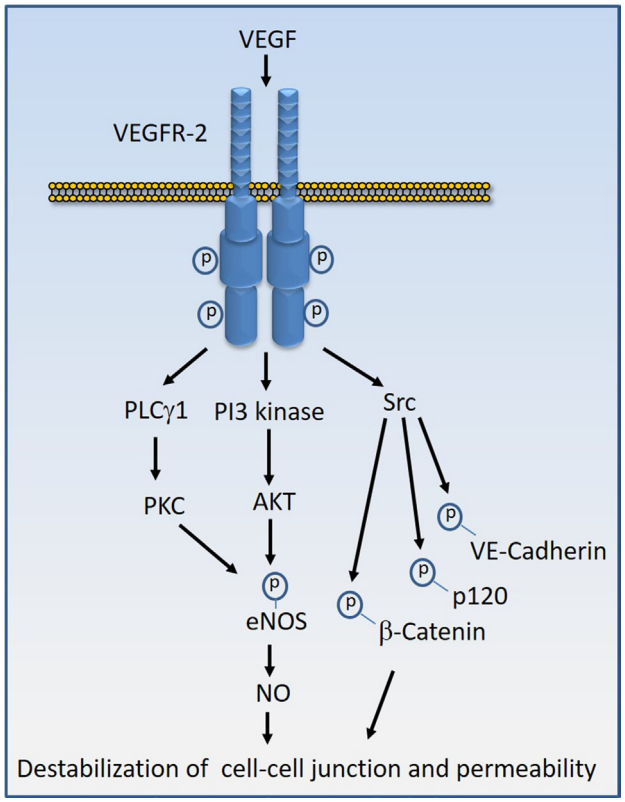

FIGURE 4 | Vascular endothelial growth factor (VEGF)-induced VEGF receptor-2 (VEGFR-2) activation signal transduction that leads to destabilization of cell–cell junctions. Stimulation of VEGFR-2 by VEGF results in the kinase activation of VEGFR-2 and recruitment of diverse signaling proteins to VEGFR-2. The key VEGFR-2 signaling proteins whose activity are linked to vascular permeability include Src family kinases, phosphoinositide 3-kinase (PI3 kinase), and phospholipase Cγ1 (PLCγ1). Src kinases in turn can phosphorylate VE-cadherin and VE-cadherin-associated proteins such as β-catenin and p120 leading destabilization of VE-cadherin mediated endothelial barrier function. Activation of PI3 kinase and PLCγ1 can lead to phosphorylation of eNOS and production of nitric oxide (NO) that leads to interruption of endothelial junctions.

VEGF's receptor, VEGF receptor-2 (VEGFR-2), predominantly mediates VEGF-mediated destabilization of endothelial junctions (16, 62). Upon activation by VEGF, VEGFR-2 undergoes various posttranslational modifications including phosphorylation and methylation, which stimulate its activation and recruitment of signaling proteins to the receptor (63–65). Activation of VEGFR-2 by VEGF stimulates diverse signaling events that affect endothelial cell migration, proliferation, tube formation, and regulation of endothelial junctions. However, the activation of Src family kinases, phosphoinositide 3-kinase (66, 67), and phospholipase Cγ1 (PLCγ1) in particular play major roles in the induction of vascular permeability (16, 67) (**Figure 4**). In addition, VEGFR-2 can also stimulate permeability by directly targeting endothelial junctional proteins such as VE-cadherin and integrins (16, 62, 68, 69), providing an additional layer of complexity to VEGF-mediated destabilization of endothelial barrier function.

Vascular endothelial cadherin (VE-cadherin also called Cadherin-5 and CD144) is considered a main transmembrane component of endothelial adherens junction (70, 71). Similar to E-cadherin, VE-cadherin binds to members of the armadillo repeat family of proteins, p120-catenin, β-catenin, and plakoglobin through its cytoplasmic C-terminal (72). Inactivation of the VE-cadherin gene in both mouse and zebrafish clearly demonstrated its key role in vascular remodeling (73, 74). VE-cadherin plays an important role in controlling endothelial monolayer permeability and angiogenesis. VEGF-induced tyrosine phosphorylation of VE-cadherin at Y658 and Y731 by Src family kinases appear to play a prominent role in the destabilization of adherens junction and increased permeability of endothelial cells (75, 76). Consistent with the regulatory role of phosphorylation on VE-cadherin, other factors such as TNF-α that stimulate permeability also target VE-cadherin through tyrosine phosphorylation at Y658 and Y731 through proline-rich tyrosine kinase 2 and Rac1/Tiam1 (77). Phosphorylation of Y658 and Y731 disrupt VE-cadherin binding with VE-cadherin-associated proteins such as p120-catenin and β-catenin (78). Underscoring the role of phosphorylation in the regulation of VE-cadherin function, several protein tyrosine phosphatases are known to associate with and dephosphorylate VE-cadherin (79, 80). In view of the fundamental role of VEGF in angiogenesis and its robust action in the destabilization of endothelial barrier function in pathological conditions, VEGF system emerged as a major challenger and provocateur of the endothelial barrier function.

IGPR-1 IS A DISTINCT CAM

Immunoglobulin (Ig) and proline-rich receptor-1 is expressed in human endothelial and epithelial cells. Unlike the classical cadherins and tight junction proteins such as JAMs, claudins family proteins, and occludin, IGPR-1 expression is restricted to higher mammalians as it is not present in rodents such as mouse or rat (81). However, its closely related protein, transmembrane and immunoglobulin domain1 is expressed in the renal epithelial cells of human and rodents (82). IGPR-1 colocalizes with VE-cadherin in endothelial cells in cell culture and mediates endothelial cell–cell adhesion and its activity is required for angiogenesis *in vitro* and regulation of cell migration (81). Further studies revealed that it plays an important role in monolayer permeability (83). IGPR-1 is composed of three major domains: extracellular, transmembrane, and intracellular. The extracellular domain of IGPR-1 contains a single Ig domain followed by a single transmembrane domain and a proline-rich intracellular domain (81). The Ig-containing extracellular domain is required for IGPR-1 to mediate endothelial cell–cell interaction and barrier function (83). IGPR-1 is typically present as a disulfide bound *cis*-dimer, which further forms a *trans*-dimer complex in a cell density-dependent manner (83) (**Figure 5A**).

Proline rich sequences (PRDs) play a major role in mediating protein–protein interaction in prokaryotes and eukaryotes (84). PRDs are highly versatile and recognize different consensus motifs or canonical sequences in their protein ligands. A conspicuous

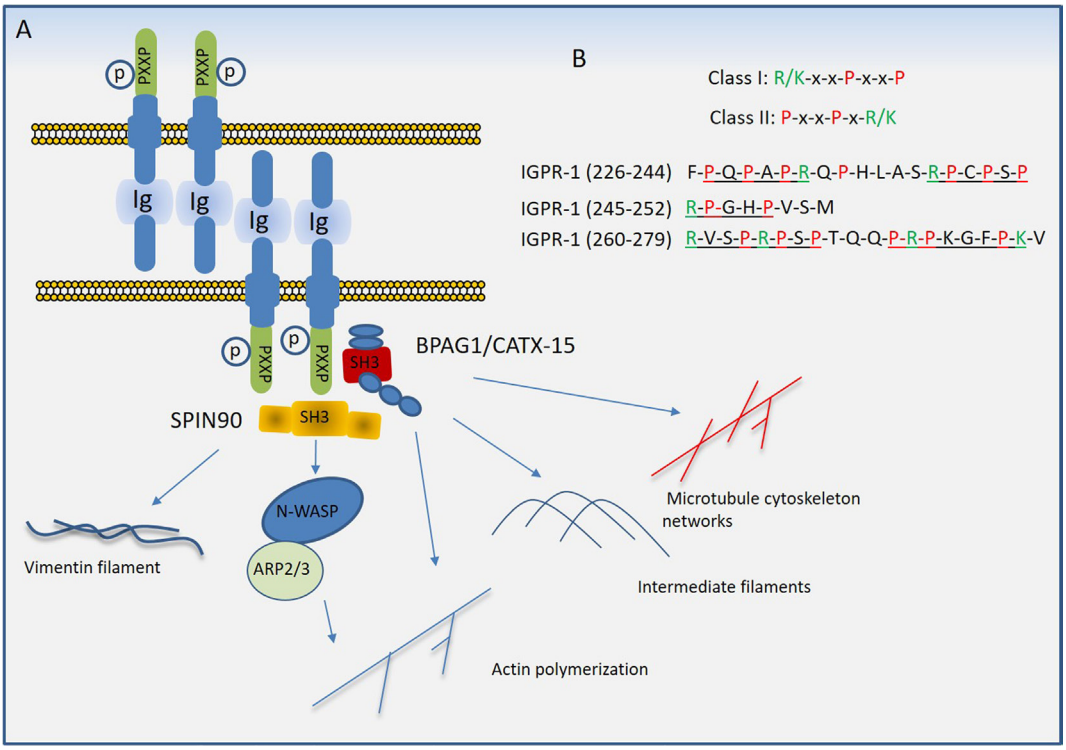

FIGURE 5 | Proposed model of immunoglobulin (Ig) and proline-rich receptor-1 (IGPR-1) mediated regulation of endothelial barrier. **(A)** *Trans*-dimeric IGPR-1 undergoes serine phosphorylation at multiple sites. IGPR-1 through its proline-rich motifs recruits SH3 containing proteins, bullous pemphigoid antigen 1 (BPAG1), and SH3 protein interacting with Nck90 (SPIN90)/WISH (SH3 protein interacting with Nck). Interaction of IGPR-1 with BPAG1 and SPIN90 links IGPR-1 to actin fibril assembly, intermediate filament formation, microtubule cytoskeleton networks, and vimentin filament assembly (see text). **(B)** The conventional proline-rich motifs and the proline-rich motifs on the cytoplasmic domain of IGPR-1 are shown.

feature of most binding motifs identified for PDRs is the presence of one or more proline residue that interact with the ligand, while residues that flank the core proline residue determines the selectivity (85). Although various protein domains are known to interact with proline-rich sequences (85), Src homology domain 3 (SH3) and WW domains are the most common domains that interact with PRDs (86). The PRDs of IGPR-1 interact with multiple SH3 domain-containing proteins including SPIN90/WISH (SH3 protein interacting with Nck) and bullous pemphigoid antigen 1 (BPAG1) (81). The cytoplasmic domain of IGPR-1 contains at least five PRDs (**Figure 5B**), which are variants of canonical class I (R/KxxPxxP) and class II (PxxPxR/K) PRD motifs (85). Furthermore, SH3 domain-containing proteins can interact with PRDs beyond the PXXP motifs, consistent with their versatility in their interaction with other proteins (85, 87).

In addition to being rich in proline residues, the cytoplasmic domain of IGPR-1 also is heavily phosphorylated at serine residues. A recent liquid chromatography–tandem mass spectrometry analysis of IGPR-1 identified seven phosphorylated serine residues on the cytoplasmic domain of IGPR-1, including Ser186, Ser220, Ser238, Ser243, Ser249, Ser262, and Ser266, five of which are located in the proline-rich region (83). Although the functional importance of these phosphorylation sites remains to be determined, phosphorylation of Ser220 is regulated by homophilic *trans*-dimerization of IGPR-1 and is required for endothelial barrier function and angiogenesis (83).

IGPR-1 SIGNAL TRANSDUCTION IN ENDOTHELIAL CELLS

Although significant work is required to fully understand the signal transduction events orchestrated by IGPR-1, recent studies, however, provide important new insights about signaling of IGPR-1 in endothelial cells (81, 83). Through the screening of a Src-homology3 (SH3) domain array, BPAG1 (or BP230), also called DST and SPIN90/WISH (SH3 protein interacting with Nck), also called NCKIPSD were identified as putative IGPR-1-binding proteins (81). The binding of BPAG1 and SPIN90 with IGPR-1 was further confirmed by recombinant GST-SH3 domain of BPAG1 and SPIN90 in a GST-pull down assay (81).

Bullous Pemphigoid Antigen 1

Bullous pemphigoid antigen 1 is a member of the plakin family proteins, which include desmoplakin, plectin, envoplakin, and periplakin, is involved in cytoskeletal organization (88). BPAG1 is a cytoskeletal linker protein that crosslinks cytoskeletal filaments

to membrane-associated complexes at cell junctions in epithelial cells and other cell types (89, 90). BPAG1 is a gigantic protein with 7,570 amino acids and an approximate molecular weight of 834 kDa. However, it is expressed in a various isoforms by mechanism of mRNA alternative splicing, which results in the transcription and translation that generates different isoforms of BPAG1 with varying molecular weights (91). Based on the human genome sequence information, there are 35 different transcripts of BPAG1, many of which are untranslated[1] and based on the available human protein sequence data[2] there are at least nine isoforms of BPAG1 (**Figure 6**). BPAG1 is a multidomain protein. It has a conserved N-terminal actin-binding domain, followed by plakin domain which consists of 4–8 spectrin repeats interrupted by a Src-homology3 (SH3) domain. This unique domain is conserved in all plakin family proteins. The C-terminal of BPAG1 composed of additional plakin repeat domains and intermediate filaments binding domain (88, 92–94).

Despite extensive studies on the functional role of BPAG1 in epithelial cells, expression and importance of BPAG1 in endothelial cells remains virtually unknown. However, analysis of publically available gene array datasets[3] indicates that BPAG1 is likely expressed in mouse and human endothelial cells. Based on various recently published gene array analyzes, BPAG1 appears to widely expressed in human vascular endothelial cells derived from lung (95), macrovascular umbilical vein endothelial cells (96), umbilical cord arterial and venous endothelial cells (97), and mouse neonatal retinal endothelial cells (98), suggesting a functional role for BPAG1 in IGPR-1-mediated signal transduction in endothelial cells. Interestingly, one of the major characteristics of epidermolysis bullosa, a neurological condition that causes the skin to blister, is caused by a genetic defect in BPAG1 and it is associated with increased pathological angiogenesis with a leaky vessel (99). Nevertheless, the role of vascular component in epidermolysis bullosa remains unexamined. While the role of BPAG1 in endothelial cells and its possible role in connecting IGPR-1 cytoskeletal filaments remains unresolved, a recent study demonstrated that plectin/epiplakin 1, a closely related protein to BPAG1, is expressed in endothelial cells, which crosslinks vimentin to the actin assembly to regulate vascular integrity (100).

SH3 Protein Interacting with Nck90

SH3 protein interacting with Nck90/WISH (SH3 protein interacting with Nck 90, also called NCKIPSD and DIP, mDia-interacting protein) is another cytoplasmic signaling protein that interacts with IGPR-1 in endothelial cells (81). SPIN90 is involved in actin polymerization *via* its interactions with Arp2/3,

[1] http://www.ensembl.org.
[2] http://www.uniprot.org/.
[3] https://www.ncbi.nlm.nih.gov/geo/.

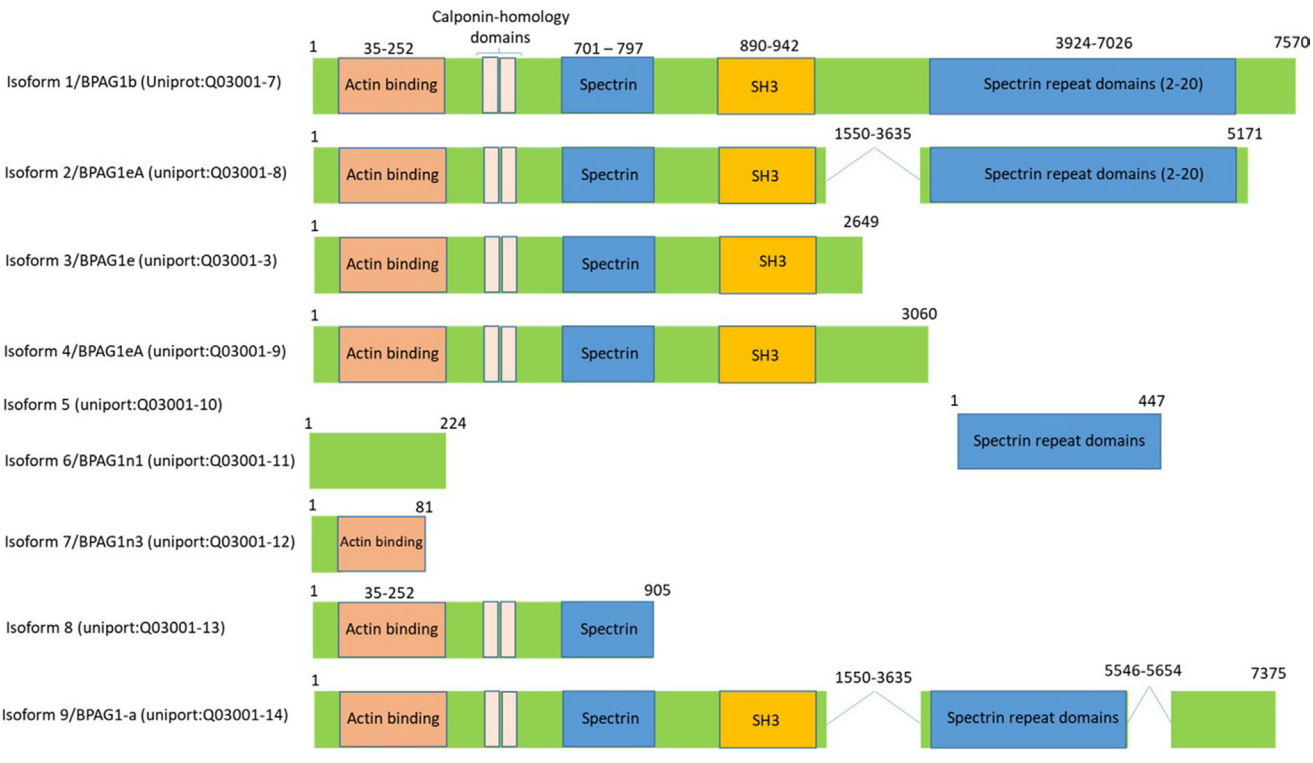

FIGURE 6 | Bullous pemphigoid antigen 1 (BPAG1) is a multidomain protein with various alternatively spliced variants. BPAG1 is a large protein with 7,570 amino acids with multiple domains including N-terminus actin-binding domain, followed by plakin domain which consists of 4–8 spectrin repeats interrupted by a Src-homology3 (SH3) domain. The C-terminal of BPAG1 is composed of additional plakin repeat domains and intermediate filaments binding domain. Various alternatively spliced variants of BPAG1 are also shown.

N-WASP, and actin (101), regulates stress fiber formation (102), Rac-mediated membrane ruffling (103), and binds to Palladin, a cytoskeletal protein that is required for organization of normal actin cytoskeleton, which is important for cell morphology, motility, and cell adhesion (104). SPIN90 is highly expressed in endothelial cells and the siRNA-mediated downregulation of SPIN90 inhibited capillary tube formation, suggesting an important role for SPIN90 in IGPR-1-mediated signaling events in endothelial cells and angiogenesis (81).

Although a significant work remains, the emerging picture of IGPR-1-mediated signal transduction in endothelial cells indicates that IGPR-1 is cross-linked to actin fibril assembly and other cytoskeletal filaments that contributes to endothelial cell adhesion, integrity, and barrier function partly through interaction with SPIN90 and BPAG1. However, deciphering the molecular mechanisms of IGPR-1 in various cell culture systems and animal models other than mouse (IGPR-1 is not expressed mouse or rat) is an important area for future research, which may lead to the discovery of new therapeutic targets for various human diseases associated with endothelial dysfunction.

CONCLUSION

Altered endothelial barrier function is a hallmark of many human disorders. Understanding the molecular mechanisms of vascular permeability could lead to new therapeutic strategies to prevent vascular leakage and improve drug delivery. Moreover, controlling integrity and function of endothelial cells in organ transplantation could reduce complications associated with transplantation medicine.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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REVIEW
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CD95/Fas, Non-Apoptotic Signaling Pathways, and Kinases

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Endothelial cells lining new blood vessels that develop during inflammatory disorders or cancers act as doors that either allow or block access to the tumor or inflamed organ. Recent data show that these endothelial cells in cancer tissues and inflamed tissues of lupus patients overexpress CD95L, the biological role of which is a subject of debate. The receptor CD95 (also named Fas or apoptosis antigen 1) belongs to the tumor necrosis factor (TNF) receptor superfamily. Its cognate ligand, CD95L, is implicated in immune homeostasis and immune surveillance. Because mutations of this receptor or its ligand lead to autoimmune disorders such as systemic lupus erythematosus (SLE) and cancers, CD95 and CD95L were initially thought to play a role in immune homeostasis and tumor elimination *via* apoptotic signaling pathways. However, recent data reveal that CD95 also evokes non-apoptotic signals, promotes inflammation, and contributes to carcinogenesis; therefore, it is difficult to dissect its apoptotic effects from its non-apoptotic effects during pathogenesis of disease. CD95L is cleaved by metalloproteases and so exists in two different forms: a transmembrane form and a soluble ligand (s-CD95L). We recently observed that the soluble ligand is overexpressed in serum from patients with triple-negative breast cancer or SLE, in whom it contributes to disease severity by activating non-apoptotic signaling pathways and promoting either metastatic dissemination or accumulation of certain T cell subsets in damaged organs. Here, we discuss the roles of CD95 in modulating immune functions *via* induction of mainly non-apoptotic signaling pathways.

Keywords: apoptosis, endothelium, FasL, Fas, lupus metastasis, migration, tyrosine kinase

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INTRODUCTION

Accumulating evidence suggests that high endothelial venule (HEV) density in the tumor stroma is a strong predictor of infiltration by $CD4^+$ T, $CD8^+$ T, and B cells. Although this statement seems obvious because the blood vessels carry oxygen, nutrients, and cells to the organs, selective infiltration by certain T cell subsets at the expense of others is a very complex process. Here, we describe recent data suggesting new roles for CD95L in this process and, more generally, new biological roles for CD95L in progression of cancer and autoimmune disorders such as systemic lupus erythematosus (SLE).

The immune system comprises specialized cells that protect the body from infection and cell transformation. Antigen (Ag)-presenting cells (APCs) survey tissues for non-self Ags and capture pathogenic agents. Ags are processed and presented at the cell surface in the context of major histocompatibility complex (MHC) molecules. Similar to macrophages and B lymphocytes, dendritic

cells are professional APCs. APCs present Ags on MHC class II molecules and not only participate in activating non-self-Ag-recognizing T cells but also contribute to elimination of T-cells that recognize self-Ags with a too strong affinity; the latter process is called peripheral tolerance. αβ T cells display either CD4 or CD8 on their surface. $CD4^+$ T helper (Th) lymphocytes bind Ag presented in the context of MHC class II molecules expressed by professional APCs and produce cytokines that drive the immune response. $CD8^+$ T-lymphocytes recognize antigenic peptides presented by MHC class I molecules expressed on the surface of all cells (apart from some transformed or infected cells), resulting in elimination of these target cells.

The long list of Th subset lineages shows the complexity of the mechanisms developed by the immune system to respond to the diversity of infectious agents and cancers. Polarization of $CD4^+$ T-cell subsets occurs *via* exposure to cytokines, microbiota, high salt concentrations, or high-fat diet (1) that induce expression of different transcription factors such as STAT1, STAT4, and T-bet (for Th1 cells), STAT6 and GATA-3 (for Th2 cells), FoxP3 (for regulatory $CD4^+$ T cells; Tregs), Bcl6 (for follicular Th cells; TFH) (2), RORγt, RORα, and STAT3 (for Th17 cells), T-bet and AhR (for Th22 cells) (3), and PU-1 for Th9 cells (4). These T cells express a set of cytokines that organize the entire immune system (innate and adaptive immune cells), so that it responds adequately (or not) to infection and transformation. $CD8^+$ cytotoxic T-lymphocytes destroy target cells and spare bystanders. Recent studies show that the immune landscape (intensity and distribution) within tumors may serve as a prognostic marker for the risk of disease progression and help to predict the efficiency of chemotherapeutic/radiotherapeutic/immunotherapeutic regimens (5).

To simplify, APCs present Ags to $CD4^+$ Th cells, which in turn orchestrate activation of the immune response *via* a cellular (Th1/Th17) or humoral (Th2) response, or by downregulating the intensity/kinetics of the adaptive immune response by promoting differentiation of Tregs. $CD4^+$ Th subsets regulate the adaptive immune response by finely tuning the response to particular infectious agents and cancer cells. In addition, the efficiency of the immune response relies on the ability of these cells to cross blood vessels and migrate within inflamed or transformed tissue to reach the target. Selectins, integrins, and chemokine receptors play a central role in T cell extravasation, which involves several different steps: arrest of cells on the endothelial surface, adhesion and spread, crawling, and transendothelial migration (**Figure 1**). Once immune function has been achieved, effector T cells are eliminated by molecular mechanisms based on death receptors (DRs) or the BH3-only factor BIM (according to the affinity of the immune cell for the stimulating Ag, i.e., low versus high affinity) (6); however, some Ag-specific memory T cells persist.

Because TCR activation induces *de novo* expression of CD95L by $CD8^+$ T-cells, this ligand was regarded as a lethal weapon used by these cells to eliminate target cells (7–9). However, novel studies revisited the biological role of CD95 and found that this receptor also activates non-apoptotic signaling pathways,

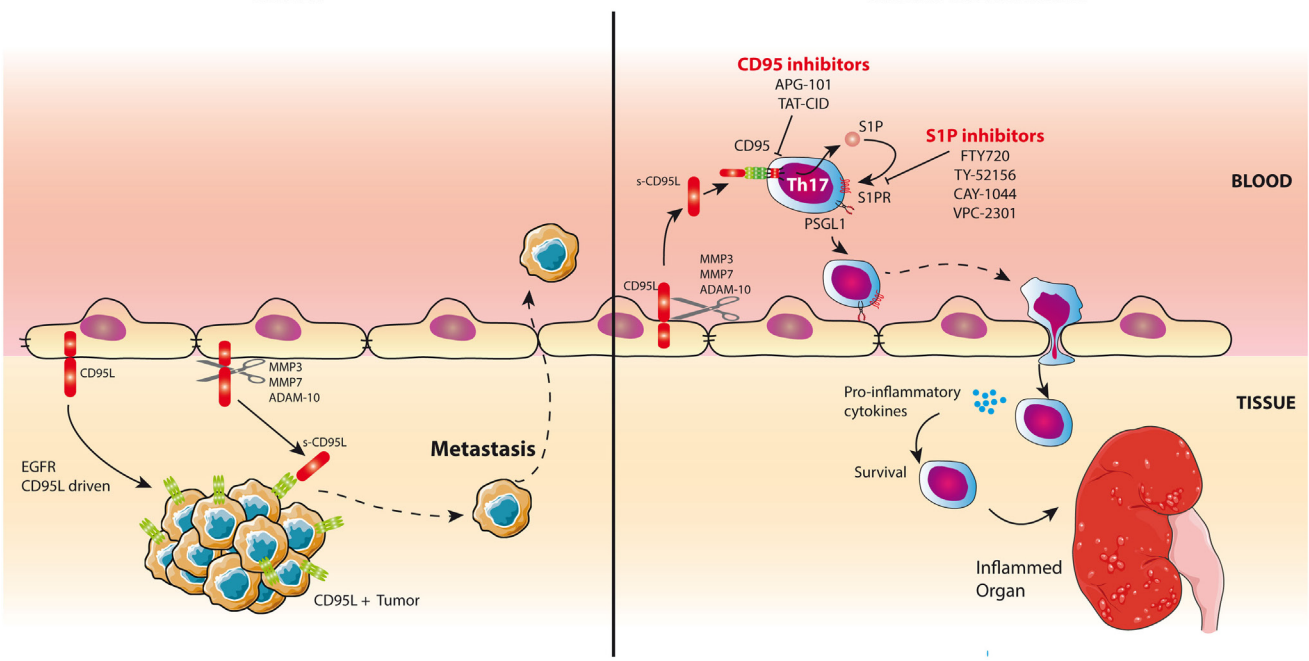

FIGURE 1 | CD95L is a chemoattractant for inflammatory T cells and triple-negative breast cancer (TNBC) cells. *Left panel:* CD95L is expressed by endothelial cells in damaged organs in lupus patients and in TNBCs. Cleavage of CD95L by metalloproteases releases a soluble form of the ligand. In TNBCs, s-CD95L acts as a chemottractant for tumor cells promoting metastasis. *Right panel:* In chronic inflammatory pathologies like systemic lupus erythematosus, s-CD95L enhances the trafficking of Th17 T cells in damaged organs through the activation of sphingosine 1 phosphate (S1P) pathway. Infiltrating Th17 cells release pro-inflammatory cytokines. Th17 recruitment can be blocked by S1P or CD95 inhibitors.

thereby enhancing pro-inflammatory functions that contribute to the severity of autoimmune disorders and cancers. For instance, CD95/CD95L participates in selective accumulation of pro-inflammatory Th17 cells in the damaged organs of lupus patients (10).

DRs BELONGING TO THE TUMOR NECROSIS FACTOR (TNF) RECEPTOR (TNF-R) FAMILY

CD95

Death receptors including CD95 (11), TNFR1 (12), DR4 (13), DR5 (14), and DR6 (15) belong to the TNF-R superfamily. These type I transmembrane proteins share common features, including extracellular amino-terminal cysteine-rich domains (16, 17) that contribute to both ligand specificity (18) and receptor preassociation (19–21) and the death domain (DD) that encompasses an 80 amino acid sequence located in the cytoplasmic tail; this domain transmits the apoptotic signal through protein–protein interactions (PPIs) (22, 23). Among them, adaptor proteins that interact with the DD, TRADD, and FADD are instrumental in implementing cell death pathways (24–27). It is noteworthy that TNF-R members do not possess enzymatic activity rather they rely on dynamic PPI formation to recruit enzymes and signals. This property accounts for the versatility and pleiotropy of these receptors in terms of triggered signaling pathways.

Based on the fact that DRs were originally identified and cloned according to their ability to elicit apoptosis, some of these receptors (mainly CD95 for historical reasons) are still considered by a part of the scientific community to be monomaniac death inducers, even though this is clearly not the case (28–31). Historically, a monoclonal antibody designated "apoptosis antigen 1" was selected for its ability to trigger cell death in tumor cells (32); this antibody recognizes Fas/CD95 (11). Accordingly, most future studies investigated the role of the CD95-mediated apoptotic signaling pathway during progression of cancers and inflammatory disorders. Nonetheless, and unlike TNFα-targeting therapeutics, all molecules acting as CD95 agonists failed to reach clinical trials. This is probably due to the fact that CD95 does not only function as a DR and that inhibiting it rather than activating it may be a more attractive therapeutic option for patients suffering from cancers and chronic inflammatory disorders. Although the molecular events that generate the apoptotic signal induced by CD95 are well defined, it is not clear how the receptor activates non-apoptotic signaling pathways (i.e., NF-κB, MAPK, and PI3K).

CD95L

Transmembrane CD95L (m-CD95L) is a potent inducer of cell death (33). m-CD95L can be cleaved by different metalloproteases and although s-CD95L interacts with CD95 it fails to trigger cell death (34, 35). It does, however, trigger non-apoptotic signaling pathways (36, 37). Until now, no-one has explained how the interaction between a unique ligand (CD95L) and a unique receptor (CD95) induces such different signaling pathways. It may be that differences in the kinetics of CD95 aggregation, or its internalization and/or membrane distribution, may account for the induction of one cue at the expense of another. Our hypothesis is that the magnitude and pace of execution of the above mechanisms may depend on the stoichiometry of the ligand interacting with CD95. Indeed, while s-CD95L exists mainly as a homotrimer (35), its membrane-bound counterpart exists as multi-aggregated homotrimers (38). This is an important feature of TNF family members; these membrane-bound ligands are processed by metalloproteases to release a soluble ligand counterpart (30). The difference in their stoichiometry (soluble and homotrimeric ligand versus membrane-bound and multi-aggregated homotrimeric CD95L) and/or their conformational effect on the receptor (natural ligand versus agonistic antibodies) might explain the differences in activated cell signaling pathways (39, 40). Similarly, TNFα can be cleaved by the metalloprotease TACE (41, 42) to release a soluble form of the cytokine (s-TNFα). Whereas s-TNFα only activates TNFR1, m-TNFα binds and activates both TNFR1 and TNFR2 (43). Activating TNFR1 induces cellular processes ranging from cell death (apoptosis or necroptosis) to cell proliferation, migration, and differentiation; implementation of these cellular responses reflects the dynamic formation of PPIs following receptor activation (44).

The transmembrane form of CD95L was originally detected at the surface of activated lymphocytes (45) and natural killer (NK) cells (46), where it not only contributes to eliminating tumor and infected cells but also kills T-cells through a process called activation-induced cell death (AICD) and dendritic cells (47). CD95L is also expressed on the surface of neurons (48), corneal epithelia, and endothelia (49, 50), where it prevents infiltration of immune cells and thereby inhibits the spread of inflammation within these sensitive organs, which are commonly referred to as "immune-privileged" sites. Of the different types of intestinal epithelial cells, Paneth cells express CD95L (51), although no expression has been reported in normal villus epithelium. However, mouse villus epithelial cells express CD95L after exposure to oxidative stress, and this expression may affect the way the immune response reacts to the microbiota (52).

More recently, different groups have shown that, even if CD95L can be detected on the surface of immune cells, the ligand is mainly found on the surface of endothelial cells lining blood vessels feeding cancer tissues (37, 53) or damaged organs in lupus patients (10, 36). Although many studies have examined expression of CD95L, few report expression of this ligand on the surface of endothelial cells, probably because they used the incorrect antibodies. Indeed, although several anti-CD95L antibodies exist, only one (clone G247-4) gives reliable results in immunohistochemistry experiments (54). In patients with inflammatory bowel diseases, G247-4 detected CD95L in HEVs expressing HEV markers such as MAdCAM-1 and MECA-79 (55). Strikingly, CD95L-expressing HEVs observed in cow's-milk-sensitive enteropathy (CMSE) and celiac disease were surrounded by lymphoid follicles. Moreover, overexpression of m-CD95L in CMSE correlated with increased concentrations of serum CD95L, enlargement of mucosal lymphoid nodules in the gastrointestinal tract (GIT), and infiltration by

intraepithelial lymphocytes (56). These data suggest that, after cleavage by metalloproteases, s-CD95L attracts leukocytes to the inflamed GIT.

We recently detected CD95L in endothelial cells lining blood vessels in inflamed skin of lupus patients and in tumor tissues from women with triple-negative breast cancer (TNBC) (10, 37). This ligand is cleaved by metalloprotease to yield s-CD95L, which is present in the serum of these patients at higher amounts than in that of healthy donors. Our initial study showed that the naturally processed ligand triggers PI3K and Ca^{2+} signaling pathways in activated T cells, which then accumulate in the inflamed organs of SLE patients (36); this raises the question of whether all T cells, or only certain Th subsets, respond to s-CD95L and contribute to SLE pathology by accumulating in inflamed organs. As aforementioned, TCR engagement leads to the overexpression of CD95 (57) and CD95L (9) rendering activated T-cells susceptible to apoptosis and contributing to their elimination by AICD (58, 59). Overexpression of m-CD95L in activated $CD4^+CD25^{high}Foxp3^+$ Tregs serves to eliminate $CD8^+$ T-cells (60). Strikingly, blood Tregs isolated from patients affected by head and neck squamous cell carcinoma and stimulated with anti-CD3 mAb and IL-2 exhibit a higher level of CD95L as compared to that present at the surface of Tregs from healthy donors, providing a potential explanation for the elimination of tumor-infiltrating $CD8^+$ effector T-cells in these cancer patients (60). Similar to Tregs, myeloid-derived suppressor cells (MDSCs) inhibit adaptive and innate immunity and accumulate in the blood of cancer patients, chronic inflammation and infection. They consist of two major subsets, granulocytic (G-MDSC) and monocytic (M-MDSC) subsets, producing immune suppressive factors including arginase I, inducible nitric oxide synthase, and cytokines and thereby, inhibiting effector T-cell functions (61). Similar to Tregs, MDSCs express CD95 and die in response to CD95L stimulation (62). Consequently, *gld* BALB/c mice, which express a mutated CD95L unable to bind its receptor, show an accumulation of MDSCs as compared to their wild-type counterpart (62) indicating that CD95/CD95L pair is instrumental in the homeostasis of this immunosuppressive cell population. Interestingly, a syngeneic graft of Lewis lung carcinoma in CD95-KO mice (C57BL/6 strain) did not reveal any increase in the number of MDSCs in the spleen or the tumor tissues as compared to wild-type mice (63). The G-MDSC subset is the prevalent population of MDSCs in different tumor models with an expected ratio of 60% of G-MDSCs (63). An in-depth analysis of LCC-infiltrating MDSCs highlighted that the loss of CD95L skewed cell populations toward the M-MDSC subset, which displayed a higher immunosuppressive activity as compared to G-MDSCs (63). Overall, these findings indicate that the loss of CD95L or CD95 might create an immunosuppressive environment by increasing the number of Tregs and MDSCs and/or by increasing in the M-MDSC/G-MDSC ratio and thereby renders mice more susceptible to tumor progression as compared to the wild-type mice.

Th17 cells contribute to kidney damage and pathology severity in SLE patients (64, 65). Although blood Tregs express higher CD95 levels than Th17 cells, this former regulatory T-cell did not respond to s-CD95L (10). On the other hand, Th17 cells underwent endothelial transmigration and accumulated in inflamed organs of lupus patients in a CD95-dependent manner. In an effort to explain the CD95-driven accumulation of Th17 cells in damaged organs, we showed that these Th17 cells exposed to s-CD95L upregulate expression of the adhesion molecule PSGL-1, which not only promotes tethering of lymphocytes to endothelial cells and subsequent rolling (**Figure 1**) but also provokes secretion of effector cytokines when highly expressed by T cells (66). Indeed, Bradley team suggests that PSGL-1 expression affects the interaction of T-cells with APCs and more specifically, among the $CD4^+$ Th1, activation of cells with the highest PSGL-1 levels are the most proliferative effectors with the greatest capacity for effector cytokine secretion (66) probably through a PI3K-driven mechanism (67). Therefore, s-CD95L might fuel the inflammatory process in lupus patients first by mediating accumulation of Th17 cells in organs and second by altering the pattern of cytokines released in the inflamed tissues (**Figure 1**). The sphingosine 1 phosphate (S1P) signaling pathway is a critical regulator of many pathophysiological processes (68). Unlike Tregs, exposure of Th17 cells to s-CD95L causes them to upregulate expression of S1P (10). Activating the S1P signaling pathway in Th17 cells exposed to s-CD95L provides a molecular mechanism by which Th17 cells transmigrate in the presence of this soluble ligand. Successful development of the S1P analog FTY720 and its use as a drug to treat multiple sclerosis (69) shows that it is possible to target S1P signaling in humans and, therefore, that S1P antagonists would be an attractive therapeutic option for preventing CD95-mediated transmigration of Th17 cells in SLE patients (**Figure 1**). Interestingly, the ectopic expression of CD95L by endothelial cells also promotes transmigration of CD95-expressing myeloid cells by tethering cells and slowing cell rolling (70).

Nonetheless, the role of endothelial CD95L remains controversial because, while Coukos et al. observed that the membrane-bound ligand served as a barrier to prevent CD8 T-cell extravasation (while sparing Treg accumulation in the tumor tissue) (53) (**Figure 1**), we found that CD95L on the surface of endothelial cells can be cleaved by metalloproteases to create a gradient that is responsible for accumulation of Th17 cells in inflamed organs (extravasation) (10, 36) or the metastatic dissemination of TNBC cells (intravasation) (37) (**Figure 1**). In agreement with our data, initial experiments with CD95L-expressing tumor cells did not promote carcinogenesis by killing tumor-infiltrating immune cells but instead caused acceleration of tumor rejection (when compared with CD95L-negative tumor cells), which was associated with massive recruitment of neutrophils to the transplanted area (71). Moreover, CD95L-expressing cells provide T cells with a survival signal (72). For instance, CD95L-expressing β-islets increase $CD8^+$ activation, leading to organ damage; also, the same experiment in NOD mice backcrossed with $MRL^{lpr/lpr}$ mice (exhibiting loss of CD95) failed to stimulate T cells, meaning that the mice did not develop diabetes (73). In summary, accumulating evidence suggests that CD95L acts as a direct chemoattractant for neutrophils, macrophages (71, 74–76), T lymphocytes (36), and malignant cells in which the CD95-mediated apoptotic signal is non-productive (77, 78). This ligand can also exert an indirect chemoattractant effect by

promoting the expression of IL-8 and MCP-1 recruiting phagocytes (79, 80). Recent data highlight that inflammatory Th17 cells fail to differentiate when CD95 expression is downregulated (81), suggesting that experiments with CD95L-expressing tumor cells may promote tumor graft rejection by inducing a Th17 differentiation-driven inflammatory environment. The cytokine microenvironment also plays a pivotal role in the CD95 signaling pathway, as highlighted by the fact that addition of TGF-β to the microenvironment of CD95L-expressing tumor cells inhibits their rejection (71). This conclusion remains difficult to reconcile with the fact that TGF-β is a crucial cytokine for Th17 cell development and maintenance (82). Nonetheless, TGF-β1 is also instrumental in Treg differentiation and thereby prevents autoimmunity (83). The current consensus is that TGF-β orchestrates the differentiation of Th17 and Treg subsets by promoting the expression of both Foxp3 and RORγt (84); but, TGF-β treatment leads exclusively to Treg differentiation, because Foxp3 inhibits RORγt transcriptional activation (84). On the other hand, the presence of IL-6 and/or IL-21 (85) abrogates this inhibition and favors the Th17 differentiation (86, 87). In addition, recent data highlight that, in the presence of TGF-β, differentiated Th17 cells can express regulatory cytokines including IL-9 and IL-10 whereas those derived from medium devoid of TGF-β possess a more inflammatory signature and express Th1-associated molecules such as IFN-γ (88). Therefore, TGF-β may favor differentiation of Tregs or "regulatory" Th17 cells and prevent elimination of CD95L-expressing tumor cells.

Some years ago, we were surprised to observe that there was no correlation between the magnitude of the CD95-mediated non-apoptotic signaling pathways and resistance to cell death, thereby ruling out the hypothesis that the CD95-mediated non-apoptotic signaling pathway counteracts the apoptotic pathway (89). Recent data show that the simultaneous induction of apoptotic and non-apoptotic signaling pathways in cells exposed to cytotoxic CD95L is necessary for an efficient immune response because CD95-mediated non-apoptotic signaling pathways in dying cells allow production of cytokines (including MCP-1 and IL-8) that in turn recruit professional phagocytes *via* a cellular inhibitor of apoptosis (cIAP)-1 and a cIAP-2-driven molecular mechanism (80). This original and cooperative interplay between apoptotic and non-apoptotic signaling pathways promotes accumulation of macrophages and phagocytes, which then engulf dying cells. These results not only explain the rapid elimination of apoptotic cells *in vivo* but also may provide a molecular link between cell death and the manner in which Ags (derived from transformed or infected cells) are presented to mount an efficient or impaired immune response.

Of note, s-CD95L can also fuel carcinogenesis by promoting angiogenesis (90, 91), probably through the stimulation of vascular endothelial growth factor (VEGF) secretion by endothelial cells (91). Because in cancer tissues, VEGF, IL-10, and prostaglandin E2 cooperatively stimulate the expression of m-CD95L by endothelial cells (53), we envision that the presence of a metalloprotease, which remains to be identified, can release s-CD95L and thereby triggers a positive feedback loop accelerating carcinogenesis.

TWO CD95L AND MANY CELL SIGNALING PATHWAYS

CD95L is a transmembrane cytokine whose ectodomain can be cleaved close to the plasma membrane by metalloproteases such as MMP3 (92), MMP7 (93), MMP9 (94), and ADAM10 (95, 96); CD95L is then released into the bloodstream as s-CD95L. Metalloprotease-mediated cleavage of CD95L releases a homotrimeric ligand that does not trigger the apoptotic program (35). Indeed, experiments show that hexameric CD95L represents the minimal level of self-association required to signal apoptosis (39). The homotrimeric ligand has long been considered an inert molecule that only competes with its membrane-bound counterpart to antagonize the death signal (34, 35). However, recent work shows that this metalloprotease-cleaved CD95L actively aggravates inflammation and autoimmunity in patients with SLE by inducing non-apoptotic signaling pathways, including the NF-κB and PI3K pathways (36, 97).

It is noteworthy that there are different forms of s-CD95L. Although the soluble form of CD95L generated by MMP7-driven cleavage of its ^{113}ELR^{115} sequence (the CD95L stalk region) induces apoptosis (93), its counterpart (cleaved between serine 126 and leucine 127) does not (35, 36, 97). For instance, s-CD95L in the bronchoalveolar lavage (BAL) fluid of patients suffering from acute respiratory distress syndrome (ARDS) is oxidized at methionines 224 and 225, thereby promoting aggregation of the soluble ligand and boosting its cytotoxic activity (98). The stalk region of CD95L, corresponding to amino acids 103–136, contains all metalloprotease cleavage sites described in the literature. Surprisingly, this region is conserved in s-CD95L detected in ARDS patients, participates in CD95L multimerization, and accounts for ligand-mediated cytotoxicity and damage to the lung epithelium (98). Of note, in ARDS BAL fluid, additional oxidation of s-CD95L occurs at methionine 121, thereby preventing cleavage of CD95L by MMP7 and potentially explaining why this cytotoxic ligand retains its stalk region and contributes to disease pathology (98). Nonetheless, preservation of this region raises the question of whether an as-yet-unidentified MMP7-independent cleavage site exists in the juxtamembrane region of CD95L, or whether the ligand detected in ARDS patients corresponds in fact to the full-length form of CD95L embedded in exosomes (99, 100). Indeed, exosome-bound CD95L can be expressed by human prostate cancer cells (i.e., the LNCaP cell line) and evokes apoptosis in activated T lymphocytes (101). In addition to MMPs, plasmin can also cleave the m-CD95L expressed by neo-vessels in cancers (102). Plasminogen activator inhibitor-1 (PAI-1) inhibits the activity of urokinase-type plasminogen activator, which converts plasminogen into its active form, plasmin. PAI-1 is a good prognostic marker in patients affected by glioblastoma or breast and colon cancers (102). Plasmin cleaves CD95L between the amino acid residues Arg144 and Lys145 within its trimerization domain and releases a soluble ligand, which surprisingly triggers cell death in endothelial cells (102). Because no analysis of the s-CD95L stoichiometry has been realized in this study, it is difficult to decipher whether the soluble plasmin-generated CD95L corresponds to an aggregate of homotrimers or whether

cleavage by plasmin releases a peculiar homotrimeric ligand, which interacts with other components accounting for its unexpected apoptotic effect.

Overall, these findings emphasize the importance of finely characterizing the stoichiometry of naturally processed CD95L in the serum of patients affected by cancers or chronic/acute inflammatory disorders to predict the biological role (i.e., apoptotic versus non-apoptotic) of this soluble ligand. Such investigations will improve our understanding of the molecular mechanisms set in motion by this ligand, and our appreciation of its downstream biological functions.

INTERPLAY BETWEEN CASPASE-8 AND KINASES IN THE CD95 SIGNALING PATHWAYS

As mentioned earlier, CD95 does not possess any enzymatic activity; however, by triggering a large number of PPIs, it activates different signaling pathways resulting in different clinical outcomes. Here, the aim is not to exhaustively describe the initial events of the CD95-mediated signaling pathway, but rather to focus on the role of kinases in these pathways. Unlike m-CD95L, whose interaction with CD95 leads to formation of the apoptotic complex *Death-Inducing Signaling Complex* (DISC), s-CD95L leads to formation of a molecular complex devoid of FADD and caspase-8, which instead recruits and activates the Src kinase c-yes *via* production of NADPH oxidase 3 and ROS (36, 78); this unconventional receptosome is designated the motility-inducing signaling complex (MISC) (36, 37). This process was also observed in apoptotic-resistant glioblastoma cells, in which CD95 stimulation triggers recruitment of a molecular complex that contains the Src tyrosine kinase c-yes (78). This complex promotes micrometastatic dissemination of glioblastoma cells. Similarly, upon injury to the central nervous system, neutrophils and macrophages overexpress CD95L, which promotes migration of these cells *via* activation of the src kinase Syk (76). Of note, while deleting CD95 from neural cells has no effect on pathology progression, deleting CD95L from myeloid cells accelerates spinal cord recovery by reducing the infiltration of neutrophils and macrophages and the magnitude of inflammation. These findings clearly show that the CD95-mediated apoptotic response

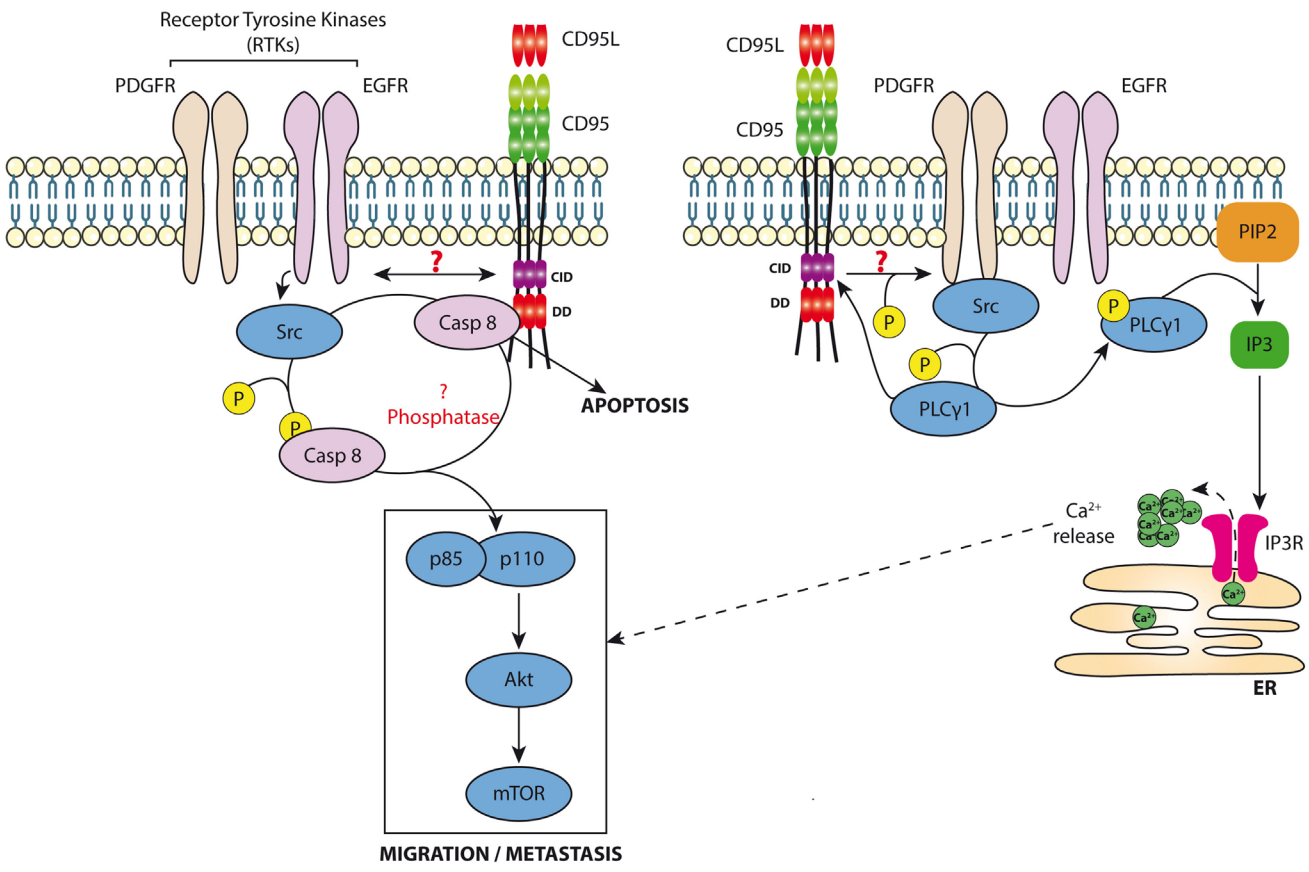

FIGURE 2 | Non-apoptotic signaling pathway induced by CD95. *Left panel:* Cancer cells exposed to s-CD95L induce the recruitment of RTKs (i.e., EGFR or PDGFR) by CD95 leading to the binding and activation of src. In turn, src kinases can phosphorylate caspase-8 neutralizing its enzymatic activity and promoting its role in the activation of the PI3K signaling pathway (i.e., recruitment of p85) and cell migration. *Right panel:* CD95 favors RTK activation leading to Src recruitment. s-CD95L interaction with CD95 induces binding of PLCγ1, which is activated through a src-dependent phosphorylation. Activated PLCγ1 hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP2), releasing inositol triphosphate (IP3), which activates IP3Rs on endoplasmic reticulum promoting calcium release into the cytoplasm that contributes to the CD95-mediated cell migration signaling pathway.

is not responsible for CD95/CD95L function in this pathological context, but that the ligand/receptor pair is required for accumulation of inflammatory cells contributing to the degradation of the organ.

Although our data showed no trace of caspase-8 in the MISC of TNBC cells exposed to s-CD95L (37), we cannot rule out the presence of trivial amounts of this protease in MISC (undetectable by western blotting). Caspase-8 is known to participate in cell migration mainly through an enzymatic-independent mechanism. Src kinases abrogate the protease activity of caspase-8 by phosphorylating it at tyrosine 380 (Y380) (103); this favors recruitment of the PI3K adaptor p85 alpha subunit, thereby activating the PI3K signaling pathway (104) (**Figure 2**). A study revealed that in hepatocytes, hyperosmolarity induces association between a receptor tyrosine kinase (RTK) [called epidermal growth factor receptor (EGFR)] and CD95 through a JNK and PKC-dependent mechanism that remains to be elucidated (105). Strikingly, this study showed that CD95/EGFR increases both DISC formation and the caspase-8-dependent apoptotic response in hepatocytes. More recent data show that EGFR phosphorylates caspase-8 at Y380 through activation of Src kinases, and that this RTK-mediated translational modification prevents triggering of the CD95-mediated apoptotic signaling pathway (103). Several reports confirm that phosphorylation of caspase-8 at Y380 inhibits its protease activity (106, 107). The death effector domains of caspase-8 are both sufficient and necessary for EGFR-dependent cell migration (108). Because caspase-8 phosphorylation at Y380 recruits Src kinases, including Fyn, Lyn, and Src kinases, it is tempting to speculate that this process constitutes a positive feedback loop, which in turn phosphorylates additional caspase-8 molecules (107) (**Figure 2**). We recently observed that exposure of TNBC cells overexpressing EGFR to s-CD95L induces association of CD95 with EGFR, thereby promoting cell migration and metastatic dissemination of these cancer cells (37). RTK-driven Y380 phosphorylation of caspase-8 might prime certain cancer cells to become unresponsive to the apoptotic signal triggered by cytotoxic CD95L, thereby diverting this cell signaling pathway toward promoting cell migration, an essential step in cancer cell metastasis (**Figure 2**). These data are in agreement with those published in a recent study showing that another RTK, PDGFR-β, is recruited by CD95 in colon cancer cells exposed to CD95L to trigger cancer cell metastasis (109). CD95 triggers phosphorylation of PDGFR at tyrosine 1021, thereby promoting recruitment of phospholipase Cγ1 (PLCγ1) (109). Of note, we recently showed that PLCγ1 directly docks onto the calcium-inducing domain of CD95 (10), suggesting that PDGFR-β-dependent recruitment of PLCγ1 might increase the amount of lipase recruited by CD95, and/or sustain its presence at the plasma membrane to increase production of inositol trisphosphate, which activates the Ca^{2+} response and diacylglycerol to stimulate conventional and novel PKCs (**Figure 2**). CD95 induces T cell migration by activating PLCγ1 (36), which necessitates its Src kinase-driven phosphorylation at tyrosine 783 (Y783) (110). Also, the RTK called hepatocyte growth factor receptor or c-Met binds and sequesters CD95 to impair its interaction with CD95L, highlighting another molecular mechanism used by RTKs to prevent apoptosis (111).

These findings show that CD95-mediated recruitment of RTKs, or *vice versa*, may correspond to a physiological process that simultaneously inhibits the apoptotic signal by phosphorylating caspase-8, thereby promoting cell motility by activating the PI3K signaling pathway. However, it is unclear how CD95 interacts with RTKs.

CONCLUSION AND PERSPECTIVES

Although the CD95/CD95L interaction can eliminate malignant cells by promoting formation of the DISC, it can also promote carcinogenesis by maintaining inflammation and/or by inducing metastatic dissemination (36, 76–78, 97, 112, 113). The molecular mechanisms underlying the switch between these different signaling pathways remain enigmatic. An important question to be addressed is how the magnitude of CD95 aggregation regulates formation of "Death"- versus "Motility"-ISCs. Answering these questions will lead to development of new therapeutic agents with the ability to prevent the spread and chronicity of inflammation, or to impinge on carcinogenesis, at least with respect to pathologies associated with increased s-CD95L, such as cancers [e.g., pancreatic cancer (114), large granular lymphocytic leukemia, breast cancer (37), and NK cell lymphoma (115)] and autoimmune disorders [e.g., rheumatoid arthritis and osteoarthritis (116), and graft-versus-host-disease (117, 118), or SLE (36, 119)].

This review highlights that CD95L is frequently detected overexpressed at the surface of endothelial cells covering blood vessels in tissues of patients affected by inflammatory disorders or cancers. Although its role remains to be elucidated, growing evidence suggest that this ectopic expression can be a biological marker whose combination with a "lethal" metalloprotease might turn out to enhance the severity of autoimmune disorders and cancers. An extensive review of the CD95/CD95L role in the function of endothelial cells indicates that besides its role in the immune system, CD95 can directly regulate angiogenesis either by implementing cell death when encountering its transmembrane ligand or by promoting survival and migration of these endothelial cells when exposed to s-CD95L. Finally, CD95 can also stimulate the production of nitric oxide and thereby control blood pressure (120) revealing its complex biological role in the context of the endothelium survival, proliferation, and function.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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