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REVIEW

The effects of sepsis on endothelium and clinical implications

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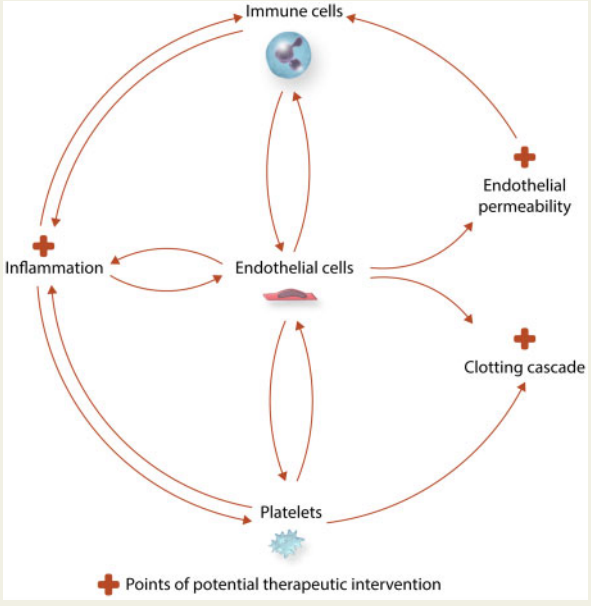
Abstract

Sepsis accounts for nearly 700 000 deaths in Europe annually and is caused by an overwhelming host response to infection resulting in organ failure. The endothelium is an active contributor to sepsis and as such represents a major target for therapy. During sepsis, endothelial cells amplify the immune response and activate the coagulation system. They are both a target and source of inflammation and serve as a link between local and systemic immune responses. In response to cytokines produced by immune cells, the endothelium expresses adhesion molecules and produces vasoactive compounds, inflammatory cytokines, and chemoattractants, thus switching from an anticoagulant to procoagulant state. These responses contribute to local control of infection, but systemic activation can lead to microvascular thrombosis, capillary permeability, hypotension, tissue hypoxia, and ultimately tissue damage. This review focuses on the role of the endothelium in leucocyte adhesion and transmigration as well as production of reactive oxygen and nitrogen species, microRNAs and cytokines, formation of signalling microparticles, and disseminated intravascular coagulation. We also discuss alterations in endothelial permeability and apoptosis. Finally, we review the diagnostic potential of endothelial markers and endothelial pathways as therapeutic targets for this devastating disease.

Graphical Abstract



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1. Introduction

Sepsis is a life-threatening organ dysfunction caused by a dysregulated host response to infection[1] associated with significant morbidity and mortality. According to the latest reports, each year at least 1.7 million adults in the USA develop sepsis and nearly 265 000 of them die. In Europe, the incidence of sepsis is estimated to be more than 3.4 million cases per year; 700 000 of these patients do not survive the hospitalization and one-third of survivors die during the first year after hospitalization.[2] Due to the lack of a quick and specific diagnostic tool and fairly non-specific clinical presentation, the definition of sepsis has undergone few modifications in recent years.[1] Clinical features of sepsis reflect the body's response to infection and include fever, tachycardia, hypotension, and leucocytosis as well as end organ dysfunction, such as acute lung injury, acute kidney injury, encephalopathy, and cardiomyopathy.[3] As sepsis is a systemic condition, it affects virtually all organs and tissues, with endothelium being one of the first cell types to encounter and respond to the insult. During sepsis, the two most pronounced roles of endothelial cells (ECs) are to amplify the immune response and to activate the coagulation system. Endothelial activation and/or dysfunction ultimately contribute to end organ damage during sepsis. Furthermore, the endothelium provides a link between local and systemic immune responses, as it is simultaneously a target and a source of inflammation.[4] Upon stimulation, ECs express adhesion molecules and produce vasoactive compounds, inflammatory cytokines, and chemoattractants, thus switching from an anticoagulant to procoagulant state.[5] While endothelial activation locally aids in fighting the source of infection, systemic activation may result in microvascular thrombosis, capillary permeability, hypotension, tissue hypoxia, and ultimately tissue damage.

2. Endothelium and the pathogen—initial encounter

When there is a breach allowing a pathogen to enter the blood stream, generalized inflammation from exposure to bacterial components and tissue breakdown products occurs. While immune cells ensure an adequate response to the insult, endothelium is also activated and is thought to direct and modulate the inflammatory response.[4] During severe inflammation, such as seen in sepsis, the activation of an inflammatory cascade by the pathogen (*Figure 1*) can lead to auto-amplifying cytokine production, the cytokine storm. Cytokines are a broad category of relatively small proteins (<40 kDa) (interleukins, chemokines, interferons, tumour necrosis factor, and growth factors[6]) produced and released predominantly by immune cells.[7] Infection leads to the activation of the cytokine network, which is comprised of pro-inflammatory cytokines and anti-inflammatory cytokines. The balance between these counter-regulatory pathways eventually determines the net inflammatory activity of the cytokine network.

NF-κB plays a crucial role in the cell (both inflammatory cells and ECs) response to cytokines or bacterial cell wall components [i.e. lipopolysaccharide (LPS)] (*Figure 2*).[8] LPS forms a complex with LPS-binding protein, MD-2, toll-like receptor-4 (TLR-4), and CD14, further initiating intracellular signalling.[9] The downstream pathways can be crudely divided in two competitive pathways: TLR4/TRIF/IRF3 and TLR4/MyD88/NF-κB. The TLR4/TRIF/IRF3 pathway involves activation of TRIF, internalization of the TLR4/TRIF complex within endosomes with subsequent activation of interferon regulatory transcription factor-3 (IRF3) and interferon production. At the same time, activation of the TLR4/MyD88/NF-κB pathway leads to phosphorylation of MyD88 and interleukin-1 receptor-associated kinases 1 and 4 (IRAK1 and IRAK4). IRAKs in turn phosphorylate TNF receptor-associated factor 6 (TRAF6), which promotes degradation of IκB and nuclear translocation of NF-κB. TRAF6 is also thought to activate mitogen-activated protein kinases (MAPKs), ultimately resulting in activation of activator protein-1 (AP-1).[9] Inflammatory cytokines, such as TNF-α, can activate similar pathways resulting in nuclear translocation of NF-κB, further increasing cytokine production.[10] Although immune cells are responsible for most cytokine production during sepsis, ECs are not only the target of cytokines, but are also, via similar pathways,[11] able to secrete such cytokines as IL-1β, IL-6 and interferon. While the exact role of endothelial-derived cytokines is unclear, current thought is that ECs contribute to ramping up and modulating the inflammatory cascade and play an important role in activation and fine tuning of the local immune response.[12]

With growing evidence on the important role of the endothelium in sepsis development, much attention has been devoted to its potential as a target for therapeutic intervention. Initially, multiple studies focused on quenching the bacterial components, thus preventing their deleterious effect on the endothelium. Due to its well-described toxic effects, quenching LPS from the membrane of the Gram-negative bacteria with various lipophilic and amphiphilic cations as well as zwitterions has been successfully explored in animal models of LPS-induced endotoxaemia and *Escherichia coli* sepsis.[13,14] However, due to the fact that most of these compounds are cationic amphipaths and potentially can exhibit membrane-perturbing activity, the implementation of most of these quenchers in clinical practice is limited.[13] Among drugs with known safety profile that are already being used in clinical practice, colistin showed promising anti-endotoxin effects *in vitro*[15] as well as in a randomized clinical trial investigating its anti-inflammatory effect in LPS-induced endotoxaemia in healthy volunteers.[16]

As noted above, LPS exerts its effect on endothelium via binding to TLR4; thus, intervening in LPS binding to TLR4 as well as targeting the downstream signalling pathways is another potential treatment for Gram-negative sepsis.[17] While designed to be protective by its nature, in cases when the stimulus becomes overwhelming, the TLR4 pathway can contribute to the pathologic downward spiral of sepsis. Therefore, several approaches were suggested for intervening in this pathway. First, a number of TLR4-antagonists were tested. For instance, Eritoran, a structural analogue of lipid A that does not exhibit TLR4-agonistic effects, competitively binds to TLR4-MD2 and prevents LPS from initiating an inflammatory response (i.e. it was shown to block NF-κB activation and TNF-α and IL-6 production in animal and human models of endotoxaemia).[18] Another direct TLR4 antagonist, TAK-242, binds directly to Cys747 in the intracellular TLR4 domain and disrupts TLR4 interactions with TIRAP and TRAM.[19] While showing promising results in animal models of sepsis,[20] it failed, however, to be effective in clinical trials.[21] A number of existing agents were shown to alter the levels of TLR4 expression (i.e. chloroquine, ketamine, nicotine, opioids, statins, vitamin D3, lidocaine, glycine, and proton pump inhibitors), with some of them showing auspicious results in animal studies. Randomized controlled

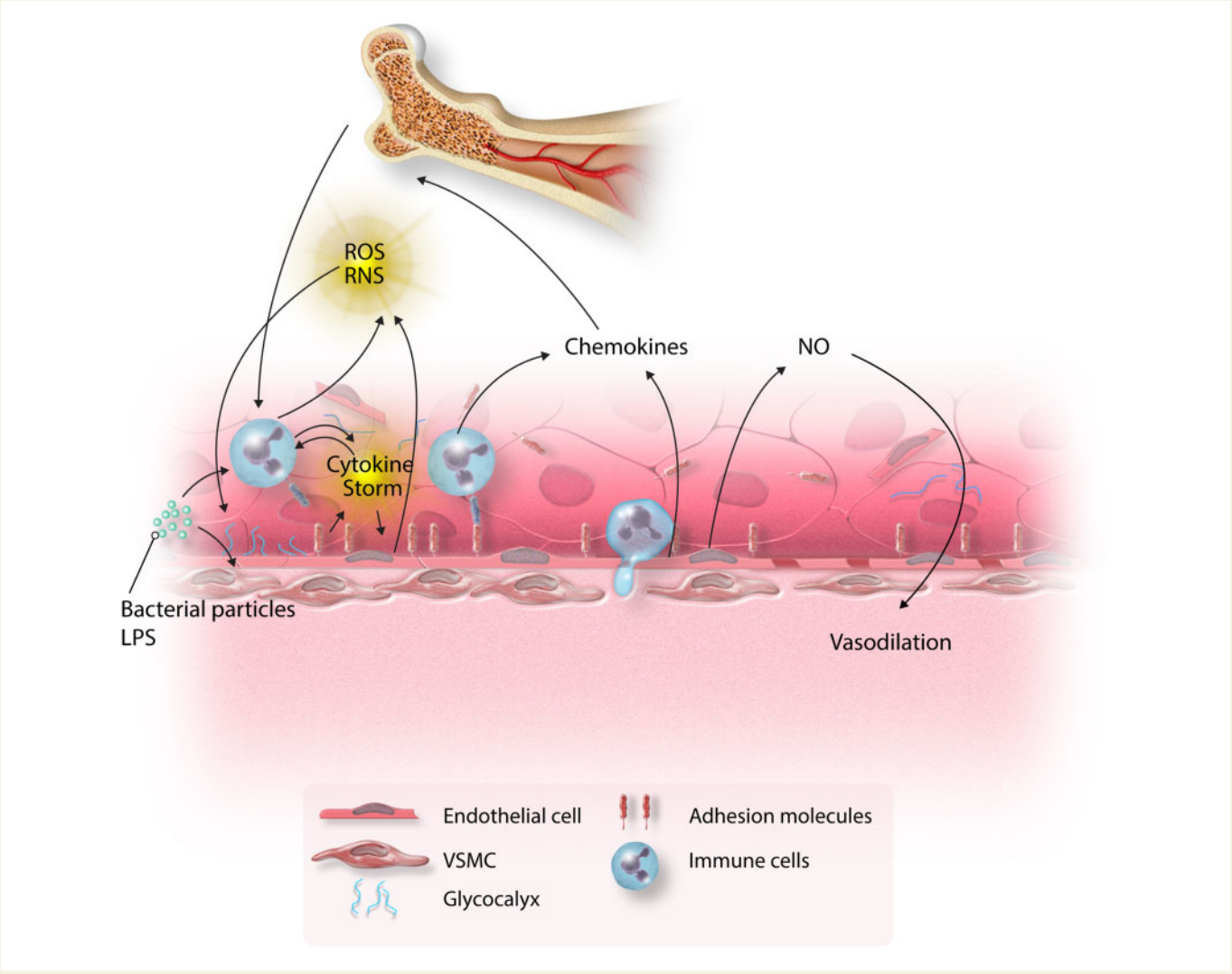

Figure 1 Inflammatory cascade in sepsis. Bacterial components activate both immune cells and endothelium inducing cytokine production, which is self-perpetuating. Endothelial cells become activated and express adhesion molecules, to which immune cells bind. This initiates the process of transmigration of immune cells to the site of injury. ROS secreted by immune cells and endothelium further augment the inflammatory response. The combination of these insults leads to shedding of glycocalyx, induction of adhesion molecules, increased endothelial permeability, and endothelial apoptosis. Chemokines secreted by immune cells and endothelium recruit immune cells from the bone marrow. The shift in the eNOS/iNOS balance results in excess NO synthesis and vasodilation.

trials are needed to evaluate the efficacy of these compounds in clinical practice.[22] Finally, an immune cell-derived protein that is highly elevated in sepsis, resistin, was found also to prevent LPS binding to the TLR4 receptor and was suggested as a potential therapeutic modality, but further studies are needed to support this possibility.[23]

Apart from blocking the TLR4 receptor itself, attempts were made to intervene with various downstream targets. For example, ARF6, a small ATPase activated by the MyD88–ARNO interaction, was shown to induce vascular leak in septicaemia[24] and blocking ARF6 with a peptide reversed this effect, improving survival in endotoxic shock.[25] Another study attempted to assess vascular permeability in response to LPS after inhibition of either MyD88 or TRIF and demonstrated that blocking TRIF, but not MyD88, had therapeutic potential.[26] A number of compounds used in traditional Chinese medicine (such as Houttuynia, tanshinones, Emodin, Ugonin M, LianQinJieDu, *Astragalus membranaceus*, and *Salvia miltiorrhiza*) were shown to intervene at various stages of TLR4 pathway and therefore were suggested as potential adjunct therapies for the treatment of Gram-negative sepsis; however, randomized clinical trials are needed to assess their efficacy in clinical setting.[17,27,28]

3. Endothelium and leucocyte adhesion and transmigration

In response to inflammatory cytokines (TNF-α and IL-1β in particular), the expression of adhesion molecules [selectins, integrins, and members of an immunoglobulin superfamily known as intercellular adhesion molecules (ICAMs) and vascular cell adhesion molecule-1

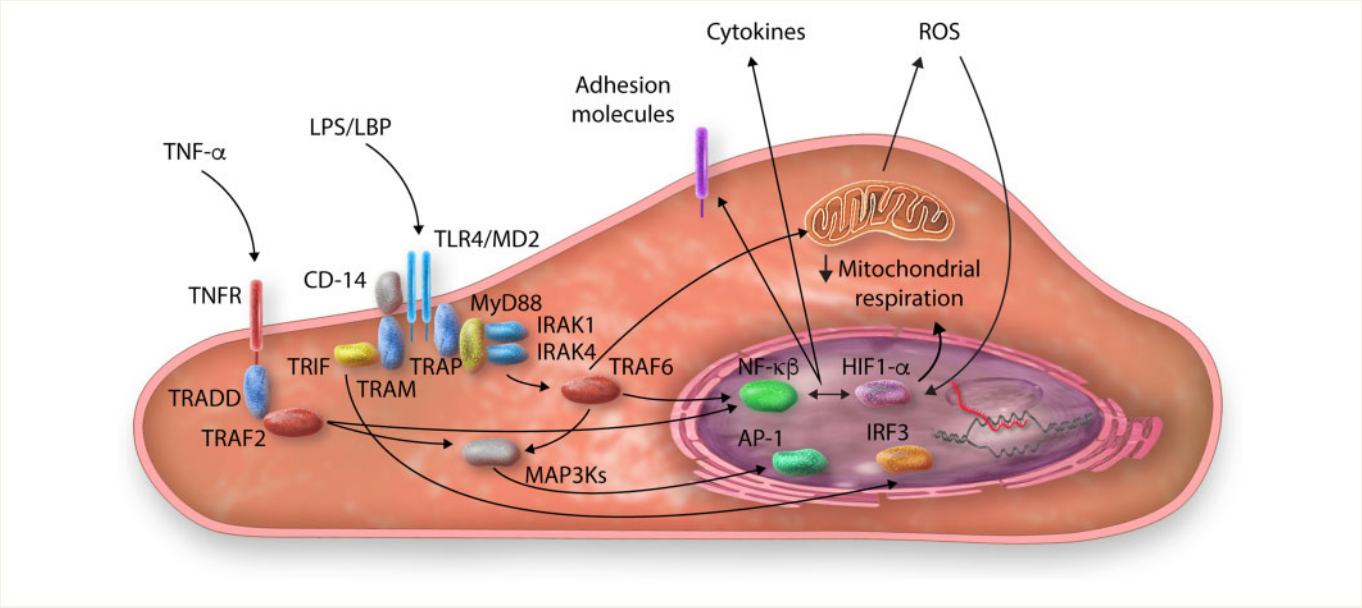

Figure 2 Examples of inflammatory pathways within endothelial cells during sepsis (TLR4 and TNF-α). LPS and cytokines trigger intracellular pathways that ultimately lead to the activation of number of transcription factors (NF-κB, AP-1, and IRF3), resulting in increased cytokine production and adhesion molecule expression. These pathways also likely contribute to mitochondrial dysfunction and ROS production (see text for details).

(VCAM-1)] on the surface of ECs increases dramatically (*Figure 1*).[29] In the initial phase, the Sialyl-Lewis carbohydrate ligands found on leucocytes loosely attach to E-selectin and P-selectin, which allows them to roll on the endothelium. In the second phase, the rolling leucocytes are activated by chemokines locally released by macrophages and ECs to express integrins on their surface, which permits firmer adhesion to ICAM-1 and VCAM-1 and initiates their transendothelial migration into the injured tissues.[30] Chemokines that are bound to the endothelium via heparan sulphates form a chemotactic gradient directing further migration of the leucocytes.[31] Moreover, the gradient of chemokines (such as CXCL8, CXCL1, CXCL2, and CXCL5) produced by the immune cells and ECs promotes recruitment of the neutrophils from bone marrow reserves and enhances neutrophil adhesion.[31] P-selectin on ECs not only captures leucocytes and promotes their rolling but also activates integrins through P-selectin glycoprotein ligand-1 (PSGL-1) and induces further leucocyte activation.[32] Other molecules that have been shown to be necessary for transmigration of leucocytes across the endothelial lining are platelet EC adhesion molecule-1 (PECAM-1) and CD99,[33,34] with blockade of PECAM-1 function severely impairing transendothelial migration.[33]

Meanwhile, as inflammatory responses progress, soluble isoforms of the leucocyte recruitment adhesion molecules are shed from cell surfaces and accumulate within the circulating blood plasma.[35] Although still a matter of controversy, increasing evidence suggests that shedding serves regulatory roles to dampen inflammation (and specifically to reduce leucocyte–endothelial interactions) and protect the host from excessive collateral damage.[35]

Measurement of these soluble adhesion molecules has also been proposed as a diagnostic tool for early detection of sepsis as well as severity and outcome prediction. Serum concentrations of adhesion molecules such as E-selectin, P-selectin, ICAM-1, and VCAM-1 increase during sepsis[36] and correlate with sepsis severity (as assessed by the Simplified Acute Physiology Score and the number of organ failures) and mortality.[37] E-selectin levels rise significantly with the onset of sepsis-related organ dysfunction and decrease after organ dysfunction has resolved.[38] In particular, E-selectin was elevated in patients with positive blood cultures indicating that bacteraemia resulted in pronounced endothelial damage and greater shedding of E-selectin.[39] When compared to such classical markers of bacterial infection as procalcitonin, measurement of either circulating E- or P-selectin levels had higher sensitivity and specificity for predicting future sepsis development in ICU patients.[40] In contrast, while soluble ICAM-1 levels were significantly elevated in patients with sepsis, they also rose (although to a lesser degree) in patients with systemic inflammation without sepsis.[41] Nevertheless, soluble ICAM-1 levels directly correlated with severity of sepsis and organ dysfunction.[41] Moreover, in patients with multiple organ dysfunction who did not have sepsis, elevation of soluble ICAM-1 levels was much less pronounced.[42] While quite sensitive to organ dysfunction and severity of sepsis, a moderate rise in levels of circulating adhesion molecules is also seen in other conditions that are associated with vascular damage (such as trauma or cardiopulmonary bypass), so their use as a biomarker must be interpreted with care.[43,44]

While adhesion molecule expression is crucial for recruiting immune cells to battle the infection, targeting adhesion molecules in treatment of sepsis remains controversial. Interestingly, one study demonstrated that in a mouse model of polymicrobial sepsis, the use of antibodies against ICAM resulted in an increased recruitment of neutrophils to the site of infection with a concurrent decrease of infiltration in other organs (such as lung, thymus, and spleen).[45] Other studies explored the idea of fusing compounds with antibodies against adhesion molecules for endothelium-specific delivery of certain drugs.[46,47] Finally, one of the proposed mechanisms of the beneficial effect of inotropes in sepsis is their ability to decrease adhesion molecule expression on the endothelium,[48] resulting in reduced transendothelial neutrophil migration.[49]

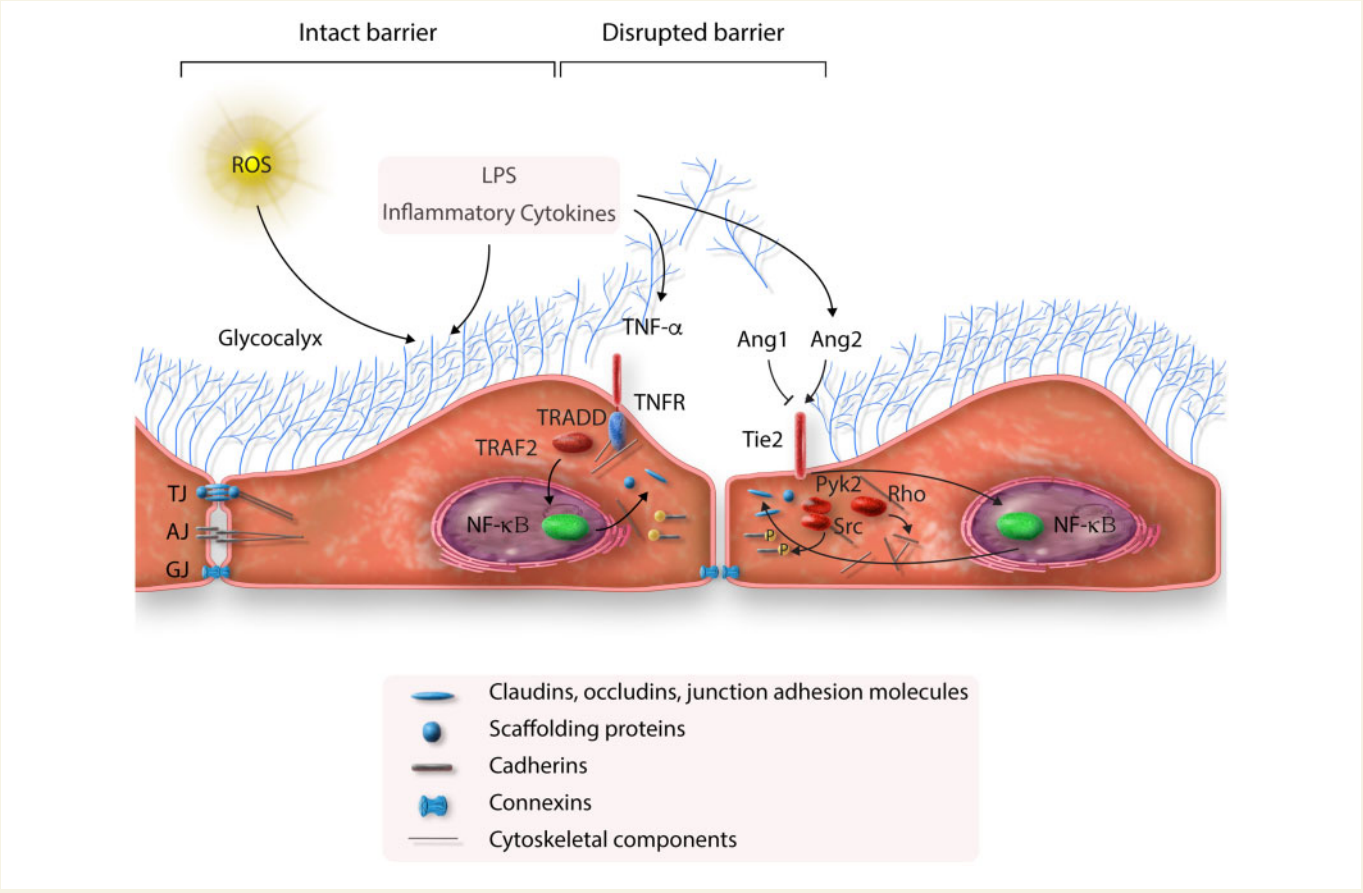

Figure 3 Endothelial permeability in sepsis. ROS and bacterial components (i.e. LPS) damage the glycocalyx. LPS and inflammatory cytokines result in disruption of tight junctions (TJ), adherence junctions (AJ), and gap junctions (GJ) via activation of TNF-α and Ang2 pathways. The above effects increase endothelial permeability (see text for details).

Overall, despite the fact that the role of endothelial adhesion molecules in sepsis is undeniable, surprisingly few studies have focused on targeting them in treatment of sepsis.

4. Endothelial permeability in sepsis

A highly selective endothelial barrier is essential to maintain tissue fluid homeostasis and to support normal organ function.[50] A main feature of the endothelium in sepsis is an increased permeability or loss of barrier function, resulting in a shift of circulating elements and tissue oedema (*Figure 3*).[51]

Vascular endothelial (VE)-cadherin is the major component of endothelial adherens junctions—tightly regulated protein complexes that join adjacent ECs and prevent leucocyte migration and vascular leak. Inflammatory mediators suppress cAMP/Rac1 signalling, promote Rho activity and activate kinases such as Src and Pyk2,[52] which results in VE-cadherin phosphorylation, dissociation from p120 catenin and induces its endocytosis.[53] Endocytosis of VE-cadherin by itself is sufficient to induce gaps between ECs, resulting in increased permeability.[54] Similarly to adherens junctions, several studies reported disruption of endothelial tight junctions in sepsis as well with reduction of protein levels of occludin and zonula occludens-1.[55,56] TNF-α, one of the main cytokines in sepsis as noted above, was shown to cause disruption of claudin-5 at cell–cell junctions of ECs through activation of the NF-κB pathway.[57] Another important player in maintenance of the endothelial barrier is gap junctions, formed by connexins, which allow cell–cell flux of small molecules and solutes.[58] Inhibition of connexins prevents the increase in endothelial permeability observed in an experimental model of lung injury.[58]

Several other mechanisms involved in maintenance of the endothelial barrier have been suggested. For example, we showed that decreased expression of DNA polymerase-δ interacting protein 2 (Poldip2) maintains the endothelial barrier in sepsis,[59,60] although more work needs to be done to fully define the mechanism. One of the most commonly implicated pathways in vascular permeability is the Ang/Tie2 pathway. Angiopoetin2 (Ang2) is highly up-regulated in the serum of patients with sepsis, correlating with disease severity.[61] Current evidence suggests a crucial role of Ang2 in vascular permeability in sepsis. Ang2 expression by ECs is increased upon stimulation by inflammatory cytokines, resulting in disruption of Ang1–Ang2 equilibrium and increasing vascular permeability.[62] Endothelial-specific Ang2 overexpression in mice leads to the development of haemodynamic changes resembling sepsis.[63] These effects can be abrogated by shifting the Ang1/Ang2 balance towards a normal ratio by injecting mice with *Angpt1*- or *Pdgfb*-encoding

adeno-associated virus. Moreover, Ang-2 neutralizing antibody decreases LPS-induced mortality,[63] and mice with heterozygous deletion of Ang2 have reduced VCAM-1 expression, inflammatory cell infiltration, and improved survival in sepsis.[61]

Both Ang1 and Ang2 are known to bind Tie2, a transmembrane endothelial tyrosine kinase receptor.[64] Ang1 acts as an agonist, phosphorylating Tie2, and, through Akt activation, inhibiting Foxo1,[65] ultimately promoting EC survival and migration and inhibiting vascular leakage.[66] Ang2, on the other hand, acts as a Tie2 antagonist by blocking the stabilizing action of Ang1.[64] Ang2 exposure results in a formation of a complex between Tie2, αvβ3 integrin, and focal adhesion kinase, leading to focal adhesion kinase phosphorylation, αvβ3 integrin internalization[67] and ultimately EC barrier destabilization. Ang2 was also shown to induce VE-cadherin phosphorylation, causing disruption of adherence junctions, although the exact molecular pathway remains unclear.[68] Activation of Tie2 with a specific ligand or introducing a stable Ang1 variant improves survival in experimental models of sepsis.[69,70] To date most work focused on the antagonistic effects of Ang2 on the Tie2 receptor; recent studies, however, have shown that Ang2 also binds β1 integrin thus destabilizing endothelium even further.[71] Moreover, targeting β1 integrin either by heterozygous deletion or inhibitory antibodies exhibited a protective effect on endotoxaemia-induced VE-cadherin redistribution and endothelial permeability.[72] These studies, taken together, indicate an important role of the Ang1/Ang2 balance in vascular permeability in sepsis and point towards potential therapeutics to modulate this equilibrium, preventing the downward spiral that is characteristic of sepsis.

Evaluating Ang1/Ang2 equilibrium by measuring levels of soluble Ang2 in the serum of patients with sepsis was investigated as a predictor of outcomes. A satellite study of the ProCESS trial demonstrated significantly higher levels of serum Ang-2 in patients who did not survive sepsis.[73] Moreover, another study investigated the predictive value of circulating levels of Ang-2 together with a few other factors (VEGF, TM, vWF) for acute respiratory distress syndrome (ARDS) development in sepsis, which further supported an important role of vascular permeability in general, and Ang2 in particular, in sepsis-induced lung injury.[74] Finally, higher levels of soluble Ang-2 were associated with higher likelihood of disseminated intravascular coagulation (DIC), one of the most morbid complications of sepsis.[75]

Despite the undeniable importance of Ang1/Ang2 equilibrium, only a handful of studies is available focusing on altering this balance in treatment of sepsis. Various compounds stimulating Tie2 (thus mimicking the effect of Ang1) were reported to improve mortality in murine models of sepsis.[69,76] However, the short half-life and high rate of non-specific binding of these compounds limits their use in systemic disease like sepsis in larger organisms.[77] On the other hand, antibodies and small-interfering RNAs against Ang2 were also shown to delay septic progression in experimental sepsis models; their efficacy, however, was variable.[78,79] Finally, a special Tie2-agonistic antibody that also oligomerized Ang2 was able to enhance the effects of Tie2 stimulation by concomitant Ang2 inhibition *in vitro* and in sepsis mouse model.[80] While promising, these results also point towards the need for further studies focusing on possible therapeutics altering Ang1/Ang2 equilibrium in sepsis.

5. Glycocalyx in sepsis

The endothelial glycocalyx has been recognized as a critical regulator of barrier integrity in the endothelium. In a unique position between the blood and vessel wall, the endothelial glycocalyx consists of a membrane-bound negatively charged network of proteoglycans, glycoproteins, glycolipids, glycosaminoglycans (i.e. heparan sulphate), and adherent plasma proteins.[81] It actively regulates barrier function via mechanotransduction, and its alteration may lead to augmentation in endothelial hydraulic conductivity and subsequent formation of pulmonary oedema.[82]

Inflammation in general and sepsis in particular result in changes in the glycocalyx, leading to endothelial damage and microvascular dysfunction.[83] Thus, the glycocalyx is an intriguing candidate for assessment of the degree of endothelial damage in sepsis. However, studies focusing on the components of glycocalyx in sepsis remain limited. Syndecan-1, a transmembrane (type I) heparan sulphate proteoglycan, for example was shown to increase in conditions resulting in endothelial damage, including sepsis.[84] Plasma levels of heparan sulphate were significantly higher in patients with septic shock[85] with markedly higher levels in non-survivors.[86] However, heparan sulphate levels were also significantly elevated in patients who underwent neurosurgery and did not have sepsis.[86] One of the most promising glycocalyx markers in sepsis is endocan (a soluble dermatan sulphate proteoglycan), which was shown to increase after LPS administration in healthy volunteers,[87] and in patients with sepsis was predictive of later development of sepsis-induced lung injury and mortality.[88]

Shedding of the glycocalyx exposes ECs for leucocyte and platelet adhesion, augmenting inflammation and activating the clotting cascade.[89] Several studies focused on maintaining the integrity of glycocalyx during sepsis. The effect of fluid resuscitation, a mainstay therapy in septic shock, on glycocalyx has been studied quite extensively. It was suggested that hypervolemia can be associated with increased glycocalyx degradation in sepsis;[90] however, other groups observed no difference in circulating glycocalyx components between patients receiving low or large volumes of fluid resuscitation.[91] Based on lower circulating components of glycocalyx in animal studies and a few clinical studies, albumin and fresh frozen plasma were offered as glycocalyx-protective options for intravenous solutions.[92,93] Whether or not the observed effect can translate into improved mortality in sepsis is yet to be determined,[94] with crystalloids (such as normal saline or Ringer's lactate solution) currently remaining a preferred means for the resuscitation in septic shock. Such widely used drugs as dexamethasone and doxycycline can exhibit their beneficial effect through suppression of metalloproteinases and decreased shedding of components of the glycocalyx.[95] Heparin is also postulated to protect the glycocalyx via inhibition of heparanase, thus decreasing activity of metalloproteinases.[96] Finally, inhibition of Ang2 has also been suggested to maintain glycocalyx integrity.[80] While it is accepted that disruption of the glycocalyx adds to the organism-wide insult in sepsis, it remains unclear if maintaining or restoring the glycocalyx by itself would have mortality benefit. The issue is complicated by the interconnection of the cascades involved, and the challenge of designing a glycocalyx-specific intervention.

6. Endothelium and the clotting cascade in sepsis

Disseminated intravascular coagulation is one of the gravest complications of sepsis and is associated with extremely high mortality.[97] While the exact mechanism and the order of triggering events remains

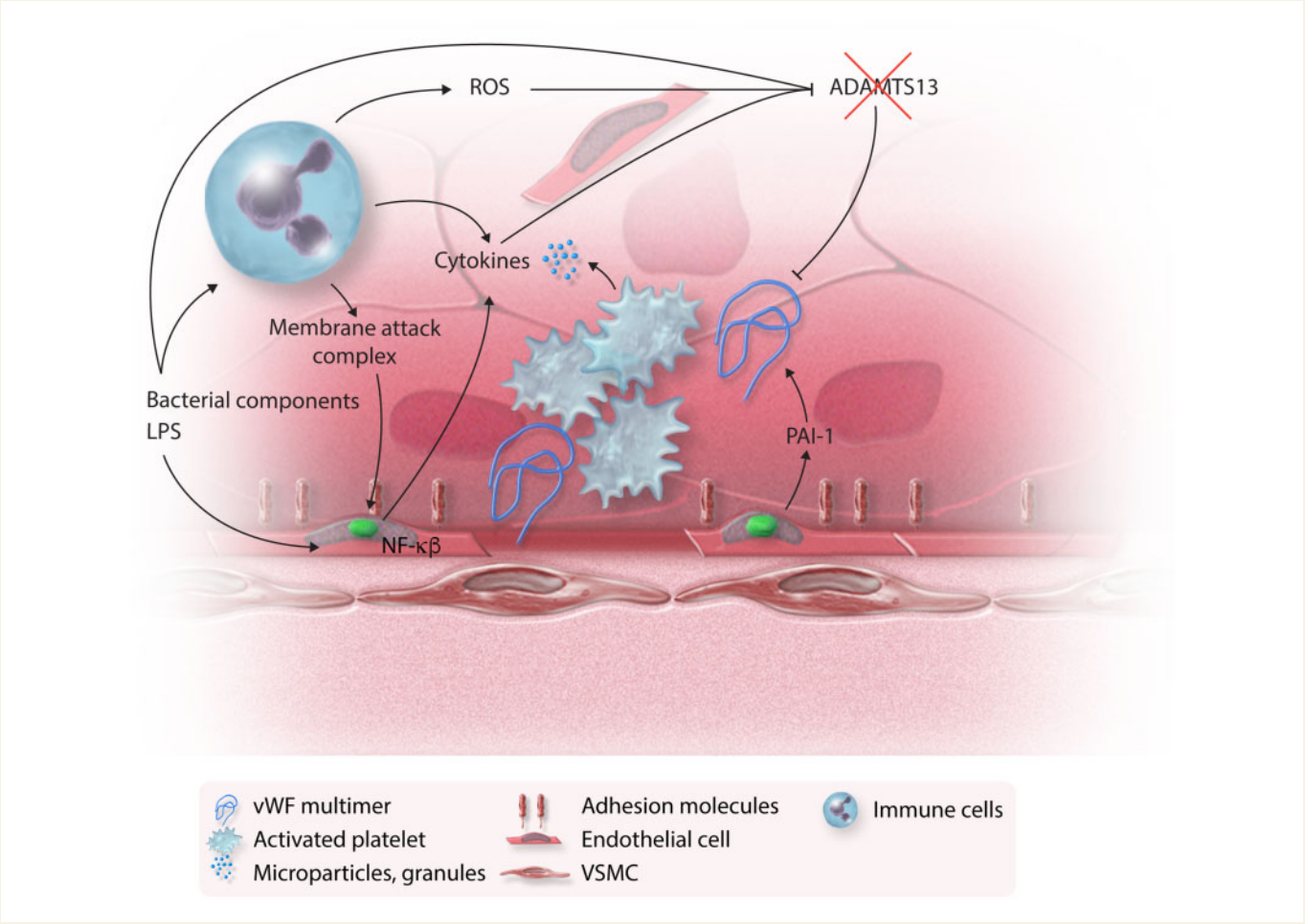

Figure 4 Endothelium and coagulation cascade in sepsis. When endothelial integrity is compromised by membrane attack complexes and endothelial apoptosis, a prothrombotic state ensues: endothelial cells express adhesion molecules and platelets become activated. Activated platelets release a number of active compounds contributing to the inflammatory cascade. Bacterial components, ROS, and cytokines lead to acquired ADAMTS13 deficiency and accumulation of vWF multimers that are secreted from the activated endothelium. Activated endothelium also secretes PAI-1, further contributing to a prothrombotic state (see text for details).

unknown, the endothelium is thought to play a major role in this process (*Figure 4*). One of the suggested mechanisms is disruption of the EC membrane by membrane attack complex,[98] which further augments inflammation and activates the microthrombotic pathway. Activation of the microthrombotic pathway mediates platelet activation and exocytosis of unusually large von Willebrand factor multimers from ECs and initiates microthrombogenesis.[98] Under normal conditions, these von Willebrand factor multimers are rapidly cleaved to less active forms by a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13 (ADAMTS-13). During overwhelming inflammation in sepsis, however, inflammatory mediators such as IL-6, plasma-free haemoglobin, VWF proteolytic fragments, Shiga toxin, and neutralizing autoantibodies can inactivate ADAMTS-13.[99,100] ADAMTS-13 can also be inhibited by plasmin, thrombin, products of activated coagulation, granulocyte elastase released by activated neutrophils as well as neutrophil-derived reactive oxygen species (ROS). Together, these events lead to an acquired ADAMTS-13 deficiency and increased risk of DIC. Finally, in sepsis the endothelium releases increased amounts of plasminogen activator inhibitor-1 (PAI-1), thus suppressing the fibrinolytic pathway. All of this creates an imbalance between prothrombotic and antifibrinolytic pathways, leading to the dissemination of fibrin-rich microvascular thrombi as observed in DIC.[101] This results in organism-wide microvascular thrombus formation, depletion of the clotting factors, and bleeding as the most classical presentation of DIC.

While ECs undoubtedly orchestrate the clotting cascade and platelet activation and aggregation, platelets themselves can secrete a number of cytokines upon activation.[102] Most of these cytokines stimulate immune cells and promote immune-cell adhesion to the endothelium.[102] A few of them, however, have very distinct effects on endothelium itself. CD40L, for example interacts with CD40 on ECs and promotes chemokine secretion and expression of adhesion molecules.[103] Activated platelets also release IL-1(α and/or β) and induce CCL2 (a chemokine) secretion and ICAM-1 expression in ECs.[104]

Apart from classic tests, such as D-dimer and fibrinogen for establishing a diagnosis of DIC, other players of the clotting cascade have been

also suggested for prediction of sepsis severity, DIC development, and mortality. For example, in the aforementioned satellite study of the ProCESS trial, the levels of haemostasis factors, such as VEGF, thrombomodulin (TM), and tissue plasminogen activator, in sepsis were evaluated and circulating levels of all three factors were significantly higher in non-survivors.[73] Higher PAI-1 levels were also associated with increased mortality and higher rates of DIC and end organ failure.[105]

Due to the enormous mortality associated with DIC,[97] attempts were made to modulate coagulation in order to treat sepsis and prevent sepsis-induced DIC. The possibility of heparin inducing further bleeding in severe sepsis as well as negative results from a few animal studies[106] limited enthusiasm for its use in sepsis. Low-dose heparin, however, showed promising results in a few randomized clinical trials as well as after meta-analysis, with reduced 28-day mortality in severe sepsis and no increase in bleeding rates.[107] Inhibition of thrombin (by thrombin inhibitors, decrease in thrombin generation, binding thrombin with AT III, or thrombin degradation by protein C or TM) has shown encouraging results in animal studies, indicating a possible effect on both coagulation and inflammatory pathways within the endothelium.[108,109] Follow-up clinical studies, however, showed variable results. Further studies are therefore needed to fully evaluate their role in the treatment of sepsis. Tissue factor pathway inhibitor (TFPI) suppresses the primary steps of thrombin generation and exhibits its anti-inflammatory effect through suppression of thrombin binding to protease-activated receptor-1.[110] Use of recombinant TFPI was suggested for treatment of sepsis; however, a few large randomized clinical trials failed to show any improvement in mortality.[111,112] Another thrombin antagonist with anti-inflammatory properties is ATIII, which apart from directly inhibiting a number of serine proteases and binding thrombin, can also induce release of prostacyclin, suppress P-selectin, and decrease the expression of inflammatory cytokines.[109,113,114] The results of clinical trials with ATIII were overall positive, with most reporting a decrease in 28-day mortality. The effect, however, varied with different doses, concomitant heparin administration, and age of the patient.[115,116]

Activated protein C inactivates factors Va and VIIIa, thus limiting thrombin generation, and is involved in lysis of the thrombin–TM complex. It also has been shown to have anti-inflammatory qualities by suppressing pro-inflammatory cytokines in activated leucocytes, exhibiting antioxidant properties, displaying antiapoptotic activity, and stabilizing the endothelial barrier.[117] The results of the randomized clinical trials with recombinant activated protein C, however, were inconsistent, with some studies showing no improvement in mortality.[118,119] While the consensus regarding its efficacy has not been reached, the production of recombinant activated protein C was nevertheless stopped.

Thrombomodulin binds to thrombin and activated protein C. It also has some anti-inflammatory effect by interfering with complement activation and inhibition of leucocyte–endothelial interaction.[120] Initial clinical studies of recombinant TM showed improved outcomes in patients with DIC and larger studies are currently underway.[121,122] Thus, intervening at various stages of thrombin synthesis and degradation harbours promising potential in treatment of sepsis and sepsis-induced DIC. One has to realize, however, that while the results of many of the clinical trials investigating thrombin inhibitors in sepsis are optimistic, differences in inclusion criteria and reported bleeding events should be noted, which might account for some of the inconsistencies in results.

7. Endothelium and ROS in sepsis

During sepsis, activated immune cells release reactive molecules that are designed to fight pathogens, but on the other hand can cause tissue damage.[123] ROS can directly attack ECs, resulting in increased vascular permeability, worsening hypotension, and decreased colloid osmotic pressure of the plasma. Furthermore, they alter oxygen consumption by the tissues, accelerating organ failure.[124] Published studies provide substantial evidence that ROS can damage the EC glycocalyx[125] and have profound negative effects on endothelial barrier function,[126] thereby promoting neutrophil recruitment and trafficking. Both *in vitro* and *in vivo* exposure of ECs to ROS results in EC cytoskeletal remodelling and an up-regulated expression of ICAM-1, PECAM-1, VCAM-1, and P-selectin and a subsequent increase of neutrophil adhesion to ECs.[127] Alterations in calcium homeostasis and activation of protein kinase C, p38MAPK, and phosphodiesterases have been proposed as mediators of the ROS-induced increase in endothelial permeability.[126,128,129] ROS have also been implicated in disruption of tight junction proteins: H_2O_2 caused redistribution of occludin on the cell surface, limiting its association with zonula occludens-1 and leading to an increase in endothelial permeability.[130]

ECs themselves also can produce ROS mediated by the mitochondrial electron transport chain, nicotinamide adenine dinucleotide phosphate hydrogen oxidases (NOXs), especially NOX2 and NOX4, uncoupled endothelial nitric oxide synthase (eNOS), and xanthine oxidase.[131] At physiological levels, ROS-induced signalling is necessary for maintaining vascular tone by the endothelium and also facilitates angiogenesis and acute inflammatory responses to fight the invading pathogens.[132] In pathological settings like sepsis, however, when the ROS response is overwhelming, reduction of ROS production by inhibition of NOX2, for example was shown to be protective against sepsis-induced organ damage.[133–135]

A main endogenous vasodilator and antiproliferative agent is nitric oxide (NO), which is synthesized in ECs by eNOS. NO stimulates soluble guanylate cyclase to increase the level of cyclic GMP in smooth muscle cells.[136] During sepsis, some of the essential cofactors necessary for eNOS activity, such as tetrahydrobiopterin, are depleted via oxidation, resulting in uncoupling of the enzyme, superoxide anion generation, and reduced NO production by eNOS.[125] At the same time, iNOS in immune cells and endothelium becomes activated, providing a large amount of NO (*Figure 1*). The reaction between NO and superoxide anion results in formation of reactive nitrogen species (RNS), such as peroxynitrite (ONOO-), nitrogen dioxide ($\bullet NO_2$), and dinitrogen trioxide (N_2O_3), which also contribute to endothelial dysfunction.[137] Deleterious cellular effects of both ROS and RNS have been attributed to their role in the oxidation of proteins, lipids, and oxidant-induced DNA damage.

Despite the overwhelming ROS production during sepsis, the utility of ROS measurement in diagnosis of sepsis and mortality prediction remains limited. This may be partly due to the instability of ROS, which reduces our ability to accurately measure them in a clinical setting, and the relative non-specificity of the ROS response. Despite those limitations, one study identified a ROS-specific gene expression signature that was able to predict mortality in patients with sepsis.[138] Another study investigated ROS-activatable nanoprobes for early diagnosis and sepsis severity prediction in mouse model of sepsis.[139]

Multiple approaches, however, have been suggested to halt the chain reaction of ROS production. Indeed, both catalase and superoxide dismutase prevented shedding of the glycocalyx and preserved

the endothelial barrier in response to hydrogen peroxide *in vitro*.[140] Interestingly, to date there are no trials investigating the therapeutic potential of these molecules. This might be partly due to the challenges with cell-specific delivery of these relatively large proteins. Those challenges are being addressed in animal studies with the use of SOD conjugated with antibodies targeted to endothelial endosomes[141] and the mitochondria-targeted antioxidant, MitoTEMPO.[142] Positive results of these studies in animal models of endotoxaemia would need, however, further evaluation before their potential use in clinical practice due to the universal role of ROS in normal physiology.

NO, particularly due to dysregulated iNOS activity, has been implicated in organism-wide vasodilatation and increased vascular permeability in sepsis. Pharmacological blockade of NO production mitigates sepsis-induced hypotension in animal models.[143,144] Unfortunately, these studies failed to translate into clinical practice after a large-scale multi-centre randomized clinical trial demonstrated that treatment with NG-monomethyl-L-arginine (L-NMMA) (a non-specific NOS inhibitor) increased mortality in sepsis while raising mean blood pressure and decreasing the vasopressor requirement.[145] More targeted approaches by attempting to act more specifically on the eNOS/iNOS balance were suggested. One of the most recent suggestions is BH_4, which is a crucial co-factor of NOS. The data on exact function of BH_4 in sepsis, however, are conflicting, with some studies showing improved mortality with inhibition of BH_4 production[146] while others show that addition of BH_4 improves microcirculation in sepsis.[147] Moreover, tetrahydrobiopterin analogues such as sepiapterin, which reduce superoxide generation via uncoupling of eNOS, were shown to protect the endothelium and organ function.[148] Therefore, more detailed studies, focusing particularly on the eNOS/iNOS balance in sepsis, are needed.

Finally, vitamin C, due to its recognized antioxidant effect, has been suggested as an adjunctive therapy in sepsis. Vitamin C scavenges ROS and RNS, and as a result becomes oxidized to ascorbate-free radical, which then dismutates to form dehydroascorbic acid.[149] Besides the direct effect on ROS and RNS, ascorbate reduces production of ROS and RNS by preventing NOX activation, decreasing expression of iNOS and increasing NO bioavailability.[150,151] By decreasing ROS, vitamin C decreases endothelial permeability.[149,152] There are also reports from *in vitro* studies that vitamin C can bind to alpha-adrenergic receptors and augment the effect of vasopressors.[153] In animal models of vascular injury due to endotoxaemia, reperfusion injury, ARDS, and burns, vitamin C administration preserved lung barrier function, prevented oedema formation in burns and decreased ROS production.[154,155] These promising experimental data combined with the fact that the levels of vitamin C in critically ill patients are markedly decreased,[156] made it a compelling candidate for adjunctive therapy in sepsis to supplement the nutrient that has been lost due to increased demand by tissues suffering from oxidative stress. A small phase I trial including 24 ICU patients with severe sepsis showed that administration of vitamin C decreased the extent of the organ failure.[157] Other studies used vitamin C in conjunction with either vitamin E[158] or thiamine and hydrocortisone[159] and demonstrated decreased vasopressor requirement and mortality. A recent large randomized trial focusing on the effects of vitamin C in septic patients with ARDS showed no improvement in organ dysfunction or inflammatory marker levels. While in that study, the 28-day mortality was significantly lower in vitamin C group compared to the placebo group, the authors point out that per study design, mortality was a secondary outcome, and some internal biases could not be excluded.[160] Thus, large randomized multi-centre studies are still needed to establish the utility of vitamin C in treatment of sepsis. The relative safety (apart from possible worsening of kidney failure[159]) and low cost of this drug make it a very attractive therapy.

8. Endothelial cell apoptosis and regeneration in sepsis

Endothelial cell apoptosis is a highly regulated process.[161] LPS from Gram-negative bacteria was shown to induce apoptosis in ECs *in vitro*; however, the effect appeared to depend on experimental conditions.[162] Interestingly, no apoptotic effect on human umbilical vein ECs was observed *in vitro* at concentrations of LPS matching those in patients' serum during sepsis.[162] Besides the direct effect of pathogen-derived substances and particles, ROS and RNS accumulating during sepsis are also toxic and proapoptotic for the endothelium.[123] Endothelial apoptosis results in a loss of normal anticoagulant properties of endothelium. The surface of the apoptotic cell exhibits increased tissue factor procoagulant activity, as well as reduced surface TM, heparan sulphate, and TFPI expression.[163] This leads to increased thrombin formation by both adherent and detached apoptotic ECs[164] and further augments the inflammatory and clotting cascade. Regeneration of ECs in sepsis is an important component of recovery from sepsis and is regulated via HIF-1α and its downstream target Sox17.[165] Thus, both EC apoptosis and regeneration play important roles in pathophysiology of sepsis and targeting them might provide future insights into the development of novel therapeutics. For example, a few small studies have shown that both circulating ECs and circulating endothelial progenitor cells were increased during sepsis, with higher numbers of progenitor cells and lower number of ECs associated with improved survival.[166] The relatively low number of both circulating ECs and progenitor cells, however, limits their utility as a diagnostic test.

9. Novel directions

9.1 Endothelium, sepsis, and microRNAs

MicroRNAs (miRs) are non-coding endogenous RNAs that target messenger RNA (mRNA) resulting in translational repression.[167] miRs have been implicated in various stages of sepsis development (*Table 1*). For example, miR-146 has been shown to prevent endothelial activation by inhibiting several inflammatory pathways, such as those regulated by NF-κB, MAPK, and AP-1. miR-146 also targets the RNA-binding protein, human antigen R, thus dampening the pro-inflammatory response.[169] In addition to miR-146, the NF-κB pathway in the endothelium is regulated by miR-181b via inhibition of importin-α3, which is essential for NF-κB nuclear translocation. miR-181b also reduces the expression of VCAM-1 and E-selectin in ECs.[170] Similar to miR-146 and -181 b, miR-155 was shown to suppress endothelial inflammation by down-regulating NF-κB p65 and adhesion molecule expression in TNFα-stimulated ECs.[171] In addition, miR-130b appears to inhibit inflammatory gene expression by down-regulating LPS-induced aberrant activation of ERK signalling.[172] A possible role of miRs in maintenance of endothelial barrier via regulation of tight junctions has also recently been suggested.[179] For instance, ECs from miR-150 deficient mice demonstrated a persistent increase in Ang2 levels, thus resulting in an irreversible increase in vascular permeability. Restoring miR-150 expression in miR-150 knockout mice prevented the production of Ang2 and preserved vascular barrier function,

Table 1 MicroRNAs with potential roles in the endothelium in sepsis

Potential therapeutic implication	MicroRNA	Pathway affected	Observed effect in sepsis models
Anti-inflammatory effect	miR-146	NF-κB, MAPK, and AP-1[168] human antigen R[169]	Decreases inflammatory response
	miR-181b	Importin-α3[170]	Inhibits nuclear translocation of NF-κB; reduces the expression of VCAM-1 and E-selectin
	miR-155	NF-κB p65[171]	Decreased expression of ICAM and VCAM
	miR-130b	Tpl2[172]	Decreased IL-6 and TNF-α expression
Inhibition of endothelial permeability	miR-150	Angiopoetin2[173]	Decreased vascular permeability
	miR-147b	ADAM15[174]	Preservation of endothelial barrier
DIC	miR-30b	PAI-1[175]	Unknown
	miR-181b	PAI-1[176]	Unknown
	miR-143/145	PAI-1[177]	Unknown
	miR-96	Aquaporin-5[178]	Unknown
	miR-330	Aquaporin-5[178]	Unknown

ultimately reducing mortality from sepsis.[173] miR-147b was also associated with maintenance of the endothelial barrier after treatment with LPS via targeting the 3′-UTR of the metalloproteinase ADAM15, which favours the increased endothelial permeability during inflammation and sepsis.[174]

PAI-1 inhibits fibrinolysis and sustains DIC in septic patients, leading to multiple organ failure.[180] PAI-1 levels are elevated in the endothelium during severe sepsis and their levels correlate with disease severity.[181] Several miRs, such as miR-30b, miR-181b, and miR-143/145 clusters, were shown to regulate PAI-1 levels.[175–177] Moreover, miR-96 and miR-330 have been found to contribute to lung injury in LPS-induced DIC in rat models.[178] This growing body of evidence pointing towards the importance of miRs in sepsis can provide insights for the development of novel therapeutic targets for maintenance of endothelial barrier and prevention of inflammatory response dysregulation.

9.2 Microparticles and endothelium in sepsis

The endothelium is not only a victim in sepsis but also an active participant that, via its interaction with other cells and tissues, is capable of modulating the inflammation. Well-orchestrated interactions between immune cells and endothelium ensure appropriate responses at times of infection and inflammation. One mechanism of cell–cell communication that has received a significant amount of attention in recent years is microparticles (MPs).[182] MPs are 0.2–2 μm cell membrane-derived particles harbouring phosphatidylserine and tissue factor on the outer leaflet and proteins, mRNAs, and miRNAs on the inside that are capable of promoting coagulation and inflammation. Sepsis-induced microvascular injury during sepsis leads to the release of MPs from ECs, red blood cells, monocytes, and platelets into the systemic circulation.[183] Specific receptors (such as Annexin I or CD36) are required for the interaction between MPs and target cells;[184,185] however, there may be other receptors that have not yet been described. Apart from their role in promoting coagulation and inflammation, MPs may also have some anti-inflammatory effect, with several studies showing decreased immune-cell activation after incubation with platelet-derived MPs.[186]

Other investigators have reported an increased number of MPs in patients with septic shock[187] as well as in animal models of sepsis.[188] Endothelium and leucocyte-derived MPs were one of the first markers to be elevated in DIC, with endothelium-derived CD105-MPs and CD31-MPs strongly associated with early DIC in multivariate analysis.[189] Overall, the relative stability of the MPs in the blood stream as well as their signalling potential make them a compelling target for developing novel diagnostic tools and therapeutic agents.

10. Conclusions

The body of evidence supporting a crucial role of the endothelium in sepsis is continuously growing. It is clear now that endothelium is involved in both physiologic and pathologic responses in sepsis and targeting those responses has great potential for developing therapeutics for management of this condition. Significant efforts have been already made to investigate the utility of targeting various endothelial pathways activated in sepsis for diagnosis and treatment of sepsis; more detailed studies are needed, however, to unravel the exact mechanisms as well as to further explore potential clinical applications. While some aspects of endothelial activation in sepsis have received much attention and have even been tested in large randomized clinical trials, the knowledge of other pathways lags behind. Translating experimental data from animal models into clinical practice has been particularly challenging, which might be a reflection of the limitations of animal models or the high variability of clinical scenarios underlying sepsis that are difficult to account for during the design of a randomized clinical trial. Overall, the endothelium undergoes dramatic changes during sepsis and remains one of the most compelling targets for therapeutic development. The modalities currently recommended for management of sepsis, however, do little to protect the endothelium or restore endothelial function, leaving much room for future drug development.

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