SEPSIS

Broken Barriers: A New Take on Sepsis Pathogenesis

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Despite intense research into the pathogenesis of sepsis, the current therapy for this devastating syndrome is primarily supportive and mortality remains high. The paucity of specific therapies is not for lack of effort; countless clinical trials in sepsis patients have failed despite promising preclinical data obtained from in vitro and animal models. Human sepsis is characterized by diffuse microvascular leak and tissue edema—features that have been largely ignored in animal models. Moreover, there have been no clinical trials of agents designed to prevent or treat leaky vasculature. Recent compelling evidence suggests that the breakdown in endothelial barrier function plays a crucial role in the pathogenesis of sepsis. In particular, these data suggest that preventing vascular leak can reduce mortality from sepsis. In this Perspective, we highlight the endothelial barrier as a new target for sepsis therapeutics, examining three potential strategies: enhancement of endothelial junctions; reinforcement of the endothelial cytoskeleton; and modulation of endothelial activation.

RETHINKING SEPSIS

"Sepsis" refers to the systemic inflammatory response after microbial infection. Unfortunately, its incidence appears to be rising, and the mortality caused by this syndrome remains between 30 and 50% (*1*). Treatments for sepsis are largely limited to surgical removal or drainage of the infected site, antibiotics, and supportive care. The lack of therapies is not for want of effort; on the basis of promising in vitro and animal studies, many drugs have entered clinical trials only to fail, resulting in the description of sepsis research as a "graveyard" of discovery (*2*). To date, activated protein C (aPC) remains the only therapy approved for use in the most severe cases of sepsis, and its mechanism of action and efficacy continue to be debated (Table 1) (*3*, *4*).

The repeated failure of clinical trials suggests that some fundamental knowledge is lacking in our current understanding of the pathogenesis of human sepsis. In particular, it suggests that animal models of sepsis, which have traditionally focused on the immune system, might neglect a key element inherent in the human syndrome. Clinicians who look after patients with sepsis are struck by the prevalence and severity of microvascular leak, which manifests as tissue and organ edema, hypotension, and shock. These characteristic features of human sepsis have been essentially absent from animal models; indeed, the importance of endothelial barrier integrity is scarcely mentioned in several recent, high-profile reviews of sepsis (*2*, *3*, *5*) despite epidemiological evidence suggesting that leak and tissue edema might be harmful (*6*, *7*).

There have been no clinical trials of therapies aimed at enhancing vascular integrity during sepsis, probably because the importance of vascular leak in this context has emerged only recently in a series of diverse and convincing experiments in animals (*8*–*10*). In mouse models of sepsis, such as cecal ligation and puncture, the amelioration of microvascular leak achieved by different investigators and by using different strategies was associated with an improvement in survival (*8*–*10*). Thus, these data strongly indicate that microvascular leak plays a defining role in the outcome of sepsis. This discovery warrants a reassessment of the pathogenesis of human sepsis and lays the groundwork for the development of novel therapies. In this Perspective, we discuss mechanisms that lead to the loss of microvascular barrier function, emphasizing the means by which the endothelial monolayer can be supported as the basis for future drug discovery.

DIRECT TARGETING OF ENDOTHELIAL JUNCTIONS

The endothelial barrier consists of cell-cell junctions as well as various noncellular components, such as the glycocalyx and

Table 1. Potential barrier-enhancing agents. Selected molecules with effects upon the endothelial barrier are listed along with their proposed mechanisms of action. This list is not exhaustive but instead emphasizes effects on intercellular junctions, the cytoskeleton, and endothelial activation.

Agent	Mechanism of action to enhance endothelial barrier
Activated protein C (aPC)	• Increases S1P production via EPCR and PAR1 activation • Inhibits endothelial apoptosis; prevents damage from circulating histones
Angiopoietin-1 (Ang1)	• Decreases NF-κB–triggered gene expression • Stimulates SK1 to increase S1P production • Inhibits endothelial apoptosis via survivin • Blocks Src phosphorylation and VE-cadherin internalization owing to VEGF signaling
Atrial natriuretic peptide (ANP)	• Activates Rac and downstream effectors (such as PAK) • Decreases inflammatory signaling through p38 mitogen-activated protein kinase and NF-κB
Slit2N	• Signals through Robo4 in order to inhibit VEGF-induced microvascular leak; stabilizes VE-cadherin at junctions by increasing p120-catenin binding; blocks Src activation
Sphingosine 1-phosphate (S1P)	• Activates S1P1 to increase cortical actin via Rac, PAK, and cortactin • Increases junctional targeting of VE-cadherin
Statins (3-hydroxy-3-methyl-glutaryl-CoA reductase inhibitors)	• Regulate RhoA activity by inhibiting lipid modification and causing increased cortical actin and decreased stress fibers. Numerous other effects for these drugs have been described, including inhibition of reactive oxygen species generation and up-regulation of endothelial nitric oxide synthase.

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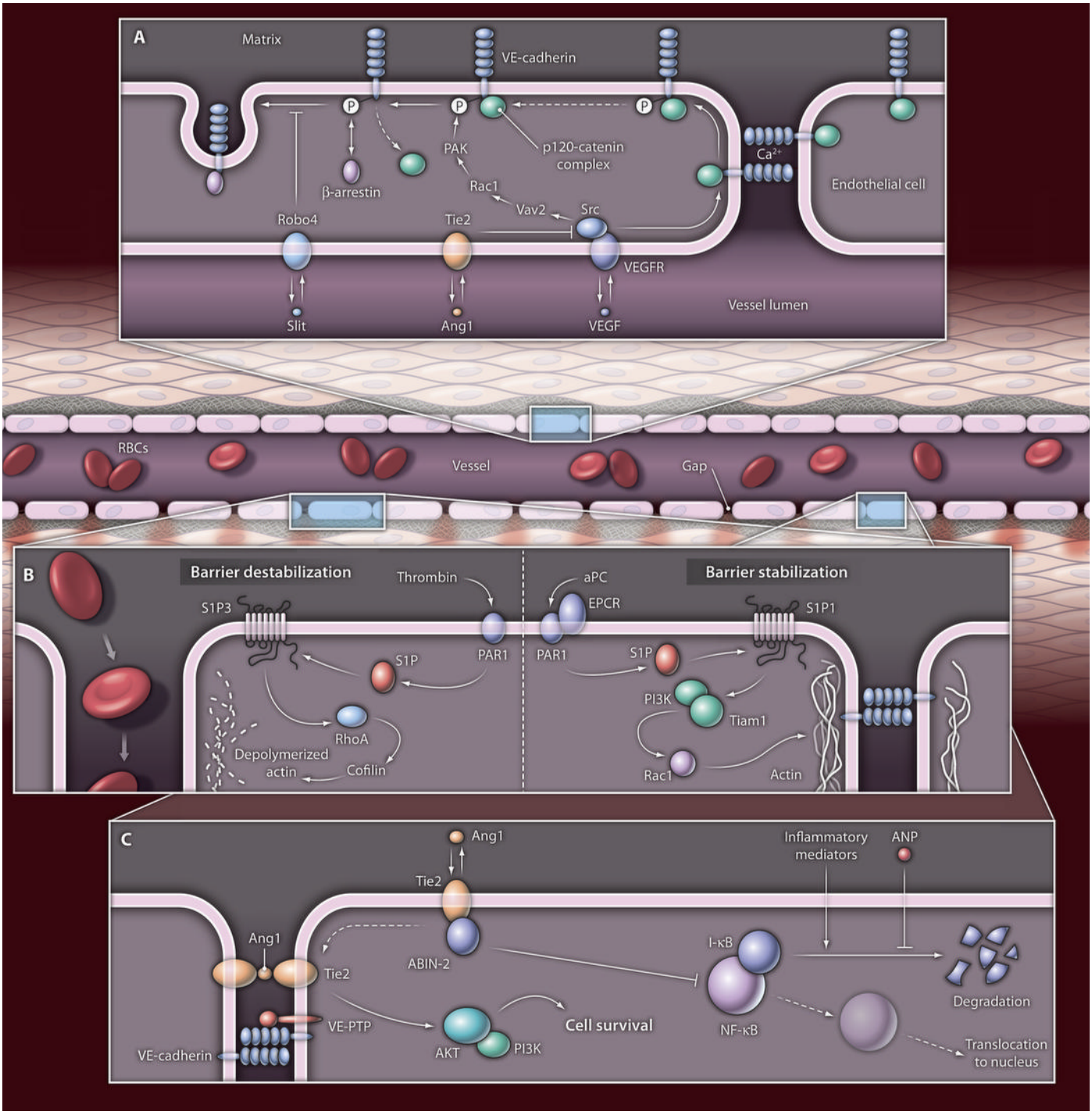

Fig. 1. Mechanisms of endothelial barrier protection. (**A**) Enhancing endothelial cell junctions. The amount of VE-cadherin in the plasma membrane is regulated by endocytosis. Phosphorylation (P) of VE-cadherin occurs directly via activation of Src as well as indirectly through downstream signaling via Vav2. Phosphorylated VE-cadherin is internalized via β-arrestin2–dependent endocytosis. Decreased VE-cadherin at the plasmalemma results in diminished vascular integrity. Ang1 inhibits VE-cadherin internalization by sequestering Src and blocking its phosphorylation in response to VEGF. Slit is barrier-protective by maintaining membrane VE-cadherin levels through reduced endocytosis. (**B**) Reinforcement of the endothelial cytoskeleton. aPC binds its receptor, which leads to activation of the S1P receptor type 1 (S1P1) and subsequent activation of PI3K/Tiam1. S1P can also bind the S1P receptor type 3 (S1P3) and activate RhoA, which alters cortical actin via cofilin. (**C**) Modulation of endothelial activation. NF-κB is retained normally in the cytosol by its inhibitor, I-κB. Inflammatory mediators lead to endothelial activation in part through I-κB degradation, allowing for NF-κB nuclear translocation; ensuing gene expression promotes vascular leak. ANP promotes barrier integrity by inhibiting NF-κB activation. In sepsis, endothelial activation is countered by Ang1/Tie2 signaling, which suppresses the NF-κB pathway through ABIN-2. Ang1 binding also induces translocation of Tie2 to the endothelial cell-cell junction, where Ang1 bridges Tie2 receptors. Tie2 receptors associate with VE-PTP and signal through PI3K/Akt to enhance endothelial cell survival.

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extracellular matrix. Although the latter contributes to the prevention of leak, these noncellular elements are beyond the scope of this discussion. Increased permeability of the endothelial monolayer is largely explained by gaps between cells (paracellular leak) or by transit through individual cells (transcellular leak). Although transcellular leak has been described [for example, the transcytosis of albumin (*11*)], its contribution to sepsis is unknown. The vast majority of research examines paracellular leak, which is the focus herein. In addition, although our discussion centers on systemic vascular leak, it is important to note that vascular beds differ in different tissues. For instance, in the lung, barrier function is shared by both endothelia and pulmonary epithelia (*12*); in the kidney, permeability is governed by the endothelium and its glycocalyx, the basement membrane, and podocytes; in the liver, sinusoidal endothelium is fenestrated (*13*). Also, receptors expressed in distinct vascular beds can result in differential signaling of many mediators of endothelial function, including the Tie-angiopoietin system discussed below (*13*, *14*).

Cell-cell junctions play an obvious and important role in preserving and regulating endothelial barrier function. Endothelial cells have several types of junctions, including adherens junctions and tight junctions. Adherens junctions are crucial for regulating endothelial permeability in the venular circulation, which is the type most prone to leakage during sepsis. Furthermore, the postcapillary venule expresses receptors for important mediators of inflammation, including tumor necrosis factor (TNF), interleukin-1 (IL-1), and vascular endothelial growth factor (VEGF) [reviewed in (*13*)]. The predominant structural element of the endothelial adherens junction is vascular endothelial cadherin (VE-cadherin)—a protein that connects neighboring cells in a calcium-dependent manner and also has important signaling functions. The intracellular juxtamembrane and carboxyl-terminal domains of VE-cadherin interact directly with p120-catenin and β-catenin proteins (*15*, *16*) and indirectly with the actin cytoskeleton. The integrity of adherens junctions is regulated by tyrosine phosphorylation of its components, in particular VE-cadherin, which can influence its binding to p120-catenin and β-catenin (Fig. 1A) (*15*). For example, the binding of p120-catenin to VE-cadherin masks a dileucine motif that normally acts as a signal for clathrin-mediated endocytosis of VE-cadherin. This in turn prevents the translocation of VE-cadherin from the cell surface, where it is required to maintain normal cell-cell adhesion (*17*). Inflammatory mediators like thrombin and VEGF induce endothelial leak by promoting the internalization of VE-cadherin (Fig. 1A) (*18*). For instance, VEGF initiates a signaling cascade involving Src kinase, Vav2, and Rac that triggers the serine phosphorylation of VE-cadherin, leading to the recruitment of β-arrestin2 and culminating in the endocytosis of VE-cadherin (Fig. 1A) (*18*). Inhibition of this VEGF signaling pathway is one of the ways that the vascular protective agent angiopoietin-1 (Ang1) prevents vascular leak (Fig. 1A) (*19*).

Although the importance of VE-cadherin to barrier integrity has been known for some time (*20*), it has only recently been appreciated that preserving VE-cadherin function can improve the outcome of inflammatory diseases. One recently described strategy involves the secreted protein Slit, which binds the receptor Robo4 (Fig. 1A). A recombinant Slit fragment, Slit2N, was found to enhance microvascular barrier integrity of lung endothelium in culture and to decrease vascular leak in mice (Table 1) (*8*). Specifically, treatment with Slit2N significantly improved survival of mice in three different models of systemic inflammation: endotoxemia, cecal ligation and puncture, and H5N1 influenza infection. By promoting the association of p120-catenin and VE-cadherin, Slit2N decreased endocytosis of VE-cadherin, stabilizing adherens junctions and maintaining barrier function (Fig. 1A). Strikingly, Slit2N treatment did not modify the profound elevation in cytokines caused by sepsis (*8*). It remains unclear how Slit2N conveys its signals to p120-catenin and VE-cadherin, and this mechanism should be a focus of future investigation to identify additional molecular targets for stabilizing the endothelial barrier. Nonetheless, this finding suggests that buttressing the endothelial barrier may be sufficient to reduce mortality due to sepsis, independently of any effect on the immune response.

REINFORCING THE ENDOTHELIAL CYTOSKELETON

Cell-cell junctional complexes do not operate in isolation. Their linkage to the cellular cytoskeleton ensures that junctional forces and cell shape are coordinated. Furthermore, this association highlights the role that the cytoskeleton itself plays in the regulation of permeability. As the "scaffolding" of the cell, cytoskeleton remodeling leads to rapid changes in cell shape; distortions of shape could introduce gaps in the endothelial monolayer, causing microvascular leak.

Actin filaments are critical components of the cytoskeleton and can be found in several distinct structures. Cortical actin is a girdle of actin bundles that runs beneath the plasma membrane in a functional ring. This structure is associated with a large group of proteins that regulate the polymerization of filaments and tethering of actin to membrane proteins [reviewed in (*21*)], such as VE-cadherin. Actin is dynamically regulated by members of the Rho family of guanosine triphosphatases (GTPases), notably Rac, cell division control protein 42 homolog (Cdc42), and Ras homolog gene family, member A (RhoA). Signaling through Rac1 and Cdc42 stabilizes cortical actin and enhances barrier stability (Fig. 1B) (*22*, *23*). In contrast, activation of RhoA, which occurs in response to the inflammatory stimuli thrombin, VEGF, or IL-1, regulates effector molecules, such as the actin-severing enzyme cofilin. This disrupts the actin cytoskeleton (*24*), which in turn destabilizes VE-cadherin at the endothelial junction and increases membrane permeability (Fig. 1B). Although most reports describe the barrier-enhancing effect of Rac1 signaling, the reality is that both too much and too little Rac activity can cause microvascular leak [for example, the Rac-mediated endocytosis of VE-cadherin described earlier (*18*)] (*25*). Similarly, although RhoA is thought primarily to destabilize the endothelial barrier, it is protective under basal conditions [reviewed in (*26*)]. Agents known to increase microvascular leak, such as thrombin, disrupt cortical actin and initiate endothelial cell contraction by increasing the inward tension exerted by actin-containing stress fibers (Fig. 1B). The balance between RhoA and Rac signaling is essential for normal endothelial barrier function, and factors influencing this balance might represent therapeutic targets. In particular, agents that promote cortical actin formation might enhance endothelial integrity.

Recent data suggest that aPC may confer its therapeutic effects in part by decreasing vascular leakage (Fig. 1B) (*9*). Considering the established role of aPC in the coagulation cascade, it was originally thought that its mechanism of benefit in sepsis was through its ability to inhibit clotting. However, an engineered mutant version of aPC

that retained less than 10% of its anticoagulant ability was just as effective as wild-type aPC in limiting death from sepsis owing to endotoxemia or bacteremia in mice (*9*). Intriguingly, this non-anticoagulant form of aPC markedly decreased vascular leak in a mouse model of sepsis (*9*). The mechanism of this protective effect has been elucidated: After binding to the endothelial protein C receptor (EPCR), aPC is capable of cleaving another receptor on endothelial cells known as the protease-activated receptor 1 (PAR1) (*27*). Cleavage activates PAR1, triggering the generation of a lysophospholipid called sphingosine 1-phosphate (S1P) (Fig. 1B). S1P is produced through the phosphorylation of sphingosine by sphingosine kinases 1 and 2 (SK1/2) and enhances cortical actin formation in endothelial cells, leading to increased barrier integrity [reviewed in (*21*)] (*28*). A relationship between S1P and vascular integrity is further supported by the finding that knockout mice that lack SK1 and SK2 exhibit increased vascular leak both in the basal state and in response to challenge with histamine or serotonin (*29*). In addition to its effects on actin, S1P signaling results in the recruitment of VE-cadherin to the endothelial cell junction (Fig. 1B), increasing the integrity of the endothelial monolayer and decreasing protein leak out of the vasculature.

The enhancing effect of S1P on barrier function in cell culture is widely accepted; however, its role in vivo is more complex. For instance, the edemagenic factor thrombin also signals through PAR1, but this may lead to engagement of a different S1P receptor, S1P3 (Fig. 1B) (*30*, *31*). Although the protective effects of S1P are mediated through S1P1, thrombin-mediated leak triggers the S1P3 receptor, leading to barrier disruption, in part through activation of RhoA (*30*, *32*). Indeed, S1P generation occurs in several compartments, including erythrocytes (a major source of plasma S1P), endothelial cells, and phagocytes (*29*, *33*). Although S1P circulating in plasma appears to be barrier-protective, S1P production in phagocytes has been shown to have a proinflammatory effect and causes increased mortality in animal models of sepsis (*33*). Given the expected protective effects of S1P on barrier function, it seems somewhat paradoxical that inhibition or knockdown of SK1 was protective in animal models of sepsis—an effect that was attributed to decreased production of proinflammatory cytokines (*33*).

The barrier-enhancing effects and proinflammatory effects of S1P are seemingly contradictory and may reflect an effect of dose or of location. For instance, macrophages may produce S1P in response to an inflammatory stimulus; in turn, signaling via S1P may lead to an increase in macrophage-mediated inflammation, but in an autocrine fashion. At the same time, other blood elements—specifically erythrocytes—are known to secrete S1P into the plasma, which then signals endothelial cells via S1P receptors. At the endothelium, signaling through S1P1 buttresses the adherens junction, whereas signaling through S1P3 weakens it (Fig. 1B). The balance between engagement of S1P1 and S1P3 might result from differing affinities of S1P for these receptors. Consistent with this notion is the finding that prolonged exposure to S1P agonists is barrier-disruptive in an animal model (*34*): Although initially protective, agonist treatment alters S1P signaling over time, which results in a loss of endothelial responsiveness to the barrier-protective effects. Clearly, S1P is an important regulator of endothelial barrier function and of the innate immune system. Further research is needed to clarify its mechanism of action and potential clinical utility.

MODULATION OF ENDOTHELIAL ACTIVATION

Another potential therapeutic strategy for treating sepsis is the attenuation of endothelial activation. For instance, the importance of endothelial nuclear factor κB (NF-κB) has been underscored by a series of elegant studies employing transgenic mice that conditionally overexpress a degradation-resistant form of the NF-κB inhibitor, I-κB, in endothelial cells, but not other cell types (*10*, *35*). In animal models of sepsis caused either by bacterial endotoxin in the blood (endotoxemia) or by cecal ligation and perforation, selective suppression of NF-κB in endothelial cells improved multiple-organ injury and survival, without affecting the clearance of bacteria from the circulation or the blood levels of cytokines, such as TNF-α, IL-1β, and IL-6 (*10*, *35*). The mechanism by which NF-κB suppression in endothelia results in improved survival is not clear; however, pathological examination revealed that mice with inhibition of endothelial NF-κB exhibited decreased endothelial permeability (*10*). Thus, although some degree of endothelial activation is likely required for host defense (*36*), excessive activation causes endothelial damage (*37*) and vascular leak (*10*).

Other barrier-enhancing agents exert their effects, at least in part, by blocking NF-κB. For instance, atrial natriuretic peptide (ANP) (Table 1) protects against microvascular leakage in the lung (*38*, *39*) by blocking NF-κB (*39*) activity (Fig. 1C), while concurrently activating Rac and enhancing VE-cadherin localization to cell-cell junctions (*38*). Another example of an endogenous barrier-protective agent is the secreted growth factor Ang1 (*40*). Binding of Ang1 to its receptor Tie2 leads to suppression of NF-κB–directed gene expression and signaling mediated by the ABIN-2 (A20-binding inhibitor of NF-κB activator-2) protein (Fig. 1C) (*41*). Ang1 binding also induces translocation of Tie2 to the interendothelial junction where multimeric Ang1 functions as a bridge between Tie2 receptors on adjacent cells (*42*, *43*). Tie2 receptors at the junction associate with vascular endothelial protein tyrosine phosphatase (VE-PTP), which itself has been shown to inhibit paracellular permeability (*44*) and signal through Akt and phosphoinositide 3-kinase (PI3K) (Fig. 1C). In addition, Ang1 has been shown to transiently stimulate SK1, which might explain part of its vascular barrier–promoting effects (*45*).

These potentially salutary effects of Ang1 are counterbalanced by Ang2—a related growth factor that is released by activated endothelial cells and that, by binding Tie2, inhibits Ang1-mediated signaling. One study demonstrated an increase in circulating Ang2 levels in septic patients and found that Ang2 levels correlated with the ability to induce endothelial leak in cell culture monolayers; administration of exogenous Ang2 to mice caused pulmonary vascular hyperpermeability (*46*). Vascular leak was attributed to Ang2-mediated stimulation of RhoA signaling and endothelial cytoskeleton rearrangement, an effect abrogated by inhibitors of downstream effectors of RhoA (*46*). Thus, agents that mimic Ang1 or inhibit Ang2 might have therapeutic potential in treating sepsis.

THERAPEUTIC STRATEGIES FOR SEPSIS

Several recent and elegant studies now provide convincing evidence that microvascular leak is not merely the byproduct of sepsis, but instead a major contributor to its morbidity and mortality. Clinicians have long recognized the problem of vascular leak but have had no tools to reverse it. The

realization that this characteristic feature of human sepsis is, in fact, crucial to its pathogenesis is likely to spur the development of new therapies. We propose that the three areas discussed in this Perspective could serve as a starting point for future work. For instance, the development of agents that reinforce intercellular junctions should be a goal of drug research. This might involve delineating the downstream players in the Slit-Robo pathway (Fig. 1A) or further developing mediators that are already known to enhance localization of VE-cadherin to the plasma membrane (such as ANP).

Another area of research can focus on stabilization of the endothelial cytoskeleton. Numerous agents, such as S1P, aPC, ANP, and HMG-CoA reductase inhibitors (statins) have been shown to either enhance the formation of cortical actin or inhibit actin stress fibers, leading to a stronger endothelial barrier (Table 1). Given the tight interplay between intercellular junctions and the cytoskeleton, it is likely that a suitable agent would enhance the endothelial barrier by working on both systems. For instance, S1P-derivatives may prove to be particularly effective therapies for sepsis because they work in multiple ways, by increasing cortical actin, by targeting VE-cadherin to the adherens junction, and by stabilizing focal adhesions. Finally, excessive activation of the endothelium during systemic inflammation appears to be harmful, in part through its contribution to microvascular leak; thus, agents such as Ang1 and ANP that modulate endothelial activation might form the basis for future therapies for sepsis.

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47. **Acknowledgments:** We apologize to the many investigators whose work we could not cite because of space limitations. We thank W. C. Liles for thoughtful review of our manuscript. **Funding:** N.M.G. is supported by a Vision Science Grant. B.E.S. is supported by M.D./Ph.D. studentships from the Canadian Institutes of Health Research and the McLaughlin Centre for Molecular Medicine. W.L.L. is supported by the Canadian Institutes of Health Research, the Ontario Thoracic Society, the Physicians Services Incorporated Foundation, and the Keenan Research Center of the Li Ka Shing Knowledge Institute, St. Michael's Hospital (Toronto). **Competing interests:** The authors declare no competing interests.

10.1126/scitranslmed.3002011

Citation: N. M. Goldenberg, B. E. Steinberg, A. S. Slutsky, W. L. Lee, Broken barriers: A new take on sepsis pathogenesis. *Sci. Transl. Med.* **3**, 88ps25 (2011).