Therapeutic Targeting of Kinases and Phosphatases to Inhibit Microvascular Leakage in Sepsis

Masterthesis by

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Abstract. Sepsis and septic shock are poorly understood and have mortality rates as high as 50% due to microvascular leakage, tissue hypoxia, and organ damage and subsequent organ failure in patients. Microvascular leakage is caused by activation of endothelial cells and dysfunction of the endothelial barrier. Currently, no drug is available to ameliorate microvascular leakage. Therefore, this review explores sphingosine-1-phosphate (S1P), Rho-associated protein kinase (ROCK), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), and tunica intima endothelial kinase 2 (Tie2) as possible pathways to restore the endothelial barrier function in sepsis. Specifically, each of these pathways encompasses kinases and phosphatases which are highlighted as possible therapeutic targets in sepsis. Finally, recommendations are made concerning the use of animal models in the development of sepsis drugs.
Introduction

Shock is a pathophysiological condition with a multitude of possible causes. Septic shock is the most notable, and is initially caused by the bodily response to infection. Regardless of the exact cause of shock, the condition is associated with both hypotension and an increased heart rate, which in turn leads to a decreased perfusion of the peripheral tissues and subsequently possible failure of one or more organs (Jean-Baptiste, 2007).

Historically, shock has not been studied as thoroughly as many other similarly threatening conditions, which can be explained by the heterogeneity of the disease, both in causes, clinical symptoms and treatment options, as well as the difficulty in determining time of onset and its general acute and overwhelming character. However, the demand for treatment options is extensive, with current mortality rates being estimated as high as 50% (Leligdowicz et al. 2014). Current treatment options include the administration of intravenous fluids to counteract hypotension, antibiotics in the case of sepsis initiated by a bacterial infection, and vasopressor drugs to normalize blood pressure (Cohen et al. 2015). Vasopressor drugs induce vasoconstriction of smooth muscle cells surrounding the arterioles and retain blood pressure, which may involve interaction with the endothelium dependent on the specific vasopressor. This is of particular interest to this literature study, as a more recent development in the field recognizing the endothelium lining the blood vessels as not merely passively adapting in response to stimuli present in the blood stream, but rather acting on the forefront as it actively controls its microenvironment and pathophysiological processes. In the treatment of shock, to which the dysregulated functioning of the endothelium heavily contributes via increased vasodilation and increased permeability of the endothelial barrier of the capillaries and post-capillary venules, the endothelium thus has more recently become an interesting therapeutic target.

Several studies have been conducted to investigate the effects of multiple endothelium modulatory agents as possible therapeutic treatment for shock, some of which have entered clinical trials (Cohen et al. 2015). In this review, I will describe signaling pathways involved in microvascular leakage in sepsis, focusing on kinases and phosphatases in these pathways that might serve as drug targets. Before that, the pathophysiology of septic shock will be discussed in more detail, with an emphasis on the altered functioning of the endothelium in this condition. Finally, I will discuss current animal models for sepsis and how they might be improved in order to decrease the current discrepancy between success of sepsis drugs in animal models on the one hand and patients on the other hand.
Pathophysiology of sepsis

Sepsis, or more commonly known as blood poisoning, has been through multiple definition changes in recent history. One of the first medically sound definitions was the one which indicated the invasion of the blood stream by pathogens as the cause of sepsis. Despite the important role these pathogens play in the pathogenesis of sepsis, as will be discussed in more detail later, this particular theory was soon invalidated by the fact that clearance of bacteria from the blood stream did not resolve all septic symptoms (Angus et al. 2013). This finding led to the theory that sepsis might in fact be caused primarily by the inflammatory response of the body to the pathogens instead of the mere presence of pathogens in the blood stream. In 1992, sepsis was officially defined as “a systemic inflammatory response to infection” (Bone et al. 1992). Since then, this definition has seen some minor revisions, primarily in an attempt to make a clearer distinction between varying severities of sepsis. The definition of sepsis remains “a systemic inflammatory response to infection”, but when this is accompanied by partial organ failure, we speak of severe sepsis. The most serious type of sepsis is septic shock, during which the hypotension is unable to be relieved by administration of intravenous fluids (Angus et al. 2013; Dellinger et al. 2013).

Instead of merely eliciting a systemic inflammatory response in the host as a response to an infection, sepsis is characterized by both pro- and anti-inflammatory responses (Angus et al. 2013). In general, a pro-inflammatory response is elicited against the pathogens or mitochondrial damage-associated molecular patterns (DAMPs) after being recognized by the immune system of the host (Zhang et al. 2010). In order to efficiently battle the infections without causing tissue damage due to an excessive pro-inflammatory response, an adequate anti-inflammatory response of the host is paramount. The combination of both of these responses is necessary for clearance of infection and tissue recovery, but can also lead to organ injury and secondary infections in case of an imbalance between both responses (Angus et al. 2013). The immediate period after the onset of sepsis is commonly characterized by a dominant pro-inflammatory response, also known as systemic inflammatory response syndrome (SIRS), which contributes heavily to tissue and subsequent organ damage (Osuchowski et al. 2006). In an attempt to counter the pro-inflammatory reaction, the immune system of the host will mount an anti-inflammatory response, also known as compensatory anti-inflammatory response syndrome (CARS) (Bone, 1996). If this anti-inflammatory response is sufficiently severe, it may result in an increased risk of infection (Osuchowski et al. 2006). The linear development from SIRS to CARS offers an accessible model to describe the immune response during sepsis. However, this model is oversimplified, as is shown by a study in which early postoperative sepsis in patients was accompanied by an increase in both pro-inflammatory IL-6 and anti-inflammatory IL-10, indicating the simultaneous occurrence of SIRS and CARS (Novotny et al. 2012). It is reasonable to assert that the immune response to sepsis is more complex than can be described within the limits of both SIRS and CARS. Nevertheless, their occurrences could still be useful in determining treatment options for patients, even though they do not necessarily develop in a predictable, linear manner.

From a therapeutic point of view, this indicates that the default administration of anti-inflammatory drugs upon identification of sepsis in patients is an unadvisable strategy, for the reason that the patient may already be in an anti-inflammatory dominant stage and this
treatment might worsen this condition. Considering this one aspect of sepsis alone makes it clear why the treatment of sepsis needs to be tailor-made to the specific needs of individual patients. This development is not only a result of the identification of different stages of systemic immune responses in the patients, but also based on differences in disease progression. Moreover, severe sepsis and septic shock are also accompanied by organ dysfunction, which can vary between the organs affected as well as in severity; both of these factors are important as well in establishing an effective treatment for the patient.

Microvascular leakage in sepsis

Another core part of the pathology of sepsis revolves around the dysregulated functioning of the endothelium (Ait-Oufella et al. 2010). The endothelium consists of a layer of cells lining the blood vessels and is receiving increased recognition as a separate organ. Under normal conditions, endothelial cells are responsible for numerous processes, including facilitation of the exchange of oxygen and nutrients in the blood with the underlying tissue, the recruitment and subsequent traversing of leukocytes from the blood through the endothelium into the tissue, the formation of a barrier to inhibit unwanted cells and proteins from crossing the endothelial layer, and regulation of vasomotor tone (Ait-Oufella et al. 2010; Vandenbroucke et al. 2008). Furthermore, balance between pro- and anti-coagulatory factors is also maintained via interactions with proteins on the endothelial cell surface (Sen et al. 2012; van Hinsbergh, 2012). The maintenance of this balance is required to prevent either unwanted blood clotting and subsequent blood vessel obstruction or bleeding complications to occur. Many of these processes are disrupted in sepsis (Ait-Oufella et al. 2010), including the disruption of the balance between pro- and anti-coagulatory factors that is being tilted towards pro-coagulation, the implications and mechanisms of which will be discussed in detail later.

Adjacent endothelial cells of the continuous endothelium form molecular structures with barrier functions to prohibit unbridled traversing of the endothelium by substances or cells present in the blood. These structures are commonly referred to as tight junctions, which is a collective term for a variety of cell-surface spanning proteins that form physical connections between cells (Vandenbroucke et al. 2008). Tight junctions are less prominent in organs with fenestrated (e.g. kidney) and discontinuous endothelium (e.g. liver), due to the necessity of increased endothelial permeability in the functioning of fenestrated and discontinuous endothelium and a lower dependency on the integrity of the endothelial barrier function (Aird, 2012). Whenever tight junctions are compromised, the functionality of the endothelial barrier is reduced. This is not only due to changes in the morphology of the endothelial cells in response to signals evoked in sepsis, which will be discussed in more detail later, but also due to sepsis-induced apoptosis of these cells (Zhou et al. 2004). The apoptosis of endothelial cells in vascular segments contributes to a compromised endothelial barrier integrity. This is due to a loss of cellular contacts between endothelial cells, enabling microvascular leakage to occur and contribute to the hypotension and subsequent tissue hypoxia commonly observed in sepsis. In addition, the observed tissue hypoxia might lead to failure of one or multiple organs and in the case of septic shock, hypotension is no longer
successfully counteracted by fluid resuscitation. Both of these processes contribute to the high mortality rates of septic shock (Bannerman et al. 2003; Leligdowicz et al. 2014). Acknowledging the impact of a compromised endothelial barrier function and microvascular leakage in sepsis, this review focuses on various therapeutic approaches that aim to restore the dysregulated endothelial barrier after the onset of sepsis, as to offer a potential strategy to improve sepsis patient status via restoration of endothelial barrier function.

**Endothelial cell heterogeneity**

In order to appreciate the complexity of the endothelial cell dysfunction during sepsis, a deeper understanding of endothelial cell heterogeneity is vital. This is true for both offering an explanation of observed symptoms and for designing a therapeutic strategy. In the previous paragraph discussing the encountered issues of endothelial cell dysfunction in sepsis, a general endothelial cell in which all the explained processes occurred was described. In reality, these processes do not always occur at the same scale or at identical points in time, let alone that they all occur in all endothelial cells. It is this heterogeneity in endothelial cells, exemplified by the fact that they do not all exhibit the same responses to a similar extent upon experiencing a similar systemic stimulus, that makes the development of anti-sepsis drugs that pharmacologically target endothelial cells a delicate matter (Molema, 2010). Furthermore, drugs interfering with endothelial cell functions in diseased tissue may also exert effects in unaffected endothelial cells and other cells, potentially reducing the effectiveness of the approach or even causing damaging side effects to the host that may nullify the positive effects of the treatment altogether. The next section will discuss how endothelial cell heterogeneity affects sepsis treatment strategies.

The various vessel segments that constitute the circulatory system - arteries, arterioles, capillaries, venules, and veins – all have important roles that vary from those of the other segments. For example, the majority of vasoconstriction and vasodilation effects are exerted via control of the smooth muscle cells of arteries and the smaller arterioles, whereas capillaries and veins contribute little to nothing to these effects. In addition, influx of leukocytes is especially prominent in post-capillary venules (Aird, 2007). This location enables the infiltration of tissue by leukocytes without compromising the integrity of capillaries and organ function (Molema, 2010). The capillaries and post-capillary venules are of particular interest with respect to sepsis, due to their role as one of the major sites of vascular leakage (Aird, 2012) and because the capillaries have few surrounding support cells, meaning that local behavior of the endothelial cells is largely dependent on the cells themselves (Langenkamp et al. 2009). Problems with altered permeability of the endothelium is most likely to occur in capillaries and post-capillary venules, whereas increased leukocyte influx primarily takes place in post-capillary venules, and hypotension in sepsis is partly due to insufficient vasoconstriction in the arteries and arterioles. Thus, the aberrant functioning of endothelial cells in sepsis includes all segments of the circulatory system, with each segment fulfilling a different role. The important question remains what underlying mechanisms cause the endothelial cell heterogeneity.
An important component of endothelial cell heterogeneity is if and to what extent endothelial cells respond to certain blood borne cytokines or ligands. This is depending on the absence or presence of receptors, which may differ between different vascular segments (Molema, 2010) and directly influence its effects. Furthermore, even if receptors are present at similar levels, downstream signaling might still differ due to different levels of kinase expression in endothelial cells (Moldobaeva et al. 2008). Moreover, these differences are not merely present between different blood vessel components, but can also exist within similar segments, for example capillaries throughout the body, and even within the same vessel. It was shown that endothelial marker gene expression differed to a large extent between capillaries from different organs, namely brain, liver, kidney, lungs and heart capillaries (Langenkamp et al. 2009). For example, intercellular adhesion molecule 1 (ICAM-1) is expressed on all organ capillaries mentioned earlier except brain capillaries, whereas VE-cadherin is expressed on all mentioned organ capillaries, but only strongly so on kidney and brain capillaries. This summary of endothelial heterogeneity is not exhaustive, and is expected to expand as ongoing research will uncover more factors constituting heterogeneity of endothelial cells. On the one hand, the heterogeneity of the endothelium offers opportunities, because the differences in origin are likely to be accompanied by a unique gene expression profile, which may be used for the targeted delivery of drugs at specific endothelial sites without affecting cells at other sites (Muzykantov, 2005). On the other hand, heterogeneity makes it difficult to offer concise treatment options interfering with endothelial dysfunction, due to the risk of unforeseen side effects on other cells when the specificity of targeted delivery proves inadequate, which could lead to the unintended effects of drugs in cells that were not the desired target.
Signal transduction pathways involved in microvascular leakage in sepsis

The aim of this section is to describe molecular targets in endothelial cells, specifically kinases and phosphatases, involved in endothelial barrier function and microvascular leakage, that are hypothesized to be therapeutically applicable for the treatment of sepsis in patients. Characterization of these targets might point out one or multiple mechanisms involving the blood vessel endothelium, including prevention of microvascular leakage. An important element to note here, is that the focus of the next part will be on molecular targets that have the potential to be relevant for patients admitted in hospitals with sepsis. Elucidation of the pathogenic molecular patterns that trigger a response of the immune system and what this response entails intracellularly is a compelling problem. However, patients admitted to the hospital are often already past the first stage of sepsis during which the initial systemic reaction to the recognition of the pathogen occurs, unless they become septic while already admitted in the hospital. This means that in order to offer immediate help to septic patients, therapies that focus on reestablishing the endothelial barrier function are more likely to have success in a clinical setting when compared to therapies that focus solely on eliminating immunogenic constituents of pathogens. At present, we have no therapies that aim to ameliorate microvascular leakage, while administration of antibiotics is a common and necessary treatment upon identification of sepsis. It is for this reason that the rest of this review puts an emphasis on molecular targets that could be targeted to restore the endothelial vascular permeability, which in theory may be of added value to improve the outcome of septic patients in the clinic in the future.

S1P ameliorates microvascular leakage via APC-dependent stimulation of PAR1

An aspect of sepsis that has only been mentioned briefly thus far, is the altered coagulation status during the disease. More specifically, there is an increase in coagulation due to an increase in production of pro-coagulatory factors and a decrease in production of anti-coagulatory factors, resulting in an increased viscosity of the blood (Levi et al. 2010). The process of altered coagulation in sepsis and the role of endothelial cells herein is reviewed in detail elsewhere (Angus et al. 2013). The aspect that is of importance in the context of this review is that the decrease in anti-coagulatory factors is associated with decreased levels of activated protein C (APC). APC has many roles in endothelial cell dependent disease development, and include anti-inflammatory, anti-coagulatory and anti-thrombotic effects. Under regular conditions, relatively high concentrations of APC (5-20 nM) along with low concentrations of the pro-coagulatory protein thrombin (40 pM) stimulate protease-activated receptor 1 (PAR1) in a way that elicits a protective effect on the endothelial barrier function (Feistritzer et al. 2005). However, the APC:thrombin ratio is decreased in sepsis due to decreased levels of APC and increased levels of thrombin, resulting in increased
microvascular leakage due to thrombin-dependent PAR1 stimulation. Activation of PAR1 by thrombin has been shown to increase permeability of the endothelial barrier via cytoskeleton rearrangement through activation of the RhoA kinase pathway (Adyshev et al. 2013), which will be discussed in detail later. This means that stimulation of the same receptor, PAR1, can have both protective and disruptive effects on the endothelial barrier function, depending on its main agonist being either APC or thrombin, respectively (Figure 1).

It is important to note that the effect of altering APC levels in human sepsis has already been investigated in the prospective recombinant human activated protein C worldwide evaluation in severe sepsis and septic shock (PROWESS-SHOCK) study. Even though the recombinant human APC, also known as drotrecogin alfa (activated) (DrotAA), was initially reported to have a beneficial effect in the treatment of sepsis (Bernard et al. 2001), it was later concluded that no evidence was found that DrotAA indeed reduced the death rate in septic patients, and instead could cause bleeding complications to occur due to the anti-coagulatory effect of DrotAA (Ranieri et al. 2012). In spite of the failure of this particular clinical trial, there may yet be ways to circumvent pathophysiological issues that troubled the PROWESS-SHOCK study, while still making use of the protective effect on the endothelial barrier induced by APC-dependent PAR1 signaling. This will be discussed later in this review.

Interestingly, even though thrombin and APC have opposite effects via the stimulation of PAR1, APC is dependent on the presence of thrombin for its formation. Thrombin converts the precursor protein C (PC) into APC, a process that is vastly accelerated upon exposure of the complex to thrombomodulin (TM) and endothelial protein C receptor (EPCR), both of which are expressed on the surface of endothelial cells (Levi et al. 2010). Once converted into its activated form, APC inhibits formation of thrombin, forming a negative feedback loop that regulates thrombin levels (Bernard et al. 2001). It has been shown that low concentrations of thrombin exert a protective effect on the endothelial barrier function in an in vitro setting (Feistritzer et al. 2005). Although this mechanism is incompletely understood, one could speculate that the protective effects of low, not high, concentrations of thrombin stem from protein C activation by thrombin while unable to exert the barrier disruptive effect via thrombin-dependent stimulation of PAR1. In contrast, considering that thrombin facilitates the formation of APC, low thrombin levels would also be expected to lead to low APC, but this is not the case. Therefore, the decreased availability of APC in sepsis is not merely due to the altered thrombin levels. Rather, inflammatory cytokines, most notably tumor necrosis factor alpha (TNFα), have been shown to reduce the expression of TM on the surface of endothelial cells during inflammation (Moore et al. 1989) via NF-κB activation (Lin et al. 2010; Sohn et al. 2005) which will be discussed in detail later. TM is recognized as a key component in the complex converting protein C to APC and its downregulation has important implications for the APC-mediated modulation of microvascular leakage. Thus, the decreased expression of TM might be a major contributor to the decreased APC levels in sepsis despite increased thrombin levels.

The next step is to further investigate APC-induced protection of the endothelial barrier function. It was established that a high APC:thrombin ratio caused PAR1 to exert a protective effect on the endothelial barrier. A proposed mechanism is the activation of sphingosine kinase (SK) by APC-dependent PAR1 signaling, specifically the SK subspecies SK1 (Feistritzer et al. 2005). SK1 intracellularly phosphorylates sphingosine, a constituent of the cell membrane, in order to form sphingosine-1-phosphate (S1P). S1P is transported out of the cell via mechanisms that are not fully understood, although possibly Spinster homolog 2
(SPNS2) plays a role in S1P exportation from endothelial cells (Hisano et al. 2012). Furthermore, the ATP-binding cassette (ABC) transporter family is involved in transporting S1P out of the endothelial cell, although which members of the ABC family are exactly involved and the extent of their contribution to the overall export of S1P is still under debate (Nishi et al. 2014). Now located in the blood, S1P associates with carriers such as albumin and high-density lipoprotein (HDL) (Aoki et al. 2005). Interestingly, HDL contains a lipocalin called apolipoprotein M (apoM) that is not only able to contain S1P within the HDL complex, but delivers it back to the endothelial cell surface to be recognized by the membrane-bound sphingosine-1-phosphate receptor 1 (S1P₁) extracellularly (Christoffersen et al. 2011). Even though it is not completely clear how activation of this pathway leads to protection of the endothelial barrier, it was shown in vitro that such a protection did not occur after inhibition and downregulation of either SK or S1P₁ prior to APC stimulation (Feistritzer et al. 2005). This implies that the protective effect of APC on the endothelial barrier is dependent on both S1P formation and S1P-dependent activation of S1P₁. It was discussed earlier how during inflammation, decreased expression of TM might contribute to decreased APC levels. Subsequently, this could lead to decreased APC-dependent PAR1-activation and thus a reduction in activation of SK1 and S1P levels, which could play a role in microvascular leakage in sepsis. S1P is also metabolized intracellularly via either cleavage by S1P lyases or dephosphorylation by S1P phosphatases, and extracellularly via dephosphorylation by lipid phosphate phosphatases (LPP) (Nishi et al. 2014). These enzymes have in common that they reduce activation of S1P₁ by S1P, by decreasing the levels of S1P presented to S1P₁ via structural modifications to S1P. A subspecies of the S1P phosphatase family, sphingosine-1-phosphate phosphatase 2 (SPP2), was shown to have an increased expression and activity under inflammatory conditions in primary human umbilical vein endothelial cells (HUVEC) after exposure to TNFα (Mechtcheriakova et al. 2007). Moreover, the same study demonstrated that SPP2 transcription was dependent on NF-κB activation, indicating that SPP2 is part of a larger pro-inflammatory pathway (Mechtcheriakova et al. 2007). This finding shows that the protective effect of S1P₁ on the endothelial barrier is being compromised during inflammation by dephosphorylation of its agonist S1P by SPP2, thus reducing activation of S1P₁. Two mechanisms have now been identified that inhibit formation of S1P, a barrier protection molecule: the first is the downregulation of TM, leading to decreased levels of APC which prevents APC-dependent activation of PAR1 and subsequent decreased activation of SK1, and the second being NF-κB induced upregulation and increase in activity of SPP2, leading to decreased S1P levels.
Figure 1. Signal transduction pathway illustrating the protective effect on endothelial barrier function by S1P. In sepsis-associated inflammation, pro-inflammatory cytokines such as TNFα are recognized by TNFR on the endothelial cell surface, leading to activation and subsequent translocation to the nucleus of pro-inflammatory transcription factor NF-κB. Protein C (PC) is converted into activated protein C (APC) by thrombin (T) in association with thrombomodulin (TM) and endothelial protein C receptor (EPCR). However, upon nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) induced gene transcription, TM is downregulated, hereby decreasing the turnover of PC to APC. APC and low concentrations of T activate protease-activated receptor 1 (PAR1), which activates sphingosine kinase 1 (SK1). SK1 phosphorylates the lipid sphingosine (Sph) to form sphingosine-1-phosphate (S1P). This process can be reversed by sphingosine-1-phosphate phosphatase 2 (SPP2), whose activity is increased upon NF-κB induced transcription of pro-inflammatory genes, and S1P can also be degraded by sphingosine-1-phosphate lyase (SPL). S1P is transported out of the cell by ATP-binding cassette (ABC) and Spinster homolog 2 (SPNS2), where it is able to associate with carrier proteins such as albumin (Alb) and high-density lipoprotein (HDL) or can be dephosphorylated by lipid phosphate phosphatase (LPP). Bloodborne S1P can activate sphingosine-1-phosphate receptor 1 (S1P1), resulting in protection of the endothelial barrier. On the other hand, high levels of T are able to activate PAR1 in an alternate manner, which results in activation of Ras homolog gene family, member A (RhoA) and ultimately disruption of the endothelial barrier. Abbreviations: ABC, ATP-binding cassette; Alb, albumin; APC, activated protein C; EPCR, endothelial protein C receptor; HDL, high-density lipoprotein; LPP, lipid phosphate phosphatase; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; T, thrombin; TNFα, tumor necrosis factor alpha; TNFR, tumor necrosis factor alpha receptor; TM, thrombomodulin; PAR1, protease-activated receptor 1; PC, protein C; RhoA, Ras homolog gene family, member A; S1P, sphingosine-1-phosphate; S1P1, sphingosine-1-phosphate receptor 1; SK1, sphingosine kinase 1; Sph, sphingosine; SPL, sphingosine-1-phosphate lyase; SPNS2, Spinster homolog 2; SPP2, sphingosine-1-phosphate phosphatase 2.
ROCK-induced contraction of stress fibers induces microvascular leakage

The integrity of the endothelial barrier has been identified as an important factor in the development of sepsis (Goldenberg et al. 2011). Even though a barrier function altering pathway has already been described above, changing of the actual physical properties of the endothelial cells has not yet been discussed. The cells of the continuous endothelium are lined up in the blood vessels in a characteristic squamous manner, in order to form a physical barrier with close cell-cell interactions between adjacent cells. This shape can be altered by changing the properties of the endothelial cell cytoskeleton, hereby potentially inhibiting adequate cell-cell interactions to take place that then compromises the integrity of the endothelial barrier function. The cytoskeleton consists of several structural elements. Here we will be focusing on the common actin filaments throughout the endothelial cell because of their involvement in barrier disruptive cytoskeleton rearrangements (Gröger et al. 2009; Hoang et al. 2011).

Special complexes of actin filaments and myosin called stress fibers are known to be regulated by Ras homolog gene family, member A (RhoA), most notably via activation of Rho-associated protein kinase (Rho kinase), also known as ROCK (Essler et al. 1998). Phosphorylation of the myosin light chain (MLC) enables the stress fibers to contract, which alters the cell structure and subsequently disrupts the endothelial barrier function (Figure 2). ROCK is responsible for the phosphorylation of myosin light chain phosphatase (MLCP) at an important inhibitory site of MLCP (Khromov et al. 2012), which reduces MLCP activity and subsequently prevents the dephosphorylation of MLC and stimulates its contractility. Furthermore, ROCK also phosphorylates MLC directly, although its overall contribution to stress fiber contractility in microvascular leakage is not clear (Totsukawa et al. 2000). Phosphorylation of peripheral MLC by myosin light chain kinase (MLCK) also leads to contraction of the endothelial cell and subsequently increased permeability (Rigor et al. 2013; Totsukawa et al. 2000). Under basal conditions however, ROCK also plays a key role in maintaining the endothelial barrier function, with inhibition of ROCK resulting in significant worsening of the integrity of the endothelial barrier in HUVEC (van Nieuw Amerongen et al. 2007).

ROCK plays a dual role in the regulation of the endothelial barrier function, at least in vitro: its activation is associated with a decreased barrier function under inflammatory conditions as well as an improved barrier function under basal conditions. A potential role of thrombin in the ROCK pathway is interesting, because thrombin levels are increased during sepsis. In addition, thrombin has been described as a potent activator of the RhoA / ROCK pathway, although the exact mechanism of this activation is not yet clear. In vitro, thrombin increased endothelial permeability, which may be an effect of the observed increased phosphorylation of myosin and formation of stress fibers (van Nieuw Amerongen et al. 2000). This might indicate that activation of ROCK by thrombin may contribute to sepsis-induced microvascular leakage. Although the exact mechanisms of both effects of ROCK activation, the decreased barrier function during inflammation and the improved barrier function under basal conditions, require further elucidation to determine their contribution to overall barrier function, the disruption of the endothelial barrier function during inflammation in sepsis may be caused by activation of the PAR1 receptor by thrombin. Indeed, downstream activation of ROCK after thrombin-dependent activation of PAR1 has been suggested in corneal epithelial and
endothelial cells *in vitro* (Narayan et al. 2010; Satpathy et al. 2004). To our knowledge, the activation of ROCK by thrombin via PAR1 has not yet been implied specifically as a potentially harmful pathway in sepsis. In our description of dysregulated APC formation in sepsis, we noted how PAR1 can be activated by both thrombin and APC. APC-dependent stimulation of PAR1 resulted in protection of the endothelial barrier function, potentially through S1P, whereas thrombin-dependent stimulation of PAR1 was shown to activate RhoA and induced a loss of endothelial barrier integrity. If this connection turns out to be present during sepsis, then the activation of ROCK might provide us with an additional explanation of microvascular leakage via the elevated thrombin levels in sepsis.

**Figure 2.** Signal transduction pathway illustrating the disruptive effect on endothelial barrier function by ROCK. Ras homolog gene family, member A (RhoA) activates Rho-associated protein kinase (ROCK), which phosphorylates myosin light chain phosphatase (MLCP), hereby inactivating MLCP. Furthermore, together with myosin light chain kinase (MLCK), ROCK phosphorylates myosin light chain (MLC). Phosphorylated MLC can be dephosphorylated by active MLCP. Upon phosphorylation, MLC induces the formation and contraction of stress fibers in the cell, causing the cell to contract, leading to reduced cell-cell contacts and microvascular leakage. **Abbreviations:** MLC, myosin light chain; MLCK, myosin light chain kinase; MLCP, myosin light chain phosphatase; RhoA, Ras homolog gene family, member A; ROCK, Rho-associated protein kinase.
NF-κB nuclear translocation induces activation of endothelial cells

During the pro-inflammatory phase of sepsis, endothelial cells can become activated by a variety of exogenous agents, most notably by lipopolysaccharide (LPS) during infection with Gram-negative bacteria. Furthermore, immune cells in the host release a variety of pro-inflammatory cytokines, providing a second pathway to induce the activation of endothelial cells (Cohen, 2002). The signal transduction pathway involved depends on the presence of either exogenous pathogen constituents or pro-inflammatory cytokines, or both. LPS for example is recognized by Toll-like receptor 4 (TLR4), which belongs to a family of pattern recognition receptors (PRR) that function as an important part of the innate immune response against invading pathogens (Akira et al. 2001). Alternatively, pro-inflammatory cytokines are recognized by the endothelial cells by specific receptors. For example, TNFα is recognized via its receptors TNFα receptor type 1 (TNFR1) and TNFα receptor type 2 (TNFR2), depending on the origin of the endothelium that responds (Zhou et al. 2007). Although different receptors and agonists are involved, activation of TLR4 by LPS as well as activation of TNFR1 by TNFα are known to induce the translocation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) to the nucleus (Akira et al. 2003; Daun et al. 2000; Zhou et al. 2007) (Figure 3). As a transcription factor, NF-κB induces the transcription of a variety of pro-inflammatory genes, which contribute to the dysregulation of the endothelium in sepsis (Kisseleva et al. 2006). Without stimulation by extracellular pro-inflammatory signals, NF-κB is retained in the cytoplasm via an interaction with inhibitor of κB (IκB). This interaction prevents NF-κB from translocating to the nucleus and altering gene transcription, due to concealment of the nuclear localization signal of NF-κB by IκB (Jacobs & Harrison, 1998). In particular the IκB subtype IκBα can be phosphorylated by IκB kinase (IKK), which is a downstream responder of TNFR1/2 and TLR4, and causes IκBα to dissociate from NF-κB and be degraded in a proteasome. Upon dissociation of IκBα, the nuclear localization signal of NF-κB is no longer concealed, enabling its translocation to the nucleus.

If the translocation of NF-κB to the nucleus can be somehow inhibited, a major contributor to the pro-inflammatory response in sepsis might be excluded (Ye et al. 2008). In addition, it has been noted that the pro-inflammatory response or SIRS is thought to be responsible for the tissue damage present in severe sepsis, which may be relieved upon prevention of the nuclear translocation of NF-κB. With the mechanism of NF-κB activation being described, an obvious target to prevent NF-κB activation would be IKK. NF-κB already has an endogenous inhibitor in IκB, so in theory, inhibiting the IKK-induced phosphorylation and subsequent degradation of IκBα could prove sufficient in preventing NF-κB translocation to the nucleus. Indeed, this strategy has been shown to be successful in a cecal ligation and puncture model (CLP), an animal model for sepsis. After onset of sepsis, mice were treated with IKK inhibitor IKK 16, a 2-benzamido-pyrimidine which decreased the activity of the IKK complex (Waelchli et al. 2006), reduced IκBα phosphorylation, nuclear translocation of NF-κB and even attenuated sepsis-associated organ dysfunction (Coldewey et al. 2013). More specifically, this treatment showed attenuation of cardiac dysfunction (systolic contractility), renal dysfunction (serum creatinine/urea), hepatocellular injury (alanine aminotransferase (ALT)/aspartate aminotransferase (AST)), and lung inflammation (myeloperoxidase (MPO)) (Coldewey et al. 2013). This was achieved by treating the mice with IKK 16 1 hour after undergoing CLP. This study indicates that specific inhibition of processes along the NF-κB...
pathway might be a promising approach for treating organ dysfunction at multiple sites in sepsis. Although Coldeway and colleagues (2013) elegantly showed the value of IKK 16 in this in vivo model with respect to ameliorating organ dysfunction, it would be interesting to see what results would be obtained upon administration of IKK 16 at a later point in time. As unfortunately, not all septic patients will be able to receive treatment within 1 hour after onset. Furthermore, whether similar results can be obtained upon extension of the period between disease onset and IKK 16 treatment remains to be investigated. Based on these studies, several statements can be made about the role of NF-κB in sepsis. First, IKK plays a key role in the regulation of NF-κB in vivo, and appears to be a suitable target for therapy. Second, reduced activation of NF-κB target genes is directly associated with an attenuation of organ dysfunction in sepsis, suggesting a role for NF-κB as an important factor in sepsis related systemic inflammation.

The importance of NF-κB in microvascular inflammation in sepsis is further illustrated by a study in which plasma from septic patients was added to HUVEC, resulting in endothelial cell activation and increased apoptosis. The NF-κB pathway was activated to a higher extent in cells treated with plasma from septic patients compared to those treated with plasma from non-septic controls, thus suggesting the existence of a link between sepsis-associated endothelial dysfunction and NF-κB activation in vitro (Liang et al. 2014). However, the same study also found that the apoptosis in the cells increased upon pretreatment with NF-κB pathway inhibitor before incubation with plasma from septic patients. It is therefore important to note here that NF-κB does not universally induce damaging and disruptive effects on the endothelium via its promotion of pro-inflammatory pathways, but that protective effects are also likely elicited via the NF-κB pathway. The extent to which either of these dual effects occur in vitro is incompletely understood and is likely to be even more complex in vivo than implied by this in vitro characterization. Moreover, organ injury and microvascular leakage were ameliorated in a transgenic mouse model, in which mice overexpressed a NF-κB inhibitor exclusively in endothelial cells (Ye et al. 2008). If apoptosis in sepsis is indeed shown to be extended through NF-κB inhibition in endothelial cells in patients as well, then this is something therapeutic strategies focusing on the reduction of NF-κB signaling need to take into account.
Figure 3. Signal transduction pathway illustrating the activation of endothelial cells following nuclear translocation of NF-κB. Pro-inflammatory cytokine tumor necrosis factor alpha (TNFα) is recognized by endothelial tumor necrosis factor alpha receptor (TNFR) and/or bacterial lipopolysaccharide (LPS) is recognized by Toll-like receptor 4 (TLR4), hereby activating IkB kinase (IKK). Inhibitor of κB (IkB) masks the nuclear translocation signal of NF-κB, thus keeping it in the cytosol and preventing nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) to induce the transcription of pro-inflammatory genes. However, upon activation of IKK, IkB is phosphorylated and dissociates from NF-κB. IkB is then enzymatically degraded by the proteasomes, whereas NF-κB is now free to translocate to the nucleus and induce the activation of endothelial cells. 

**Abbreviations:** IkB, inhibitor of κB; IKK, IkB kinase; LPS, lipopolysaccharide; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; TLR4, Toll-like receptor 4; TNFα, tumor necrosis factor alpha; TNFR, tumor necrosis factor alpha receptor.

**Angpt1-induced stimulation of Tie2 protects endothelial barrier function**

Another target that the sepsis field has put its attention to in more recent years, is the activation of tunica intima endothelial kinase 2 (Tie2) in endothelial cells. In the context of sepsis, two ligands appear to be playing a key role in regulating Tie2 signaling. First, binding of the agonist angiopoietin-1 (Angpt1) to Tie2 induces anti-apoptotic (DeBusk et al. 2004), anti-inflammatory (Hughes et al. 2003) and anti-permeability effects (Gavard et al. 2008) (Figure 4). The second ligand is the antagonist angiopoietin-2 (Angpt2), which competitively binds to Tie2 and prevents interaction between Angpt1 and Tie2, thus inhibiting protective effects on endothelial cells to occur (Maisonpierre et al. 1997). Two of the three protective
effects of Tie2 activation by Angpt1, anti-inflammation and anti-permeability, are realized via pathways that have already been described in this review. Tie2 activates the phosphoinositide 3-kinase (PI3K) / AKT pathway, which is reviewed in detail elsewhere (Franke, 2008). PI3K/AKT exerts its anti-inflammatory effect through inhibition of NF-κB (Zheng et al. 2013) and its anti-permeability effect via a decreased activation of ROCK, although this mechanism is incompletely understood (David et al. 2013; Li et al. 2004). The third effect, prevention of apoptosis of endothelial cells, is elicited via PI3K/AKT pathway activation as well (DeBusk et al. 2004; Kim et al. 2000) and enhances the survival of endothelial cells.

Angpt1/Tie2 signaling is disrupted during sepsis, due to increased levels of the antagonist Angpt2 (David et al. 2012), resulting in increased inflammation, apoptosis and microvascular leakage (Augustin et al. 2009). Furthermore, Tie2 is downregulated after LPS injection mice in several organs, including brain, kidney and liver (Kurniati et al. 2013). A recent study in renal biopsies of septic patients with renal dysfunction has shown that mRNA levels of both Angpt1 and Angpt2 decreased in sepsis, with Angpt1 levels being decreased to a greater extent (Aslan et al. 2014). Moreover, the same study showed that mRNA levels of Tie2 were also significantly decreased in the renal microvasculature sepsis (Aslan et al. 2014), indicating that therapeutic intervention via this pathway is likely to be more complex than merely altering Angpt1/2 levels.

Due to the ability of Angpt1 and Angpt2 to bind competitively to Tie2, it is expected that either increased levels of Angpt1 or decreased levels of Angpt2 or both would improve endothelial cell functioning via increased Tie2 activation. In vivo mouse studies showed that Angpt2 depletion due to gene knockout in a LPS/endotoxin model failed to prevent kidney dysfunction, although a decrease in inflammatory endothelial cell activation was observed (Kurniati et al. 2013). Although this one animal study does not indicate that a therapy using an Angpt2 depletion strategy has no chance to succeed in septic patients, it does suggest that more success may be obtained when other routes along this pathway are also explored. For example, the combined effect of increased Angpt1 levels and decreased Angpt2 levels has the additional advantage of not merely reducing the inhibitory effect of Angpt2 on Tie2, but also to increase Angpt1-dependent activation of Tie2. One study demonstrated how increased Angpt1 levels could be obtained with the more potent Tie2 activator cartilage oligomeric matrix protein-angiopoietin-1 (COMP-Angpt1) (Kim et al. 2009). COMP-Angpt1 was administered via a recombinant adenovirus, which caused COMP-Angpt1 levels to peak after 3 days, at which point LPS was added to the mice. Indeed, COMP-Angpt1 was shown to have a beneficial effect in organ dysfunction, specifically via attenuation of renal dysfunction, in an LPS/endotoxin model in mice (Kim et al. 2009). In addition, it would be interesting to determine whether COMP-Angpt1 treatment also ameliorates the dysfunction of organs other than the kidney. Furthermore, studying whether the protective effects of COMP-Angpt1 persist upon administration after LPS injection, instead of administrating LPS at the peak in COMP-Angpt1 levels, would also reveal more about the potential clinical applicability of this compound. Nevertheless, a dual therapy of both COMP-Angpt1 or similar treatment and a reduction of Angpt2 levels is an interesting prospect for now that may be worth investigating further.
Figure 4. Signal transduction pathway illustrating the diverse effects on endothelial cells following Tie2 activation. Angiopoietin-1 (Angpt1) activates tunica intima endothelial kinase 2 (Tie2), whereas angiopoietin-2 (Angpt2) competitively binds to Tie2, thus preventing Angpt1-dependent activation of Tie2. Activation of Tie2 leads to activation of the phosphoinositide 3-kinase/AKT (PI3K/AKT) pathway, which causes: 1) inhibition of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), leading to an anti-inflammatory effect, 2) anti-apoptotic effect and 3) direct or indirect decreased activation of Rho-associated protein kinase (ROCK), leading to protection of the endothelial barrier. **Abbreviations:** Angpt1, angiopoietin-1; Angpt2, angiopoietin-2; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; PI3K/AKT, phosphoinositide 3-kinase/AKT; ROCK, Rho-associated protein kinase; Tie2, tunica intima endothelial kinase 2.

**Overview of endothelial barrier altering pathways**

The overview shown in Figure 5 provides an extensive yet incomplete signal transduction scheme concerning the disruption of the endothelial barrier function during sepsis. Simplifications have been made in this figure for the sake of clarity or because more exact molecular mechanisms that are involved in these processes are still incompletely understood. Nevertheless, it depicts how the pathways that have already been described individually in sepsis have both separate and overlapping molecular routes through which their effects on the endothelial barrier function are exerted. For example, it is interesting to see that thrombin-dependent activation of PAR1 stimulates the RhoA/ROCK pathway, ultimately leading to increased contraction of cellular stress fibers and microvascular leakage. ROCK is inhibited as well by activation of Tie2 through unknown mechanisms (David et al. 2013; Li et al. 2004). Tie2 activation also inhibits nuclear translocation of NF-κB (Zheng et al. 2013), which in theory prevents both NF-κB induced downregulation of TM (Lin et al. 2010; Sohn et al. 2005) and the occurrence of increased levels of SPP2 (Mechtcheriakova et al. 2007). In theory, the effect of Tie2 with respect to the APC pathway is twofold. On the one hand, prevention of the downregulation of TM should lead to an
increased conversion of PC to APC, which would increase both APC-dependent activation of PAR1 and the blocking of thrombin by APC (Feistritzer et al. 2005). On the other hand, by inhibiting the upregulation of SPP2, increased amounts of S1P are produced by the stimulated SK1, which increases the availability of S1P to exit the cell and stimulate the S1P1 receptor. S1P1 activation will then exert a protective effect on the endothelial barrier (Feistritzer et al. 2005). This illustrates how inactivation of NF-κB affects both the availability of APC as an agonist to PAR1, and the effectiveness of the pathway further downstream due to the prevention of upregulation of SPP2.

When studying the endothelial barrier in sepsis, researchers tend to alter the expression or activation of one minor constituent of one individual pathway in an attempt to uncover a possible pharmaceutical target to attenuate the barrier dysfunction. However, considering the sheer complexity of the involved pathways and the signaling between the pathways themselves as described earlier, it seems improbable that such a target will be found anytime soon, if it exists at all. Therefore, a more promising approach might be to both stimulate barrier protective pathways, for example the S1P pathway, while simultaneously inhibiting the pathways that contribute to microvascular leakage, such as ROCK. This may contribute to the restoration of the endothelial barrier function to a greater extent than either approach would be able to realize separately. Moreover, targeted delivery of pharmaceutical agents might be equally important in guiding the response of different cell types. For example, the clinical trial with recombinant human APC (PROWESS-SHOCK) might have failed because of interactions of APC with cells other than endothelial cells. It cannot be excluded that APC did have a protective effect on the endothelial barrier function in sepsis in the PROWESS-SHOCK trials, but that this effect was overshadowed by other effects caused by interactions of APC with other cell types, for example monocytes (Stephenson et al. 2006) and dendritic cells (Matsumoto et al. 2015). Targeted delivery of pharmaceutical agents might reduce the impact of possible side effects while allowing researchers to study the effect of the drug on the cells of interest – affected endothelial cells – more closely. An interesting tool for targeted intracellular endothelial cell delivery of drugs might be lipid based particles called SAINT-O-Somes, which are liposomes supplemented with Synthetic Amphiphile INTeraction (SAINT-C18). SAINT-O-Somes are able to efficiently and with low toxicity deliver drugs to selected cell subsets (Adrian et al. 2010). Selectivity of the SAINT-O-Somes can be assured through supplying their surface with an antibody against the endothelial cell’s exclusive E-selectin molecule. By encapsulating a drug within the SAINT-O-Somes, it is released intracellularly in the endothelial cell cytoplasm via an incompletely understood mechanism (Adrian et al. 2010). Although more research is required to further verify the viability of the SAINT-O-Somes as a drug delivery strategy, it seems to be a promising approach to affect expression and/or activity of kinases and phosphatases involved in microvascular leakage in sepsis. Indeed, SAINT-O-Somes have already been shown to efficiently deliver functional short interfering RNA (siRNA) in HUVEC and human aortic endothelial cells (HAEC) as well as in vivo (Kowalski et al. 2013), which might be an interesting therapeutic strategy to selectively deliver drugs to inflamed endothelial cells in sepsis.
Figure 5. Overview of signal transduction pathways illustrating their effects on endothelial cell activation and endothelial barrier function. The red, yellow, blue and green backgrounds represent the NF-κB, S1P, Tie2 and ROCK signaling pathways, respectively. Their individual descriptions can be found in the legends of Figure 1-4. This overview illustrates the complexity of microvascular leakage in sepsis as well as the crosstalk between. After exposure to inflammatory cytokines or lipopolysaccharide (LPS), NF-κB can translocate into the nucleus and cause the activation of endothelial cells as well as hinder the barrier protective S1P pathway. This activity of NF-κB is inhibited by Angpt1/Tie2 dependent activation of PI3K/AKT, which is also known to have anti-apoptotic effects and is also linked to inhibition of the barrier disruptive ROCK-pathway. Alternatively, high levels of thrombin are able to activate PAR1 in an alternate manner, which results in activation of RhoA and ultimately disruption of the endothelial barrier via ROCK activation. **Abbreviations:** ABC, ATP-binding cassette; Alb, albumin; Angpt1, angiopoietin-1; Angpt2, angiopoietin-2; APC, activated protein C; EPCR, endothelial protein C receptor; HDL, high-density lipoprotein; IkB, inhibitor of κB; IKK, IkB kinase; LPP, lipid phosphate phosphatase; LPS, lipopolysaccharide; MLC, myosin light chain; MLCK, myosin light chain kinase; MLCP, myosin light chain phosphatase; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; PAR1, protease-activated receptor 1; PC, protein C; PI3K/AKT, phosphoinositide 3-kinase/AKT; RhoA, Ras homolog gene family, member A; ROCK, Rho-associated protein kinase; S1P, sphingosine-1-phosphate; S1P1, sphingosine-1-phosphate receptor 1; SK1, sphingosine kinase 1; Sph, sphingosine; SPL, sphingosine-1-phosphate lyase; SPNS2, Spinster homolog 2; SPP2, sphingosine-1-phosphate phosphatase 2; T, thrombin; TNFα, tumor necrosis factor alpha; Tie2, tunica intima endothelial kinase 2; TLR4, Toll-like receptor 4; TNFα, tumor necrosis factor alpha; TNFR, tumor necrosis factor alpha receptor; TM, thrombomodulin.
Animal models in sepsis

Experiments in animal models make up a large part of sepsis studies and are of importance for the translation of *in vitro* data to *in vivo* data. In sepsis, two models are commonly used. The endotoxemia model is based on the systemic administration of LPS to the animal, causing an inflammatory response through activation of TLR4. However, sepsis associated with fungal, viral or Gram-positive bacterial infection (van der Poll et al. 2008) is not represented by this model, because of its sole focus on the constituent of Gram-negative bacteria. Furthermore, endotoxemia does not accurately mimic changes in human sepsis with respect to cytokine profiles, as is exemplified by the strong and sudden increase in a variety of pro-inflammatory cytokines that does not occur to the same extent in humans (Dejager et al. 2011).

The cecal ligation and puncture (CLP) model takes a different approach than the endotoxemia model. In CLP, the cecum is ligated and then punctured according to a fixed protocol (Cuenca et al. 2010), which causes fecal matter to leak into the abdomen of the animal, thus inducing sepsis. CLP is generally considered to be the best sepsis model currently available, in part due to the occurrence of a cytokine profile similar to human sepsis. However, CLP also has several limitations. After cecal puncture, it is difficult to precisely control the extent of bacterial invasion into the blood stream, potentially causing the animals to develop sepsis of varying degrees of severities. Furthermore, the puncture itself is difficult to standardize due to experimental variations in the method of operation. In addition, Dejager et al. (2011) composed an extensive list with discrepancies between the CLP model and patients that may be relevant in the translation from animal models to clinical therapy, which until now has been suboptimal at best. This list includes age, gender, weight, comorbidities, and supportive care such as resuscitation (Dejager et al. 2011; Ward, 2012). One discrepancy that requires additional attention, is the start of experimental treatment. Although this has already been discussed briefly, it is important to stress the importance of administering the drug of choice after onset of sepsis. Many studies using CLP have started drug treatment simultaneously with sepsis onset, or even before that. While this allows studying the mechanisms contributing to sepsis, it can be detrimental when determining the viability of a new drug for sepsis treatment. In the clinic, patients always develop sepsis before being able to receive treatment, not vice versa. Therefore, while it might be interesting and insightful to observe an attenuation of sepsis symptoms after pretreatment with a particular drug, its clinical relevancy will be limited due to the timing of the intervention. Initiation of drug intervention after CLP is more likely to provide more information at an earlier stage of development about the applicability of the drug to patients who follow a similar disease progression. This approach was already used by a study, which showed positive results in mice survival upon administration of IL-17A antibodies 12 hours after CLP (Flierl et al. 2008).

Despite these difficulties, CLP is still the animal model of choice for studying sepsis and has contributed immensely to the recent increase in knowledge about the mechanisms involved in the development of sepsis. However, CLP can be further optimized by following the aforementioned recommendations concerning alterations in the model in timing of drug intervention, in order to increase the probability that endothelial barrier enhancing drugs or anti-sepsis drugs in general that have good outcomes in animal studies are also applicable in human sepsis.
Discussion

The aim of this review was to create an overview of endothelial barrier dysfunction in sepsis, including causative mechanisms, effects on sepsis development, and potential druggable kinases and phosphatases to attenuate microvascular leakage. More specifically, emphasis was placed on identifying molecular mechanisms contributing to microvascular leakage that were both well-defined and involving one or multiple potentially druggable kinases or phosphatases. The pathways that were described in this review were S1P, ROCK, NF-κB and Tie2 pathways, which were all summed up in Figure 5. Although this figure shows the signal transduction route of these pathways, including signaling between the different pathways and potential druggable targets, it encompasses several simplifications. Furthermore, some links between the pathways were excluded, due to controversy concerning the existence of these links or because they simply did not fit in with the other pathways. Regardless of this, it is interesting to mention them to further illustrate the complexity of the maintenance of the endothelial barrier. For instance, Angpt1-dependent Tie2 activation is shown to decrease endothelial cell permeability, via stimulation of SK1 activity and a subsequent increase in S1P concentrations (Gamble et al. 2000; Li et al. 2008). SK1 is also stimulated by APC-dependent PAR1 activation (Feistritzer et al. 2005), thus implying that the barrier protective effect of both APC and Tie2 activation might be partially induced in a similar way. Furthermore, S1P has also been linked to controlling MLCK activity, an important player in stress fiber contraction in endothelial cells (Rigor et al. 2013). In addition, ezrin, radixin, and moesin (ERM) are actin binding proteins that are involved in S1P-dependent barrier enhancement (Adyshev et al. 2011). Moreover, ERM proteins have been suggested to be phosphorylated via thrombin-dependent PAR1 activation, which also stimulated the ROCK pathway. Interestingly, the ERM proteins appear to have opposing effects on the endothelial barrier, with depletion of moesin resulting in attenuation of endothelial barrier dysfunction, whereas radixin depletion contributes to endothelial barrier dysfunction (Adyshev et al. 2013). Elucidating the exact roles of these proteins and their involvement in the other, as of now, better-described pathways in barrier disruption might be promising in gaining a better understanding in the cytoskeletal movements in endothelial cells in sepsis.

Another interesting finding is the protective effect of the drug imatinib on endothelial barrier function and edema formation, which functions through inhibition of Abl-related gene kinase (Arg) (Aman et al. 2012). Arg has not been described in microvascular leakage in sepsis in any of the other pathways described here, and although its exact functioning is not known, the successful imatinib treatment in vitro and in vivo illustrates the potential value of treating sepsis via attenuation of endothelial barrier dysfunction.

The next step is to transition from the molecular mechanisms to the actual patients. In the treatment of septic patients, sensitive, specific biomarkers that are easily detected at a low cost have yet to be discovered. In addition, it is difficult to find biomarkers that are able to discern between septic inflammation and local inflammation, as well as biomarkers being able to reliably predict organ specific vascular leakage. A recent study proposes to use the morphology, mechanics and motility of neutrophils to make a more accurate sepsis diagnosis (Zonneveld et al. 2015). More specifically, neutrophils become less granular, more rigid, migrate to a lower extent and increase in total number in sepsis when compared to healthy
individuals. Moreover, the degree of changes in neutrophil parameters are related to severity and mortality in sepsis. To study neutrophils for this purpose, Zonneveld proposes the use of automated hematology cell analyzers and microfluidic devices.

Furthermore, the recent increase in knowledge concerning the mechanisms behind the development of sepsis makes it even less likely that the ideal biomarker will ever be found. For when sepsis is considered as an overarching term for conditions that include infections by vastly different pathogens – bacteria, fungi, viruses – that originate from infections in different organs – lungs, intestine, bladder – it seems near impossible that one biomarker would exist that completely covers this heterogeneity. Instead, a more fruitful approach might be to determine in which of the previously described immunological stages of sepsis – SIRS or CARS – the patient is currently in. This would allow for the administration of anti-sepsis drugs based on the needs of the patient. For example, drugs that exert an anti-inflammatory effect might be beneficial in SIRS, but not in CARS where the anti-inflammatory effect is already dominant. Monitoring cytokine profiles in septic patients seems to be the most straightforward approach to gain further insight in which stage the patient is currently in. However, determining cytokine levels in the clinic is currently not common practice and requires more testing before its value in determining immunological sepsis stages can be ascertained. Moreover, extensive studies are required to find out how each stage is associated with the development of microvascular leakage in sepsis, in order to determine which endothelial barrier altering treatment might be most beneficial at which stage.

Summarizing, this review describes several pathways involved in endothelial barrier dysfunction in sepsis, which is a major regulator in sepsis associated hypotension, edema formation, and organ dysfunction. These pathways include S1P, ROCK, NF-κB and Tie2 pathways, all of which include kinases or phosphatases are interesting targets to attenuate microvascular leakage in sepsis. The functioning of these pathways and their interactions with one another are only known in part, and future research on this subject is expected to describe in more detail which targets are most suitable for targeted development. To ensure specific drug delivery to endothelial cells in order to prevent unintended effects on other cells, the lipid based SAINT-O-Somes might prove to be a promising delivery system. In order to make the translation from animal models like CLP to actual septic patients run more fluently, changes to the CLP model might be necessary to more accurately replicate the conditions in patients. Finally, adequate anti-sepsis drugs have long eluded scientists and clinicians alike. Attenuating microvascular leakage is one of the more novel approaches in the treatment of sepsis, and has yet to prove its value in improving the condition of patients. With our growing understanding of the molecular mechanisms underlying microvascular leakage, we will be increasingly able to intervene in ongoing processes at the right point in time. If the timing and functioning of these drugs can be optimized, the protection and restoration of the endothelial barrier function might help septic patients in the foreseeable future toward a quicker recovery.
References


