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Mending leaky blood vessels: The Angiopoietin-Tie2 pathway in sepsis

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Non-standard abbreviations:

ARDS - adult respiratory distress syndrome

DIC - disseminated intravascular coagulopathy

PI3K - phosphatidylinositol 3-kinase

GTP - Guanosintriphosphat

MSC - mesenchymal stem cells

LPS - Lipopolysaccharide

COMP - cartilage oligomeric matrix protein

HMVECs - human microvascular endothelial cells

TNF - tumor necrosis factor

CLP - cecal ligation and puncture

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ABSTRACT

Sepsis is a systemic inflammatory response to infection. A common end-feature these patients regularly suffer from is the so-called multiple organ dysfunction syndrome, an often fatal consequence of organ hypo-perfusion, coagulopathy, immune dysregulation, and mitochondrial dysfunction. Microvascular dysfunction critically contributes to the morbidity and mortality of this disease.

The angiopoietin (Angpt)/Tie2 system consists of the transmembrane endothelial tyrosine kinase Tie2 and its circulating ligands (Angpt1, 2 and 3/4). The balance between the canonical agonist Angpt-1 and its competitive inhibitor Angpt-2 regulates basal endothelial barrier function, and the leakage and vascular inflammation that develop in response to pathogens and cytokines. Here we summarize recent work in mice and men to highlight the therapeutic potential in this pathway to prevent or even reverse microvascular dysfunction in this deadly disease.

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Sepsis is a systemic inflammatory response of the organism to an -often local- infection. Most serious manifestations of the disease are severe sepsis and septic shock with organ dysfunction and hypotension. Sepsis is a major healthcare problem, affecting millions of individuals around the world each year (Martin et al., 2003). Unfortunately, its incidence appears to be rising, and the mortality caused by this syndrome remains between 38 and 59% (Dombrovskiy et al., 2007). Despite the ability to mechanically "replace" the function of the lungs and kidney, mortality rates when acute lung injury (ALI) and/or acute kidney injury (AKI) complicate sepsis range between 40-80% (Ware and Matthay, 2000; Schrier and Wang, 2004).

The pathophysiology of the disease is highly complex and includes diverse facets from an altered immune system including coagulopathy and endothelial dysfunction. Of numerous investigated pathways, only 5 randomized controlled clinical trials improved survival in septic patients about a decade ago (Brower et al., 2000; Bernard et al., 2001; Rivers et al., 2001; van den Berghe et al., 2001; Annane et al., 2002). Three of those have been disproven in larger studies with activated protein C (APC) being the latest and most prominent candidate that was removed from the market in late 2011 (Ranieri et al.). The lack of targeted therapies is not for wont of effort, but probably a result of focusing on elucidating the humoral and immune pathways over the last decades. The immune response in septic patients is at least bimodal, with an early hyper-/ and late hypo-inflammatory response, a fact that alone would require tailored patient-specific treatment regimens. Particularly hard to achieve however, is the monitoring of the highly complex and redundant immune system. As an illustrating example, the efficacy of (later failed) anti-TNF therapeutics is highly dependent on the immune status the septic organism is confronted with at the actual time of treatment. What might help one patient might actually harm another. Therefore, optimal stratification of study populations for randomized clinical trials is particularly challenging for this disease.

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The observation that clinical manifestations of sepsis (shock, ARDS, and disseminated intravascular coagulopathy [DIC]) have a strong vascular component (permeability, loss of vascular tone, disturbed endothelial – platelet interaction) suggests that further study of sepsis as a state of profound vascular dysregulation may yield additional insights. Within the vasculature, it is the endothelium that is in direct contact with the circulating blood, thereby being involved in an adaptive response to various environmental stimuli. The endothelium controls vasomotor tone, regulates cellular oxygen and nutrients trafficking, maintains blood fluidity, contributes to the local balance in pro-inflammatory and anti-inflammatory mediators, and participates in generation of new blood vessels. All of these physiological functions can be altered in sepsis. As a net result, the endothelial phenotype is changed from a more or less quiescent state to an over-activated state characterized by pro-coagulant, pro-inflammatory and hyper-permeable properties. Hyperpermeability mechanistically contributes to the impaired gas exchange characteristic for acute lung injury and ARDS by increasing the distance essential nutrients and waste products must diffuse. Moreover, it gives rise to hypovolemia aggravating hemoconcentration, stasis of blood flow, and shock.

In short, it is clear that several of the most critical clinical manifestations of sepsis arise from dysfunction and injury to blood vessels. Thus, pharmacological strategies that address the septic circulation have the potential to ameliorate sepsis-associated morbidities without incurring the risks inherent to immunomodulation during severe infection.

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The Angiopoietin / Tie2 system

The Angiopoietin (Angpt)/Tie2 system consists of the transmembrane endothelial tyrosine kinase Tie2 and its 4 circulating ligands; of those Angpt-1 and Angpt-2 have been studied most comprehensively. The activation (i.e. phosphorylation) state of Tie2 regulates baseline endothelial quiescence and its response to an injurious stimulus. In endothelial cells, Tie2 phosphorylation is largely controlled by the ratio that exists between the agonistic ligand Angpt-1 and competitive inhibitor Angpt-2 (Maisonpierre et al., 1997; Yuan et al., 2009). Their mutually antagonistic properties are supported by functional data. For example, during embryonic development, Angpt-1 and Tie2 global knockout phenotypes are indistinguishable from each other and phenocopy Angpt-2 transgenics. In inflammatory diseases, Angpt-1 and Angpt-2 appear to have opposing functions as well; the former mitigates vascular inflammation and leakage whereas the latter sensitizes the endothelium to inflammatory cytokines. From the signalling point of view, Angpt-1 ligation to Tie2 results in tyrosine phosphorylation that maintains vessel integrity, inhibits vascular leakage, suppresses inflammatory gene expression, and prevents recruitment and transmigration of leukocytes mainly by activation of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway (*31*) and by regulating small GTPase proteins (e.g. RhoA, Rac1) (*27*). Together, the endothelial effects of Angpt-1/Tie2 signaling can be summarized as anti-inflammatory, anti-apoptotic, anti-permeable and pro-survival (**Figure 1**).

As Angpt-1 and Angpt-2 are circulating proteins, they are accessible for detection in patients' blood. We and others found in septic individuals that circulating levels of Angpt-2 can increase up to ~ 50 times while Angpt-1 remains more or less unchanged (Kumpers et al., 2008; Lukasz et al., 2008). It is important to understand that Tie2 is a *growth factor receptor* that – in contrast to other family members – is constitutively

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activated in quiescent mature vasculature. While yet to be formally demonstrated, it is widely believed that tonic Tie2 signaling may mediate the quiescent, healthy state of blood vessels. In sepsis, the disturbed Angpt-1/-2 homeostasis may deactivate baseline Tie2 signalling, thereby actively contributing to the devastating over-activated endothelial phenotype. Consistent with this hypothesis, circulating Angpt-2 levels of septic individuals are strongly associated with severity of illness, markers of endothelial inflammation and most importantly with adverse outcome (Kumpers et al., 2008; Kumpers et al., 2010).

Based on these observations, several groups have investigated whether modulation of Tie2 signalling might be beneficial in experimental sepsis (**Table 1**). Two major approaches have been used:

1. The Tie2 Agonist perspective

In 2005 Witzenbichler et al. showed that excess Angpt-1 was protective in endotoxic shock (Witzenbichler et al., 2005). To achieve high levels of circulating Angpt-1, they used an adenoviral gene transfer approach. They showed improved hemodynamic function, reduced lung injury, and a lesser inflammatory response accompanied by improved survival. Although, adenoviral transduction is generally not feasible in humans and leads to a very high expression of Angpt-1 (up to 1 µg/mL) the authors demonstrated here a potential role of Angpt-1 as an adjunctive agent for the treatment of septic shock and opened a new avenue for specific sepsis therapeutics. Remembering Paracelsus' assumption, one might wonder if excess Angpt-1 could have negative side effects. The available data is controversial and mostly limited to the fine-print. In 2000 Thurston et al. report parenthetically that up to 1 month of transgenic Angpt-1 overexpression had no adverse effect on vessel morphology (Thurston et al.,

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2000). On the other hand, Dumont's group showed that prolonged transgenic Angpt-1 over-expression, even post-developmentally, was locally injurious in the liver (Ward et al., 2004).

In 2007 Mei et al. provided further evidence for the protective potential of Angpt-1. They showed in a set of *in vivo* experiments the effect of mesenchymal stem cells (MSC) over-expressing Angpt-1 for the prevention of LPS-induced ALI. Administration of MSC transfected with Angpt-1 resulted in a nearly complete reversal of LPS-induced pulmonary hyperpermeability, as reflected by reductions in IgM and albumin levels in bronchoalveolar lavage (Mei et al., 2007). Unfortunately, neither adenoviral nor modified-stem cell delivery are feasible techniques to translate these finding from mice to men.

Kim et al. determined the positive effect of an increased Angpt-1 / Tie2 signalling in LPS-induced AKI using a slightly different approach. Mice were pretreated with an engineered variant of native Angpt-1, the so-called "*Angpt-1 with cartilage oligomeric matrix protein"* or COMPAngpt-1, which is more potent in phosphorylating Tie2 than native Angpt-1 (Kim et al., 2009). It is important to note that the delivery form of this engineered Angpt-1 variant was again an adenoviral one. Their findings demonstrate a protective effect of COMPAngpt-1 pretreatment against endotoxic AKI. A comparable experiment with a rescue application to simulate a more realistic setting was not provided in this work (probably due to feasibility reasons using viral transfer), but would have been highly desirable.

Another way to augment Tie2 phosphorylation is the administration of Vasculotide (VT), a PEGylated 7-mer peptide (HHHRHSF) which was recently identified by screening a phage display library for binding to the Tie2 receptor (Tournaire et al., 2004). In human microvascular endothelial cells (HMVECs), VT completely prevents characteristic morphological and functional changes induced by LPS. From a pharmaceutical point of view VT may hold promise as a drug-like compound. We

therefore assessed VT *in vivo* in a model of endotoxemic ALI. And indeed, VT treatment was sufficient to prevent the LPS-induced development of lung vascular leakage, and improved survival of endotoxemia by 41%, an effect that was completely abolished in Tie2 heterozygous knockout mice. Indicating the high specificity of VT for Tie2 (David et al., 2011a). In an additional study, Kümpers et al. showed that prophylactic as well as therapeutic (i.e. rescue) administration of VT was sufficient to ameliorate AKI and reduce mortality in a clinically relevant surgical model of sepsis, cecal ligation and perforation (Kumpers et al., 2011).

2. The Tie2 antagonist perspective

Studies from independent labs in models of *in vivo* inflammation, tissue injury, and bacterial infections (including sepsis) suggest that Angpt-2, a context-dependent antagonist of the endothelial receptor Tie-2, exacerbates a wide spectrum of pernicious manifestations (**Table 1**). In 2006, Parikh, et al. reported that circulating Angpt-2 was elevated in subjects with severe sepsis and showed that Angpt-2 administration to otherwise healthy adult mice resulted in lung injury and vascular leakage (Parikh et al., 2006). These results provided the first direct evidence suggesting that Angpt-2 was both a marker and mediator of vascular injury in sepsis. This study also showed that septic human serum disrupted the integrity of microvascular endothelium in a fashion reversible by Angpt-1, suggesting a mechanism by which excess Angpt-2 could be pathogenic.

In the same year, Fiedler et al. used intravital microscopy in models of sterile chemical peritonitis to show that gene deletion of Angpt-2 dose-proportionally attenuated leukocyte-endothelial interactions. They found that TNF-α-induced upregulation of inflammatory adhesion molecules by endothelial cells required Angpt-2 expression by

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those cells. (Fiedler et al., 2006). Shortly thereafter, Bhandari et al. reported that hyperoxia-induced acute lung injury in mice was ameliorated by genetic or siRNA depletion of Angpt-2 and showed that brochoalevolar lavage fluid from individuals with acute lung injury contained high levels of Angpt-2 (Bhandari et al., 2012). Of note, the authors argued for an injurious role of Angpt-2 in the alveolar epithelial necrosis characteristic of this model.

In contrast to these findings, Tzepi, et al. recently reported in this journal that systemic delivery of recombinant human Angpt-2 (rhAngpt-2), but not rhAngpt-1, improved survival in mice challenged with live Pseudomonas (Tzepi et al., 2012). How are these findings to be reconciled with the existing literature and our current understanding of Angpt/Tie2 signalling? First, the use of this particular bacterium is new. To mitigate concerns of unique model-specific factors, the authors also demonstrated a protective effect of rhAngpt-2 in E. coli bacteremia. Curiously, however, they did not observe an Angpt-2-dependent survival benefit in mice injected with E. coli LPS. Second, from our point of view the experimental approach may be critical. The applied dose of rhAngpt-2 results in a circulating concentration exceeding those known from humans with septic shock by 5-10-fold. Supra-physiological concentrations of Angpt-2 appear to activate Tie2 in mice, as shown by Daly et al. (Daly et al., 2006). Measurement of Tie-2 phosphorylation in the work by Tzepi, et al., would have added to our understanding of Pseudomonal bacteremia's molecular pathogenesis and to the underlying mechanism driving rhAngpt-2's benefit in this model.

Our own results with vasculotide in CLP and E. coli LPS clearly demonstrate the protection conferred in experimental sepsis by enhancing Tie2 phosphorylation. The intriguing results of Tzepi and co-workers could also reflect other putative mechanisms—e.g., stimulation of non-endothelial cells and/or signalling through non-Tie2 receptors

(Felcht et al., 2012). In our opinion, the simplest test of whether Angpt-2 induction is pathogenic (or protective) in sepsis would target the *endogenous* protein by inhibiting its production, blocking its release, or neutralizing its effects. Such a study should also characterize the signalling consequences, ideally in multiple organ beds and hematopoietic cells and at sequential times after model induction.

Kurniati and coworkers recently used Angpt-2 (-/-) mice and their wildtype (+/+) littermates to analyze the role of Angpt-2 in the development of endotoxemic AKI (Kurniati et al., 2012). Surprisingly, they found that Angpt-2 deficient mice were not protected from the development of septic renal dysfunction. Moreover, this group found that lack of Angpt-2 was associated with higher circulating and tissue cytokine levels. The authors speculate that early excess Angpt-2 could actually represent a compensatory attempt in suppressing cytokine production. From our point of view another explanation might be worth mentioning here. As the authors note in their discussion, homozygote Angpt-2 null mice have a phenotype that is dominated by severe lymphatic defects (Gale et al., 2002). Given the contribution of the lymphatics in the complex physiology of the immune response, one should keep in mind that this could dramatically influence the hosts LPS response. Secondly, from our experience (Tran et al., 2011), sustained overt renal failure is difficult to achieve in endotoxemia because a strong dose of LPS results in early lethality whereas a weak dose produces mostly a “pre-renal” response that reflects renal ischemia without substantial structural injury to the kidney. Furthermore, traditional assays for serum creatinine become falsely positive from the ketosis that accompanies sepsis. As a result, the authors may have been compelled to use markers of AKI that are not the gold standard.

We recently applied models of sepsis in mice lacking just one allele of Angpt-2 (+/-). Compared to wildtype littermates (+/+), these mice exhibited less tissue inflammation, less renal failure, less lung injury, and better survival (David et al., 2012).

Moreover, severe morphological changes induced in EC culture by co-incubation with serum from septic patients were completely abrogated by treatment with an Angpt-2 function-blocking antibody. Finally, we studied patients presenting to the emergency department with suspicion of infection. We found that circulating Angpt-2 measured within the first hour of emergency department admission was proportionally elevated to the future severity of sepsis and was predictive of septic shock and death with cut-off values that performed equally well in two independent cohorts of similar subjects. These *in vivo* and *in vitro* experiments provide evidence that Angpt-2 might directly contribute to the adverse outcomes in sepsis and might therefore be a promising candidate for future evaluation.

As the work of Tzepi and Kurniati suggest, we should not view Angpt-2 as purely harmful. Indeed, it may be necessary for the early adaptive response to infection—e.g., by facilitating the egress of cellular and humoral mediators of immunity from blood vessels to neutralize pathogens at the portal of entry. However, when this locally adaptive response is applied throughout the circulatory system, the resulting vascular leak and systemic inflammation may well be detrimental to the host.

Together data from one decade of *in vitro* and *in vivo* research suggest that both an Angpt-1 mimetic as well as an Angpt-2 depleting or inhibiting strategy might be a useful and specific pharmaceutical tool to treat sepsis-induced vascular barrier breakdown.

AUTHORSHIP CONTRIBUTION

Wrote the manuscript: SD, PK, PvS and SMP

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FIGURE 1 LEGEND

Angiopoietin (Angpt) / Tie2 signaling. Angpt-1 is continuously secreted from pericytes and vascular smooth muscle (VSM) cells. Angpt-1 ligation activates the Tie2 receptor, promoting downstream signaling via the phosphatidylinositol 3-kinase (PI3K)/Akt pathway to propagate anti-inflammatory, anti-permeability and anti-apoptotic signals. Upon stimulation with various cytokines, endothelial cells release pre-stored Angpt-2 from Weibel Palade bodies (WPB). Angpt-2 competitively antagonizes Angpt-1/Tie2 signaling. Downstream of Tie-2, inflammation is regulated by inhibition of surface adhesion molecule expression (intercellular adhesion molecule-1 [ICAM-1], and vascular cell adhesion molecule-1 [VCAM-1]) and the transcription factor NF-kappaB. Moreover, PI3K/Akt signaling promotes an anti-apoptotic, pro-survival signal. Barrier defense against permeability mediators is achieved by signals to the actin cytoskeleton and junctional adhesion complexes through simultaneous inhibition of the Rho kinase and activation of the small GTPase Rac1 via binding to IQ-motif containing GTPase activating protein-1 (IQGAP1). Direct effects on the adherens junction protein VE-cadherin via src have also been reported.

TABLE 1

Tie2 modulating therapeutic strategies in different systemic inflammatory models from different groups using different models. The "standard" hypothesis refers to the canonical understanding of Angpt-1 being a protective Tie2 agonist, whereas Angpt-2 is antagonistic. The alternatives hypothesis supports the opposite idea, i.e. Angpt-2 being protective.

Year	Authors	Treatment	Injury Model	Results	supp. Hypothesis
1999	Thurston (Thurston et al., 1999)	Ad Angpt-1	VEGF	leakage resistant vessels	standard
2000	Thurston (Thurston et al., 2000)	rhAngpt-1	VEGF	leakage resistant vessels	standard
2005	Witzenbichler (Witzenbichler et al., 2005)	Ad Angpt-1	LPS	improved survival & cardiac function	standard
2005	Hall (Hall and Brookes, 2005)	rhAngpt-1	LPS	increased arteriolar vasoconstriction	standard
2006	Parikh (Parikh et al., 2006)	rhAngpt-2	--	spontaneous leak	standard
2006	Fiedler (Fiedler et al., 2006)	Angpt-2 (-/-) KO	peritonitis	ameliorated infl. response to TNFα	standard
2007	Mammoto (Mammoto et al., 2007)	Ad Angpt-1	LPS	reduced vascular leakage	standard
2009	Hwang (Hwang et al., 2009)	COMP-Angpt-1	LPS	ameliorated leukocyte adhesion	standard
2009	Kim (Kim et al., 2009)	COMP-Angpt-1	LPS	protects against acute kidney injury	standard
2010	Tabruyn (Tabruyn et al., 2010)	Angpt-2 inhibition	M. pulmonis	reduced local inflammation	standard
2011	David (David et al., 2011b)	rhAngpt-1 i.v.	CLP	improved survival, better organ function	standard
2011	Kümpers	Tie2 agonist	CLP	improved survival, better organ function	standard

	(Kumpers et al., 2011)				
2011	David (David et al., 2011a)	Tie2 agonist	LPS	improved leakage, organ funct., survival	standard
2012	Ghosh (Ghosh et al., 2012)	Angpt-2$^{+/-}$ / Ad A1	Anthrax	improved leakage, organ funct., survival	standard
2012	Kurniati (Kurniati et al., 2012)	Angpt-2(-/-)	LPS	worse kidney function	*alternative*
2012	Tzepi (Tzepi et al., 2012)	rhAngpt-2	PSA sepsis	improved survival	*alternative*
2012	David (David et al., 2012)	Angpt-2 (+/-)	CLP / LPS	improved organ function and survival	standard

Abbreviation: Angpt = Angiopoietin, Ad = adenovirus, VEGF = vascular endothelial growth factor, rh = recombinant human, LPS = lipopolysaccharides, KO = knockout, TNFα = tumor necrosis factor alpha, COMP = cartilage oligomeric matrix protein, CLP = cecal ligation and puncture, PSA = pseudomonas aeruginosa

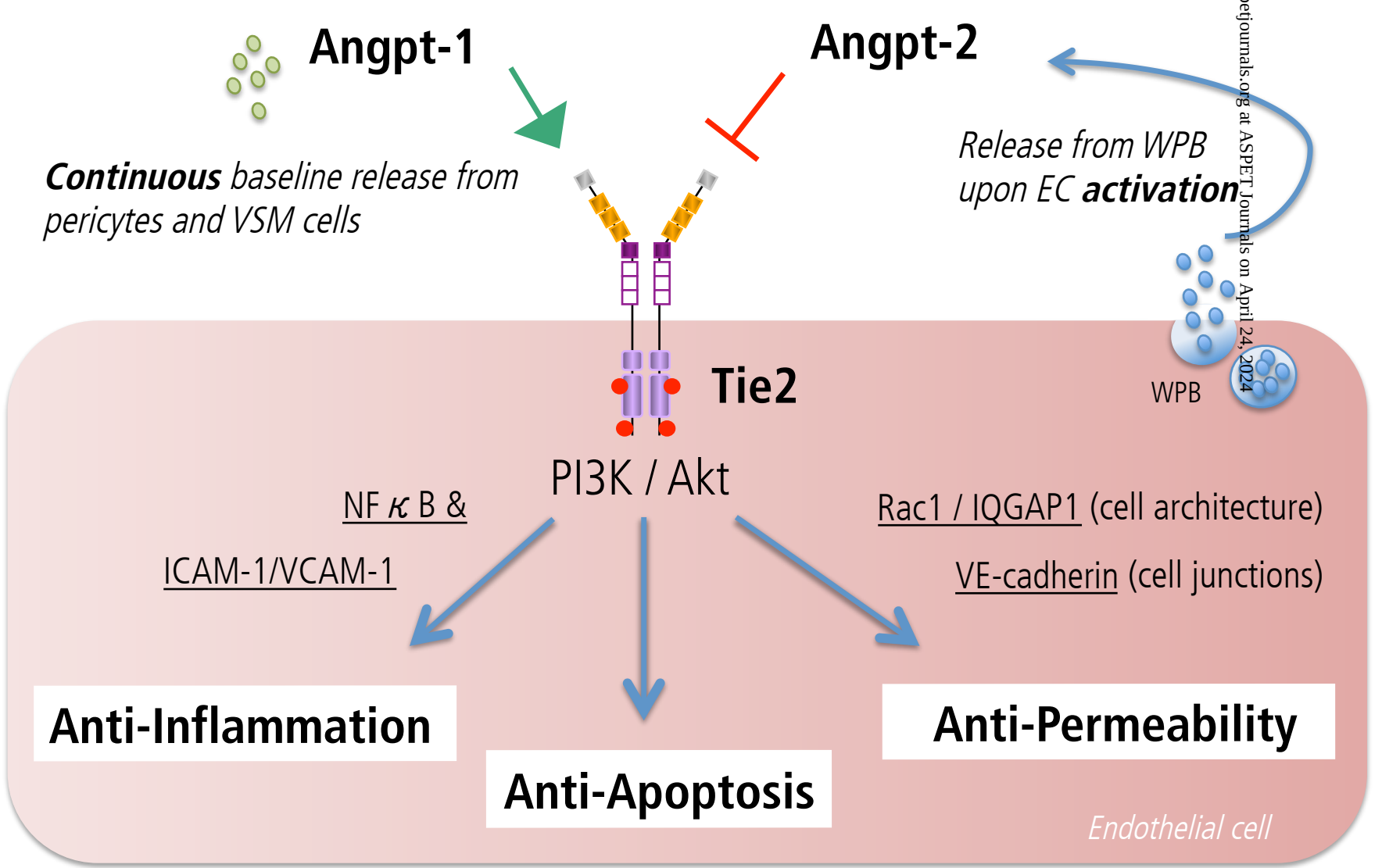

Figure 1