Chapter 8
The Lung Endothelial Barrier in Acute Inflammation

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Non-Standard Abbreviations

$[Ca^{2+}]_i$	Intracellular calcium concentration
ALI	Acute lung injury
AM	Adrenomedullin
Ang	Angiopoietin
ARDS	Acute respiratory distress syndrome
COX	cyclooxygenase
DAMP	Danger-associated molecular pattern
eNOS	Endothelial nitric oxide synthase
HMGB1	High mobility group box-1
ICAM-1	Intercellular adhesion molecule 1
IL	Interleukin
iNOS	Inducible nitric oxide synthase
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MLC	Myosin light chain
MLCK	Myosin light chain kinase
NF-κB	Nuclear factor-kappa B
PAF	Platelet activating factor
PAMP	Pathogen-associated molecular pattern
PMN	Polymorphonuclear leukocyte

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PRR   Pathogen recognition receptor
RNS   Reactive nitrogen species
ROS   Reactive oxygen species
S1P   Sphingosine-1-phosphate
S1P1−5   S1P receptor 1–5
S1PL   S1P lyase
SM   Sphingomyelin
Sph   Sphingosine
SphK   Sphingosine kinase
TLR   Toll-like receptor
TNFα   Tumour necrosis factor alpha
TRPC   Transient receptor potential canonical
TX   Thromboxane
TXR   Thromboxane receptor
VE-cadherin Vascular endothelial cadherin
VEGF   Vascular endothelial growth factor
VILI   Ventilator-induced lung injury

8.1   Introduction

Blood vessels are lined on their inner surface by a continuous layer of endothelial cells, closely connected by interendothelial junctions, thereby forming a semipermeable barrier between blood and interstitium. Transcellular transport mechanisms and paracellular extravasation of solutes and proteins is actively regulated by endothelial cells, thereby controlling tissue fluid homeostasis. Adherent junctions consisting of vascular endothelial (VE)-cadherin and catenin maintain the tight interendothelial connections (Predescu et al. 2007).

In the lungs, alveolar endo- and epithelial cells and their merged basal laminae form a less than 1 µm-thin membrane, which allows rapid and effective gas exchange between the alveolar and vascular compartment. Nevertheless, this extremely thin structure provides a barrier against inhaled particles and pathogens and importantly contributes to pulmonary metabolic and immunologic functions.

Pulmonary endothelial barrier function may be affected by infectious or sterile inflammatory stimulation via either the alveolar (e.g. in pneumonia, mechanical ventilation) or the vascular lumen (e.g. in bacteremia and sepsis). This may lead to increased permeability with protein-rich fluid extravasation, lung edema, and finally acute respiratory distress syndrome (ARDS; reviewed in Muller-Redetzky et al. 2014a; Fig. 8.1). Depending on severity, ARDS is associated with mortality rates ranging from 27 to 45 % (Ranieri et al. 2012).

Pneumonia is the most common infectious disease worldwide, and the third most common cause of death (World-Health-Organisation 2013). Further, pneumonia is the most frequent cause of sepsis, a systemic inflammatory response to infection
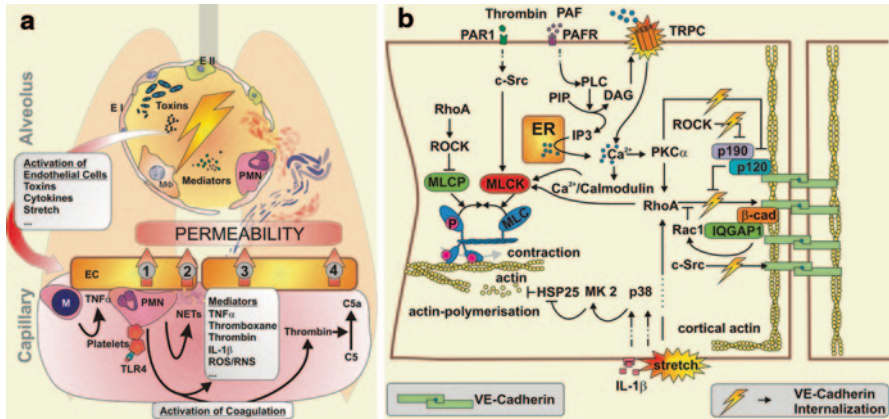
Fig. 8.1 **a** Airspace-derived activation of the endothelium by mediators, bacterial toxins or physical stress due to mechanical ventilation starts a complex interplay of various inflammatory cascades resulting in vascular permeability. Monocytes (*M*) are recruited to the endothelium (*EC*) and facilitate its further activation by secretion of *TNFα*, thereby augmenting the recruitment of neutrophils (*PMN*). Activated platelets stimulate *PMN*. Endothelium-*PMN* contact leads to permeability (*1*). Upon stimulation *PMN* undergo netosis, liberating neutrophil extracellular traps (*NETs*) consisting of DNA and histones that cause endothelial toxicity and barrier breakdown (*2*). Specific soluble mediators also increase permeability (*3*). Neutrophil–platelet complexes activate blood coagulation. Central effector proteases like thrombin directly mediate vascular permeability. Further, *thrombin* activates complement factor *C5* to *C5a*—a permeability increasing anaphylatoxin (*4*). TNF tumour necrosis factor; *IL-1β* Interleukin-1β; *ROS/RNS* reactive oxygen and nitrogen species. **b** Intracellular signalling regulates endothelial permeability. Endothelial contraction results from actin myosin interaction after *MLC* phosphorylation, which is regulated by myosin light chain kinase (*MLCK*) and myosin light chain phosphatase (*MLCP*). *MLCP* is inhibited by *RhoA–ROCK* signalling while *MLCK* is activated by *c-Src*, *RhoA* and Ca^{2+}/*calmodulin*. Ca^{2+} enters the cytosol from endoplasmatic reticulum (*ER*) or extracellular space. Downstream of platelet activating factor (*PAF*) and *PAF* receptor (*PAFR*), phospholipase C (*PLC*) hydrolyses posphatidyl inositol bisphosphate (*PIP*) into inositol 1,4,5-triphosphate (*IP3*) and diacylglycerol (*DAG*). *IP3* mediates Ca^{2+} liberation from the *ER* while *DAG* opens transient receptor potential canonical (*TRPC*) channels in the cellular membrane. The resulting increase of intracellular Ca^{2+} leads to the activation of protein kinase C (*PKC*) *α*, to further *RhoA* activation and to Ca^{2+}/calmodulin complexes, altogether finally leading to *MLCK* activation. Actin polymerization forms stress fibres associated with endothelial contraction. Various stimuli like *IL-1β* or mechanical force activate mitogen-activated protein kinase (*MAPK*) *p38* (*p38*), which activates *MAPK* activated protein kinase 2 *(MK2)*, which phosphorylates heat shock protein 25 (*HSP25*) leading to actin polymerization. Adherence junctions (*AJ*) are mandatory for the sealing of intercellular contacts. *VE-cadherin* is anchored in peripheral cortical actin to the cytoskeleton. *VE-cadherin* phosphorylation leads to *VE-cadherin* internalization and thereby to increased endothelial permeability. *RhoA* and *c-Src* phosphorylate *VE-cadherin*. *Rac-1* and 190RhoAGAP (*p190*) functionally antagonize *RhoA* activity. p190RhoAGAP is recruited to the AJ by p120-catenin (*p120*), which itself inhibits *VE-cadherin* internalization. *ROCK* inhibits p190RhoAGAP and *PKCα* inactivates *p120*-catenin, thereby augmenting destabilization of *AJ*. IQ domain GTPase-activating protein 1 (*IQGAP1*) recruits and stabilizes *Rac-1*, protecting against *VE-cadherin* internalization. (reprinted from Müller-Redetzky et al., Cell Tissue Res (2014) 355:657–673 with kind permission of the publisher)

(Matthay et al. 2012). Both pneumonia and sepsis originate due to an innate immune reaction to invading pathogens. Host–pathogen interactions lead to complex inflammatory responses including secretion of inflammatory mediators, leukocyte recruitment and activation, as well as activation of complement and coagulation cascades that may contribute to pulmonary hyperpermeability and development of ARDS.

Mechanical ventilation is an essential component of the care of patients with ARDS, but it may also perpetuate the inflammatory response and further aggravate pulmonary endothelial barrier dysfunction (Verbrugge et al. 2007). Currently, there are no specific pharmacologic therapies improving endothelial barrier function in patients with pneumonia, sepsis and/or ARDS. However, recent experimental studies unraveled endothelial pathomechanisms, which contribute to the development of ARDS, thereby providing the basis for the development of novel therapeutic strategies (reviewed in Muller-Redetzky et al. 2014a). This chapter gives an overview on recent insights into the mechanisms of pulmonary endothelial barrier dysfunction in acute inflammation.

8.2   Pulmonary Endothelial Barrier Disruption

Pathogens that enter the alveolar compartment and liberated pathogenic factors are detected by pattern recognition receptors (PRRs). The recognition constitutes a crucial step of host defence during infection. PRRs include Toll-like receptors (TLRs), cytosolic NOD-like receptors (NLRs), RIG-I-like RNA receptors (RLRs), and DNA sensors (Opitz et al. 2010). PRRs sense evolutionarily conserved structures on pathogens called pathogen-associated molecular patterns (PAMPs), and specific endogenous molecules released after cell injury called danger-associated molecular patterns (DAMPs). In the alveolar compartment, PRRs are expressed in macrophages, epithelial and endothelial cells, dendritic cells and in recruited immune cells. PRRs play a key role in the inflammatory response to microbial infection and sterile tissue injury. PRR activation triggers production of inflammatory cytokines, interferons and chemokines on transcriptional and post-translational levels (Opitz et al. 2010), leading to the activation of local cells and recruitment of macrophages and neutrophils. At best, these inflammatory cascades will lead to pathogen clearance and finally survival. However, ongoing PAMP and DAMP release from dying bacteria and injured cells, respectively, may lead to an overactivation of the immune response. This over-activation results in uncontrolled production of cytokines, chemokines and lipid mediators, accumulation and activation of leukocytes, inappropriate activation of complement and coagulation cascades and eventually, endothelial barrier disruption.

In addition to host-dependent inflammatory mechanisms, pathogens may also directly induce endothelial injury resulting in pulmonary hyperpermeability. As one of many examples, the pneumococcal exotoxin pneumolysin may induce calcium influx as well as secretion of platelet activating factor (PAF) followed by thromboxane (TX) release (Witzenrath et al. 2007; Lucas et al. 2012). Both increased

intracellular calcium concentration ($[Ca^{2+}]_i$) and TX receptor (TXR) ligation lead to activation of myosin light chain kinase (MLCK) via protein kinase C (PKC) alpha- and Rho-kinase-dependent signalling (Hippenstiel et al. 1997; Witzenrath et al. 2007; Lucas et al. 2012). Phosphorylation of MLC induces actomyosin contractility leading to disruption of adherens junctions, interendothelial gap formation and paracellular permeability (Shen et al. 2010). Furthermore, pneumolysin belongs to the cholesterol-dependent cytolysins and is able to kill endothelial cells by forming pores in their cell membranes (Tilley et al. 2005).

8.3   Role of Neutrophils, Monocytes and Thrombocytes in Pulmonary Endothelial Barrier Disruption

Upon acute inflammation, neutrophils are the first cells to be recruited into the lungs (Grommes and Soehnlein 2011). In contrast to other vascular beds, in the lungs neutrophils transmigrate across the capillary endothelium. Stimulation of neutrophils with various inflammatory agents causes cytoskeletal rearrangements, thereby forming peripheral actin rims, leading to neutrophil stiffening and trapping in the capillary bed (Yoshida et al. 2006). Although the initial trapping is independent from cell surface expression of integrins and selectins, further neutrophil recruitment may depend on these cell adhesion molecules in distinct scenarios (reviewed in Grommes and Soehnlein 2011). Interestingly, adhesion of neutrophils to the endothelium and alveolar recruitment of neutrophils do not necessarily cause a significant change in vascular permeability (Martin et al. 1989; Rosengren et al. 1991). In human umbilical vein endothelial cells (HUVECs) or cremaster vessel preparations, it was shown that during the process of neutrophil transmigration the endothelium forms "transmigratory cups/endothelial domes" that completely encapsulate the emigrating neutrophils, thereby retaining barrier function (Carman and Springer 2004; Phillipson et al. 2008). Furthermore, ring-like structures of neutrophil leukocyte integrin lymphocyte function-associated antigen 1 (LFA-1) and endothelial intercellular adhesion molecule 1 (ICAM-1) were shown to be associated with neutrophil transmigration (Shaw et al. 2004).

However, under pathologic circumstances neutrophils contribute to vascular barrier dysfunction by secretion of soluble mediators causing endothelial contraction, neutrophil–endothelial adhesion-mediated mechanisms, generation of reactive oxygen species (ROS) and liberation of neutrophil extracellular traps (NETs).

One important soluble mediator secreted by neutrophils is tumour necrosis factor (TNF)α, which is known to induce vascular permeability. TNFα leads to MLCK and Rho kinase (ROCK)-dependent actin stress fibre generation in endothelial cells and induces p38 mitogen-activated protein kinase (MAPK)-dependent disarrangement of the microtubule system and thereby loss of intercellular VE-cadherin. However, only blocking microtubule breakdown strongly protected against barrier failure induced by TNFα, whereas inhibiting ROCK or MLCK did not increase transcellular resistance (Petrache et al. 2001). Other soluble mediators of neutrophils involved in endothelial barrier disruption include: (1) thromboxane A2 (TXA2), which is

processed from neutrophil-derived arachidonic acid by endothelial cyclooxygenase-2 (COX2) and binds to the TXR (Kim et al. 2010); (2) leukotriene A4 (LTA4), which is metabolized into LTC4 by endothelial LTC4 synthetase and binds to the endothelial cysteinyl LT receptor subtype 2 (CysLT2R); and (3) CXC chemokine ligands CXCL1, -2, -3 and -8 which bind to CXC chemokine receptor CXCR2 (reviewed in DiStasi and Ley 2009).

Endothelial contraction resulting in hyperpermeability is also caused by the interaction of neutrophils with the endothelium via LFA-1/macrophage receptor 1 (Mac-1) and ICAM-1. This neutrophil–endothelial interaction leads to rapid increase of endothelial $[Ca^{2+}]_i$, which mediates actin polymerization and cytoskeletal reorganization as well as phosphorylation of VE-cadherin causing junctional disruption. Further, neutrophil–endothelial contact and neutrophil activation by leukotriene B4 (LTB4) release lead to secretion of heparin-binding protein (HBP) which in turn increases endothelial permeability (for detailed review see DiStasi and Ley 2009).

Neutrophil-derived ROS may also induce barrier failure. ROS generation upon TNFα stimulation was shown to be mediated by class IA phosphoinositide 3 (PI3) kinase regulating CD11b/CD18 integrin-dependent neutrophil adhesion and NADPH oxidase activation and finally generation of ROS, causing vascular leakage (Gao et al. 2007).

Another defence mechanism of neutrophils is the formation of NETs, which constitute an extracellular fibrous web of DNA–histone complexes to which antimicrobial peptides and proteases like neutrophil elastase (NE) or myeloperoxidase (MPO) are attached in high concentrations (Brinkmann et al. 2004). NETs can trap and kill bacteria but on the other hand may also cause harm. NETs and particularly NET-bound histones possess high cellular cytotoxicity, leading to destabilization of endothelial and potentially epithelial barrier function (Saffarzadeh et al. 2012). In the lungs, this may result in high permeability lung edema. NET formation has been observed in lung injury induced by lipopolysaccharide (LPS), transfusion, influenza virus or *Klebsiella pneumoniae* (Papayannopoulos et al. 2010; Narasaraju et al. 2011; Caudrillier et al. 2012; Saffarzadeh et al. 2012). The induction of NET formation by neutrophils is highly accelerated by binding of activated thrombocytes to neutrophils and is involved in organ failure in septic mice (Clark et al. 2007). Accordingly, NET formation was ameliorated by antiplatelet therapy in transfusion-related lung injury (TRALI) (Caudrillier et al. 2012). To date, the significance of pulmonary NET formation in acute lung injury (ALI) has not been determined, but remains a field of interest as degradation of NETs by DNAse, neutralization of histone-mediated cytotoxicity by for example, polysialic acids, or reduction of netosis by targeting thrombocytes represent potential therapeutic approaches in humans. However, further preclinical evidence is needed to assess the significance and rationale of therapeutic NET targeting in ARDS.

Platelets directly and indirectly mediate vascular permeability. Upon activation, they secrete various factors, including TXA2, which was shown to increase endothelial permeability in vitro and in vivo through upregulation of interleukin (IL)-8 (Kim et al. 2010). Furthermore, in infection and inflammation they activate neutrophils, thereby indirectly contributing to barrier dysfunction (He et al. 2006; Zarbock

et al. 2006; Looney et al. 2009). The formation of permeability-mediating platelet–neutrophil complexes was shown to be associated with TXA2 formation (Zarbock et al. 2006). In contrast, vascular permeability was not increased by neutrophils solely attached to the endothelium after activation by TNFα (He et al. 2006). In a murine model of TRALI, pulmonary neutrophil sequestration was platelet-dependent, and platelet inhibition or depletion protected animals from development of lung injury (Looney et al. 2009). As outlined above, in TRALI activated platelets induce the formation of NETs (Caudrillier et al. 2012) that can trap and kill bacteria (Brinkmann et al. 2004), but may also contribute to lung endothelial permeability (Clark et al. 2007; Caudrillier et al. 2012; Saffarzadeh et al. 2012).

Among the heterogenous population of peripheral blood monocytes, the murine Gr-1^{high}/CCR2^{+}/CX$_3$CR1^{low} monocyte subset is actively recruited to sites of inflammation (Geissmann et al. 2003). These inflammatory subset monocytes rapidly home in the pulmonary microcirculation upon LPS infusion or the onset of high-stretch mechanical ventilation and prime the lung for the development of pulmonary edema towards further stimuli like LPS, zymosan or ventilator-induced lung injury (VILI; O'Dea et al. 2009; Wilson et al. 2009). However, the mechanism by which marginated monocytes contribute to endothelial barrier dysfunction is not fully understood. O'Dea et al. demonstrated that lung-marginated monocytes express TNFα and directly activate pulmonary endothelial cells via TNF- and contact-dependent mechanisms (O'Dea et al. 2005). In addition, blood monocytes were shown to be involved in the regulation of neutrophil recruitment in ALI (Dhaliwal et al. 2012).

A lot of research effort is being made to unravel the mechanisms underlying leukocyte-mediated barrier disruption. However, therapeutic strategies based on depletion or blocking of cell recruitment to attenuate ALI should raise concerns as neutrophils and monocytes are central in the pulmonary and systemic innate immune responses, and therapeutic intervention at this level might induce functional immunosuppression.

8.4 Complement and Coagulation Systems and Vascular Permeability

The complement system consists of plasma and membrane-bound proteins and plays an important role in host defence and inflammation (Mastellos et al. 2003). There are three different complement activation pathways, that all converge at the central molecule C3 (Markiewski and Lambris 2007). The classical pathway is activated by antigen–antibody complexes binding to C1q, thereby activating C1s which then cleaves C4 and C2 to form the C3 convertase enzyme complex C4bC2a. In the lectin pathway, mannose binding lectins (MBL) associated with mannose-binding lectin proteases (MBLP) 1 and 2 bind to PAMPs on bacteria, thereby leading to the generation of the C3 convertase C4bC2a. The alternative pathway is triggered after contact with, for example, bacterial surfaces by spontaneous hydrolysis of C3 leading to generation of a conformationally altered C3 capable of binding factor B,

thereby forming the CbBb complex, the alternative C3 convertase. Both C3 convertases lead to cleavage of C3 to generate C3a and C3b. C3a is an anaphylatoxic peptide and C3b contributes to the assembly of the C5 convertase that processes C5 to the anaphylatoxin C5a and to C5b which is part of the membrane attack complex C5b-9. While C5b-9 causes cell lysis, C3a and C5a induce inflammatory responses and vascular permeability. In endothelial cells, both anaphylatoxins evoked stress fibre generation and thereby cell contraction, although with a different response kinetic. C3a stimulation led to a transient increase in stress fibre generation, while the response after exposure to C5a was delayed and prolonged (Schraufstatter et al. 2002). Notably, C3a and C5a activate different signal transduction cascades in endothelial cells. While C5a-induced permeability was dependent on activation of phosphatidylinositol-3 kinase, Src kinase and epidermal growth factor (EGF) receptor, C3a triggered signal transduction pathways controlled by ROCK (Schraufstatter et al. 2002).

In murine models of ALI and systemic inflammatory responses, silencing of C5a resulted in reduced permeability in multiple organs including the lungs (Liu et al. 2010). Interestingly, C3 deficiency in mice was not associated with attenuated immune complex-mediated lung injury and vascular permeability, while blocking of C5a was shown to be protective. These findings suggest a different complement activation pathway in the absence of C3 with thrombin acting as C5 convertase (Huber-Lang et al. 2006). This compensatory adaptive pathway was also demonstrated to be active in C3 knockout mice suffering from acute tracheal allograft rejection that showed aggravated microvascular injury, while C5a inhibition was protective (Khan et al. 2013).

Another system organized into proteolytic cascades and involved in tissue injury and inflammation is the coagulation system. Intrapulmonary fibrin deposition resulting from abnormal fibrin turnover is a characteristic feature of ALI and some components of the coagulation system may correlate with disease severity (Prabhakaran et al. 2003; Glas et al. 2013). Elevated fibrin turnover is caused by activation of the tissue factor VII pathway, increased plasminogen activator inhibitor 1 (PAI-1) levels and therefore decreased fibrinolytic activity and reduced antithrombin III levels (Prabhakaran et al. 2003; Hofstra et al. 2011). Further, decreased activity of the anticoagulant protein C contributes to intrapulmonary fibrin deposition. This decreased protein C activity is based on reduced protein C production as well as shedding of the cell surface protein thrombomodulin which is an important cofactor for the activation of protein C (Ware et al. 2003). Pulmonary coagulopathy is observed after severe pulmonary permeability edema with alveolar flooding, but it may also promote inflammation and vascular permeability and worsen ALI. The serine protease thrombin is the key enzyme in the coagulation cascade converting fibrinogen into fibrin. Protease activated receptor (PAR) ligation mediates proinflammatory effects of thrombin including release of cytokines and vascular endothelial growth factor (VEGF), which increases vascular permeability (Hippenstiel et al. 1998). Moreover, thrombin induces activation of endothelial MLCK and thereby cell contraction, and acts as C5 convertase generating the anaphylatoxic peptide C5a, which induces inflammatory responses and vascular permeability (Cirino et al. 1996; Huber-Lang et al. 2006; Liu et al. 2010; Glas et al. 2013; Khan et al. 2013).

8.5   Role of Toll-like Receptor 4 in the Regulation of Pulmonary Vascular Permeability

As outlined above, pattern recognition receptor signalling is involved in the development of barrier dysfunction. More specifically, and as an example, TLR4 signalling has been intensively studied regarding its impact on endo–epithelial barrier dysfunction. TLR4 is activated by both exogenous (e.g. LPS; Chow et al. 1999) and endogenous (e.g. high mobility group box-1 (HMGB1) protein, oxidized phospholipid) pro-inflammatory stimuli (Park et al. 2004; Imai et al. 2008). Several in vitro and in vivo studies demonstrated the permeability-enhancing effect of LPS (Mehta and Malik 2006). Further, elevated systemic LPS levels were associated with the severity of sepsis and related organ failure (Marshall et al. 2004). The critical role of TLR4 in regulating vascular permeability was convincingly demonstrated by the observation that mice lacking TLR4 were protected against lung injury due to various stimuli including LPS, oleic acid, cecal ligation and puncture and gut or lung ischemia/reperfusion injury (Imai et al. 2008; Zanotti et al. 2009; Hilberath et al. 2011; Ben et al. 2012; Tauseef et al. 2012).

TLR4-mediated signalling predominantly activates the nuclear factor-kappa B (NF-κB) pathway which induces inflammatory gene transcription (Chow et al. 1999). LPS binds to the TLR4/MD2 receptor complex and NF-κB activation is induced via myeloid differentiation factor 88 (MyD88), interleukin-1 receptor-associated kinase 1 (IRAK1), IRAK2 and IRAK4 (Medvedev et al. 2002; Kawagoe et al. 2008). Furthermore, intracellular diacylglycerol (DAG) levels were increased after recognition of LPS by TLR4, leading to direct activation of transient receptor potential canonical 6 (TRPC6) channels. The resultant TRPC6-mediated calcium influx into endothelial cells activated MLCK and thereby phosphorylation of MLC leading to cell contraction. Further, MLCK also promoted LPS-induced NF-κB-related inflammatory responses that contribute to lung vascular barrier dysfunction (Tauseef et al. 2012). Binding of LPS to TLR4 was also shown to activate the Src family kinases leading to phosphorylation of zonula adherens proteins and consequently disruption of endothelial barrier integrity (Gong et al. 2008).

Whereas the receptor for advanced glycation end products (RAGE) was suggested to be the main endothelial HMGB1 receptor for induction of endothelial permeability (Wolfson et al. 2011), signalling through TLR4 was also shown to play a role in HMGB1-induced inflammatory responses in monocytes which again were found to be MyD88-, IRAK1,2,4- and NF-κB-dependent (Park et al. 2004). Further, HMGB1 was suggested to contribute to the development of VILI (Ogawa et al. 2006).

Oxidized phospholipids as further endogenous stimuli contributed to pulmonary edema by inducing TLR4-mediated activation of TRIF (TIR domain-containing adapter-inducing interferon-β) and TNF receptor-associated factor 6 (TRAF6) leading to NF-κB-mediated IL-6 secretion (Imai et al. 2008).

Since TLR4 was shown to play a crucial role in regulating pulmonary endothelial permeability, targeting TLR4-mediated signalling seemed to be a promising therapeutic approach. In this context, the synthetic TLR4 antagonist eritoran was

developed that inhibits binding of LPS to MD2 and therefore suppresses TLR4/MD2-mediated signalling. Indeed, eritoran was shown to reduce pulmonary inflammation in different animal models (Mullarkey et al. 2003) as well as in healthy humans exposed to a bolus infusion of LPS (Lynn et al. 2003). In a phase II, placebo-controlled trial, patients with severe sepsis and high predicted risk of death that were treated with eritoran showed a trend towards lower mortality (Tidswell et al. 2010). However, in a recent phase III trial of patients with severe sepsis, administration of eritoran had no significant impact on mortality or relevant secondary outcome parameters (Opal et al. 2013), questioning the rationale for the therapeutic use of TLR4 antagonists in sepsis and related organ failure including ARDS. Since various DAMPs and PAMPs are recognized by many PRRs leading to NF-κB-mediated transcription of inflammatory genes, targeting downstream effectors in the inflammatory cascade may be more reasonable (Opal et al. 2013).

8.6   The Angiopoietin/Tie2 System

The angiopoietins (Ang-1 to Ang-4) are ligands for the receptor tyrosine kinase Tie2 which is abundantly expressed by endothelial cells, but also present in polymorphonuclear leukocytes (PMNs) and a subpopulation of monocytes (Wong et al. 2000; Lemieux et al. 2005). Ang-1 and Ang-2 are currently the best described angiopoietins and well-known regulators of angiogenesis, inflammation and vascular leakage (reviewed in David et al. 2013; Eklund and Saharinen 2013). Constitutive Ang-1 expression is found in different cell types including pericytes, vascular smooth muscle cells, fibroblasts, thrombocytes and megakaryocytes (Eklund and Saharinen 2013). Activation of Tie2 by Ang-1 leads to endothelial quiescence and stabilization of barrier function. In contrast, Ang-2 is primarily expressed by endothelial cells and stored in Weibel–Palade bodies (Fiedler et al. 2004), from where it can be rapidly released upon endothelial activation by inflammatory stimuli including TNFα and thrombin (Fiedler et al. 2004; Fiedler et al. 2006). Ang-2 acts as a functionally antagonistic ligand at the Tie2 receptor, thereby destabilizing the quiescent endothelium and promoting inflammation and vascular permeability (Scharpfenecker et al. 2005; Fiedler et al. 2006).

The expression of Ang-2 is transcriptionally upregulated by different factors including thrombin, hypoxia and VEGF (Oh et al. 1999; Huang et al. 2002). In a murine model of endotoxemia induced by LPS injection, functional inhibition of the Ang-1/Tie-2 pathway was observed with increased pulmonary expression and synthesis of Ang-2 (Mofarrahi et al. 2008). In patients with sepsis, Ang-2 serum levels were higher than in healthy volunteers and even more increased in patients with sepsis-associated ARDS (Parikh et al. 2006). Further, plasma Ang-2 levels were shown to have prognostic value for mortality in non-infection-related, but not in infection-related ALI (Calfee et al. 2012). In two different experimental models of sepsis, Ang-2 heterozygous mice with reduced Ang-2 levels were protected against lung injury suggesting that Ang-2 contributes to the pathogenesis of sepsis

besides predicting disease severity (David et al. 2012). This conclusion was further supported by a recent study in which Ang-2 inhibition by antibody treatment reduced microvascular alterations, hypotension and mortality in a murine model of LPS-induced systemic inflammation (Ziegler et al. 2013). In vitro studies showed that expression of endothelial adhesion molecules as well as PMN adhesion were increased by Ang-2 and inhibited by Ang-1 (Gamble et al. 2000; Fiedler et al. 2006). Ang-2 was also shown to induce recruitment of monocytes expressing Tie2 to sites of inflammation (Murdoch et al. 2007). Moreover, in experimental models of ALI, transgenic Ang-1 overexpression or treatment was associated with reduced pulmonary cytokine and adhesion molecule expression, PMN recruitment and endothelial permeability (Witzenbichler et al. 2005; Mammoto et al. 2007; McCarter et al. 2007; Xu et al. 2008). The protective anti-inflammatory Ang-1 effect seems to involve the direct interaction of Tie2 with the NF-κB inhibitor A20 binding inhibitor (ABIN)-2 (Hughes et al. 2003).

Ang-1 was also shown to directly improve endothelial integrity. Tie2 phosphorylation by Ang-1 reduces F-actin stress fibre formation and enhances endothelial barrier function via PI3 kinase leading to the activation of Rac1 and inhibition of RhoA, both linked by the p190 Rho GTPase-activating protein (p190 RhoGAP; Mammoto et al. 2007). The importance of this pathway was further demonstrated by the observation that downregulation of pulmonary p190 RhoGAP expression abolished the protective anti-permeability effect of Ang-1 (Mammoto et al. 2007). In addition, IQ domain GTPase-activating protein 1 (IQGAP1) was suggested to be required for Ang-1-mediated Rac1 activation, thereby contributing to the regulation of endothelial permeability (David et al. 2011b).

Further molecular mechanisms underlying the barrier-protective effect of Ang-1 include (i) impeding the interaction of the inositol triphosphate (IP3) receptor with TRPC1, thereby inhibiting the VEGF-induced, TRPC1-dependent Ca^{2+} influx and reducing endothelial permeability (Jho et al. 2005); (ii) counteracting VEGF-induced Src activation and signalling thereby preventing phosphorylation and internalization of VE-cadherin and disassembly of interendothelial adherens junctions (Gavard et al. 2008); and (iii) activating sphingosine kinase 1 (SphK1) through the extracellular signal-regulated kinase (ERK) 1/2-dependent pathway thereby tightening endothelial cell junctions (Li et al. 2008).

Several studies confirmed the hypothesis that excess Ang-1 would effectively protect against vascular leakage in experimental sepsis and ALI (Witzenbichler et al. 2005; Mammoto et al. 2007; McCarter et al. 2007; Mei et al. 2007; Huang et al. 2008; David et al. 2011c). However, the methods of Ang-1 application such as gene therapy or cell-based delivery were far from proof of clinical efficacy. Recently, a short synthetic peptide (later termed vasculotide) was discovered that activates the Tie2 receptor, thereby completely inhibiting binding of both ligands Ang-1 and Ang-2 (Tournaire et al. 2004). Vasculotide was then proven to have therapeutic potential by preventing or counteracting vascular barrier disruption and reducing mortality in endotoxemia and established abdominal sepsis in mice (David et al. 2011a; Kumpers et al. 2011).

8.7   Sphingosine-1-Phosphate and other Biologically Active Sphingolipids

Sphingolipids form one class of membrane lipids, but are more than just structural components of biological membranes. Some sphingolipids including sphingomyelin (SM), ceramide, sphingosine (Sph) and sphingosine-1-phosphate (S1P) are biologically active and have been implicated in the regulation of diverse signalling pathways. The multiple roles of the four mentioned and other sphingoid bases in the pathophysiology of lung injury have been recently summarized in detail (Natarajan et al. 2013; Uhlig and Yang 2013).

Ceramide is synthesized from serine and palmitoyl coenzyme A (CoA) by multiple enzymatic reactions or derived from SM by sphingomyelinase (SMase). Through the action of ceramidases, ceramide is deacylated to Sph (Canals et al. 2011), which in turn may be phosphorylated to S1P by SphK-1 and -2. S1P is either dephosphorylated to Sph by S1P phosphatases 1 and 2 (S1PPase) or by lipid phosphate phosphatases (LPP), or irreversibly cleaved by S1P lyase (S1PL) to ethanol–amine phosphate and trans-2-hexadecenal.

The effects of ceramide and S1P are often contradictory. While S1P improves barrier integrity, ceramide increases paracellular permeability. Interestingly, the Gram-negative endotoxin LPS and the pneumococcal exotoxin pneumolysin induce microvascular leakage by a PAF-dependent mechanism (Witzenrath et al. 2007; Uhlig and Yang 2013), with PAF increasing endothelial permeability via acid SMase (aSMase)-dependent generation of ceramide (Goggel et al. 2004). Briefly, ceramide generated by aSMase leads to recruitment of caveolin-1, endothelial nitric oxide synthase (eNOS) and TRPC6 channels into caveolae. TRPC6 channels are usually blocked by nitric oxide (NO), but caveolin-1 inhibits NO synthesis by eNOS, leading to TRPC6 activation followed by increase of $[Ca^{2+}]_i$, MLCK activation, MLC phosphorylation, endothelial cell contraction and finally increase in vascular permeability (Uhlig and Yang 2013).

Various cell types are able to generate S1P including platelets, erythrocytes, hematopoietic and vascular endothelial cells (Yatomi et al. 1995; Tani et al. 2005; Hanel et al. 2007; Venkataraman et al. 2008). Coordinated activities of the biosynthetic and biodegradative enzymes maintain S1P concentrations in the ranges required for optimal physiological activities including regulation of cell proliferation, migration, differentiation, survival, morphogenesis and barrier function (Le Stunff et al. 2004; Natarajan et al. 2013). Camerer et al. suggested that basal plasma S1P levels maintain vascular integrity. Mutant mice engineered to selectively lack S1P in the plasma displayed increased vascular leak and enhanced susceptibility to PAF stimulation, which could be reversed by S1P transfusion (Camerer et al. 2009).

S1P acts as both an intracellular messenger (Le Stunff et al. 2004) and an extracellular ligand of five cell surface receptors ($S1P_{1-5}$) that are differentially expressed and coupled to various G proteins (Uhlig and Yang 2013). Vascular endothelial cells primarily express $S1P_1$, $S1P_2$ and $S1P_3$ (Hla et al. 2001). Physiologic plasma S1P concentrations (0.5–1 µM) maintain microvascular integrity via ligation of the G_i-coupled $S1P_1$ (Wang and Dudek 2009), and exogenously added

S1P to pulmonary endothelial cells increased monolayer integrity rapidly and dose-dependently through $S1P_1$ (Garcia et al. 2001). Ligation of endothelial $S1P_1$ induces activation of Rac GTPase, phosphorylation of MLC and cytoskeletal alterations, thereby mediating the barrier-protective effect of S1P (Garcia et al. 2001). In influenza pneumonia, endothelial $S1P_1$ was identified to be critically involved in the regulation of immune responses. $S1P_1$ receptor activation attenuated cytokine storm, suppressed immune cell recruitment and reduced mortality during murine infection with a human pathogenic strain of influenza virus (Teijaro et al. 2011) suggesting that under these circumstances endothelial cells are controlling the innate immune response (Iwasaki and Medzhitov 2011).

In addition to receptor-dependent extracellular S1P signalling, intracellular S1P protected endothelial barrier function, and mice deficient of SphK1 are much more susceptible to LPS-induced lung injury compared to wildtype (WT) controls (Wadgaonkar et al. 2009). Likewise, LPS increased expression of the S1P cleaving enzyme S1PL, thereby decreasing S1P levels in lung tissue. Inhibiting S1PL expression or activity reduced LPS-induced lung injury and inflammation in vitro and in vivo (Zhao et al. 2011). Moreover, in different murine and canine in vivo models of lung injury, including ischemia/reperfusion, pancreatitis and endotoxin challenge, S1P infusion was shown to reduce pulmonary vascular leakage and inflammation (McVerry et al. 2004; Peng et al. 2004; Okazaki et al. 2007; Liu et al. 2008). However, at supraphysiologic concentrations (>5 μM) S1P induces RhoA-dependent barrier disruption through binding to $S1P_2$ and $S1P_3$, which couple to G_i, G_q and $G_{12/13}$ (Siehler and Manning 2002; Wang and Dudek 2009; Sammani et al. 2010). Further, S1P stimulates human airway smooth muscle cell contractility (Rosenfeldt et al. 2003), triggers murine airway hyperresponsiveness (Roviezzo et al. 2007), and induces bradycardia through $S1P_3$ (Forrest et al. 2004). The latter findings implicate a rather narrow therapeutic window for S1P, which may limit the therapeutic potential of S1P and drugs designed to increase S1P production or reduce S1P catabolism.

For this reason, S1P receptor agonists have aroused considerable interest. For example, intratracheal or intravenous application of the selective $S1P_1$ agonist SEW-2871 reduced lung permeability upon endotoxin injection (Sammani et al. 2010). Further, in a murine influenza infection model lung permeability and mortality were shown to be improved after intratracheal treatment with the S1P receptor 1 and 3–5 agonist AAL-R (Walsh et al. 2011). More widely used in clinical application is the S1P receptor modulator fingolimod (FTY720), a derivative of the fungal metabolite myriocin, which shows close structural homology to Sph and has been approved as an immunomodulatory drug for the treatment of multiple sclerosis (Brinkmann et al. 2010). FTY720 besides being immunosuppressive, exerts endothelial barrier-protective effects in vitro (Sanchez et al. 2003) and in vivo (Dudek et al. 2007) and attenuated lung injury in mice following LPS stimulation (Peng et al. 2004; Natarajan et al. 2013). However, similar to S1P, FTY720 does affect endothelial integrity dose-dependently. We recently observed that FTY720 at lower concentrations improved barrier integrity in endothelial cell monolayers (0.01–1 μM FTY720) and in mechanically ventilated mice (0.1 mg/kg FTY720). However, higher concentrations of FTY720 induced apoptosis and barrier disruption in vitro (10–100 μM FTY720)

and in mechanically ventilated mice (2 mg/kg FTY720), but not in spontaneously breathing mice (Müller et al. 2011). Trying to translate these experimental findings into the clinical setting, it cannot be ruled out that in FTY720-treated, ventilated patients with multiple organ dysfunction syndrome and disturbed hepatic metabolism of FTY720, increased FTY720 plasma concentrations might harm lungs that are sensitized by mechanical ventilation towards barrier-disruptive effects of the drug.

It is well-known that FTY720 phosphate is the active metabolite mediating the barrier-protective effect in vivo; however, the underlying mechanisms are not well understood. SphK2 was identified to be the predominant enzyme in generating FTY720 phosphate, thereby increasing its affinity to S1P receptors (Billich et al. 2003; Sanchez et al. 2003). But inhibition of VEGF-induced vascular permeability by FTY720 was independent from $S1P_1$ expression (Sanchez et al. 2003), and $S1P_1$ internalization and degradation by FTY720 has been suggested (Cyster 2005). Further concepts that may explain the barrier-protective effect of FTY720 have recently been reviewed (Natarajan et al. 2013). However, similar to S1P, FTY720 induces bradycardia and dyspnea along with decreases in forced expiratory volume in 1 s (FEV_1) (Kappos et al. 2006), thereby limiting its therapeutic use as a barrier-protective agent in critically ill patients.

8.8 Role of Reactive Oxygen and Nitrogen Species in the Pathogenesis of Lung Injury

ROS and reactive nitrogen species (RNS) are critically involved in the regulation of cellular function. ROS include superoxide anions, hydroxyl radical, hydrogen peroxide, hypochloric acid and other partly reduced derivatives of molecular oxygen, while metabolites of the NO metabolism like nitrite or peroxynitirite with oxidative power are summarized as RNS. Levels of ROS and RNS are tightly controlled by the protective actions of antioxidants such as superoxide dismutase or glutathione. However, an imbalance of both systems due to either excessive ROS/RNS production or critical reduction of antioxidants leads to oxidative stress which contributes to the pathogenesis of lung injury and particularly vascular permeability.

ROS generated during mitochondrial oxidative phosphorylation can modulate cellular processes by reacting with redox-reactive cystein residues on proteins, thereby altering enzyme activities and controlling cell regulatory pathways (Ray et al. 2012). Upon inflammation, endothelial cells produce ROS/RNS involving various enzymatic systems such as NADPH oxidases, xanthine oxidase, COX and eNOS. Neutrophils generate even higher amounts of ROS due to NADPH oxydase activity, which are partly converted to the potent oxidant HOCl by myeloperoxidase. Under inflammatory conditions, neutrophils also generate RNS by inducible NO synthase (iNOS; Boueiz and Hassoun 2009). Increased levels of ROS and RNS contribute to the pathogenesis of ALI upon different insults. Perfusion of isolated rabbit lungs with H_2O_2 induces barrier dysfunction resulting in pulmonary edema formation (Seeger et al. 1995; Hippenstiel et al. 2002). Exposure of endothelial

cells to H_2O_2 led to a rapid and dramatic decrease in cAMP content, and adenylate cyclase activation inhibited H_2O_2-induced permeability increase in endothelial cells (Suttorp et al. 1993b). Mechanical ventilation was shown to induce enzymatic activity of xanthine oxidoreductase (XOR), and inhibiting XOR protected mice from development of pulmonary hyperpermeability (Abdulnour et al. 2006). Thereby, the MAPK-dependent pathway is involved in permeability induction in rodents subjected to VILI (Abdulnour et al. 2006; Dolinay et al. 2008; Park et al. 2012). The underlying mechanisms are pro-inflammatory functions of this pathway as well as heat shock protein 25 (HSP25) phosphorylation leading to stress fibre formation and endothelial contraction (Abdulnour et al. 2006; Dolinay et al. 2008; Damarla et al. 2009). Furthermore, aggravated permeability and lung injury in VILI were demonstrated in mice lacking the transcription factor Nrf2 that regulates expression of several antioxidant enzymes, and antioxidant supplementation was able to rescue from exacerbation of lung injury (Papaiahgari et al. 2007).

NO is a reactive free radical and also a highly diffusible gas that is produced in the lungs from L-arginin in endothelial cells by constitutively expressed eNOS and in macrophages by iNOS. Activity of eNOS can be enhanced while its expression generally stays constant. In contrast, expression of iNOS is inducible, while its activity is more or less constant. Upon inflammation, different stimuli induce NO production and release, such as pore-forming bacterial exotoxins (Suttorp et al. 1993a). NO has multiple biological activities including control of vascular tone and permeability, regulation of mitochondrial respiration and adhesion of platelets and leukocytes. NO protects cells against oxidant injury and microbial threats, but it can also have detrimental effects, for example activation of inflammatory processes, enzyme inhibition and DNA damage. It is suggested that these varying cellular responses are highly dependent on the relative NO concentrations (Thomas et al. 2008).

The molecular mechanisms underlying NO activities are (i) reaction with transition metals like iron, copper and zinc, (ii) nitrolysation of cystein residues, and (iii) reaction with superoxide anion and formation of the oxidant peroxynitrite which leads to nitration of proteins involved in the regulation of cellular function (Korhonen et al. 2005).

Inhalation of NO is applied as rescue therapy in individual cases of hypoxic respiratory failure in adults, children and newborns along with respiratory support and other therapeutic measures. By inhalation, the potent vasodilator selectively decreases pulmonary arterial pressure without causing systemic vasodilation and redistributes pulmonary blood flow towards ventilated lung regions, thereby reducing intrapulmonary shunting and improving arterial oxygenation (Raoof et al. 2010). Nonetheless, although inhaled NO results in a transient improvement of oxygenation during the first 24 h of treatment, it does not increase ventilator-free days or survival of ARDS patients (Afshari et al. 2011).

Besides being a vasodilator, NO exerts endothelial barrier-regulating effects in the lungs; however, contradictory results have been reported. Inhaled NO was shown to maintain endothelial integrity in isolated perfused and ventilated murine lungs upon oxidative stress or ischemia/reperfusion (Kavanagh et al. 1994; Poss

et al. 1995; Schutte et al. 2001b). Further, inhaled NO decreased pulmonary trans-vascular albumin flux in patients with ALI (Benzing et al. 1995). How exactly NO exerts this barrier-stabilizing effect is largely unknown, but it is suggested that an increase in cyclic guanosine monophosphate (cGMP) through activation of guanylate cyclase (GC) may play a role. In rabbit lung ischemia/reperfusion, NO-induced barrier protection was associated with enhanced cGMP production and could be further increased by inhibition of the cGMP-specific phosphodiesterase (PDE) 5 (Schutte et al. 2001b). cGMP-dependent mechanisms mediating barrier protection could also be demonstrated in lung endothelial cells upon H_2O_2 treatment (Seeger et al. 1995; Suttorp et al. 1996), in endothelial cells and perfused mouse lungs stimulated with thrombin (Seybold et al. 2005), and in mice with severe pneumo-coccal pneumonia (Witzenrath et al. 2009). The barrier-protective effect of NO and cGMP can be partly explained by a negative feedback loop that regulates specific endothelial TRP channels (Yin et al. 2008), some of which are crucial for $[Ca^{2+}]_i$ increase, EC contraction and pulmonary hyperpermeability in response to different stimuli (Tiruppathi et al. 2002; Alvarez et al. 2006; Hamanaka et al. 2007; Jian et al. 2008; Yin et al. 2008; Boueiz and Hassoun 2009; Kuebler et al. 2010).

In contrast to its barrier-protective effect, endogenous NO was also shown to contribute to lung injury in isolated rabbit lungs upon hypoxic ischemia/reperfusion (Schutte et al. 2001a). Further, iNOS expression was upregulated in mice subjected to mechanical ventilation, and ventilated iNOS-deficient mice as well as mice treated with an iNOS inhibitor showed reduced permeability and lung inflammation compared to control WT mice (Peng et al. 2005). Similarly, development of pulmonary hyperpermeability in response to mechanical ventilation in rats was prevented by pharmacologic inhibition of NOS (Choi et al. 2003). Murine gain and loss of function studies verified a contribution of soluble GC to VILI (Schmidt et al. 2008). Moreover, inhaled NO increased endothelial permeability in a rat model of *Pseudomonas aeruginosa* pneumonia and this effect was not related to an enhanced inflammatory response (Ader et al. 2007). Consequently, the individual effects of NO on pulmonary endothelial barrier function might be determined by local NO concentrations and the prevalent pathologic circumstances.

8.9   Adrenomedullin (AM) and Endothelial Barrier Function

Adrenomedullin (AM) is a multifunctional, endogenous peptide that was shown to have endothelial barrier-stabilizing properties. It is expressed by different cell types including endothelial and epithelial cells, vascular smooth muscle cells, cardiomyocytes and leukocytes. AM is derived from a larger precursor peptide (prepro-AM) by posttranslational processing including amidation by peptidogly-cine alpha-amidating monooxygenase (PAM; Temmesfeld-Wollbruck et al. 2007b). AM is a ligand for the calcitonin receptor like receptor (CRLR), which associates with receptor activity modifying proteins (RAMP)-2 and RAMP-3. AM receptor

binding in endothelial cells leads to accumulation of the second messenger cAMP and activation of different kinases including protein kinase A (PKA), PKC, MAP kinases (Hippenstiel et al. 2002; Temmesfeld-Wollbruck et al. 2007b).

The importance of AM for vascular barrier integrity was highlighted by the demonstration that mice deficient for AM, CRLR, PAM or RAMP2 die prematurely of hydrops fetalis (Caron and Smithies 2001; Czyzyk et al. 2005; Dackor et al. 2006; Ichikawa-Shindo et al. 2008). Further, expression of AM is increased under inflammatory conditions like sepsis or ALI (Matheson et al. 2003; Cheung et al. 2004; Agorreta et al. 2005) and mice heterozygous for the AM gene exhibit an aggravated inflammatory response upon LPS challenge (Dackor and Caron 2007).

In different in vitro, ex vivo and in vivo models, AM reduced pulmonary endothelial hyperpermeability induced by various stimuli such as hydrogen peroxide, LPS or *Staphylococcus aureus* alpha-toxin and protected against VILI in mice with and without pneumonia (Hippenstiel et al. 2002; Itoh et al. 2007; Temmesfeld-Wollbruck et al. 2007a; Müller et al. 2010; Muller-Redetzky et al. 2014b). In a two-hit model of relatively high clinical relevance, mice were subjected to pneumococcal pneumonia before being mechanically ventilated. Further, treatment with exogenous AM protected against gut barrier disruption after *Staphylococcus aureus* alpha-toxin stimulation and in ischemia/reperfusion injury, and improved blood–brain barrier function (Kis et al. 2003; Brell et al. 2005a; Brell et al. 2005b; Honda et al. 2006; Higuchi et al. 2008; Temmesfeld-Wollbruck et al. 2009).

There are at least two central mechanisms by which AM mediates endothelial barrier stabilization. The first is based on AM capability to block generation of actin stress fibres and actin myosin interaction thereby leading to endothelial cell relaxation (Temmesfeld-Wollbruck et al. 2007b). Briefly, AM was shown to increase intracellular cAMP levels in endothelial cells, thereby inhibiting MLC phosphorylation and actin myosin interaction and finally cell contraction induced by thrombin or hydrogen peroxide in vitro, or caused by mechanical ventilation in vivo (Hippenstiel et al. 2002; Brell et al. 2005b; Hocke et al. 2006; Müller et al. 2010; Muller-Redetzky et al. 2014b). However, in gut epithelial cells AM was shown to exert barrier-stabilizing effects independently of cAMP (Temmesfeld-Wollbruck et al. 2009). The second mechanism underlying AM-mediated barrier stabilization is through an increase of intercellular adherence. In a rat model of isolated perfused ileum, *Staphylococcus aureus* alpha-toxin-induced loss of the junctional protein VE-cadherin was prevented by treatment with AM (Hocke et al. 2006). In vitro, AM protected against *Staphylococcus aureus* alpha-toxin- or thrombin-mediated loss of VE-cadherin and occludin, and increased expression of claudin-5 in brain microvascular endothelial cells (Hocke et al. 2006; Honda et al. 2006). AM was also shown to exert immunomodulatory effects (Gonzalez-Rey et al. 2006), however we observed that the AM-mediated improvement of barrier function was not associated with a downregulated inflammatory response (Müller et al. 2010; Muller-Redetzky et al. 2014b). Although the obviously cell specific mechanisms underlying AM-mediated barrier protection are only partly understood, the impressive effects observed in complex experimental models regardless of the stimulus and uncoupled to immunosuppressive properties suggest a high translational potential for AM.

8.10   Stem Cells in Lung Injury

In lung injury, inflammatory signalling cascades are activated and chemoattractant factors released, including granulocyte-macrophage stimulating factor (GM-CSF), granulocyte colony stimulating factor (G-CSF), and stromal cell-derived factor 1α (SDF-1α) and its receptor CXCR4, which results in mobilization of stem cells from bone marrow to the injured lung (Maron-Gutierrez et al. 2014). Stem cells are able to renew themselves and to differentiate into multiple cell types, which may contribute to regeneration of damaged organs (Cribbs et al. 2010). In lung injury, stem cells engraft into tissue and interact with neighbouring cells, thereby directly contributing to epithelial and endothelial repair. However, the levels of engraftment seem to be too low to account for all the observed positive effects. Indeed, stem cells may additionally alter the immune response in a beneficial manner to diminish destructive inflammation while preserving the host's ability to combat pathogens (Cribbs et al. 2010). Alterations of immune responses and improvement of cellular functions seem to result in particular from of delivery of paracrine mediators and mitochondrion-containing microvesicles by stem cells (Bhattacharya and Matthay 2013). Allogeneic human mesenchymal stem cells were also shown to restore compromised alveolar fluid clearance ex vivo in human lungs rejected for transplantation due to lung injury and dysfunction after prolonged ischemia (McAuley et al. 2014).

Endothelial progenitor cells (EPCs) are a subtype of hematopoietic stem cells, which exclusively differentiate into endothelial cells and can be derived from mononuclear cells from bone marrow, cord blood and circulating blood. Patients with ARDS had increased numbers of circulating EPCs, and higher EPC counts correlated with improved survival (Burnham et al. 2005). Notably, EPCs engrafted into endothelial tissue of injured, but not of healthy lungs, again implying that injured lung tissue produces chemoattractants that mobilize EPCs specifically to damaged tissue areas.

However, although EPC recruitment was enhanced in patients with sepsis, proliferation, adhesion and migration of endogenous EPCs were reduced (Luo et al. 2009; Patschan et al. 2011). Two different experimental strategies were pursued to enhance beneficial functions of EPCs. First, autologous transplantation of EPCs from healthy donors was performed in rabbit and rat models of ALI, and exogenous EPCs reduced lung edema and hyaline membrane formation most likely due to reendothelialization of damaged lung vasculature (Lam et al. 2008; Mao et al. 2010). Second, autologous EPC transplantation was recently combined with SDF-1α treatment, which enhanced functional properties of EPCs (Fan et al. 2014). Exogenous EPCs and SDF-1α synergistically improved survival in mice with polymicrobial sepsis (Fan et al. 2014).

In summary, stem cells probably have prognostic value and provide a therapeutic perspective in ALI. Currently, bone marrow-derived human mesenchymal stem cells are tested as adjuvant therapy for the treatment of ARDS in a clinical phase I (NCT01775774), and in a multicenter phase II trial (NCT02097641).

8.11   Conclusions and Future Perspectives

ARDS may result from acute inflammatory diseases such as pneumonia or sepsis and still carries an unacceptably high mortality rate. In various studies, basic pathomechanisms involved in the development of ARDS were unraveled leading to improvements in therapy including ventilation and resuscitation strategies. Pulmonary endothelial cells have long been identified as key players in the pathogenesis of ARDS and researchers have discovered numerous central mechanisms underlying increased vascular permeability (Muller-Redetzky et al. 2014a). However, most therapeutic approaches based on the understanding of pathomechanisms underlying pulmonary endothelial barrier dysfunction have been frustrating so far. Nevertheless, these drawbacks should not discourage scientists from further research, quite the contrary they should be understood as important sources of perception. In this context, it is worth considering some general aspects when moving forward in this field.

First, to reverse a barrier dysfunction of an already injured endothelium may be a hardly achievable goal. Notably, the only therapeutic approaches reducing ARDS mortality so far, reduction of tidal volume and probably early prone positioning, short term use of neuromuscular blockers, and esophageal pressure-guided PEEP adjustment (Network ARDS 2000; Talmor et al. 2008; Papazian et al. 2010; Guerin et al. 2013), are aiming at prevention of further inflammatory stress by mechanical ventilation, thus being rather preventive. To focus on therapeutic approaches that slow down the development of pneumonia or sepsis to ARDS seems to be more promising than trying to reverse severe tissue inflammation and injury. Therefore, clinical and biological predictors of development towards ARDS are required, and future therapeutic strategies should be introduced before full-blown ARDS has developed. However, this notion should not tempt researchers to perform experimental studies that focus on preventive strategies with the start of the specific treatment before onset of the preceding disease (pneumonia or sepsis in this case), because they are far from translation into clinics.

Second, the real-life scenario should always be considered. Due to numerous simultaneous incidents, ICU patients are often susceptible to the development of ARDS. This is in contrast to experimental studies using, for example, LPS-treated mice, implicating that many redundant pathways may be differentially involved and should probably be targeted therapeutically at once. Furthermore, important interindividual differences need to be reflected.

Third, complexity is an aspect that should not be underestimated. The greater our understanding of the pathomechanisms contributing to lung injury is, the more aware we become of the differential pathomechanistic roles one and the same signalling system may have. For example, S1P was shown to differentially affect endothelial barrier function, depending on S1P concentration, receptor expression and the specific local cellular environment, which adds a further dimension to the big picture of mechanisms involved in endothelial barrier disruption. Presumably, systems biology combined with mathematical multi-scale models that integrate knowledge from experimental studies (in vitro, in vivo and in silico), clinical trials and clinical and biological predictors of individual patients will facilitate development of successful novel therapies and improvement of ARDS prevention.

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