Chapter 63
Genomics of Acute Lung Injury and Vascular Barrier Dysfunction

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Abstract Acute lung injury (ALI) is a devastating syndrome of diffuse alveolar damage that develops via a variety of local and systemic insults such as sepsis, trauma, pneumonia, and aspiration. It is interestingly to note that only a subset of individuals exposed to potential ALI-inciting insults develop the disorder and the severity of the disease varies from complete resolution to death. In addition, ALI susceptibility and severity are also affected by ethnicity as evidenced by the higher mortality rates observed in African-American ALI patients compared with other ethnic groups in the USA. Moreover, marked differences in strain-specific ALI responses to inflammatory and injurious agents are observed in preclinical animal models. Together, these observations strongly indicate genetic components to be involved in the pathogenesis of ALI. The identification of genes contributing to ALI would potentially provide a better understanding of ALI pathobiology, yield novel biomarkers, identify individuals or populations at risk, and prove useful for the development of novel and individualized therapies. Genome-wide searches in animal models have identified a number of quantitative trait loci that associate with ALI susceptibility. In this chapter, we utilize a systems biology approach combining cellular signaling pathway analysis with population- based association studies to review established and suspected candidate genes that contribute to dysfunction of endothelial cell barrier integrity and ALI susceptibility.

Keywords Alveolar capillary permeability • Lung injury • Genomics • Genetics • Bioinformatics • Polymorphism

1 Introduction

Acute lung injury (ALI) is a devastating syndrome of diffuse alveolar damage that develops via a variety of local and systemic insults such as sepsis, trauma, pneumonia, and aspiration [1]. Deranged alveolar capillary permeability, profound inflammation, and extravasation of edematous fluids into the alveolar spaces are critical elements of ALI, reflecting the substantial surface area of the pulmonary vasculature needed for alveolar gas exchange. ALI, together with its severest form, acute respiratory distress syndrome (ARDS), afflicts approximately 190,000 patients per year in the USA and has a mortality rate of 35–50% [2, 3]. It is interestingly to note, however, that only a subset of individuals exposed to potential ALI-inciting insults develop the disorder and the severity of the disease varies from complete resolution to death. In addition, ALI susceptibility and severity are also affected by ethnicity as evidenced by the higher mortality rates observed in African-American ALI patients compared with other ethnic groups in the USA [4]. Moreover, marked differences in strain-specific ALI responses to inflammatory and injurious agents are observed in preclinical animal models [5]. Together, these observations strongly indicate genetic components to be involved in the pathogenesis of ALI.

The role that genetics plays in determining ALI risk or the subsequent severity of the outcome is one of the many unanswered questions regarding ALI pathogenesis and epidemiology. The identification of genes contributing to ALI would potentially provide a better understanding of the pathogenic mechanisms of ALI, yield novel biomarkers, identify individuals or populations at risk, and prove useful for the development of novel and individualized therapies. However, a traditional genetic approach to studies using family linkage mapping is not feasible given the sporadic nature of ALI and the necessity of an extreme environmental insult. Further, genetic studies of ALI are challenging owing to the substantial phenotypic variance in critically ill patients, diversity in the lung injury evoking stimuli, presence of varied comorbid illnesses common in the critically ill patient, complex gene–environment interactions, and potentially incomplete gene penetrance [6, 7]. Despite these inherent challenges, the unrivaled progress made in the post-human genome era combined with the utilization of sophisticated bioinformatics and high-throughput methods have allowed significant advances to be made. For example, these tools are now linked to escalating knowledge of the

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molecular mechanisms of lung endothelial permeability, a hallmark of ALI and an attractive target for the design of novel therapies, to identify candidate genes whose variants are potentially involved in ALI susceptibility. Genome-wide searches in animal models have identified a number of quantitative trait loci that associate with ALI susceptibility [8].

In this chapter, we utilize a systems biology approach combining cellular signaling pathway analysis with population-based association studies to review established and suspected candidate genes that contribute to dysfunction of endothelial cell barrier integrity and ALI susceptibility. The integration of high-throughput gene expression profiling in preclinical models of ALI with bioinformatics has led to the identification of differentially expressed genes in response to ALI whose variants are potentially involved in ALI susceptibility and severity. This approach confirmed long-suspected ALI-associated candidate genes, but more importantly, identified novel genes not previously implicated in ALI. Increasing knowledge of the molecular mechanisms of endothelial-barrier-regulatory pathways has also enhanced the ability to find novel ALI candidate genes. The analysis of the molecular pathways involving the cytoskeletal scaffolding and the dynamic cytoskeletal changes driving cell shape alterations, a key feature of vascular permeability, has identified additional genes contributing to the development and severity of ALI, thereby providing novel therapeutic targets in this devastating illness. Genes encoding proinflammatory cytokines, growth factors and mediators, receptors for barrier-regulatory agonists, and mechanical-stress-sensitive genes expressed in endothelium which regulate inflammatory responses also serve as attractive ALI candidate genes and are representative of the diverse but fertile areas of exploration for candidate SNPs affecting ALI susceptibility and severity.

2 Candidate Genes in Acute Lung Injury: Vascular Barrier Regulatory Cytokines, Growth Factors, and Mediators

2.1 Angiotensin-Converting Enzyme

Angiotensin-converting enzyme (ACE) is a member of the rennin–angiotensin system (RAS), balancing the levels of angiotensin I and angiotensin II, with significant expression in lung vascular endothelium as compared with other vascular beds [9]. The RAS is considered to be an important regulator of inflammation that contributes to ALI by altering vascular permeability, vascular tone, fibroblast activation, and endothelial–epithelial cell survival [10–12]. For example, angiotensin II activates inflammatory processes by upregulating proinflammatory cytokines and chemokines via type I and type II angiotensin II receptors that subsequently activate the nuclear factor κB (NF-κB) pathway [13, 14]. The RAS is also involved in the fibrotic response to ALI via induction of transforming growth factor expression [15]. The most compelling evidence for RAS involvement in ALI has come from the effective attenuation of ALI pathobiology by ACE inhibitors or angiotensin receptor blocking drugs [16, 17] and ACE knockout mice in preclinical models of ALI [18].

An intronic insertion (I) or deletion (D) of a 287-bp Alu repeat sequence in the human ACE gene, located on chromosome 17q35, has been associated with ACE levels and activity in serum [19, 20]. The D allele possesses a higher enzyme activity which parallels the higher gene expression in individuals with DD genotype [21]. The initial association of the DD genotype in the ACE gene with increased ALI mortality provided the impetus for subsequent studies to more firmly establish a genetic basis of ALI and to identify ALI candidate genes [22]. Caucasian patients with ARDS show significantly higher frequencies of the DD genotype and the D allele as compared with ventilated intensive care unit (ICU) patients without ARDS, patients after coronary artery bypass surgery, or healthy controls. Moreover, ARDS patients with DD genotype show markedly higher mortality (54%) in comparison with the II genotype (11%) or strike "4" ID genotype (28%) [22]. The higher mortality rate in ARDS patients with DD or ID genotype as compared with II genotype was subsequently confirmed in Han Chinese patients in Taiwan, although the frequency of the D allele is significantly lower in the Chinese population as compared with Western populations [23]. Compared with Caucasians, a higher frequency of D allele has been reported among Africans (Nigerian and African-American populations) [24, 25], potentially contributing to the observed disparity in ALI-associated higher mortality rates in African-Americans [4]. However, to date, no association study of ACE polymorphisms and lung injury has been performed in African-Americans. In contrast, Mexican and Amerindian populations have slightly lower allelic frequencies of the D allele [25]. Thus, ACE represents a highly viable endothelial candidate gene and attractive target in acute inflammatory lung disease.

2.2 Tumor Necrosis Factor

Tumor necrosis factor (TNF) α, an early mediator of ALI development, is a potent proinflammatory cytokine which dramatically increases endothelial cell permeability, cytokine production, and a variety of cytotoxic and proinflammatory compounds which lead to subsequent vascular leakage and disturbed lung water balance. Both TNFα and TNFβ subtypes appear in the circulation, in bronchoalveolar lavage (BAL) fluid and in pulmonary edema fluid during the onset of

lung injury. As such, the elevated levels of TNF and its soluble receptors are commonly used as markers of inflammation and are associated with morbidity and mortality in ALI patients [26]. Both the TNFα and TNFβ genes lie in close proximity within the major histocompatibility complex, with several polymorphisms described in this region. The -308G/A promoter polymorphism in the TNFα gene and the NcoI restriction fragment length polymorphism in the TNFβ gene appear to influence the expression of TNFα. The carriers of the -308A allele and homozygotes for the $TNFβ_2$ allele exhibit increased TNFα expression and have increased susceptibility and mortality to sepsis [27, 28]. In patients with ARDS, the -308A allele is also associated with increased 60-day mortality, with the strongest association found among younger individuals [29]. However, in ARDS patients with direct or indirect pulmonary injury, these SNPs are associated with alterations in ALI susceptibility (TNFα -308 G/A SNP only in the direct pulmonary injury group, and TNFβ NcoI only in the indirect pulmonary injury group). Owing to the extent of linkage disequilibrium in the region, it remains unclear as to whether these are regulatory SNPs or if the TNF protein level is modulated by a third locus or a haplotype [30]. Promoter SNPs within the TNFα gene (-238 G/A, -857 C/T) have been associated with inflammatory bowel disease along with the -308 G/A SNP [31]. Thus, the role of TNF variants in inflammatory disorders is apparent and indicates a need for further study of other TNF variants in association with ALI.

2.3 Interleukin-6

Interleukin-6 (IL-6) is an acute-phase response cytokine that plays a key role in the activation of B and T cells. Inflammatory cytokines, including IL-6, are essential for the immune system homeostasis; however, when IL-6 production is exaggerated as observed in inflammatory lung disorders including ALI [22, 32], clearly detrimental outcomes are observed. ALI-related increased levels of IL-6 have been established in the BAL fluid of critically ill patients with ARDS, sepsis, and trauma [33, 34] in association with ALI adverse outcome [35] and development of multisystem organ failure [36]. In prior reports, we observed significantly higher expression of IL-6 and the IL-6 receptor genes across multiple-species ALI models and in human lung endothelium exposed to ventilator-induced mechanical stress as well as in differential region-specific expression in lungs of the canine ALI model [37–39]. On the basis of these data, the IL-6 gene constitutes an excellent candidate gene to understand the genetic basis underlying ALI. A functional polymorphism in the IL-6 gene promoter region at the -174 position (G-174C) has been associated with alterations in both gene expression and IL-6 levels and lower circulating IL-6 concentrations and lower mortality rates in patients with acute respiratory failure admitted to the ICU [22]. The contrasting correlation between G-174C alleles and circulating IL-6 levels has also been reported [32]. The haplotype involving -174 G/C, 1753C/G, and 2954G/C is associated with higher mortality (and other secondary clinical outcomes) in a cohort of septic patients of European descent [40].

We further evaluated 14 IL-6 gene tagging SNPs covering the entire gene for potential association in sepsis and ALI patients of European descent [32]. No single SNP was identified as significantly associated with ALI; however, a common haplotype (comprising -1363G/-572G/-174G/1208A/1305A/4835C) with a frequency of 63% in cases and 49% in controls showed a significant association with ALI susceptibility. In addition, homozygote carriers of the risk haplotype are twice as frequent in ALI cases (44.8%) than in controls (22.9%), yielding a highly significantly increased odds ratio for developing ALI (odds ratio 2.73; 95% confidence interval, 1.39–5.37; $p=0.003$). This haplotype spans the entire IL-6 gene including the G allele at position -174, i.e. the risk allele for susceptibility to ALI noted above. These data support the association of the IL-6 gene with ALI susceptibility and illustrate the value of haplotype analysis as a robust approach in association studies.

2.4 Vascular Endothelial Growth Factor

Vascular endothelial growth factor (VEGF) is an endothelial-cell-specific mitogen that regulates angiogenesis, migration, and cell permeability [41]. VEGF plays an important role in several organs by directly regulating vascular permeability to water and proteins. Lung overexpression of VEGF induces increased pulmonary vascular permeability, resulting in marked pulmonary edema [42], and plasma VEGF levels are significantly elevated in ALI patients [43]. Several studies have reported the association of low levels of VEGF with the severity of ARDS and elevated levels with the recovery from ARDS, indicating a role for VEGF in the repair process of lung injury [44]. Several polymorphisms have been described in the VEGF gene, primarily in association with cancer susceptibility and severity. The C/T SNP at position 936 of the 3′ untranslated region (UTR) of the gene has been associated with higher VEGF plasma levels in healthy subjects [45]. Recently, the C936T SNP in the VEGF gene has been associated with ARDS susceptibility and severity (increased mortality) in subjects of European descent [46, 47]. The haplotype TCT at position C-460 T, C+405G, and C+936 T was significantly associated with a higher rate of mortality in ARDS patients and higher plasma levels of VEGF [47]. These studies highlight the VEGF gene as an attractive barrier-regulatory ALI candidate gene and molecular target in ALI therapeutic strategies.

Table 1 Genes with significant differential expression in multispecies models of acute lung injury and number of PubMatrix citations

Genes	Gene symbol	Fold change ($p<0.05$)	PubMatrix (tool for multiplex literature mining, http://pubmatrix.grc.nia.nih.gov), April 2009					
			Acute lung injury	Endothelium	Endothelial permeability	Sepsis	Inflammation	Lung diseases
Interleukin-1β	IL-1b	1.53	168	829	66	523	4,219	649
Interleukin-6	IL-6	1.84	459	1,571	168	1,906	14,178	2,446
Tissue factor/thromboplastin	F3	1.52	39	971	34	452	792	530
Plasminogen activator inhibitor type I	PAI-1	1.47	54	1270	23	189	888	303
Cyclooxygenase II	COX2	1.79	2	278	11	60	717	107
Interleukin-13	IL-13	1.3	16	85	12	52	1,467	1,070
Aquaporin 1	AQP-1	1.3	11	74	52	1	18	19
Plasminogen activator urokinase receptor	PLAUR	1.47	10	285	8	21	165	122
Interleukin-1 receptor antagonist	IL-1RA	2	24	83	4	208	1,200	166
Pre-B cell colony enhancing factor	PBEF	2.82	16	16	5	10	59	17
Chemokine receptor 4	CXCR4	1.62	24	316	8	90	1,265	394
Growth arrest DNA damage inducible α	GADD45α	1.71	0	1	1	0	11	7

2.5 Chemokine Receptor 4

Chemokine receptor 4 (CXCR4) is an α-chemokine receptor specific for stromal-derived factor 1 (SDF-1; also known as CXCL12) that plays an important role in cell migration, inflammation, B lymphocyte development, angiogenesis, and human immunodeficiency virus (HIV) infection (HIV coreceptor) [48–50]. Chemokine receptors are G-protein-coupled receptors, which trigger diverse signaling cascades including activation of G proteins and the phosphatidylinositol 3-kinase, Janus kinase/signal transducer and activator of transcription, Rho-p160 Rho kinase, and mitogen-activated protein kinase signaling pathways [51]. The activation of these signaling pathways is often accompanied by the internalization of chemokine receptors and their trafficking back to the plasma membrane. This intracellular turnover determines the leukocyte responsiveness to chemokines [52]. Nonmuscle myosin II A is a molecular motor that binds with the cytoplasmic tail of CXCR4 and CCR552 and participates in the SDF-1-dependent endocytosis of CXCR4 via dynamic interaction with α-arrestin, a key component of the CXCR4 internalization pathway [50]. The CXCR4 gene was identified as a novel candidate gene in ALI as it survived two filtering strategies dedicated to identifying ALI-susceptibility genes associated with elevated levels of mechanical stress as observed in mechanical ventilator-associated lung injury (VALI). Our orthologous gene approach determined ALI-specific gene ontologies – coagulation, inflammation, chemotaxis/cell motility, and immune response [38] – involving recognized genes likely to participate in ALI pathogenesis [IL-6, aquaporin 1 (AQP-1), plasminogen activator inhibitor type I (PAI-1)], as well as novel genes not previously known to be mechanistically involved in ALI, including CXCR4 [38] (Table 1). We subsequently utilized a consomic rodent approach with introgression of rat chromosomes 2, 13, 16, and 17, which contained the highest density of VALI-responsive genes [39]. Introgression of the VALI-sensitive Brown Norway (BN) rat chromosome 13, containing several genes, including CXCR-4, into the VALI-resistant Dahl salt-sensitive (SS) rat resulted in conversion of the SS consomic rats to a VALI-sensitive phenotype [39]. Surface expression of CXCR4 is downregulated by interleukin-4, interleukin-13, and granulocyte–macrophage colony-stimulating factor and upregulated by interleukin-10 and transforming growth factor-β (TGFβ) [53], suggesting that CXCR4 may also play a role in the fibrotic response to ALI via TGFβ signaling.

Polymorphisms in the CXCR4 gene have not yet been reported; however, a SNP in the 3′ UTR of the SDF-1 gene (G801A), is associated with susceptibility to AIDS and type 1 diabetes [54, 55]. We are currently exploring CXCR4 as a potential ALI-associated candidate gene as suggested by the density of PubMatrix citations relating CXCR4 to inflammation (1,151 published papers), endothelium (297 published papers), ALI (28 published papers) and endothelial permeability (eleven published papers). PubMatrix is a Web-based tool that allows simple text-based mining of the NCBI literature search service PubMed using any two lists of keywords terms, resulting in a frequency matrix of term co-occurrence.

3 Strategies to Identify New Genes and Biomarkers in Acute Lung Injury

The advent of high-throughput gene sequencing and expression technologies, and complete genome sequencing of model organisms, now provides the tools to perform large-scale analyses of the genome in complex disorders such as ALI. Whole genome scans, in silico approaches, utilization of consomic rats, and a candidate gene approach involving expression profiling and pathway analysis are proving exceptionally useful in identifying novel candidate genes and genetic variations (Fig. 1).

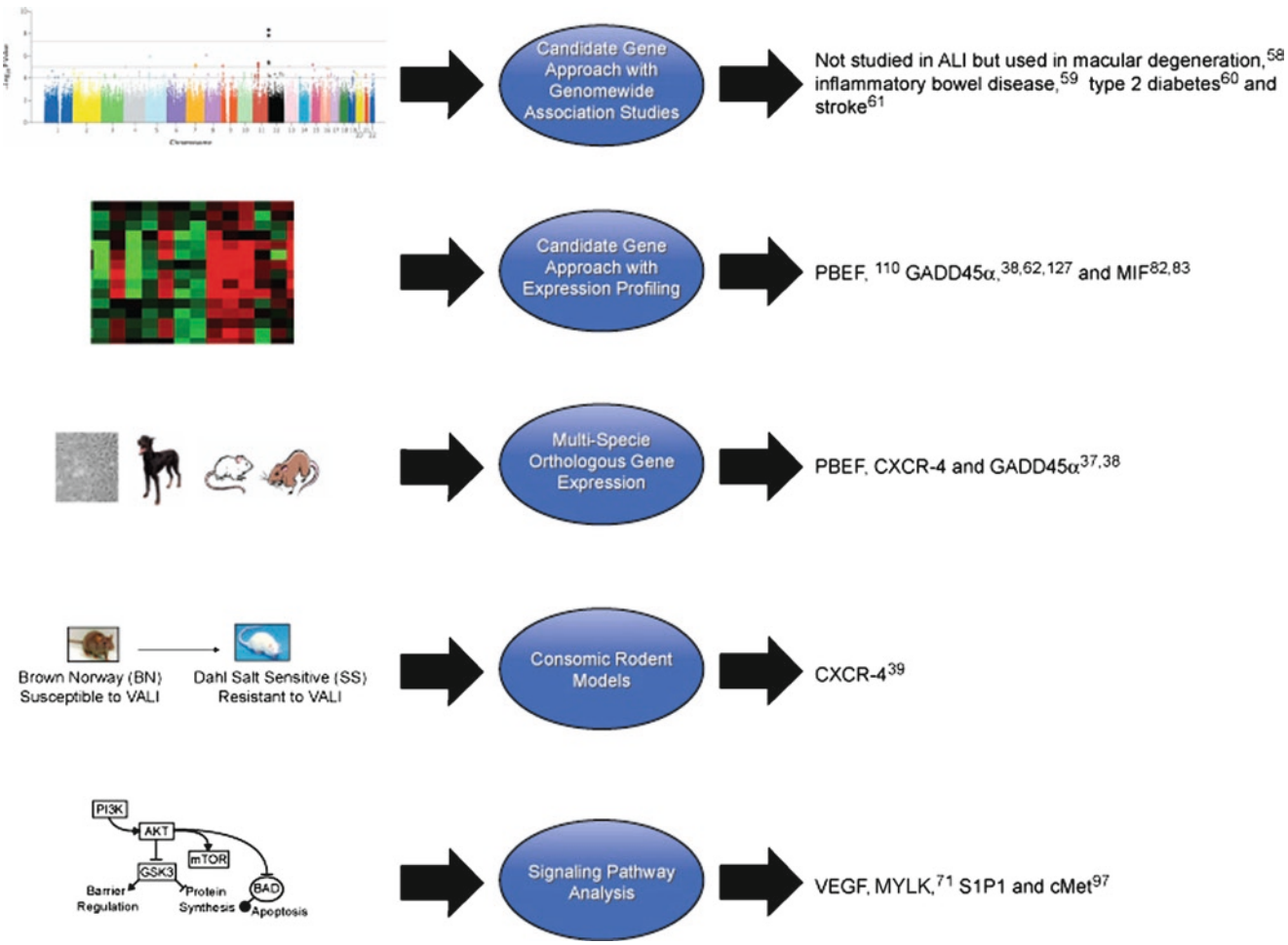

Fig. 1 Novel approaches to identify acute lung injury (*ALI*) genes. High-throughput gene sequencing and expression technologies, and complete genome sequencing of model organisms, now provide the tools to perform large-scale analyses of the genome in complex disorders such as ALI. Genome-wide association study (*GWAS*) platforms are effective and have been successfully used in diverse disorders, but although this approach has yet to be employed in either sepsis or ALI, the application of GWAS to the disease is clearly imminent. The differential gene expression between lung apex/base regions as well as between gravitationally dependent/nondependent regions of the lung base in a canine model of ventilator-associated lung injury (*VALI*) identified ALI-implicated lung genes in response to local mechanical stress within the lung. This approach identified the already established ALI gene macrophage migration inhibitory factor and novel genes such as growth arrest DNA damage inducible (*GADD45*) and pre-B cell colony enhancing factor (*PBEF*). Our multispecies orthologous gene approach in human (endothelial cells), rat, mouse, and canine models of VALI exhibits expression of common ALI-implicated evolutionarily conserved genes (orthologues) across the species. The genes with a unidirectional 1.3-fold change ($p > 0.05$) are found to reside in high density on rat chromosomes 13 and 16, the chromosomal loci used to develop the consomic rodent model. Together, these approaches identified novel ALI genes such as PBEF, chemokine receptor (*CXCR-4*) GADD45. Interrogating the prospective pathways involved in endothelial permeability and correlation with these differentially expressed genes in VALI models identified the most putative ALI genes such as myosin light chain kinase (*MYLK*), sphingosine 1-phosphate receptor 1, cMet, and vascular endothelial growth factor (*VEGF*)

High-throughput whole genome scanning technology has recently emerged as a powerful tool, particularly in detecting disease-susceptibility genes with modest effects. The Haplotype Mapping Project [56], which identified blocks of SNPs associated with each other, has allowed selection of the most informative SNPs for further disease association studies [57]. Currently, the most commonly used high-throughput SNP platforms involve assessment of over one million SNPs spanning the genome, i.e. genome-wide association studies (GWAS). GWAS platforms are effective and have been successfully used in diverse disorders such as age-related macular degeneration [58], inflammatory bowel disease [59], type 2 diabetes [60], and stroke [61]. Although this approach has yet to be employed in either sepsis or ALI, the application of GWAS to the disease is clearly imminent.

Another method to identify ALI candidate genes is an orthologue gene in silico approach. The basis of this approach is the hypothesis that patients with ALI and preclinical animal models of ALI would exhibit commonality in expression of evolutionarily conserved genes across species. For example, profiling results from more than 50 Affymetrix microarray chips obtained from ventilator-associated ALI models (human, rat, mouse, canine) identified 3,077 genes whose expression was altered across all four species in response to ventilator-associated

mechanical stress [37, 38]. Filtering these results for a unidirectional change in gene expression with greater than 1.3-fold change in expression refined the list to 69 genes, reflecting specific ALI-associated gene module/ontology categories: coagulation, inflammation, chemotaxis/cell motility, and immune response. This approach identified multiple genes already recognized as ALI genes (such as IL-6, AQP-1, and PAI-1), but also identified several novel genes that were not previously known to be mechanistically involved in ALI [38]. Complementing the in silico approach described above, a consomic rat approach can also be utilized to identify novel ALI gene candidates. In an experimental study, two strains of inbred rodents were determined to have differing susceptibility to VALI (20 mL/kg, 4 h): VALI-sensitive BN rats and the VALI-resistant Dahl SS rats. Using microarray analysis and a bioinformatic-intense candidate gene approach, we identified 245 differentially expressed potential VALI genes with ontologies such as transcription, chemotaxis, and inflammation. Because chromosomes 2, 13, 16, and 17 were found to contain the highest number of VALI-response genes, consomic SS rats containing substituted BN chromosome 13 were exposed to VALI mechanical stress, resulting in conversion of the resistant SS rat to VALI sensitivity [39].

Extensive expression profiling across preclinical ALI models can extend the identification of ALI gene candidates to determination of allelic frequencies of gene polymorphisms (SNPs) that may confer ALI risk or severity. This "candidate gene approach" has identified several candidates with hypothesized significant mechanistic roles in lung injury, inflammation, or repair in the setting of ALI and VALI [62]. Further, given the availability of sophisticated bioinformatic methods and increasing knowledge of the molecular and cellular mechanisms of lung injury, candidate genes can also be identified via analysis of cellular pathways involved in ALI pathogenesis [63, 67].

4 Novel Acute Lung Injury Candidate Genes and Biomarkers

The application of the novel techniques described in the previous section is proving to be exceptionally useful in identifying novel candidate genes and genetic variations in the study of the pathobiology of ALI. These novel gene and biomarkers are discussed in this section.

4.1 Myosin Light Chain Kinase

Myosin light chain kinase (MLCK) is an enzyme that phosphorylates regulatory myosin light chains, which allows myosin cross-bridging interactions with F-actin. In endothelial cells, the contraction of the actomyosin complex generates a stronger centripetal force that overcomes the force keeping the adjacent endothelial cell tethered, leading to endothelial retraction, decreased intercellular adhesion, and increased vascular permeability [68, 69]. This phenomenon is physiologically relevant as evidenced by nonmuscle MLCK (nmMLCK) isoform knockout mice [which retain the smooth muscle MLCK (smMLCK) isoform] that are less susceptible to lipopolysaccharide (LPS)- and ventilator-induced ALI [70, 71]. Further, treatment with a MLCK inhibitor prior to LPS exposure in the wild-type mice attenuates endothelial cell barrier dysfunction and inflammation [70]. Thus, the myosin light chain kinase gene (*MYLK*), which encodes for MLCK, is an excellent ALI candidate gene.

Since initial cloning of the highly expressed nmMLCK in endothelium in our laboratory [72], we have identified substantial roles of nmMLCK in cytoskeleton rearrangement of endothelial cells regulating vascular barrier function [64, 68], angiogenesis, and leukocyte diapedesis [73], consistent with a potential mechanistic role for MLCK in the genesis of ALI. The human *MYLK* gene is located on chromosome 3q21 and encodes three proteins, including nmMLCK, smMLCK, and telokin. We sequenced exons, exon–intron boundaries, and 2 kb of the 5′ UTR of *MYLK* in healthy individuals, patients with sepsis alone, and patients with sepsis-associated ALI, all of European and African-American descent [66], and identified 51 SNPs (ten exonic, 31 intronic, nine in the 5′ UTR, and one in the noncoding exon 1), of which 28 were chosen for further linkage disequilibrium studies. Five of the ten coding *MYLK* SNPs confer an amino acid change (Pro21His, Pro147Ser, Val261Ala, Ser1341Pro, and Arg1450Gln) in MLCK. Subsequently, association analysis of both single SNPs and haplotypes demonstrated very strong associations in both ethnic groups [66]. In European Americans, the rs3845915A/MYLK_037C haplotype was associated with more than a fivefold increase in the risk of developing ALI and sepsis. In contrast, the haplotype MYLK_021G/MYLK_022G/MYLK_011T conferred specific risk for ALI but not sepsis [66]. The 5′ haplotype of the *MYLK* gene also conferred ALI-specific risk in both European- and African-descent subjects; however, the 3′ region haplotype was associated with ALI only in African-descent subjects. In African-Americans, the haplotype hcv1602689**C**/MYLK_037**A**/rs11707609**G** is substantially more prevalent in ALI (11%) as compared with sepsis (1%). This CAG haplotype is not found in European Americans, suggesting a potential genetic contribution to the observed ethnicity-specific differences in ALI/ARDS prevalence and susceptibility [4]. We noted similar findings in association studies involving a cohort with trauma-induced ALI [74]. We have also evaluated the association of 17 *MYLK* genetic variants with severe asthma in both European American and African-American populations and identified a SNP highly associated with severe asthma in

African-Americans [75] consistent with data linking this chromosomal locus (*MYLK*, 3q21.1) to asthma and asthma-related phenotypes [75]. Taken together, these data strongly implicate *MYLK* genetic variants as risk variants in inflammatory lung disorders, such as ALI and asthma.

4.2 Macrophage Migration Inhibitory Factor

Macrophage migration inhibitory factor (MIF) is an ALI candidate gene and recognized biomarker, initially discovered as a soluble product of activated T cells and named for its role in inhibiting random macrophage migration [76]. MIF is a proinflammatory cytokine which binds to CD44 and CD74 and is produced by many cell types, including monocytes/macrophages, pituitary cells, vascular endothelium, and respiratory epithelium [77, 78]. MIF may serve as a delicate regulator of the cytokine balance between immunity and inflammation as MIF counterregulates the immunosuppressive effects of glucocorticoids [79]. The role of MIF as an endogenous prosurvival factor has been demonstrated in vitro. LPS-mediated induction of Flice-like inhibitory protein (FLIP) by MIF confers resistance to LPS-mediated endothelial cell death [80]. Suppression of MIF by RNA interference induces cell death and sensitivity to apoptotic stimuli [80]. In addition, MIF interacts with the multidimensional nmMLCK [81] isoform which regulates TNF-mediated apoptosis in addition to its potent effects on endothelial cell barrier dysfunction as discussed earlier [68, 69]. Together, these findings implicate the role of MIF in regulation of nonmuscle cytoskeletal dynamics and vascular pathophysiology, which is evident from the enhanced MIF levels in the serum, BAL fluid, and alveolar endothelium of patients with ARDS as compared with other critically ill patients [76, 78, 82]. We found significant increases in MIF transcript and protein levels in murine and canine models of ventilator-induced lung injury (VILI) (using high mechanical ventilation and endotoxin exposure, respectively) [82] and in human lung endothelium cells exposed to 48 h of cyclic stretch [83]. MIF deficiency or immunoneutralization appears to protect mice or rats from fatal endotoxic shock or other inflammatory diseases [84] although these results are not without controversy [85] and our own studies in 8–12-week-old mice failed to demonstrate a VILI/ALI-related phenotype which was different from controls (data not shown). MIF also upregulates the expression of AQP-1, the water channels expressed in alveolar endothelial and epithelial cells, and a candidate gene we identified in models of VILI-associated mechanical stress [38]. MIF may serve to modulate fluid movement into alveolar spaces, a cardinal feature of ALI [86].

To extend the likelihood that MIF serves as a putative candidate gene in ALI and sepsis, we studied the association of eight MIF polymorphisms, including the most studied MIF promoter G/C SNP at position -173, in a sepsis-induced ALI cohort ($n = 506$) of African- and European-descent cases [82]. No individual SNP showed a significant association with either ALI or sepsis; however, the carriers of the CC genotype (rs755622) and the carriers of the TT genotype (rs2070767) showed more than twofold increased risk of developing sepsis and ALI, respectively. This association was lost, however, after age and gender adjustment in a logistic regression model. In contrast, MIF haplotypes at the 3′ region of the gene display strong association with ALI and sepsis, conferring both protection as well as susceptibility to ALI, in European and African populations [82]. Furthermore, the haplotype at the 5′ promoter region of the gene involving a short tandem repeat at position -794 (CATT)5 and the -173 G allele show significant association with both ALI and trauma [82]; however, no association was found between promoter region haplotypes and MIF levels. Rheumatoid arthritis patients with the -173C allele have higher levels of MIF in the serum and synovial fluid than the carriers of the G allele and have a higher probability of developing idiopathic arthritis [87]. Thus, given these diverse MIF functions, MIF remains an attractive target in inflammatory diseases including the lung.

4.3 Sphingosine 1-Phosphate Receptor 1

The bioactive sphingolipid metabolite sphingosine 1-phosphate (S1P) is an important lipid mediator that enhances endothelilal cell barrier function in vivo and in vitro by ligating S1P receptor 1 (S1P1), which is encoded by an endothelial differentiation gene (*EDG1* or *S1P1*) [88, 89]. S1P1 is a pertussis-toxin-sensitive, G_i-coupled receptor which induces Rac GTPase-dependent substantial increases in cortical actin polymerization critical to endothelial cell barrier enhancement [88, 90]. S1P1 activation enhances the organization and redistribution of vascular endothelial cadherin and β-catenin in junctional complexes in endothelium by phosphorylation of cadherin as well as p120-catenin and inducing the formation of cadherin/catenin/actin complexes [91]. Understanding the role of S1P in enhancing endothelial cell barrier function underscores its importance as a therapeutic target in reversing loss of endothelial cell barrier integrity. In vivo administration of selective S1P1 competitive antagonists induces a dose-dependent disruption of barrier integrity in pulmonary endothelium [92, 93], whereas S1P1 agonists, SEW2871 and FTY720, promote vascular endothelial barrier function [94–96]. A compelling argument for S1P1 as an attractive ALI candidate gene is not only its ability to transduce signals which restore barrier integrity but also that S1P1 is the target for transactivation by receptors for other potent barrier-protective agonists. These include EPCR (receptor for activated protein C) [65], c-Met [receptor for hepatocyte growth factor (HGF)] [97], CD44 (receptor for high molecular

weight hyaluronan) [67], and the ATP receptor [98]. We recently resequenced the S1P1 gene (14 African-Americans and 13 European Americans) to search for common variations in the *EDG1* gene and identified 39 SNPs in the *EDG1* gene, with several promoter SNPs associated with asthma, another inflammatory lung syndrome [99].

4.4 c-Met (Hepatocyte Growth Factor Receptor)

The role of HGF and its tyrosine kinase receptor c-Met has been investigated in lung development, inflammation, and repair [100] as well as in neoplastic processes such as cellular transformation, neoplastic invasion, and metastasis [101, 102]. SNPs causing underexpression of c-Met have been associated with autism and c-Met SNPs/mutations appear to be linked to lung cancer disparities in different ethnic groups. These include an N375S mutation in the HGF-binding domain of c-Met, an R988C SNP/mutation in the juxtamembrane domain, and an activating M1268T mutation in the tyrosine kinase domain (exon 19), all linked to development of solid tumors such as lung cancer, renal cancer, gastric cancer, and hepatocellular carcinoma [102]. HGF influences morphogenesis in epithelial cells from a variety of organs, including lungs, where HGF antisense oligonucleotides block alveolar and branching morphogenesis [103]. HGF expression and activity increase after 3–6 h of lung injury with intratracheally administered hydrochloric acid, suggesting that HGF plays a role in reparative responses to lung injury [104]. c-Met expression on type II pneumocytes is likely involved in increased type II pneumocyte proliferation and restoration of an intact alveolar epithelium [105]. c-Met is composed of a 50-kDa extracellular α subunit and a 145-kDa transmembrane β subunit [106] which contains tyrosine kinase domains, tyrosine phosphorylation sites, and tyrosine docking sites [107]. We demonstrated that HGF-mediated c-Met phosphorylation and c-Met recruitment to caveolin-enriched microdomains (CEMs) protects against the LPS-induced pulmonary vascular hyperpermeability that is regulated by high molecular weight hyaluronan (CD44 ligand) [108]. Our novel findings indicate that HGF/c-Met-mediated, CD44-regulated CEM signaling promotes Tiam1 (a Rac1 exchange factor)/dynamin 2 dependent Rac1 activation, and peripheral recruitment of cortactin (an actin cytoskeletal regulator), processes essential for endothelial cell barrier integrity. Understanding the mechanism(s) by which HGF/c-Met promotes increased endothelial cell barrier function may lead to novel treatments for diseases involving vascular barrier disruption, including inflammation, tumor angiogenesis, atherosclerosis, and ALI. However, on the contrary, the higher mortality rate in ALI patients with increased levels of HGF in BAL fluids [109] and in pulmonary edematous fluids [110] indicates severer injury and inflammation in response to increased HGF levels. It has now become increasingly clear that HGF plays an important role in normal and injured lung and may have a therapeutic potential in lung diseases.

4.5 Pre-B Cell Colony Enhancing Factor

Pre-B cell colony enhancing factor (PBEF), was first identified by Samal and colleagues in 1994 as a protein secreted by activated lymphocytes in bone marrow stromal cells that stimulate early stage B cell formation in conjugation with stem cell factor and interleukin-7. A large body of work has now highlighted the power of a systems biology approach in the search for novel disease-susceptibility genes and potentially novel biomarkers, with PBEF serving as an excellent example of this approach (Fig. 2). We first identified marked upregulation of PBEF via microarray analyses of murine and canine models of VILI/ALI with increased gene/protein expression in BAL fluid and serum samples from critically ill ICU patients with ALI and sepsis [111]. With only a total of eight papers in PubMed at that time, we next directly sequenced the PBEF gene in 36 subjects with ALI, sepsis, and healthy controls and conducted a PBEF SNP-based association study in ALI subjects of European and African-American descent [111]. We identified 11 SNPs in the *PBEF* gene with two promoter SNPs, T-1001G and C-1543 T, associated with ALI and sepsis. Genotyping of PBEF C-1543 T and T-1001G SNPs revealed significant associations of sepsis and ALI, with the strongest association found with the -1543C/-1001G haplotype. Univariate analysis found carriers of the G allele (T1001G) to have 2.75-fold higher risk of developing ALI as compared with controls ($p = 0.002$) [111]. These results were subsequently confirmed in a comparable but distinct replicate ALI population [112]. Interestingly, the -1543G/-1001C haplotype was also associated with increased ICU parient mortality, whereas the -1543 T/-1001 T haplotype was associated with fewer ventilator days and decreased ICU patient mortality [112].

A key challenge in genomic explorations is the ability to confirm the contribution of a SNP to a dysfunctional-gene-involved disease process. Additional reports have highlighted the capacity for the PBEF gene to have an influence far beyond any B-cell regulatory function, with a key role in regulating vascular permeability [113] as well as inhibiting neutrophil apoptosis [114]. To further explore mechanistic participation of PBEF in ALI and VILI, we focused on the contribution of PBEF to endothelial function. Our prior immunohistochemical staining of canine-injured lung tissues localized PBEF expression to vascular endothelial cells, in addition to infiltrating neutrophils and type 2 alveolar epithelial cells [111]. Our in vitro studies showed that expression

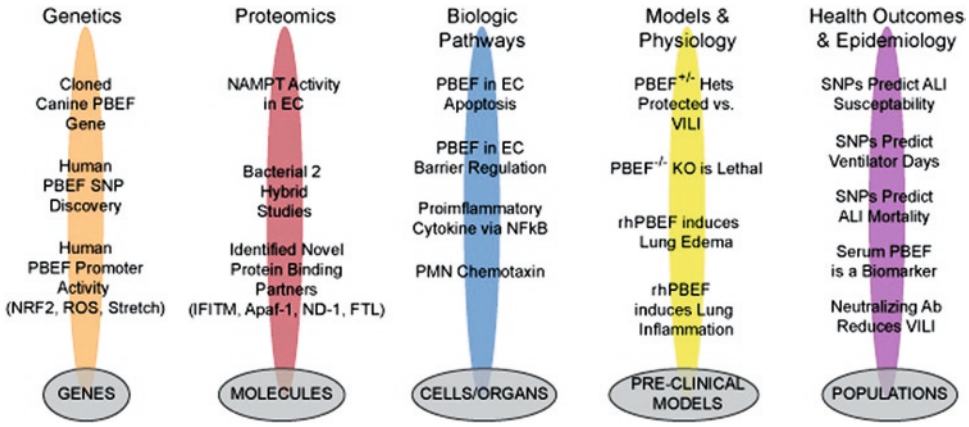

Fig. 2 Systems biology approach in defining the role of *PBEF* in ALI and ventilator-induced lung injury (*VILI*). Each systems biology compartment has been utilized to address PBEF involvement across the entire systems biology spectrum. This includes genes (microarrays, SNP discovery, small interfering RNAs, microRNAs, promoter luciferase assays), proteins (bacterial two hybrid studies, recombinant human PBEF (*rhPBEF*), cell signaling, site-directed mutagenesis), organelles (nuclear events, mitochondrial function, cytoskeleton), cells (endothelial cells, neutrophils), organs (lung), preclinical animal models (genetically engineered $PBEF^{+/-}$ mice, VILI, and sepsis models), and populations [bronchoalveolar lavage (*BAL*) studies, biomarker studies in intensive care unit (*ICU*) subjects, SNP association studies]

of PBEF in pulmonary artery endothelial cells increases thrombin-mediated vascular permeability [111], suggesting that enhanced PBEF expression may mediate the early increase in vascular permeability that is characteristic of ALI. Neutrophils harvested from the circulation of septic and ALI patients show marked inhibition of the apoptotic process in association with evidence of enhanced respiratory burst capacity [115, 116], with both activities largely restored with administration of PBEF antisense oligonucleotides. Our initial in vitro studies further demonstrated recombinant human PBEF (rhPBEF) as a direct rat neutrophil chemotactic factor, with in vivo studies demonstrating marked increases in BAL fluid leukocytes (polymorphonuclear leukocytes, PMNs) following intratracheal injection in C57BL/6 J mice [117]. These changes were accompanied by increased BAL fluid levels of PMN chemoattractants (KC and MIP2) and modest increases in lung vascular and alveolar permeability. We also noted synergism between rhPBEF challenge and a model of limited VILI and observed dramatic increases in BAL fluid PMNs, BAL protein, and cytokine levels (IL-6, TNFα, KC) compared with either challenge alone. Gene expression profiling identified induction of ALI- and VILI-associated gene modules (NF-κB, leukocyte extravasation, apoptosis, toll-receptor pathways). Heterozygous $PBEF^{+/-}$ mice were significantly protected (reduced BAL fluid protein levels, BAL fluid IL-6 levels, peak inspiratory pressures) when exposed to a model of severe VILI (4 h, 40 mL/kg tidal volume) and exhibited significantly reduced gene expression of VILI-associated modules. Finally, strategies to reduce PBEF availability (neutralizing antibody) resulted in significant protection from VILI [117]. PBEF is now recognized as associated with modestly increased risk of type 2 diabetes and elevated levels of acute-phase proteins [118] and a C-948G SNP has been associated with an increased diastolic blood pressure in obese children [119]. These studies implicate PBEF, now associated with a number of inflammatory disorders such as inflammatory bowel disease, multiple sclerosis, cystic fibrosis, and asthma [120–122], as a key inflammatory mediator intimately involved in both the development and the severity of ventilator-induced ALI.

4.6 *Growth Arrest DNA Damage Inducible α (GADD45α)*

Growth arrest DNA damage inducible α (GADD45α), a member of an evolutionarily conserved gene family, is implicated as a stress sensor that modulates the response of mammalian cells to genotoxic or physiological stress [123, 124]. GADD45α is a small 21-kDa predominantly nuclear protein that interacts with other proteins implicated in stress responses, including proliferating cell nuclear antigen, p21, Cdc2/cyclin B1, MEKK4, and p38 kinase [125, 126]. GADD45 induces cell cycle arrest and apoptosis in most of cells as well as promoting DNA repair functions and survival [126]. Growth Arrest and DNA Damage gene (GADD45) also maintains genomic stability in a p53-responsive manner [127]. Despite the multiple known functions of GADD45, its role ALI, endothelial/epithelial barrier dysfunction, or repair of injured lung is unknown [38]. GADD45 exhibited differential expression in orthologous global gene expression profiling, in multispecies ALI models [38], in region-specific lung tissue expression profiling [62], and was markedly upregulated in response to the VILI [128]. We explored the mechanistic involvement of GADD45α in endotoxin (LPS)- and ventilator-induced inflammatory lung injury (VILI) by comparing multiple biochemical and genomic parameters of

inflammatory lung injury in wild-type C57Bl/6 and GADD45α$^{-/-}$ knockout mice exposed to high tidal volume ventilation (VILI) or intratracheally administered LPS [129]. GADD45α$^{-/-}$ mice were modestly susceptible to LPS-induced injury but were profoundly susceptible to VILI, demonstrating increased inflammation and increased microvascular permeability. VILI-exposed GADD45α$^{-/-}$ mice manifested striking neutrophilic alveolitis with increased BAL fluid levels of protein, IgG, and inflammatory cytokines. Expression profiling of lung homogenates revealed strong dysregulation in the B cell receptor signaling pathway in GADD45α$^{-/-}$ mice, suggesting the involvement of phosphatidylinositol 3-kinase/Akt signaling components. Western blots confirmed a threefold reduction in Akt protein and phosphorylated Akt levels observed in GADD45α$^{-/-}$ lungs. Electrical resistance measurements across human lung endothelial cell monolayers transfected with small interfering RNAs to reduce GADD45α or Akt expression revealed significant potentiation of LPS-induced endothelial barrier dysfunction which was attenuated by overexpression of a constitutively active Akt1 transgene. Whereas other lung injury studies failed to demonstrate a role for GADD45 in hyperoxic lung injury [130, 131], our studies validate GADD45α as a novel inflammatory lung injury candidate gene and a significant participant in vascular barrier regulation via effects on Akt-mediated endothelial signaling [132]. Thus, both Akt and GADD45 are extremely attractive ALI candidate genes. The human GADD45α gene contains 25 validated SNPs (National Center for Biotechnology Information SNP database) whose role in ALI pathogenesis is completely unknown [123]. We are currently pursuing further characterization of the role of GADD45α and its association of genetic variants with sepsis and ALI.

5 Novel Therapeutics

The identification of novel pathways involved in the pathobiology of ALI also opens doors for the exploration of new therapeutic targets for the disease. As such, the use of agents that attenuate the endothelial barrier dysfunction and the inflammatory response characteristic of ALI have shown promise in preclinical studies which will hopefully lead to their use in trials of human ALI (Fig. 3).

5.1 Sphingosine 1-Phosphate

S1P, an important lipid mediator generated by the phosphorylation of sphingosine by sphingosine kinase, decreases endothelial permeability to both water and solute via cytoskeletal reorganization and adherens junction assembly

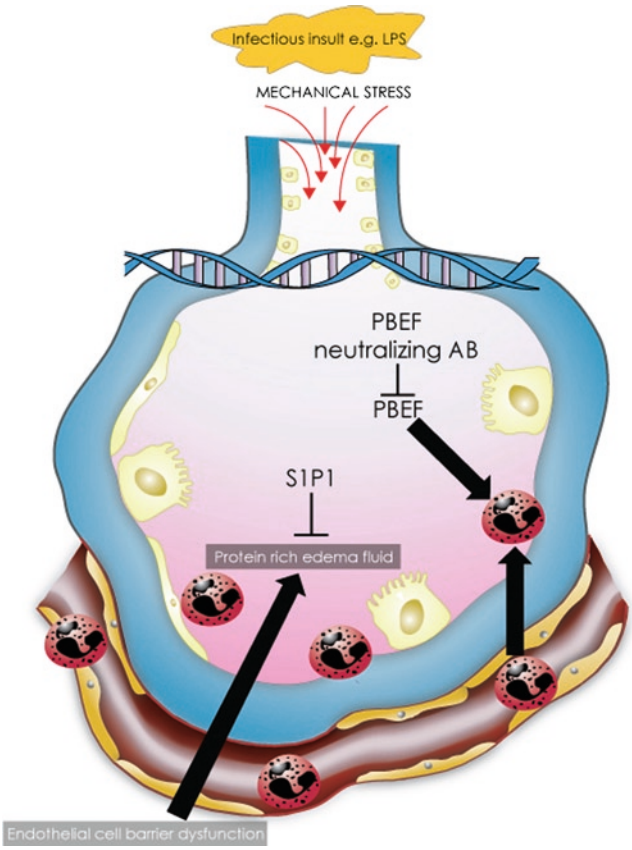

Fig. 3 Mechanism-based novel therapies for ALI. The identification of novel pathways involved in the pathobiology of ALI also facilitates the exploration of new therapeutic targets. Sphingosine 1-phosphate (S1P1) attenuates the endothelial barrier dysfunction associated with ALI, whereas blocking of PBEF attenuates VALI

[88, 89]. S1P-induced barrier protective effects could serve to attenuate the increased pulmonary vascular permeability essential factor in the development of ALI. The S1P analogue FTY720 (0.1 mg/kg),when administered to C57BL/6 mice with endotoxin-induced lung injury, decreases lung edema formation, solute transport across the alveolar capillary endothelium, and inflammatory cell infiltration into lung parenchyma [94]. Similarly, the prophylactic administration of S1P attenuates both alveolar and vascular barrier dysfunction while significantly reducing shunt formation associated with lung injury in rodent and canine models of ALI induced by combined intrabronchial endotoxin administration and high-tidal-volume mechanical ventilation [133]. In a recent study of a canine model of ALI, we demonstrated that when bacterial endotoxin was instilled intratracheally followed in 1 h by intravenous administration of S1P (85 μg/kg) or vehicle and 8 h of high-tidal-volume mechanical ventilation [134], S1P treatment attenuated the severity of ALI-induced increases in shunt fraction and the presence of both protein and neutrophils in BAL fluid compared with vehicle controls. Interestingly, BAL fluid cytokine production was not altered

significantly by intravenous administration of S1P and S1P potentiated the endotoxin-induced systemic production of the inflammatory cytokines TNFα, C-X-C chemokine ligand-1, and IL-6, without resulting in end-organ dysfunction. These data suggest that S1P may represent a viable therapy for the prevention and treatment of ALI.

5.2 Pre-B Cell Colony Enhancing Factor Blockage

As previously described in this chapter, PBEF appears to play a central role in the promotion of several pathogenetic aspects of ALI and VALI. Therefore, interventions aimed at attenuating the effects of PBEF could have a potential therapeutic effect in these disorders. To begin to address the potential for PBEF to serve as a therapeutic target in ameliorating VILI, we assessed the effect of PBEF neutralizing antibody on rhPBEF-stimulated lung inflammation [117]. Simultaneous instillation of rhPBEF and PBEF neutralizing antibody produced dramatic reductions in rhPBEF-induced neutrophil recruitment. Further, the intratracheal delivery of PBEF neutralizing antibody (30 min before high-tidal-volume mechanical ventilation) abolished VILI-induced increases in total BAL fluid cell counts and significantly decreased neutrophil influx into the alveolar space as well as VILI-mediated increases in the level of lung tissue albumin.

6 Summary

ALI is a major cause of morbidity and mortality in critically ill patients. Given the unacceptably high mortality rate observed in ALI and the paucity of novel therapies and biomarkers, it is essential to recognize molecular targets associated with ALI to identify individuals at risk and to develop novel therapeutic targets and biomarkers. It is clear that derangements in endothelial cell barrier regulation play a major role in the pathobiology of ALI and genetic variants regulate endothelial cell barrier function, thereby determining ALI risk or subsequent severity of outcome. High-throughput gene sequencing and expression technologies, and complete genome sequencing of model organisms, have allowed for the performance of large-scale analyses of the genome in ALI. In this chapter, we have highlighted how global gene expression profiling in multispecies ALI models served to broaden our net knowledge of ALI-implicated genes and provide a basis for hope that increased insights and therapies may be forthcoming. As genotyping becomes more rapid and easily accessed, combining advanced bioinformatics techniques with high-throughput methods will be the future practice of personalizing treatment strategies. Continued challenges will be the gene–gene and gene–environment interactions, which add complexity to our understanding of the genome. These novel genetic approaches may prove exceptionally useful in ushering in the era of personalized medicine for critically ill individuals.

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