IMPACT OF DIABETES ON ACUTE LUNG INJURY: UNRAVELLING MECHANISMS TO IMPROVE OUTCOMES

by

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ABSTRACT

Diabetes mellitus (DM) is a common comorbidity in acute lung injury (ALI) patients. Despite its well-established prevalence and known complications affecting many organs, our understanding of the lung as a target organ in DM remains limited, relying heavily on inconsistent findings from observational data. This thesis aims to investigate the effects of DM on lung health and ALI development and outcomes using a combinatorial approach incorporating clinical, cellular, and pre-clinical data. The Fluids and Catheters Treatment Trial (FACTT) dataset and plasma samples were analyzed to assess the impact of pre-existing diabetes on ALI patients. Additionally, the molecular effects of advanced glycation end-products (AGEs) were examined in human microvascular lung endothelial (HMLE) and epithelial (A549) cells, while the influence of DM on lung health and ALI severity was evaluated in 12-week-old type 1 DM mice with or without sepsis. Clinically, we found that ARDS patients with DM had lower survival rates and longer hospital and ICU stays compared to those without DM. Mechanistically, AGE treatment (50 µg/ml) modulated tight junction proteins and impaired barrier function. AGE exposure also activated key inflammatory pathways (AKT and P38 MAPK) and elevated the expression of inflammatory cytokines (TNF-α, IL-1β, IL-10, and IL-6). Experimentally, mouse DM lungs exhibited significant inflammation and edema compared to non-DM lungs. RNA sequencing identified genes involved in inflammation and endothelial barrier dysfunction, supporting the link between DM and lung injury. Furthermore, DM increased the risk of worsening ALI in sepsis mice compared to on-DM sepsis. Overall, our findings indicate that DM is a risk factor for lung inflammation and worsened ALI progression. This study highlights the potential for improving ALI outcomes by targeting diabetes-associated inflammatory and metabolic pathways, offering new insights for therapeutic interventions in diabetic patients at risk for ALI.

INDEX WORDS: ARDS; diabetes mellitus; inflammation; cytokines; advanced glycation end products; lung endothelial barrier.

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DEDICATION

This thesis is wholeheartedly dedicated to my father, Hammad, who has meant and continues to mean so much to me. Although he is no longer in my life, his memories and encouraging words continue to support and regulate my life.

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CHAPTER 1

The impact of diabetes mellitus on blood-tissue barrier regulation and vascular complications: Is the lung different from other organs?

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Abstract

Diabetes Mellitus presents a formidable challenge as one of the most prevalent and complex chronic diseases, exerting significant strain on both patients and the world economy. It is recognized as a common comorbidity among severely ill individuals, often leading to a myriad of micro- and macro-vascular complications. Despite extensive research dissecting the pathophysiology and molecular mechanisms underlying vascular complications of diabetes, relatively little attention has been paid to potential lung-related complications. This review aims to illuminate the impact of diabetes on prevalent respiratory diseases, including chronic obstructive pulmonary disease (COPD), acute respiratory distress syndrome (ARDS), idiopathic pulmonary fibrosis (IPF), tuberculosis (TB), pneumonia infections, and asthma, and compare the vascular complications with other vascular beds. Additionally, we explore the primary mechanistic pathways contributing to these complications, such as the expression modulation of blood-tissue-barrier proteins, resulting in increased paracellular and transcellular permeability, and compromised immune responses rendering diabetes patients more susceptible to infections. The activation of inflammatory pathways leading to cellular injury and hastening the onset of these respiratory complications is also discussed.

Keywords: diabetes mellitus; inflammation; vascular permeability; pneumonia; acute respiratory distress syndrome; asthma; chronic obstructive pulmonary disease; pulmonary fibrosis

1. Introduction

Diabetes Mellitus (DM) is a complex systemic disorder characterized by persistent hyperglycemia, often accompanied by inflammation and oxidative stress[1]. It stands as one of the most prevalent and formidable chronic diseases, exerting a substantial burden on individuals and economies worldwide[1]. The International Diabetes Federation reports a concerning annual rise in DM incidence, reaching a staggering 536.6 million cases in 2021, projecting a surge to 783.2 million in 2045[2]. While type-2 DM accounts for over 90% of the cases, it was estimated that 8.4 million were living with type-1 DM worldwide in 2021[3]. Furthermore, DM is recognized as one of the most prevalent comorbid conditions among critically ill patients, contributing to approximately 3.7 million deaths worldwide. Notably, about 40% of patients admitted to intensive care units have pre-existing DM[4]. Morbidity and mortality in patients with DM are presumably attributed to endothelial dysfunction, and subsequent vasculopathy, including retinopathy, nephropathy, neuropathy, accelerated atherosclerosis, and cerebrovascular accidents, are significant complications of DM[5]. The pathophysiology and molecular mechanisms underlying micro- and macro-vascular complications caused by DM have been extensively investigated[6]. Yet, scant attention has been directed toward the lung as a potential target organ for DM, leaving this aspect inadequately investigated. Diverse conclusions have been drawn from several discordant studies in the literature regarding the effect of DM on lung disorders. While some studies suggest that DM does not impact lung function[7], several others indicate the contrary[8, 9]. Consequently, comprehensive studies investigating the association between DM and respiratory diseases at both bench and clinical phases are required to elucidate such a relationship. Additionally, understanding the impact of co-morbid diseases on the pathophysiology of respiratory diseases might provide valuable guidance for optimal treatment strategies. Hence, this review focuses on the potential mechanisms underlying the impact of DM on physiological functions such as immunity, vascular health, and inflammation and explores the relationship between DM and prevalent respiratory diseases, encompassing chronic obstructive pulmonary disease (COPD), acute respiratory distress syndrome (ARDS), idiopathic pulmonary fibrosis (IPF), tuberculosis (TB), pneumonia, and asthma.

2. The influence of DM on physiological functions

a. DM and immunity

It is believed that the susceptibility of DM patients to infections is attributed to several dysregulations of the immune system, mainly due to the impact of hyperglycemia on immunity[10]. Although limited studies have been conducted to investigate the exact mechanistic pathways behind the immune alterations, potential mechanisms such as decline in inflammatory cytokine production upon immune system activation and an upsurge in production with no identifiable triggers have been demonstrated in both mice and patients associated with DM[11-13]. In summary, the complex interplay between hyperglycemia and immune dysregulation underscores the increased susceptibility of DM patients to infections, shedding light on potential mechanistic pathways that warrant further investigation.

b. DM and vascular health

DM unveils several vascular complications, which are categorized into microvascular and macrovascular, with a much higher prevalence observed in the former[14]. Maintaining homeostasis relies on vascular permeability to sieve solutes and molecules, enabling vital exchange between vessels, tissues, and organs[15]. However, it can be altered in several disease conditions, particularly in DM. The prevailing belief is that microvascular complications are the main contributors to abnormal permeability[16]. The endothelial glycocalyx plays a crucial role in regulating vascular permeability by hindering cell margination towards the vessel wall, highlighting its significance in controlling this aspect of vascular function[17, 18]. Hyperglycemia has been identified to impair the glycocalyx function, leading to upregulated vascular permeability, as demonstrated in multiple in vitro and in vivo studies[19-21]. Research conducted on human umbilical vein endothelial cells demonstrated that hyperglycemia significantly increased monocyte adherence, expression levels of cell adhesion molecules, and reactive oxygen species production, ultimately leading to impaired vascular permeability[22]. Exposure to high glucose concentrations in human umbilical vein endothelial cells increased vascular endothelial cadherin (VE-cadherin) phosphorylation and subsequent dissociation of the VE-cadherin–β-catenin complex, disrupting endothelial Adherens junctions (AJs), with this effect being attenuated by inhibiting protein kinase c- β (PKC-β)[20]. Multiple research studies have consistently indicated that lung inflammation destabilizes the cells, AJs, specifically VE-cadherin–β-catenin, and compromises the endothelial barriers[23, 24]. A consensus has been reached regarding the involvement of disrupted tight junctions (TJs), compromised membrane integrity, and heightened vascular permeability, all of which can be held accountable for the pathologies of DM[25].

c. Diabetic microvascular complications

The primary microvascular complications of DM comprise nephropathy, neuropathy, and retinopathy[26]. Throughout the progression of the disease, most diabetic patients will experience one or more of these manifestations. These complications arise from a complex interplay of factors including hyperglycemia, endothelial damage, and oxidative stress, culminating in disturbances in blood flow, endothelial permeability, and the deposition and coagulation of extracellular proteins, ultimately leading to organ dysfunction[26, 27].

Diabetic retinopathy (DR) stands as the primary cause of vision impairment among the working-age population in developed nations. Studies suggest that the lifetime risk of DR reaches up to 90% in individuals with type-1 DM and approximately 50–60% in those with type-2 DM[28]. Remarkably, up to 40% of individuals diagnosed with type-2 DM already display some degree of retinopathy at the time of diagnosis[29]. Key factors contributing to the progression of DR include the DM type, duration of DM, glycemic control, and blood pressure levels[30]. A prior study indicated that disruption of NOTCH1 signaling in DR leads to endothelial junction destabilization and elevated vascular permeability through activation of the Src signaling pathway, resulting in the dissociation of VE-cadherin and β-catenin[31]. In the retina, pericyte disruption is a major contributor to microvascular remodeling and endothelial dysfunction[32]. Methyltransferase-like 3, a mammalian protein-coding gene whose overexpression spiked the hyperglycemia-induced pericyte dysfunction illustrated through decreased cell viability and impaired permeability in vitro, knocking out pericyte-specific methyltransferase-like 3 led to a successful reduction of retinal pericyte dysfunction and vascular complications in a streptozotocin (STZ)-induced type-1 DM mouse model[33]. In DR murine and human retinal and vitreous samples, overexpression of soluble epoxide hydrolase levels, an enzyme that causes the annihilation of pericytes and contributes to the deterioration of the endothelial barrier, was noted[34]. In a DR in vitro model, it was reported that human pericyte-like adipose mesenchymal stem cells improved the expression levels of VE-cadherin and ZO-1, resulting in the preservation of retinal barrier integrity in human retinal endothelial cells[35].

Diabetic nephropathy (DNeph) emerges from the synergistic effects of hyperglycemia and hypertension, which result in glomerular damage[36]. Worldwide, diabetic kidney diseases constitute the primary cause of end-stage renal disease[37]. Although not all diabetic patients develop DNeph, the progression varies among those affected[38]. The underlying pathological alterations entail thickening of the basement membrane, atrophy, interstitial fibrosis, and arteriosclerosis[26]. Clinically, numerous studies have identified vascular abnormalities in the glomeruli of individuals with long-term type-1 and type-2 DM[39, 36]. As DM advances, an in-depth examination of 3D images in patients with DNeph unveiled the presence of abnormal blood vessels, suggesting potential dysfunction of endothelial cells and a gradual decline in endothelial thickness, alongside observable swelling[40].

Like other microvascular complications, the risk of developing diabetic neuropathy (DNeuro) correlates with both the intensity and duration of hyperglycemia, with genetic factors influencing susceptibility in certain patients[41]. DNeuro encompasses a spectrum of neurological disorders associated with DM, resulting from damage to the peripheral and autonomic nervous systems[26]. This complication significantly impacts patients by inducing pain, increasing the risk of falls, and thereby diminishing their quality of life[42]. While various genes are implicated in the pathogenesis of DNeuro, research has primarily focused on polymorphisms in angiotensin-converting enzyme (ACE) and methylenetetrahydrofolate reductase (MTHFR)[42]. In a study utilizing capillary sodium fluorescein leakage as a measure of capillary permeability, it was observed that the leakage was significantly higher in diabetic patients with confirmed peripheral neuropathy than without peripheral neuropathy[43]. Together, these findings underscore the multifaceted nature of microvascular dysfunction in DM and highlight the importance of targeted interventions to preserve microvascular endothelial integrity and mitigate the progression of diabetic complications.

d. Diabetic macrovascular complications

Several preclinical studies have been conducted to illustrate the central role of vascular leakage in the development of diabetic macrovascular complications[44]. While the microvascular complications associated with DM can have severe consequences, diabetic cardiomyopathy stands out as a leading cause of mortality in diabetic patients, primarily due to its impact on myocyte loss and fibrosis[45, 46]. Diabetic cardiomyopathy is defined as abnormal ventricular function and myocardial structure without the presence of typical cardiac risk factors, such as hypertension, dyslipidemia, or coronary artery disease in diabetic patients[47]. A study revealed that diabetic patients were 39% more likely to experience heart failure compared to those without diabetes (23%)[48]. Hyperglycemia leads to the buildup of advanced glycation end products (AGEs), which cause myocardial structural abnormalities by provoking proinflammatory responses, promoting myocardial collagen and fibronectin, as well as matrix protein and connective tissue production through the activation of Janus kinase (JAK) and mitogen-activated protein kinase (MAPK) pathways[49]. The extended endothelial insult brought about by DM causes endothelial cells to undergo a phenomenon known as endothelial-to-mesenchymal transition (EndMT)[50], which is regarded as a hallmark of diabetic cardiac fibrosis[51, 52]. The overexpression of KLK8, a member of the tissue kallikrein-related peptidase (KLK) multigene family[53], potentially causes cardiac fibrosis[54]. By knocking down KLK8 in human coronary artery endothelial cells, a previous study uncovered substantial mitigation of hyperglycemia-induced endothelial damage, perivascular cardiac fibrosis, EndMT in the heart, and impaired permeability[55]. It was also reported that adipsin, an adipokine secreted by adipose cells, played a significant role in mitigating diabetic cardiomyopathy triggered by both hyperglycemia and exposure to palmitic acid by inhibiting VE-cadherin phosphorylation and internalization, thus reversing endothelial hyperpermeability in diabetic mice[56]. With DM having a notorious impact on different parts of the body, the lungs, being a vital organ in the body, are particularly affected by DM, making diabetic patients more prone to developing COPD, fibrosis, and lung cancer[57]. Lung fibrosis is predominantly driven by the deposition of ECM proteins by lung fibroblasts and inflammatory myofibroblasts. In a study using a diabetic rat model, it was found that diabetic rats developed lung fibrosis, while the fibrotic lung tissue demonstrated increased amounts of N-cadherin and alpha-smooth muscle actin proteins linked to epithelial to mesenchymal transition (EMT), implying that DM is implicated in promoting the loss of polarity and differentiation of epithelial cells via EMT[58]. In diabetic mice and human pulmonary microvascular endothelial cells, proinsulin C-peptide has been shown to mitigate the pulmonary fibrosis induced by hyperglycemia, where it impeded the expression of fibrosis-related proteins and, along with hindering vascular endothelial growth factor (VEGF)-induced adherens junctions (AJ) disruption, vascular leakage, hyperglycemia-induced inflammation, and apoptosis, actively counteracts the progression of pulmonary fibrosis[59].

3. Effect of DM on respiratory infections

Diabetic individuals have been observed to experience an increased occurrence of respiratory infections, coupled with a more intricate progression of such infections associated with these patients[60]. It has been observed that *Mycobacterium tuberculosis*, influenza virus, and *Streptococcus pneumoniae* are the most predominant pathogens that are responsible for respiratory infections in diabetic patients[61]. The influence of DM on respiratory infections has been evaluated in several studies **(Table 1)**. A study by *Miller* et al. aimed to identify the most common pre-existing diseases and risk factors for complications linked to H1N1, a pandemic influenza virus infection, found that DM was among the common co-morbidities contributing to severe complications and poor outcomes in affected individuals[62]. Tuberculosis (TB), a highly contagious infection, has been documented to disproportionately affect the lungs of diabetic patients compared to non-diabetic individuals. Research indicates that patients with a history of DM not only have a tripled likelihood of TB infection but also face an elevated risk of mortality[63]. A meta-analysis study validated a substantial positive correlation between DM and TB infection. It revealed that individuals with DM face an increased risk of contracting TB, with a two- to four-fold increase compared to those without DM[64]. Similarly, another systematic review, encompassing 13 observational studies, established that DM significantly raises the risk of TB infection, with a relative risk of 3.11[65]. Pneumonia is another common infection that DM patients are recognized as increasingly susceptible to developing it. It has been reported that the risk of developing pneumonia is found to be more elevated in people with DM, with an adjusted hazard ratio of 1.75 in comparison to people without DM[66]. In a separate study assessing mortality risk in DM patients diagnosed with pneumonia, it was discovered that mortality rates at both 30 and 90 days were markedly higher for individuals with DM, among other comorbidities[67]. Likewise, a study by *Hamilton et al.* unveiled that DM patients are at a higher risk of prolonged hospitalization due to community-acquired pneumonia[68]. In line with earlier findings, a multicenter prospective study demonstrated that undiagnosed DM was frequently encountered in individuals with community-acquired pneumonia, which significantly impacted survival rates, with a higher likelihood of death observed in patients with DM compared to those without DM[69, 70]. A multitude of research endeavors employing various study designs have been undertaken to comprehend the severity of comorbid DM in conjunction with COVID-19, a global pandemic that emerged within the past four years. Although some of these studies reported a negative or no effect of DM on patients with COVID-19[71], the majority of the analysis indicated that DM is a notable risk factor that significantly contributes to the worsening of COVID-19 severity, insufficient outcomes, and increased mortality[72, 73, 74].

Table 1. Summary of available studies on the impact of DM on respiratory infection

Study name	Study design	Study population	Effect of DM on respiratory infections
Miller et al, 2012	Retrospective	Patients with H1N1 influenza	• Increased risk of H1N1 • No data or mortality
Al-Rifai et al, 2017	Meta-analysis	Patients with TB	• Increased risk of active and latent TB • No data on mortality
Jeon et al, 2008	Systematic review		• Increased risk of active TB • No data on mortality
Kornum et al, 2007	Retrospective	Patients with HAP	• Increased risk of hospital-acquired pneumonia (HAP) • Increased the mortality rate
Benfield et al, 2007	Retrospective	Diabetic patients	• Increased risk of pneumonia infection • No association with mortality rate
Hamilton et al, 2013	Retrospective	Diabetic patients	• Increased risk of community-acquired pneumonia (CAP) • No data on mortality
Jensen et al.2017	Prospective	Undiagnosed diabetic subjects with CAP	• Increased risk of CAP infection • Increased mortality rate
Liu et al, 2020	Retrospective	COVID-19	• Increased severity of COVID-19 • No association with mortality rate
Kumar et al, 2020	Meta-analysis		• Increased severity of COVID-19 • Increased mortality rate
Huang, Ian et al,2020	Meta-analysis		

4. Effect of DM on ARDS

ARDS presents as an acute onset of lung infiltrates accompanied by diffuse damage to the alveoli and is a frequent and serious complication observed in intensive care units, often resulting in respiratory failure[75]. Published reports consistently show a substantial mortality rate linked with ARDS, typically ranging from 30% to 40%[76]. Increased endothelium capillary permeability, alveolar epithelium disruption, and neutrophil activation along with the release of proinflammatory cytokines, including TNF-α, IL-1, and IL-6 are key attributes of ARDS[77]. Multiple studies investigating the link between DM and the onset of ARDS have produced varied findings, with some confirming a connection, others disputing it, and some showing no discernible impact **(Table**

2). In a multicenter study assessing the prevalence of ARDS in septic shock patients with pre-existing DM, it was observed that the occurrence of ARDS was lower among individuals with DM. However, there was no significant difference in mortality rates compared to those without DM[7]. Likewise, another study demonstrated a protective effect, suggesting that the presence of DM possibly contributed to reduced risk of ARDS development triggered by varying clinical conditions encompassing sepsis, trauma, aspiration, and hyper-transfusion. Nevertheless, the mortality rate in this study was not different between people with DM and those with other conditions[8]. Although the precise reasons behind the observed protection effects of DM remain unclear, the activation of proinflammatory signaling in the lungs might play a role in deterring ARDS development. Conversely, another study identified a significant association between DM and ARDS, noting that post-surgery patients with pre-existing DM had an increased risk of ARDS progression[78]. This aligns with our very recent study where ARDS patients with DM exhibit a lower survival rate, longer hospitalization duration, and extended stays in the ICU when compared to their non-DM counterparts[79]. Additionally, data from a retrospective-longitudinal cohort supports the earlier study by affirming that individuals affected by DM are at significant risk of developing ARDS[9]. Furthermore, an analysis examining mortality rates among patients with respiratory disorders prescribed antidiabetic medication revealed a heightened risk of death associated with a history of DM[80]. Combining the data revealed that patients diagnosed with DM often present respiratory symptoms more frequently compared to the general population[81].

The disparities noted in the aforementioned studies could have been adversely affected by either uncontrolled or potentially biased research designs. Given that DM is associated with various health complications[1], including micro-macro complications, hyperglycemia, and compromised immune function, it is plausible that it impacts lung function and exacerbates the severity of lung disorders, resulting in poor outcomes and contributing to higher mortality rates, as evidenced in aforesaid epidemiological studies. However, a thorough examination of DM and its connection with lung disorders necessitates well-designed preclinical and clinical research.

Table 2. Summary of available studies on the effect of DM on ARDS outcomes

Study name	Study design	Study population	Effect of DM on ARDS development and mortality
Moss et al, 2000	Prospective	Patients with septic shock	• Decreased risk of ARDS development • No significant difference in mortality rate
Gong et al, 2005	Prospective	Patients with sepsis, trauma, aspiration, and hyper transfusion	
Kor et al., 2011	Prospective	Patients with post-surgery	• Increased risk of ARDS development • No data on mortality
Trillo-Alvarez et al, 2011	Retrospective	Patients with sepsis, trauma, and Shock	• No association • No data on mortality

5. Effect of DM on COPD

The global prevalence of COPD is estimated to be approximately 10.3% among individuals aged 30-79 years, representing a substantial population of 391.9 million patients worldwide[82]. This progressive condition is distinguished by chronic bronchitis, emphysema, alveolar wall thickening, and mucus hypersecretion, resulting in poorly reversible airway limitation[82, 83]. In understanding the pathophysiology of COPD, two critical mechanisms to note are the constant presence of inflammation and the imbalance between oxidants and antioxidants[84]. The process of inflammation involves various cell types, such as macrophages, lymphocytes, and neutrophils, which stimulate the release of inflammatory mediators like cytokines, chemoattractants, and proteolytic enzymes, which sustain an unregulated inflammatory cascade[85]. Recent reports suggest that DM might also have a detrimental impact on airway obstruction **(Table 3)**. A recent cross-sectional study examining the prevalence of restrictive pulmonary disorders in diabetic patients found that both longstanding and newly diagnosed individuals with DM exhibited a statistically significant decline in lung function measures such as forced vital capacity (FVC) and total lung capacity (TLC) compared to nondiabetic patients[86]. Mortality rates among COPD patients with pre-existing DM have been evaluated in multiple analyses. A recent cohort study concluded that the presence of DM in patients with COPD was significantly linked to a lower survival rate compared to subjects without DM[87]. Similarly, another study found that a higher hazard ratio for mortality was detected in acute exacerbations of COPD patients, attributing the impaired pulmonary functions to the consequence of hyperglycemia. Notably, these patients also exhibited unfavorable clinical outcomes[88]. In a prospective analysis, DM has been identified as an exacerbating factor and significantly contributing to mortality when compared to non-diabetic individuals[89]. Recent extensive prospective research has reaffirmed previous findings regarding the significant association between DM and COPD. It reveals that individuals with confirmed DM are not only more prone to developing COPD and have lower survival rates compared to non-diabetic subjects, but also pre-diabetic individuals may have a heightened risk of COPD development, consequently impacting survival rates[90]. Moreover, several additional studies strongly indicate that DM serves as an additional risk factor, resulting in prolonged hospital stays, increased mortality rates, and subsequent respiratory complications in COPD patients[91, 92]. Based on the consistent findings across numerous studies, we strongly assert that DM has an adverse impact on COPD. Hence, it is reasonable to speculate that DM, together with numerous other factors, represents a prominent etiological factor contributing to the development of COPD. Additionally, its inimical outcomes on other respiratory diseases cannot be overlooked, considering the scarcity of pre-clinical research in the literature.

Table 3. Summary of available studies on the effect of DM on COPD outcomes

Study name	Study design	Study population	Effect of DM on COPD development and mortality
Ho et al, 2017	Retrospective	Patients with COPD	• Increased hazard ratio for mortality
Baker et al, 2005		Patients with acute exacerbations of COPD	• Increased relative risk of death • Increased hospitalization days • Increased adverse outcomes
Castañ-Abad et al, 2020	Prospective	Patients COPD	• Increased COPD exacerbations • Increased risk of death
Su, Jian et al. 2023		Patients with COPD	• Increased risk of COPD development • Increased mortality rate
Monsour et al 2021	Retrospective	Patients with acute exacerbation of COPD	• Increased ICU admission rates • No significant difference in mortality rate

6. Effect of DM on IPF

Idiopathic pulmonary fibrosis, also referred to as cryptogenic fibrosing alveolitis, is a progressive lung disease that manifests with radiographically apparent interstitial infiltrates and is accompanied by inflammation, shortness of breath, and declining lung function[75]. Several disease origins have been proposed, including smoking exposure, and chronic viral infections, though genetic predisposition and aging are the most compelling factors[93]. Several rare mutations of the genes in telomerase reverse transcriptase (TERT) and TERC, the telomerase RNA component (TERC), have been identified in IPF patients, causing shortened telomeres that impact type-2 alveolar epithelial cells, which are the main cell type affected in IPF[94, 95]. Recent epidemiological research indicated that the prevalence of IPF ranges from 10 to 42% in patients with co-morbidities such as DM[96]. The association between DM and IPF has been marked by a few inconsistent studies **(Table 4)**. Bai et al. revealed that DM emerged as a significant risk factor for the progression of IPF in a meta-analysis aimed at exploring its relationship with DM[97]. Supporting the previous assertion, another group reported that the likelihood of developing IPF was not significantly reduced in patients with DM[98]. However, the precise causal relationship has yet to be fully established. Similarly, in a case-control study, DM was independently identified as a significant risk factor for IPF[99]. Interestingly, individuals with DM demonstrated a lower incidence of pulmonary fibrosis and a prolonged survival rate, according to findings from a comprehensive retrospective study. This contrasts with the results observed in those without DM, which reported incidences of 8.2% and 9.9%, respectively[100]. Based on the consensus within the literature, it is plausible to argue that the evident correlation between IPF and DM implies that individuals with a history of DM might be more susceptible to, or experience suboptimal clinical outcomes related to IPF.

Table 4. Summary of available studies on the effect of DM on IPF outcomes

Study name	Study design	Study population	Effect of DM on IPF development and mortality
Bai et al, 2021	Meta-analysis and systematic review	IPF patients	• Increased risk of IPF development • No data on the mortality rate
Li et al, 2021	Meta-analysis		
Jeganathan et al, 2020	Retrospective		• Decreased IPF development. • No difference in mortality rates
García-Sancho Figueroa et al, 2010	Retrospective		• Increased risk of IFP development

7. Effect of DM on asthma

Asthma is described as a heterogeneous disease characterized by reversible airway obstruction, attributed to bronchial hyperresponsiveness resulting from increased smooth muscle cell contraction and heightened mucus secretion[101]. The presence of T-helper cells in asthmatic patients leads to the release of cytokines such as IL-4, 5, and 13, which trigger mast cell activity, promote leukocytosis, and enhance B-cell immunoglobulin E (Ig-E) production, all of which contribute to the characteristic airway remodeling associated with asthma [102]. Studies have detected a higher prevalence of asthma among diabetic patients compared to those without DM **(Table 5)**[9]. A study investigating the connection between DM and asthma found that the odds of asthma development were notably greater among patients with DM after controlling for age and obesity[103]. Remarkably, insulin use was noticed as a substantial contributor to increased risk of asthma in diabetic patients[103]. A similar observation was noted in another recent study on Korean adults with pre-existing DM, aimed at evaluating the potential progression of asthma. The investigators revealed that individuals with DM, as well as those with elevated hemoglobin A1C and insulin levels, were more likely to develop asthma compared to those without DM[104]. In addition to this, several studies have pointed out that the diagnosis of DM as a concurrent illness is accountable for increasing the severity of asthma, recurring exacerbations, and levels of mortality[105-107]. Yuh-Lih et al. discovered that a history of DM, along with other conditions such as pneumonia and arrhythmia, served as a statistically significant independent risk factor for the increased mortality rate in subjects experiencing asthma exacerbation[108]. A wealth of evidence strongly supports the positive association between asthma and DM in various research studies. However, a few reports have also suggested a negative or inverse association[109], leading to controversy surrounding the subject.

The impact of DM extends beyond minor capillaries to major target organs by modulating the activity of TJ and AJ proteins, resulting in increased membrane permeability[110]. Additionally, it compromises the body's immune response, rendering diabetic patients more susceptible to infections[10]. Moreover, DM activates various inflammatory cytokines, leading to cellular injury and accelerating the onset of complications[111]. Below, we delineate significant literature concerning the major mechanistic pathways that underlie diabetic complications.

Table 5. Summary of available studies on the effect of DM on asthma outcomes

Study name	Study design	Study population	Effect of DM on asthma development and mortality
Chen et al, 2017	Retrospective	Patients with asthma	• Increased risk of asthma development • No data on mortality
Lee et al, 2020	Retrospective		
Price et al, 2016	Retrospective		• Increased asthma exacerbation
Koskela et al, 2015	Prospective		• Increased risk of late mortality
Chang et al, 2020	Retrospective		• Increased risk of death

8. Mechanisms of DM-induced inflammation in various tissues

Our current knowledge of diabetic complications in the lungs is very limited. Unlike the brain, kidneys, and retina, lung complications in diabetics with no other comorbidities or infections are extremely rare, making this a highly understudied area. Hence, in this section, we compare the limited literature available on lung inflammation and associated mechanisms in diabetic lungs with the more extensive research on other organs.

a. NF-κB pathway in DM-associated micro- and macro-vascular complications

An important regulatory protein in the nucleus, NF-κB plays a major role in inflammation[112]. NF-κB levels were found to be highly expressed in a study conducted on diabetic rats, resulting in increased inflammation, apoptosis, and angiogenetic processes with the ultimate progression to DR[113]. Metformin, an oral hypoglycemic, showed protective effects against DR, as demonstrated by a reduction in retinal thickness and disruption of retinal structures in streptozotocin (STZ)-induced diabetic rat models via suppression of NF-κB expression[114]. The administration of Oleanolic acid partially alleviated diabetic nephropathy and inflammation by inhibiting the expression of NF-κB in STZ diabetic rats[115]. By negatively regulating NF-κB signaling, melatonin treatment effectively improved renal functions and the preservation of healthy epithelial lining and brush borders in diabetic rats[116]. Through a dosage-dependent suppression of NF-κB signaling, tocotrienol, a compound exhibiting vitamin E activity, has been able to alleviate nephropathy and restore kidney functions in STZ-induced diabetic rats[117]. In STZ-induced diabetic spinal cord neuropathy, curcumin effectively suppressed both neuronal cells and microglial activation by downregulating NF-κB expression[118]. Varied mechanisms have been implicated in the connection between DM and cardiac fibrosis as well as vasculopathy[119, 120]. Nonetheless, there has perpetually been a lack of research on diabetic-induced lung complications. The release of certain inflammatory cytokines, such as IL-3 and IL-4, along with the activation of transcriptional factors like NF-κB, is believed to be the cause of lung fibrosis[121]. The inhibition of the NF-κB pathway was reported to have contributed to a decrease in inflammatory response in diabetic lung fibrosis in STZ-induced diabetic rats[58]. Diabetic-induced dysregulation in the gut microbiota has been linked to increased NF-κB signaling pathway activity, leading to pulmonary alveolar thickening and the promotion of lung fibrosis in mice with DM[122]. As a potential treatment for diabetic-induced airway inflammation, saxagliptin provides promising results by reducing the upregulation of NF-κB signaling, resulting in decreased leukocyte count, total nitrate production, and protein concentration in bronchoalveolar lavage fluid in ovalbumin-induced allergic asthma in mice[123]. Several preclinical and clinical studies have shown that the activation of NF-κB in cases of acute respiratory distress syndrome (ARDS) leads to increased expression of cytokines and proinflammatory mediators, while its suppression can alleviate the severity of ARDS[124-126]. Taken together, the Suppression of NF-κB signaling activation emerges as a solution to conquer DM complications. Thus, conducting further clinical investigations that complement the existing preclinical findings is imperative.

b. Advanced glycation end products in DM complications

While comprehending the entire pathophysiology of DM may be challenging, advanced glycation end products (AGEs) play a significant role in both the progression of the disease and its associated complications, given their production that stems from uncontrolled hyperglycemia.[41, 127]. As multi-functional molecules, AGEs can bind to a variety of receptors. Nevertheless, it is especially notable that they primarily interact with the advanced glycation end products receptor (RAGE)[128, 129]. Although achieving early euglycemia is impartial in minimizing DM complications, the longstanding impact of the AGEs accumulated over time is detrimental as it sustains an overexpression of RAGE, activates NF-κB, and contributes to pronounced inflammation, hence accelerating diabetic complications, often referred to as metabolic memory[130]. Retinal explants from non-diabetic rats showed pronounced retinal cell apoptosis, neuroglial degeneration, and lesions when treated with glycated bovine serum albumin, emphasizing the contribution of the AGEs/RAGE axis as a potentiator in DR[131]. Preclinical studies also indicate that the abundant accumulation of AGEs in diabetic rat retinas and neuroglial cells plays a significant role in the degree of lesions and changes to cell structures[132, 133]. In a previous study, AGE-RAGE binding in a proximal tubule of diabetic rats resulted in dose-dependent myofibroblast trans-differentiation by upregulating the transforming growth factor β (TGF-β) pathway, which was subsequently reversed by reducing the expression of Tubular AGE and TGF-β[134]. It was reported that following prolonged exposure to advanced glycated albumin product, the activation of extracellular signal-regulated kinase (ERK) was inhibited by angiotensin (1-7), resulting in a reduction of AGE-induced kidney injury, tubular hypertrophy, and myofibroblast transition[135]. It is also worth noting that angiopoietin-1, an endothelial protective agent, was reported to inhibit AGE-induced perturbation of VE-cadherin-β-catenin complex, enhancing the expression of VE-cadherin, preserving the endothelial integrity and reversing AGE-induced hyperpermeability in HUVECs[136]. Numerous clinical studies have been conducted to investigate the detrimental effects of AGEs on complications associated with DM, including micro and macrovascular issues as well as atherosclerosis, cardiovascular, COPD, and ARDS diseases[137-140], which points out that the progression of diabetic complications can be primarily attributed to the upregulation of AGEs. Nonetheless, further preclinical research is required to fully comprehend the significance of AGE-RAGE axes in diabetic-induced lung complications, given that the majority of available data is based on observational studies.

c. Akt and P38 MAP kinase signaling in inflammation

PI3Kinase-Akt signaling plays a key role in maintenance of the cell growth, proliferation, migration, and metabolism, and dysregulation of this signaling axis is associated with a whole spectrum of pathological conditions, namely cancer, DM, neurodegenerative diseases, among others[141]. A study performed on mice with cigarette smoke-induced COPD showed significant activation of PI3Kinase signaling in the alveolar macrophages, which in turn augmented the inflammatory responses evidenced by high pro-inflammatory cytokine concentrations, including TNF-α and IL-1β[142]. Furthermore, it was observed that the PI3Kinase inhibitor LY-294002 at different concentrations was efficacious in inhibiting the ovalbumin-induced airway hyperresponsiveness in a mouse model of asthma[143]. P38 mitogen-activated protein kinase (p38 MAPK) pathway is known to affect the endothelial barrier integrity in pulmonary ischemia and related reperfusion injuries as well as in various inflammatory processes[144]. It was revealed that inhibiting p38 MAPK signaling reversed the deleterious effects caused by DM-induced activation of this pathway in a rat lung ischemia-reperfusion injury model. These effects included an increase in pro-inflammatory markers like tumor necrosis factor alpha (TNF-α), interleukin-6, higher lung injury scores, and an elevated lung wet-to-dry weight ratio[145]. A follow-up study showed that in vitro knockdown of p38 MAPK attenuated the lung ischemia-reperfusion injury by upregulating the expression of the permeability proteins, including aquaporin 1 (AQP1), endothelial nitric oxide synthase (eNOS), and junctional proteins Zonula occludens-1 (ZO1) and VE-cadherin promoting barrier integrity[146]. By concurrently targeting both Akt and P38 signaling pathways, there exists a potential to alleviate the complications linked to DM-induced lung inflammation.

d. Role of TNF-α signaling in diabetic-induced inflammation

TNF-alpha is a transmembrane protein that is primarily expressed in macrophages and lymphocytes[147]. Its dysregulation increases the risk of numerous diseases, including cancers and inflammatory disorders[148]. Research indicates that TNF-α activation causes glucose intolerance and tissue insulin resistance[149]. Moreover, the serum levels of TNF-α have been reported to be elevated in overweight prediabetic patients, leading to an increased risk of type-2 DM development[150]. A cross-sectional study showed that patients with inadequate glucose control had significantly higher TNF-α levels and impaired lung functions than those with adequate glucose control subjects[151]. In animal studies, DM has been reported to induce an upregulation of inflammatory cytokines, including TNF-α and IL-6, in the lung tissues of mice despite utilizing a protective mechanical ventilation technique[152]. Another study also indicated that type-2 diabetes caused injury to lung tissue by attracting immune cells and promoting neutrophilic leukocytosis within the pulmonary niche by increasing the levels of TNF-α, ICAM-1, and VCAM-1 in a diabetic rat model[153]. Ginsenoside Rb1, the active constituent of ginseng, has been found to have a protective effect on the lungs of diabetic rats as it significantly reduced the levels of proinflammatory cytokines IL-6, IL-1α, and TNF-α and restored the normal structure of the lungs[154]. According to the above findings, inhibiting TNF-α may offer promising outcomes to reduce DM-induced lung injury.

9. Summary and Conclusions

Despite its well-established prevalence and impact on various organs, including the cardiovascular system, kidneys, and eyes, the association of DM with respiratory health has received comparatively less attention. This comprehensive review aims to explore the intricate relationship between DM and respiratory diseases, shedding light on potential mechanisms underlying this association and the implications for clinical management.

The influence of DM extends beyond its classical complications, impacting physiological functions such as immunity and vascular health. DM compromises immune function, rendering individuals more susceptible to respiratory infections. Moreover, it engenders vascular complications, affecting micro- and macro-vascular integrity, which can exacerbate respiratory diseases. Mechanisms like endothelial dysfunction, glycocalyx impairment, and disruption of endothelial junctions contribute to vascular complications and may play a role in respiratory pathology. The review highlights the impact of DM on various respiratory infections, indicating increased susceptibility and poorer outcomes in diabetic individuals. Furthermore, DM exacerbates COPD, ARDS, IPF, TB, pneumonia infections, and asthma. These findings underscore the need for tailored approaches to managing respiratory diseases in diabetic patients. Pathways such as NF-κB, advanced glycation end products (AGEs), and Akt/P38 MAP kinase signaling contribute to inflammation, endothelial dysfunction, and tissue damage, exacerbating diabetic complications, including those affecting respiratory health **(Figure 1)**.

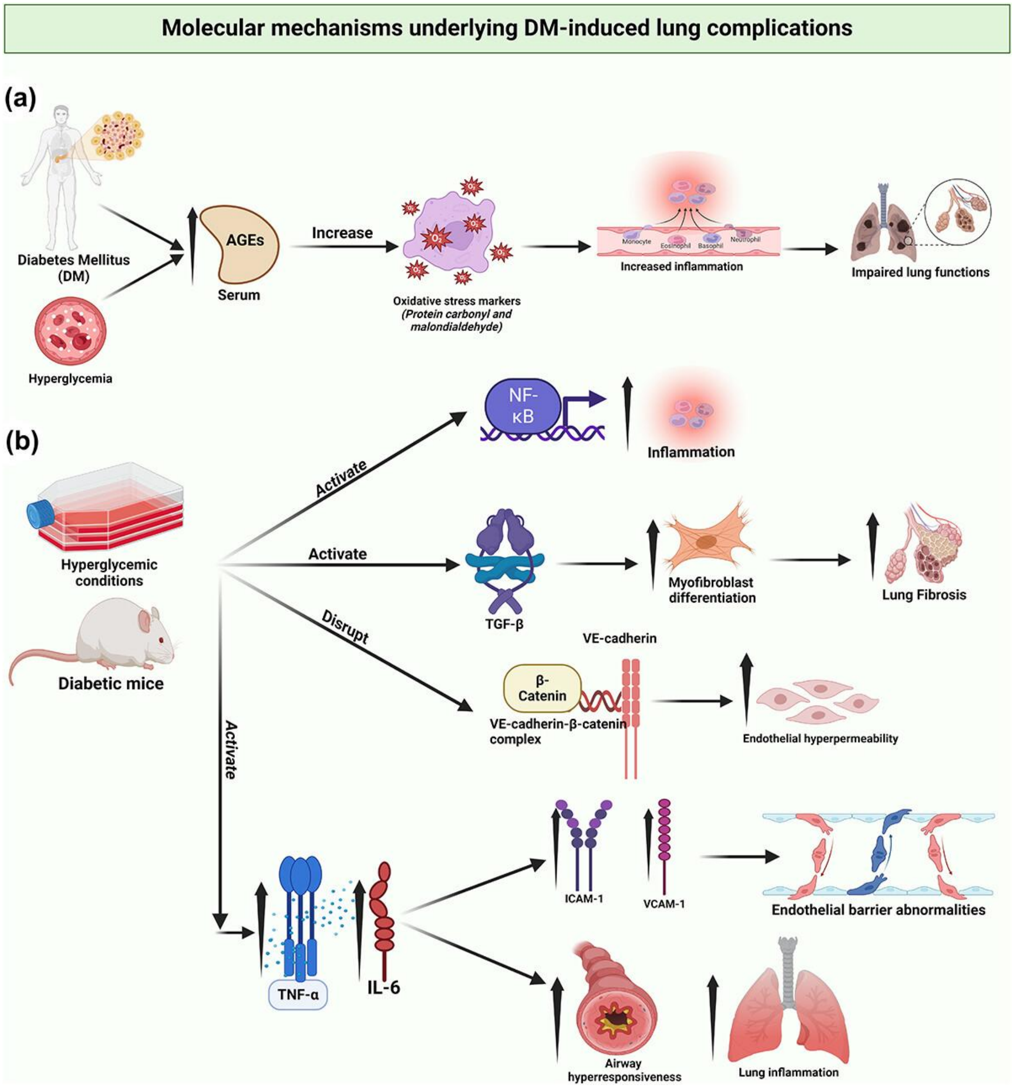

Figure Legend:

Figure 1: Despite the well-established impact of DM on organs like the cardiovascular system, kidneys, and eyes, its association with respiratory health has received comparatively less attention. **(A)** The literature offers compelling evidence that elevated levels of AGEs in diabetic lungs precipitate oxidative stress and activate inflammatory cells, thereby contributing to vascular complications. **(B)** Under hyperglycemic conditions and in diabetic mice, literature indicates that activation of the NF-κB pathway leads to lung inflammation, while activation of the TGFβ

pathway promotes myofibroblast differentiation in lung fibrosis. Disruption of the VE-cadherin and β-catenin complex in the AJs contributes to vascular permeability in acute lung injury. Activation of TNFα and IL-6 leads to increased inflammatory responses in endothelial cells, which in turn results in the upregulation of ICAM-1 and VCAM-1, causing lung injury and arterial remodeling that ultimately leads to pulmonary arterial hypertension. Further research is needed to elucidate precise molecular pathways and identify novel therapeutic targets.

DM exerts a profound influence on respiratory health, impacting susceptibility to infections and exacerbating chronic respiratory diseases. The mechanisms underlying this association involve immune dysregulation, vascular dysfunction, and inflammation, which collectively contribute to respiratory pathology. Understanding these mechanisms is vital for developing targeted interventions to improve respiratory health outcomes in diabetic individuals. Further research is warranted to elucidate the precise molecular pathways linking DM and respiratory diseases and to identify novel therapeutic targets. Additionally, integrated management strategies that address both DM and respiratory conditions are essential for optimizing clinical outcomes and enhancing the quality of life for affected individuals. By recognizing the intricate interplay between DM and respiratory health, healthcare providers can better tailor interventions to meet the specific needs of this vulnerable population.

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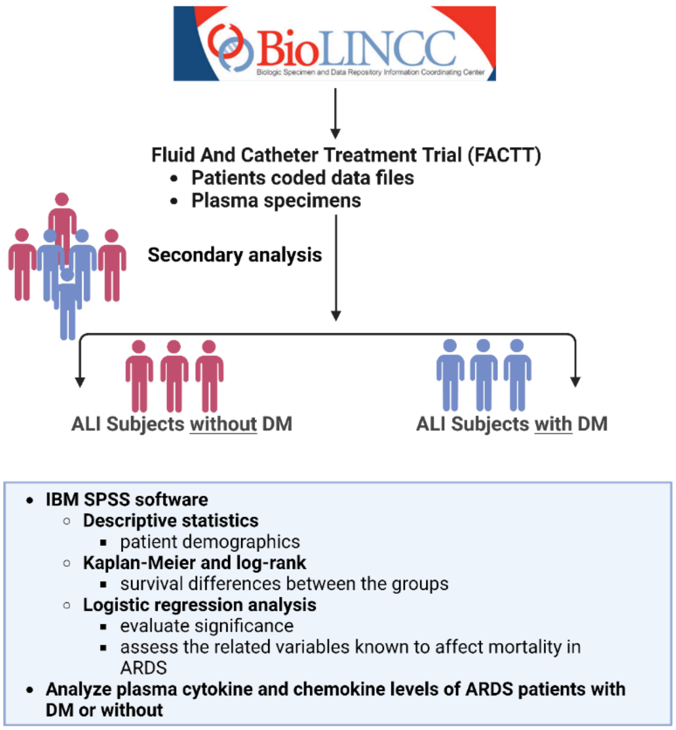
OBJECTIVES AND CENTRAL HYPOTHESIS

Diabetes mellitus (DM) is a chronic condition characterized by high blood glucose levels, inflammation, and oxidative stress. It is highly prevalent among critically ill patients, including those with acute respiratory distress syndrome (ARDS). Emerging evidence suggests that DM may affect the lungs, potentially influencing outcomes in pulmonary disorders. However, the impact of DM on respiratory diseases remains uncertain, with studies reporting both protective and detrimental effects. While vascular complications of DM are extensively studied, its effects on the lungs are still largely unexplored. Currently, there remains a ***gap in understanding*** the clinical implications of this interplay, and the underlying mechanisms are not yet fully understood. The objectives of this study are to investigate the effects of DM on the lungs, both with and without acute lung injury (ALI), and to explore the mechanisms by which DM causes lung impairment. Our central hypothesis is that DM negatively impacts lung function, increasing susceptibility and worsening outcomes in ALI patients. We propose that advanced glycation end-products (AGEs), as DM-associated factors, induce pathological gene expression changes in lung endothelial and epithelial cells. We also ***hypothesize*** that DM adversely affects the lungs and exacerbates ALI severity in a murine sepsis model. The ***significance*** of this study lies in the potential to enhance the understanding the impact of DM on respiratory diseases, providing insights that could improve management and treatment approaches.

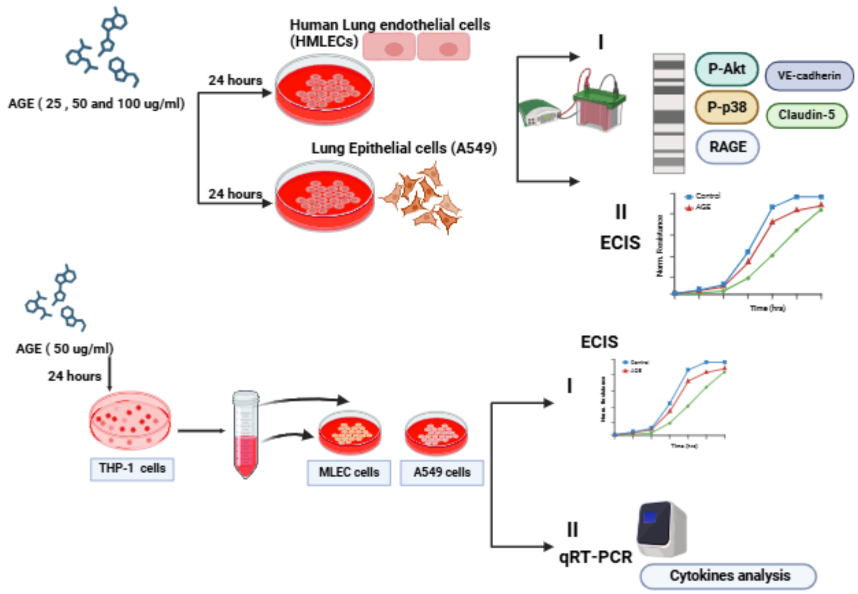
SPECIFIC AIMS

Specific aim 1: Investigate the impact of pre-existing DM on patients with ARDS using FACTT clinical data sets

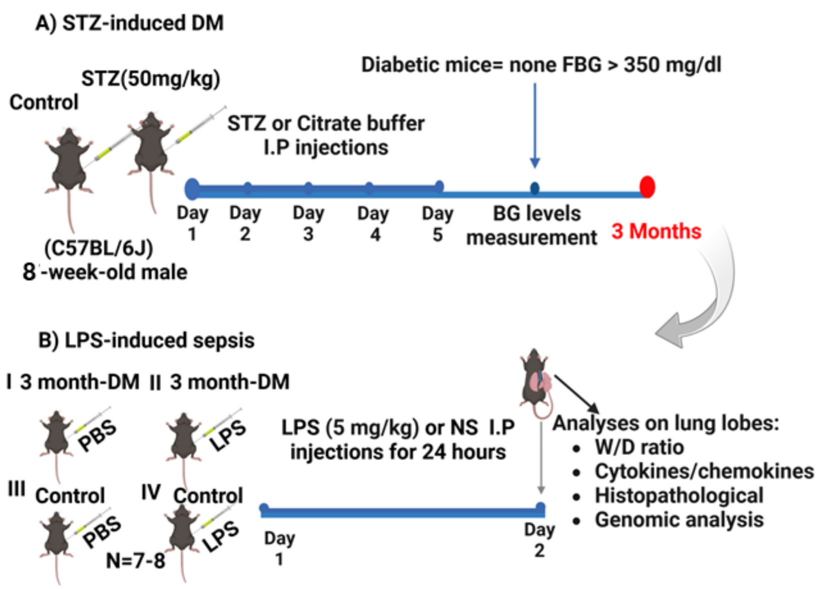


Specific Aim 2: Test the hypothesis that pre-existing DM will exacerbate ARDS outcomes in vivo

- **Aim 2A:** Determine the effect of advanced glycation end-products (AGEs) treatment as DM-associated factors on primary lung endothelial, epithelial, and macrophage (THP-1) cell function, molecular pathways, gene expression changes, and cytokine analysis.



- **Aim 2B:** Investigate the effect of DM independently on lungs and its effect on the severity of ALI in the lipopolysaccharide (LPS)-induced sepsis model in mice



CHAPTER 2

Fluids and Catheters Treatment Trial (FACTT) data reveal poor clinical outcomes in acute respiratory distress syndrome patients with diabetes.

Alanazi, Abdulaziz H et *al. "Secondary Analysis of Fluids and Catheters Treatment Trial (FACTT) data reveal poor clinical outcomes in acute respiratory distress syndrome patients with diabetes."* Respiratory medicine *vol. 223 (2024): 107540. doi:10.1016/j.rmed.**2024**.107540.* Reprinted here with permission of publisher

Abstract

Objectives: Conflicting reports exist about the link between diabetes mellitus (DM) and acute respiratory distress syndrome (ARDS). Our study examines the impact of pre-existing DM on ARDS patients within the Fluid and Catheter Treatment Trial (FACTT). **Design:** Conducting a secondary analysis of FACTT data, we incorporated 967 participants with identified DM status (173 with DM, 794 without DM) and examined outcomes like 90-day mortality, hospital and ICU stays, and ventilator days until unassisted breathing. The primary outcome of hospital mortality at day 90 was evaluated through logistic regression using IBM SPSS software. Additionally, we assessed plasma cytokines and chemokines utilizing a human magnetic bead-based multiplex assay. **Results:** Patients with pre-existing DM exhibited a lower survival rate compared to non-DM patients (61.3 vs. 72.3%, p=0.006). Subjects with DM experienced significantly longer hospital lengths of stay (24.5 vs. 19.7 days; p=0.008) and prolonged ICU stays (14.8 vs. 12.4 days; p=0.029). No significant difference was found in ventilator days until unassisted breathing between the two groups (11.7 vs. 10; p=0.1). Cytokine/chemokine analyses indicated a non-significant trend toward heightened levels of cytokines (TNF-α, IL-10, and IL-6) and chemokines (CRP, MCP-1) in DM patients compared to non-DM on both days 0 and 1. Notably, lipopolysaccharide-binding protein (LBP) exhibited significantly higher levels in DM compared to non-DM individuals. **Conclusions:** ARDS patients with DM suffered worse clinical outcomes compared to non-DM patients, indicating that DM may negatively affect the respiratory functions in these subjects. Further comprehensive clinical and pre-clinical studies will strengthen this relationship.

Keywords: Acute respiratory distress syndrome; acute lung injury; diabetes mellitus; mortality; cytokine.

Introduction

Acute respiratory distress syndrome (ARDS), a common contributor to respiratory failure, is distinguished by hypoxemia, alveolar-capillary membrane permeability, edema, and compromised gas exchange[1]. The annual incidence of ARDS is estimated to fall within the range of 64.2 to 78.9 cases per 100,000 individuals[2]. This syndrome is associated with elevated rates of morbidity, mortality, and higher utilization of critical healthcare resources [3]. While it is established that predisposing injuries such as pneumonia, sepsis, and aspiration, elevate the likelihood of ARDS development [4, 5] and certain factors like transfusions, diabetes, and obesity can influence risk [6, 7] the precise interplay between these variables and their impact on ARDS progression remains an active area of research. Further investigation is needed to comprehensively understand the intricate connections between diabetes and ARDS pathogenesis, enhancing our ability to formulate targeted interventions and management strategies.

Diabetes mellitus (DM) stands out as a prevalent comorbidity among critically ill patients, including those grappling with conditions like ARDS. Several studies have identified it as an adjunctive risk factor, heightening susceptibility. Observational data underpinning this reveal that a pre-existing diabetes diagnosis is evident in approximately 40% of patients admitted to intensive care units (ICUs) [8]. Furthermore, DM is intricately associated with an array of micro- and macrovascular complications spanning diverse organ systems [9]. Because ARDS is marked by dysfunction of the microvascular endothelium and alveolar epithelium of the lung [10], understanding the role of pre-existing conditions on this pathophysiology may shed light on treatment strategy. Growing evidence suggests that the lung may be a potential target organ of DM [11]. However, the literature on the effect of pre-existing DM, primarily on ARDS outcomes, has been conflicting. Many clinical studies have noted a correlation between severe hyperglycemia and an increased production of inflammatory cytokines, which subsequently contributes to the progression of tissue injury [12, 13]. Furthermore, maintaining well-managed blood glucose levels has demonstrated a positive correlation with improved outcomes among DM patients afflicted by ARDS [14]. Conversely, a subset of studies has proposed that diabetes mellitus (DM) could potentially offer protective effects for individuals with ARDS, while alternate analyses indicate that DM might exacerbate the outcomes for ARDS patients [11, 15]. Consequently, elucidating this intricate relationship holds the promise of offering valuable insights into refining management approaches and facilitating prompter diagnostic procedures.

Fluid And Catheter Treatment Trial (FACTT) is a prospective, randomized clinical trial that compared the advantages and drawbacks of two distinct fluid management approaches—conservative and liberal—for patients diagnosed with Acute Lung Injury (ALI) [16]. The present study sought to assess the impact of pre-existing DM on individuals with ARDS who were enrolled in the FACTT trial. We hypothesized that ARDS patients with pre-existing DM would experience worsened outcomes, irrespective of whether they were subjected to conservative or liberal fluid strategies, in contrast to those without DM.

Materials and Methods

Study plan

The study is a secondary analysis of the FACTT data (Clinical Trials.Gov # NCT00281268), a clinical randomized controlled trial carried out in a 20-healthcare organization set up [16]. FACTT was originally aimed to compare the efficacy of conservative or liberal fluid approaches in participants who had acute lung injury (ALI)/ARDS. The inclusion and exclusion criteria, along with the outcomes, have been published beforehand [16]. Our study primarily focused on patients with DM compared to non-DM subjects, thus patients with unclear DM status were not included in our secondary analysis.

Biosamples

Plasma specimens from the patients and their respective coded data files from those who registered in FACTT were sourced from the National Heart, Lung, and Blood Institute and Biological Specimen and Data Repository Information Coordinating Center (BioLINCC). A comprehensive panel of cytokines and chemokines, comprising CRP, IL-10, IL-6, LBP, MCP-1, and TNF-α were assessed on days 0 and 1 utilizing human magnetic bead-based multiplex assay (R&D Systems, Catalog # LXSAHM, Minneapolis, MN), and strictly following the manufacturer's instructions.

Statistical analysis

IBM SPSS Statistics Version 29 was used to conduct all the statistical analyses. Descriptive statistics were applied to evaluate the patient demographics. Continuous variables were assessed by student t-test with Welch's correction for parametric data and Mann-Whitney *U* test for nonparametric data. Kaplan-Meier and log-rank tests were employed for survival analysis. Logistic regression analysis was used to evaluate the significance, assess the relevant variables known to affect mortality in ARDS, and created a regression to consider the independent impact of DM on ARDS patients. GraphPad Prism version 9 was used to design the figures. A statistical significance was considered with an alpha level of < 0.05.

Results

Patient Characteristics

Of the total 1000 participants, 33 were not included in this secondary analysis as their DM status was unknown. Among the 967 participants evaluated, 173 patients had DM and 794 did not have DM. Baseline characteristics showed a significant difference in age between the two groups. The average age in the DM group was 53.9±1.1 years old, while the non-DM group had an average age of 48.9±0.57 years old. Around 92 (53.2%) and 359 (45.2 %) subjects were female, respectively.

Demographic characteristics are reported in Table 1. The Acute Physiology and Chronic Health Evaluation III (APACHE III) score was higher in DM patients compared to the non-DM group (98.4 vs. 93.1; p= 0.038); II), and platelet counts were significantly elevated with pre-existing DM subjects versus those without DM (218.6 vs. 195.4 1000 /mm^3, p= 0.02). The group with DM had elevated levels of glucose, blood urea nitrogen (BUN), creatinine (1.6±0.07 vs. 1.2±0.028 mg/dL, p= 0.001), and total protein (5.2±0.08 vs. 5.0±0.03 g/dL, p= 0.02) in comparison to the group without DM (**Table 1**).

Table 1. Acute lung injury Patient Characteristics and Outcomes in Presence and Absence of DM			
Characteristics	**ALI Subjects with DM (N= 173)**	**ALI Subjects without DM (N= 794)**	***P* Value**
Age (years)	53.86±1.1	48.88±0.57	0.001
Female sex	53.2 %	45.2	0.5
APACHE III score	98.39 ±2.2	93.14±1.1	0.038
Mean Airway Pressure (cm H_2O)	15.68±0.5	15.48±0.2	0.7
F_iO_2	0.66±0.01	0.64±.007	0.23
PaO_2 (mmHg)	89.45±2.9	90.35±1.5	0.78
PaCO2 (mmHg)	39.5±0.8	40.61±0.36	0.21
Arterial pH	7.35±00.7	7.35±.003	0.8
SpO2 %	95.08±0.29	94.60±0.18	0.17
Hgb (g/dL)	10.27±0.12	10.42±.0.07	0.29
WBC (mm^3)	13057.64±786.9	14121.19±467.9	0.24
Platelets (1000 /mm^3)	218.65±9.4	195.41±4.4	0.02
Sodium (mEq/L)	139.42±0.5	138.74±0.1	0.2
Potassium (mEq/L)	3.9±.0.05	4±0.02	0.9
Glucose (mg/dL)	168.84±6.6	134.01±2.4	0.001
Chloride (mEq/L)	107.61±0.62	107.65±0.23	0.9
BUN (mg/dL)	29.9±1.47	22.47±0.62	0.001
Creatinine (mg/dL)	1.57±0.07	1.20±0.028	0.001
Serum Bicarbonate(mEq/L)	21.5±0.40	22.4±0.18	0.027
Total Protein (g/dL)	5.22±0.08	5.01±0.03	0.023
Albumin (g/dL)	2.25±0.05	2.20±0.02	0.3
Ratio of Pao2 to Fio2, n %	134.49±+5.1	130.86±2.1	0.5
Outcomes (%)			
Probability of Survival % **Male** **Female**	61.3 53.1 68.5	72.3 71 73.8	0.006 0.002 0.3
Hospital length of stay, d, mean (SE)	24.53±1.7	19.73±0.5	0.008
ICU length of stay, d, mean (SE)	14.89±1	12.47±0.39	0.029
ventilator days until Unassisted Breathing (UAB) at 90 d, mean (SE)	11.71±0.9	10±0.3	0.1

Data demonstrated as means ± SE or n (%). *Abbreviations*: APACHE III score, Acute Physiology, Age and Chronic Health Evaluation; FiO2, fraction of inspired oxygen; PaO2, Partial pressure of oxygen; PaCO2 partial pressure of carbon dioxide; Hgb, hemoglobin; WBC, white blood cells; BUN, blood urea nitrogen.

Patient outcomes

Our primary outcome was death at day 90, while the secondary outcomes werehospital length of stay, ICU length of stay, and ventilator days until unassisted breathing at day 90. Our analysis indicated that regardless of the treatment group, patients with DM had a lower survival rate than patients without pre-existing DM (61.3 vs.72.3 %; *p*=0.006) (Table 1 and Figure 1).

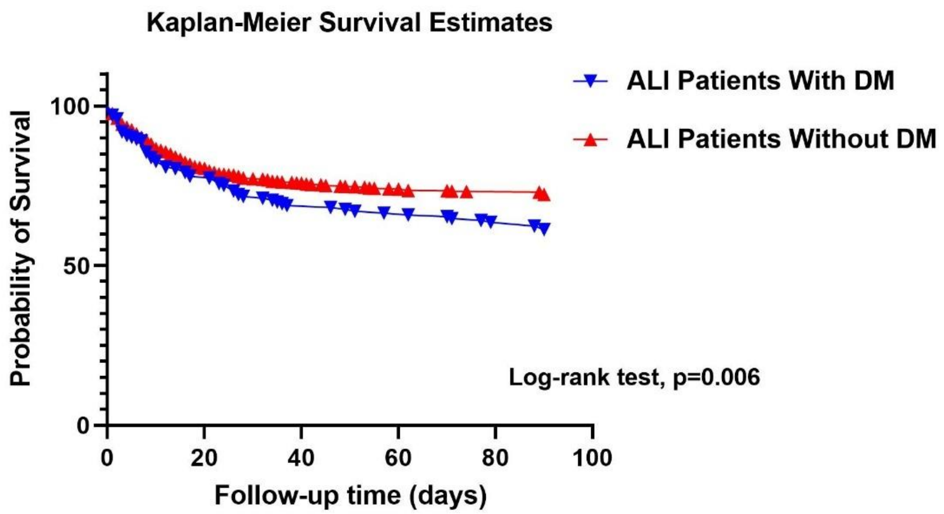


Figure 1. Kaplan-Meier survival curves stratified by the presence and absence of DM in ARDS patients showing a difference in survival. ARDS Patients with DM demonstrated significantly lower survival rates than non-DM patients.

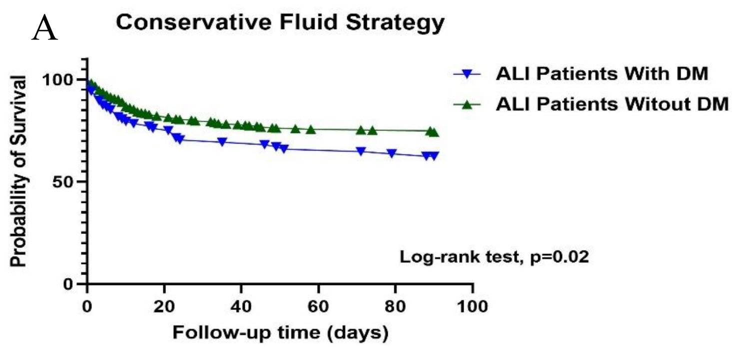
In a gender-specific analysis, a non-significant trend toward a shorter survival rate was found in the female DM group against females with no DM (68.5 vs. 73.8 %; *p*= 0.3). Nevertheless, a lower likelihood of survival was detected at a statistically significant level in DM male patients compared to males without a history of DM (53.1vs. 71 %; *p*=0.002) (Table 1).Upon univariate logistic regression, pre-existing diabetes was significantly associated with mortality (odds ratio 1.65 [95% CI,1.170-2.324]). Our multivariate logistic regression controlling for APACHE III, and conservative treatment also showed that pre-comorbidity with DM is an important factor contributing to the high mortality rate (odds ratio, 1.49 [95% CI, 1.028-2.152]) (Table 2). The probability of survival was also computed after participants were stratified by the treatment group (liberal and conservative fluid). In the conservative fluid strategy, a significantly lower survival rate was linked to DM (62.5 vs. 74.2 %; p=0.02) (Figure 2A). In contrast, DM subjects who underwent liberal management had a trend towards a decrease in survival rate, but the data was not statistically significant (60.0 vs. 70.4 %; p=0.1) (Figure 2B).

Table 2. Logistic regression analyses for the presence of DM and mortality

Variable	**Odds ratio (95% confidence interval)**
Univariate analysis	
Diabetes Mellitus	1.649 (1.170, 2.324)
Multivariate analysis	
Constant	.026
Diabetes Mellitus	**1.487 (1.028,2.152)**
APACHE III	1.029 (1.023,1034)
Conservative treatment	0.864 (.641,1.165)

Abbreviations: APACHE III: Acute Physiology and Chronic Health Evaluation

A



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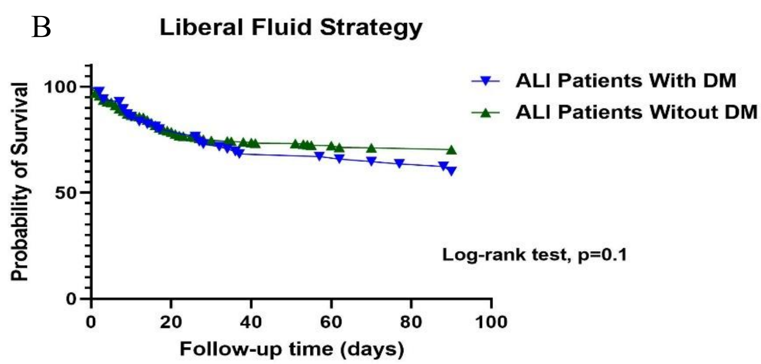

Figure 2. Kaplan-Meier survival curves stratified by fluid treatment strategies showing the probability of survival. **(A)** Conservative treatment shows a significant negative effect of DM on ARDS patients. **(B)** Liberal treatment demonstrates a similar trend in negative effect (not significant) of DM in ARDS patients compared to non-DM.

Similar to survival analysis, patients with pre-existing DM experienced significantly longer hospital lengths of stay (24.5 vs. 19.7 days; p= 0.008) and prolonged ICU stays (14.8 vs. 12.4 days; p=0.029). However, there was no difference in ventilator days until unassisted breathing at day 90 within the groups (11.7 vs. 10; p=0.1) (Table 1). Our subgroup analysis was constrained by the available sample size from BioLINCC. This limitation could have influenced the lack of statistical significance. Nevertheless, our subgroup analysis yielded outcomes consistent with the primary analysis (Table S1).

Supplemental Table. Subgroup analysis of FACTT

Characteristics	**ALI Subjects with DM (N=15)**	**ALI Subjects without DM (N= 50)**	***P* Value**
Age (years)	56.3±3.2	46.7±2.4	0.2
Female sex	46.7	52.0	0.7
APACHE III score	104.4±7.7	88.2±3	0.7
Outcomes (%)			
Probability of Survival % **Male** **Female**	66.7 50 85.7	76 75 76.9	0.5 0.2 0.6
Hospital length of stay, d, mean (SE)	23.5±4.1	16.13±1.5	0.1
ICU length of stay, d, mean (SE)	11.4±1.6	10.9±1.2	0.8
Ventilator days until Unassisted Breathing (UAB) at 90 d, mean (SE)	10.2±2.2	10±1.5	0.9

Plasma cytokine and chemokine analysis

Our analysis of plasma cytokines and chemokines on day 0, when treatments were not started, showed a non-significant tendency toward increased levels of cytokines (TNF-α and IL-10, and IL-6) and chemokines (CRP, MCP-1) in DM patients compared to non-DM (Figure 3). A similar pattern was also observed on the first day after randomization of the treatment strategies

(Figure 4). Upon analysis of lipopolysaccharide-binding protein (LBP), an acute-phase protein, a significantly higher level was detected and associated with DM on both days 0 and 1 compared to non-DM patients.

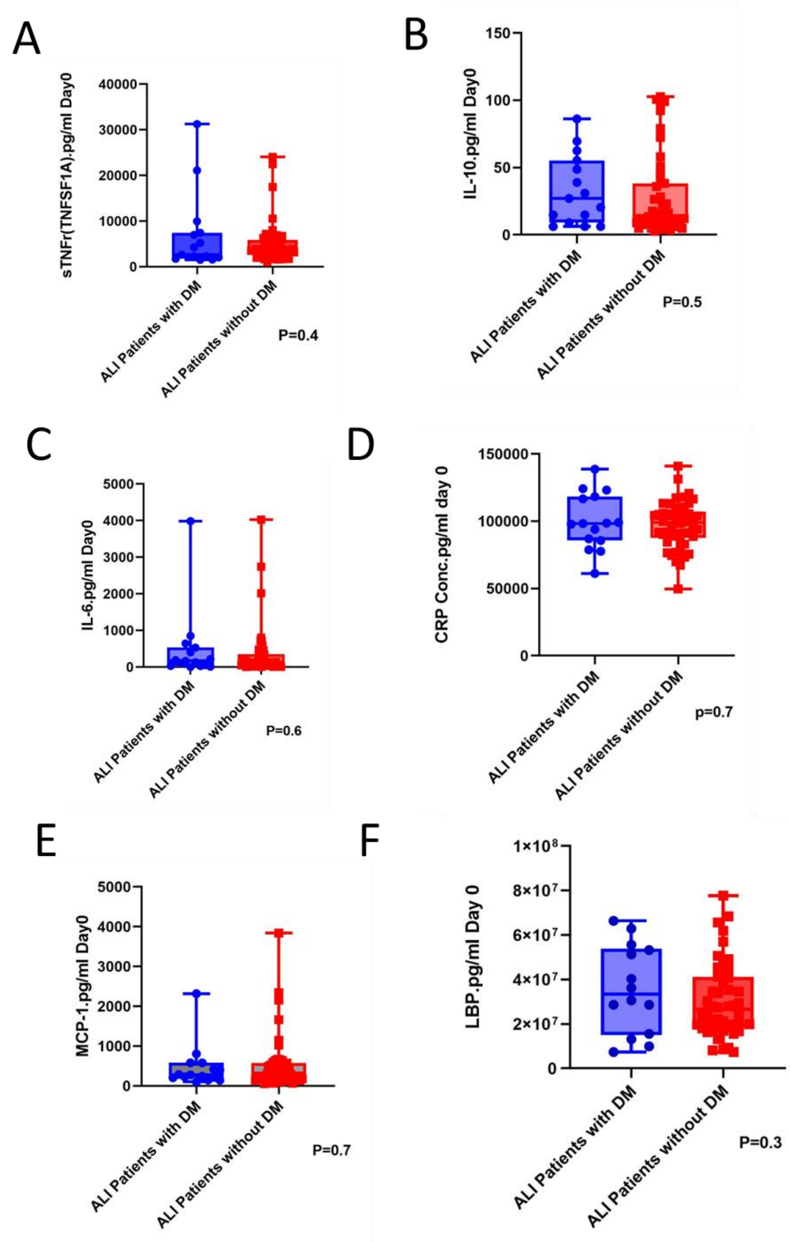

Figure 3. Plasma cytokine and chemokine levels of ARDS patients with and without DM before starting the treatments (day 0). **(A)** TNF-α, Tumor necrosis factor-alpha **(B)** IL-10, interleukin-10 **(C)** IL-6, interleukin-6 **(D)** MCP-1, monocyte chemoattractant protein-1 **(E)** CRP, C-reactive protein **(F)** LBP, lipopolysaccharide-binding protein.

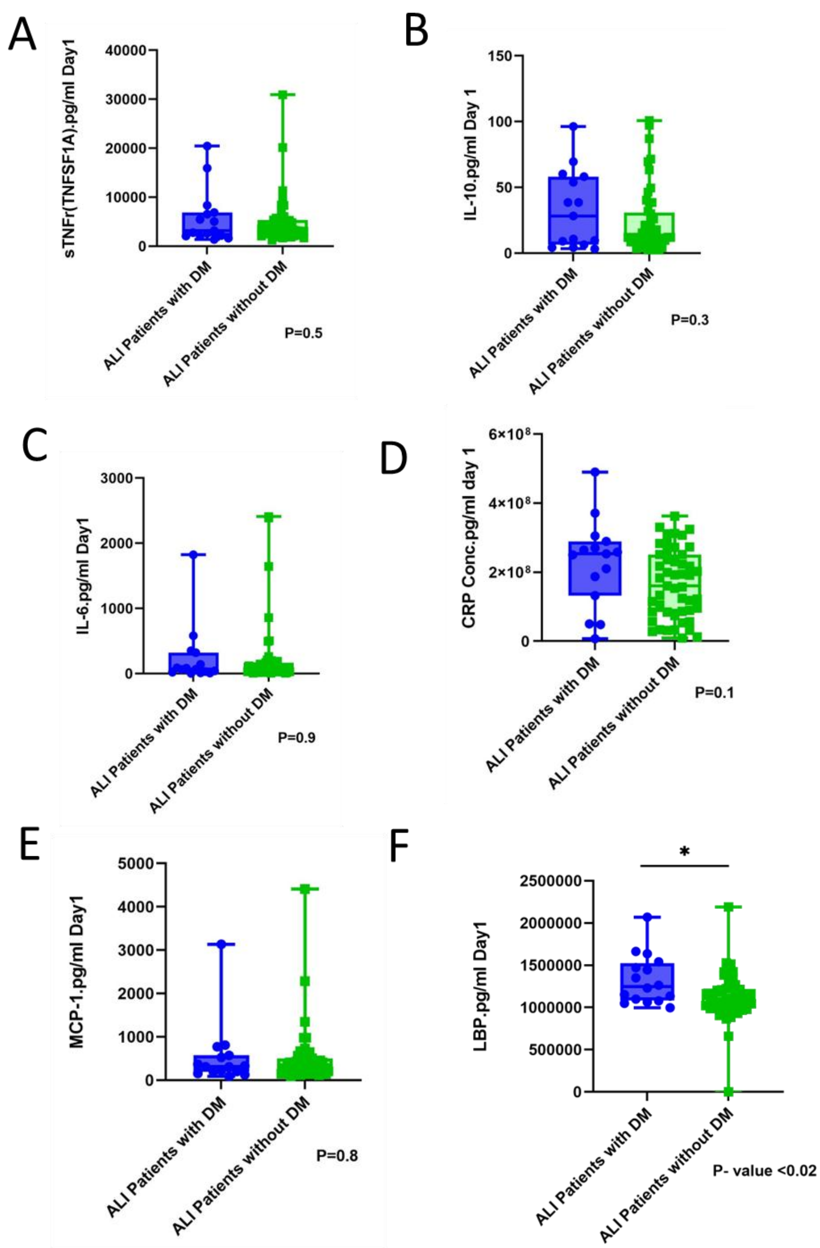

Figure 4. Plasma cytokine and chemokine levels of ARDS patients with and without DM on Day 1 post-treatment. **(A)** TNF-α, Tumor necrosis factor-alpha **(B)** IL-10, interleukin-10 **(C)** IL-6, interleukin-6 **(D)** MCP-1, monocyte chemoattractant protein-1 **(E)** CRP, C-reactive protein **(F)** LBP, lipopolysaccharide-binding protein.

Discussion

Our secondary analysis of the FACTT dataset revealed that DM might be a risk factor for ARDS susceptibility as well as adverse outcomes. Notably, individuals with DM exhibited considerably elevated mortality rates, prolonged hospital stays, and a reduced number of ICU-free days in comparison to ARDS patients without DM. Furthermore, a gender-specific analysis indicated that both men (at a statistically significant level) and women exhibited heightened odds of mortality when compared to non-DM patients, implying that DM might exert an influence on ARDS outcomes across both genders. Neither conservative nor liberal fluids management strategy reduced the mortality ratio in ARDS patients with DM compared to non-DM patients.

The relationship between DM and lung diseases has been marked by conflicting findings (Table 3). A potential protective effect of DM was identified in a prospective multicenter study that aimed to investigate whether the presence of pre-existing DM could alter the incidence of ARDS in patients with septic shock [15]. Conversely, another study that explored the progression and impacts of ARDS in participants revealed that DM was associated with a diminished risk of ARDS development [6]. Likewise, a different study indicated that individuals with DM did not face a heightened risk of developing ARDS compared to those without DM (25% vs. 47%; odds ratio = 0.33) [17]. Conversely, some studies have proposed that DM raises the likelihood of ALI/ARDS and is associated with elevated mortality rates [11]. Daryl et al. identified that DM significantly contributes to the risk of ARDS in post-surgery patients [18]. Likewise, a meta-analysis reached the conclusion that DM appeared to act as a risk factor for ICU admission and fatalities among COVID-19 patients who presented with ARDS[19]. Surprisingly, DM was reported as not a risk factor for ICU admission in COVID-19 patients [20]. Furthermore, a retrospective study conducted on individuals with miliary tuberculosis revealed that the presence of DM independently increased the risk of developing ARDS [21]. Amidst these contradictory reports, our findings lend support to the plausible link between DM and its role as a risk factor in the development of ARDS.

Several of the aforementioned studies also indicated the possibility of a connection between DM and increased mortality associated with ARDS. Although one study indicated a notably reduced prevalence of ARDS in comparison to patients without a history of DM, no significant distinction in hospital mortality was observed between individuals with ARDS who had DM and those without DM [15]. In an alternate study, the mortality rate remained consistent among individuals with ARDS regardless of their DM diagnosis, although an association between DM and a reduced risk of ARDS development was observed. [6]. Furthermore, another study did not report any difference in the likelihood of mortality between the two cohorts [17]. In contrast, a meta-analysis provided evidence that the existence of DM has been linked to a notable increase in both the mortality rate and the severity of ARDS among critically ill COVID-19 patients, signifying statistical significance ($p < 0.001$) [20]. Moreover, the retrospective analysis of individuals with miliary tuberculosis demonstrated that the presence of DM independently correlated with higher mortality rates ($p < 0.01$) [21]. Despite the conflicting nature of these reports, our findings strengthen the potential association between DM and its role as a factor influencing mortality in ARDS patients.

The discrepancies in findings across various studies regarding mortality outcomes might stem from the fact that a reduced incidence of ARDS, attributed to minimized lung injury, could logically correspond with a decrease in the mortality rate. We also contend that the clinical outcomes in individuals with diabetic-ARDS may hinge on specific disease contexts. To illustrate, protective effects or no discernible association with DM have predominantly been documented in patients with sepsis, whereas unfavorable outcomes have been more commonly observed in cases of ARDS stemming from different causes such as infections or post-surgery conditions. Our study was designed to assess the impact of pre-existing DM, without delving into the causal link with ARDS. Therefore, factoring in the actual underlying cause of ARDS is of paramount importance for refining therapeutic responses and ultimately enhancing patient outcomes.

Our findings, as revealed through the secondary analysis of FACTT data, align with studies that propose a potential association between pre-existing DM and an elevated mortality rate as well as a increased likelihood of ICU admission among ARDS patients. The presence of a low-grade chronic inflammation has been acknowledged in DM patients, playing a crucial role in the initiation and progression of ALI/ARDS [22]. While some studies have noted a decrease in inflammatory response and lower cytokine production among individuals with DM, suggesting a possible mitigation of ALI/ARDS progression in severe cases [9], contrasting results have been reported, indicating that levels of cytokines and chemokines were elevated in ARDS patients with pre-existing DM compared to those without DM[23]. Our study demonstrates a trend where individuals with DM exhibited increased concentrations of cytokines and chemokines, consistent with the emergence of negative clinical outcomes.

Despite a strong and statistically significant association observed between DM and non-DM patients on ARDS outcomes within our analysis, the study comes with several limitations.

Firstly, there exists an imbalance in sample sizes between the groups, impeding the generalizability of our findings. Secondly, due to constraints, we were unable to stratify the study based on the underlying cause of ARDS or the specific type of diabetes, be it type I or type II. Thirdly, our analysis of cytokines and chemokines is restricted by the availability of plasma samples obtained from BioLINCC (n=15 for DM and n=50 for non-DM), which could potentially contribute to the lack of significance observed in certain analyses.

Table.3 Summary of available studies on effect of DM on ARDS outcomes

Study name	Intervention/Study design	Study population	Outcomes	
			ARDS risk in pre-existing DM	**Association of DM with ARDS mortality rate**
Moss et al 2000	None /Prospective	ICU-Septic shock patients	Negative, P=0.03	No association, $P = 0.5$
Iscimen et al 2008	None/Prospective	ICU-Septic shock patients	Negative, P=0.07	No data
Gong et al 2005	None /Prospective	Patients with sepsis, trauma, aspiration, and hyper-transfusion	Negative, P=0.02	No association, $P = 0.1$
Yu et al 2013	None /Prospective	Patients with sepsis, pneumonia, trauma, aspiration	Negative, P= 0.01	Not associated with 60-day mortality, $P < 0.02$
Trillo-Alvarez et al 2011	None/Retrospective	ICU patients with > 4 predisposing ARDS condition	No association, P=0.63	No data
Luo et al 2017	None/Retrospective	ICU patients	No data	Not associated with mortality, P=0.02
Ian et al 2020	Meta-analysis	COVID-19 patients	Positive, P= <0.01	Associated with mortality, $P < 0.001$
Daryl J et al 2017	None/ retrospective	Post-surgical patients	Positive, P= < 0.01	No data
Loris et al 2020	None/ Meta-analysis	COVID-19 patients	Positive, P=<0.01	Associated with mortality, $P < 0.001$
Deng et al 2012	None/ retrospective	Miliary tuberculosis patients	Positive, P =<0.01	Associated with mortality, $P < 0.01$

Negative; DM association with ARDS development is statistically non-significant.
Positive; DM association with ARDS development is statistically significant.

Conclusions

Our secondary analysis of FACTT data revealed that pre-comorbidity with DM might aggravate the clinical outcomes in ALI patients. Therefore, the clinical-biological relations in DM lungs require further extensive preclinical and clinical studies.

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CHAPTER 3

Advanced Glycation End-Products Induce Cytokine Dysregulation and Weaken Lung Epithelial and Endothelial Barrier Integrity

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Abstract

Diabetes Mellitus is a systemic disease characterized by chronic hyperglycemia, persistent inflammation, and oxidative stress. While the vascular complications of diabetes are well-documented, their impact on lung barrier integrity remains underexplored. In this study, we investigated the molecular mechanisms by which advanced glycation end-products (AGE) compromise the integrity of lung endothelial and epithelial barriers. Using human lung microvascular endothelial cells and epithelial (A549) cells, we assessed the impact of AGE on the tight junction protein claudin-5, adherens junction protein VE-cadherin, and key signaling molecules including the receptor for AGE (RAGE), phosphorylated Akt, and p38 MAPK as well as a panel of pro-inflammatory cytokines. Our findings demonstrated that AGE exposure (50 μg/mL) significantly activated Akt and p38 MAPK, upregulated Claudin-5 and RAGE, and downregulated VE-cadherin, correlating with reduced transendothelial electrical resistance in vitro. Notably, we observed similar effects on lung epithelial cells. Moreover, AGE-treated conditioned media from THP-1 macrophages induced a pronounced increase in inflammatory cytokines, amplifying the disruption of lung barrier integrity. These findings reveal a potential mechanism linking diabetes-induced vascular dysfunction and immune activation to compromised lung barrier function, emphasizing the need for further research into diabetes-associated lung complications.

Keywords: diabetes mellitus; advanced glycation end products; inflammation; lung endothelial-barrier, macrophages, cytokines.

1. Introduction

Diabetes mellitus is a chronic condition characterized by persistent hyperglycemia, vascular injury, inflammation, and oxidative stress[1]. Despite the extensive capillary network in the lungs, diabetes-associated pulmonary complications remain underexplored compared to other vascular complications[2]. We recently reported that patients with diabetes suffering from ARDS exhibit worse clinical outcomes than those without diabetes, reinforcing the link between diabetes and impaired respiratory function[3]. Although considerable research has focused on vascular complications in diabetes, emerging evidence suggests that the lungs may also be adversely impacted, particularly in individuals with pulmonary disorders such as acute lung injury (ALI)[2-4.]

Advanced glycation end products (AGE), a diverse group of substances, are formed through non-enzymatic reactions between glucose or its by-products and proteins or lipids[5,6]. Substantial evidence highlights that unmanaged hyperglycemia promotes AGE accumulation, which plays a pivotal role in the development of many diabetes-related complications[7], a significant health burden that has increased rapidly over the last few years8. These complications are classified into two categories: microvascular complications primarily affecting the kidneys, eyes, and neurons, and macrovascular complications involving the heart and brain[8]. The effect of AGE on lung-resident cells is unknown. We sought to address this gap by being the first to investigate the potential effects of AGE on lung epithelial and endothelial cells and their cross-talk with macrophages in mediating inflammation.

In this study, we investigated the impact of AGE on barrier functions in human microvascular lung endothelial (HMLE) cells and alveolar type II epithelial (A549) cells, focusing on the integrity of cell-cell junction proteins. We also examined the activation of key pro-inflammatory pathways, including Akt and P38 MAP kinase, in response to AGE exposure. Recognizing the critical role of inflammation in diabetes, where macrophages play a pivotal role[9], we further investigated both the direct and indirect effects of AGE on macrophage activation, which, in turn, influences endothelial and epithelial barrier integrity and cytokine production. This included analyzing the expression of cytokines and chemokines such as tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), interleukin-10 (IL-10), and interleukin-4 (IL-4) in lung endothelial and epithelial cells. Our findings offer new insights into how diabetes-induced vascular alterations and immune activation may compromise lung barrier integrity, providing a foundation for developing targeted therapies to prevent or alleviate lung complications in diabetes patients.

2. Materials and Methods:

2.1. Cell Culture and Reagents

Human microvascular lung endothelial (HMLE) cells were cultured in a complete human endothelial cell medium (Cat# H1168, Cell biologics, Chicago, IL, USA), while the human type II alveolar epithelial cells (A549) were maintained in a Gibco Dulbecco's Modified Eagle Medium (DMEM) enriched with 1% penicillin/streptomycin (ATCC, Manassas, VA), and 10% fetal bovine serum (FBS) (Atlanta Biologicals, Atlanta, GA). Cells between passages around 14-21 were utilized for the experiments. The human THP-1 cell line was bought from ATCC, Manassas, VA. These cells were cultured in an RPMI-1640 medium containing 10% FBS before being differentiated to be macrophage-like cells using 150 nM of PMA treatment for 24 hours. Plastic culture wares, chemicals, and all other reagents used in the study were obtained from Fisher Scientific, Hampton, NH, USA. BSA-AGE (Cat N. 22968, Cayman Chemical, Ann Arbor, MI) at three different doses, incorporating (25, 50, and 100 μg/ml) was tested on the above-mentioned cell lines.

2.2. Experimental design

Our study was designed to comprehensively investigate the molecular mechanisms of diabetes-induced lung consequences. We first investigated the effect of multiple doses of AGE treatment on HMLE and A549 cells. The experimental groups were classified as follows: (1) Untreated HMLE or A549 cells serving as controls, (2) HMLE or A549 cells treated with a dose of 25 µg/ml, 50 µg/ml, and 100 µg/ml for 24 hours. The potential role of immune-mediated inflammation was significantly addressed. We investigated how AGE affects the activation of macrophage cells by culturing AGE-treated conditioned media (CM) from differentiated THP-1 macrophages and subsequently incubating it with HMLE and A549 cells for 24 hours. For this experiment, the groups were as follows: (1) untreated control groups of HMLE or A549 cells, (2) THP-1 CM without AGE treatment, and (3) THP-1 CM treated with (AGE) at a dose of 50 µg/ml for 24 hours.

2.3. Western blot analysis

The cellular monolayers were scratched and lysed using a mixture of protease and phosphatase inhibitors in RIPA buffer (Roche, Basel, Switzerland). The protein concentration was determined using a DC protein assay (Bio-Rad, Hercules, CA), and approximately 30–40 µg of proteins in Laemmli buffer were used for the experiments. Western blotting was performed as previously described10-12. The antibodies used and their respective dilution ratios were as follows: Anti-pSer473-Akt (1:1000, Cat No. 4060S), anti-Akt (1:1000, Cat No. 4691S), anti-pP38 MAPK (1:1000, Cat No. 4511S), anti-P38 MAPK (1:1000, Cat No. 9212S), VE-Cadherin (1:1000, Cat No. 2158S), and RAGE (1:1000, Cat No. 6996S). All these antibodies were obtained from Cell Signaling Technology (Danvers, MA). The monoclonal anti-β-actin antibody (1:10000, Cat No. 5174) was purchased from Sigma. Anti-Claudin-5 (Cldn-5) (1:1000, Cat No. 4C3C2) was obtained from Thermo Scientific (Waltham, MA). The secondary antibodies, including anti-mouse (Cat No.

170-6516) and anti-rabbit (Cat No. 170-6515), were procured from Bio-Rad (Hercules, CA). Band intensity quantifications were performed using the National Institutes of Health (NIH) ImageJ software.

2.4. Assessment of Epithelial and Endothelial-Barrier Resistance

The integrity of both endothelial and epithelial barriers was assessed by measuring the electrical resistance of the cell monolayer using the Electric Cell-Substrate Impedance Sensing (ECIS) technique (Applied Biophysics, Troy, NY, USA). This assay was performed as previously described13-15. Before initiating the assay, the cells were stabilized for 24 hours to ensure monolayer formation and constant resistance. Following stabilization, the experimental cells were exposed to a dose-dependent treatment of AGE at 25, 50, and 100 µg/ml for 24 hours or as specified. Multiple frequencies in the real-time mode were used to evaluate barrier resistance.

2.5. RNA Isolation and Quantitative RT-PCR

QIAzol Reagent (Qiagen, Hilden, Germany) was used to lyse the cells. The miRNeasy Mini Kit (Qiagen) was utilized to isolate total RNA. A NanoDrop Lite Spectrophotometer (ND-LITE-PR, Thermo Fisher Scientific, Waltham, MA, USA) was used to measure the concentrations of the extracted RNA. cDNA was synthesized from approximately 700–1100 ng of RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA, USA). The StepOnePlus™ Real-Time PCR System (4376600, Applied Biosystems), with Power SYBR Green Master Mix (Applied Biosystems), was employed to perform the quantitative PCR. GAPDH was used as the normalizing control. The ΔΔCt method was applied to estimate fold changes. The sequences of primers used in the analysis are listed in Table 1.

Table 1. List of primes utilized in the study

Gene name	Forward Primer	Reverse Primer
IL-1β	5′-CCACAGACCTTCCAGGAGAATG-3′	5′-GTGCAGTTCAGTGATCGTACAGG-3′
IL-6	5′-ACTCACCTCTTCAGAACGAATTG-3′	5′-CCATCTTTGGAAGGTTCAGGTTG-3′
IL-10	5'-TCAAGGCGCATGTGAACTCC-3'	5'-GATGTCAAACTCACTCATGGCT-3'
TNF-α	5′-GGTCCCCAAAGGGATGAGAA-3′	5′-TGAGGGTCTGGGCCATAGAA-3′
GAPDH	5'-TGCACCACCAACTGCTTAGC-3'	5'-GGCATGGACTGTGGTCATGAG-3'

2.6. Statistical analysis

All data are presented as the Mean ± SEM. Statistical analysis was performed on results from at least three independent experiments using Student's unpaired t-test or one-way ANOVA followed by Tukey's post hoc test. All analyses were conducted using GraphPad Prism software version 11.0. A p-value < 0.05 was considered statistically significant.

3. Results

3.1. AGE treatment activates AKT and P38 MAPK pathways in lung-resident cells

We examined the potential of AGE treatment to activate pro-inflammatory pathways, Akt and P38 MAPK, in both the lung-resident cells. Epithelial (A549) cells treated with 50 µg/ml AGE for 24 hours revealed a significant increase in the expression of Akt phosphorylation (Figures 1A and C). More importantly, the level of phosphorylated P38 MAPK was significantly elevated with the same dose (50 µg/ml AGE) (Figures 1A and E). Collectively, a dose of 50 µg/ml of AGE showed a consistent effect in activating Akt and P38 MAPK pathways in both HMLE and A549 cells. Likewise, after 24 hours of AGE exposure, HMLE cells exhibited a significant increase in Akt phosphorylation at a concentration of 50 µg/ml, while a higher dose of 100 µg/ml led to a statistically insignificant, modest increase in Akt phosphorylation (Figures 1B and D). Surprisingly, the expression of phosphorylated P38 MAPK was significantly increased at only 50 µg/ml dose, and no changes were observed with other doses. (Figures 1B and F).

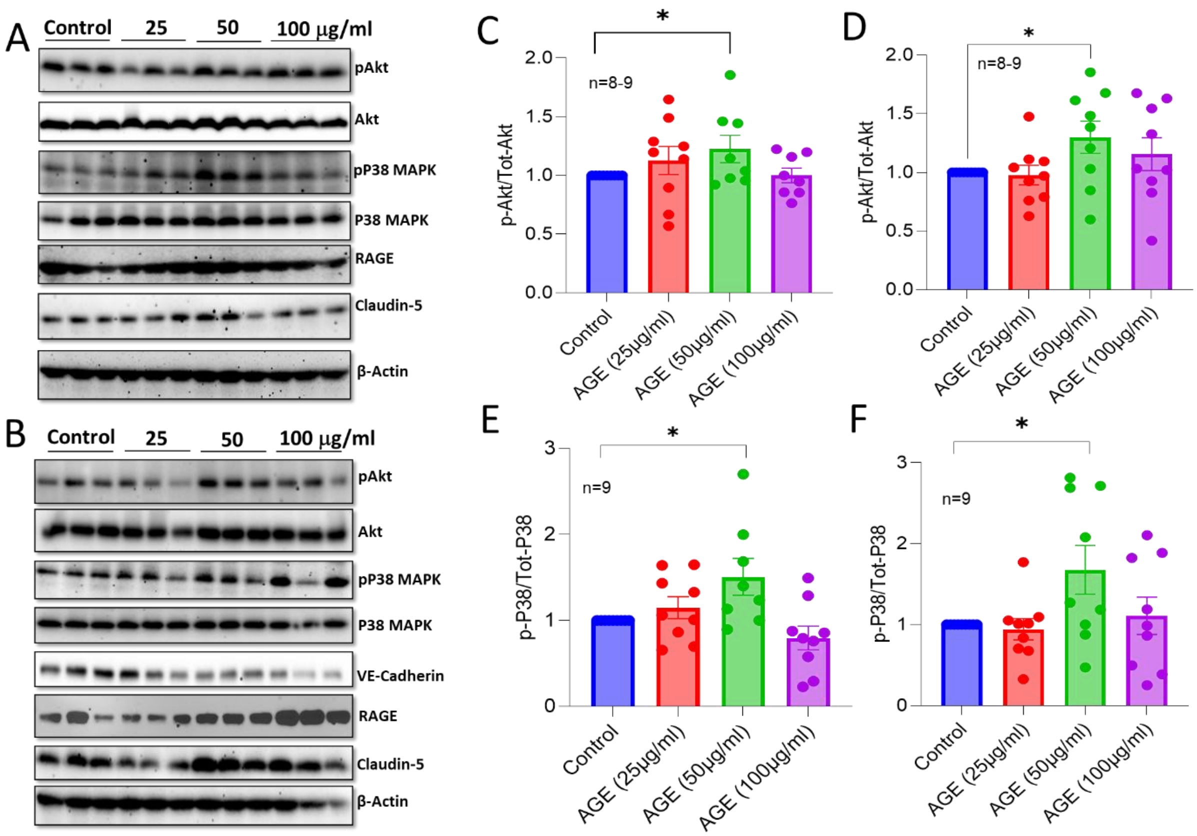

Figure 1: Treatment of A549 and HMLE cells with AGE activates the Akt and P38 MAPK pathways while modulating the expression of cell-cell junction proteins. (A and B) Representative Western blot images of A549 and HMLE cell lysates treated with AGE at three doses (25, 50, and 100 µg/mL) for 24 hours, showing changes in pAkt and pP38 MAPK. (C and E) Quantitative analysis of Western blot data presented as bar graphs indicating increased pAkt and pP38 MAPK expression in A549 cells following AGE treatment compared to controls. (D and F) Bar graphs from densitometry analysis demonstrating the effect of AGE treatment on pAkt and pP38 MAPK levels in HMLE cells compared to controls. Data presented as Mean + SEM. *p<0.05; **p<0.01; #p<0.001.

3.2. AGE modulates the expression of cell-junction proteins in lung-resident cells

Since AGE is one of the main contributors to complications of diabetes, we first aimed to evaluate the extent to which it impacts tight junction (TJ) protein Cldn-5 in A549 and HMLE cells, and adherens junction (AJ) protein VE-Cadherin in HMLE cells. Our findings demonstrated that incubating HMLE cells with a 50 µg/ml dose of AGE for 24 hours resulted in a statistically significant increase in Cldn-5 levels exclusively in HMLE cells (Figures 1B and 2B). Notably, no changes in Cldn-5 expression were observed in epithelial cells under the same conditions (Figures 1A and 2A). When we measured VE-Cadherin levels, specifically expressed in HMLE cells, we observed a downward trend with AGE concentrations of 25 and 50 µg/ml. However, exposure to a higher dose of AGEs (100 µg/ml) for 24 hours led to a statistically significant reduction in VE-Cadherin expression (Figures 1B and 2C). Considering the importance of the receptor for advanced glycation end products (RAGE), we examined its expression in response to AGE exposure for 24 hours. Although our results showed upregulation in a dose-dependent manner in both cell lines (Figures 2D and E), only the epithelial cells exhibited a significant upregulation following administration of 50 µg/ml, as opposed to the endothelial cells (Figures 1A and 2D). Despite the differences observed in the impact of various doses of AGEs, the results indicated that a concentration of 50 µg/ml modulates TJs through upregulation of Cldn-5 protein expression in HMLE cells and increased levels of RAGE in epithelial cells.

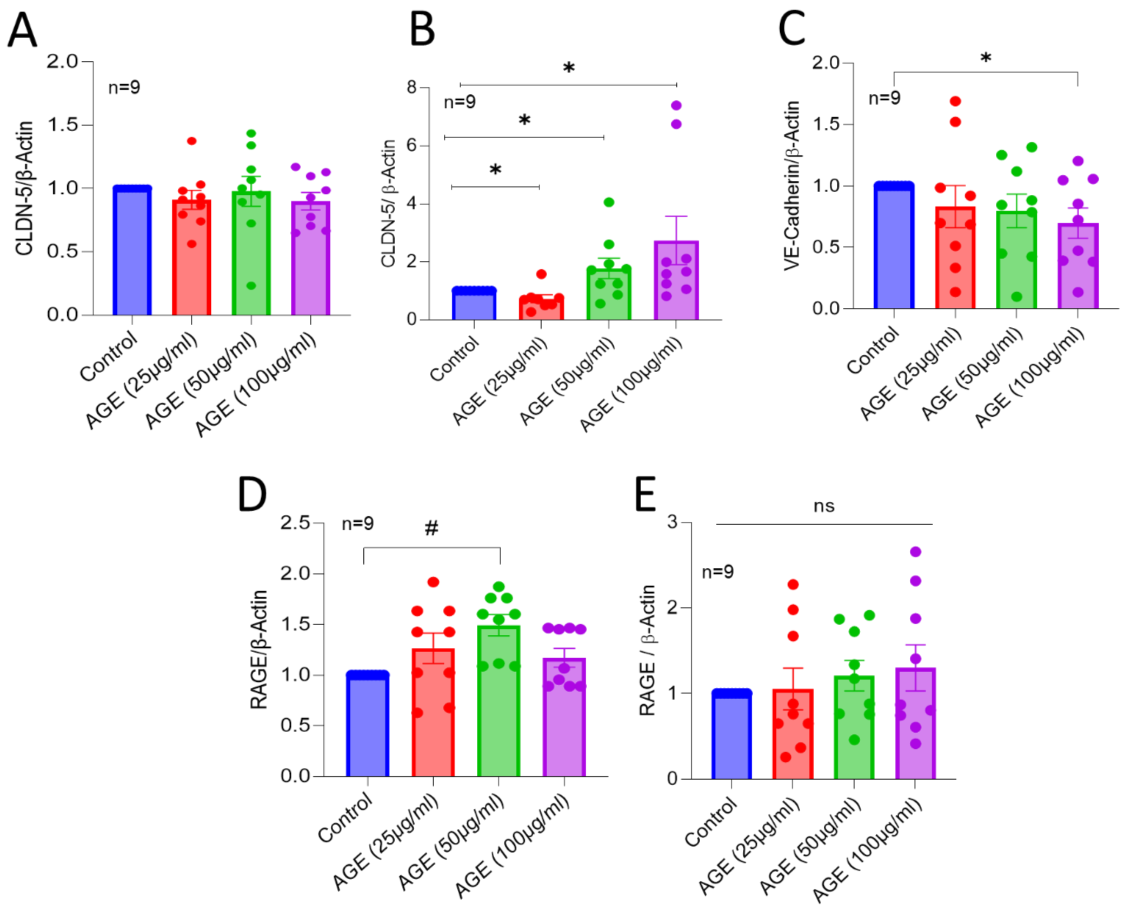

Figure 2: AGE treatment induces cell-cell junction protein turnover in A549 and HMLE cells. (A and B) Bar graphs showing quantitative analysis of Western blots demonstrating expression changes in Cldn-5 in AGE (50 µg/ml)-treated A549 and HMLE cells, respectively. (C) Bar graphs with quantitative analysis of Western blots showing VE-Cadherin expression in AGE-treated A549 and HMLE cells, respectively in comparison to the control group. (D and E) Bar graphs showing quantitative analysis of Western blots exhibiting activated RAGE compared to controls in AGE-treated A549 and HMLE cells, correspondingly. Data presented as Mean + SEM. *p<0.05; **p<0.01; #p<0.001.

3.3. AGE exposure reveals distinct cytokine profiles in pulmonary epithelial and endothelial cells

Next, we explored the direct effect of the AGE insult on cytokine expressions in both cell lines. We subjected these cells to AGE 50 μg/ml for 24 hours, and then we determined the relative expression of key pro-inflammatory cytokine mRNAs, including TNF-α and IL-6, IL-10, and IL-1β. We found that Incubating HMLE cells directly with 50ug/ml of AGE demonstrated a significant reduction in IL-10 expression, while TNF-α and IL-6 expression showed no significant differences compared to the control (Figures 3B, F, and H). Unexpectedly, the level of IL-1β was significantly lowered in the presence of AGE treatment compared to the control group (Figure 3D). Unlike HMLE cells, lung epithelial cells showed a statistically significant, considerable increase in the levels of IL-10 and IL-6 (Figures 3E and G), while TNF-α tended to increase in levels compared to the untreated group (Figures 3A).

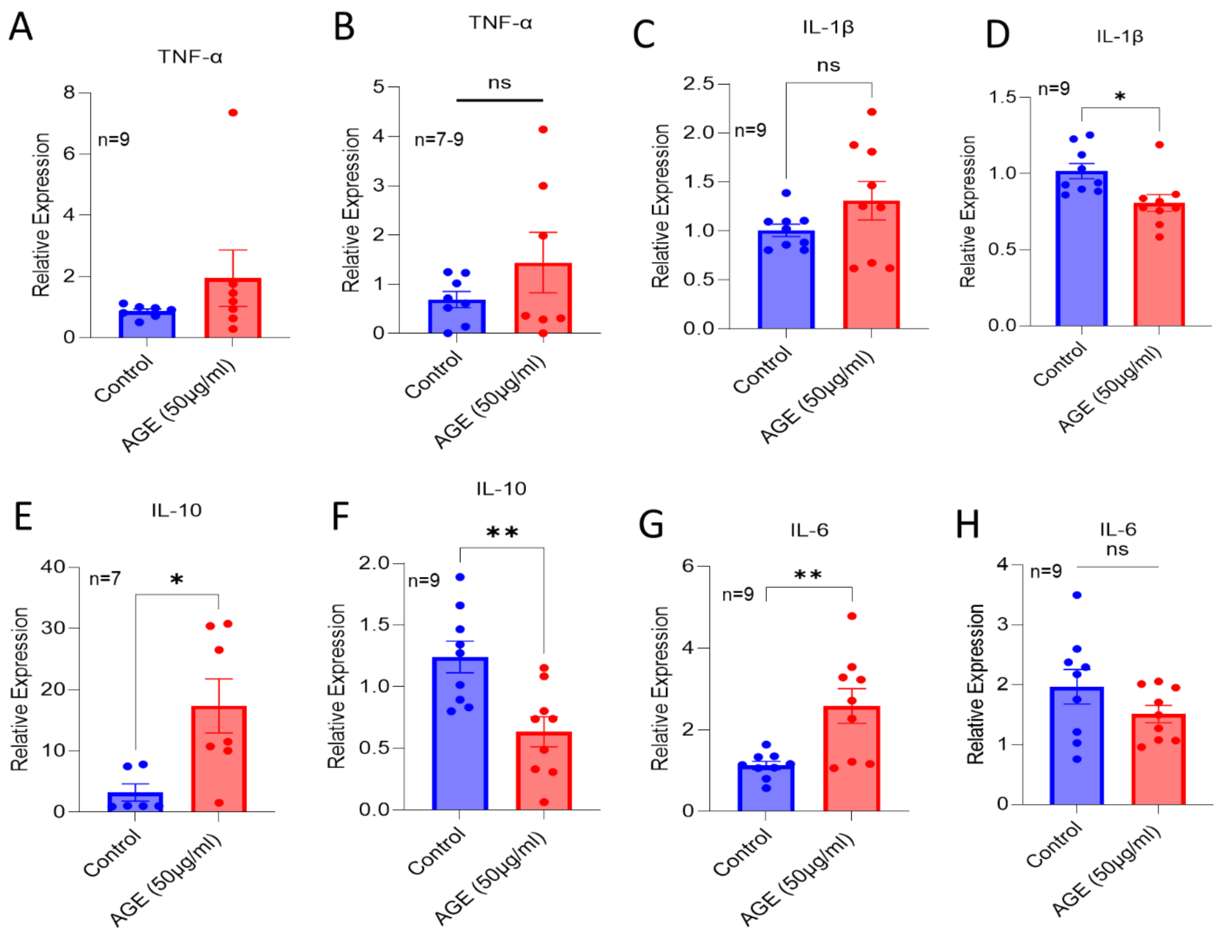

Figure 3: AGE treatment alters the pro-inflammatory cytokine levels in A549 and HMLE cells. (A, C, E, and G) Bar graphs showing qRT-PCR data of A549 cells after 24 hours incubation with AGE (50 µg/ml) showing changes in mRNA expression of pro-inflammatory cytokines TNFα, IL-1β, IL-10, and IL-6. (B, D, F, and H) Bar graphs showing qRT-PCR analysis of HMLE cells post 24 hours treatment with AGE (50 µg/ml) showing changes in mRNA relative expression of pro-inflammatory cytokines TNFα, IL-1β, IL-10, and IL-6, normalized to GAPDH. Data presented as Mean + SEM. $^*p<0.05$; $^{**}p<0.01$

3.4. AGE treatment modulates HMLE and A549 cell barrier function

To further evaluate the influence of AGEs on A549 and HMLE cell barriers, an ECIS assay was performed to measure barrier resistance, which measures even subtle changes in barrier function in response to various stimuli. In a manner comparable to Western blotting experiments, the cells were treated with AGE in three doses (25, 50, and 100 µg/ml) for 24 hours. A concentration of 50 µg/ml significantly downregulated the epithelial barrier resistance in contrast to the control group (Figure 4A). The monolayer barrier of HMLE cells was significantly decreased with 25 and 100 µg/ml for 24 hours, with a severe reduction observed with the latter dose compared to the control group (Figure 4B). Conversely, when HMLE cells were exposed to 50 µg/ml, the cells displayed a modest decrease in barrier strength, which was deemed non-significant. A concentration of 50 µg/ml was found to be the optimal dose for further investigations, as 25 µg/ml showed inconsistent outcomes, while 100 µg/ml seemed to affect the health of the cells.

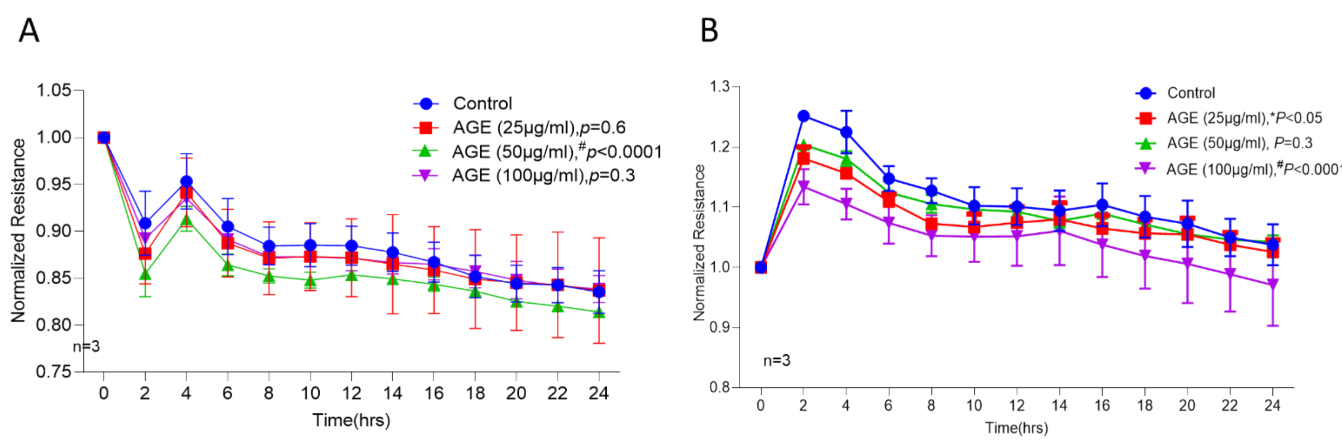

Figure 4: Treatment of A549 and HMLE cells with AGE modulates barrier resistance. (A) ECIS analysis showing modulated A549 cell-barrier resistance upon treatment with AGE (25, 50 and 100 µg/ml) for 24 hours compared to the untreated control group. (G) ECIS analysis showing modulated HMLE cell-barrier resistance upon treatment with AGE (25, 50 and 100 µg/ml) for 24

hours compared to the untreated control group. Data presented as Mean + SEM. *p<0.05; #p<0.001.

3.5. Conditioned media from AGE-treated THP-1 macrophages induce pro-inflammatory cytokine secretion in lung-resident cells

We aimed to examine the impact of AGE-treated conditioned media (CM) from differentiated THP-1 macrophages on HMLE and epithelial barriers. Our findings demonstrated that incubation of both cell lines with THP-1 CM pre-treated with 50 µg/ml of AGE for 24 hours significantly decreased transendothelial and transepithelial electrical resistance (TEER) values, compared to cells treated with THP-1 CM (pre-treated with vehicle) and from the no CM control group. These results suggest that AGE-treated THP-1 CM compromises the integrity of the epithelial and barriers (Figures 5A and B).

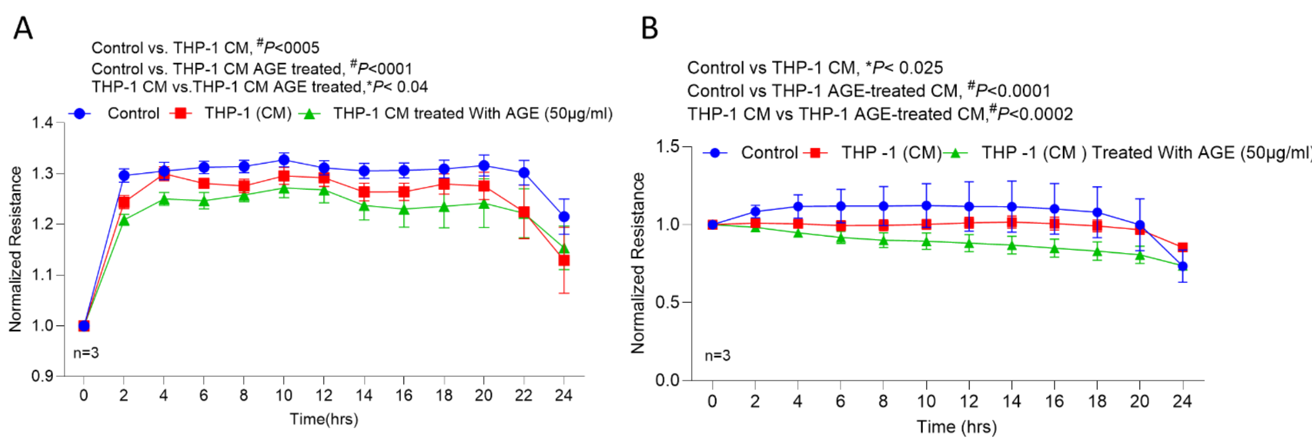

Figure 5: AGE-treated THP-1 macrophage conditioned media modulates lung epithelial and endothelial barrier integrity. (A) ECIS analysis showing real-time changes in A549 cell-barrier resistance upon treatment with THP-1 conditioned media (CM) with and without AGE (50 µg/ml) for 24 hours compared to the untreated control. (B) ECIS analysis showing real-time changes in

HMLE cell-barrier resistance upon treatment with THP-1 CM with and without AGE (50 μg/ml) for 24 hours compared to the untreated control.

We further investigated the inflammatory responses in HMLE and epithelial cells incubated with THP-1 CM pre-treated with 50 μg/ml of AGE for 24 hours, compared to THP-1 CM without AGE treatment and the no CM control group. In HMLE cells, we observed a significant upregulation of IL-1β expression in AGE-enriched THP-1 CM compared to THP-1 CM alone and the control. Although increased levels of TNF-α, IL-10, and IL-6 were observed in both AGE-treated and non-AGE-treated THP-1 CM compared to the control (Figures 6B, D, and F), no significant difference was found between these two CM-treated groups. Conversely, lung epithelial cells exposed to THP-1 CM with 50 μg/ml of AGEs showed a substantial increase in IL-1β, IL-10, and IL-6 levels compared to THP-1 CM without AGE treatment and the control groups (Figures 6C, E and G) However, TNF-α expression remained unchanged compared to the control group (Figure 6A). These results indicate that under diabetic conditions, macrophages significantly promote inflammatory activation in lung epithelial and endothelial cells, thereby contributing to the impairment of barrier integrity.

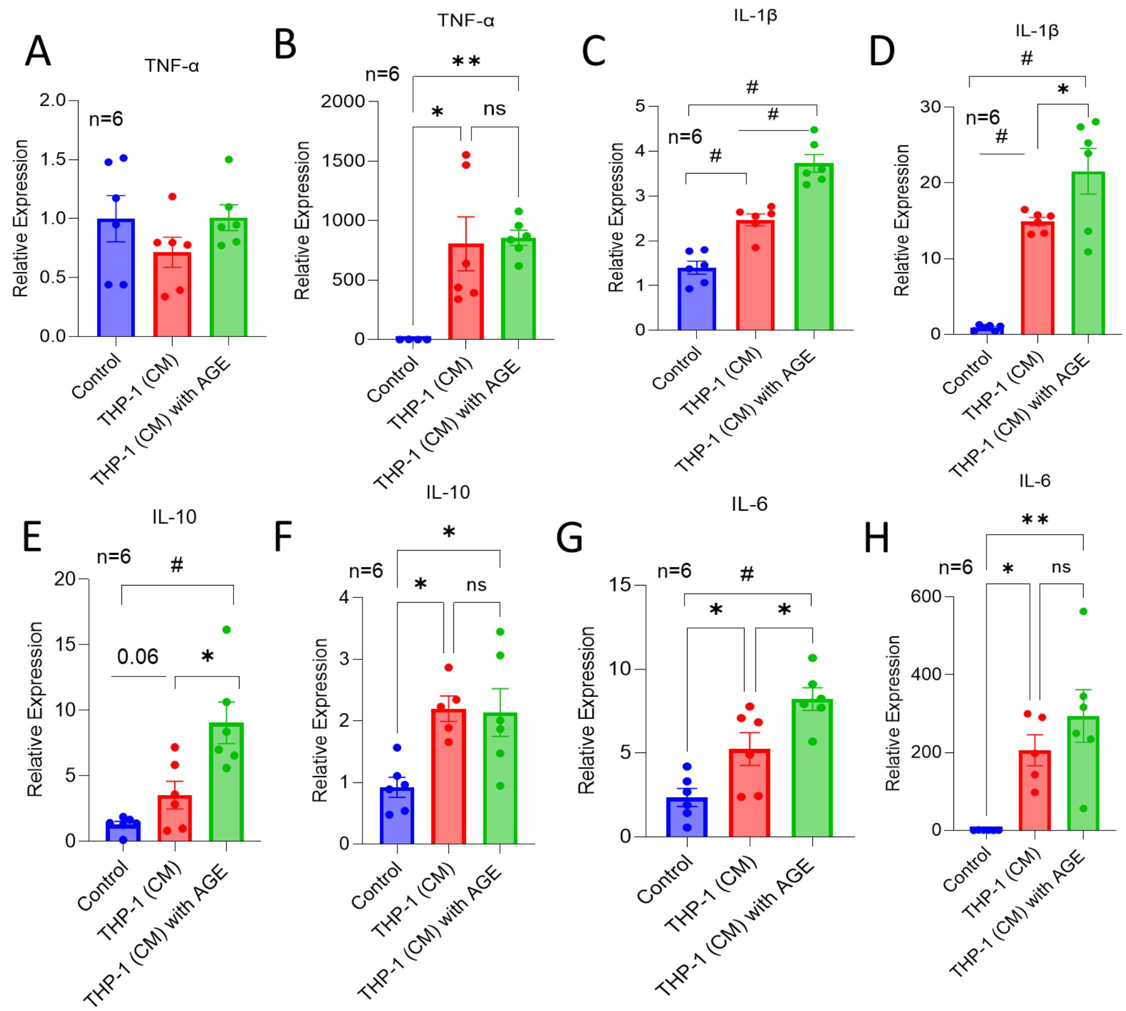

Figure 6: AGE-treated THP-1 macrophage conditioned media triggered pro-inflammatory cytokines production in A549 and HMLE cells. qRT-PCR analyses of A549 (A, C, E, and G) and HMLE cells (B, D, F, and E) revealing changes in mRNA relative expression of TNFα, IL-1β, IL-10, and IL-6 (normalized to GAPDH) following 24-hour treatment with AGE-treated THP-1 CM compared to vehicle-treated THP-1 CM. Data presented as Mean + SEM. *p<0.05; **p<0.01; #p<0.001.

Discussion

Diabetes is a significant risk factor for developing both microvascular and macrovascular complications resulting in high morbidity and mortality[16,17]. These vascular changes primarily arise from the long-term effects of chronic hyperglycemia18. AGE, formed through non-enzymatic reactions between sugars and proteins, lipids, or other sugar derivatives, plays a pivotal role in this process[7]. While AGE production occurs at a slow rate under normal glucose conditions, hyperglycemia accelerates their formation, with elevated AGE levels strongly linked to the progression of diabetes-related complications such as vascular damage[19,20]. Notably, high AGE levels have been detected in patients with diabetes, further establishing their role as a harmful factor in promoting vascular dysfunction[21-23]. Although the relationship between AGE and traditional microvascular and macrovascular complications has been extensively studied[24,25], the impact of diabetes on lung health remains underexplored. Our recent studies indicated that diabetes can adversely affect lung function, leading to poorer clinical outcomes, particularly in the presence of respiratory diseases[2,3]. This highlights an urgent need to investigate the molecular mechanisms underlying diabetic lung complications.

The current study highlights the profound impact of AGEs on lung-resident endothelial (HMLE) and epithelial (A549) cells, offering insights into the molecular mechanisms contributing to diabetic lung complications. We observed significant, cell-specific effects of AGE exposure on pro-inflammatory signaling, cell-junction protein expression, cytokine secretion, and barrier integrity. The RAGE (receptor for AGE) plays a pivotal role in mediating pulmonary and vascular inflammation and is well-documented to be activated in response to AGE exposure[26]. Previous studies have reported significantly elevated RAGE mRNA expression in diabetic patients with microvascular and macrovascular complications compared to healthy controls, highlighting its detrimental effects on vascular health[26]. Consistent with these findings, our study demonstrated that AGE treatment increased RAGE expression in both lung-resident cell lines, underscoring its potential role in promoting inflammatory responses and barrier dysfunction in diabetic lung pathology. Our findings expand on the established literature on AGEs and diabetic complications, addressing the underexplored role and pathological effects of AGE in pulmonary pathophysiology.

AGE treatment activated the Akt and P38 MAPK pathways in both cell types, with consistent Akt phosphorylation and significant P38 MAPK activation observed in epithelial cells. These pathways are central to cellular stress and inflammatory responses and have been implicated in AGE-induced damage in vascular and epithelial tissues[27]. Prior studies from our lab have demonstrated the role of the P38 MAPK and Akt pathways in promoting inflammation and vascular permeability[2,15,28-35]. Akt activation has also been associated with oxidative stress and endothelial dysfunction[26-29,36]. Our findings align with these reports and suggest that differential activation of these pathways may contribute to the distinct responses of lung-resident cells to AGE exposure.

Beyond these pathways, AGE treatment influenced the expression of key cell-junction proteins, with differential effects observed between cell types. In HMLE cells, AGE treatment upregulated Cldn-5 expression, which aligns with recent reports indicating that increased Cldn-5 expression in endothelial cells contributes to vascular pathology in various vascular beds[10,35,37]. Intriguingly, elevated Cldn-5 expression in lung epithelium has been reported to be associated with increased permeability, susceptibility to edema, and exacerbated lung injury[38,39]. However, in lung epithelial cells, AGE treatment did not induce significant changes in Cldn-5 expression, further emphasizing cell and stimuli-specific variations in junctional remodeling. Conversely, the reduction in VE-Cadherin expression at higher AGE doses supports evidence that AGEs disrupt AJs, contributing to vascular permeability[15,31,34,40]. A reduction in VE-Cadherin and other junctional proteins, such as β-catenin, has also been observed in various vascular pathologies[10,11,31,41-43]. It has also been reported that diabetes significantly induces retinal vascular hyperpermeability, which is associated with a decrease in VE-Cadherin expression44. Similarly, reduced VE-Cadherin levels contribute to disrupted cellular borders in human lung and umbilical vein endothelial cells in vitro and sepsis-induced lung injury in humans[45]. Thus, our findings suggest that endothelial barriers are particularly susceptible to AGE-induced dysfunction, consistent with the vascular complications commonly associated with diabetes.

Cytokine profiling revealed distinct inflammatory responses in lung resident cells. The upregulation of cytokines in epithelial cells aligns with prior studies showing AGE-induced amplification of pro-inflammatory cytokines in epithelial tissues[6,18,20,23,26,46]. Interestingly, while AGE stimulated the expression of IL-10 and IL-6 in lung epithelial cells, it reduced IL-10 and IL-1β expression in HMLE endothelial cells. The immune system is generally well-regulated, with pro-inflammatory stimuli often triggering an anti-inflammatory response to counterbalance the reaction[47]. This feedback loop helps resolve inflammation and prevents tissue damage caused by an overactive immune response. This differential regulation partly explains our observation of increased expression of the anti-inflammatory cytokine IL-10 alongside pro-inflammatory cytokines in AGE-induced epithelial cells. While such regulatory mechanisms primarily respond to strong stimuli like infections or injuries, weaker but persistent triggers can lead to chronic low-grade inflammation, potentially resulting in inadequate anti-inflammatory responses[48].Conversely, the reduction in IL-10 in HMLE cells may reflect impaired anti-inflammatory signaling, which has been linked to endothelial dysfunction in diabetes and coronary artery disease[49,50]. The upregulation of IL-1β in endothelial cells exposed to AGE-treated THP-1 macrophage-conditioned media, in turn, compromising the lung epithelial and endothelial barriers further supports the role of macrophage-derived inflammatory mediators in exacerbating endothelial damage, as previously documented in diabetic models[51]. From a clinical perspective, these findings emphasize the importance of targeting AGEs in mitigating diabetes-associated pulmonary complications. Therapies such as RAGE antagonists, glycation inhibitors, and glycation breakers have shown promise in preclinical studies[52-54].

Although the study presents significant findings regarding the impact of AGE treatment on lung endothelial and epithelial cells, several weaknesses and limitations must be considered. Firstly, while the experiments highlight the role of AGE in activating pro-inflammatory pathways such as Akt and P38 MAPK and in modulating lung endothelial and epithelial cell-barrier integrity, the observed effects are modest compared to those in vascular beds such as the diabetic retina and brain, where the impact is far more pronounced. This may partly explain why the effects of diabetes on lung health are less prominent compared to its well-documented impacts on the retina, kidneys, and brain. The differential regulation of cytokines, such as IL-10 and IL-1β in HMLE cells, suggests complex, cell-specific responses to AGE treatment that warrant further validation. To ensure translational relevance, these findings should be corroborated across a broader range of lung-resident cells and in vivo models. Furthermore, the study's focus on short-term effects (24 hours) limits its ability to capture the chronic and cumulative impacts of AGE exposure, which are critical in the context of diabetes-related complications. Another limitation lies in the interpretation of AGE-mediated modulation of barrier integrity and cell-junction protein expression. While the ECIS assay and protein analyses reveal changes in barrier function, the mechanistic links between these alterations and downstream pathophysiological outcomes remain speculative. For instance, the reduction in VE-Cadherin and the selective upregulation of Cldn-5 in lung endothelial cells indicate AGE-induced disruptions, yet the study does not explore compensatory mechanisms or the interplay between lung endothelial and epithelial layers in maintaining lung barrier function. Moreover, the exclusive reliance on in vitro models, although informative, limits the applicability of the findings to the complexity of diabetic pulmonary pathophysiology. Factors such as systemic inflammation, oxidative stress, and immune cell interactions, which are integral to diabetes-induced tissue damage, are not addressed. To address these gaps, future studies should prioritize in vivo validation, extended exposure times, and the inclusion of diabetic models to better contextualize the role of AGEs in lung health. Such efforts could also help refine therapeutic strategies targeting RAGE and associated pathways, potentially mitigating diabetes-associated pulmonary complications.

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CHAPTER 4

Pre-existing Diabetes Aggravates Inflammation and Worsens Lung Injury in Sepsis

Alanazi, Abdulaziz H. et al., **Manuscript to be submitted *to Diabetologia***

Abstract

Background: Diabetes mellitus (DM) is a systemic disease known for its cardiovascular complications. However, its impact on lung health remains underexplored. We aimed to determine the effect of DM on lung health and its effect on acute lung injury (ALI) progression.

Methods: RNA sequencing of streptozotocin-induced non-DM and DM mouse lungs was performed, followed by gene enrichment and bioinformatics analysis. Lung injury and inflammation were assessed in lipopolysaccharide-induced sepsis mice through lung Wet/Dry ratio measurements, H&E staining, RT-qPCR, and analysis of inflammatory markers.

Results: RNA-sequencing revealed distinct gene expression changes in DM lung tissues, emphasizing upregulated inflammatory pathways and impaired endothelial barrier function. Pre-existing DM exacerbated inflammation and injury in septic mouse lungs, suggesting its contribution to poorer ALI outcomes. DM lungs exhibited increased production of inflammatory cytokines (TNF-α, IL-1β, MCP-1, CXCL-1), elevated fluid accumulation, and structural alterations indicative of ALI.

Conclusion: DM significantly exacerbated lung inflammation and worsened ALI outcomes. This study highlights the potential for improving ALI outcomes by targeting DM-associated inflammatory and metabolic pathways, offering new insights for therapeutic interventions in DM patients at risk for ALI.

Keywords: diabetes mellitus; inflammation; sepsis; cytokines; acute lung injury; RNA-seq; lung injury; lung disease.

Introduction

Diabetes Mellitus (DM) is an intricate systemic disease marked by chronic hyperglycemia, usually associated with inflammation and oxidative stress [1]. It is one of the most common and challenging chronic conditions, significantly burdening individuals and economies globally [2]. According to the International Diabetes Federation, the incidence of DM is increasing at a concerning annual rate, with a total of 536.6 million cases reported in 2021, and is anticipated to reach 783.2 million in 2045 [3]. Over 90% of the cases are attributed to type-2 DM, while an estimated 8.4 million individuals had a type-1 DM diagnosis worldwide in 2021 [3]. DM is determined to be the most common co-morbidity among critically ill patients; about 40% of intensive care unit (ICU) subjects have pre-existing DM, including those suffering from respiratory disorders like acute respiratory distress syndrome (ARDS) [4].

Morbidity and mortality linked to DM patients are thought to be a result of endothelial dysfunction, which in turn leads to vascular complications, including retinopathy, nephropathy, neuropathy, accelerated atherosclerosis, and cerebrovascular accidents [5]. The pathophysiology and molecular mechanisms underlying the complications have largely been examined [6]. Although the lungs have a large capillary network, little attention has been given to the lung as a target organ for DM, leading to inadequate understanding of the potential pulmonary complications of DM. Despite the presence of numerous observational studies, the literature reports diverse and often inconsistent findings regarding the association between DM and lung disorders [7-11]. ARDS is a severe and common complication causing respiratory failure in ICU patients and is associated with 30% to 40% mortality [12, 13].

While DM is a risk factor contributing to adverse outcomes in patients with respiratory diseases, some reports are contradictory [7, 14]. In a clinical study, we previously scrutinized the relationship between DM and ARDS disease [15], revealing DM as a potential risk factor significantly attributed to unfavorable outcomes involving high death and ICU rates in ARDS patients compared to ARDS subjects with no history of DM [15]. Considering the importance of our robust clinical findings and the critical role of combinatorial studies in validating such a positive association, we expanded our research to thoroughly investigate DM lungs in a well-designed streptozotocin (STZ)--induced mouse model. Consequently, this current study evaluated the consequence of DM on lung health in mice and investigated the impact of pre-existing DM on the exacerbation of acute lung injury (ALI) outcomes in the lipopolysaccharide (LPS)-induced sepsis model and characterized the associated molecular mechanisms involving RNA sequencing, bioinformatics, and biochemical analysis.

Methods

Mice

All the animal studies were carried out in the Charlie Norwood Veterans Affairs Medical Center, Augusta and approved by the Institutional Animal Care and Use Committee (Animal Component of Research Protocol # 1604232 approved on 01/28/2023). Seven-week-old male C57BL/6J mice with body weights around 23g were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and maintained for one week before STZ treatment. For the animal studies, all mice were allocated to experimental groups without randomization. Each set of experiments was conducted simultaneously to minimize variability.

STZ-induced DM in mice

STZ-induced type 1 DM, a well-established DM model, was utilized in our study [16]. Male C57BL/6 mice were subjected to intraperitoneal (I.P) STZ injections of 50 mg/kg dissolved in citrate buffer once a day [17] for five consecutive days, with control groups receiving only citrate buffer injections. Blood samples were extracted via the tail vein to measure blood glucose levels at least twice a month using a glucometer (Alpha TRAK2 blood glucose monitoring system, Fisher Scientific, Pittsburgh, PA, USA). Mice whose blood glucose levels were above 350 mg/dL were considered DM. Mouse body weights were frequently recorded during the study period. Mice were then euthanized after 12 weeks of being DM, and lungs were isolated for analysis. To assess the impact of pre-existing DM on the severity of ALI, we utilized a clinically relevant model of sepsis-induced ALI. For this experiment, 12-week DM mice were intraperitoneally (I.P) challenged with LPS at a dose of 5 mg/kg, and non-DM mice who served as positive controls received LPS-only [17]. After 24 hours of LPS treatment, mice were sacrificed, and lungs were extracted for examination. The final group allocation of our experiment was as follows: (1) Control, (2) DM, (3) LPS-treated controls (LPS), and (4) LPS-treated DM mice (DM + LPS). A flow chart of the experimental protocol is illustrated in **Figure 1a.**

Lung Wet/Dry weight ratio analysis

To measure lung edema, lungs from all the experimental mice were retrieved, and the upper right lobes were freshly weighed before incubating them in an oven for 72 hours at 80°C. Following that, dry weights were measured, and final wet/dry ratios were calculated to estimate the water content in the lungs.

Histopathological analysis and lung injury scoring

To examine and compare the pathological changes associated with DM or DM with sepsis (DM + LPS) mice compared to their respective controls, lungs were collected, immediately fixed in 4% paraformaldehyde, and sectioned before being stained with hematoxylin and eosin (H&E). Following that, lung tissues were assessed using microscopy, and lung injury was blindly scored based on already developed criteria [18]. Briefly, a scale of 1-5 was utilized to score all the samples, with 1 being normal to minimal damage lung, whereas 5 indicating critical lung injury evidenced by diffuse (occurs in more than 50 % of lung section) consolidation concurrent with inflammatory cell infiltration.

qRT-PCR analysis of inflammatory cytokines in lung tissues

Lung RNA analysis was performed as mentioned previously [18]. Briefly, RNA was isolated from mice lung tissues utilizing a miRNeasy mini kit (Qiagen). The quality of the RNA was checked via a Nanodrop 2000 spectrophotometer (Thermo Scientific). Around 1000 ng of the total RNA was used for Complementary DNA (cDNA) synthesis employing a High-Capacity cDNA Reverse Transcript Kit (Applied Biosystems, Waltham, MA, USA). Quantitative real-time PCR (qRT-PCR) was conducted using Power SYBR Green Master Mix (Applied Biosystems) in StepOnePlus™ Real-Time PCR System (Applied Biosystems). All mouse primers included in the analysis are listed in Supplemental Table 1.

Supplemental Table 1. List of primes utilized in the analysis.

Gene name	Forward primer	Reverse primer
IL1β	CCAAGCAACGACAAAATACC	GTTGAAGACAAACCGTTTTTCC
IL10	GCTCTTACTGACTGGCATGAG	CGCAGCTCTAGGAGCATGTG
IL6	AGACAAAGCCAGAGTCCTTCAG	TGCCGAGTAGATCTCAAAGTGA
IL4	GGTCTCAACCCCCAGCTAGT	GCCGATGATCTCTCTCAAGTGAT
GAPDH	TGGTGAAGGTCGGTGTGAAC	CCATGTAGTTGAGGTCAATGAAGG
TNFα	GGTCCCCAAAGGGATGAGAA	TGAGGGTCTGGGCCATAGAA
CXCL-1	CTGGGATTCACCTCAAGAACATC	CAGGGTCAAGGCAAGCCTC

RNA-Sequencing and bioinformatics analysis

Lung tissues from DM and non-DM (control) mice were taken out, RNA was isolated, and then quality control tests were performed to ensure these samples were pure and integrated using NanoDrop 2000 (Thermo Scientific) and the Agilent 2100 bioanalyzer, respectively. Total RNAs that passed the quality control test were further processed for cDNA library preparation employing Illumina NovaSeq 6000 and X-Plus Sequencing Platform with PE150 strategy, followed by bioinformatics analysis. All differentially expressed genes (DEG) among DM vs. non-DM arms were subjected to comprehensive gene set enrichment analysis (GSEA) and gene ontology (GO). Several databases for pathway enrichment analysis, such as KEGG and Reactome, were included in our analysis to detect signaling pathways related to DEGs. R program and SRplot were used to carry out gene distribution analysis, incorporating PCA, volcano plot, and heatmaps[19]. A Venn diagram was created to show genes that were uniquely expressed in each group, along with upregulated and down-regulated genes in the DM group. While the PCA was used to demonstrate that the DM group did not overlap with the control group, volcano plots, and heatmaps were performed to reveal the top DEGs in DM lung tissues. The violin plots were created using GraphPad software. The interactions between DEGs were assessed utilizing STRING [20].

Statistical analysis

All the data are depicted as means ± SEM, and the 'n' value for each dataset indicates the sample size used for that experiment. All the data were assessed by parametric testing using Student's unpaired t-test or one-way ANOVA. In bioinformatic analysis, DEGs with fold change values > 0.5 or <0.5 are deemed to be upregulated and downregulated, respectively. In all the analyses, a *p*-value < 0.05 is considered statistically significant.

Results

STZ-induced diabetes impaired lung health and worsened LPS-induced lung injury in mice. To confirm the successful induction and maintenance of diabetes by STZ throughout the experiment, blood glucose levels were measured at random intervals. As expected, DM mice showed a significant increase in blood glucose levels, ranging from 300 to 600 mg/dL **(Fig. 1b)**. Additionally, body weights were regularly monitored, revealing a modest but significant weight reduction in DM mice compared to control groups **(Fig. 1c)**.

To assess the impact of DM on the lungs and its influence as a comorbid condition in sepsis-induced ALI, we performed histopathological examinations on lung sections from all experimental groups. Our analysis revealed a significant increase in lung injury scores in DM mice compared to non-DM mice **(Figs. 1d-e)**. Histological evaluation showed inflammatory cell infiltration and mild interstitial congestion in the lungs of DM mice **(Figs. 1d-e)**. When assessing the effect of pre-existing DM in septic mice, we observed that lung tissues from DM mice with sepsis exhibited increased inflammatory cell infiltration, thickened alveolar walls, and more pronounced interstitial congestion and edema compared to non-DM sepsis or DM-only mice (DM + LPS vs. LPS or DM). Although lung injury scores were elevated, the differences did not reach statistical significance **(Figs. 1d-e)**. Our H&E staining data revealed substantial histopathological changes in DM lung tissues. Moreover, these alterations were worse in mice with both DM and sepsis, highlighting that DM compromises lung health and structure, rendering the lungs more susceptible to ALI.

Since pulmonary edema is a key indicator of ALI, we assessed lung water content using the wet-to-dry (W/D) ratio to further evaluate the impact of DM alone and as a comorbid condition in sepsis-induced ALI. Our results showed a significantly higher water content in DM mouse lungs compared to non-DM lungs, suggesting that DM increases capillary permeability, facilitating fluid leakage into the interstitial and air spaces **(Fig. 1f)**. As expected, sepsis mice (LPS) exhibited significantly increased pulmonary edema compared to controls **(Fig. 1f)**. When evaluating the extent of edema in septic mice with pre-existing DM compared to those with only DM or sepsis, we observed a further increase in lung fluid content. However, these differences did not reach statistical significance **(Fig. 1f)**.

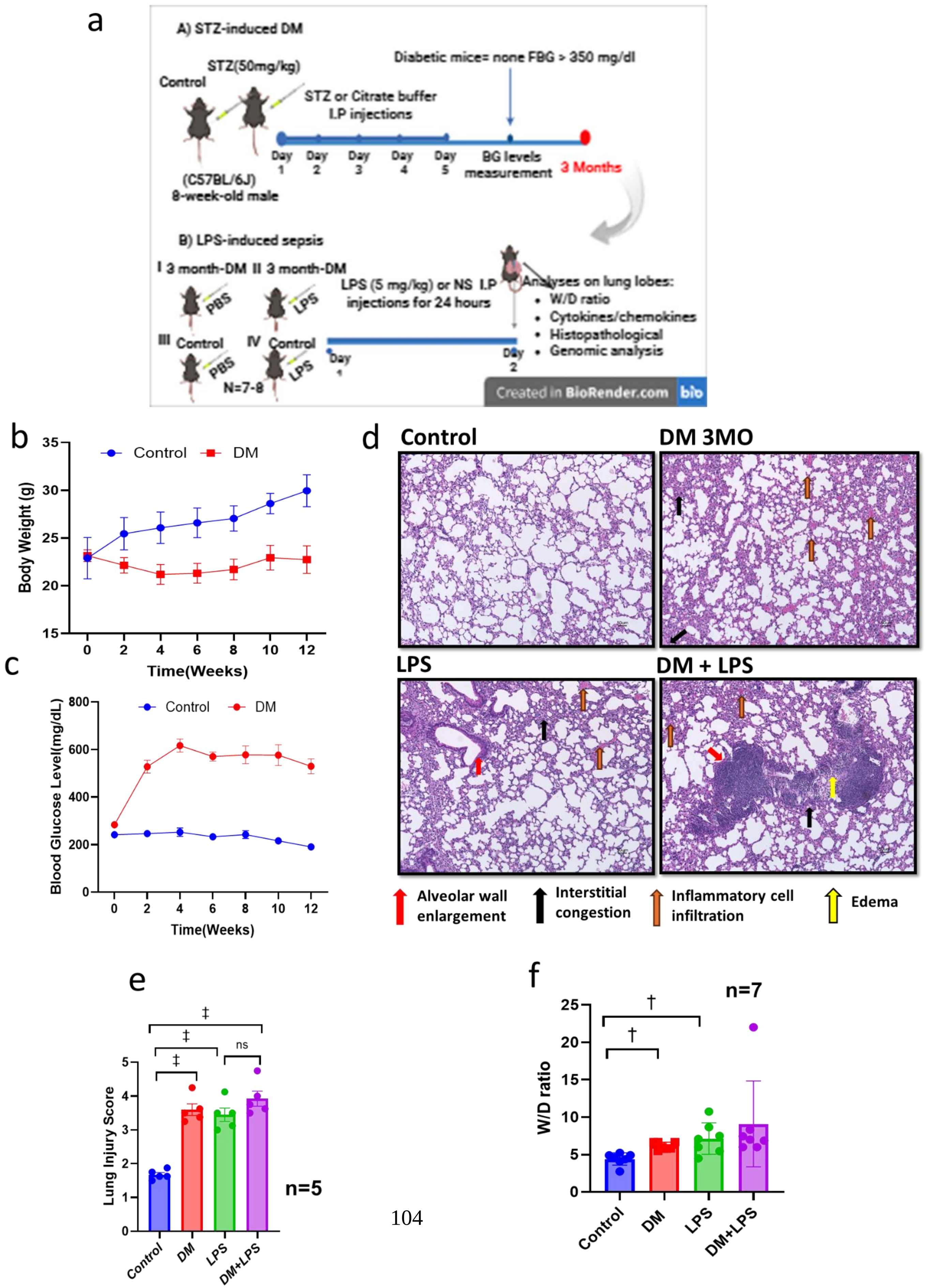
a
A) STZ-induced DM
STZ(50mg/kg)
Control
Diabetic mice= none FBG > 350 mg/dl
STZ or Citrate buffer
I.P injections
Day 1
Day 2
Day 3
Day 4
Day 5
BG levels measurement
3 Months
(C57BL/6J)
8-week-old male
B) LPS-induced sepsis
I 3 month-DM
II 3 month-DM
PBS
LPS
LPS (5 mg/kg) or NS I.P
injections for 24 hours
Analyses on lung lobes:
• W/D ratio
• Cytokines/chemokines
• Histopathological
• Genomic analysis
III Control
IV Control
Day 1
Day 2
N=7-8
Created in BioRender.com
b
Control
DM
Body Weight (g)
Time(Weeks)
c
Blood Glucose Level(mg/dL)
Time(Weeks)
d
Control
DM 3MO
LPS
DM + LPS
Alveolar wall enlargement
Interstitial congestion
Inflammatory cell infiltration
Edema
e
Lung Injury Score
n=5
ns
Control
DM
LPS
DM+LPS
f
W/D ratio
n=7
Control
DM
LPS
DM+LPS

Fig. 1 STZ-induced diabetes compromises lung health and exacerbates lps-induced lung injury in mice. **(a)** A flow chart of the experimental protocol. **(b)** Blood glucose levels (once every two weeks) in STZ-induced DM mice compared to non-DM mice (n=7). **(c)** Body weight measurements (once every two weeks) in STZ-induced DM mice compared to non-DM mice (n=7). **(d** Representative images of H&E staining demonstrating pathological alterations in LPS-treated, 3 month DM, and 3 month DM mice treated with LPS, compared to control (vehicle-treated) non-DM mice (n=7). **(e)** Bar graph showing quantification (blinded analysis) of lung injury scores LPS-treated, 3 month DM, and 3 month DM mice treated with LPS, compared to control (vehicle-treated) non-DM mice (n=5). **(f)** Bar graph showing wet/dry ratio of lung tissues in LPS-treated, 3 month DM, 3 month and DM mice treated with LPS, compared to control (vehicle-treated) non-DM mice (n=7). †$p<0.01$, ‡$p<0.001$

RNA sequencing analysis of DM lungs shows activation of pathways involved in inflammation and vascular injury

Given the intriguing observations in diabetic lungs, we conducted RNA-seq analysis to explore the transcriptional effects of DM on lung tissue compared to non-DM mouse lungs. The principal component analysis (PCA) revealed a clear segregation between DM and non-DM lung samples, suggesting that DM is associated with a distinct gene expression signature **(Fig. 2a)**. Furthermore, the transcriptomic analysis identified 14,141 genes commonly expressed in both DM and non-DM lungs, with 501 genes exclusively detected in diabetic lungs **(Figs. 2b-c)**. Differential gene expression analysis revealed significant alterations, with 203 genes upregulated and 671 genes downregulated in diabetic lungs compared to controls, as illustrated in the volcano plot **(Fig. 2b)**.

To further highlight the most differentially expressed genes (DEGs) in diabetic lungs, we generated a heatmap displaying the top upregulated and downregulated genes **(Fig. 2d)**.

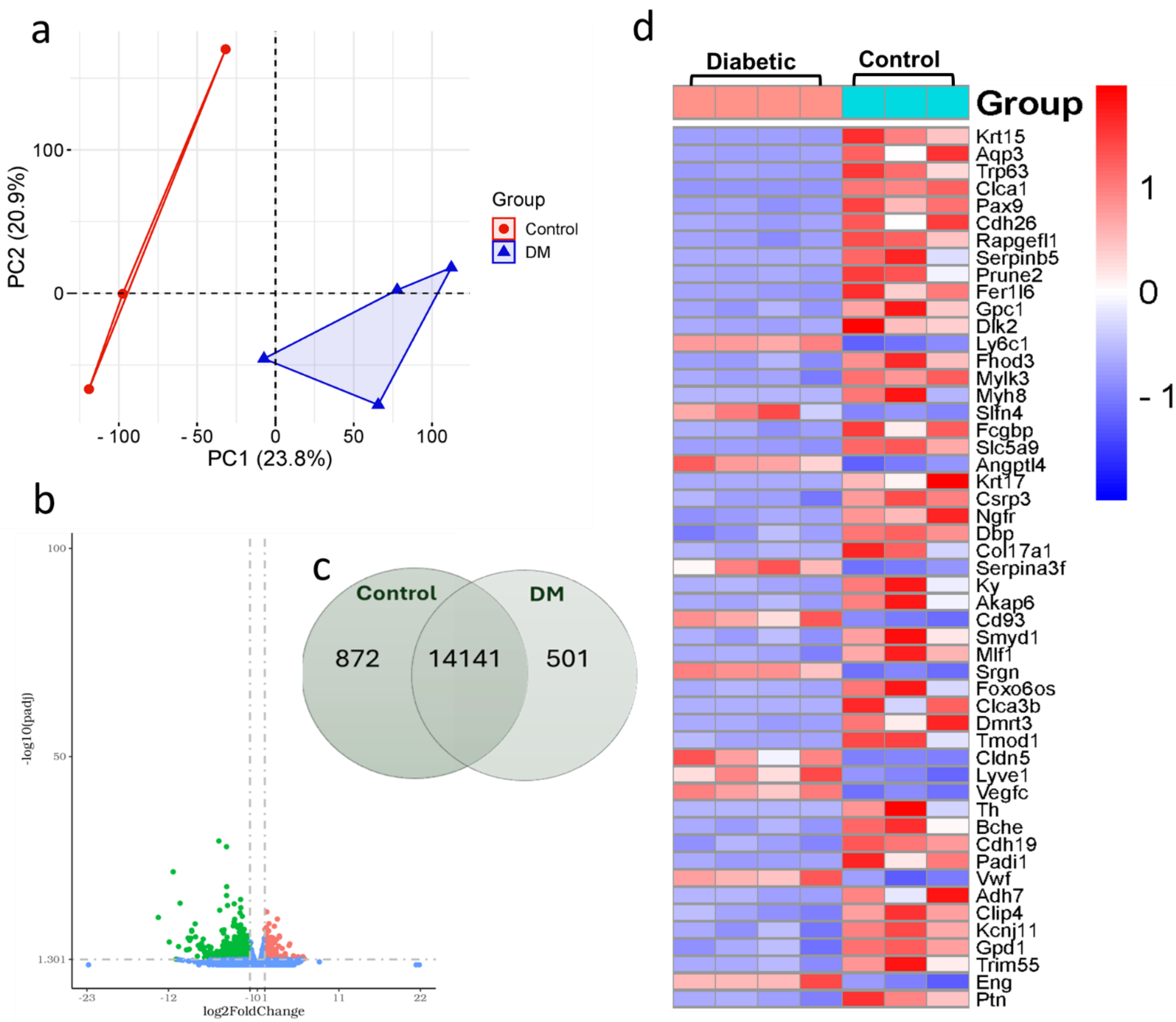

Fig. 2 RNA sequencing analysis showing differential gene expression in DM mouse lungs compared to non-DM mouse lungs. **(a)** Principal component analysis (PCA) reveals a significant separation of gene expressions between DM and non-DM lung samples. **(b)** The volcano plot shows DEGs in DM compared to non-DM mouse lung tissues. **(c)** Venn diagram showing the

number of identified genes between the two groups. **(d)** The heatmap displays the top DEGs in the DM and non-DM lung tissues (n=3-4).

Following our initial genomic profiling, we conducted a detailed analysis of differentially expressed genes (DEGs) in DM lungs compared to non-DM lungs. Our findings revealed a significant upregulation of genes involved in inflammatory responses **(Fig. 3a)**. Notably, Ly6c1, predominantly expressed in the lung, was significantly elevated in DM lung samples. Additionally, Slfn4, a vascular inflammatory modulator, and Angptl4, associated with chronic low-grade inflammation, were markedly increased, indicating increased immune activation. In DM lungs, genes regulating endothelial and epithelial barrier integrity, such as Cldn5 and VE-Cadherin, were upregulated, suggesting DM-induced vascular dysfunction. Conversely, several genes showed reduced expression in the DM group compared to controls **(Fig. 3b)**. For instance, Aqp3, which regulates water movement and maintains fluid homeostasis in the lungs, was significantly downregulated in diabetic lung tissues. Moreover, our RNA-seq data revealed alterations in several pro- and anti-inflammatory genes, including TNF-α, IL-1β, IL-6, CXCL-1, IL-10, and IL-4, as depicted in donut heatmaps and violin plots **(Figs. 3c, e-f)**. Additionally, the receptor for advanced glycation end products (AGER), a key contributor to DM-related complications, was significantly increased in DM lungs **(Figs. 3c-d)**. Collectively, these findings provide strong evidence of inflammation and edema in lungs affected by DM.

a

Top 10 Upregulated genes in diabetic lung vs normal sample

#	Gene ID	Gene name	Symbol	p value
1	ENSMUSG00000079018	lymphocyte antigen 6 complex	Ly6c1	1.04E-16
2	ENSMUSG00000000204	schlafen 4	Slfn4	7.16E-15
3	ENSMUSG00000002289	angiopoietin-like 4	Angptl4	1.06E-13
4	ENSMUSG00000066363	serine peptidase inhibitor	Serpina3f	6.09E-13
5	ENSMUSG00000027435	CD93 antigen	Cd93	1.31E-12
6	ENSMUSG00000020077	serglycin	Srgn	4.22E-12
7	ENSMUSG00000041378	claudin 5	Cldn5	1.12E-11
8	ENSMUSG00000030787	lymphatic vessel endothelial hyaluronan receptor 1	Lyve1	1.13E-11
9	ENSMUSG00000031520	vascular endothelial growth factor C	Vegfc	1.22E-11
10	ENSMUSG00000001930	Von Willebrand factor	Vwf	9.03E-11

b

Top 10 Downregulated genes in diabetic lung vs normal sample

#	Gene ID	Gene name	Symbol	p value
1	ENSMUSG00000054146	keratin 15	Krt15	1.98E-118
2	ENSMUSG00000028435	aquaporin 3	Aqp3	1.53E-34
3	ENSMUSG00000022510	transformation related protein 63	Trp63	5.40E-33
4	ENSMUSG00000028255	chloride channel accessory 1	Clca1	7.61E-27
5	ENSMUSG00000001497	paired box 9	Pax9	3.55E-23
6	ENSMUSG00000039155	cadherin-like 26	Cdh26	5.67E-21
7	ENSMUSG00000038020	Rap guanine nucleotide exchange factor (GEF)-like 1	Rapgefl1	5.71E-20
8	ENSMUSG00000067006	serine (or cysteine) peptidase inhibitor, clade B, member 5	Serpinb5	5.39E-19
9	ENSMUSG00000039126	prune homolog 2	Prune2	8.34E-19
10	ENSMUSG00000037106	fer-1-like 6	Fer1l6	2.32E-18

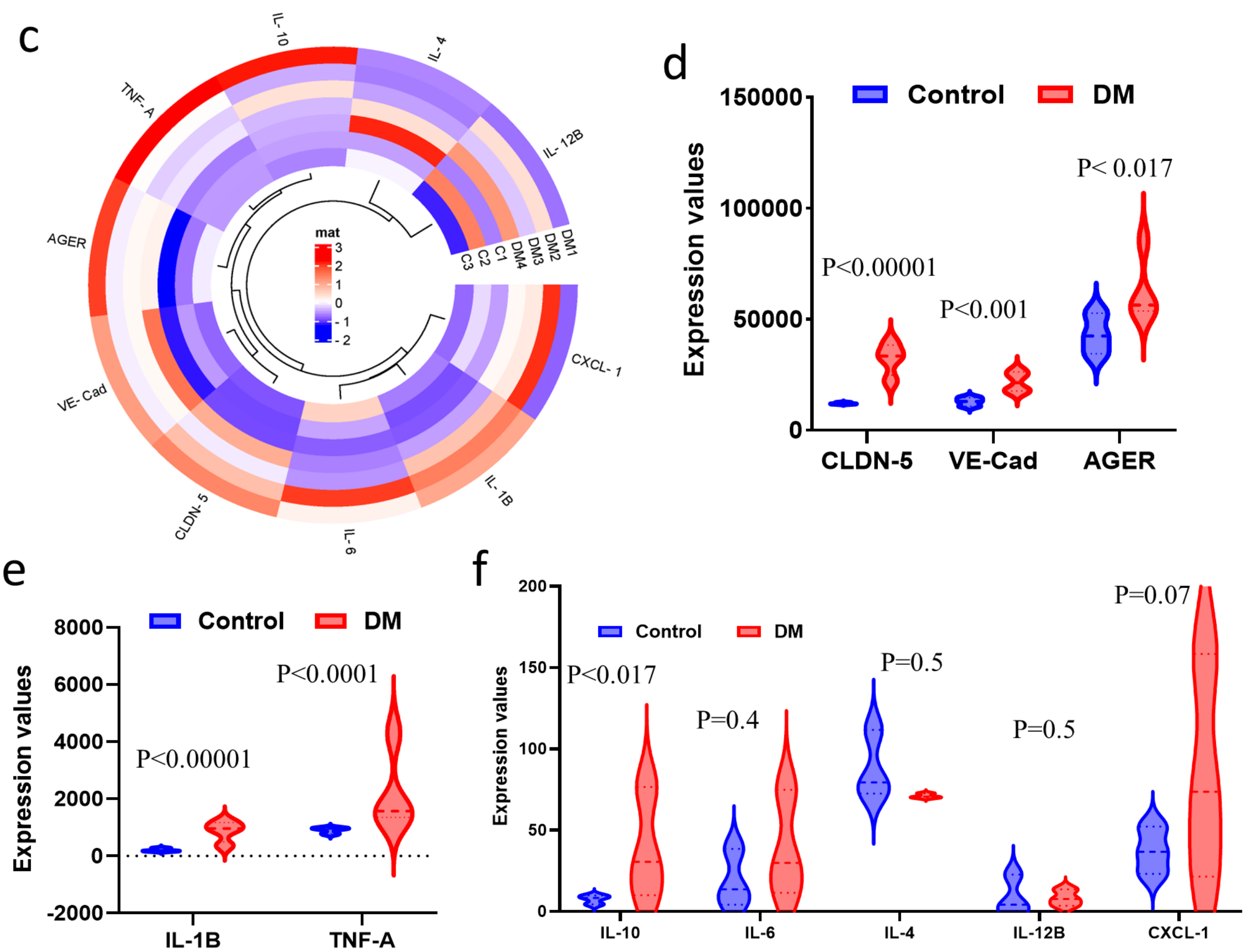

Fig. 3 The top DEGs in DM mouse lungs suggests inflammation and endothelial cell abnormalities. **(a-b)** List of the top upregulated and downregulated genes in DM lung tissues compared to non-DM lung tissues, respectively. **(c)** Donut heatmap representing the expression of the top inflammatory and endothelial genes in DM lungs compared to non-DM lungs. **(d-f)** Violin plots demonstrating expression changes in Cldn5, VE-Cadherin, AGER, IL1β, TNF-α, IL-10, IL-6, IL-4, IL-12B, and Cxcl-1 in DM compared to non-DM lungs (n=3-4). $^*p<0.05$, $^\dagger p<0.01$, $^\ddagger p<0.001$.

GO, KEGG and Reactome analysis reveal activation of metabolic and inflammatory pathways in DM lungs

To identify deregulated signaling pathways associated with DEGs in DM mouse lungs, we conducted a comprehensive pathway analysis using multiple databases, including KEGG and Reactome. KEGG analysis revealed significant alterations in metabolic pathways, such as beta-alanine metabolism, Apelin signaling, and insulin signaling, including its resistance mechanisms. Additionally, pro-inflammatory pathways, such as adipocytokine signaling and immune responses, including *Staphylococcus aureus* infection, were significantly involved in DM lungs **(Fig. 4a)**. Consistent with KEGG findings, Reactome pathway analysis further confirmed the involvement of DEGs in metabolic and inflammatory pathways. Specifically, dysregulated metabolic pathways were linked to ion homeostasis, glucose metabolism, and gluconeogenesis, while immune-related pathways, such as neutrophil degranulation and antimicrobial peptide activity, were significantly affected in DM lungs. Notably, DEGs in DM lungs also contributed to the disruption of gap junction assembly **(Fig. 4b)**. Collectively, these findings indicate that DM lungs undergo significant metabolic disturbances, immune activation, and gap junction dysregulation. Given these observations, a more detailed analysis of these altered pathways and their associated DEGs is essential to uncover potential therapeutic strategies for mitigating DM-induced lung injury.

Next, we performed GO and Gene Set Enrichment Analysis (GSEA). GO analysis revealed that the top molecular functions (MFs) of altered genes were associated with signaling receptor regulation, peptidase inhibitor/regulator activity, ion channel regulation, gap junction channel function, and cytokine activity, as shown in **(Fig. 4c)**. Cellular component (CC) analysis identified key structural and functional elements, including contractile fibers, myofibers, cell-cell contact zones, glycoprotein complexes, and adherens junctions **(Fig. 4d)**. Meanwhile, enriched biological processes (BPs) of DEGs were linked to myofiber assembly, antimicrobial humoral responses, glycogen metabolism, and leukocyte chemotaxis/migration **(Fig. 4e)**. A comprehensive list of CCs and BPs is presented in **Figs. 4d-e**. Additionally, we explored interactions among DEGs, revealing multiple gene-gene interactions in DM lungs **(Fig. 5)**.

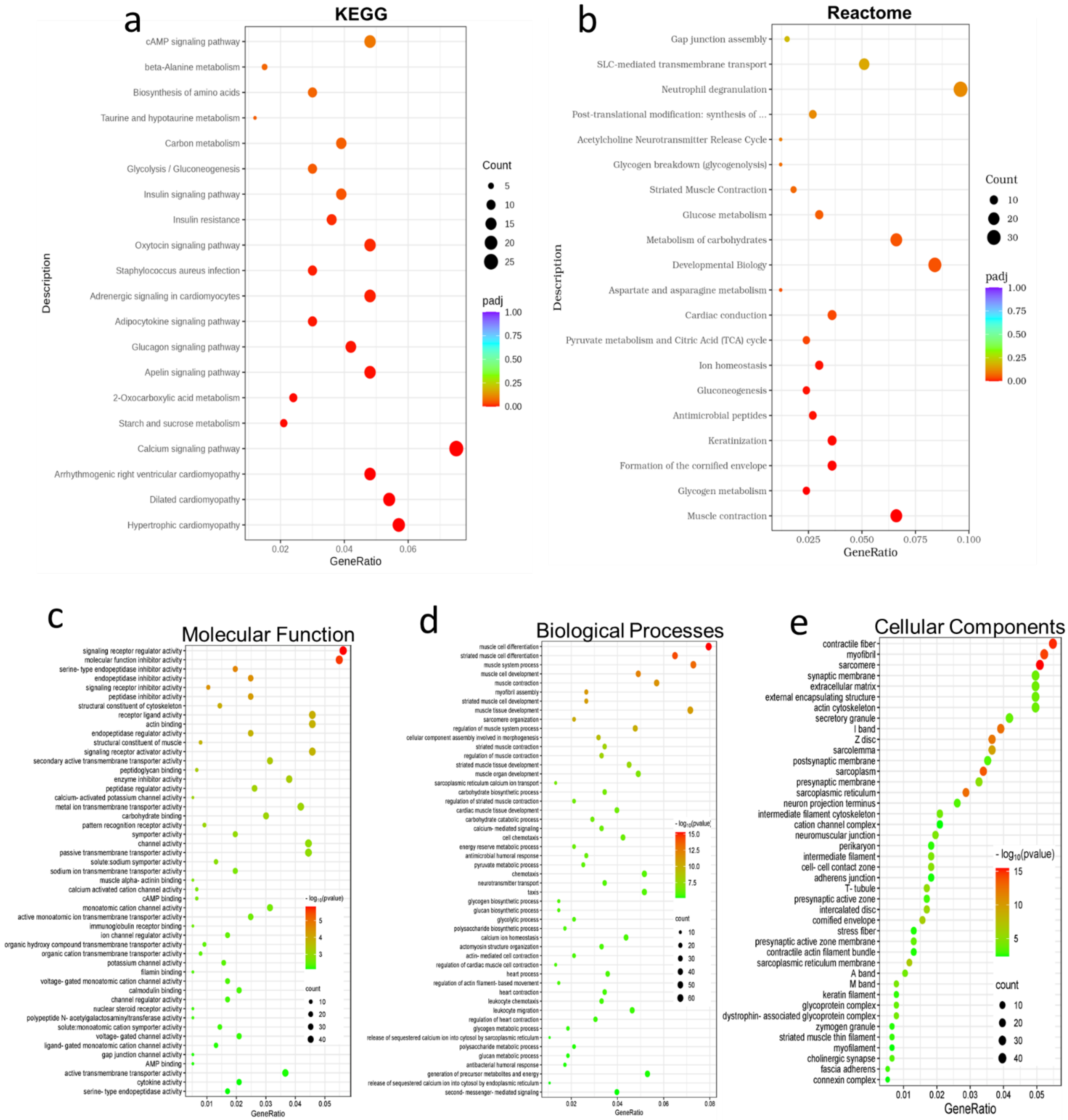

Fig. 4 Gene enrichment analysis show activation of pathways regulating inflammation, metabolism, and vascular abnormalities in DM compared to non-DM lungs. (a-b) KEGG and Reactome pathway analysis plots demonstrating top altered molecular pathways in the DM lungs.

(c-e) GO analysis shows DEGs involved in molecular function, biological processes, and cellular components, respectively.

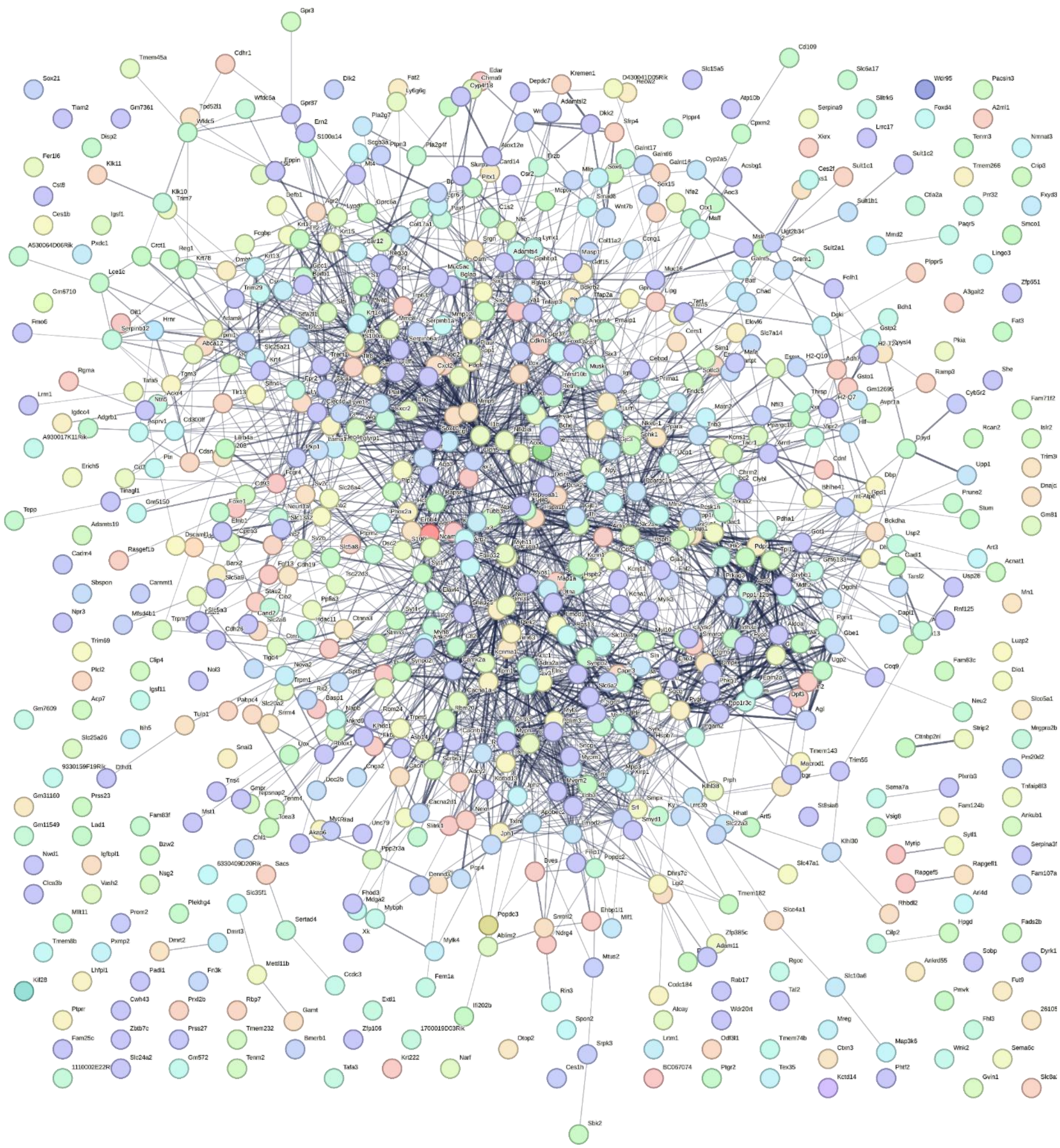

Fig. 5 STRING network analysis illustrating the interaction among all the identified DEGs in DM lungs.

GSEA revealed the enrichment of several inflammatory-related signaling pathways, including chemokine signaling, TNF-α, PI3K-AKT, and AGE-RAGE pathways, in the lungs of DM mice compared to non-DM mice **(Figs. a-d)**. Interestingly, genes involved in the development of endothelial barriers, adherens junctions, and vasculature were notably modulated in DM lungs **(Figs. 6e-f)**. Overall, these findings highlight an overactive immune response and disrupted metabolic processes in diabetic lung tissue, both of which could contribute to the development of vascular dysfunction.

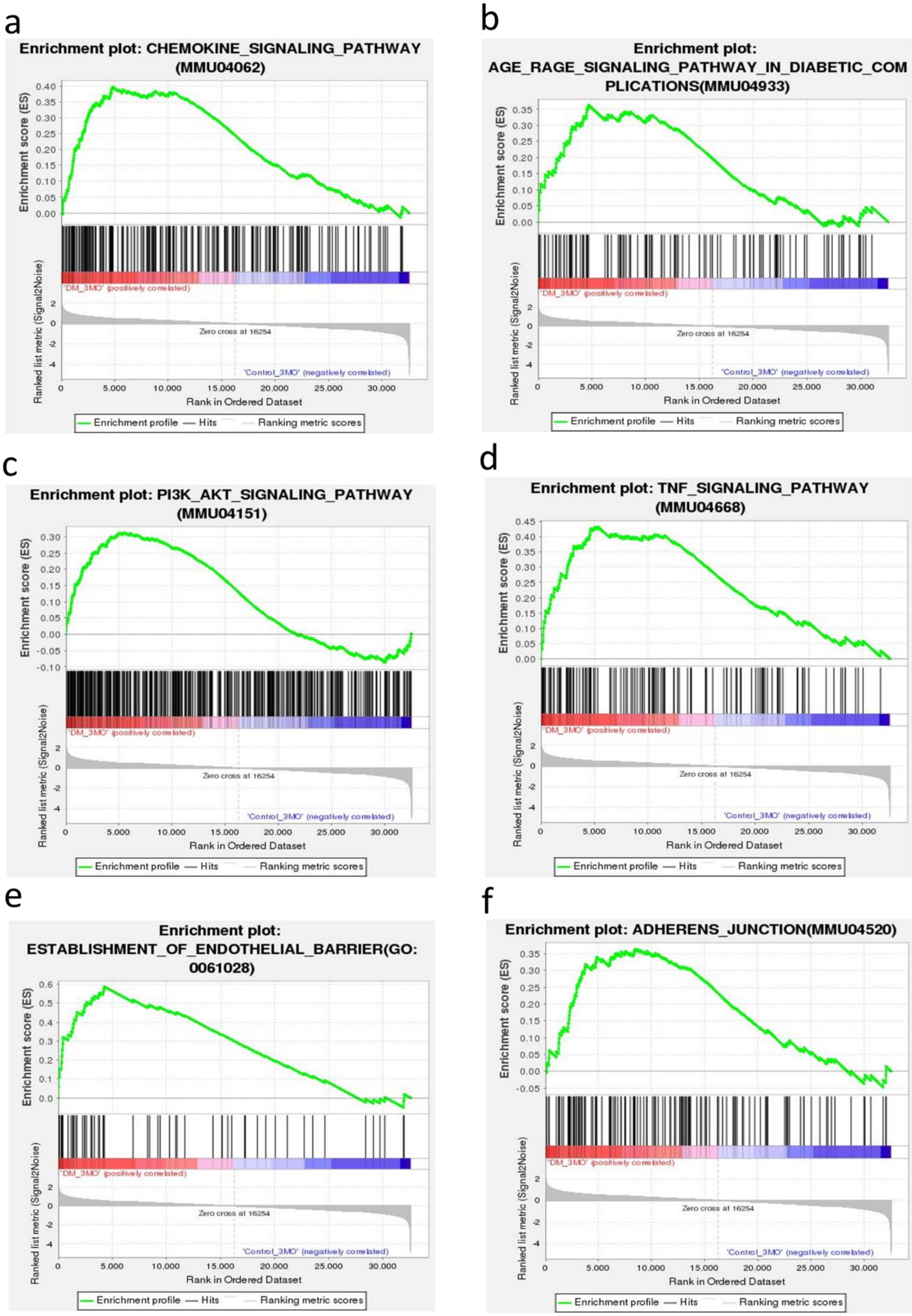
a
Enrichment plot: CHEMOKINE_SIGNALING_PATHWAY (MMU04062)
b
Enrichment plot: AGE_RAGE_SIGNALING_PATHWAY_IN_DIABETIC_COMPLICATIONS(MMU04933)
c
Enrichment plot: PI3K_AKT_SIGNALING_PATHWAY (MMU04151)
d
Enrichment plot: TNF_SIGNALING_PATHWAY (MMU04668)
e
Enrichment plot: ESTABLISHMENT_OF_ENDOTHELIAL_BARRIER(GO:0061028)
f
Enrichment plot: ADHERENS_JUNCTION(MMU04520)
Enrichment score (ES)
Ranked list metric (Signal2Noise)
'DM_3MO' (positively correlated)
Zero cross at 16254
'Control_3MO' (negatively correlated)
Rank in Ordered Dataset
Enrichment profile
Hits
Ranking metric scores

Fig. 6 Gene set enrichment analysis (GSEA) of DEGs in the tissue of DM lungs. **(a-f)** Enrichment plots profiling the positive correlation between altered genes and the molecular pathways in DM lungs including Chemokine signaling **(a)**, AGER signaling **(b)**, PI3 Kinase-Akt signaling **(c)**, TNF-α signaling **(d)**, endothelial-barrier regulation **(e)**, and Adherens junction regulation **(f)**.

DM mouse lungs generated increased inflammatory cytokines, worsened by sepsis.

Given the significant pathological changes in the lungs of DM mice and the exacerbation of ALI by pre-existing DM in septic mice, we aimed to assess the degree of inflammation in lung homogenates across experimental groups. Using a panel of inflammatory cytokines and chemokines, including TNF-α, IL-1β, IL-6, IL-10, IL-4, IL-12B, MCP-1, and CXCL-1, we first compared the inflammatory levels between DM and non-DM mouse lungs. We found that DM mouse lungs had significantly higher levels of pro-inflammatory markers, such as TNF-α, IL-1β, MCP-1, and CXCL-1, compared to non-DM lungs. Conversely, anti-inflammatory cytokines like IL-4 were significantly downregulated in DM lungs **(Figs. 7a-h)**. These results strongly suggest that the lung environment is inflamed and adversely affected by DM. Next, we evaluated whether pre-existing DM increased the risk or severity of ALI in sepsis mice. While not reaching statistical significance, our data indicated that DM with sepsis exhibited amplified inflammatory responses, with higher expressions of pro-inflammatory markers, such as IL-12B, CXCL-1, and TNF-α, compared to sepsis mice without DM **(Figs. 7a-h)**. Interestingly, IL-1β was significantly higher in the LPS-only group compared to the DM + LPS group **(Fig. 7d)**. Anti-inflammatory cytokines, such as IL-4 and IL-10, showed a strong trend toward downregulation in DM + LPS mice compared to the LPS-only group **(Figs. 7e-g)**. Lastly, when comparing DM mice with and without sepsis (DM + LPS vs. DM only), we observed that most pro-inflammatory markers, including

TNF-α, IL-1β, MCP-1, and IL-6, were significantly elevated in the presence of both DM and sepsis. While IL-12B and CXCL-1 levels were higher in sepsis DM mice compared to DM-only mice, these differences were not statistically significant. Additionally, anti-inflammatory cytokines, IL-4 and IL-10, tended to be downregulated in DM + LPS mice compared to DM-only mice **(Figs. 7a-h)**. Overall, our findings suggest that DM acts as a risk factor that exacerbates inflammation in the sepsis mouse lungs, worsening the severity of ALI compared to non-DM mice.

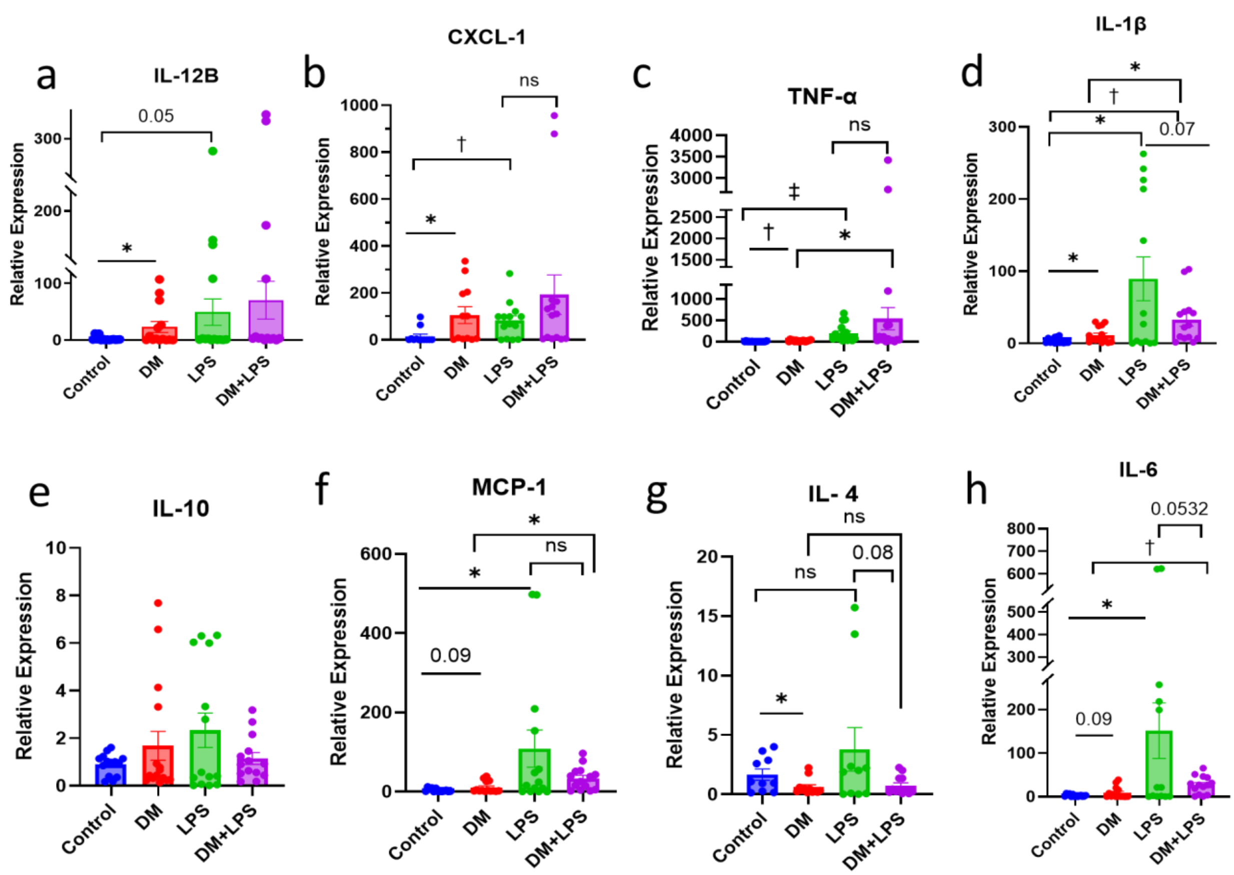

Fig. 7 Analysis of lung homogenates showing increased expression of cytokines in DM mouse lungs. (a-h) Bar graphs showing changes in mRNA levels of IL-12B **(a)**, CXCL-1 **(b)**, TNF-α **(c)**, IL-1β **(d)**, IL-10 **(e)**, MCP-1 **(f)**, IL-4 **(g)**, and IL-6 **(h)** in lung tissues with DM copared to non-

DM lungs, and LPS-treated DM lungs compared to vehicle-treated DM mice (n=7). Data are shown as mean ± SEM. *p<0.05, †p<0.01, ‡p<0.001.

Discussion

DM, characterized by persistent hyperglycemia and inflammation, is a widespread and complex chronic disease that significantly affects both individuals and economies worldwide [21]. While vascular complications of DM are well-documented, pulmonary complications remain inadequately studied, with inconsistent findings [22, 23]. Acute respiratory distress syndrome (ARDS), a severe and prevalent complication, often leads to respiratory failure in ICU patients [24]. Given that nearly half of ICU patients have DM as a comorbid condition, it is crucial to investigate how DM influences respiratory disorders, particularly diseases with high mortality rates, such as ARDS, which carries a mortality rate of around 40% [24]. Our recent clinical study suggested a positive relationship between DM and ARDS [15], but literature on lung inflammation and the effect of comorbid DM on lung disease remains limited. Therefore, our study aimed to explore how pre-existing DM affects lung health and exacerbates sepsis-induced ALI. Our results demonstrate significant alterations in inflammatory cytokine production, injury and edema in DM mouse lungs compared to controls associated with deregulated metabolic, inflammatory, and vascular pathways.

A recent study reported significant immune cell infiltration in the lungs of 33-week-old type 2 DM mice, with an increase in polymorphonuclear-myeloid-derived suppressor cells (PMN-MDSCs) [25]. In contrast, DM rat serum analysis showed reduced neutrophil migration, indicating compromised immune function [26]. Our findings in type 1, STZ-induced DM mice after three months align with these results. Despite differences in experimental models, our data support the role of inflammation in DM lungs. Consistent with another report [27], we observed significant upregulation of IL-6 expression in DM lungs, suggesting that systemic inflammation in DM extends to the lungs.

The relationship between DM and ALI is characterized by diverse and inconsistent findings [22, 23]. While some studies suggest that DM may reduce the risk of ARDS progression without affecting mortality, others report that DM significantly increases the risk of ARDS compared to non-DM individuals [23]. A large cohort analysis supports the hypothesis that DM elevates the likelihood of ARDS development [28]. These discrepancies might arise from research designs that were not fully controlled or may have limitations. Our recent secondary analysis of the Fluid and Catheter Treatment Trial (FACTT) also found a significant association between pre-existing DM and poor outcomes in ARDS patients [15]. In preclinical studies, hyperglycemia caused significant lung tissue damage in an LPS-induced sepsis rat model [29]. Similarly, studies on both type 1 (STZ, 4 weeks) [30] and type 2 DM [31] mice infected with SARS-CoV-2 found higher inflammation and pulmonary injury compared to non-DM mice. Alingning with these observations, our RNA sequencing and GSEA analyses identified deregulated TNF-α, PI3K-AKT, AGER, and endothelial barrier signaling pathways, and revealed disruptions in metabolic, inflammatory, and vascular pathways. These findings are consistent with a previous study showing that immune system impairment in DM mice increases their susceptibility to sepsis [32]. Septic DM mice had elevated levels of pro-inflammatory markers, including IL-12B, CXCL-1, and TNF-α, and reduced IL-4 levels compared to mice with sepsis or DM alone. Other upregulated genes include Ly6c1, which regulates inflammation and complement activity, and Slfn4, implicated in systemic inflammation and atherosclerosis [33, 34]. Angptl4, a multifunctional cytokine involved in inflammation and endothelial injury [35], with higher serum levels linked to reduced lung function and systemic inflammation in COPD patients [36], and correlated with disease severity and mortality in ARDS patients [37-39], was highly upregulated in DM lungs, highlighting its role in lung inflammation. Additionally, upregulation of Serpina family genes is associated with lung disorders like pulmonary fibrosis and COPD [40-42]. These results indicate increased vulnerability of the DM lungs to ALI.

Pathological assessments of DM and septic DM mouse lungs suggest potential dysfunction in the pulmonary vascular endothelium. Genes CD93 and claudin-5, both primarily expressed in endothelial cells, were among the top upregulated genes, suggesting their role in modulating vascular permeability in DM lungs. Increased Cldn5 expression has been shown to impair vascular structure, leading to lung damage due to enhanced permeability and edema [43, 44]. Elevated Cldn5 expression in lung epithelium is associated with increased permeability, promoting edema and contributing to lung damage [45-47]. Additionally, we previously published that upregulation of Cldn5 is associated with DM retinopathy, a disease marked by retinal vasculature impairment and inflammation [48, 49]. In contrast, genes like Krt15 and Aqp3 were significantly downregulated in DM lungs. Krt15 knockout mice exhibited severe lung injury and inflammation in a COPD model [50], and the reduced expression of Krt15 in DM lungs may contribute to increased lung vulnerability. Similarly, Aqp3, essential for respiratory function, has been shown that its deficiency negatively impacts lung health [51]. These dysregulated genes could serve as potential prognostic markers or novel therapeutic targets for mitigating DM-related lung injury.

One limitation of this study is the use of the STZ-induced type 1 DM mouse model, which may not fully replicate the complexities of human DM, particularly in terms of long-term disease progression and comorbidities. Additionally, while significant inflammation and injury were observed in DM lungs, the temporal dynamics of these changes and the potential reversibility of lung damage with therapeutic interventions were not explored. RNA sequencing, while useful, may overlook some gene interactions or regulatory mechanisms not captured by mRNA expression alone. The reliance on a sepsis model to induce ALI also limits the generalizability of results, as it may not fully represent other types of lung injury seen in DM patients, such as those caused by infections or environmental factors. Furthermore, the influence of other comorbidities commonly associated with DM, like cardiovascular disease, was not investigated, which could be relevant in understanding the broader impact of DM on lung health. Despite these limitations, our study underscores that DM worsens lung health, with pre-existing DM exacerbating sepsis-induced ALI through inflammation and endothelial dysfunction. Key genes involved in inflammatory, metabolic, and vascular pathways increase the susceptibility of DM lungs to injury, highlighting the need for further research into the mechanisms linking DM to lung complications and potential therapeutic targets.

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CHAPTER 5

Overall summary and conclusions

Diabetes mellitus (DM) is a chronic and debilitating condition characterized by elevated blood glucose levels and systemic inflammation [1]. The prevalence of DM is rising rapidly, with projections estimating around 783 million cases by 2045, posing a substantial burden on both individuals and economies [2, 3]. In intensive care units (ICUs), DM is frequently diagnosed as a comorbidity in patients with severe illnesses, including acute respiratory distress syndrome (ARDS), a leading cause of respiratory failure in ICUs [4]. The relationship between ARDS and DM remains controversial, with conflicting reports. Some studies suggest that DM may have a protective role against ARDS development, while others indicate that it exacerbates disease severity and worsens outcomes for ARDS patients [5-8]. However, most previous studies rely on observational data, which are subject to significant limitations, including bias. To resolve these discrepancies, there is a critical need for integrative research that combines well-designed clinical, preclinical, and cellular studies to clarify the association between DM and lung injury and to investigate the molecular mechanisms by which DM may contribute to pulmonary damage. This thesis aimed to test the hypothesis that DM negatively impacts lung function, increasing susceptibility and worsening outcomes in ARDS patients compared to those without a history of DM. In vitro, we proposed that advanced glycation end-products (AGEs), known contributors to vascular complications, will induce pathological gene expression changes in human microvascular lung endothelial (HMLE) and human type II alveolar epithelial (A549) cells. In vivo, we hypothesized that DM will exacerbate ALI severity in a murine sepsis model, further highlighting its detrimental effects on lung health.

Our analysis of the Fluids and Catheters Treatment Trial (FACTT) clinical data, which includes a large sample of DM patients, revealed that DM is significantly associated with worse outcomes in ARDS patients, as evidenced by lower survival rates, and prolonged ICU and hospital stays. Additionally, we measured plasma cytokine and chemokine levels in ARDS patients with and without DM, finding elevated concentrations of tumor necrosis factor-alpha (TNF-α), interleukin-10 (IL-10), interleukin-6 (IL-6), C-reactive protein (CRP), monocyte chemoattractant protein-1 (MCP-1), and lipopolysaccharide-binding protein (LBP) in ARDS patients with DM. These findings suggest an increased inflammatory response in ARDS patients when DM is present as a comorbidity.

Given that DM is known to cause various vascular complications, including neuropathy, nephropathy, retinopathy, and heart disease [9], its molecular impact on pulmonary complications remains largely unexplored. We investigated the role of advanced glycation end-products (AGEs), a key DM-associated factor implicated in multiple complications, in lung injury using human microvascular lung endothelial (HMLE) and alveolar epithelial (A549) cells. A dose-response study with AGE concentrations of 25, 50, and 100 μg/ml revealed that a moderate dose (50 μg/ml) over 24 hours significantly impaired lung endothelial and epithelial barrier integrity. This disruption was associated with the activation of key inflammatory pathways, Akt and p38 MAPK, along with increased expression of Claudin-5, a tight junction protein, and its receptor RAGE in both cell lines. Additionally, AGE-treated conditioned media (CM) from THP-1 macrophages significantly elevated inflammatory markers, including TNF-α, IL-1β, IL-6, and IL-10, while reducing barrier function, as measured by Electric Cell-Substrate Impedance Sensing (ECIS) assays, compared to both positive controls (THP-1 CM-treated cells) and untreated controls. These findings suggest that AGE treatment compromises lung cell permeability by disrupting junctional proteins, activating inflammatory pathways, and impairing immune function, providing a potential mechanism by which diabetes-induced vascular changes and immune activation weaken lung barrier integrity. This study offers critical insights into DM-related lung complications and lays the groundwork for developing targeted therapies to mitigate pulmonary damage in DM patients.

In our pre-clinical study, we investigated the impact of DM as an independent disease on lung health using the streptozotocin (STZ)-induced type 1 DM mouse model, as well as its role as a pre-existing condition exacerbating ALI outcomes in a lipopolysaccharide (LPS)-induced sepsis model. Histological analysis and edema assessments revealed that DM mice exhibited significant lung injury with increased pulmonary water accumulation compared to control mice. Additionally, qRT-PCR analysis showed a statistically significant upregulation of inflammatory cytokines, including TNF-α, IL-1β, MCP-1, and CXCL-1, in DM mice compared to non-DM controls. Notably, when DM was present as a comorbidity in ALI secondary to sepsis, lung injury was exacerbated, with higher levels of these cytokines and chemokines than in mice with ALI alone. Furthermore, RNA sequencing analysis identified several differentially expressed genes associated with diabetic lungs, many of which are implicated in inflammation, immune activation, and vascular permeability, suggesting potential biomarkers or therapeutic targets for DM-induced lung damage. Overall, our findings demonstrate that diabetic lungs experience significant inflammation, leading to pathological changes and edema, and that pre-existing DM in ALI further amplifies inflammation, resulting in more severe ALI compared to either condition alone. These results establish DM as a critical factor contributing to pulmonary complications and facilitating ALI progression. Looking ahead, our research has laid out a promising roadmap to further unravel the complexities of diabetic lung disease. Future studies will include functional assessments of diabetic lung mechanics using the FlexiVent system and detailed analysis of bronchoalveolar lavage fluid to identify novel biomarkers of lung injury. Additionally, characterizing the roles of differentially expressed genes identified in diabetic lung tissue may reveal new, druggable targets. These efforts aim to translate bench-side insights into bedside therapies, ultimately improving clinical outcomes for ARDS patients with pre-existing diabetes.

In conclusion, this thesis highlights the detrimental effects of diabetes mellitus on lung health, demonstrating that DM exacerbates inflammation, disrupts lung barrier integrity, and worsens ALI, providing crucial insights into potential therapeutic approaches to mitigate pulmonary complications in diabetic patients.

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