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„Mechanism of drug-induced pneumonitis by mTOR inhibitors“

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Sevdican Üstün

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Betreut von: Ass. Prof. Priv. Doz. Dr. Thomas Weichhart
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Summary

The PI3K (phosphoinositide 3-kinase) pathway promotes AKT-mediated activation of mammalian target of rapamycin (mTOR) which is an important kinase mediating cell growth, proliferation, survival and inflammation. Inhibitors of mTOR, including everolimus, are used to prevent allograft rejection after solid organ transplantation and in the treatment of several cancers. However, they have a known side effect, which is known as noninfectious pneumonitis. The molecular mechanism how mTOR inhibitors promote lung injury remains elusive. In this study the effects of the mTOR inhibitor everolimus and the novel dual PI3K/mTOR inhibitor NVP-BEZ235 treatment were tested in two murine models of acute lung injury. The effects of the different treatments on ALI were analyzed by measuring the cell numbers and the levels of inflammatory cytokines in the bronchoalveolar lavage (BAL) fluid, and histopathology of the lungs at different time points. Intratracheal LPS administration induced a significant increase in total cell, neutrophil and erythrocyte numbers in the BAL fluid after six, 12 and 24 hours. In the histological examination we observed a serious lung injury shown by interstitial edema, vascular congestion and mononuclear cell infiltration in these mice after 24 hours. Everolimus and NVP-BEZ235 did not appreciably affect the overall histopathology of the lungs in the lung injury model. However, NVP-BEZ235 enhanced IL-6 and TNF-α expression after 24 hours. In contrast, everolimus did not affect IL-6 and TNF-α levels. Interestingly, both inhibitors reduced inflammatory cytokines in an LPS/oleic acid-induced lung injury model. In summary, both inhibitors had distinct effects on inflammatory cytokine expression in the lung but the histopathological severity of the ALI model was not significantly affected.
Zusammenfassung

1 INTRODUCTION
1.1 **Acute Respiratory Distress Syndrome (ARDS) and Acute Lung Injury (ALI)**

The Acute Respiratory Distress Syndrome (ARDS) was first described by Ashbaugh and colleagues in 1967, in 12 adult patients who were hospitalized in the intensive care unit of the Colorado General Hospital (1). ARDS is the leading cause of mortality and morbidity income defined as a clinical pattern including dyspnea, tachypnea, cyanosis resistant to oxygen therapy, loss of lung compliance and diffuse infiltrates on chest radiographs (1). Ashbaugh and colleagues also observed; (i) hyaline membranes, (ii) diffuse interstitial inflammation, (iii) interstitial and intra-alveolar edema and (iv) hemorrhage in the autopsy finding on lung microscopy (1).

In 1994 the American-European Consensus Conference (AECC) published a new report and accepted that ARDS is the severe form of acute lung injury (ALI). To distinguish between ARDS and ALI, the committee suggested to use the value of arterial blood oxygenation ($\text{PaO}_2/\text{FI}_O2$) which is the ratio between partial pressure of arterial oxygen ($\text{PaO}_2$) and the fraction of inspired oxygen by ventilator support ($\text{FI}_O2$) (2). New diagnostic criteria were formalized for the differentiation between ARDS and ALI: (i) acute and sudden onset of severe respiratory distress; (ii) bilateral infiltrates on frontal chest radiograph; (iii) the absence of left atrial hypertension - a pulmonary capillary wedge pressure $<18$ mmHg or no clinical signs of left ventricular failure; (iv) ALI defined as $\text{PaO}_2/\text{FI}_O2$ ratio $\leq 300$ and (v) ARDS defined as $\text{PaO}_2/\text{FI}_O2$ ratio $\leq 200$ (Table 1) (2).

<table>
<thead>
<tr>
<th></th>
<th>ARDS</th>
<th>ALI</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Timing</strong></td>
<td>Acute onset</td>
<td>Acute onset</td>
</tr>
<tr>
<td><strong>Chest X-Ray</strong></td>
<td>Bilateral infiltrates</td>
<td>Bilateral infiltrates</td>
</tr>
<tr>
<td><strong>PWP</strong></td>
<td>$\leq 18$ mm Hg or no clinical data of left arterial hypertension</td>
<td>$\leq 18$ mm Hg or no clinical data of left arterial hypertension</td>
</tr>
<tr>
<td><strong>Oxygenation</strong></td>
<td>$\text{PaO}_2/\text{FI}_O2 \leq 200$ mm Hg</td>
<td>$\text{PaO}_2/\text{FI}_O2 \leq 300$ mm Hg</td>
</tr>
</tbody>
</table>

Table 1 AECC criteria for ARDS and ALI. Adapted from Bernard et al. (2).
These criteria belong to humans (Table 2) and cannot be directly translated to experimental animals (Table 3), because of the absence of adequate laboratory equipment and/or the incompatibility with experimental setups (3). The ideal way to observe human ALI mechanisms and consequences in the model system is to reproduce the same evolution way for the injury and the repair mechanisms.

<table>
<thead>
<tr>
<th>Clinical features</th>
<th>Acute onset</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Diffuse bilateral alveolar damage</td>
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<tr>
<td></td>
<td>Acute exudative phase</td>
</tr>
<tr>
<td></td>
<td>Repair with fibrosis</td>
</tr>
<tr>
<td>Physiological changes</td>
<td>Ventilation/perfusion ratio abnormalities</td>
</tr>
<tr>
<td></td>
<td>Severe hypoxemia</td>
</tr>
<tr>
<td></td>
<td>Decreased compliance</td>
</tr>
<tr>
<td></td>
<td>Impaired alveolar fluid clearance</td>
</tr>
<tr>
<td>Biological changes</td>
<td>Increased endothelial and epithelial permeability</td>
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<tr>
<td></td>
<td>Increase in cytokine concentrations in the lungs</td>
</tr>
<tr>
<td></td>
<td>Protease activation</td>
</tr>
<tr>
<td></td>
<td>Coagulation abnormalities</td>
</tr>
<tr>
<td>Pathological changes</td>
<td>Neutrophilic alveolar infiltrates</td>
</tr>
<tr>
<td></td>
<td>Intra-alveolar coagulation and fibrin deposition</td>
</tr>
<tr>
<td></td>
<td>Injury of the alveolar epithelium with denudation of the basement membrane</td>
</tr>
</tbody>
</table>

Table 2 Physiological and pathological changes in the human lung injury. Adapted from Matuto-Bello, G. et al. (4)

| Histological changes        | Neutrophil accumulation in the lung              |
|                            | Hyaline membrane formation                      |
|                            | Alveolar space filled with proteinaceous debris |
|                            | Thickenig of the alveolar wall                  |
| Alveolar capillary barrier changes | An increase in interstitial lung water content |
|                                | Accumulation of an exogenous protein in the airspaces or the extravascular compartment |
|                                | Increase in total BAL protein concentration     |
| Inflammatory changes         | Increase in the number of neutrophils in BAL fluid |
|                                | Increase in lung myeloperoxidase activity       |
|                                | Increase in the concentration of proinflammatory cytokines in lung tissues or BAL fluid |
| Physiological changes        | Hypoxemia                                        |
|                                | Increased alveolar-arterial oxygen difference   |

Table 3 Features and measurements of acute lung injury in animals. Adapted from Matuto-Bello, G. et al. (3)
ALI and its more severe form ARDS can arise in patients of all ages caused by different injuries and conditions of direct (pulmonary) and indirect (extrapulmonary) injuries to the parenchyma or vasculature of the lungs (Table 4) (5). Lung bacterial and viral infection and gastric content aspiration cause direct injury to the airway, alveolar epithelium and other parts in the airspaces (6). On the other hand, systemic infections, which can cause sepsis, blood transfusions as well as systemic medications and illicit drugs are part of indirect factors (6).

<table>
<thead>
<tr>
<th>Direct</th>
<th>Indirect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung infection</td>
<td>Sepsis</td>
</tr>
<tr>
<td>Aspiration</td>
<td>Multiple trauma with shock</td>
</tr>
<tr>
<td></td>
<td>Large volume blood replacement</td>
</tr>
</tbody>
</table>

Table 4 Direct and indirect injuries in the ALI/ARDS. Adapted from Matthay, A.M. and G.A. Zimmerman (7).

Sepsis or pneumonia initiate lung injury in animal models by bacterial lipopolysaccharide (LPS) and activate toll like receptor signaling (TLRs) (8; 9). mTOR is involved in TLR signaling as a signaling intermediate in the PI3K-AKT-mTOR pathway (8) (Figure 1). mTOR and its role in LPS activation is uncertain but mTOR inhibitor class of drugs associated non-infectious pneumonitis (NIP) is an iatrogenic lung disease observed in cancer patients as well as in transplant patients. Noninfectious drug-induced pneumonitis (DIP) is characterized by nonmalignant infiltration of the lungs with several respiratory symptoms (10). The incidence of clinically identified pneumonitis has been reported 5-15% in sirolimus (rapamycin) receiving solid organ transplant recipients and 13,5% in everolimus receiving renal carcinoma patients (11).
Figure 1 The mTOR pathway and ALI. Adapted from Sudarsanam, S. and D.E. Johnson (12) and Weichhart, T. and M.D. Säemann (13)
1.2 The Mammalian Target of Rapamycin

1.2.1 mTOR Structure

The serine-threonine protein kinase TOR (mTOR in mammals, also known as FRAP, RAFT, or RAPT) is a conserved member of the PI3K-related kinase (PIKK) family with a predicted molecular weight of 290 kDa (14; 15). mTOR is a key signaling kinase acting downstream of PI3K-AKT pathway regulating cell growth, cell survival, cellular metabolism, inflammatory response and the immune system by coordinately controlling the translational effectors p70S6K1 (p70 ribosomal protein S6 kinase 1) and 4E-BP1 (eukaryotic initiation factor 4E-binding protein 1) (15; 16) (Figure 1).

mTOR interacts with various proteins to form two distinct complexes called mammalian TOR complex 1 (mTORC1) and 2 (mTORC2) (Table 5). Sensitivity to rapamycin is variable in the mTOR-containing complexes, depending on mTOR upstream input and also downstream output (17).

mTORC1 has six known protein components, which are mTOR, raptor (regulatory-associated protein of mTOR), PRAS40 (proline-rich AKT substrate 40 kDa), mLST8 (mammalian lethal with Sec13 protein 8), Deptor (DEP-domain-containing mTOR-interacting protein) and the Tti1/Tel2 complex (17). mTORC2 has seven known protein components which are mTOR, rictor (rapamycin-insensitive companion of mTOR), mSIN1 (mammalian stress-activated protein kinase interacting protein), Protor1/2 (protein observed with Rictor 1 and 2), mLST8, Deptor and the Tti1/Tel2 complex (17).

<table>
<thead>
<tr>
<th>mTORC1</th>
<th>mTORC2</th>
</tr>
</thead>
<tbody>
<tr>
<td>mTOR</td>
<td>mTOR</td>
</tr>
<tr>
<td>Raptor</td>
<td>Rictor</td>
</tr>
<tr>
<td>Pras_{40}</td>
<td>mSIN_{1}</td>
</tr>
<tr>
<td>mLST8</td>
<td>mLST8</td>
</tr>
<tr>
<td>Deptor</td>
<td>Deptor</td>
</tr>
<tr>
<td>Tti1/Tel2 complex</td>
<td>Tti1/Tel2 complex</td>
</tr>
<tr>
<td></td>
<td>Protor1/2</td>
</tr>
</tbody>
</table>

Table 5 Differences between mTORC1 and mTORC2.
1.2.2 mTOR SIGNALING PATHWAY

mTOR regulates cell growth, proliferation and the immune system (13). mTORC1 upstream regulators are cytokines, growth factors, amino acids, insulin, and TLR ligands, energy levels, and oxygen to control major mechanisms, such as protein and lipid synthesis and autophagy (15; 17). mTORC2 is regulated by growth factors but it does not respond to nutrients (17).

The best way to activate mTOR is via the PI3K pathway. Insulin or insulin-like growth factors (IGFs) activate tyrosine kinase adaptor molecules at the cell membrane and PI3K bound to this receptor complex (13; 15). PI3K leading to phosphorylation of PIP$_2$ (phosphatidylinositol 4,5-bisphosphate) to generate PIP$_3$ (phosphatidylinositol 3,4,5-triphosphate) and activate protein kinase AKT (13). AKT also called PKB (protein kinase B), which is a negative regulator of TSC2 (tuberous sclerosis complex protein 2) and positive regulator of mTORC1 and its downstream targets (13; 15) (Figure 2).

mTORC1 activation has some downstream effects in mRNA translation via the phosphorylation and inactivation of the repressor of 4E-BP1, and via the phosphorylation and activation of p70S6K (18) (Figure 2). Active mTORC1 has several effects in autophagy suppression, ribosome biogenesis, and activation of transcription controls the cellular metabolism and adipogenesis (15).

mTORC2 phosphorylates AKT at Ser473 to regulate cellular survival and it also regulates cytoskeletal dynamics, ion transport and growth (15) (Figure 2).

Deviant mTOR signaling is involved in different types of disease including cancer, cardiovascular disease, and metabolic disorders (19).
Figure 2 PI3K/AKT/mTOR pathway. Adapted from Sudarsanam, S. and D.E. Johnson (12) and Weichhart, T. and M.D. Säemann (13)
1.2.3 mTOR INHIBITORS (mTOR-I)

mTOR is important for translational control and progression of cell-cycle from G0/G1 to S phase (20). Rapamycin and its analogues arrest cells in the G1 phase and delay G1-S cell cycle progression, sensitizes cells to apoptosis, modulates the cellular metabolism, inhibits tumor growth, metastasis and angiogenesis. Because of their antiproliferative activities mTOR inhibitors have been evaluated for clinical use.

1.2.3.1 Rapamycin

On the Easter Island also known as Rapa Nui, in 1975, the macrolide rapamycin was isolated from the bacterial strain *Streptomyces hygroscopicus* and subsequently turned out to be a potent anti-fungal with immunosuppressive and anti-proliferative properties (21). Rapamycin also known as sirolimus, has been approved by the FDA (Food and Drug Administration) in 1999 for the prevention of transplanted allograft organ rejection (13). Moreover, rapamycin is also used to inhibit endothelial proliferation and vessel restenosis after angioplasty (21; 22). Rapamycin binds to an intracellular protein FKBP-12 (FK506-binding protein-12) and this complex inhibits mTOR (23).

1.2.3.2 Everolimus

Everolimus (RAD001) is a rapamycin analogue (rapalog), which functions similar as rapamycin and inhibits mTOR in complex with FKBP-12 (23; 24). Prevention of allograft rejection after organ transplantation, treatment of RCC and other tumors are indications for everolimus (11; 25). Everolimus also causes serious side effects, including NIP, stomatitis/oral mucositis and an increased risk of infection (11) (Figure 3).
1.2.3.3 *NVP-BEZ235*

NVP-BEZ235 is an oral, ATP-competitive dual kinase inhibitor of mTOR and PI3K, which inhibits the catalytic activity at their ATP-binding site (26). NVP-BEZ235 is currently in phase I/II clinical trials for the treatment of cancer (25; 12) (Figure 3).

Figure 3 mTOR inhibitors. Adapted from Sudarsanam, S. and D.E. Johnson (12).
1.2.4 **Drug-Induced Pneumonitis (DIP)**

Interstitial lung disease is a general term for several lung disorders, including *idiopathic pulmonary fibrosis (IPF)*, *desquamative interstitial pneumonia*, *respiratory bronchiolitis (RB)* interstitial lung disease, *lymphoid interstitial pneumonia (LIP)*, *cryptogenic organizing pneumonia (COP)*, *diffuse alveolar damage (DAD)*, *acute interstitial pneumonia (AIP)*, and *non-specific interstitial pneumonia (NSIP)* (20).

Drug-induced interstitial lung disease is a class effect of mTOR inhibitors (mTOR-I), which is characterized by noninfectious, nonmalignant cellular infiltrates and called as NIP (11). mTOR-inhibitor induced interstitial pneumonitis was first observed in renal, liver, and heart transplant recipients with rapamycin treatment (27). NIP was later also observed in everolimus-treated metastatic renal cell carcinoma and breast cancer patients (10; 28). The incidence of NIP is 14-39% with rapalogs in recent retrospective analysis (29).

**1.3 Clinical Markers Of mTOR-I Induced Pneumonitis**

mTOR inhibitor-associated pulmonary toxicity was observed in the form of diffuse interstitial pneumonia, which can be recognized with the common clinical symptoms cough and dyspnea followed by fever and fatigue (11). The most common radiological findings of mTOR-I associated pulmonary abnormalities are ground glass opacity and lung patchy consolidation, especially in the lower lobes of the lungs (10). Physiological abnormalities of the lungs with decreased diffusing capacity and abnormal alveolar-arterial oxygen gradient, without an infectious disease and neoplasm are features of NIP (20). Also histopathological changes in the lung parenchyma with fibrosis and inflammation can be observed in mTOR inhibitor induced pneumonitis (20).
1.3.1 HISTOLOGICAL MARKERS OF mTOR-I INDUCED PNEUMONITIS

Histological features of the bronchoalveolar fluid and the lung biopsy are lymphocytic alveolitis, lymphocytic interstitial pneumonitis, bronchiolitis obliterans organizing pneumonia, interstitial pneumonitis with or without fibrosis, alveolar hemorrhage or a combination of these (27). TNF-α is involved in the induction of apoptosis, which causes disruption of the alveolar epithelial and capillary endothelial cells basement membranes and therewith contributes to pulmonary permeability edema (30). The disease can further extended into the alveolar space, acini, bronchiolar lumen and bronchioles (20). Collection of the data and diagnosis of drug reaction are observed with bronchoalveolar lavage (BAL) or bronchoscopic biopsy.

1.3.1.1 BRONCHOALVEOLAR LAVAGE FLUID (BALF)

In drug induced lung injury, the bronchoalveolar lavage fluids (BALF) include cytotoxic changes with unusual hyperchromatic cells, pulmonary hemorrhage and lymphocytic alveolitis (31). Alveolitis refers to an inflammation of the alveoli in the lungs and the accumulation of macrophages, neutrophils and lymphocytes within the lower respiratory tract (20). However, in the noninfectious origin of DIP, BALF microbiological evaluation is negative for many bacteria, fungi, parasites, or viruses (32).

1.3.1.2 LUNG PATHOLOGY

In the interactive phases of ALI, the acute phase (exudative phase) is followed by the fibro-proliferative phase. Diffuse alveolar damage, microvascular injury with pulmonary edema, type I pneumocyte necrosis and accumulation of inflammatory cells and release of mediators are the features of the exudative phase (33). Fibroblast proliferation, type II pneumocyte hyperplasia and lung repair are belongs to the second phase of ALI (33).

The pathologic characteristics of drug-induced lung disease are interstitial and intra-alveolar oedema, hyaline membrane formation and interstitial inflammation (20).
1.3.2 CELL SPECIFIC MARKERS OF DIP

The exudative and fibro-proliferative phases of ALI are regulated by the innate immune system, which includes myeloid cells such as neutrophils, monocytes, macrophages (alveolar and interstitial), eosinophils and dendritic cells (34).

1.3.2.1 MACROPHAGES

Macrophages and their circulating precursors, monocytes, are important in innate and adaptive immunity and effective in the elimination of microbes (35). Lung macrophages are found either in the interstitium as interstitial macrophage or in the alveoli as alveolar macrophages (36). Upon activation by TLRs alveolar macrophages release cytokines, which stimulate alveolar epithelial cells and tissue-resident macrophages to mediate the recruitment of neutrophils, exudates macrophages and lymphocytes to the site of infection, finally resulting in the clearance of the invading pathogens (36).

1.3.2.2 NEUTROPHILS

Neutrophils, polymorphonuclear leukocytes, are produced in the bone marrow and mediate the earliest phases of inflammatory responses (35). Acute lung injury is characterized by the accumulation of neutrophils in the lungs, development of interstitial edema and a pulmonary inflammatory response (37). The neutrophils that penetrate the lungs and migrate into the airways express proinflammatory cytokines, such as interleukin (IL)-1β and tumor necrosis factor (TNF)-α, whose transcription is dependent on NF-κB and leads to injury and loss of epithelial integrity (38). PI3K and Akt are activated by LPS-exposed neutrophils leading to increased nuclear accumulation of NF-κB (38). For this reason blocking PI3K decreases LPS-induced increases in the activation of NF-κB and the production of cytokines (38).
1.3.2.3 **LYMPHOCYTES**

T and B lymphocytes are central cells for adaptive immunity. T lymphocytes produce cytokines in cell mediated immunity and B lymphocytes produce antibodies in humoral immunity.

mTOR blockage inhibits T and B cell proliferation, differentiation and clonal expansion as a immunosuppressive potent (39; 40). The conversion of thymocytes from CD4/CD8 double-negative to double-positive T cells is blocked by long-term rapamycin treatment (41).

1.3.3 **MARKERS OF ACUTE INFLAMMATION**

1.3.3.1 **CYTOKINES**

Cytokines are polypeptides that can be produced by different cell types including macrophages, monocytes, neutrophils, endothelial and epithelial cells in response to microbes and other antigens.

The physiological and cellular basis of lung injury is mediated by a complex interaction that occurs between diverse proinflammatory mediators including LPS, complement products, cytokines (TNF-α, IL-1β, IL-6), chemokine (IL-8), reactive oxygen species, eicosanoids and anti-inflammatory (IL-10, IL-1-RA, PGI2, IL-13) mediators (33; 42). Two of the most important early-response cytokines in innate immunity are IL-1 and TNF-α.

TNF-α is found in both plasma and BALF in the early phase of ALI. It is produced and released by alveolar macrophages and monocytes, and mediates the recruitment of neutrophils, monocytes and lymphocytes into the alveolar space (33). IL-6 contributes also together with other proinflammatory cytokines in acute inflammatory responses (43). It is critical for B-cell differentiation and maturation with secretion of immunoglobulins, cytotoxic T cell differentiation, macrophage and monocyte function and production of acute phase proteins (33).
1.3.4 Animal Models Of ALI

Animal studies make it possible to test hypotheses of human studies and their relationship within complete living systems. Many cellular signal transduction pathways are similar in humans and mice, which makes it possible to test the function of kinase inhibitors in animal models of human disease. Animal models are very important to understand the correlation between health and disorder in human clinical studies.

Animal models of ALI are based on clinical disorders that are associated with ALI/ARDS in humans. There are some similarities and differences between each model and human lung injury. The main characteristics of experimental ALI in animals are rapid onset (within 24 h) and (i) histological evidence of tissue injury, (ii) alteration of the alveolar capillary barrier, (iii) an inflammatory response, (iv) evidence of physiological dysfunction (3). The main animal model types of ALI are induced by mechanical ventilation, lipopolysaccharide (LPS), oleic acid (OA), hyperoxia, bleomycin, cecal ligation and puncture, administration of live bacteria and acid aspiration (4). In the murine model of ALI, the lung macrophages damage the lung parenchyma after activation by several proteases, reactive oxygen and nitrogen species and transmigrated neutrophils in the interstitial and alveolar compartments (44). Additional features are microvascular injury and diffuse alveolar damage with intrapulmonary hemorrhage, edema, and fibrin deposition (44).
1.3.5 AGENTS TO INDUCE LUNG INJURY IN MODEL SYSTEMS

1.3.5.1 LIPOLYSACCHARIDE (LPS)

LPS is a glycolipid present in the outer membrane of gram-negative bacteria, composed of a polysaccharide core (lipid A) and an O-antigen side chain (45). The plasma LPS-binding protein (LBP) binds endotoxin to enhance different LPS-stimulated responses that can lead to ARDS. It regulates CD18, promotes neutrophil adhesion to the endothelium, and cytokine production (e.g., TNF-α by alveolar macrophages) (46). The LPS/LBP complex interacts with the CD14/TLR4/MD2 receptor complex on monocytes, macrophages, and other cells, triggering the production of inflammatory mediators (35). Murine LPS-induced ALI has similar pathophysiological phenotype as human ALI. The LPS provokes acute pulmonary inflammation, through the production of local proinflammatory mediators, and exacerbates the alveolar-capillary leak (8). Also, the neutrophilic inflammatory response with an increase in intrapulmonary cytokines is similar between the model and human lung injury (4).

1.3.5.2 OLEIC ACID

Oleic acid, an 18-carbon unsaturated fatty acid with a single double bond, induces ARDS in experimental models, and lung injury correlates with elevated free fatty acid levels (47; 48). Acute and repair phases in animal models of lung injury have similar histopathological and physiological features with the human ARDS (4).
2 Aim of the study
At present, everolimus has not been investigated in a murine model of pneumonitis/ALI. Whether NVP-BEZ235, a novel active site PI3K/mTORC1/2 inhibitor, has the potential to induce DIP, is also completely unknown. Because of these reasons we would like to determine the outcome of everolimus and NVP-BEZ235 treatment in the murine models of pneumonitis/ALI.

Therefore, we will pretreat wild type mice with everolimus, NVP-BEZ235, or vehicle and then induce ALI with oleic acid and LPS. Six, 12 and 24 hours after the induction of ALI, we will sacrifice the mice with CO$_2$ and obtain the bronchoalveolar lavage fluid (BALF). The lungs will be removed and be either fixed in formalin or snap-frozen in liquid nitrogen for biochemical analyses. The spleens will be removed. The immune cells in BALF, whole lung or spleen single cell suspension will be analyzed by flow cytometry. We will analyze lung histology by H&E and immunohistochemical staining methods. In BALF and blood serum, we will measure the cytokines TNF-$\alpha$, IL-6 and IL-12. In lung lysates, we will determine the activation status of mTORC1 and mTORC2 by immunoblotting p-S6K1, p-S6, and p-Akt (Ser473).

We perform these studies to better understand the molecular causes of noninfectious DIP in order to better manage or interfere with this side effect of everolimus and NVP-BEZ235 in the future.
3 Material and methods
3.1 Media, buffers and solutions

3.1.1 Flow Cytometry

Flow Cytometry Buffer (FACS Buffer)
- 1x PBS
- 2% BSA
- 2mM EDTA

10x RBC (Red Blood Cell) Lysis Buffer pH 7.3
- 82.6g Ammonium Chloride
- 10g Potassium Bicarbonate
- 0.37g EDTA
- HCl to adjust pH
- Fill up to 1l with dH₂O
- Sterile filter

3.1.2 ELISA

Wash Buffer
- 100 ml 10x PBS
- 0.5 ml Tween 20
- 899.5 ml dH₂O

Stop Solution
- 2N H₂SO₄

3.1.3 Western Blot Buffers

1x PBS
- 100 ml 10x PBS (without Ca & Mg) (PAA)
- 900 ml dH₂O

1x PBS - Tween (PBS-T)
- 100 ml 10x PBS
- 1 ml Tween 20 (aplichem)
- 899 ml dH₂O
50x Phosphatase inhibitor
- 500 mM NaF
- 100 nM Na₄P₂O₇
- 100 mM Glycerophosphate
- 10 nM Na₃VO₄
- Store at 4 °C

10x Protease inhibitor
- 1 Protease inhibitor cocktail tablet
- 2 ml dH₂O
- Freeze aliquots at -20°C

2x Hepes buffer
- 20 mM Hepes
- 140 mM NaCl
- 2 mM EDTA
- pH 7.9

Tx-100 Lysis Buffer (10 ml)
- 5 ml 2x Hepes Buffer
- 1 ml 10x Protease Inhibitor
- 0.2 ml 50x Phosphatase Inhibitor
- 1 ml 10% Tx-100
- 2.8 ml dH₂O

4x Reducing Sample Buffer
- 250 mM Tris-HCL pH 6.8
- 40% Glycerol
- 8% SDS
- 400 mM Dithiothreitol, added fresh
- Stain with bromphenolblue

5x Running Buffer
- 15.14 g/L Tris
- 72.07 g/L Glycin
- 5 g/L SDS
- dH₂O add 1000 ml
1x Running Buffer
- 200 ml 5x Running Buffer
- 800 ml dH₂O

1x Transfer Buffer (Harlow Buffer)
- 29 g Tris-Base
- 145 g Glycine
- 25 ml 20% SDS
- 1 l Methanol
- 4 l dH₂O
- Store at 4°C

3.1.4 Acrylamide gels

<table>
<thead>
<tr>
<th></th>
<th>Stack (10ml)</th>
<th>10% (10ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30% AA/Bis (Sigma)</td>
<td>1.33 ml</td>
<td>3.33 ml</td>
</tr>
<tr>
<td>0.5 M Tris-HCl, pH6.8</td>
<td>2.50 ml</td>
<td>-</td>
</tr>
<tr>
<td>1.5 M Tris-HCl, pH8.8</td>
<td>-</td>
<td>2.50 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.10 ml</td>
<td>0.10 ml</td>
</tr>
<tr>
<td>dH₂O</td>
<td>6.07 ml</td>
<td>4.07 ml</td>
</tr>
<tr>
<td>TEMED (Biorad)</td>
<td>10 μl</td>
<td>10 μl</td>
</tr>
<tr>
<td>10% APS (Amersham)</td>
<td>50 μl</td>
<td>50 μl</td>
</tr>
</tbody>
</table>

Table 6 Recipes for stacking and resolving gels used in SDS-PAGE.

4% PFA in PBS
- 1.85 g Paraformaldehyde
- 3.5 ml dH₂O
- 10 μl 10 M NaOH
- 65°C water bath
- Dilute in 41.25 ml PBS
3.2 Animals

Female C57BL/6JRj bom mice (age: 8-10 weeks) (Janvier Labs, France) were used in all experiments. The mice were housed under specific pathogen-free conditions corresponding to FELASA guidelines. All animal experiments were discussed and approved by the Ethics and Animal Welfare Committee of the University of Veterinary Medicine Vienna, conform to the guidelines of the national authority (the Austrian Federal Ministry for Science and Research) as laid down in the Animal Science and Experiments Act (Tierversuchsgesetz- TVG; refs BMWF-68.205/0159-II/3b/2013) and were performed corresponding to the guidelines of FELASA and ARRIVE by Dr. Caroline Lassnig from the University of Veterinary Medicine Vienna.

3.2.1 Preparation and administration of drugs

Female mice (~20g) were pretreated once per day on 2 consecutive days with a dose of 10 mg Everolimus (Novartis) per kilogram body weight and 20 mg NVP-BEZ235-AN (Novartis) per kilogram body weight administered orally (p.o.) with an application volume of 10 mL/kg. Control groups were given the same volume of placebo solution. The drugs were prepared according to the guidelines of Novartis. PBS-mice were orally (p.o.) fed 200μl PBS.

3.2.2 LPS challenge

We first established the most consistent ALI model. The model were started one hour after the second application of the inhibitors. We used different concentrations of LPS O55:B5 (Escherichia coli, Sigma) and Oleic acid (Sigma, O1008) to compare the ALI models. In the first model, the animals were exposed to 0.1 μg LPS (dissolved in 50 μl PBS) by intranasal (i.n.) administration for 6 hours. In the second model, we used 10 mg LPS per kilogram body weight by the intratracheal (i.t.) route or with 2% oleic acid (dissolved in 0.1 % BSA in PBS) 5 μl/g per mouse intravenously (i.v.) 30 minutes after intratracheal (i.t.) administration of 10mg/kg LPS. PBS-mice got
50µl PBS intratracheal (i.t.). We used three different time points for analysis after the LPS administration: 6 hours, 12 hours and 24 hours later.

3.2.3 Isolation of BALF and organs

All mice were anaesthetized twice with Ketasol/ Xylasol. first before the intratracheal LPS application and second before collecting blood. After the induction of ALI, the mice were killed by CO₂ asphyxiation. BALF was obtained by cannulating the trachea and then lavaging the lungs two times with 0.8 ml saline.

3.3 Cytospin

BALF samples of each mouse were pooled and centrifuged at 350xg for 7 minutes at 4°C, the supernatant was separated and the cell pellet was washed with ice cold FACS buffer. After another round of centrifugation and discarding the supernatant, the cells were resuspended in 1 ml FACS buffer. BALF cells were counted with a hemocytometer and 3.10⁴ cells from each animal were used per microscopy slide. The BALF cells were centrifuged onto glass slides at 550x rpm for 5 minutes using Shandon Cytospin 2. The cytospun cells were fixed with cold methanol and stained with 10% Giemsa before counting the number of neutrophils, macrophages and monocytes per high-power field light microscopy.

3.4 Single cell suspension of the spleen

The spleens were isolated and put into ice cold PBS. The tissue was transferred to a 70 µm cell strainer (BD Falcon) on top of a 50 ml tube. We used the back of a 5 ml syringe plunger (Braun) and macerate the cells with PBS through the filter. The single cell suspension was centrifuged at 350x g for 7 minutes at 4°C. The cells were washed twice with ice cold PBS and then resuspended in 3 ml Erythrocyte Lysis Buffer for 5 min. Lysis was stopped by the addition of 10 ml FACS buffer.
3.5 Single cell suspension of the lung

The lungs were isolated, put into ice-cold HBSS and chopped into 1-mm fragments with a scissor. Lung fragments were incubated in 5 ml solution containing 125 u/ml collagenase (Gibco-Type I) and 60 u/ml Deoxyribonuclease I (Dnase I) (Life Technologies) at 37 °C for 60 minutes with an agitation of 200x rpm. The resulting suspension was transferred to a 70 µm cell strainer (BD Falcon) on top of a 50 ml tube and debris was removed. The filter was rinsed with PBS and the tube was centrifuged at 300x g for 20 minutes at 4°C, supernatant was discarded and cells were washed twice with ice cold PBS. Red blood cell lysis was conducted as described for the spleen single cell suspension.

3.6 Flow cytometry

Cells were counted with Casy (Roche-applied-science) and 5.10⁵ cells from each animal were used per staining. All further steps were proceeded on ice. The cells were transferred to FACS-tubes (BD), centrifuged at 350x g for 7 minutes at 4°C and re-suspended in 50 µl FACS buffer. After washing the cells once more with FACS buffer, they were blocked with 1µl TrueStain fcX (anti-mouse CD16/32, bioLegend, clone 93) in 9 µl FACS buffer for 15 min. Afterwards, fluorescent labelled antibodies were added into the tubes and incubated for 20 min in the dark (Table 7). The cells were washed with 2 ml FACS buffer, centrifuged again at 350x g for 7 minutes at 4°C and re-suspended in 300 µl FACS buffer for analysis. Cells obtained from BALF, spleen and lungs were analyzed with a FACSCalibur Flow Cytometer (Beckton Dickinson, Franklin Lakes, NJ, USA) using Cellquest (Version 6.0 BD Immunocytometry Systems) to acquire the raw data. FlowJo (Version 7.5.5) was used for analysis.
## 3.7 Histology

### 3.7.1 Preparation for Hematoxylin and Eosin staining

The lungs were fixed in 4% paraformaldehyde, dehydrated and embedded in paraffin before being sectioned. The paraffin was melted for 20 min at 60°C and the tissues were deparaffinized and rehydrated using the following series of washes: two xylene washes (10 min each), followed by isopropanol (5 min) followed subsequently by 96%, 70% and 50% ethanol (2 min) and brief ddH2O washes. Slides were stained in Mayer’s hematoxylin solution (Roth, 2 min) and changed to water 2 times. The slides were incubated in 50% and then 70% ethanol (2 min), counterstained in eosin solution (Roth, 1 min) and run in the reverse manner from the run down, taken back through 70%, 96% ethanol, isopropanol, two changes of xylene and mounted with xylene based mounting medium (merckmillipore).

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Conjugate</th>
<th>Clone</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4</td>
<td>APC</td>
<td>RM4-5</td>
<td>BioLegend</td>
</tr>
<tr>
<td>CD8a</td>
<td>PerCP</td>
<td>53-6.7</td>
<td>BioLegend</td>
</tr>
<tr>
<td>CD19</td>
<td>FITC</td>
<td>6D5</td>
<td>BioLegend</td>
</tr>
<tr>
<td>I-Ab</td>
<td>PE</td>
<td>AF6-120.1</td>
<td>BioLegend</td>
</tr>
<tr>
<td>Ly-6G/Ly-6C (GR1)</td>
<td>PE</td>
<td>RB6-8C5</td>
<td>BioLegend</td>
</tr>
<tr>
<td>CD11c</td>
<td>FITC</td>
<td>N418</td>
<td>BioLegend</td>
</tr>
<tr>
<td>CD11b</td>
<td>PerCP</td>
<td>M1/70</td>
<td>BioLegend</td>
</tr>
<tr>
<td>F4/80</td>
<td>APC</td>
<td>BM8</td>
<td>BioLegend</td>
</tr>
</tbody>
</table>

Table 7 Antibodies used for flow cytometry.
### 3.7.2 Preparation for Immunohistochemistry

Lungs were in 4% paraformaldehyde, and embedded in paraffin before being sectioned. Slides were incubated 20 min at 60°C and deparaffinized and rehydrated using the following series of washes: two xylene washes (10 min each), followed subsequently by isopropanol, 96%, 70% and 50% ethanol (2 min) and washed by ddH$_2$O (5 min). To unmask the antigens, the slides were treated with an antigen retrieval solution (Dako, pH 6.0) and heated using a steam cooker. After cooling down for 20 minutes, slides were treated with 1% H$_2$O$_2$ to inactivate endogenous peroxidase. 0.1% Triton X in PBS was used for tissue permeabilization. Tissues were blocked in vector horse serum. The primary antibody was diluted to the appropriate concentration in PBS, applied to each section and incubated O/N at 4°C (Table 8). After washing with PBS, the sections were incubated with the appropriate secondary HRP-conjugated antibody (diluted in PBS) for one hour at room temperature (Table 8). Slides were incubated in novocastra streptavidin-HRP (Leica) and AEC-high sensitivity substrat chromogen (Dako) and counterstained with Hematoxylin.

<table>
<thead>
<tr>
<th>Primary antibodies</th>
<th>Dilution</th>
<th>Origin</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-S6 (Ser240/244)</td>
<td>1:600 in PBS</td>
<td>Rabbit</td>
<td>Cell Signaling</td>
</tr>
<tr>
<td>p-AKT (Ser473)</td>
<td>1:400 in PBS</td>
<td>Rabbit</td>
<td>Cell Signaling</td>
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</table>

<table>
<thead>
<tr>
<th>Secondary antibodies</th>
<th>Dilution</th>
<th>Origin</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biotinylated Anti-Rabbit IgG (H+L)</td>
<td>1:500 in PBS</td>
<td>Horse</td>
<td>Vector</td>
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</tbody>
</table>

Table 8 Primary and secondary antibodies used for immunohistochemistry.

### 3.7.3 Visualization

Analysis was performed using high-power field by light microscope.

### 3.8 Enzyme-linked immunosorbent assay (ELISA)

Mouse TNF-α and IL-6 matched antibodies for ELISA kit (BioLegend-ELISA MAX Deluxe Sets) were used for ELISA analysis of mouse BALF supernatant and mouse blood serum.
3.9 Western-Blot

Half of the lavaged lungs were removed, snap frozen in liquid nitrogen, and stored at -80°C prior to protein extraction. Lungs were mechanically disrupted in lysis buffer using Precellys tissue homogenizer. Homogenates were kept 30 min on ice and cleared by centrifugation at 10000x g for 10 min at 4°C. Supernatants were assayed for protein content by Bradford assay. Equal amounts of protein were separated by SDS-PAGE (Table 6) and transferred to nitrocellulose membrane before immunoblotting with primary antibodies (Table 9). The membranes were incubated with anti-rabbit or anti-mouse IgG-h+l HRP conjugated antibodies (Table 9) and developed using peroxide-luminol enhancer solution (Thermo Fisher Scientific) with a medical x-ray processor machine (Kodak).

<table>
<thead>
<tr>
<th>Primary antibodies</th>
<th>Dilution</th>
<th>Origin</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>pAKT (Ser473)</td>
<td>1:2000 in PBS-T + 0.02% NaN3</td>
<td>Rabbit</td>
<td>Cell Signaling</td>
</tr>
<tr>
<td>panAKT</td>
<td>1:1000 in PBS-T + 0.02% NaN3</td>
<td>Mouse</td>
<td>Cell Signaling</td>
</tr>
<tr>
<td>pS6 (Ser240/244)</td>
<td>1:2000 in PBS-T + 0.02% NaN3</td>
<td>Rabbit</td>
<td>Cell Signaling</td>
</tr>
<tr>
<td>PanS6</td>
<td>1:1000 in PBS-T + 0.02% NaN3</td>
<td>mouse</td>
<td>Cell Signaling</td>
</tr>
<tr>
<td>4EBP1</td>
<td>1:1000 in PBS-T + 0.02% NaN3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Secondary antibodies</th>
<th>Dilution</th>
<th>Origin</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-rabbit</td>
<td>1:10000 in 4% dry milk+ PBS-T</td>
<td>Goat</td>
<td>Bethyl laboratories</td>
</tr>
<tr>
<td>Anti-mouse</td>
<td>1:10000 in 4% dry milk+ PBS-T</td>
<td>Goat</td>
<td>Bethyl laboratories</td>
</tr>
</tbody>
</table>

Table 9 Primary and secondary antibodies used for Western Blot.
4 Results
4.1 Evolution of the LPS-induced murine acute lung injury model

In the beginning of this study, we tested three different models to induce acute lung injury. C57BL/6Jrj mice were exposed to LPS first 5 ng/g intranasally (i.n.), second 10 μg/g intratracheally (i.t.) and third 100 μl 2% oleic acid (OA) solution intravenously (i.v.) after 10 μg/g (i.t.) LPS administration.

After six hours we lavaged the BAL fluid from lung and compared the number of neutrophils. We could not find significant numbers of neutrophils in the BAL of mice treated with PBS or (i.n.) LPS (Figure 4 A, B). On the other hand, we observed high neutrophil influx into the BAL in mice treated with (i.t.) LPS or LPS with OA (Figure 4 A, B). Furthermore, we detected increased expression of the proinflammatory cytokine TNF-α in the BAL of the (i.t.) LPS or LPS with OA induced mice in contrast to PBS or (i.n.) LPS-induced mice (Figure 4 C)

![Figure 4 Establishment of acute lung injury model.](image)

After 6h, saline or 5ng/g (i.n.), 10μg/g LPS (i.t.), 2% oleic acid (dissolved in 0.1% BSA in PBS) 5μl/g per mouse administrated intravenously (i.v.) 30 minutes after (i.t.) administration of 10μg/g LPS. Panel A shows the representative Giemsa stained sections of BALF cytospin slides. Panel B shows the neutrophil numbers in BALF. Panel C shows the TNF-α levels in BALF were quantified by ELISA. (n=3 mice for PBS group, n=5 mice per group).
4.2 Treatment with mTOR inhibitors after ALI for 6 hours

Most of the previous studies on the mTOR inhibitor rapamycin were practiced on mice stimulated with (i.t.) administration of LPS. On account of this we chose to evaluate everolimus and NVP-BEZ235 with (i.t.) LPS alone.

We pretreated mice with placebo, everolimus and NVP-BEZ235 by oral route on two consecutive days. The six hours ALI model started after the second application of the inhibitors and vehicle controls. As discussed before the administration of LPS resulted in increased total cell numbers and an increase in the production of the proinflammatory cytokines TNF-α and IL-6 in the BAL fluids of mice (Figure 5 A, B, C). However, the inhibitors did not change or even slightly reduced these levels (Figure 5 A, B, C).

Figure 5 Treatment with mTOR inhibitors after ALI for 6 hours.
Mice were pretreated with saline, placebo, everolimus and NVP-BEZ 235. After 6 h intratracheal (i.t.) administration of 10μg/g LPS. Panel A shows the total cell numbers counted in BALF with hemocytometer. Panel B and C show TNF-α and IL-6 levels in the BALF. n=3 mice for PBS group, n=5 mice per group).
4.3 ALI for 24 hours changes the effects of the drugs

After the 6 hours experiment we wanted to examine a more advance lung injury in histological examination and cytokine levels. Because of this reason we choose to evaluate LPS-induced ALI after 24 hours. As for the 6 hours ALI model, intratracheally (i.t.) administered LPS resulted in serious lung injury with the influx of neutrophils and erythrocytes into the BALF after 24 hours (Figure 6 A, B, C). The influx of neutrophils into the BAL was elevated by everolimus and did not change with NVP-BEZ235 (Figure 6 A). The number of erythrocytes was not modulated by mTOR inhibitors (Figure 6 C). When we compared the cytokine levels, in contrast to the 6 hours ALI model everolimus had no effect on cytokine expression, whereas, NVP-BEZ235 trend towards promoted expression of TNF-α in the BAL fluid, IL-6 in the BAL fluid and IL-6 in the serum but not IL-12 in the BAL fluid compared to purely LPS administered mice, but these are not statistically significant (Figure 6 D, E, F, G).

Figure 6 24 hours LPS-induced ALI changes the effects of the drugs. Mice were pretreated with saline, placebo, everolimus and NVP-BEZ 235. After 24 h (i.t.) administration of 10μg/g LPS. Panel A shows the Giemsa stained sections of BALF cytospin slides. Panel B shows the total cell numbers counted in BALF with hemocytometer. Panel C shows the flow cytometric results of the BALF for TER-119. Panel D and E show TNF-α and IL-6 levels in BALF, F shows IL-6 levels in serum, G shows IL-12 levels in BALF were quantified by ELISA. n=6 mice for PBS group, n=6-9 mice per group)
Flow cytometric analysis of T-cells, B-cells, macrophages or dendritic cells in the spleen of the injured mice showed no statistically significant differences between the groups (Figure 7 A-H).

Figure 7 24 hours LPS-induced ALI changes the effects of the drugs. Mice were pretreated with saline, placebo, everolimus and NVP-BEZ 235. After 24 h (i.t.) administration of 10μg/g LPS. Flow cytometric results of the spleen; A-CD4, B-CD8, C-CD19+Ia+b, D-CD11b+CD11c+, E-F4/80+CD11b+, F-F4/80+CD11b–, G-GR+CD11c+, H-F4/80+CD11c– (n=6 mice for PBS group, n=6-9 mice per group)
Histological examination of the lung damage was observed by H&E and immunohistochemical staining with pS6 and pAKT. In the 24 hours LPS-induced ALI model, LPS treated mice showed the results of lung injury, characterized by a strong thickening of the alveolar septum, infiltration of inflammatory cells and hemorrhage (Figure 8A). PBS-induced mice did not exhibit features of lung injury (Figure 8A). There were no appreciable effects of everolimus and NVP-BEZ235 in LPS-induced lung injury by histopathology (Figure 8A). Administration of (i.t.) LPS significantly increased the levels of S6 phosphorylation, consistent with the activation of mTOR, in the cells of the alveolar endothelium (Figure 8B). Comparing the different mTOR inhibitors, everolimus, but not NVP-BEZ235, abolished alveolar phosphorylation of S6. (Figure 8B). Similarly in bronchial cells the S6 phosphorylation was blocked by everolimus, but remained unchanged by NVP-BEZ235 compared to the PBS control mice (Figure 8B). We also observed Akt phosphorylation at the residue Ser473 in the bronchial cells; however, neither LPS, everolimus, nor NVP-BEZ235 changed the Akt phosphorylation levels in our experimental setup (Figure 8C).

Figure 8 Histological examination of the lung in the 24 hours (i.t.) LPS-induced ALI model. Mice were pretreated with saline, placebo, everolimus and NVP-BEZ 235. After 24 h (i.t.) administration of 10μg/g LPS. Panel A shows H&E stained sections of lung slides. Panel B and C show immunohistochemical stained sections of lung slides with pS6 and pAkt antibodies.
4.4 LPS and oleic acid induced lung injury model

Finally we also tested our mTOR inhibitors with another lung injury model with 10 μg/g amount of (i.t.) LPS (12 hours) together with the administration of (i.v.) oleic acid. LPS/OA-induced mice had high cell infiltration in the BALF as expected (Figure 9 A). Everolimus attenuated the cell infiltration in the BALF whereas NVP-BEZ235 distinctively enhanced cellular influx (Figure 9 A). Erythrocyte accumulation in the BAL was not effected by mTOR inhibitors (Figure 9B). When we analyzed cytokine levels, mTOR inhibitors decreased the expression of TNF-α and IL-6 (Figure 9 C, D).

Figure 9 LPS and OA induced lung injury model. Mice were pretreated with saline, placebo, everolimus and NVP-BEZ235. 2% oleic acid (dissolved in 0.1% BSA in PBS) 5μl/g per mouse administrated intravenously (i.v.) 30 minutes after intratracheal (i.t.) administration of 10μg/g LPS. After 12 h, panel A shows shows the total cell numbers counted in BALF with hemocytometer. Panel B shows the flow cytometric results of the BALF for TER-119. Panel C and D show TNF-α and IL-6 levels in the BALF. (n=3 mice for PBS group, n=5 mice per group).
By western blot analysis of whole lung homogenates, LPS/OA resulted in an increase of phospho S6, total S6, phospho AKT and total AKT levels (data not shown). Everolimus abolished phospho S6 phosphorylation whereas NVP-BEZ235 did not (Figure 10 E). In both everolimus and NVP-BEZ235 treatment, we detected a shift of the alpha and β isoforms to the γ isoform of 4EBP1 compared to LPS/OA alone (Figure 10 E).

![Western Blot Analysis](image)

Figure 10 WB analysis with LPS and OA induced lung injury model. Mice were pretreated with placebo, everolimus and NVP-BEZ 235. 2% oleic acid (dissolved in 0.1% BSA in PBS) 5μl/g per mouse administrated intravenously (i.v.) 30 minutes after intratracheal (i.t.) administration of 10μg/g LPS for 12 h.

In the spleen of the mice, we again observed no difference in the number of major immune cells between the groups (Figure 11 A, B, C, D).

![Flow Cytometry Analysis](image)

Figure 11 Flow cytometry analysis with LPS and OA induced lung injury models. Mice were pretreated with placebo, everolimus and NVP-BEZ 235. 2% oleic acid (dissolved in 0.1% BSA in PBS) 5μl/g per mouse administrated intravenously (i.v.) 30 minutes after intratracheal (i.t.) administration of 10μg/g LPS for 12 h. Flow cytometric results of the spleen; A-CD4, B-CD8, C-CD19+Iaαβ+, D- F4/80+CD11c+. (n=3 mice for PBS group, n=5 mice per group).
4.5 Lung flow cytometry analysis

Flow cytometry analysis of BAL fluid and spleen showed no statistically significant differences between the groups (Figure 6, 7, 9, 11). Because of this reason, we analyzed whole lungs. The gating strategy was adapted (49) according to published data for an improved detection of the major immune cells. However, also with the improved settings, we did not find any significant effects of the inhibitors in the distribution of cells in the lungs of the mice (Figure 12 A, B, C, D).

Figure 12 Whole lungs flow cytometry analysis.
Mice were pretreated with saline, placebo, everolimus and NVP-BEZ 235. After 24 h intratracheal (i.t.) administration of 10μg/g LPS. Flow cytometric results of the lung; A-CD4, B-CD8, C-CD19+Ia+b+, D-F4/80+CD11c+. (n=3 mice for PBS group, n=5 mice per group).
5 Discussion
The most generally accepted definition of ALI and its more severe form ARDS was published by the AECC in 1994 after three decades later Ashbaugh and colleagues described ARDS (2). The pathogenesis of ALI is complex and was defined by several interconnected features with loss of the alveolar-capillary barrier, alveolar epithelium injury, neutrophil and macrophages influx and fibrin deposition (50). LPS is the most widely used endotoxin to generate ALI in animal models and is recognized by TLR4 (4).

PI3K/AKT/mTOR pathway which is a signaling pathway, regulates metabolism, growth, proliferation and apoptosis, is involved in TLR signaling (8; 21). However, the exact role of mTOR pathway in TLR activation by LPS is not known yet and only a few studies have explored these mechanisms in ALI with mTOR-inhibitors (8; 9; 22).

mTOR is a serine/threonine protein kinase and a link between oncogenic PI3K signaling and its downstream pathways (23). mTOR kinase exists in two distinct multiprotein complexes called mTORC1 and mTORC2 (15). During the last decades, researchers used rapamycin, which is a fungicide and forms a complex with FKBP12 then binds to and inhibits the activity of mTORC1 (21). They also showed that mTORC1, a complex including mTOR and raptor, controls mRNA translation via S6K1 and 4E-BP1 by effecting cell growth, ribosome biogenesis, autophagy and cellular metabolism, as well as the innate immune system (15; 16). Besides this mTORC2, a complex including mTOR and rictor, regulates the activity of kinase AKT on S473 (serine473) and directly phosphorylates TSC2 to inhibit TSC1/TSC2 complex, thus upregulating mTORC1 activity (21).

mTOR inhibitors, such as rapamycin and its derivatives, are clinically used to prevent allograft rejection after solid organ transplantation and in the treatment of several cancers because of their antiproliferative and immunosuppressive effects (21). However, they have a side effect, known as drug-induced pneumonitis (11).
The development and severity of ALI is related to an interaction between mTOR complex and toll-like receptor complex-4 (TLR-4) (22). Previous studies have shown that LPS exposure was associated with mTOR and activated it during the development of ALI models (8; 9; 22). Our experiments are consistent with these findings, we proved that with S6, which is an important downstream target of mTOR, and used to examine whether LPS stimulation activate mTOR through TLR-4.

In the studies of mTOR biology, rapamycin is the commonly used pharmacological tool to inhibit mTORC1 (18). Previous studies have shown, that i.t. LPS administration activated mTOR and rapamycin blockage plays an important role in inflammatory processes and in the lung tissues of mice (8; 9; 22). However, the effects of rapamycin administration are different with each other in these studies. Wang et al. reported that LPS increased the phosphorylation of S6 but rapamycin blocked the enhancement of phosphorylation, reduced the levels of proinflammatory cytokines in the BAL fluid (TNF-α and IL-6) and decreased the severity of ALI (8). In contrast, another study revealed that rapamycin increased LPS-induced lung injury and apoptosis, but reduced neutrophils and TNF-α in the BAL fluid (9). In the study of Lorne et al. the production of proinflammatory mediators is increased by mTORC1 activation and the severity of lung injury is decreased by rapamycin (22).

Rapamycin has been implicated in the development of ALI, however, everolimus has not been evaluated in a murine model of pneumonitis. Everolimus is an other inhibitor of mTOR and currently in use after solid organ transplantation to prevent allograft rejection and in the treatment of advanced RCC (11). Moreover, it is completely unknown whether the novel active-site PI3K/mTOR inhibitor NVP-BEZ235 shares the DIP-inducing activities with classical mTOR inhibitors. Thus, we used the LPS-induced ALI murine model to investigate the role of everolimus and NVP-BEZ3235 in ALI. The molecular mechanism how mTOR inhibitors promote lung injury remains elusive.
In this study, 8-10 week old C57/6 female mice were treated with everolimus (10mg/kg/day), NVP-BEZ235 (20mg/kg/day) or placebo (PBS) by oral gavage on two consecutive days before the induction of murine ALI. After six hours, we found high neutrophil influx in LPS (i.t) or LPS with OA compared to PBS or LPS (i.n.). Because of this reason we chose to evaluate mTOR inhibitors with i.t. LPS alone. We found that LPS and LPS with OA induced a significant increase in total cell, neutrophil and erythrocyte number in the bronchoalveolar lavage fluid after six, 12 and 24 hours.

Besides, in histological examinations, we observed serious lung injury after 24 hours. However, mTOR inhibitors did not affect the severity of ALI in our models. We observed strong pS6 staining in the bronchial cells of the PBS and (i.t.) LPS treated mice. This increase was inhibited by everolimus, but not by NVP-BEZ235. Basal phosphorylation of AKT was detected in some of the bronchial cells, but none of the treatment or LPS can modulate the staining. In contrast, they exerted distinct effects on the expression of the proinflammatory cytokines TNF-α and IL-6 in the lung. After six hours, TNF-α and IL-6 cytokine levels decreased with mTOR inhibitors in the LPS-induced ALI injury model and consistent with other studies (8; 9; 22). We also observed similar results in our 12 hours experiments in the combined LPS and OA model. However, after 24 hours, cytokine levels were enhanced by NVP-BEZ235. In contrast, everolimus did not change or reduce the cytokine levels.

On the western-blots, everolimus dosage was found to be adequate to inhibit mTORC1 which observed by decreased pS6 levels upon LPS/OA administration. In contrast, NVP-BEZ235 was not inhibit neither pS6 nor pAKT levels. After activation of neutrophils with LPS and appearence of the higher molecular weight form of 4EBP-1 after LPS/OA stimulation by TLR-4 we observed shift of 4EBP-1 α and β isoforms to the γ isoform which shows activation of mTORC1. In contrast after treatment we observed α and β isoform separately again.
Flow cytometry analysis in the BALF, spleen and whole lung cells did not show any significant effects in the number of major immune cells between the groups.

In summary, our results proved that mTOR was activated by LPS in our LPS-induced ALI model and both mTOR inhibitors had distinct effects on inflammatory cytokine expression in the lung. In our models the histopathological severity of the ALI was not affected by the tested mTOR inhibitors.
6 List of abbreviations
NSIP  non-specific interstitial pneumonia
OA    oleic acid
p70 S6K ribosomal protein S6-kinase, 70 kDa
pAKT phosphorylated v-Akt murine thymoma viral oncogene homolog
PBS  phosphate buffered saline
PDK1 3-phosphoinositide dependent protein kinase-1
PFA paraformaldehyde
pH  potentiometric hydrogen ion concentration
PI3K phosphoinositide 3-kinase
PIKK phosphoinositide 3-kinase related kinase
PIP2 phosphatidylinositol 4,5-biphosphate
PIP3 phosphatidylinositol 3,4,5-triphosphate
PKB protein kinase B
p.o. orally
PRAS40 proline-rich AKT substrate of 40 kDa
Protor1/2 protein observed with rictor 1 and 2
pS6 phosphorylated ribosomal protein S6
raptor regulatory associated protein of mTOR
RB  respiratory bronchiolitis interstitial lung disease
RCC renal cell carcinoma
Rheb ras-homologue enriched in brain
rictor rapamycin insensitive companion of mTOR
RNA ribonucleic acid
rpm rounds per minute
S serine
S6 ribosomal protein S6
S6K1 ribosomal protein S6 kinase 1
S6K2 ribosomal protein S6 kinase 2
SDS sodium dodecyl sulfate
SDS-PAGE sodium dodecyl sulfate polyacrylamide gel electrophoresis
sec. seconds
siRNA small interfering RNA
TEMED N, N, N', N'-tetramethylethylenediamine
TLRs toll-like receptor signaling
TNF-α tumor necrosis factor-α
TOR target of rapamycin
TSC tuberous sclerosis complex
TSC1 tuberous sclerosis complex 1
TSC2 tuberous sclerosis complex 2
U enzyme unit
μ micro
μg microgram
μl microliter
μM micromol
7 References


8 Curriculum vitae
Personal Information

Name: Sevdican Üstün
Date of birth: 1st March, 1985
Place of birth: Istanbul, Turkey
Nationality: Turkish

Education:

03/2013 - 06/2014
Master thesis at the group of Assoc. Prof. Dr Thomas Weichhart
Institute of Medical Genetics, Medical University of Vienna, Vienna, Austria

2010 – 2014
Master study in Genetics and Developmental Biology
Vienna University, Vienna, Austria

2004 - 2008
Bachelor study in Biology
Ankara University Faculty of Science, Ankara, Turkey