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mTOR inhibitors“

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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

Der PI3K (Phosphoinositid-3-Kinase)-Signalweg aktiviert über AKT die mTOR (mammalian target of rapamycin)-Kinase, welche eine wichtige Rolle für Zellwachstum, -Proliferation und -Überleben und in Entzündungen spielt. Inhibitoren von mTOR, wie beispielsweise Rapamycin, werden zur Vorbeugung gegen die Abstoßung von Organtransplantaten sowie in der Krebstherapie verwendet. Ein bekannter Nebeneffekt ist nichtinfektiöse Pneumonitis. Die molekularen Mechanismen dieser mTOR-Inhibitor-induzierten Lungenschädigungen sind noch nicht erforscht. In dieser Studie wurden der bereits etablierte mTOR-Inhibitor Everolimus sowie der neuartige duale PI3K/mTOR-Inhibitor NVP-BEZ235 in einem Mausmodell von akutem Lungenversagen (ALI) auf ihre Wirkung untersucht. Der Effekt der unterschiedlichen Inhibitoren auf akutes Lungenversagen wurde zu unterschiedlichen Zeitpunkten mittels Quantifizierung von unterschiedlichen Zelltypen und proinflammatorischen Zytokinen in der Bronchoalveolären Lavage bestimmt, sowie durch histopathologische Untersuchungen der Lungen. Durch intratracheale Verabreichung von LPS kam es nach 6, 12 und 24 Stunden zu einem signifikanten Anstieg der absoluten Zellzahl, sowie von Neutrophilen und Erythrozyten in der BAL-Flüssigkeit. Die histologische Untersuchung der Lungen nach 24 Stunden zeigte eine schwerwiegende Lungenschädigung durch interstitielle Ödeme und Infiltration von mononukleären Zellen. In unserem Modell von akutem Lungenversagen konnten wir keine histologischen Veränderungen durch Everolimus und NVP-BEZ235 feststellen. Jedoch verstärkte NVP-BEZ235, nicht aber Everolimus, die Expression von IL-6 und TNF- α nach 24 Stunden. Interessanterweise reduzierten beide Inhibitoren in einem alternativen, durch LPS/Oleinsäure induzierten Modell von akutem Lungenversagen die Ausschüttung proinflammatorischer Zytokine. Zusammenfassend hatten die beiden untersuchten Inhibitoren unterschiedliche Auswirkungen auf die Zytokinexpression in der Lunge, wohingegen die histopathologischen Merkmale des akuten Lungenversagens weitgehend unverändert blieben.

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 ($\text{PaO}_2/\text{FiO}_2$) which is the ratio between partial pressure of arterial oxygen (PaO_2) and the fraction of inspired oxygen by ventilator support (FiO_2) (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 $\text{PaO}_2/\text{FiO}_2$ ratio ≤ 300 and (v) ARDS defined as $\text{PaO}_2/\text{FiO}_2$ ratio ≤ 200 (Table1) (2).

	ARDS	ALI
Timing	Acute onset	Acute onset
Chest X-Ray	Bilateral infiltrates	Bilateral infiltrates
PWP	≤ 18 mm Hg or no clinical data of left arterial hypertension	≤ 18 mm Hg or no clinical data of left arterial hypertension
Oxygenation	$\text{PaO}_2/\text{FiO}_2 \leq 200$ mm Hg	$\text{PaO}_2/\text{FiO}_2 \leq 300$ mm Hg

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.

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

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
	Thickening 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).

Direct	Indirect
Lung infection	Sepsis
Aspiration	Multiple trauma with shock
	Large volume blood replacement

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).

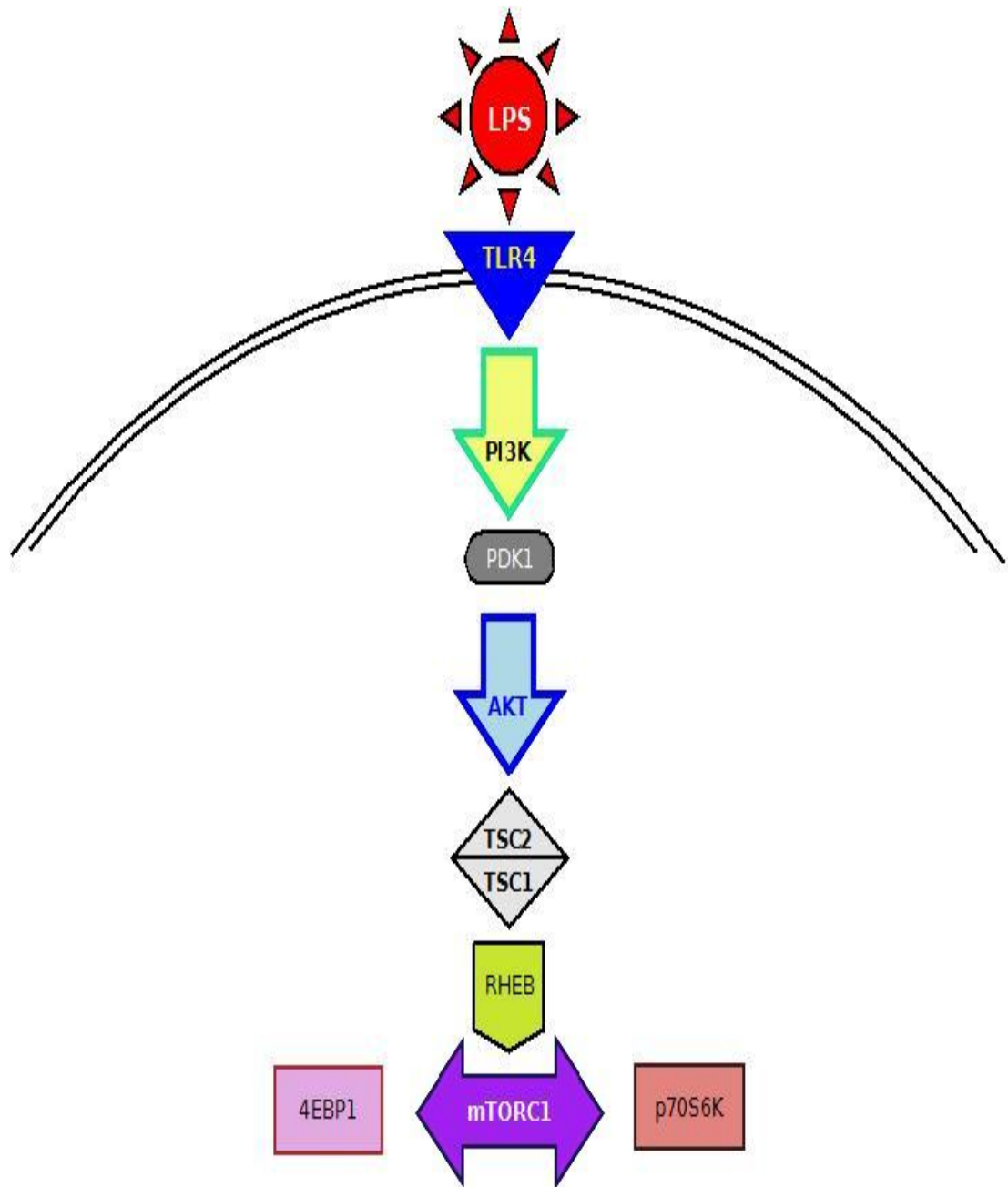


Figure 1 The mTOR pathway and ALI.
 Adapted from Sudarsanam, S. and D.E. Johnson (12) and Weichhart, T. and M.D. Säemann (13)

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).

mTORC1	mTORC2
mTOR Raptor Pras ₄₀ mLST8 Deptor Tti1/Tel2 complex	mTOR Rictor mSIN ₁ mLST8 Deptor Tti1/Tel2 complex Protor1/2

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 PIP₂ (phosphatidylinositol 4,5-bisphosphate) to generate PIP₃ (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).

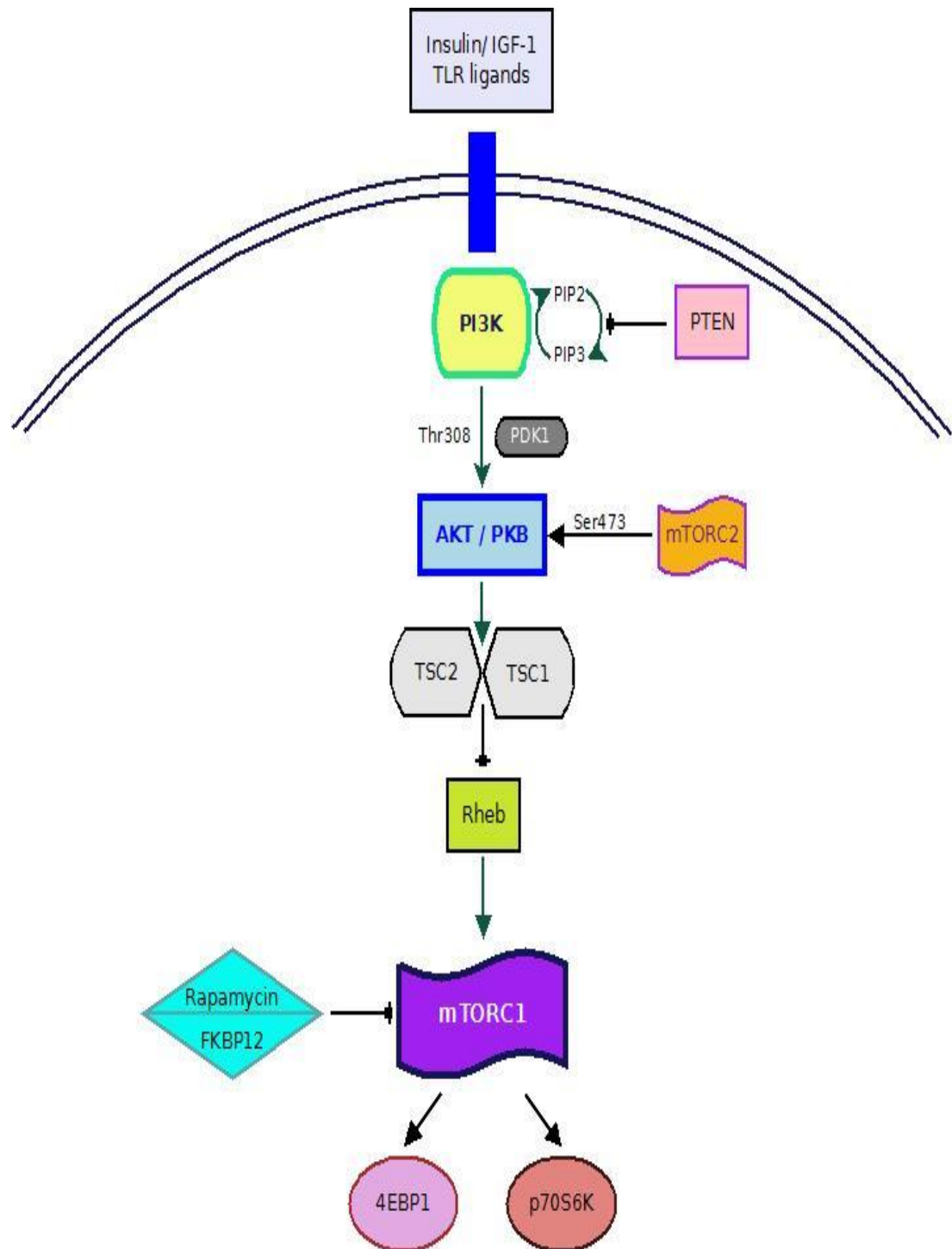


Figure 2 PI3K/AKT/mTOR pathway.
 Adapted from Sudarsanam, S. and D.E. Johnson (12) and Weichhart, T. and M.D. Sæmann (13)

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).

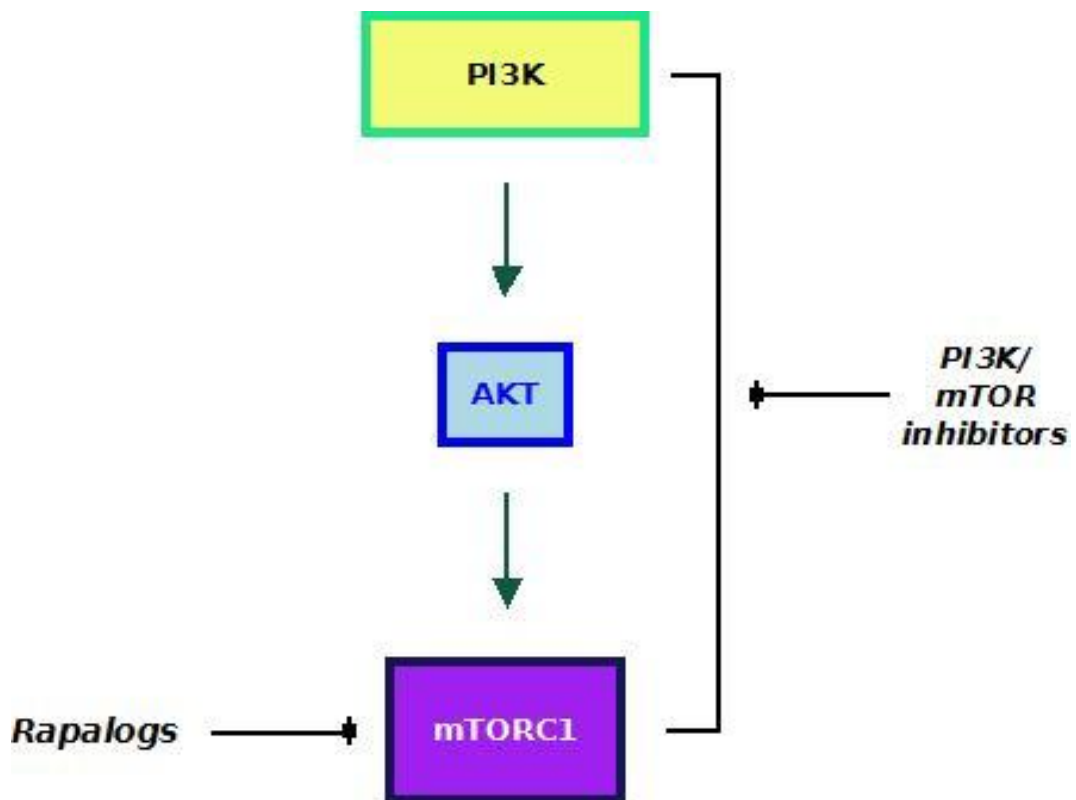


Figure 3 mTOR inhibitors.
Adapted from Sudarsanam, S. and D.E. Johnson (12).

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)-1 β and tumor necrosis factor (TNF)- α , whose transcription is dependent on NF- κ B 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- κ B (38). For this reason blocking PI3K decreases LPS-induced increases in the activation of NF- κ B 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-1 β , 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 *LIPOPOLYSACCHARIDE (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 CO₂ 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- α , 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

10xRBC (Red Blood Cell) Lysis Buffer pH7.3

- 82.6g Ammonium Chloride
- 10g Potassium Bicarbonat
- 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 (*applichem*)
- 899 ml dH₂O

50x Phosphatase inhibitor

- 500 mM NaF
- 100 nM $\text{Na}_4\text{P}_2\text{O}_7$
- 100 mM Glycerophosphat
- 10 nM Na_3VO_4
- Store at 4°C

10x Protease inhibitor

- 1 Protease inhibitor cocktail tablet
- 2 ml dH_2O
- 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 dH_2O

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
- dH_2O 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

	Stack (10ml)	10% (10ml)
30% AA/Bis (Sigma)	1.33 ml	3.33 ml
0.5 M Tris-HCl, pH6.8	2.50 ml	-
1.5 M Tris-HCl, pH8.8	-	2.50 ml
10% SDS	0.10 ml	0.10 ml
dH ₂ O	6.07 ml	4.07 ml
TEMED (Biorad)	10 µl	10 µl
10% APS (Amersham)	50 µl	50 µl

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 Ketazol/ Xylazol. 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 $5 \cdot 10^5$ 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.

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

Table 7 Antibodies used for flow cytometry.

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).

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 ddH₂O (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% H₂O₂ 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.

Primary antibodies	Dilution	Origin	Company
p-S6 (Ser240/244)	1:600 in PBS	Rabbit	Cell Signaling
p-AKT (Ser473)	1:400 in PBS	Rabbit	Cell Signaling
Secondary antibodies	Dilution	Origin	Company
Biotinylated Anti-Rabbit IgG (H+L)	1:500 in PBS	Horse	Vector

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+I HRP conjugated antibodies (Table 9) and developed using peroxide-luminol enhancer solution (Thermo Fisher Scientific) with a medical x-ray processor machine (Kodak).

Primary antibodies	Dilution	Origin	Company
pAKT (Ser473)	1:2000 in PBS-T + 0.02% NaN ₃	Rabbit	Cell Signaling
panAKT	1:1000 in PBS-T + 0.02% NaN ₃	Mouse	Cell Signaling
pS6 (Ser240/244)	1:2000 in PBS-T + 0.02% NaN ₃	Rabbit	Cell Signaling
PanS6	1:1000 in PBS-T + 0.02% NaN ₃	mouse	Cell Signaling
4EBP1	1:1000 in PBS-T + 0.02% NaN ₃		Cell Signaling
Secondary antibodies	Dilution	Origin	Company
Anti-rabbit	1:10000 in 4% dry milk+ PBS-T	Goat	Bethyl laboratories
Anti-mouse	1:10000 in 4% dry milk+ PBS-T	Goat	Bethyl laboratories

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)

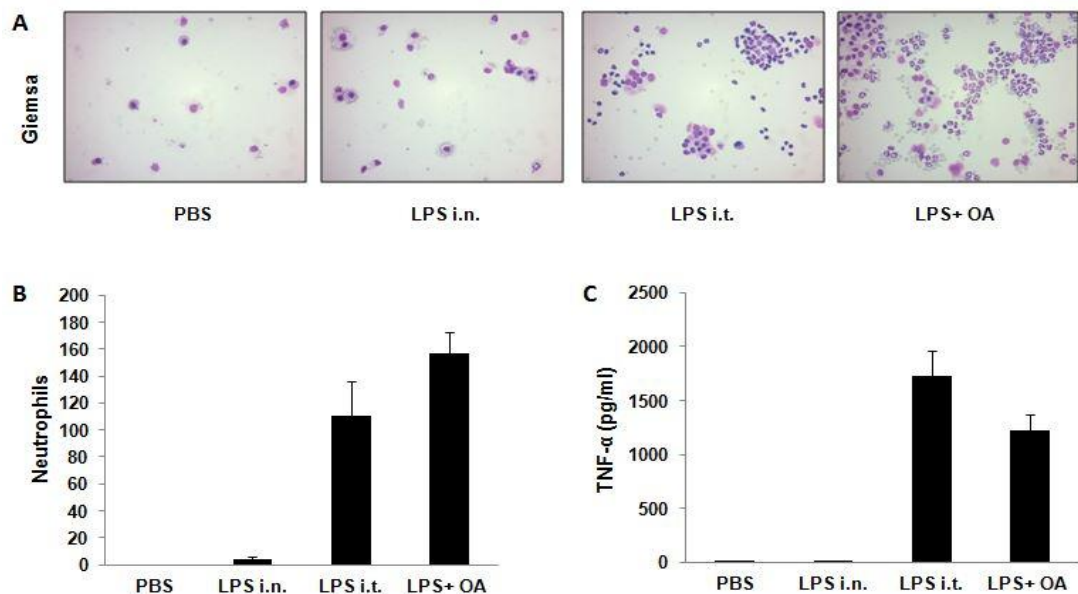


Figure 4 Establishment of acute lung injury model. After 6h, saline or 5ng/g (i.n.), 10µg/g LPS (i.t.), 2% oleic acid (dissolved in 0.1% BSA in PBS) 5µl/g per mouse administrated intravenously (i.v.) 30 minutes after (i.t.) administration of 10µg/g LPS. Panel A shows the representative Giemsa stained 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).

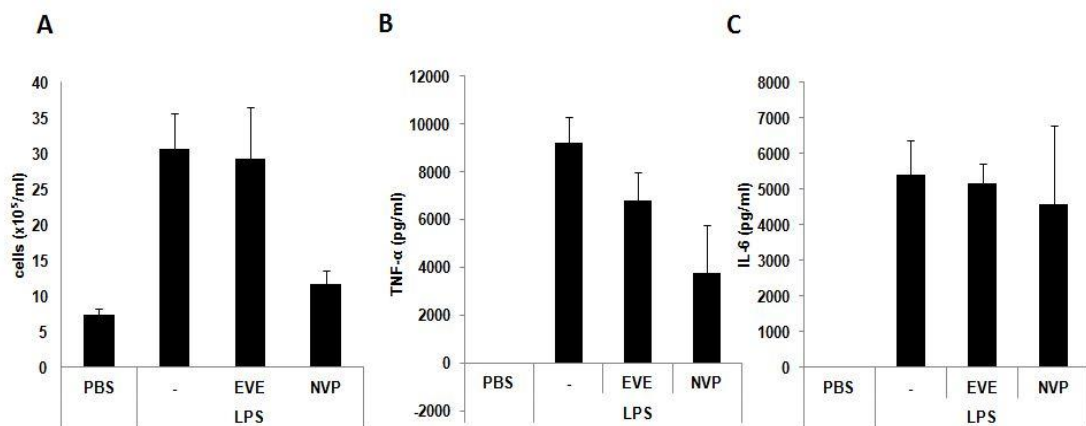


Figure 5 Treatment with mTOR inhibitors after ALI for 6 hours.

Mice were pretreated with saline, placebo, everolimus and NVP-BEZ 235. After 6 h intratracheal (i.t.) administration of 10 μ g/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).

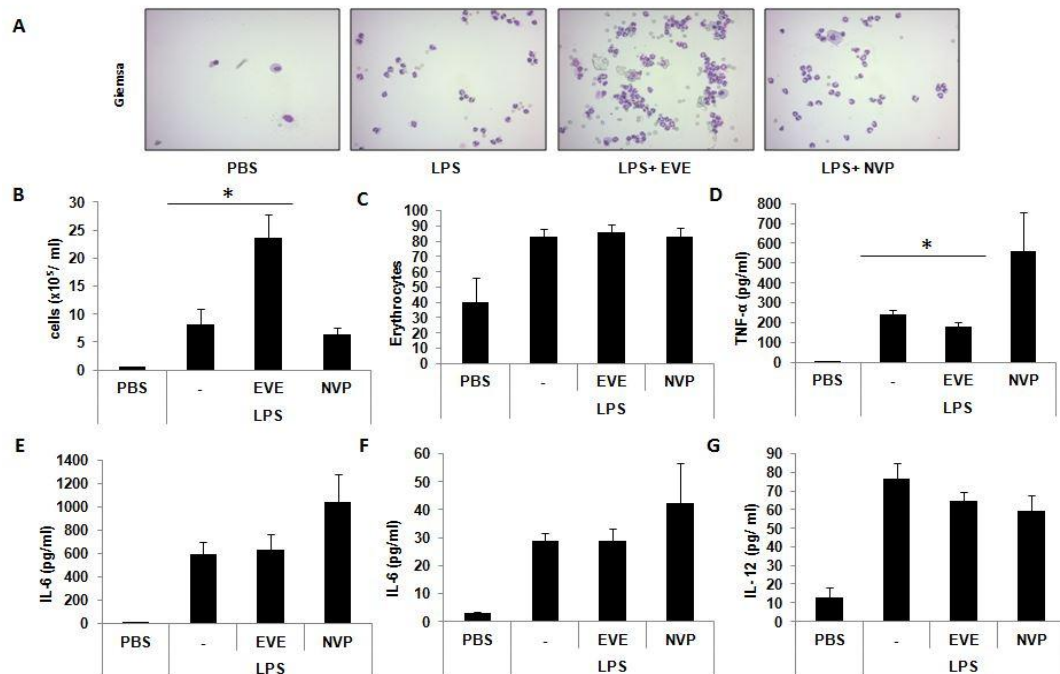


Figure 6 24 hours LPS-induced ALI changes the effects of the drugs. Mice were pretreated with saline, placebo, everolimus and NVP-BEZ 235. After 24 h (i.t.) administration of 10 μ g/g LPS. Panel A shows the Giemsa stained sections of BALF cytospin slides. Panel B shows the total cell numbers counted 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).

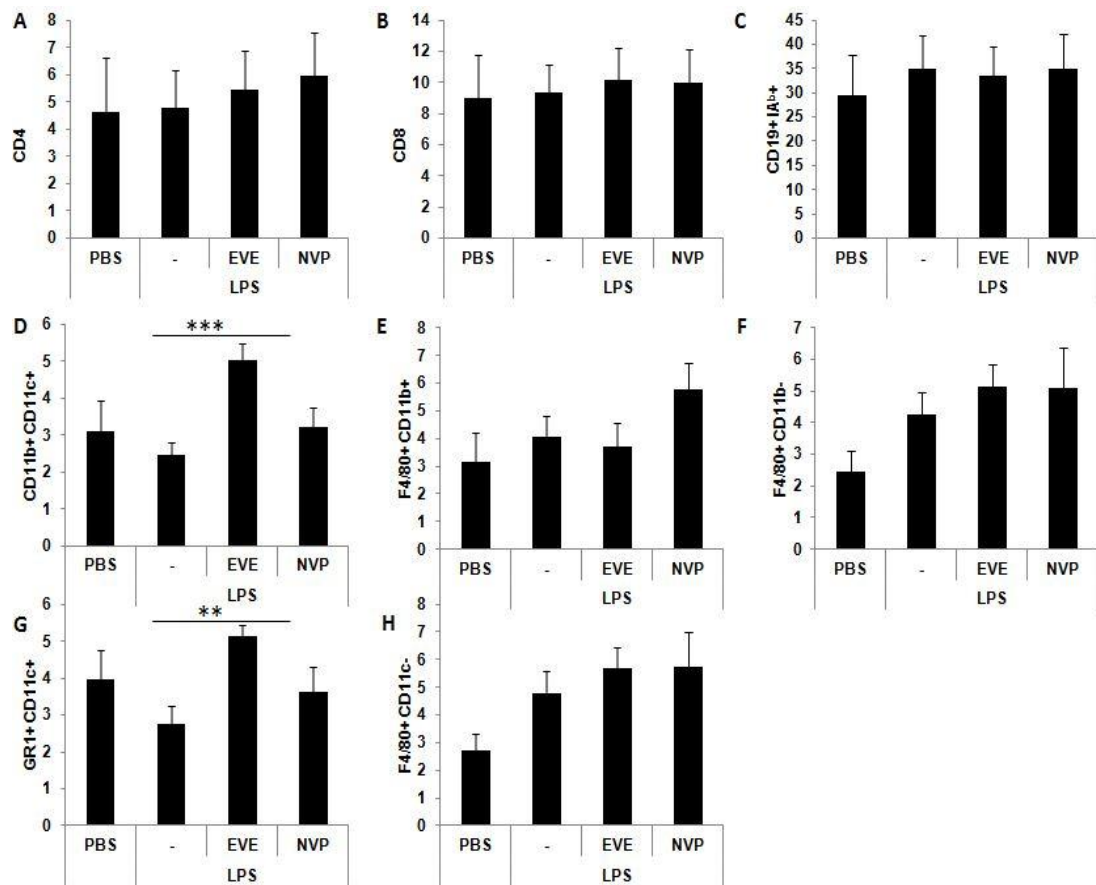


Figure 7 24 hours LPS-induced ALI changes the effects of the drugs. Mice were pretreated with saline, placebo, everolimus and NVP-BE235. After 24 h (i.t.) administration of 10µg/g LPS. Flow cytometric results of the spleen; A-CD4, B-CD8, C-CD19+Ia^b+, D-CD11b+CD11c+, E-F4/80+CD11b+, F-F4/80+CD11b-, G-GR+CD11c+, H-F4/80+CD11c- (n=6 mice for PBS group, n=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).

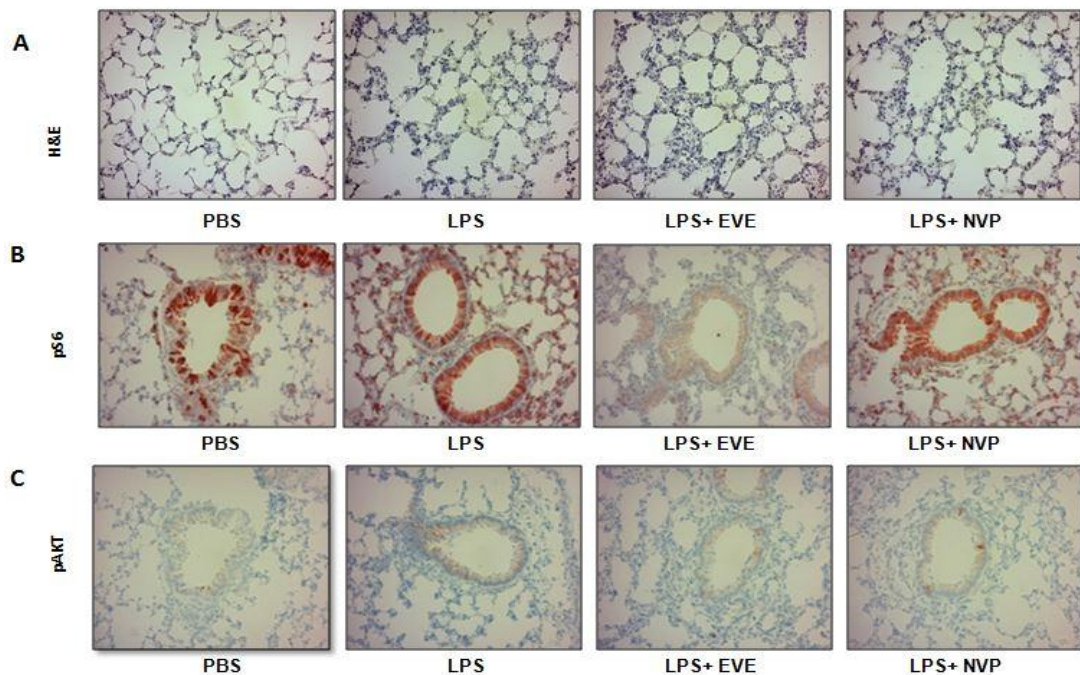


Figure 8 Histological examination of the lung in the 24 hours (i.t.) LPS-induced ALI model. Mice were pretreated with saline, placebo, everolimus and NVP-BEZ 235. After 24 h (i.t.) administration of 10µg/g LPS. Panel A shows H&E stained sections of lung slides. Panel B and C show immunohistochemical 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-BE235 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).

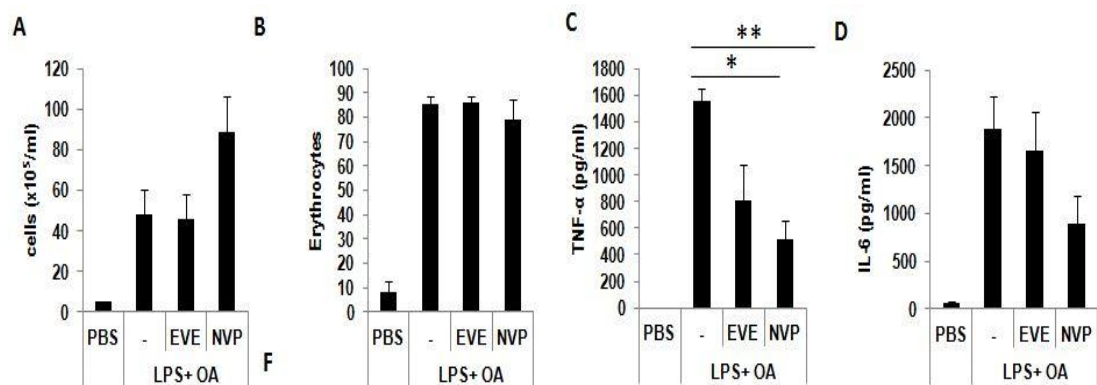


Figure 9 LPS and OA induced lung injury model.

Mice were pretreated with saline, placebo, everolimus and NVP-BE235. 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 α and β isoforms to the γ isoform of 4EBP1 compared to LPS/OA alone (Figure 10 E).

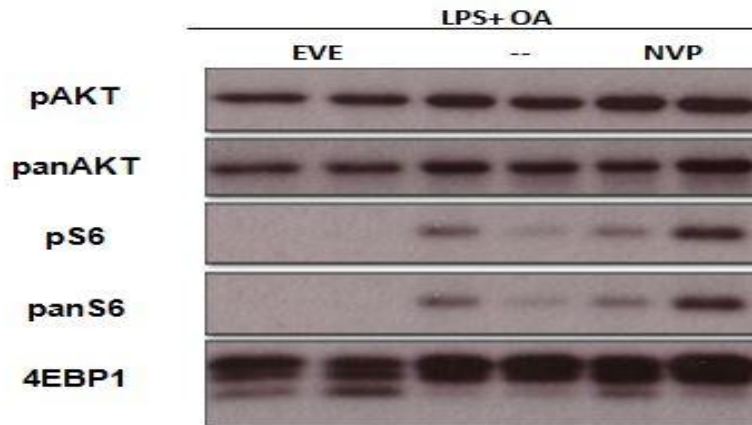


Figure 10 WB analysis with LPS and OA induced lung injury model. Mice were pretreated with placebo, everolimus and NVP-BEZ 235. 2% oleic acid (dissolved in 0.1% BSA in PBS) 5 μ l/g per mouse administrated intravenously (i.v.) 30 minutes after intratracheal (i.t.) administration of 10 μ g/g LPS for 12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

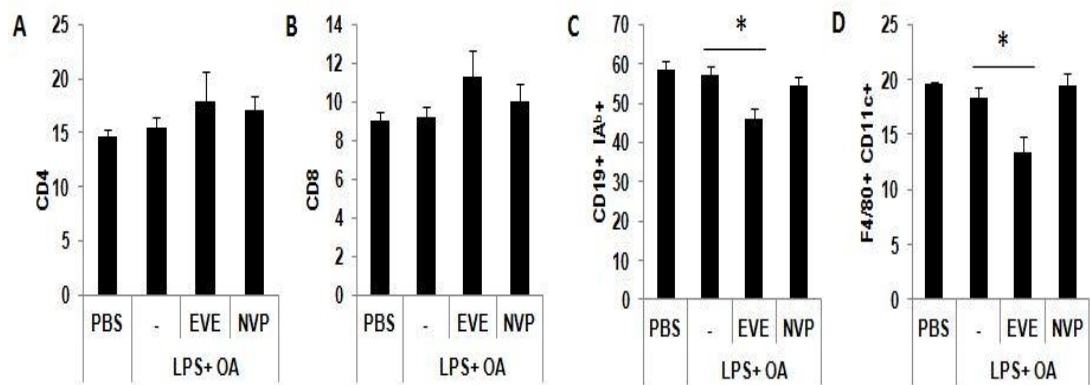


Figure 11 Flow cytometry analysis with LPS and OA induced lung injury models. Mice were pretreated with placebo, everolimus and NVP-BEZ 235. 2% oleic acid (dissolved in 0.1% BSA in PBS) 5 μ l/g per mouse administrated intravenously (i.v.) 30 minutes after intratracheal (i.t.) administration of 10 μ g/g LPS for 12 h. Flow cytometric results of the spleen; A-CD4, B-CD8, C-CD19+Ia^b+, 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).

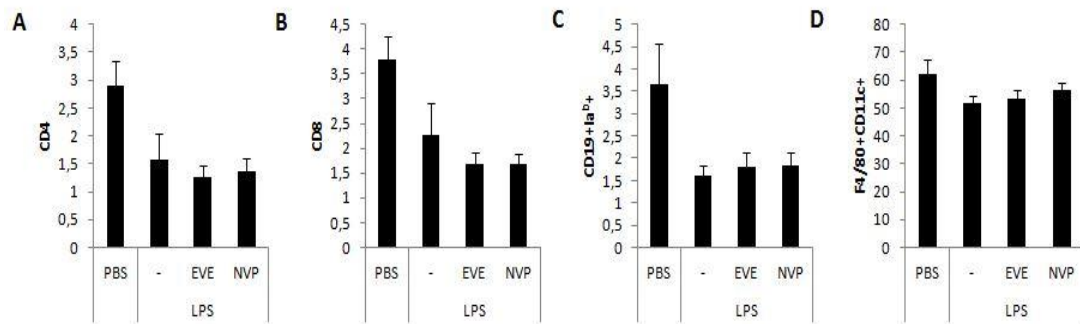


Figure 12 Whole lungs flow cytometry analysis.

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-BEZ235 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 appearance 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

%	percentage
°C	degree Celsius
4E-BP1	eukaryotic initiation factor 4E (eIF4E) binding protein 1
AECC	American-European consensus conference
AIP	acute interstitial pneumonia
ALI	acute lung injury
ARDS	acute respiratory distress syndrome
BAL	bronchoalveolar lavage
BALF	bronchoalveolar lavage fluid
COP	cryptogenic organizing pneumonia
DAD	diffuse alveolar damage
Deptor	DEP-domain-containing mTOR-interacting protein
DIP	drug-induced pneumonitis
DNA	deoxyribonucleic acid
eIF4E	eukaryotic translation initiation factor 4E
FACS	fluorescence activated cell sorting
FDA	food and drug administration
FKBP12	FK506-binding protein 12
FRB domain	FKBP12-rapamycin binding domain
g	gram
g	g-force
Gβ1/mLST8	G-protein β-subunit like protein/mammalian LST8
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IGFs	insulin-like growth factors
IL-6	interleukin-6
IL-12	interleukin-12
i.n.	intranasal
i.v.	intravenous
i.t.	intratracheal
IPF	idiopathic pulmonary fibrosis
kDa	kilodalton
l	litre
LBP	lipopolysaccharide-binding protein
LIP	lymphoid interstitial pneumonia
LPS	lipopolysaccharide
M	Mol
mg	milligram
min.	minutes
ml	millilitre
mM	millimol
mLST8	mammalian lethal with sec13 protein 8
mRNA	messenger RNA
mSin1	mammalian stress-activated protein kinase interacting protein 1
mTOR	mammalian target of rapamycin
mTOR-I	mTOR inhibitor
mTORC 1	mammalian target of rapamycin complex 1
mTORC 2	mammalian target of rapamycin complex 2
MW	molecular weight
NIP	non-infectious pneumonitis

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-bisphosphate
PIP3	phosphatidylinositol 3,4,5-trisphosphate
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
μ g	microgram
μ l	microliter
μ M	micromol

7 References

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8 Curriculum vitae

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