universität wien

MASTERARBEIT / MASTER'S THESIS

Titel der Masterarbeit / Title of the Master 's Thesis

„Model of the inflamed lung epithelium using Calu-3 cells "

verfasst von / submitted by

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angestrebter akademischer Grad / in partial fulfilment of the requirements for the degree of

Magistra pharmaciae (Mag. pharm.)

Wien, 2023 / Vienna, 2023

Studienkennzahl lt. Studienblatt / degree programme code as it appears on the student record sheet:	UA 066 605
Studienrichtung lt. Studienblatt / degree programme as it appears on the student record sheet:	Masterstudium Pharmazie
Betreut von / Supervisor:	Ass.-Prof. Dipl.-Ing. Karin Ortmayr, PhD

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Abstract

The lung is among the most important organs of the human body and at the same time frequently affected by bacterial and viral infections. There are several available models, both *in vivo* and *in vitro*, that can be utilized for the investigation of potential bioactive substances targeting lung infections. However, in many cases they do not simulate the effect of inflammation on host cells like the lung epithelium.

Therefore, the aim of this work was to establish a model that simulates the inflamed lung epithelium and on which plant extracts and various substances can be tested. To that end, the epithelial cancer cells Calu-3 were grown in high-density monolayer cultures, following an approach commonly used in lung models. Inflammation was induced by lipopolysaccharides (LPS), which are components of the cell wall of gram-negative bacteria and can lead to the release of cytokines such as IL-6, IL-8, and TNF-α through activation of different signalling pathways such as NF-κB. IL-6 concentrations were determined using ELISA as a marker for inflammation. The measurements showed that the LPS concentration of 10 µg/mL produced the desired effect of IL-6 production, and this concentration also led to a reduction in cell number and influenced viability.

Moreover, this work investigated whether this model can be used to test bioactive natural compounds. For this purpose, Sanggenon C, Sanggenon D, and MAC 130, which are derived from the root bark of *Morus alba*, were applied on the Calu-3 monolayer with and without LPS. IL-6 measurements showed that Sanggenon D and MAC 130 can reduce the IL-6 concentration, but Sanggenon C leads to an increase in the concentration of IL-6. Overall, our findings demonstrate that in vitro models of the lung epithelium can respond to inflammatory stimuli and allow studying the impact of active compounds on complex inflammatory response, enabling the discovery inflammatory modulators.

Zusammenfassung

Die Lunge ist eines der wichtigsten Organe des Körpers, ist jedoch auch häufig der Ort von bakteriellen und viralen Infekten. Für die Untersuchung von möglichen Wirkstoffen zur Behandlung von Lungeninfektionen stehen *in vivo*- und *in vitro*-Modelle zur Verfügung, jedoch ist eine begrenzte Auswahl an *in-vitro*-Modellen für die Erforschung von entzündetem Lungenepithel verfügbar. Ziel dieser Arbeit war es, ein Modell zu etablieren, das das entzündete Lungenepithel simuliert, um die antiinflammatorische Aktivität von potentiellen Wirkstoffen zu testen. Für dieses Modell wurde die epitheliale Krebszelllinie Calu-3 verwendet, die sehr gut charakterisiert ist und häufig in Lungenmodellen eingesetzt wird. Lipopolysaccharide (LPS) wurden verwendet, um in dem Modell eine Entzündung auszulösen, da diese Bestandteile der Zellwand gramnegativer Bakterien über die Aktivierung verschiedener Signalwege, wie zum Beispiel NF-κB, zur Freisetzung von Zytokinen wie IL-6, IL-8 und TNF-α in den Calu-3-Zellen führen. Als Merkmal der zellulären Entzündungsantwort wurde die Konzentration von IL-6 mittels ELISA bestimmt. Anhand der Messungen war erkennbar, dass eine LPS-Konzentration von 10 µg/ml eine Steigerung der IL-6 Produktion in Calu-3 Zellen hervorrufen kann, zudem wurde eine Verringerung der Zellzahl und Viabilität der Zellen beobachtet.

Weiters wurde ermittelt, ob das Model des entzündeten Lungenepithels zur Analyse der antiinflammatorischen Wirkung von Naturstoffen verwendet werden könnte. Aufgrund dessen wurden die Calu-3 Zellen mit den prenylierten Flavonoiden Sanggenon C, Sanggenon D und MAC 130, einem mit Sanggenonen angereicherten *Morus alba*-Extrakt, mit und ohne LPS behandelt. Anhand der IL-6 ELISA Messungen wurde erkennbar, dass Sanggenon D und MAC 130 die IL-6 Konzentration reduzieren, Sanggenon C diese hingegen steigert.

Im Großen und Ganzen zeigen unsere Ergebnisse, dass *in vitro*-Modelle des Lungenepithels auf Entzündungsreize reagieren können und es ermöglichen, die Auswirkungen von Wirkstoffen auf komplexe Entzündungsreaktionen zu untersuchen, was die Entdeckung von Entzündungsmodulatoren ermöglicht.

1. Introduction

1.1. Lung function, structure, and diseases

The lung is among the most important organs of our body. The main task of the lung is to supply the body with oxygen and to excrete carbon dioxide.[1,2,3] The lung obtains oxygen from the environment, and use it for the aerobic cellular respiration, where ATP is produced for further processes.[1] After ATP production, CO_2 is excreted with other metabolic products.[1,2]

The lung is organized in different compartments to exert its several functions. It consists of a left and right part, which are divided into two and three lobes, respectively.[3,4,5,6] These lobes are subdivided again, with each lobe consisting of many small alveoli where gas exchange takes place.[3,4] Within the lobes, bronchial arterial segments can be delineated, which are supplied by a centrally located bronchus. The segments can be further divided into lobules, which are then subdivided into bronchioles and acini.[3,4,6] Acini are the entirety of the alveoli and are connected to a bronchiolus terminalis.[3,4] Due to this multitude of compartments, different points of attack and lung diseases are present.

In particular, the lung is frequently affected by infectious diseases which can lead to severe course of disease and death.[7,8] Several infectious lung diseases are known, including bacterial and fungal pneumonias, parasitic diseases and viral infections. Bacterial pneumonias are amongst the most widespread infectious diseases in the lung.[7,8] *Streptococcus pneumonia*, *Haemophilus influenzae*, and *Klebsiella species* are the most common causes of bacterial pneumonias, which exhibit different symptoms and severeness.[7,9] *Mycobacterial pneumonias* also play an important role.[7,8] Mycobacteria are divided into two groups, namely the *Mycobacterium tuberculosis* complex and non-tuberculous mycobacteria (NTM).[7,10] While the number of NTM infections rise worldwide, the tuberculosis rate decreases.[7,10] However, infections caused by *M. tuberculosis* and *Mycobacterium avium-intracellulare* remain the most common mycobacterial pneumonias.[7] *M. tuberculosis* is the most virulent form, with which one third of the population world-wide is infected and nearly 13.7 million (0,17% of the population) of them show an active disease.[7] The active form is mostly seen in African and Asian countries, Western countries like the United States and Western Europe exhibit low rates.[7,10]

One of the main causes of mortality and morbidity are parasitic diseases such as pulmonary schistosomiasis, dirofilariasis, paragonimiasis, strongyloidiasis, and echinococcosis, showing increased numbers due to illnesses like HIV but also because of climatic factors.[7] Parasitic organisms can have an impact on the pulmonary system during their migration phase without even reaching a specific destination.[7] Furthermore, viral infections can also cause lung diseases.[9] Viruses are separated into two groups: DNA- and RNA viruses.[9,11] Examples for the DNA viruses are *adenoviruses*, *herpesviruses* and *bocaviruses*.[9,11] *Orthomyxoviridae*, *paramyxoviridae*, *picornaviridae* and coronaviruses are RNA viruses, which can be responsible for lung diseases.[9,11] Among the *Orthomyxoviridae* are Influenza viruses, which can be divided in 4 groups (A, B, C and D) and are responsible for seasonal epidemics.[9,11] Sporadic pandemics can be caused by type A of the *influenza virus* which is mostly found in aquatic birds.[9,11] The majority of the influenza-cases are self-limiting and mostly limited to the upper respiratory tract.[11] *Paramyxoviridae* like the human metapneumovirus (hMPV) cause infections usually in the upper respiratory tract, but infections in the lower respiratory tract can also be seen.[9,11]

The most common cause of viral infectious diseases in the lung are *picornaviridae*, which are divided into enteroviruses and parechoviruses.[9] These viruses are the most important cause of common cold, and the symptoms are usually mild and limited to the upper respiratory tract.[9] However, an association with exacerbation of asthma and chronic bronchitis has been seen.[9] Another RNA virus is the coronavirus, which causes 10% to 25% of the recorded colds, but mostly doesn't show a severe course of infection.[9] The group of coronaviruses, which has gained notoriety due to the SARS-Cov-2 pandemic starting in 2019, consists of seven human coronavirus types.[12] Five of the seven types have a mild course (229E, NL63, OC43, and KHU1).[12] The remaining three types (MERS-CoV, SARS-CoV, SARS-CoV-2) can cause severe respiratory infections.[12] The characteristic symptoms are fever and myalgia, which often lead to cough, dyspnoea and also acute respiratory distress syndrome.[9,12]

DNA viruses, such as the adenovirus, can also be the reason for the lung diseases.[9,11] Adenoviruses are often the reason for croup, bronchitis, pneumonia, and epidemic infective conjunctivitis.[9,11] The human bocaviruses, which belong to the category of DNA viruses, can cause respiratory and gastrointestinal illnesses.[9,11] *Herpes simplex* virus (HSV),

cytomegalovirus (CMV), Epstein-Barr virus (EBV), and the *varicella-zoster* virus (VZV) belong to the group of herpesviruses, that can have a lifelong impact on the infected person.[11] The most common form is herpes simplex virus (HSV-1 and HSV-2), which can be found in nearly 70% of the population, whereby HSV-1 itself only affects 20% of the people.[11,13]

1.2. Mechanism of the lung inflammation

Diverse pathogens are inhaled, with the airway epithelium being the first encounter of inhaled pathogens.[14] Mucins, lysozymes, defensins, nitric oxide and lactoferrin are produced by the epithelial cells to unselectively protect against foreign substances such as microbial organisms.[14,15] To mobilise the immune cells, mediators such as reactive oxygen species, cytokines (e.g., TNF-α and IL-1β) and platelet-activating factor are secreted by the epithelial cells.[7]

Furthermore, arachidonic acid is released, leading to the production of eicosanoids, which cause tissue inflammation and mucus secretion from the goblet cells.[14]

In addition to the mentioned mediators, surfactant proteins, which are produced by alveoli, have an important role in the defence.[14] Through the binding of the surfactant proteins on bacterial surface molecules and the modulation of the activation of leukocytes, the opsonization of pathogens is carried out.[14] Opsonisation is used to tag the pathogens for subsequent elimination by phagocytosis.[16] Antigen-presenting cells (APCs) like the Macrophages and Dendric cells belong to the first line defence system, they recognise the pathogens and are responsible for the antigen presentation to the resident T-cells and therefore for the response of the immune system.[14] The second-line defence includes neutrophils, which are the recruited after an injury or infection.[14,17] Neutrophils have the ability to kill the pathogens by secreting degradative enzymes, reactive oxygen species and antimicrobial proteins.[14,17] During a pulmonary infection, the neutrophiles migrate from the pulmonary capillaries into the air space and can carry out their task of eliminating pathogens.[14,17]

Lymphocytes, including T-cells and B-cells, are also responsible for the adaptive immune system (T_H2 cells and T_H1 cells).[14,18] T_H2-cytokines for example promote IgE production and the eosinophilic response in atopy.[14] The immunoglobulin IgE is responsible for the induction of degranulation and the release of proinflammatory mediators like

prostaglandins and histamine, which increase vascular permeability, inflammatory cell infiltration and bronchoconstriction.[14,19] Another protection produced by the plasma cells is the immunoglobulin IgA, which has antiviral and antimicrobial effects in the epithelial.[14,20] It is seen that COPD patients have a decrease of IgA, which is a part of the first line defence, in their bronchial secretions and saliva.[14,20]

Inflammation of the lungs occurs through several mechanisms and active substances that are released by the cells. However, the exact mechanism, as well as the influences of the different cytokines and immunoglobins, is not yet fully understood. In addition, biological and chemical substances can have an impact on the release and activation of the protective mechanisms, which can be investigated with the help of various *in vitro* models.

1.3. *In vitro* models for the simulation of the lung epithelium

Because the human lung has a complex mechanism, there are various systems available *in vivo* and *in vitro* to simulate the lung function and to develop active substances and different types of drugs. The majority of the *in vivo* experiments are based on rodent studies or tissue samples obtained from human lungs, which are often difficult to interpret or apply to the general public due to the impairment caused by lifestyle and various biological factors.[21] Because of this, there are several *in vitro* models that should circumvent the problem of individualism and give a general picture of the impact of candidate compounds on the lung epithelium during inflammation. The most common types of *in vitro* models of the lung and lung epithelia are 2D cell cultures, self-assembling 3D cultures, lung-on-chips, biological scaffolds, and bioengineered scaffolds.[21,22,23]

1.3.1. 2D cell culture

The cultivation of lung epithelial cells in a two-dimensional monolayer is one of the oldest methods to simulate a functional epithelium, with the main advantage that 2D cell cultures are fast and easy to generate.[21,23,24] To that end, cell lines are grown on a plastic surface submerged in media and can then be used for experiments, e.g. to test the impact of added bioactive substances on epithelial function and/or health.[21,23,24]

To create different 2D *in vitro* models, e.g. Transwell inserts can be used.[24,25] In Transwell inserts, lung cell mono- or co-cultures are grown and maintained under water or an aqueous milieu.[24,25] Using this method, cells of the air-liquid interface (ALI) can

be simulated. The ALI in the lung plays a crucial role in maintaining a functional barrier between the atmosphere and the fluid-containing tissues, by allowing the exchange of oxygen and carbon dioxide between the lungs and the bloodstream.[21,24] The complex structure of the lung, including the branched airways and blood vessels, provides a large surface area for this exchange to occur efficiently.[21,24] The ALI barrier is recreated in *in vitro* models to gain a simulation of the epithelium.[21,24] Cells are cultured on a porous filter in the ALI system.[21,24] This filter acts as a physical barrier between the lung epithelial tissue and the underlying media.[21,23] This arrangement enables the cells' surface to encounter the surrounding air, while the basal surface of the cells receives nutrients and other media additives through diffusion across the porous membrane.[21,23,24]

1.3.2. Scaffold based cell culture

The extracellular matrix (ECM) within the natural lung is highly complicated, playing a crucial role in shaping the environment of cells.[21,24] It regulates the availability of soluble growth factors, gives structure, and governs cell migration and adhesion.[21,23] *In vitro* lung models, natural or artificially engineered 3D scaffolds can be utilized to facilitate cell organization, proliferation, and differentiation, thereby creating a physiologically relevant microenvironment.[21,23,24]

Cell-free ECM can be obtained from the whole lung or from sections and fragments of the lung, and can subsequently be re-populated with cells of the epithelium or endothelium.[21,24] Afterwards, the recellularized matrices can be cultured in dedicated bioreactors.[21,23,24] Bioreactors are used for the controlled cultivation of living cells in a specifically generated environment depending on the cultivated cell type.[26] Scaffold-based cell culture methods thus allow obtaining a realistic ECM environment and a close imitation of lung geometry.[21,24] The use of cell-instructive material is an advantage because of the possibility to control the cellular reactions.

Self-assembling 3D cultures are an advanced version of scaffold-based cell culture. The technology for creating organoids, or more specific for the lung bronchospheres, is based on the isolation of basal stem cells from human or mouse epithelial tissue.[21,24,27] These cells are then embedded in a 3D ECM gel and after a short time spherical clonal colonies (organoids) form in the culture.[21,24] This method of creating organoids makes

it possible to study organ development and function as well as intercellular communication trough the simulation of physiological processes of the lung and the 3D architecture of the tissue.[24,27]

1.3.3. Lung-on-Chip Models

Organ-on-chip (OOC) models are new developments that enable the creation of a three dimensional microfluidic system that can simulate the biological system and cell structure of different organs, including the lung.[24,28,29,30] By using specific cell types, the replication of the functional units of the lung can be achieved.[24,28,30] Alveolar epithelial cells and lung endothelial cells can be cultivated on both sides of a thin membrane, which is utilized as a cultivation surface.[24,28] When they come into contact with the blood and air flow, mechanical stretching takes place, imitating physiological respiration.[24,28] These models can be modified to simulate disease states to study defense mechanisms and can be applied in therapeutic screening.[29,30] In addition, OOC microsystems are suitable for the 3R principle (reduce, replace, refine), as they are non-animal models, but a reproducible model of the organs, as well as the lungs, can be acquired.[24]

The first generation of OOC systems was based on silicone, however, these models were more complicated and expensive to produce than the newer ones.[24,30] The new generation of OOC models are mostly based on polymers like polydimethylsiloxane (PDMS), as this is particularly suitable for tissue and cell culture due to its chemical-physical properties.[24,29,30] Especially in lung models, where gas permeability is desired, this can be ensured with the help of this polymer, which also has an optical transparency that is useful for visualising living cells.[24]

All in all, OOC models and especially lung-on chip models are gaining popularity due to the large number of advantages and the possibility to avoid or reduce the usage of laboratory animals.

1.3.4. Negative and positive aspects of *in vitro* models of the lung epithelia

A variety of different in vitro models for simulating the lung epithelium are available. These are used depending on the need and the aspect being investigated.[24,28] The advantages and disadvantages of these are illustrated in table 1.

Table 1. Advantages and limitations of different In vitro models of the lung epithelium.

In vitro models	Advantages	Limitations	References
2D-cell culture model	• Simple, responsible, and low cost • No ethical issues • High-throughput and real-time monitoring • Unlimited cell supply • Possibility to mimic lung ALI	• Biochemical and biomechanical limitations • Usage of single cell types • Simulation of 3D tissue is not possible	[24,28]
Self-assembling 3D cultures/ organoids	• Applicable to multiple cell types • Presence of cell-cell and cell-ECM communication • Simulation of physiological processes of the lung and the 3D architecture of the tissue • No ethical issues • Real-time monitoring	• Expensive • Absence of haemodynamic system • Standard protocols are not available and therefore no unity • Absence of immune system and mechanical simulation • Fully differentiated phenotype difficult to achieve	[24,28]
Scaffold based cell culture	• Variations in the chemical composition and geometry of 3D matrices • Control of the cellular reaction using cell-instructive materials • Numerous diverse synthetic and natural materials for manufacture	• Complications in the artificial replication of the pulmonary 3D structure • Impairment of the biocompatibility of the scaffold due to the use of organic solvents • Expensive	[24]
Lung-on chip models	• Imitation of the lung microenvironment • Haemodynamic and immune system available • Sensory monitoring of biological parameters • Visualisation of multiple compartments of lung tissue • Possibility of inter-organ communication • No ethical issues	• Relatively new model • Immune compartment often absent • Highly complex • low throughput • Co-culture with multiple cell types is challenging • Standardization of the protocols is missing	[24,28]

1.4. Cell lines used in *in vitro* lung models

A diversity of continuous epithelial cell lines, which are immortalized or tumour cells, are available for *in vitro* experiments.[25,31] Continuous cell lines are derived from one donor and have a prolonged lifespan compared to primary cells.[31] Often used epithelial cell lines are the tumour cell lines Calu-3 and A549 and the bronchial epithelial cell lines 16HBE14 and BEAS-2B.[31,32,33]

The immortalized BEAS-2B cell line was generated from human bronchial epithelial cells by the usage of AD12-SV40 virus.[31] The 16HBE14 cells were obtained from human bronchial epithelial through transformation with SV40.[31] A549 cells derived from type II pneumocyte lung tumour have the characteristics of type 2 alveolar epithelial cells (AEC2).[31,33] They are not able to produce surfactant but are often used as a model for alveolar epithelial cells. In addition, no tight junctions are formed upon ALI exposure.[31,33]

The Calu-3 cell line (American Type Culture Collection, ATCC HTB-55), obtained from a 25-year-old Caucasian and in use since 1975, is a well-characterised cell line and is derived from human bronchial mucosa. [25,34,35] Mucins, surface fluids and immunologically active substances are present in the submucosal glands of the lung and also in Calu-3 cells.[25] The Calu-3 cells, which originate from the submucosal adenocarcinomas, can form confluent monolayers with tight junctions and desmosomes, and the expression of the CFTR protein is recorded.[25,34] In addition, the induction of several CYP enzymes is possible, including CYP2E1, CYP1A1 and CYP2B6, but not CYP3A4.[34] These properties are essential for the experiments, as they simulate the cell properties of epithelial cells, such as intracellular transport, the immune system and enzymatic reactions. In general, Calu-3 *in vitro* epithelial and lung models are used for studies of drug transport and metabolism, due to the formation of tight junctions between the cells and the paracellular transport, differentiation of the layers is present, which is essential for drug absorption and metabolism.[25,35]

1.5. Induction of inflammation and markers

The induction of inflammation in cell culture can be achieved by different methods that depend on the cell type and equipment. Both bacterial and viral infections can be simulated using a variety of stimuli, including bacterial DNA, LPS, flagellin, LTA, and bacteria

in general, whereby it has been observed that components of gram-negative bacteria are mainly responsible for inflammation.[36,37,38,39] Viral infections can be recognised trough the presence of dsRNA, which leads to the activation of multiple pathways like NF-κB, protein kinase R pathway, and mitogen-activated protein kinase (MAPK) pathways.[40] For the measurement of the inflammation various markers can be used. Examples for proinflammatory mediators are IL-6, IL-8, tumor necrosis factor (TNF)-α, IL-1β and the intercellular adhesion molecule-1 (ICAM-1).[36,37,41]

1.5.1. Lipopolysaccharides

Lipopolysaccharides (LPS) are part of the outer membrane of gram negative bacteria like *Escherichia coli*.[36,42] LPS act as pyrogens (i.e. substances that cause fever), and can induce inflammation through the activation of different pathways like the NF-kB pathway.[42,43] Other endotoxins originating from the bacterial cell wall include peptidoglycans and muramyl peptide derivatives.[43]

LPS can bind the Lipopolysaccharide binding protein (LBP), forming a complex that can interact and activate Toll-like receptor 4 (TLR-4).[42] The activated TLR-4 can now bind the cytosolic adapter protein MyD88, which leads to the activation of IκB kinase (IKK) through the recruitment of the kinase IRAK and the activation of TRAF6.[42,41] IKK is a kinase which phosphorylates IκB and enables the release of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB).[42] NF-κB is now able to translocate to the cell nucleus, where it activates genes.[42,36] Their products have an impact on the adaptive and innate immune system and, therefore, on the production of cytokines and the production of IκB, which binds NF-κB and ends the cascade.[42,36]

1.5.2. Interleukin 6

Interleukin-6 is a cytokine which plays a crucial role in the regulation of cell differentiation, proliferation, survival and has an impact on multiple cellular functions.[44,45] IL-6, along with other cytokines, regulates signalling pathways like signal transducer and activator of transcription 3 (STAT3).[44,45] Therefore, IL-6 is stimulated during inflammation and was traditionally seen as an acute phase response activator and a lymphocyte stimulatory factor until an effect on the immune response and

immunity was observed.[45] It can be used as a marker of inflammation, which is the case in the experiment described in this thesis.

1.6. Anti-inflammatory activity of plants

Plants and plant extracts, which are an important component of traditional medicine, are a resource of therapeutically useful compounds that have an anti-inflammatory effect, e.g., by scavenging free radicals or inhibiting lipoxygenase (LOX) or cyclooxygenase (COX).[46,47] Examples of plants that are used based on their anti-inflammatory activity are listed in table 2.

Table 2. Active compounds of Hypericum perforatum, Allium cepa, Juglans regia, Zingiber officinale and Glycyrrhiza glabra.

Medical plants	Active compounds	References
Hypericum perforatum	Hyperforin, pseudohypericin, hypericin, flavonoids (quercetin, amentoflavone) and tannins	46, 48
Allium cepa	Flavonoids (quercetin, fisetin), saponins, tannins, phenolic acids, thiosulfinates, allicin	46,49
Juglans regia	Phenolic acids, tannins, flavonoids(quercetin), ascorbic and malic acid, phosphate and calcium, naphthoquinone derivatives and essential oils	46,50
Zingiber officinale	Alkaloids, terpenoids, saponins and phenolic compounds: curcumins, flavonoids(quercetin), paradols, tannins, gingerols and shogaols	47,51
Glycyrrhiza glabra	Flavonoids, triterpenic saponins (glycyrrhizin), sterols, different sugars, saponoids, amino acids starches, essential oils, and gums	46,52

A diversity of active substances is responsible for the inhibition or reduction of proinflammatory mediators. *Allium cepa* (Onion), for example, contains various compounds like flavonoids, saponins, and tannins.[46,49] Moreover, thiosulfinates and cepaenes are also found in *Allium cepa*, which have an anti-inflammatory activity through the inhibition of cyclooxygenase and lipoxygenase enzymes and can inhibit the chemotaxis of human polymorphonuclear leukocytes.[46,49] Quercetin, which is found in *Allium cepa* but also in *Juglans regia* and other plants, is a substance which shows an anti-inflammatory effect through the reduction of IL-1a, IL-4 and TNF-α production.[46,49]

Glycyrrhiza glabra (Liquorice) can be used against inflammation.[46,52] Studies showed that bioactive compounds of *Glycyrrhiza glabra,* like flavonoids and triterpenic saponins, can inhibit the IL-6, IL-8, IL-16 and TNF-α response of macrophages which is induced by LPS.[46,52]

Like the examples above, many known and unknown plants contain important ingredients that can be helpful in the therapy of inflammation and are a good way of targeting several parameters.

1.6.1. Morus alba

The root bark of the white mulberry, *sang bai pi,* is used in traditional Chinese medicine (TCM) for its beneficial effects on bacterial and viral lung diseases.[53,54] It is generally used for the treatment of inflammatory diseases of the bronchial and epithelial system.[53,54] *Morus alba* (white mulberry) belongs to the Moraceae family.[53,54] The fruits, leaves, bark, branches and root of this plant contain bioactive substances, such as anthocyanins, flavonols, phenolic acids, flavonoids, minerals, macronutrients, vitamins, and volatile aromatic compounds, which give it its anti-inflammatory, antioxidative and antimicrobial properties seen *in vivo* and *in vitro*.[54,55] However, the major active constituents of the white mulberry root bark are Sanggenon C and Sanggenon D.[56,53,57] These are categorised as Diels-Alder adducts due to their prenylated flavonoid unit and they have an antiviral and antibiotic potency without harming lung epithelial cells.[56,53,57] Sanggenon C is an inhibitor of the NF-κB pathway and of iNOS expression, whereby Sanggenon C and D inhibit the NO production, resulting in an anti-inflammatory effect.[56,58,59] Nitric oxid (NO), which is produced by nitric oxide synthases (NOSs) like iNOS, is an important signalling molecule which is part of the defence mechanism of the immune system against pathogens.[56,60] It is important

for the inflammatory response and the innate immune system.[56,60] However, a high NO production can lead to diseases like diabetes, cardiac dysfunction and cancer.[56,60]

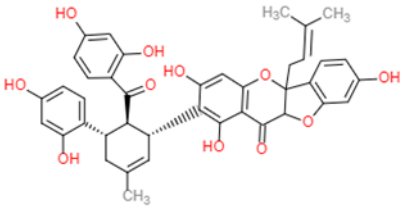

Figure 1. Sanggenon C

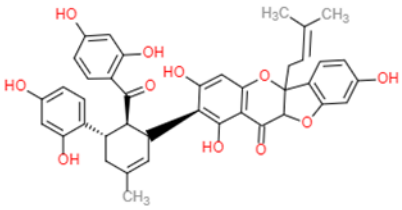

Figure 2. Sanggenon D

2. Methods and Materials

2.1. Cell cultivation

Calu-3 cells were obtained from the American Type Culture Collection (ATCC, cat. no. HTB-55, purchased via LGC Standards). The cells were maintained in DMEM/F-12 cell culture medium (cat. no. 31331028, Thermo Fisher Scientific), supplemented with 10% fetal bovine serum (FBS) (cat. no. F2442 from Sigma-Aldrich) to supply growth factors, and. 1% Penicillin/Streptomycin (P/S) (10kU-10 mg/mL P4333-100ML from Sigma Aldrich) to prevent microbial contamination.

For the washing procedure Phosphate buffered saline (PBS, pH 7.4, cat. no. 10010 from Thermo Fisher Scientific) and for the passaging process Trypsin-EDTA (Trypsin-EDTA (0.25%), phenol red, cat. no. T4049-100ML from Sigma Aldrich) solution were used. Besides, the cells were at first cultivated in T25 flask (cat. no. 734-2311P from VWR) and afterwards in a T75 flask (cat. no. 734-2314P from VWR) and T182.5 flask (cat. no. 734-2315P from VWR).

Cultivated cells above the passage number three were used in experiments. Bevor the cells were seeded inside of a 24 well plate (cat. no. 734-2325 from VWR), cell counting was done, and the suspension was diluted to a cell concentration of 1.3×10^6 cells/mL to achieve a reproducible cell density of 2D cell cultures.

2.2. Thawing cell lines from preserved stock

The first step of the cultivation of cells was the thawing of the cell line from long-term storage in liquid nitrogen. Frozen aliquots of the Calu-3 cell line (containing medium with 5% DMSO as cryoprotectant) were picked up from the liquid nitrogen tank and thawed in

the water bath at 37°C. The thawed stock was pipetted slowly into 9 mL pre-warmed medium (37°C). This ensures that the DMSO in the stock is diluted, to avoid toxic effects on the cell line. Cells were subsequently centrifugated at 400 g for 5 minutes to deposit cells at the bottom of the tube. Afterwards, the medium was aspirated without disturbing the pellet, resuspended it in 5 mL fresh medium and then transferred to a fresh T25 cell culture flask and incubated (5% CO_2, 37°C).

2.3. Passaging adherent human cells

PBS and Trypsin solution were used for the passaging of the adherent cells. Trypsin is a protease, which causes the digestion of the extracellular matrix. The extracellular matrix is the reason for the adhesion of the cells and after the digestion with trypsin, the cells can be transferred.

The medium was aspirated, the cells were washed once with PBS before adding trypsin to induce cell detachment. Calu-3 cells typically detached within 20 to 30 minutes. Fresh medium was added to inactivate trypsin, and the cell suspension centrifugated at 400 g for 5 minutes. Afterwards, the medium is aspirated, the pellet is resuspended in fresh medium, added in a new flask, and incubated (5% CO_2, 37°C).

2.4. Cell counting

Counting cells is an important step in setting up the experiment, as a certain number of cells is required to achieve the desired confluence. After trypsin-mediated cell detachment (see section “Passaging adherent human cells”), the cell pellet was resuspended in 2-3 mL of fresh medium. 12 µL was taken from this resuspension for counting and added to an Eppendorf tube with 12 µL of trypan blue (1:1) and homogenised using a pipette. Trypan blue was used for cell counting, as it cannot penetrate the cell membrane of living cells, showing how many living cells are present and alive. The suspension was pipetted into the chamber of a counting chip and the cells were counted using the EVE™ automated cell counter. This made it possible to determine how much of the cell suspension was needed to reach the desired cell count of 1.3×10^6 cells/mL.

2.5. Design of experiments

2.5.1. Determination of inflammation inducing LPS concentration

The following experiment was set up to determine the LPS concentration capable of inducing inflammation. For this purpose, the IL-6 concentration secreted into the medium was used as a marker and measured by human IL-6 ELISA. The determined concentration was used in the preparation of the inflamed epithelium model and to measure the impact of the isolated compounds of the *Morus alba* root bark on the IL-6 production.

The experiment was set up using cells with a passaging number above three. For cell seeding, 400 µL of a cell suspension with 1.3×10^6 cells/mL was transferred into separate wells of a 24-well plate. This was followed by a 72-hour attachment and equilibration period, where a monolayer with tight junctions was formed and the cells had the possibility to adapt to the environment. After the 72 hours, the medium was aspirated, and fresh warmed up medium was added. LPS was added after approximately one hour.

A solution of LPS was previously prepared by dissolving 1 mg LPS (L4391 from Sigma Aldrich) in 1 mL PBS. Appropriate quantities were taken from these tubes and diluted with medium to the desired concentration. The following LPS concentrations were used for the experiments: 0 µg/mL, 0.05 µg/mL, 0.1 µg/mL, 1 µg/mL, 10 µg/mL, 25 µg/mL, 50 µg/mL, and 100 µg/mL. After adding LPS to cell culture supernatants, the cultures were incubated for 24 hours. After this time, aliquots of the culture supernatants were transferred to fresh tubes for measurements of IL-6 concentrations by ELISA.

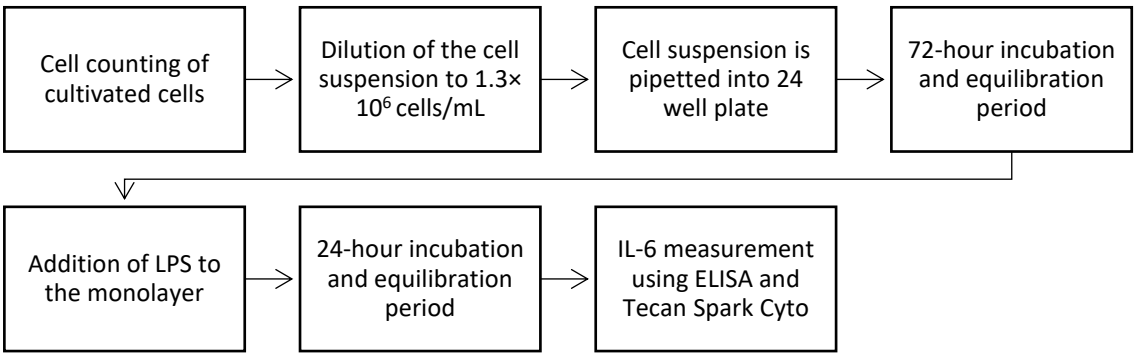

Figure 3. Design of experiment to determine the inflammation causing LPS concentration.

2.5.2. Effect of the simultaneous application of LPS and the isolated compounds of white mulberry root bark on the inflammation

In this setup the anti-inflammatory properties of Sanggenon C, Sanggenon D and MAC 130 on the inflamed lung epithelium were analysed. Sanggenon C and D are prenylated flavonoids, which have antiviral and antibiotic activity, and MAC 130 is an enriched extract which contains sanggenons. These components were isolated from the *Morus alba* root bark by Prof. Judith Rollinger's team at the Division of Pharmacognosy. Now, in the following experiment, it was investigated whether it is possible to inhibit or reduce the inflammatory effect of 10 µg/mL LPS with the help of the isolated substances through additional administration of the *Morus alba* constituents.

Two 24-well plates were used for this approach. For both, cultured cells were pipetted into the wells, whereby the cell count was 1.3×10^6 cells/mL. After a 72-hour attachment and equilibration period Sanggenon C, D and MAC 130 were added at a concentration of 50 µg/mL in triplicates in separate wells of a 24-well plate with Calu-3 cells. In the second plate, 10 µg/mL LPS was applied first, and then 50 µg/mL of either Sanggenon C, Sanggenon D or MAC 130 were pipetted to the LPS-stimulated cells. Both plates included control conditions without addition of *Morus alba* compounds, either with or without LPS stimulation. This was followed by a 24-hour incubation and equilibration period, before samples were taken for the ELISA measurement of the IL-6 concentration produced by the stimulated cells.

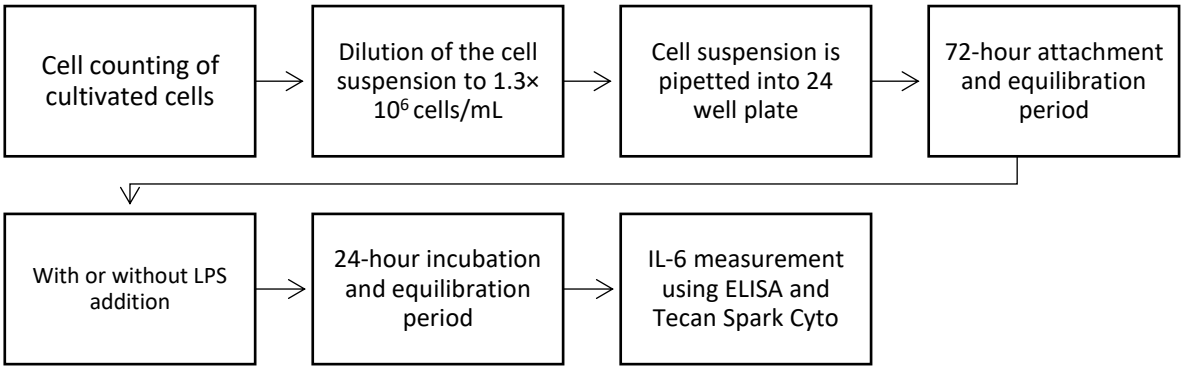

Figure 4. Design of experiment to determine the impact of 50 µg/mL Sanggenon C, 50 µg/mL Sanggenon D and 50 µg/mL MAC 130 on the LPS induced IL-6 production.

2.6. ELISA

Enzyme-linked Immunosorbent Assay is a method to quantify the concentration of inflammatory markers like IL-6. ELISA is used to determine whether a certain antigen is present. For this purpose, the analysed substance is applied on a plate coated with the antibody against the desired antigen and afterwards the antibody binds to the antigen.[42,61] Through a washing procedure, the non-specific unbound material is removed, and a second antibody is pipetted to the wells.[42,61] After a specified binding period (one hour in the used kit), the excess material is aspirated, washed with the wash buffer and conjugate(streptavidin-HRPO) is added.[42,61] The enzymatic colour change occurs through the addition of the substrate (tetramethylbenzidine; TMB).[42,61] This colour change can be measured photometrically, and the unknown concentration of the analyte can be determined from a calibration curve generated with existing standards.[42,61]

Here, we used a Human IL-6 ELISA kit (cat.no. BI-IL6, Biomedica Medizinprodukte GmbH). The kit includes a plate with microtiter strips coated with recombinant IL-6 antibody specific for human IL-6, wash buffer, assay buffer, human serum based recombinant IL-6 standards (0 / 3.125 / 6.25 / 12.5 / 25 /50 / 100 / 200 pg/ml), human serum-based control A and B, polyclonal IL-6 antibody specific for human IL-6, streptavidin-HRPO conjugate, substrate and stop solution.[61]

The determination by ELISA was performed as described below:[61]

1. The prewarmed wash buffer concentrate was diluted 1:20 with distilled water.
2. Each standard was diluted with 100 µL prewarmed medium and homogenised with a pipette.
3. The standards and samples were added to the wells and after the incubation time, the remaining medium was aspirated and the wells were washed with the washing buffer. The washing procedure was repeated each step, apart from the addition of the stop solution.
4. 100 µL antibody, conjugate, substrate and 50 µL stop solution were pipetted sequentially into the wells, each with one hour incubation time, whereby the STOP solution was added after a 30-minute incubation time.
5. The measurement was carried out by the plate reader Tecan Spark Cyto at 450/630 nm.

3. Results and Discussion

3.1. Cultivation protocol for Calu-3 cells in an in vitro model of the lung epithelium

In the course of this work, the aim was to establish a model of the inflamed lung epithelium to investigate the influence of various substances on the inflammation, as different models are available, but these usually consider the non-inflamed epithelium. In comparison to other already existing models, augmented cell model that includes an inflammatory stimulus (LPS) is used in the following experiments, whereby the anti-inflammatory effect of different bioactive compounds can be examined. To this end, in this work Calu-3 cells are used to simulate the lung epithelium based on their cell properties to generate tight monolayers through the production of tight junctions.[25,34]

To generate a cell-based model of the lung epithelium, Calu-3 cells were cultivated at high cell density in a 2D cell culture setup in 24-well plates. Cells were seeded at a density of 1.3×10^6 cells/mL and allowed to attach and equilibrate for 72 hours. During this time, the cells form a monolayer and cell-cell connections (tight junctions) with specific protein production. As seen in Figure 4, tight junction protein Occludin was detected after 72 hours, indicating a successful equilibration of the epithelial cells.

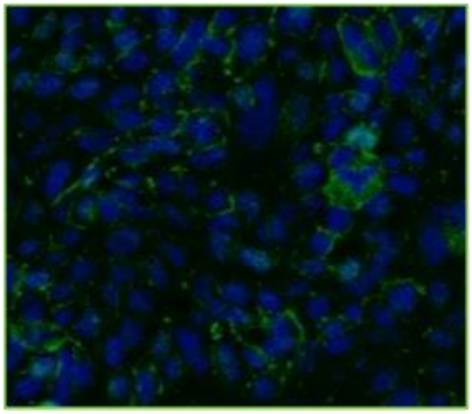

Figure 5. Antibody staining of tight-junction protein Occludin in Calu-3 cell monolayers. Green staining represents Occludin and blue staining nuclei. Cell density of 4e4 cells/cm2 and 10x magnification were used for imaging with the Tecan Spark Cyto. Images acquired by Karin Ortmayr, 2021, unpublished.

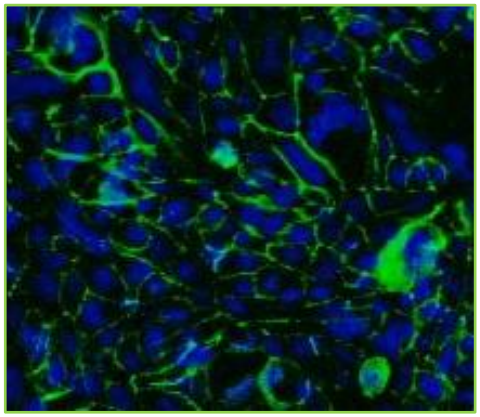

Figure 6. Antibody staining of tight-junction protein Occludin in Calu-3 cell monolayers after 72 h adhesion period. Green staining represents Occludin and blue staining nuclei. Cell density of 4e4 cells/cm2 and 10x magnification were used for imaging with the Tecan Spark Cyto. Images acquired by Karin Ortmayr, 2021, unpublished.

To induce inflammation, Lipopolysaccharides (LPS), which are a part of the cell wall of gram-negative bacteria, were used based on several experimental studies that have

already demonstrated that LPS can induce an inflammatory response in cultured cells, e.g., production of cytokines like interleukin-6 (IL-6), which was used as an inflammatory marker in these experiments.[25,62] To determine a concentration of LPS that induces a reproducible inflammatory response in Calu-3 cells LPS concentrations of 0 µg/mL, 0.05 µg/mL, 0.1 µg/mL, 1 µg/mL, 10 µg/mL, 25 µg/mL, 50 µg/mL, 75 µg/mL, and 100 µg/mL were applied to the monolayer after the 72 hour equilibration phase. At 24 hours after LPS addition, samples of the culture medium (supernatants) were taken for later measurement of IL-6 concentration.

An enzyme-linked immunosorbent assay (ELISA) kit was used to determine the IL-6 concentration in the culture supernatants. In this assay, it is possible to determine the concentration of antigen present in the sample through the antigen-antibody interaction and following enzymatic reaction. The IL-6 in the culture supernatants binds to the recombinant IL-6 antibody inside the microtiter strips, which is specific for human IL-6. After a washing procedure, the polyclonal IL-6 antibody is added, which binds the conjugate streptavidin-HRPO and after the addition of the substrate tetramethylbenzidine (TMB), an enzymatic colour change occurs, which was detected spectrophotometrically by absorbance measurement at 450 nm against a reference measurement at 630 nm in a plate reader (Tecan Spark Cyto). To quantify the IL-6 concentration in the cell culture media and to confirm the reproducibility of the experiments, a calibration with IL-6 standards in the concentration range of 0 to 200 pg/mL was performed. The linear regression (R^2) within the experiments indicates the presence of linearity and correlation between the values.

In the concentration range that the supernatant samples are expected to fall into, i.e., between 0 pg/mL and 50 pg/mL, the calibration curves showed a high overlap, indicating that the assay is reliable and reproducible (Figure 8 to Figure 11). However, there are subtle differences in the slope of the curve at the higher concentrations, which is why each experiment (supernatant samples) was evaluated with its daily calibration curve.

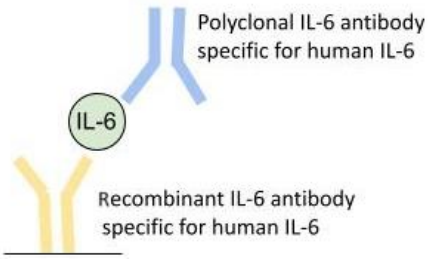

Figure 7. Illustration of the human IL-6 ELISA

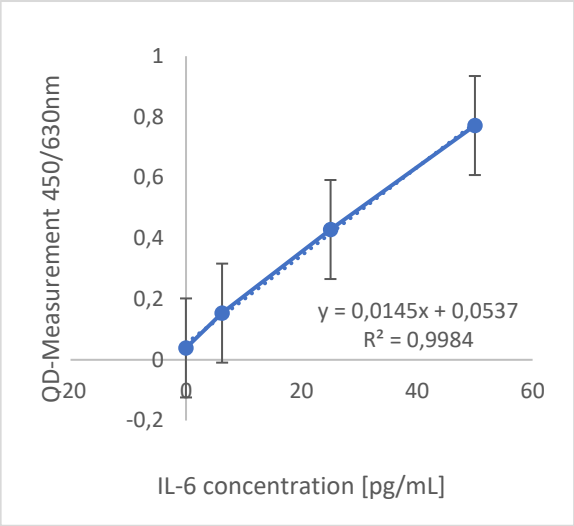

Figure 8. Calibration curve of the first experiment determined through human IL-6 ELISA.

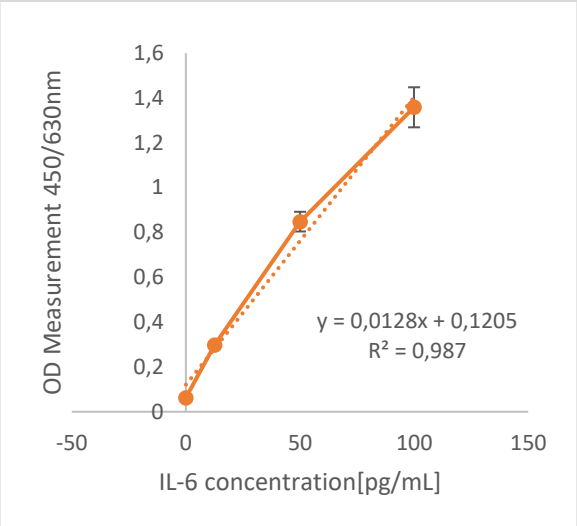

Figure 9. Calibration curve of the second experiment determined through human IL-6 ELISA.

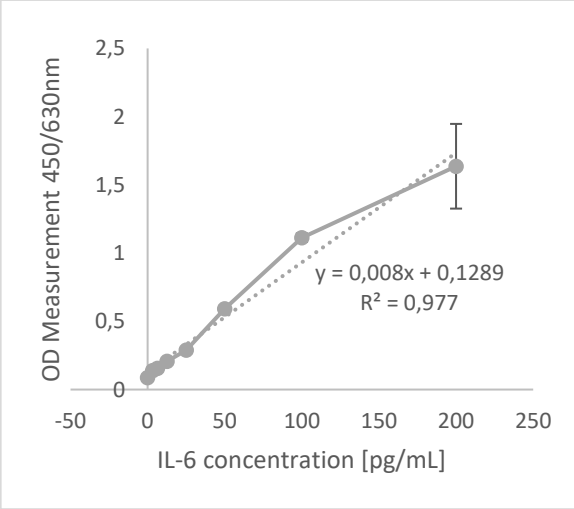

Figure 10. Calibration curve of the third experiment determined through human IL-6 ELISA.

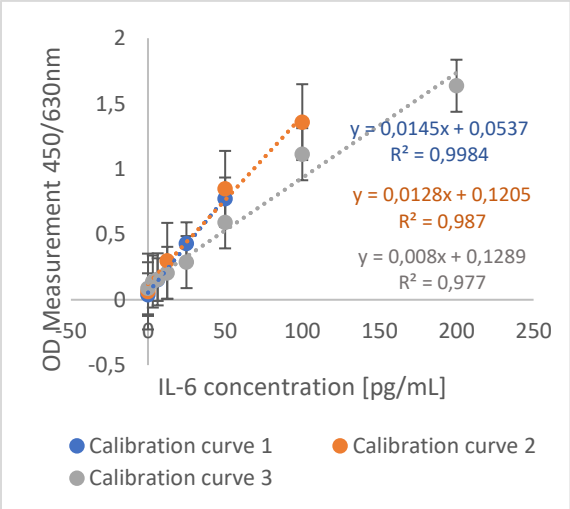

Figure 11. Comparison of the calibration curves shown in Figure 8 to 10.

3.2. Optimization of LPS concentration to induce an inflammatory response in culture Calu-3 cells

The aim of this work was to create an in vitro model with LPS stimulated inflamed Calu-3 cells. Therefore, different concentrations of LPS were applied to the Calu-3 monolayer and the required LPS concentration for the desired IL-6 production was determined. The first experiment was for training purposes and, in contrast to the following approaches, was carried out after an equilibration period of 48 hours due to time constraints (Figure 12). As seen in Figure 6, Calu-3 cells need 72 hours to produce cell-specific proteins, which have an impact on the results, making the results not entirely representative. The LPS

concentrations 0 µg/mL and 0.05 µg/mL were added to the cell monolayer and after 24 hours culture supernatants were sampled for later analysis with the IL-6 ELISA. The Calu-3 cells were able to produce IL-6 even without the inflammatory induction of LPS (Figure 12).

However, there was not a significant difference between 0 µg/mL and 0.05 µg/mL LPS observed, i.e., the standard deviation is overlapping. The experiment was repeated using an equilibration time of 72 hours to allow the Calu-3 cells to form a monolayer and to produce tight junctions to simulate the lung epitheliums properties. Additional to the LPS concentrations 0 µg/mL and 0.05 µg/mL, the concentration of 0.1 µg/mL was used. Nevertheless, a significance was not reached between the applied concentrations (Figure 13). Interestingly, we observed again that Calu-3 cells produced IL-6 even in absence of LPS as inflammatory stimulus.

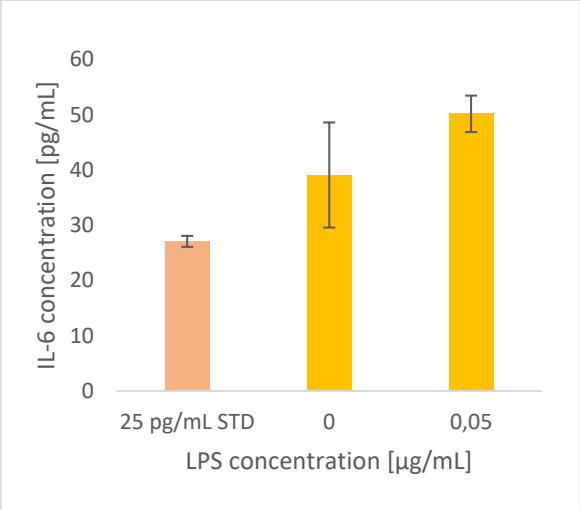

Figure 12. Measured IL-6 concentrations in Calu-3 cell culture supernatants at 24 hours after LPS addition, determined using human IL-6 ELISA. The standard (STD) was prepared using distilled water.

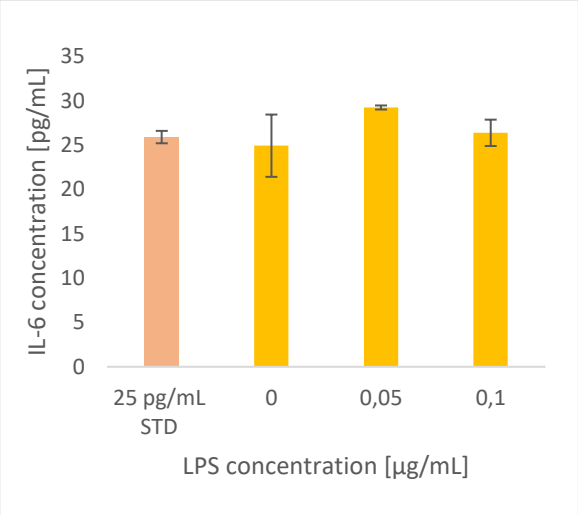

Figure 13. Measured IL-6 concentrations in Calu-3 cell culture supernatants at 24 hours after LPS addition, determined using human IL-6 ELISA. The standard (STD) was prepared using distilled water.

Based on the results of the first two experiments, we further increased the LPS concentration in the range also reported in the literature.[63,64] Therefore, the LPS concentrations 0 µg/mL, 1 µg/mL and 10 µg/mL were applied to the cells after the 72-hour equilibration period and after 24 hours the supernatants were taken to determine secreted IL-6 concentrations by ELISA. The collected supernatants were stored at -20 C° until the assay could be carried out. To assess the impact of LPS exposure on cell health, we additionally measured cell viability and determined the number of cells at 24 hours after LPS exposure. To that end, after removal of supernatant samples, cells were detached using

trypsin and subjected to automated cell counting after staining with trypan blue. This determined the viability and the cell number, which showed the influence of LPS on the cells (Figure 16 and Figure 17). Trypan blue stains dead cells because it can pass through the cell membrane of dead cells, which is not the case with living cells, making the living cells detectable and the cell viability measurable.

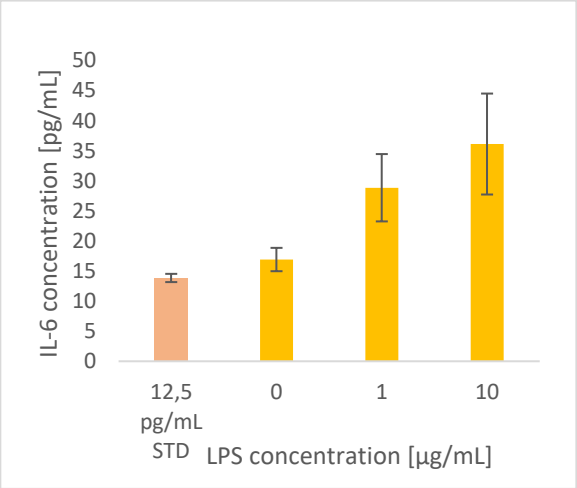

Figure 14. Measured IL-6 concentrations in Calu-3 cell culture supernatants at 24 hours after LPS addition, determined using human IL-6 ELISA. The standard (STD) was prepared using medium.

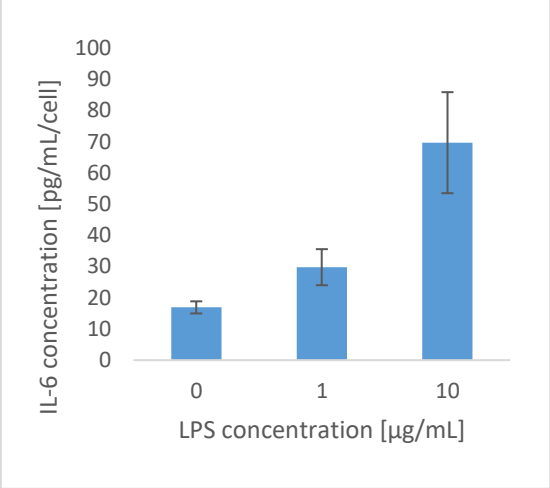

Figure 15. The IL-6 concentration after LPS addition, adjusted to the cell number measured via EVE™ automated cell counter.

As a result of the use of higher LPS concentrations, an increased IL-6 production of cells stimulated with 10 µg/mL LPS in comparison to 1 µg/mL and unstimulated cells (0 µg/mL LPS) was measured (Figure 14 and Figure 15). Moreover, the measurements uncovered a reduction in cell count and viability at 24 hours after exposure to 10 µg/mL LPS, which was not observed with 1 µg/mL LPS or in untreated cells (Figure 16 and Figure 17). By normalizing the measured IL-6 amount to the number of cells in the culture, it became apparent that 10 µg/mL LPS led to an increased IL-6 production compared to unstimulated cells, indicting a measurable stimulation of inflammation (Figure 15).

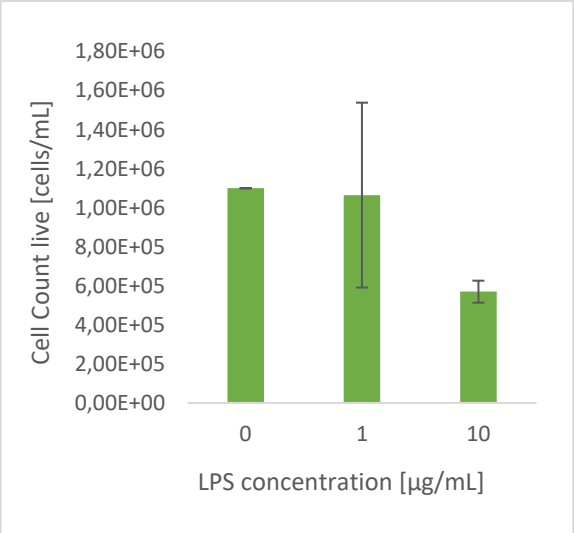

Figure 16. The influence of LPS on the Cell Count of live cells. The measurement was performed by using the EVE™ automated cell counter.

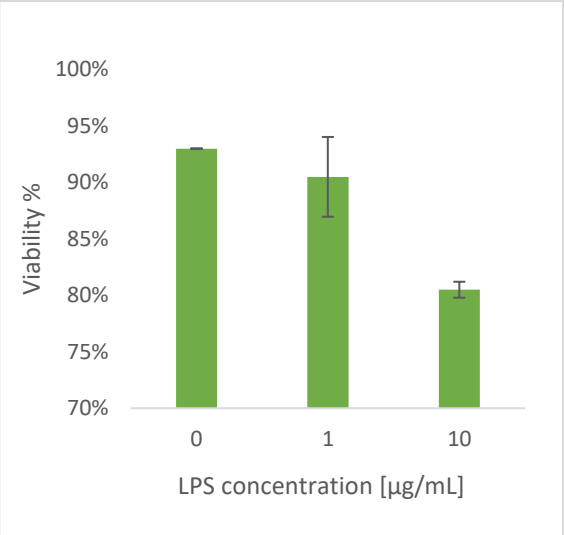

Figure 17. Comparison of the decrease of the viability through LPS. The measurement was performed by using the EVE™ automated cell counter.

Based on this outcome, higher concentrations of LPS were subsequently used to investigate whether the inflammatory response, i.e., IL-6 production, could be further increased with even higher LPS concentrations. To that end, equilibrated Calu-3 cell monolayers were exposed to LPS concentrations from 0 µg/mL to 100 µg/mL, and IL-6 concentrations as well as viability and cell numbers were determined after 24 hours. IL-6 concentrations were comparable between 10 µg/mL and 100 µg/mL doses (Figure 18), indicating that LPS concentrations higher than 10 µg/mL do not lead to a considerable increase in stimulated IL-6 production.

Furthermore, the cell count showed that the LPS concentration of 10 µg/mL had an influence on viability and cell number (Figure 19 and Figure 20), but this influence could not be increased by increasing the LPS concentration as indicated in literature. 25 µg/mL LPS and 75 µg/mL LPS even showed a higher viability and cell number than 10 µg/mL (Figure 19 and Figure 20).

Overall, it is observed that 10 µg/mL LPS causes an increase in IL-6 secretion and shows a measurable difference to the amount of IL-6 produced by unstimulated Calu-3 cells. Therefore, in the following experiments, the LPS concentration 10µg/mL was used to induce inflammation in the Calu-3 cells.

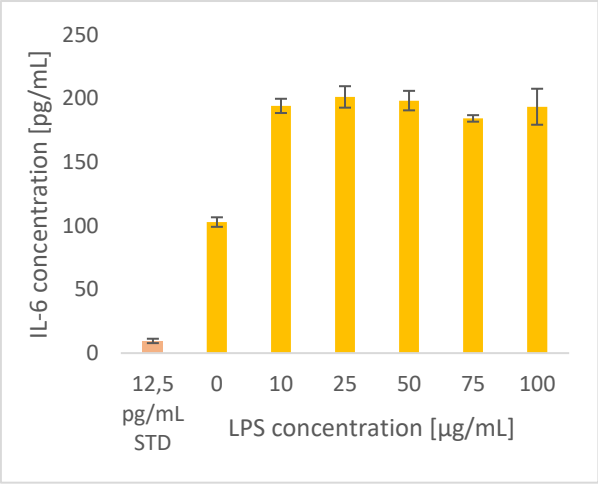

Figure 18. Measured IL-6 concentrations in Calu-3 cell culture supernatants at 24 hours after LPS addition, determined using human IL-6 ELISA. The standard (STD) was prepared using medium.

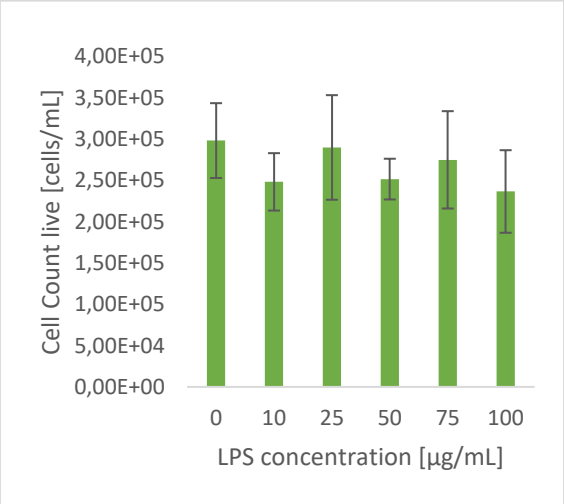

Figure 19. The impact of LPS on the Cell Count of live cells. The measurement was performed by using the EVE™ automated cell counter.

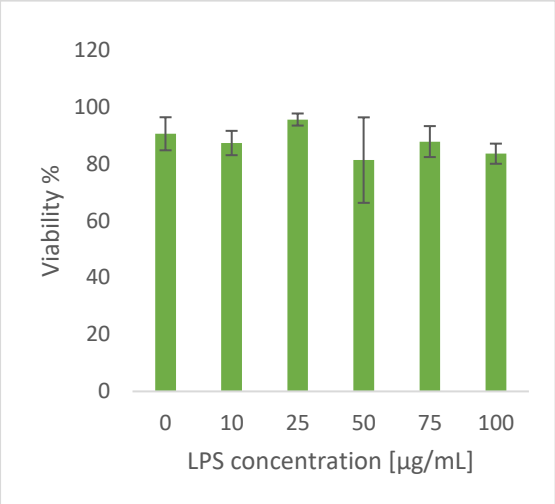

Figure 20. The reduction of the viability caused by LPS. The measurement was performed by using the EVE™ automated cell counter.

3.3. Analysis of the influence of isolated substances from white mulberry root bark and the LPS-induced inflammatory response of Calu-3 cells

The *Morus alba* root bark, which is used in traditional Chinese medicine (TCM) for lung diseases, contains prenylated flavonoids that have antibiotic and antiviral effects.[53] The sanggenons have an anti-inflammatory effect through the NF-κB inhibitory activity of Sanggenon C and the inhibition of the NO production of Sanggenon C and Sanggenon D.[56,58,59] Due to their anti-inflammatory activity, a reduction of IL-6 production was expected with and without LPS stimulation because Calu-3 cells were able to produce IL-6 even without LPS stimulation.

To test the impact of bioactive compounds from *Morus alba* on the inflammatory response of Calu-3 cells, Sanggenons or MAC 130 were added to equilibrated Calu-3 cell monolayers with or without LPS stimulation. A comparison of IL-6 concentrations in the different conditions showed that although Sanggenon C is an inhibitor of the NF-κB, an increase of IL-6 production was observed both with and without LPS stimulation (Figure 21), indicating that it induces the inflammatory activity of the cells.[56] Even higher concentrations of IL-6 were measured with the co-application of Sanggenon C and LPS (Figure 21). In contrast to Sanggenon C, the addition of Sanggenon D and MAC 130 caused a reduction of IL-6 concentration in LPS stimulated (Sanggenon D by 48.41% and MAC 130 by 34.02%) and non-stimulated cells (Sanggenon D by 16.5% and MAC 130 by 6.53%) (Figure 21).

In summary, the hypothesis that sanggenons reduce IL-6 production and have an anti-inflammatory effect on Calu-3 cells due to their inhibitory effect was partly confirmed since Sanggenon C showed an increase but Sanggenon D and MAC 130 a reduction of IL-6. It should be noted that cell number and viability were not considered in this experiment, thus the exact cell number responsible for IL-6 production remains unknown. Therefore, it is possible that the monolayer where Sanggenon C was applied, showed a higher cell number resulting in increased IL-6 production compared to Sanggenon D and MAC 130, which is an important aspect to consider in future experiments.

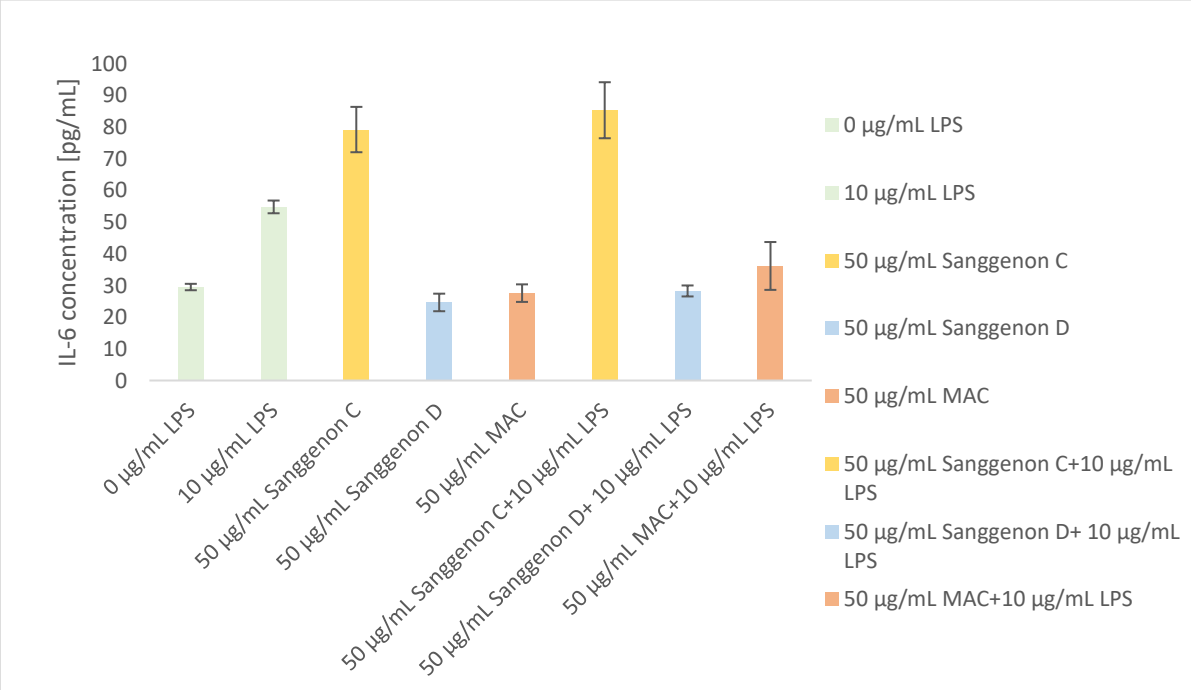

Figure 21. Impact of 50 µg/mL Sanggenon C, 50 µg/mL Sanggenon D and 50 µg/mL MAC 130 on LPS stimulated and not stimulated Calu-3 cells.

4. Conclusion

The aim of the work was to establish a model of the inflamed lung epithelium using LPS-stimulated Calu-3 cells that can be used in future experiments to investigate the anti-inflammatory effects of natural products and other substances. Models of Calu-3 cells are used in different approaches and are often used because of the lung epithelium-specific properties. It was now investigated whether it is possible to induce inflammation in Calu-3 cells using LPS, which has been used in different sources to induce inflammation.

Here, we found that the addition of LPS to the Calu-3 cells can lead to an increase in IL-6 production and thus to the induction of inflammation. It was also evident in the literature that the addition of LPS leads to increased cytokine production, but the concentrations given were too low for the minimum set-up and often different cytokines such as IL-8 and TNF-α were measured.[25,62,63] Because of this, it was possible to start from an approximate LPS concentration for the induction, but the concentration used for further experiments was higher, as noted in the literature. This could be due to the different equilibration times that could affect the differentiation process of Calu-3 cells in an epithelium-like culture, as in the experiments 72 hours were given for equilibration after cell seeding and then LPS was added and measured after 24 hours. Besides, the stimulation of the cells in other experiments happened in a shorter time periods, which can also have an impact on the outcome.[62,63]

To investigate whether the Calu-3 model is suitable for studying the impact of bioactive compounds on the inflammatory response of Calu-3 epithelial cell cultures, we tested an extract and purified compounds from *Morus alba* root bark. Sanggenon C, Sanggenon D, and MAC 130 were now applied to the LPS-stimulated and non-stimulated Calu-3 cells, and the impact on cytokine production (IL-6 as inflammation marker) was determined. We found that Sanggenon C, led to increased IL-6 production, which raises the question as to why this result was obtained, since Sanggenon C has been reported to inhibit NF-κB and to have anti-inflammatory properties. Sanggenon D and MAC 130 however have shown a reduction of IL-6 and thus an anti-inflammatory response in the Calu-3 model. Therefore, it would be interesting to investigate whether this means that the anti-inflammatory activity of Sanggenon C in comparison to the other substances is not strong enough or that there is an enhanced immune response due to the increased concentration of the cytokine,

or whether other factors are responsible for the increase in IL-6 concentration, such as other cytokines and immune responses of the Calu-3 cells.

Overall, this is an approach to create a model to simulate the inflamed lung epithelium, in that it produced the expected increase in cytokine secretion, which could be used as a readout for effects of bioactive compounds. Nevertheless, *in vitro* models have limited ability to truly represent the full functional repertoire of lung tissue. Therefore, the question arises, which other factors have an influence on the immune response of the Calu-3 cells and how the simulation of the lung epithelium can be improved and made more comparable. For this purpose, the estimation of further lung epithelium characteristic proteins and substances is interesting. Furthermore, it would be intriguing to conduct a re-evaluation of Sanggenon C, Sanggenon D, and MAC 130 in terms of cytokine production, considering both cell viability and cell number. This is interesting because these compounds share similar pathways but yield different outcomes. The question arises as to whether the observed increase in IL-6 production with Sanggenon C was a chance occurrence or if there is a specific reason behind it. Therefore, the enhancement of the execution of the experiments, e.g., by measuring the cell number after the stimulation period, as well as the reproducibility should be aimed at by repeating the experiments under the same but more controlled conditions so that possible adjustments can be made to improve the model. Moreover, incorporating additional control parameters, such as other markers of inflammation, can provide further support for the conclusions and findings.

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