# TECHNISCHE UNIVERSITÄT MÜNCHEN

## Lehrstuhl für molekulare Allergologie

Evaluation of Anti-inflammatory Potential of Tesalin® on Airway Epithelium and Innate Immune Effector Cells

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*Gewidmet an* **Angelika Steiert und Oliver Wagner**

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**Mainzer Allergie Workshop, Mainz, Germany, March 2015**

# Abbreviations

| | |
|---|---|
| µg | Microgram |
| AHR | Airway hyper-responsiveness |
| AIT | Allergy immunotherapy |
| BisTris | 1,3-bis(tris(hydroxymethyl)methylamino)propane |
| CCL | C-Chemokine ligand |
| CCR | C-Chemokine receptor |
| CD | Cluster of differentiation |
| CO | Carbon oxide |
| COPD | Chronic obstructive pulmonary disease |
| CXCL | CX-Chemokine ligand |
| DC | Dendritic cells |
| DMEM | Dulbecco's Modified Eagle Medium |
| D-PBS | Dulbecco's phosphate-buffered saline |
| EDTA | Ethylenediaminetetraacetic acid |
| ELISA | Enzyme linked immune absorbent assay |
| FACS | Fluorescence-activated cell sorting |
| GAPDH | Glycerinaldehyd-3-phosphat-Dehydrogenase |
| G-CSF | Granulocyte colony stimulating factor |
| GM-CSF | Granulocyte macrophage colony-stimulating factor |
| H | Hours |
| HMDS | Hexamethyldisilazan |
| HNEC | Primary human nasal epithelial cells |
| HRP | Horseradish peroxidase |
| ICAM-1 | Intercellular adhesion molecule 1 |
| ICS | Inhaled corticosteroids |

| | |
|---|---|
| IFN | Interferon |
| Ig | Immunoglobulin |
| IL | Interleukin |
| IP | Interferon gamma-induced protein |
| Jak | Janus kinase |
| LABA | Long-acting $\beta_2$ agonist |
| LDH | Lactate dehydrogenase assay |
| LPS | Lipopolysaccharide |
| $LTB_4$ | Leukotriene B4 |
| MCP | Monocyte chemoattractant protein |
| MDA | Melanoma differentiation-associated protein |
| Min | Minutes |
| ml | Millilitre |
| NF-κB | Nuclear factor kappa-light-chain-enhancer of activated B cells |
| PAF | Platelet-activating factor |
| PAMPs | Pathogen-associated microbial patterns |
| PBMC | Peripheral blood mononuclear cells |
| PCR | polymerase chain reaction |
| PEG | Polyethylenglycol |
| PGE | Prostaglandin |
| PM | Petasin mixture |
| PMN | Human neutrophil granulocytes |
| PNEC | Pulmonary neuroendocrine cells |
| PolyIC | Polyinosinic:polycytidylic acid |
| PVDF | Polyvinylidenfluorid |
| RIG | retinoic acid-inducible gene |
| RNA | Ribonucleic acid |

| | |
|---|---|
| RPMI | Roswell Park Memorial Institute medium |
| RSV | Respiratory Syncytial Virus |
| SCIT | Subcutaneous immunotherapy |
| SD | standard deviation |
| SEM | standard error of the mean |
| SIT | Specific immunotherapy |
| SLIT | Sublingual immunotherapy |
| STAT | Signal transducer and activators of transcription |
| Th | T helper cell |
| TLR | Toll-Like-Receptor |
| TNF | Tumor necrosis factor |
| TSLP | Thymic stromal lymphopoietin |
| VEGF | Vascular endothelial growth factor |

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# 1 Introduction

## 1.1 Inflammation and allergy

### 1.1.1 Inflammation

The general cardinal signs of inflammation in the human body are rubor (redness), tumor (sweeling), calor (heat), dolor (pain) and the loss of function. This effect is caused by the release of immune mediators and infiltrating immune cells, which process the whole mechanism of an inflammation.

In the airways, epithelial cells regulate inflammatory response by the production of several major classes of inflammatory products such as interleukin-6 (IL-6), IL-11, thymic stromal lymphopoietin TSLP, type I and II interferon (IFN), tumor necrosis factor-α (TNF-α), granulocyte colony-stimulating factor G-CSF, granulocyte-macrophages colony-stimulating factor (GM-CSF) or vascular endothelial growth factor (VEGF). Additionally, important chemokines such as IL-8, chemokine ligand 5 (RANTES), chemokine ligand 3 (MIP-1α) and chemokine ligand 4 (MIP-1β) and chemokine ligand 2 (MCP-1) are secreted by the epithelium amongst others. All these mediators trigger an inflammatory response. This leads to the recruitment of immune cells such as neutrophils, eosinophils, macrophages, lymphocytes and monocytes (Proud and Leigh, 2011). In case of a chronic airway disease or allergy the inflammatory response remains on a constant, activated level leading to a chronic inflammation. Around the world, over hundred millions of people suffer from preventable chronic respiratory diseases including asthma, chronic obstructive pulmonary disease (COPD) or respiratory allergies (WHO, 2015). In case of asthma, 300 million people of all ages are suffering from this disease worldwide, 250 000 people die of asthma every year due to lack of proper treatment. The allergen sensitization, occurring in allergic patients, is a risk factor for the development of asthma. 400 million people suffer of allergic rhinitis and the number is increasing every year. Therefore, new therapeutic treatments for these chronic diseases are of important need and research should focus on the development of new treatments, also including Phytodrugs.

### 1.1.2 Allergy

In case of an allergic reaction the human body is exposed to a harmless antigen or allergen. It gets in contact with the antigen or allergen for the first time and responds with the production of immunoglobulin E (IgE) antibodies. This process is called sensitization; the body recognizes the allergen as foreign protein or glycoprotein without any allergic reaction. If the body gets in contact with the same antigen or allergen again, a possible hyper-reaction of the immune system can then lead to an allergic reaction (Janeway et al., 2002).

The epithelium plays a crucial role in the sensitization process. The immune mediators IL-33, IL-25 and TSLP and additionally alarmins such as defensins or cathelicidin are secreted by the epithelium to induce chemotactic migration and activate antigen-presenting cells (Chertov et al., 1996; Ganz et al., 1985; Van Wetering et al., 1997; Yang et al., 1999). IL-33, IL-25 and TSLP have shown to implicate the activation of type 2 innate lymphoid cells (ILC2) within the epithelium (Suzukawa et al., 2012; Wills-Karp et al., 2012). Furthermore, T helper 2 cells (Th2), IgE, mast cells and eosinophils play a major role in the development of an allergic reaction. Dendritic cells (DCs) capture the entered antigens, process them and transport them to draining lymph nodes. Here, naïve $CD4^+$ T cells recognize the DC-presented peptides of the antigen on the surface and differentiate into Th2 cells and follicular helper T cells (TFh). Naïve $CD4^+$ T cells can polarize into different Th-cell subsets through implementing signals from their T-cell receptor, co-stimulatory molecules and cytokine receptors. This enables the host immune system to react on a broad range of pathogens and to specialise the clearance of those pathogens (Holgate, 1998; Holt et al., 1990). The presence of IL-4, produced by the activated Th2 cells itself and various surrounding cell types as basophils, further drives the differentiation into Th2 cells. Th2 cells, ILC2s and TFH cells activate B cells that are specific for allergens through the actions of cluster of differentiation 40 (CD40) ligand and the cytokines IL-4 and IL-13. IL-4, IL-13 and IL-5 are all cytokines produced by the activated Th2 cell. The activated ILC2 are able to produce IL-5 and IL-13 (Neill et al., 2010; Price et al., 2010). Now, the activated B cells undergo heavy chain isotype switching from IgM to IgE or IgG2 to IgG4 in humans. There are four types of hypersensitivity: type I-IV, whereby type I hypersensitivity is associated with isotype switch from IgM to IgE. The produced IgE is then present in the plasma, circulates in the system and binds to high affinity IgE receptors (FcεRI) present, amongst others, on tissue mast cells and circulating basophilic granulocytes. IgE binds to the Ig-like domain of the α-chain of the polypeptide chain structure. The β- and γ-chain mediates the signal transduction responding to the IgE binding. The resting IgE-coated mast cell gets activated by the cross-linking of bound IgE upon repeated antigen ex-

posure. The antigen binds to IgE/FcεRI complex and finally activates the mast cell (Abbas et al., 2012).

This process begins with the first exposure to the allergen and the activation of the Th2 cells and the stimulation of B cells. This results in the production and secretion of IgE from the B cells, continuing with the binding of IgE to the FcεRI on mast cells. The activation of these mast cells after a repeated exposure to the allergen is called immediate hypersensitivity. This reaction happens minutes after the repeated exposure to the antigen. But there is also a late phase reaction, occurring 2-4 hours after the repeated exposure.

The physiological reaction of the body in the immediate reaction is the wheal and flare reaction, which is dependent on IgE and mast cells. This reaction appears when the mast cell gets activated by the antigen- IgE-FcεRI complex described above, resulting in the release of the mediator histamine. Subsequently, histamine binds to the histamine receptors on endothelial cells, leading to the synthesis and release of lipid mediators such as prostaglandin I2 (prostacyclin, $PGI_2$), leukotriene $C_4$ ($LTC_4$), nitric oxide (NO) and platelet-activating factor (PAF). All these mediators cause vascular leakage and vasodilation, and trigger leukocyte extravasation. This inflammatory response, occurring a few hours after the immediate reaction, is the so-called late-phase reaction. This phase includes the release of cytokines such as TNF-α, IL-4, IL-13, IL-3, GM-CSF and chemokines such as MIP-1α and the already mentioned lipid mediators $LTC_4$, $LTD_4$, $LTE_4$ and PAF. TNF-α activates endothelial cells to upregulate leukocyte adhesion molecules such as E-selectin and intracellular adhesion molecule-I (ICAM-1) to accumulate inflammatory leucocytes, thereby promoting inflammatory reactions. IL-4 and IL-13 promote the differentiation towards Th2 cells, IL-3, IL-5 and GM-CSF trigger the development and activity of eosinophils. MIP-1α recruits monocytes, macrophages and neutrophils to the side of inflammation. The release of leukotrienes causes contraction of the smooth muscles (e.g. bronchial construction or intestinal construction) (Dahlén et al., 1980), increase mucus production and maintain inflammatory processes in the tissue (Henderson et al., 1996). PAF is chemotactic for leukocytes; it increases the production of lipid mediators and activates neutrophils and eosinophils. Therefore, typical leucocytes of late-phase reaction are eosinophils, Th2 cells and neutrophils (Abbas et al., 2012; Eyerich and Zielinski, 2014; Janeway et al., 2002).

The most common forms of allergic diseases nowadays are bronchial asthma, atopic dermatitis (eczema), allergic rhinitis and food allergy (NIH, 2015). All forms of the allergic reaction result in the described pro-inflammatory process in the body leading to pain and overreactivity of the immune system. Beside the common treatments of allergy, novel drug devel-

opments and strategies focus on drugs which interrupt these inflammatory processes to prevent an over-reaction of the immune system.

### 1.1.3 Current treatment of allergic diseases

In case of bronchial asthma, a repeated immediate-phase hypersensitivity and late-phase allergic reaction of the lower airways causes the disease. It often coexists with bronchitis or emphysema, which can lead to a severe damage of the lung tissue. Viral and bacterial infections in the respiratory tract can promote the development of asthma or exacerbate pre-existing asthma. The current Global Initiative for Asthma guidelines (GINA) recommend stepwise treatment to reach optimal asthma control. The treatment starts with inhaled corticosteroids (ICS), followed by an increase in the ICS dose or the addition of other controller therapy, including long-acting $\beta_2$-agonists (LABAs), leukotriene modifiers, or theophylline (methylxanthine drug) to achieve control of resistant diseases. If the state of asthma is too severe and add-on therapies cannot be achieved, anti-immunoglobulin E or oral glucocorticosteroids are recommended for the treatment (McIvor, 2015). Glucocorticosteroids (also called glucocorticoids, corticosteroids or steroids) are the most common and effective therapy for patients suffering from asthma. They are acting on various cellular levels, e.g. suppressing the production of adhesion molecules and chemotactic mediators and therefore reducing the recruitment of inflammatory cells, including eosinophils, T cells, mast cells and dendritic cells into the airways. Epithelial cells are one of the important players of the immune defence in the airways, and regular treatment with ICS re-establishes the integrity of the epithelial barrier of patients suffering from severe asthma (Kharitonov et al., 2002; Lilly et al., 1997).

A key problem are patients suffering from a mild uncontrolled asthma, which have a high risk of future exacerbations of the disease. Additionally, there are different impacts that can influence the progress of asthma as allergen exposure such as allergic rhinitis, concurrent smoking or incorrect inhaler techniques. The different allergic asthma subgroups show a broad range of variance in the pathophysiology of the disease. There are emerging therapeutic options for the treatment of asthma in different development phases (1-3), including anti-interleukin agents, chemoattractant receptor-homologous molecules expressed on T-helper type 2 lymphocytes (CRTH2) antagonist, phosphodiesterase-4 inhibitors, and long-acting muscarinic antagonists (LAMAs). A recent study suggested that the long-acting muscarinic antagonist Tiotropium could be a promising option for the treatment of asthma and, in addition for the treatment of chronic obstructive pulmonary disease (COPD), helps as an add-on

therapeutic to the common ICS (McIvor, 2015). LAMAs block the muscarinic acetylcholine receptor and therefore have an impact on smooth muscle contraction, mucus secretion and vasodilation (Beakes, 1997).

### 1.1.4 Allergy immunotherapy (AIT) using specific immunotherapy (SIT)

To control the progress of allergic asthma very few therapies are available. Current therapies focus on controlling the inflammatory processes and controlling the symptoms. During allergy immunotherapy (AIT), a treatment using a specific immunotherapy (SIT), patients get a gradually increasing dose of a specific allergen over a long time period. This approach can potentially modify the basic allergic mechanism of the disease. Indeed, in patients with mild and moderate asthma, associated with allergic rhinoconjunctivitis and controlled by pharmacotherapy, subcutaneous immunotherapy (SCIT) and sublingual immunotherapy (SLIT) can be a useful therapeutic regimen. In 1911, there was the first reported use of SCIT in the treatment of allergic rhinitis by Leonard Noon, but in 1986, the British Committee of Safety of Medicines reported 26 deaths connected with SCIT, thus different delivery methods (oral, nasal and sublingual) had to be analysed in randomized controlled trials (Aboshady and Elghanam, 2014; Brozek et al., 2010; Cox et al., 2006; Jutel et al., 2015; Wilson et al., 2003). After years of research on different delivery methods, SLIT has been identified as a therapeutic option for patients suffering from seasonal rhinitis (hay fever) or perennial rhinitis (house dust-mite), and patients uncontrolled by pharmacological treatments or patients that are affected with systemic reaction from drug treatments. In many clinical studies, SLIT has shown to modulate allergen-specific immune responses by induction of IL-10 producing allergen-specific regulatory T cells, associated with a reduced IgE to IgG4 ratio. The blocking antibody IgG competes with the present IgE antibody and prohibits the inflammatory response to IgE release (Strait, 2006). Thus, SIT can lead to a shift in the immune response from Th2 to Th1/ Tregs (Figure 1).

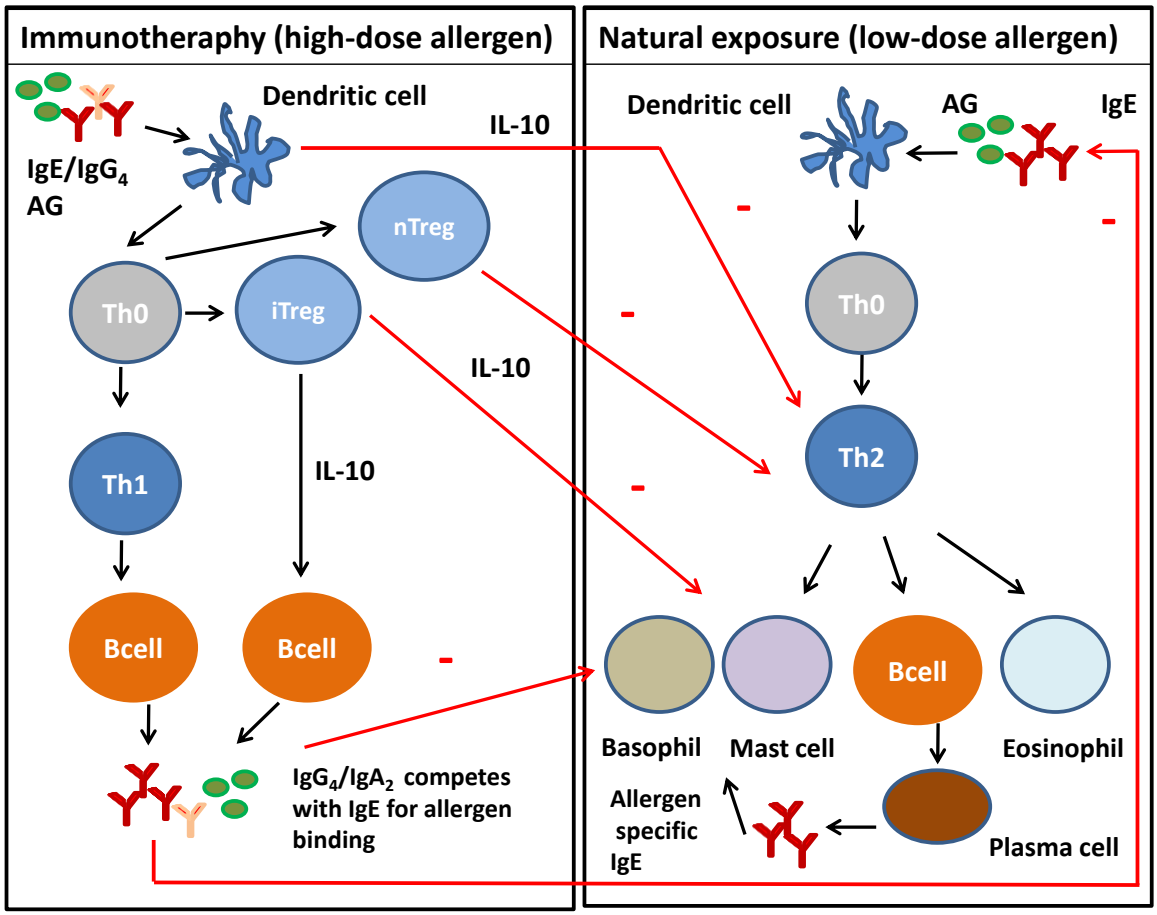


**Figure 1: Mechanism of high-dose allergen and low-dose allergen at mucosal surface in atopic individuals (Modified by Matsuoka et al., 2013).** The immune system of patients receive high-dose allergen by SLIT or SCIT shifts from a Th2 driven response to a Th1 driven response. Additionally, natural Treg cells (nTreg) and inducible Treg cells (iTreg) were induced, suppressing Th2 response and activating B cells to produce competitive IgG4 and IgA2 for allergen binding**.**

The reason why sublingual immunotherapy is preferred in comparison to subcutaneous immunotherapy is the pain-free application and the elevated safety; in the sublingual mucosa there are less mast cells present. Additionally, another benefit for the patients is the application at home.

As mentioned before, specific immunotherapy, and especially SLIT, can be applied for the causal treatment of allergic rhinitis, which is the most common allergic disease in Japan, Western Europe and the United States of America (Bauchau and Durham, 2004, 2005). Due to the high presence of serine, cysteine protease activity and nicotinamide adenine dinucleotide phosphate oxidase in the pollen of ragweed, an annual herbaceous flowering plant, ragweed pollen are one of the major causes of allergic rhinitis (Dharajiya et al., 2008; Gunawan et al., 2008). Amb a 1 and Amb a 2 are the secreted, acidic, non-glycosylated, single-chain major allergen proteins of ragweed (Gadermaier et al., 2004). For the symptomatic treatment

of ragweed-induced rhinitis, first-generation H1-antihistamine, diphenhydramine and the third-generation H1-antihistamine, desloratadine, applied orally, were effective in reducing nasal allergic symptoms. This was evaluated in several clinical trials (Wilken et al., 2003). Beside the oral application of antihistamines, a topical treatment with glucocorticosteroids is the most commonly used and clinically effective treatment for allergic rhinitis. But the treatment with glucocorticosteroids and antihistamine can also cause side effect such as osteoporosis, growth retardation, croakiness or liver stress. To avoid this, new strategies beside the conventional medicine strategies have to be developed.

### 1.1.5 Phytotherapy

An alternative way to treat allergies and inflammatory diseases such as asthma is the use of phytodrugs, also called phytochemicals. Phytodrugs represent the active compounds of plant products, gained via different procedures. Plants have been the basis of many medicinal regimens worldwide for the last thousands of years and researchers still discover new active beneficial compounds in plants. Newly found chemical plant substances are the basis to develop novel innovative drugs (Jachak S and Saklani A, 2007). The first isolated active compound in the early 19$^{th}$ century was morphine from opium, followed by the isolation of early drugs as cocaine, codeine, digitoxin and quinine (Mann, 1999; Wainwright, 1991), of which some are still in use. 250,000 flowering plants species exist worldwide. Half of them are found in the tropical forest. A big success in the field for drug discovery from plants, and the proven beneficial effects in humans resulted in the development of anti-cancer and antibacterial agents, e.g. chemotherapeutic agents such as paclitaxel, hycamptamine or 9-aminocamptothecin (Cragg et al., 1993) and the antibacterial filtrate penicillin by Fleming in 1928 (Joklik, 1996). To discover the active drug compounds from plants, various methods of analysis and multidisciplinary approaches are involved, including botanical, ethnobotanical, phytochemical and biological techniques (Jachak S and Saklani A, 2007). Plants produce a wide range of diverse organic compounds, classified into primary and secondary metabolites. The primary metabolites, such as phytosterols, acyl lipids, nucleotides, amino acids and organic acids are found in all plants and are essential for growth and development. The secondary metabolites are not directly involved in the process of growth and development, but appear in a wide variety within the plants (Croteau et al., 2000). Due to their biosynthetic origins, phytochemicals can be classified into terpenoides (carotenoids), phenolics, alkaloids, nitrogen-containing compounds and organosulfur compounds (Liu, 2004). Nowadays, several studies worldwide have proven the protective effects of phytochemicals present in plants

against acute, chronic and degenerative diseases (Arya and Arya, 2011; Nichenametla et al., 2006).

Table 1 summarizes phytochemicals with anti-inflammatory activities and their mechanism in the human system.

| Class | Compounds | Source | Examples Effects and Uses |
|---|---|---|---|
| *Polyphenols* | | | |
| Flavonoids | Kaempferol, Quercetin, Myricetin, Luteolin, Agipenin | Onion leaves, semambu leaves, black tee, papaya shoots, guave | Promoting antioxidant enzymes activity, inhibition of tyrosine kinase on activated macrophages, inhibition of arachidonic metabolism, inhibition of NF-κB activation, inhibition of production of pro-inflammatory cytokines (e.g. IL-6, IL-8 and TNF-α) |
| Non-Flavanoids | Gallic acid, curcumin, caffeic acid, secoisolariciresinol, resveratrol | Apple, virgin olive oil, blackcurrants, red wine, soyabean | Inhibition pro-oxidant enzymes activity (e.g. activation of iNOS), promoting antioxidant enzymes activity, inhibition TNF-α production |
| *Alkaloids* | | | |
| | Quinine, morphine, tryptanthrin, piperlactam S | Cinchona tree, opium straw, Isatis tinctoria L. Piper kadsura, | Antipyretic and treatment of malaria, pain medication, inhibitor of cyclooxygenase-2 (COX-2) and lipoxygenase-5 (LOX-5) and inhibition of $LTB_4$ production, inhibition cytokine production (IL-1β, IL-6, TNF-α) |
| *Terpene* | | | |
| Monoterpen | Myrcene, menthol, catalposide | Ylang-ylang, peppermint, *Catalpa ovata* | Analgesic effects and anti-inflammatory potential, antibacterial, prevention of TNF-α, IL-1β and IL-6 production in LPS-activated macrophages |

| | | | |
|---|---|---|---|
| Triterpenoid | Celastrol, ginsenosides Rb1 | *Tripterygium wilfordii, Panax ginseng C.A.* | Decreased production of TNF-α and IL-1β in monocytes and macrophages |
| Sesquiterpene | Cynaropicrin, Reynosin, Petasin | *Saussurea lappa, Petasites hybridus* | Inhibition of TNF-α in LPS-activated mouse macrophages, Petasin: inhibits LT biosynthesis (inhibits activation of $PLA_2$), inhibits release of eosinophil cationic protein; used to treat allergic rhinitis |
| *Antioxidant vitamins* | | | |
| Carotenoids | Lycopene, Lutein, β-cryptoxanthin, α-carotene β-carotene | Tomato Spinach Papaya, apple Carrot, sweet potato | Anti-oxidant, Regulation of different transcription factors (NF-κB), reduction of production of inflammatory cytokines, reduce induction of IGF-1 |
| Vitamin C | Ascorbic acid | Lemon | Anti-oxidant |
| Vitamin E | Tocopherols (α, β, γ and δ) | Olive and sunflower oils | Hypo-peroxide scavenger |

**Table 1: Anti-inflammatory activation of phytochemicals** (Modified from Bellik et al., Kulka et al., Calixto et al. and Park et al. (Bellik et al., 2012; Calixto et al., 2003; Kulka, 2009; Park et al., 2010)).

Due to their beneficial effect, phytochemicals could be of use for patients suffering from allergic rhinitis or other inflammatory diseases. The application could be given orally, but the application topical into the nose is also a possible option. The nasal epithelial cells are the first line of response to drug and have a huge impact on the following immune response of the body, and are therefore very important players of the immune system.

## 1.2 Cellular players in the airway epithelium

### 1.2.1 Cells of the airway epithelium

As already mentioned, the immune system provides an enormous variety of cellular components protecting the host and regulating the immune reactions. The epithelial cells of the airways regulate both the innate and the adaptive immune system by controlling the production of immune molecules and by managing the physical interaction with cells of the immune system. To protect the airways from environmental hazards, the airway epithelium provides a vital protective layer between the internal milieu of the lung and the external environment (Boucher, 1994). Regarding the difference in function, ultrastructure and biochemical criteria, the distinct epithelial cell types can be classified in three categories: basal, ciliated and secretory cells (Figure 2). Dependent on epithelium-produced mediators, inflammatory cells, immune cells and phagocytic cells are allowed to transmigrate to the lumen (Schon-Hegrad et al., 1991). The most frequent cell types in the airways are the columnar ciliated epithelial cells. They arise from either basal or secretory cells and represent 50% of all epithelial cells. The surface of the cells is covered with up to 300 cilia per cell, indicating their main function which is the directional transport of mucus from the lung to the throat.

Mucous cells, also called goblet cells, are important for the production and secretion of acid mucin to prevent the airways from invading pathogens and noxious particles. The correct amount of mucus and the viscoelasticity are important for efficient mucociliary clearance. An increased mucus release can be caused by the inhalation of noxious substances including sulphur dioxide, occurring with coal combustion, or tobacco smoke. In chronic airway inflammatory diseases, such as asthma or chronic bronchitis, the disease pattern shows a mucous cell hyperplasia and metaplasia, which is thought to be a reason for abundant cough and elevated mucus production, accompanying these diseases (Lumsden et al., 1984).

Basal cells play an important role in the structure of the airway epithelium, they are ubiquitous and the only cells that are firmly attached to the basement membrane (Evans and Plopper, 1988; Hicks et al., 1997). Thus, they are important for the attachment of other cells to the basement membrane via hemidesmosomal complexes (Evans et al., 1989), and basal cells are the only cells expressing hemidesmosomes. The interepithelial attachment between the cells of the epithelium is mediated by desmosomes. Mucous and ciliated cells arise from basal cells in the large airways, although basal cells are less present in the smaller airways and clara cells also perform the stem cell role in these segments. Beside the progenitor and structural role of the basal cells, they also have a functional impact via secreting bioactive

molecules including neutral endopeptidase and 15-lipoxygenase products (Knight and Holgate, 2003; Nadel et al., 1991; Ohkubo et al., 1993).

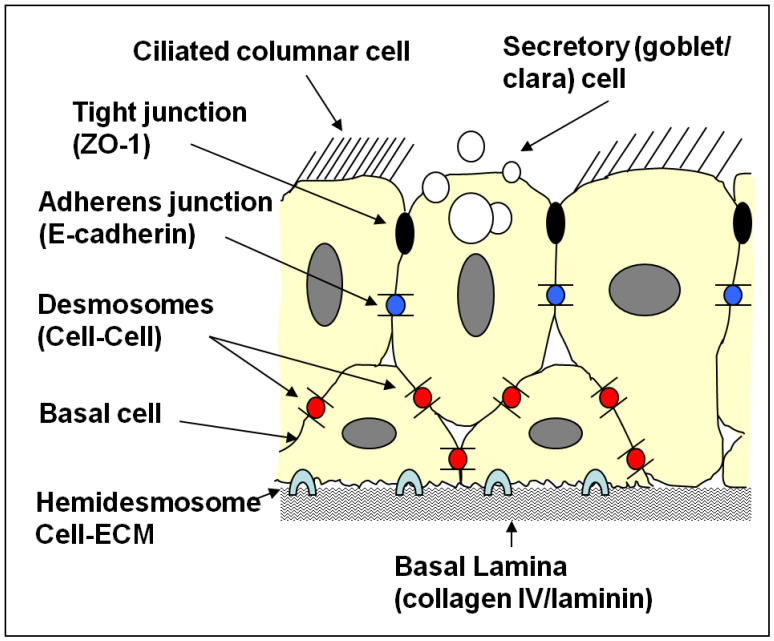


**Figure 2: Cellular players of the airway epithelium.** Adapted from Wadsworth et al. (Wadsworth et al., 2012).

Clara cells, also called “nonciliated bronchiolar secretory cells”, are located in the large, as well as in the small airways of the human airways (Widdicombe and Pack, 1982). To regenerate the bronchial epithelium, clara cells can act as stem cells by multiplying and differentiating into ciliated cells (Atkinson et al., 2008). However, the main function is the protection of the epithelium via synthesis and secretion of defence products into the lumen, including pulmonary surfactant as glycosaminoglycan and proteinase-inhibitors. By the action of p450 mono-oxygenase within the clara cells, inhaled organic harmful substances can be metabolized.

Pulmonary neuroendocrine cells (PNEC) were the first cells discovered to be specialized cells within the airway epithelium, appearing in the very early stage of embryonal development. PNECs possess dense core granules with a variety of biogenic amines and neuropeptides and therefore play an important role in foetal lung growth and airway functions (Cutz et al., 2003; Sunday et al., 2004).

The cells of the airway epithelium have a tendency to form adhesive contact with adjacent epithelial cells to generate epithelial cell-cell junctions, thus they form a physical barrier. This is another important mechanism of defence against inhaled pathogens.

### 1.2.2 The physical barrier of the airway epithelium

The apical junction complex of the physical barrier of the cells consists of tight junctions and adherens junctions. The tight junctions are intracellular junctions, located at the lateral membrane surface at the apical end of the cell. The central function consist of the variable permeable selectively barrier, the gate function, controlling the passage of ions, water and various macromolecules, and the fence function, assigning the polarity of the epithelium through function as a demarcation between the apical and basolateral domains of the membrane (Sawada et al., 2003). The transmembrane proteins of the tight junctions consist of occludin, claudins and ZO-1 (Zonula Occludens), ZO-2 and ZO-3 which are linked to the actin cytoskeleton (Fanning et al., 1998). The permeability of tight junctions can be modified by cytokines such as TNF-α, IFN-γ, interleukins and growth factors. The second part of the apical junction complex, the adherens junctions, is composed of E-cadherin, linked to the actin cytoskeleton via the α- and β-catenin adapter complex (Figure 2). Adherence junctions play an important role in the development of the epithelial layer and tight junction formation (Fristrom, 1988). This barrier function of the epithelium is deficient in patients suffering from asthma, as a post-transcriptional loss of junctional ZO-1 and occluding was observed in lung biopsies of asthma patients. Furthermore, in differentiated bronchial epithelial cells from donors with severe asthma, a reduced level of E-cadherin and an increased epithelial macromolecular permeability was detected (Xiao et al., 2011). Additionally, the barrier of cell-cell junctions can prohibit the entrance and dissemination of virus particles through the epithelium (Yoon and Spear, 2002).

Beside the physical barrier of the airway epithelium, there is also a chemical barrier to protect the host from pathogens. The production of mucus is the most important part of this chemical defence against microbes.

### 1.2.3 The chemical barrier of the airway epithelium

As mentioned before, goblet cells are the source of mucus, which creates a semipermeable barrier at the epithelial surface. The exchange of nutrients, water and gases is enabled, but most pathogens are not capable to penetrate the mucus. Beside a large variety of different

protective proteins such as antimicrobial peptides, cytokines and antioxidant substances, mucus is rich of mucins, extensively glycosylated proteins, which vary in cellular location, complex formation and glycosylation states (Williams et al., 2006). Nowadays, 11 mucins have been detected in human airways. However, MUC5AC and MUC5B are the important mucins in human sputum and are mainly regulated by nuclear factor κB (NF-κB) and/or Specificity Protein 1 (Sp1) (Rose et al., 2001). MUC5B represents the principal mucin under normal condition, whereas in asthma, MUC5AC production appears to be up-regulated leading to an asthmatic mucus hypersecretion (Evans et al., 2009). New therapeutic strategies for patients suffering from a mucus overexpression due to an airway disease are of big need. Treatments could interrupt the signalling pathway of NF-κB or Sp1 and therefore regulate the mucus production.

### 1.2.4 The immunologic barrier of the airway epithelium

When inhaled pathogenic substances penetrate the physical and chemical barriers of the airway epithelium, cells of the airways have the ability to recognize pathogen-associated molecular pattern (PAMPs) rapidly through pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) and intracellular receptors. PAMPs, which are recognized by TLRs, are pathogen specific molecules, derived from viruses, fungi, bacteria and parasites (Poltorak, 1998; Underhill et al., 1999). The recognition of PAMPs and the initiation of immune responses via binding and activation of the TLR pathways are essential for the respiratory epithelial defence. TLRs trigger their signal pathway via several key transcription factors such as NF-κB and interferon regulatory factor 3 and 7 (IRF-3 and IRF-7) or via activating mitogen-activated protein kinase (MAP), resulting in the induction of numerous pro-inflammatory cytokines, chemokines and type I and type III interferons (IFNs) (Iwamura and Nakayama, 2008).

The localisation of the TLRs differs depending on their preference for intra- or extracellular pathogens: TLR1, TLR2, TLR4, TLR5 and TLR6 are expressed on the cell surface of the epithelium, whereas TLR3, TLR7, TLR8 and TLR9 are located in intracellular vesicles. The signalling cascade of TLR7 and TLR9 act via using the adaptor MyD88, the myeloid differentiation primary response gene 88, for the activation of NF-κB and IFR-7 to initiate the production of pro-inflammatory cytokines such as IL-6 and TNF-α, and the production of type I interferons (Honda et al., 2004). Except for TLR3 signalling, all Toll-like receptors signal via MyD88/IRAK-dependent pathway. TLR3 signalling instead functions via the TRIF pathway (Figure 3).

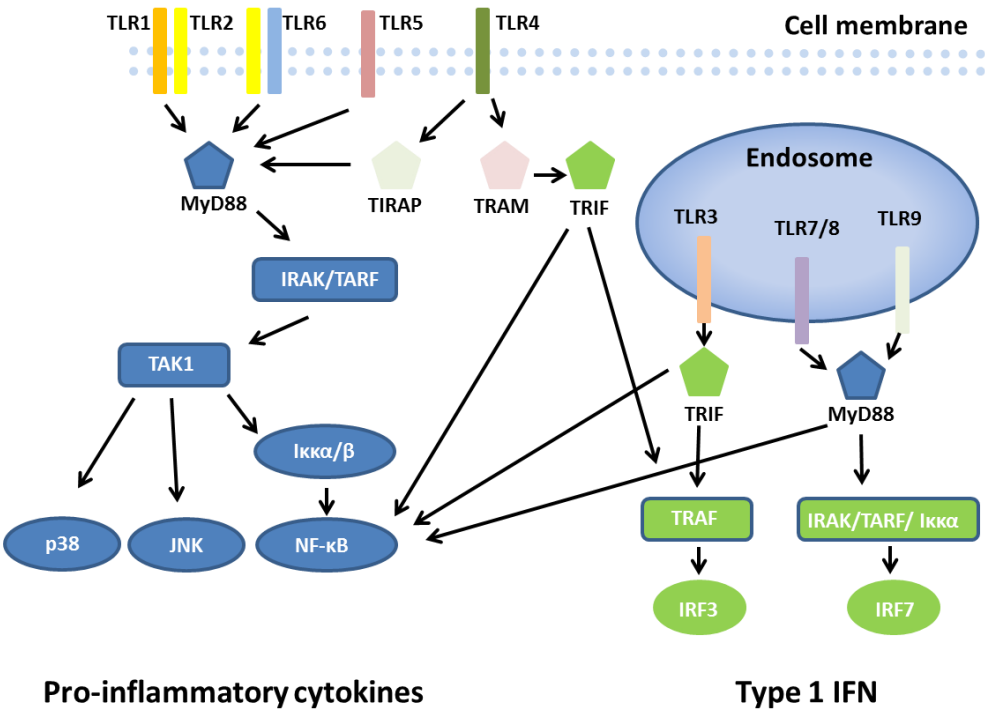


**Figure 3: The different Toll-like receptors of the epithelium and their corresponding signalling pathways in the cell.** Modified by Kumar 2009 (Kumar et al., 2009)

TLR2, TLR4 and TLR6 are typically known to be activated by bacterial products, such as LPS from gram-negative bacteria (TLR4), peptidoglycan from gram-positive bacteria (TLR2), mycobacteria (TLR1/2), flagellin from flagellated bacteria (TLR5) and genomic DNA rich in unmethylated CpG DNA from bacteria and viruses (TLR9) (Hemmi et al., 2000; Latz et al., 2004). Viral RNA can also be detected by intracellular viral sensors, members of the RIG-I-like RNA helicase family, such as RIG-I and melanoma differentiation-associated gene 5 (MDA5). RIG-I and MDA5 are located in the cytosol of the cells and have the potential to detect intracellular double-stranded RNA (dsRNA) and single-stranded RNA (ssRNA), whereas MDA5 has been shown to detect small RNA viruses (e.g. rhinovirus), resulting in the regulation of IFN-β, IFN-λ and other pro-inflammatory cytokines. However, the viral sensor RIG-I has shown the capability to bind ssRNA viruses (e.g. influenza virus or RSV) and therefore plays an important role in the antiviral reaction (Kato et al., 2006). The activation of RIG-I and MDA5, conditioned by the binding of viral ligands leads to the recruitment and activation of protein kinases and therefore to the phosphorylation of IRF-3.

### 1.2.5 The infiltrating immune cells of the airway epithelium

In addition to the protection of the host via the mechanisms of the airway epithelium itself, infiltrating immune cells play a major role in the barrier defence. The coordination and regulation of migrating immune cells is dependent on the presence of chemokines. Chemokines do not only control the migration of immune cells, but also the interaction between the immune cells. The rhodopsin-like seven-transmembrane-spanning chemokine receptors are G-protein-coupled and located at the surface of the cells. The chemokine receptors have a different specificity of binding to their ligands, meaning that CC chemokine ligands (CCL) only bind to the CCR chemokine receptors whereas CXC chemokine ligands (CXCL) can only bind to CXCR receptors.

To promote an efficient antiviral response, the recruitment of various immune cells is essential. If the airway epithelium is infected by a virus, secreted chemokines and cytokines are crucial to promote neutrophil, eosinophil, NK cell and monocyte recruitment from the blood stream to the side of infection in the tissue.

Early in response, neutrophils are recruited in response to IL-8/CXCL8, Gro-α/CXCL1 and ENA-78/CXCL5. Pizzichini et al. showed an increase of neutrophils in induced sputum at day 4 of a natural cold in asthmatics and non-asthmatics in line with an increasing IL-8 level within the sputum (Pizzichini et al., 1998). A recent study reported that CXCR4 signalling may promote the accumulation of neutrophils in the lung vasculature so that they can be rapidly mobilized (Devi et al., 2013). However, the up-regulation of CXCR4 on neutrophils may also promote re-entry into the bone marrow and therefore eliminate the neutrophils via apoptotic cell death (Martin et al., 2003).

The recruitment and activation of eosinophils to the lung epithelium is dependent on the influence of RANTES/CCL5, eotaxin-1/CCL11, eotaxin-2/CCL24, granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-5. The antiviral, cytotoxic effect of eosinophils is due to the release of eosinophil-derived neurotoxin and eosinophil cationic protein (ECP) which are both members of the ribonuclease A superfamily (RNase A) (Kimpen et al., 1992; Rosenberg and Domachowske, 2001; Slifman et al., 1986). Levels of RANTES are increased in nasal secretions of children with natural virus-induced asthma (Teran et al., 1999) and in primary nasal epithelial cell cultures, infected with respiratory syncytial virus (RSV) (Saito et al., 1997).

The function of NK cells is to eliminate infected target cells and to modulate the adaptive immunity towards an effective antiviral response. NK cells itself rapidly produce pro-inflammatory cytokines, such as IFN-γ, at an early point of an anti-viral immune response,

which is important for the antigen-independent activation of antigen-presenting cells. The recruitment and activation of NK cells at the site of infection is dependent on IFN-α/β and macrophage inflammatory protein-1α (MIP-1α/CCL3) (Biron et al., 1999).

The production of IL-1β, MIP-1α/CCL3, monocyte chemoattractant protein 1 (MCP-1)/CCL2 and TNF-α by the airway epithelium during viral infection lead to the recruitment of monocytes/macrophages. Macrophages play a central role in antiviral immune responses by internalizing and processing viral antigens for presentation to cells of the adaptive immune system. Additionally, the release of cytokines such as IL-12 and IL-10 leads to different immune modulations such as NK cell activation, regulation of Th1 responses and elevated B-cell survival, proliferation and antibody production (D'Andrea et al., 1992, 1993; See and Wark, 2008).

Beside the regulation of the innate immune response via the airway epithelium, the control and modulation of the adaptive immune response through interaction of DCs, T cells and B cells is also concerted by the epithelium.

Infiltrating and local DCs have an important role in the initiation of the adaptive immune response via inducing the proliferation and activation of T cells in the lung epithelium. There are two major types of pulmonary DCs, the conventional DCs (cDCs or myeloid DCs/mDCs) and plasmacytoid DCs (pDCs) (Siegal, 1999). Viral infections increase the numbers of cDCs and pDCs in the lung (Smit et al., 2006). The main task of cDCs is the uptake of foreign antigens, leading to the maturation process of the cDCs. Starting with the down-regulation of chemokine receptors, involved in homing to the lungs, cDCs up-regulate the expression of CCR7, which is a chemokine receptor that leads to the migration of the cDCs to the lymph nodes. Reaching the lymph nodes, cDCs mature into antigen-presenting cells characterized by the expression of CD80, CD86 and major histocompatibility complex (MHC) I and II to stimulate $CD8^+$ and $CD4^+$ T cells. The secretion of airway epithelium derived cytokines, such as IL-15 or TSLP plays an important role in the differentiation and maturation of DCs. IL-15 was shown to induce the differentiation of DCs from monocytes, while TSLP has been shown to induce DC-mediated Th2 differentiation (Rate et al., 2009; Regamey et al., 2007). cDCs themselves are major producers of IL-12. Compared to the immune function of cDCs, pDCs lack the ability to process and present antigens. Instead, the main function of pDCs is the production of IFN-α in large amounts. The produced IFN-α triggers a powerful innate immune response and therefore limits the viral replication. pDCs also migrate into draining lymph nodes where they secrete type I IFNs and IL-12, thus promoting a protective Th1 response (Cella et al., 1999; Smit et al., 2008). pDCs and cDCs also differ in the expression of TLRs on their surface: human cDCs express primarily TLR2 and TLR4, responding to viral proteins

(e.g. RSV fusion protein), whereas pDCs express TLR7 and TLR9 and therefore recognize single-stranded RNA and viral DNA.

In case of a viral infection, the airway epithelium recruits T cells into the mucosa by producing RANTES/CCL5 and IP-10/CXCL10 for the infiltration of Th1 cells. Additionally, CCL1 and CCL17/TARC are produced to induce the migration of Th2 cells (Montes-Vizuet et al., 2006; Saito et al., 1997; Sauty et al., 1999; Spurrell et al., 2005). Th1 and Th2 cells are both important for the antiviral adaptive immune response, Th1 cells via production of IFN-γ, IL-12, TNF-α and IL-2. Macrophages get activated by IFN-γ to efficiently kill intracellular microbes, to produce co-stimulatory molecules and to up-regulate the expression of MHC-II receptors. Additionally, IL-12 and IFN-γ are important for the differentiation of Th1 cells. Infiltrated Th2 cells in the airway epithelium produce IL-4, IL-5 and IL-13, which initiates the activation of mast cells and eosinophils and induces mucus secretion. Furthermore, secreted Th2 cytokines play an important role in humoral immunity against viruses by co-activating B cells. The upstream regulation of IL-4, IL-5 and IL-13 is dependent on epithelial derived cytokines such as IL-25, IL-33 and TSLP, which are important mediators in allergic inflammation (Schmitz et al., 2005; Soumelis et al., 2002; Wang et al., 2007). The airway epithelium has been identified as a major source of these cytokines. When the epithelium responds to an environmental exposure, released IL-33 is activating mast cells in the epithelium and additionally promotes the development of type 2 innate lymphoid cells (ILC2s) in combination with IL-25. Activated ILC2s secrete a Th2 cytokine profile, including IL-9, IL-15 and IL-13, thus inducing a Th2 response (Angkasekwinai et al., 2007). In case of severe asthma, levels of TSLP, IL-33 and IL-25 are elevated in the airways of the patients (Ballantyne et al., 2007; Préfontaine et al., 2010; Ying et al., 2005). In a controlled and normal immune system, there is an important balance between Th1 and Th2. The normal T-cell response to virus infection is regulated by a Th1 response of the host.

A structural change of the airway epithelium in patients suffering for airway diseases such as asthma, is called airway remodelling. It is characterised by changes including disruption and thickening of the epithelium together with goblet cell metaplasia (Aikawa et al., 1992; Benayoun et al., 2003). These modifications result downstream in changes regarding the deposition of collagens and other extracellular matrix proteins. This includes amongst others smooth muscle hyperplasia and hypertrophy, generation of myofibroblasts, subepithelial fibrosis and increasing angiogenesis (Siddiqui et al., 2007). In the histopathology in the lungs of asthmatics, all these structural changes are present, thus airway remodelling is a hallmark of asthma and often occurs in parallel with airway inflammation (Busse, 2010). The possibility

to development asthma in early childhood (first 2-3 years of life) is significantly promoted by the presence of viral infections (Sigurs et al., 2000).

Infiltrating immune cells, which migrate into the tissue in case of an inflammation, control and regulate the response of the immune system. If there is an imbalance due to a chronic or inflammatory disease, the overreaction leads to an abnormal recruitment of immune cells and in a second consequence it can lead to airway remodelling. Thus, the regulation of the migration rate of immune cells, direct or indirect by mediators, represents an interesting approach in the design of new drug developments.

## 1.3 Cytokine signalling in airway epithelium

As described before, the recognition of pathogens is mediated via binding of the molecules to PRRs on innate immune cells or the epithelium, which triggers a rapid intracellular signal transduction. These complex processes alter and educate the host immune system. One of the most essential responses initiated by PRR signalling is the synthesis and secretion of cytokines. Cytokine receptors can be classified in six groups; Interleukin type I cytokine receptor (1), interleukin type II cytokine receptor (2), immunoglobulin (Ig) superfamily (3), tumour necrosis factor receptor (TNFR) family (4), IL-17 family receptor (5) and the chemokine receptors that couple to G proteins (6) (Abbas et al., 2012; Yao et al., 1997).

Cytokines binding to the type I cytokine receptors are 4-α-helical bundle proteins, such as G-CSF, IL-3, IL-5, IL-6 and IL-11, but there is also IL-2 that binds to the type I receptor, which requires the common γ chain of the receptor. The cytokine release after binding and activation of the common γ chain receptor play an important role in B and T cell development and polarization and therefore in the immune defence against pathogens.

Cytokines with the ability to bind to type II cytokine receptors can be divided into four subgroups; IL-10 family members, type I, II and type III interferon family members (e.g. IFN-α, IFN-β, IFN-γ and IFN-λ). Members of the IL-10 family including IL-19, IL-20, IL-22 and IL-24 have been shown to have various effects on the skin such as proliferation and differentiation of keratinocytes (Blumberg et al., 2001; Kunz et al., 2006; Poindexter et al., 2010).

The Ig superfamily receptor provides an extracellular immunoglobulin (Ig)-like domain, and IL-1 family cytokines can bind to the receptor. The IL-1 family members IL-1α and IL-1β have been shown to play a role in epidermal differentiation and skin disease (Wood et al., 1992; Yano et al., 2008). A member of the IL-1 family, IL-33, has the ability to bind to the IL-1 receptor related protein ST2, which is specifically expressed on Th2 cells. This leads to the

enhanced production of Th2 cell cytokines including IL-4, IL-5 and IL-13 (Schmitz et al., 2005).

TNF receptors bind ligands belonging to the TNF family, expressed by various cells of the immune system including B cells, T cells, NK cells, dendritic cells and monocytes.

IL-17A and IL-25 (IL-17E) bind to the IL-17 family receptors, both cytokines are important regarding the skin barrier.

The signalling of all these receptors through their intracellular parts activates important immune pathways: JAK/STAT pathway, MAP kinases, PI3K/AKT pathway and NF-κB pathway (Figure 4).

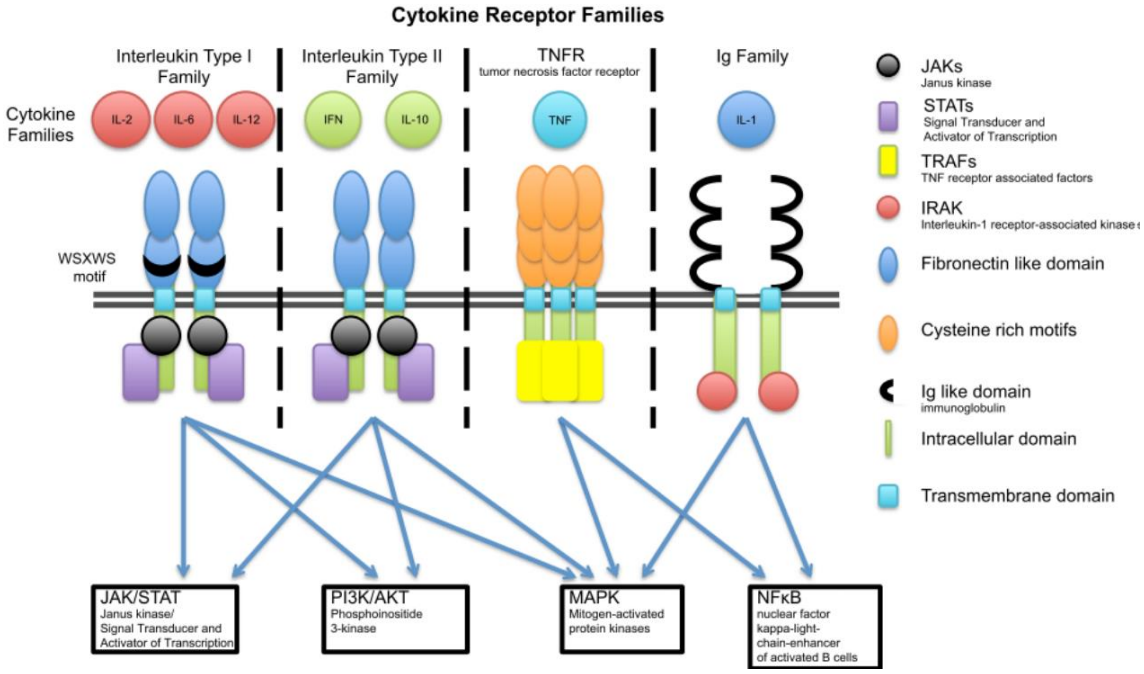


**Figure 4: The different cytokine receptor family members and their attendant agonists.** Adapted from Hänel et al. (Hänel et al., 2013).

### 1.3.1 The nuclear factor κB pathway and its functions in airway cells

NF-κB is a pleiotropic transcription factor, which is present in almost all cell types (Sen and Baltimore, 1986). It is involved in many biological processes in the host, including inflammation, immunity, differentiation, cell growth, tumorigenesis and apoptosis. NF-κB is comprised of homo-or heterodimers of five NF-κB family members: NF-κB1/p50, NF-κB2/p52, RelA/p65, Rel/c-Rel and RelB. Different dimer combinations function as activators or repressors and

this is dependent on post-translational modifications and subcellular location as well as on the interaction with co-factors and co-repressors. If NF-κB binds to a family member of the NF-κB inhibitor (IκB), it remains at an inactive state and is hold in the cytoplasm of the cell. The conversion into the active form occurs if IκB is phosphorylated by IκB kinase (IKK) and therefore dissociates from the NF-κB complex (DiDonato et al., 1997; Mercurio et al., 1997). This leads to the translocation into the nucleus where the complex binds to the promoter of NF-κB, followed by the transcription of various important genes. The signalling of NF-κB can be mediated through the classical or the non-classical pathway. The classical pathway involves IKK and is activated by TNF-α, the non-classical pathway is IKK complex independent and is triggered by cytokines such as lymphotoxin, CD40 ligand or the Epstein-Barr virus. Notably, beside these two pathways, NF-κB activation can also be triggered by divergent stimuli including IL-2, IL-4, IL-8, IL-12, LPS, bacteria, dsRNA or if the epithelium is exposed to environmental hazards such as cigarette smoke, lead or extreme pH (Batra et al., 2011).

In airway diseases including asthma and COPD, infiltrating immune cells express specific mediators, such as cytokines, chemokines and cell adhesion molecules which act in an autocrine or paracrine fashion on the inflammatory cells themselves, but in addition also act on epithelial cells. NF-κB plays an important role in regulating the expression of inflammatory genes such as IL-6, IL-8, MIP-2 and TNF-α in these airway cells (Collart et al., 1990; Driscoll et al., 1995; Harant et al., 1996; Lee et al., 2004; Poynter et al., 2002; Sancéau et al., 1995; Shakhov et al., 1990).

Infiltrated lymphocytes have important immune-modulating effects in different asthma endotypes. In neutrophilic asthma, there is a dominant Th1 and Th17 response; Th17 cells produce elevated levels of IL-17A, IL-17F and IL-22. IL-17A has been shown to induce epithelial mucus production through the NF-κB pathway (Fujisawa et al., 2011), whereas IL-22 is involved in the survival of airway smooth muscle cells, triggered through the NF-κB pathway (Chang et al., 2012). The production of IL-4, IL-5 and IL-13 by Th2 cells in the airways of patients suffering from eosinophilic allergic asthma is NF-κB dependent. The expressed cytokines lead then to an enhanced IgE production by stimulated B cells. The enhanced production of IL-8, TNF-α and intercellular adhesion molecule (ICAM)-1 through eosinophils is also dependent on the NF-κB pathway. The recruitment of neutrophils in airway diseases is amongst others IL-8 mediated, which is a NF-κB regulated gene. The reduction of IL-8 expression, as well as the downregulation of the transcriptional activity of NF- κB, could be of benefit for patients suffering from inflammatory airway diseases and are therefore an interesting point of action for therapeutic strategies.

### 1.3.2 The signal transducer and activator of transcription pathway and its function in airway cells

Other important pathways of the immune system are the signal transducer and activator of transcription (STAT) pathways. To date, seven STAT family members in mammals are known: STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B and STAT6. The activation of STATs is dependent on tyrosine Janus kinases (JAKs), which are associated with several cytokine receptors. The activated tyrosine kinase phosphorylates tyrosine residues within the intracellular domain of the receptors. This modulation provides docking sites for the SH2-domain of STAT proteins, which are localised in the cytoplasm of the cell. After the association with the receptor, STATs undergo Jak-mediated tyrosine phosphorylation, which leads then to the formation of hetero- and homodimers of the STATs via SH2-phosphotyrosine interaction. Now, the dimerized STATs translocate into the nucleus where they bind to a specific DNA binding site and regulate the expression of various genes for cytokine response (Bazan, 1990; Darnell et al., 1994; Ihle et al., 1997; Xu et al., 1996).

As described before, members of the interferons (IFNs) and IL-10 family have the ability to interact with the class II receptors, which then can activate the JAK/STAT cascade (Greenlund et al., 1994; Lackmann et al., 1998; Schindler and Darnell, 1995). Certainly, there is also a negative feedback loop mechanism to regulate the activated STATs and the subsequent protein expression. A group of proteins, called negative regulator suppressor of cytokine signalling (SOCS), inhibit STAT activation in different ways such as by associating with cytokine receptors, blocking the recruitment of STATs to the receptor, inactivating the enzymatic activity of JAK through binding to JAK or by targeting STATs for proteosomal degradation (Narazaki et al., 1998; Nicholson et al., 1999).

In several cancer cell lines, as well as in many tumour species, STAT1, STAT3 and STAT5 has often been found constitutively phosphorylated and activated (Kirito et al., 2002; Watson and Miller, 1995; Weber-Nordt et al., 1996). The transcription factors STAT3 as well as STAT5 may be directly involved in tumorigenesis, as they promote the expression of several genes, which are important for cell proliferation, cell growth and angiogenesis. Of note, Bromberg et al. identified STAT3 as an oncogene (Bromberg et al., 1999). STAT3 has also been shown to induce the transcription of many genes beneficial for tumour cells, such as vascular endothelial growth factor (VEGF), cyclin D1 or chemokines and is therefore frequently discussed as a target in tumour defence (Yue and Turkson, 2009). Instead, STAT1 has been shown to induce tumour suppressor genes downstream of IFN signalling and therefore was thought to be protective in tumour cells. But another study suggested, that

under certain cellular environmental conditions, STAT1 can also promote tumour growth and development (Kovacic et al., 2006).

STAT6 is activated by interaction of IL-4 and IL-13 with their receptor. Elevated levels of IL-13 and IL-4 are detected in airway smooth muscle cells of patients suffering from asthma as mentioned before. STAT6 is involved in the differentiation of naïve T cells into Th2 cells. In fibroblasts, smooth muscle cells and airway epithelial cells, STAT6 regulates the IL-4 and IL-13 induced production of various type 2 cytokines and eotaxin (Mathew et al., 2001; Matsukura et al., 2001). STAT6 plays a major role in the development of airway inflammation and airway hyperresponsiveness (AHR); an increased sensitivity of the airways, and mucus production (Hoshino et al., 2004; Kuperman et al., 1998; Mathew et al., 2001). It could be shown that a cell-penetrating STAT6 inhibitor protein, which binds to the SH2 domain of STAT6 with high affinity, inhibits STAT-6 dependent production of IL-4 and IL-13 from antigen-stimulated primary murine splenocytes. Additionally, the STAT6 inhibitor reduces IL-4 induced production of eotaxin-3 in cultured bronchial epithelial cells. It could also be demonstrated that the STAT6 inhibitor is specific to inhibit Th2 cytokine production as it reduces the ovalbumin-induced production of IL-4 and IL-13, but did not modulate the production of IFN-γ (McCusker et al., 2007). Moreover, in a murine model of allergic rhinitis and asthma, a STAT6 inhibitor was applied directly into the nose. This led to a reduced mucus production, accumulation of eosinophils, lung inflammation and IL-13 production in bronchial alveolar fluids (BAL) (McCusker et al., 2007). This local application of a cell-penetrating STAT6 inhibitor peptide demonstrates the high potential of this peptide in reference to the common treatment of asthma or allergic rhinitis.

New therapeutic approaches within the field of inflammatory airway diseases or chronic airway diseases have to be developed. The point of action for a drug could be in modulation of the important mediators which trigger inflammation. As described above, inflammatory mediators such as IL-8, IL-6, IL-1α or $LTB_4$ play a major role in the immune response and the selected regulation of these cytokines could be of benefit for several inflammatory diseases. Additionally, the interruption of specific significant pathways within an inflammation could be a target for a new drug. The selective regulation of specific STAT signalling pathways within an inflammatory process could represent an interesting target for upcoming therapeutic strategies. Beside synthetic drugs, the use of phytodrugs in various inflammatory diseases have proven their strong potential as a therapy concept.

# 2 Aim of the study

## 2.1 The nasal epithelium – an important player in innate immunity

The nasal epithelial cell represents an important component of the airway epithelium. Nasal epithelial cells express an array of various pattern recognition receptors (PRRs) such as TLR1/2, TLR3, TLR5, TLR7, TLR9, RIG-I and MDA-5 (Lin et al., 2007; Tengroth et al., 2014). These PRRs have the ability to sense multiple PAMPs and metabolites such as synthetic triacylated lipopeptide Pam3CSK4 (TLR1/2), dsRNA Poly I:C (TLR3), flagellin (TLR5), R848 (imidazoquinoline compound) (TLR7), CpG DNA (TLR9) and viral RNA (RIG-I/MDA5) (Sha et al., 2004), thereby contributing to the initiation and control of immune responses. In the context of inflammation, immune cell-derived cytokines, such as IL-4, IFN-γ and IL-6 act on activated epithelia via their respective receptors, providing a feedback mechanism for the adjustment of epithelial immune responses (Galy and Spits, 1991; Maloney and Gao, 2015; Voehringer et al., 2004; Zissler et al., 2015).

A disease-driven change in the expression of several cytokine and chemokine genes can lead to an abnormal modification of the immune response in nasal epithelium cells or other airway epithelial cells.

However, little is known about direct effect of medical treatment on human nasal epithelial cells, and the implication on the immune response. The nasal epithelium plays an important role but the local immunological mode of action of many drugs is poorly discovered yet. Brashier et al. showed an improvement in diabetes treatment through inhaled insulin (Brashier et al., 2015), Boateng et al. shows off the advantages of a drug delivery system of the mucosal routes (Boateng et al., 2015). All these finding indicates that strategies in drug development should include topic application and improve the understanding of mechanism of the immune responses in the cells of the nasal epithelium.

## 2.2 Biochemistry and pharmacology of the phytodrugs Ze 339

Nowadays, patients suffering from allergic or non-allergic diseases are searching for alternative treatments to combat immune over-reactivity. Due to their high acceptance in the population, plant-derived drugs (“phytodrugs”) have a great potential for medical innovations. Ze 339 is an carbon dioxide extract obtained from the leaves of butterbur (*Petasites hybridus*), a plant that has been applied therapeutically for more than 2000 years for the treatment of gas-

trointestinal colics, spasms of the urogenital tract, asthma, cough and dysmenorrhea (Debrunner B, Meier B., 1998). In the past, specific sesquiterpenes, the petasins, have been identified as active substances within Ze 339. Amongst other compounds, 30% of the Ze 339 complex consists of petasins, composed of the isoforms isopetasin, neopetasin and petasin (Figure 5). Petasins showed to decrease intracellular $Ca^{2+}$ transients and inhibit the activation of 5-lipoxygenase (5-LO), thereby limiting the synthesis of leukotriene $B_4$ in neutrophils, eosinophils and basophils (Bickel et al., 1994; Dumitru et al., 2011; Thomet et al., 2001). In a recent human randomized double placebo controlled clinical trial, Ze 339 treatment led to faster recovery from nasal obstruction and decreased the local production of IL-8 and $LTB_4$ measured in nasal lining fluid of allergic patients (Dumitru et al., 2011). Under the name of Tesalin® Ze 339 is currently approved for the treatment of allergic rhinitis in Switzerland and South America. Notably, the pro-inflammatory actions of IL-8 and $LTB_4$, which were modulated by Ze 339 in the experimental allergen challenge study, are not limited to allergic airway inflammation. Instead both mediators are involved in acute immune responses to microbial pathogens and environmental stressors (Crooks et al., 2000; DiPersio et al., 1988; Keshari et al., 2012).

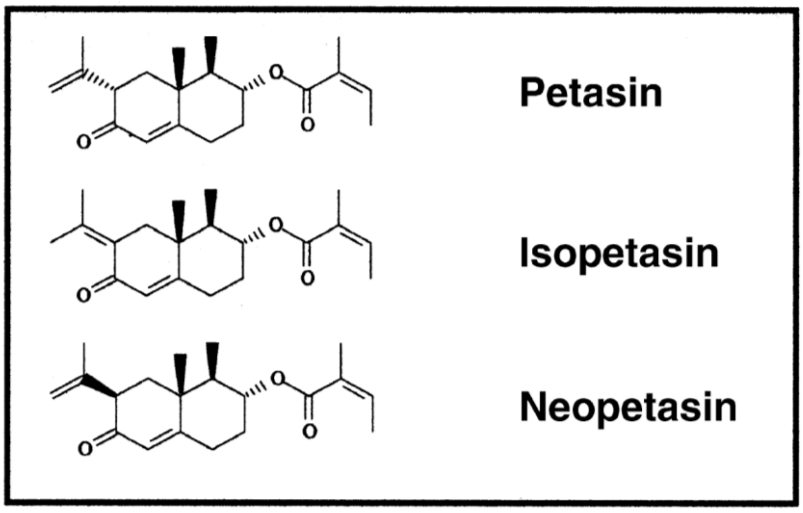


**Figure 5: Petasin isoforms: Petasin, Isopetasin and Neopetasin** (Adapted from Thomet et al., 2001).

Aim of this study was therefore to investigate immune modulatory effects of Ze 339 on primary human nasal epithelial cells (HNECs). For the generation of primary HNECs, biopsies of patients that underwent turbinoplastic surgery were processed and cultured to achieve single culture of primary HNECs. As a first experimental setup, the immune modulating effect of Ze

339 and the single components were analysed on the level of IL-8 production by HNECs. Furthermore, the cytotoxicity of Ze 339, or the single isoforms, for HNECs was determined, in the absence and presents of the viral mimic PolyIC. Subsequently, we treated the cultured HNECs with different inflammatory settings, such as viral, bacterial and cytokine stimulation to analyse immune modulation regarding specific cytokines and chemokines produced by HNECs. Additionally, a microarray analysis of pre-stimulated HNECs with Ze 339 and PolyIC was performed to assess changes on gene expression level for detectable genes involved in the immune response. HNECs treated with the leukotriene receptor antagonist Montelukast served as a control drug. To verify the received data quantitative Real time-PCR of modulated genes was processed.

To date, little is known about the signalling pathway of Ze 339. Therefore, the effect of Ze 339 on cytokine induced phosphorylation and intracellular localisation of STAT1, STAT3 and STAT6 was assessed. The results of this study could lead to a better understanding of the immune modulating effects and signalling pathway of Ze 339 in human primary epithelial cells and therefore assess whether the compound could be applied for treatment of inflammatory conditions other than allergic airway disease, e.g. non-allergic rhinitis or sinusitis.

# 3 Methods and material

| Reagent | Supplier |
|---|---|
| 10x D-PBS w/o Ca/Mg | Gibco, Life Technologie Gmbh, Darmstadt, Germany |
| 2-Mercapto-Ethanol | Sigma Aldrich, München, Germany |
| 20x NuPAGE Running Buffer | Life Technologie Gmbh, Darmstadt, Germany |
| 20x NuPAGE Transfer Buffer | Life Technologie Gmbh, Darmstadt, Germany |
| 4′,6-Diamidin-2-phenylindol (DAPI) | Life Technologie Gmbh, Darmstadt, Germany |
| Airway Epithelial Cell Basal Medium | Promocell, Heidelberg, Germany |
| Airway Epithelial Cell Growth Medium | Promocell, Heidelberg, Germany |
| Albumin from bovine serum (BSA) | Sigma Aldrich, München, Germany |
| Antibiotic/antimycotics | Life Technologie Gmbh, Darmstadt, Germany |
| Aqua ad injectabilia | Laboratori Diaco Biomedicali, Trieste, Itlay |
| $C_2H_8O_7$ | Roth, Karlsruhe, Germany |
| Cell Fractionation Kit | Cell Signaling Technologie, Danvers, MA, USA |
| Citric acid (0.1 M) | Merck, Darmstadt, Germany |
| Comassie Plus Protein Assay | Thermo Fisher Scentific, Waltham, USA |
| CpG oligonucleotide 5'-tcgtcgttttgtcgttttgtcgtt-3' | Invivogen, San Diego, USA |
| DEPC treated water (pyrogen free) | Invivogen, San Diego, USA |
| Dimethylsulfoxid (DMSO) | Merck, Darmstadt, Germany |
| ECL Select Western Blotting Detection Reagent | GE Healthcare, München, Germany |

| | |
|---|---|
| EDTA (0.5 M, pH 8.0) | Gibco, Life Technologie Gmbh, Darmstadt, Germany |
| Ethanol absolute | Merck, Darmstadt, Germany |
| Fast Start Universal SYBR Green Master | Roche, München, Germany |
| Flagellin from *pseudomonas aeruginosa* | Invivogen, San Diego, USA |
| Gentamycin | Life Technologie Gmbh, Darmstadt, Germany |
| Glutaraldehyde | Serva, Heidelberg, Germnay |
| $H_2O_2$ | Sigma Aldrich, München, Germany |
| $H_2SO_4$ | Merch, Darmstadt, Germany |
| Hexamethyldisilanzane | Polyscience, Warrington, USA |
| Histopaque 1119/1077 | Sigma Aldrich, München, Germany |
| Human IL-6 | Promokine, Heidelberg, Germany |
| Human Interferon (IFN)-γ | Promokine, Heidelberg, Germany |
| Human Interleukin (IL)-4 | Promokine, Heidelberg, Germany |
| Laemmli loading buffer 4x | Amresco, Solon, USA |
| Methanol absolute | Roth, Karlsruhe, Germany |
| Multiplex Assay Human Cytokine/Chemokine Magnetic Bead Panel | Merck KGaA, Darmstadt, Germany |
| NaCl | Roth, Karlsruhe, Germany |
| Non-essential amino Acids | Gibco, Life Technologie Gmbh, Darmstadt, Germany |
| Osmiumtetroxyd | Polyscience, Warrington, USA |
| Pam3CSK4 | Invivogen, San Diego, USA |
| Penicillin-Streptomycin | Gibco, Life Technologie Gmbh, Darmstadt, Germany |
| Poly(I:C) LyoVec | Invivogen, San Diego, USA |

| | |
|---|---|
| Polyethylenglycol (PEG) | Sigma Aldrich, München, Germany |
| Polyinosinic:polycytidylic acid (Poly I:C) | Invivogen, San Diego, USA |
| RNase free DNase Set | Qiagen, Hilden, Germany |
| RNeasy Mini Kit | Qiagen, Hilden, Germany |
| RPMI 1640 + L-Glutamin | Gibco, Life Technologie Gmbh, Darmstadt, Germany |
| Streptavidin-horseradish peroxidase | R&D Systems, Abingdon, U.K. |
| Tetramethylbenzidin (TMB) | Sigma Aldrich, München, Germany |
| Trizma base | Sigma Aldrich, München, Germany |
| Trypanblue 0.4% solution | Life Technologie Gmbh, Darmstadt, Germany |
| Trypsin 0.05% EDTA | Sigma Aldrich, München, Germany |
| Tween 20 detergent | Calbiochem, San Diego, USA |

| **Antibody/ELISA Kit** | **Supplier** |
|---|---|
| *ELISA* | |
| IL-8 ELISA Kit | BD, Bioscience, Heidelberg, Germany |
| Chemokine (C-C Motif) Ligand (CCL)-5 ELISA Kit | R&D Systems, Abingdon, U.K. |
| | |
| *Westernblot/ Immunofluorescence* | |
| Anti-STAT-1 rabbit antibody | Santa Cruz, Dallas, USA |
| Anti-STAT-3 rabbit antibody | Cell Signaling Technologie, Danvers, MA, USA |
| Anti-STAT-6 mouse antibody | Abcam, Cambridge, USA |
| Polyclonal anti-phospho-tyrosine-STAT -1 | Cell Signaling Technologie, Danvers, MA, |

| | |
|---|---|
| antibody | USA |
| Monoclonal anti-phospho-tyrosine-STAT -3 rabbit antibody | Cell Signaling Technologie, Danvers, MA, USA |
| Polyclonal anti-phospho-tyrosine-STAT -6 rabbit antibody | Cell Signaling Technologie, Danvers, MA, USA |
| Anti-Histone H3 | Cell Signaling Technologie, Danvers, MA, USA |
| Alexa Fluor 488 goat anti-mouse IgG antibody | Life Technologie Gmbh, Darmstadt, Germany |
| Alexa Fluor 568 goat anti-rabbit IgG antibody | Life Technologie Gmbh, Darmstadt, Germany |
| Anti-α- tubulin mouse | Cell Signaling Technologie, Danvers, MA, USA |
| Polyclonal goat anti-rabbit IgG HRP antibody | Santa Cruz, Dallas, USA |
| Polyclonal goat anti-mouse IgG HRP antibody | Santa Cruz, Dallas, USA |

## 3.2 Media

All media and buffers were sterile filtered and stored at 4°C.

| **Medium** | |
|---|---|
| Airway Epithelial Cell Growth Medium with supplements | 0.004 ml/ml bovine pituitary extract |
| | 10 ng/ml human epidermal growth factor |
| | 5 µg/ml insulin |
| | 0.5 µg/ml hydrocortisone |
| | 0.5 µg/ml epinephrine |
| | 6.7 ng/ml triiodothyronin |
| | 10 µg/ml transferrin |
| | 0.1 ng/ml retinoic acid |
| | 0.01ml/ml antibiotic/antimycotic |
| | 50 µg/ml gentamycin |
| | 500 ml Basal Airway Epithelial Cell Medium |
| Migration medium | 0.5% BSA |
| | 2.5 ml Penicilin/Streptavidin |
| | 2.5 ml Glutamin |
| | 2.8 ml Non-essential Aminoacids |
| | 2.8 ml Natriumpyruvat |
| | 250 µl Mercaptoethanol |
| | 240 ml RPMI |

| Instrument | Supplier |
|---|---|
| Agilent G 2545A Hybridization Oven | Agilent, Santa Clara, CA, USA |
| Bioanalyzer 2100 | Agilent, Santa Clara, CA, USA |
| Biofuge pico | Kendro, Hanau, Germany |
| Centrifuge | Thermo scientific, Waltham, USA |
| Centrifuge Megafuge | Thermoscientific, Waltham, USA |
| Herasafe 12 Biological Saftey Cabinet | Kendro, Hanau, Germany |
| Ikamag Reo | IKA works, Wilming, USA |
| Incubator Heracell | Thermoscientific, Waltham, USA |
| Intas ECL Chemocam Imager | INTAS Science Imaging Instruments, Göttingen, Germany |
| JSM 6300 scanning microscope | Jeol, Eching, Germany |
| Julabo SW 21 waterbath | Julabo, Seelbach, Germany |
| Labcycler Sensoquest | Biolab products, Gödenstorf, Germany |
| LSR Fortessa flow cytometer | BD, Bioscience, Heidelberg, Germany |
| Microplate spectrophotometer Epoch | BioTek Instruments, Winooski, USA |
| Microscale | Mettler Toledo |
| Microscope Axiovert | Zeiss, Jena, Germany |
| MS1 Minishaker | IKA works, Wilming, USA |
| Multichannel Pipettes | Eppendorf, Hamburg, Germany |
| PCR Cycler TC412 | Techne, Staffordshire, UK |
| Pipettes with disposable tips | Eppendorf, Hamburg, Germany |
| Power Easy 90W | Life Technologie Gmbh, Darmstadt, Germany |
| Spectrophotometer Nanodrop ND 1000 | Peqlab, Erlangen, Germany |
| Tecan Hydrospeed platewasher | Tecan, Grödig, Austria |
| Thermoshaker TS 100 | Kisker Biotech, Steinfurt, Germany |

| | |
|---|---|
| Titramax 101 plateshaker | Heidolph, Schwabach, Germany |
| Unitwist RT | Unieuip, Martinsried, Germany |
| Vortex Genie-2 | Scientific Industries, NY, USA |

| **Material** | **Supplier** |
|---|---|
| Bioplex Pro 96well flat bottom | Bio-Rad Laboratories, Hercules CA, USA |
| Cell culture dish (10cm) | Greiner bio-one, Frickenhausen, Germany |
| Cellstrainer 100µm Nylon | Fisher Scientific, Pittsburgh, USA |
| Chamber slides | BD Bioscience, San Jose, USA |
| Cover slip | Menzel, Braunscheig, Germany |
| Cryotubes (1.8ml) | Nunc, Roskilde, Denmark |
| Extra thick Western Blotting filter paper | Thermoscientific, Waltham, USA |
| FACS tubes | Greiner bio-one, Frickenhausen, Germany |
| Falcon tubes (15ml, 50 ml) | Fisher Scientific, Pittsburgh, USA |
| Immobilon-P transfer membrane | Merck KGaA, Darmstadt, Germany |
| Micro tubes (1.5; 2.0 ml) | Sarstedt Eppendorf, Newton, USA |
| NuPage 4-12% BisTris gel | Life Technologie Gmbh, Darmstadt, Germany |
| Pipette tips | Sarstedt Eppendorf, Newton, USA |
| Pipettes (1, 5, 10 and 25 ml) | Greiner bio-one, Frickenhausen, Germany |
| Pipetus | Hirschmann Laborgeräte, Eberstadt, Germany |
| Single-use syringe (10 ml) | Braun, Melsungen, Germany |
| Sterile filter device (500 ml) | Millipore, Billerica, USA |
| Tissue culture plates (96 well, 24 well, 6 well) flat/U-bottom | Fisher Scientific, Pittsburgh, USA |

## 3.3 Isolation of primary human nasal epithelial cells

Primary human nasal epithelial cells were isolated from inferior turbinates of patients who underwent turbinoplastic surgery, median age 31 years. Nasal biopsies were rinsed thoroughly with sterile D-PBS plus antibiotics/antimycotics, before cut in 2-3mm pieces. The pieces were then digested for 12-24 hours with trypsin-EDTA (0.05%) at 37°C, 5%CO2. On the next day pieces were passed through a 70µm pore-size cell strainer with the plunger of a 10ml syringe and centrifuged (10 min, 1200 rpm, 21°C). Single cell suspensions were seeded into tissue culture plates in supplemented Airway Epithelial Cell Growth Medium plus 1% antibiotics/antimycotics and 0.5% gentamycin (Life Technologies, Carlsbad, USA) and grown to 80% confluence at 37°C, 5%CO2. Fresh supplemented medium was given to the cells every 48h. For the experiments cells from passage 1-2 were used. Written informed consent was obtained from all participants.

## 3.4 Scanning electron micrograph

To phenotype the primary human nasal epithelia cells, primary nasal epithelial cells were seeded onto glass slides at a density of 100,000 cells per 12-well and grown for 2-5 days before fixed with 2% GA in cacodylatbuffer. For dehydration cells were incubated in 1% $OsO_4$ in 1M cacodylatpuffer (pH 7.2) for 1h at 4°C, before washed twice with cacodylatbuffer. Afterwards, cells are treated with 50%, 70%, and 100% ethanol for 10 min each before treatment with HMDS plus ethanol to dehydrate the cells. Pure HMDS is added to the cells over night for total drying before scanning with scanning electron microscope. The cells form a dense monolayer on the slide. The scanning electron microscope generates images from the surfaces of the cells. An electron beam is scanning the surface of the cells, thereby ejecting the second electrons, what forms the signals. The electrons are collected by a detector and converted to an electric signal that appears on the monitor. The scanning was done with the JSM 6300 scanning microscope.

## 3.5 Nasal epithelial cell stimulation

For the stimulation cells were seeded on 6-well culture plates (250 000-170 000 cells/well) or 24-well culture plates (62 000 - 42 000 cells/well). If confluence of 80% was reached, cells were cultured in 1 ml basal medium and incubated for at least 12h before stimulation. Then, cells were cultured for 24h in basal medium (untreated) or stimulated with 10µg/ml Poly(I:C), 4µg/ml Poly(I:C) LyoVec, 10µg/ml R-848, 1-5µM CpG oligonucleotide 5'-

tcgtcgttttgtcgttttgtcgtt-3', 5µg/ml purified flagellin from *pseudomonas aeruginosa*, 200ng/ml synthetic triacylated lipoprotein Pam3CSK4 (Invivogen, San Diego, USA), 15ng/ml IFN-γ, 50ng/ml Interleukin (IL)-4, 800U/ml IL-6 (Promokine, Heidelberg, Germany) with or without addition of Ze 339. The high pressure carbon dioxide ($CO_2$) extract of Petasites hybridus (Ze 339) and its isolated active compounds, petasin, neopetasin and isopetasin, were provided by Max Zeller Söhne AG (Zeller, Analytical Department, Romanshorn, Schwitzerland).

## 3.6 Lactate dehydrogenase assay

To measure the cytotoxicity level of the cells after stimulation with indicated stimuli, we cultured the cells until 80% confluent. Afterwards, cells are cultured for 24h in basal medium (untreated) or stimulated with indicated concentrations of Ze 339, or the isolated active compounds petasin, neopetasin and isopetasin in the absence or presence of PolyI:C (10µg/ml). 100µl supernatant was taken and 100µl reaction mixture was added and LDH activity was quantified with Cytotoxicity Detection Kit. For this absorbance of the samples at 490 nm using ELISA reader was measured.

## 3.7 Isolation neutrophil granulocytes

For the isolation of human neutrophil granulocytes (PMN), 50 ml EDTA-whole blood was taken form healthy volunteers. PMNs were separated via density gradient centrifugation. For this, 13 ml Histopaque 1119 and 13ml Histopaque are carefully layered in a 50 ml falcon. On top, 20 ml of the EDTA-whole blood was layered before centrifuged for 45 min 1900 rpm without brake to separate the cells based on their different density. Whole blood gets separated in plasma, mononuclear cells, Histopaque 1077, polymorphonuclear cells, Histopaque 1119 and erythrocytes. The top three layers are getting discarded, polymorphonuclear cells in Histopaque 1119 are carefully collected with a pipette and transferred in a 50 ml falcon. Cells were washed with D-PBS with centrifugation for 20 min 1400 rpm 20°C. Supernatant was discarded; cells were washed again with D-PBS and centrifuged for 10 min at 1200 rpm 20°C. Afterwards, to lyse erythrocytes, cells were resuspended in 3 ml aqua injectabilia for 25 sec. Directly after this 3 ml 1.8 % NaCl is added to the cells to neutralize. Cells were washed again with D-PBS and centrifuged for 10 min 1200 rpm 20°C. Cells were resuspended in neutrophil migration medium and counted with Trypanblue 0.4% solution in a Neubauer chamber. Cells were adjusted to 1 million per 1 ml migration medium for neutrophil migration assay.

## 3.8 RNA extraction kit

For RNA extraction confluent pre-stimulated cells harvested with trypsin-EDTA (0,05%) are collected in 350µl RLT-buffer/ 2% β-mecaptoethanol and homogenised by vortexing 1min. Then cells were pipetted on RNeasy Spin column collection tube and centrifuged 15 sec 10000 rpm. Flow-through got discarded and 350 µl RW1 buffer was added on the tube. Tubes were centrifuged for 15 sec 10000 rpm. 10 µL Dnase and 70 µL RDD buffer was added on the tube and incubated on room temperature for 15 min. Afterwards, 350 µL RW1 buffer were added on the tube and centrifuged 15 sec 10000 rpm. Flow-through got discarded, 500 µL RPE buffer was added on the tube before centrifuged 15 sec 10000 rpm. Flow-through got discarded, 500 µL RPE buffer was added and tube was centrifuged for 2 min 10000 rpm. Column was put on a fresh tube and centrifuged again with maximal speed for 1 min to dry the column. Then, 30 µL RNase free water was given on the column before centrifugation for 1 min 10000 rpm to elute the RNA from the column. The Flow-through was collected and the RNA concentration was measured by spectrophotometer Nanodrop.

## 3.9 Comassie Protein Assay

To quantify the protein concentration of the samples used for Westernblotting, “Comassie Plus Protein Kit” was used in accordance with the manufacturer's data (Thermo Scientific).

## 3.10 Microarray

RNA was isolated as described above. Quality and integrity of RNA samples was checked with the Agilent 2100 Bioanalyzer platform following the manufacturer’s protocol (Agilent Technologies). 25 ng of each total RNA sample was amplified and Cyanine-3 CTP-dye labelled using the Agilent Low RNA Input Quick Amp Labeling Kit One Color (Agilent Technologies) following the manufacturer’s protocol. The hybridization procedure was performed according to the Agilent microarray processing protocol using the Agilent Gene Expression Hybridization Kit and Agilent Whole Human Genome Microarrays 8 x 60K (Agilent Technologies). Fluorescence signals of the hybridized arrays were detected using Agilent’s Microarray Scanner System (Agilent Technologies). The Agilent Feature Extraction Software was used to read out and process the microarray image files, and differential gene expression Feature Extraction Software–derived output data files were further analyzed using the Gene Spring data analysis system (GeneSpring GX Software).

## 3.11 Neutrophil migration assays

Neutrophil migration assays were performed in 96-well format using ChemoTX transwell plates consisting of two elements, a well for the supernatant to be analysed and a 3µm membrane above the well. Freshly isolated neutrophils were added to the membrane to migrate towards the underlying supernatant. To analyse the chemotactic activity of the stimulated cells, 30 µL supernatant, IL-8 or D-PBS was added into the wells. Then, the membrane was given on top of the wells and 50 µL freshly isolated neutrophils were added. The 96-well plate was incubated at 37°C for 1h. Afterwards, the membrane was discarded and the plate was centrifuged for 30sec 1000 rpm. The cells collected in the wells were resuspended in 200µL FACS-Puffer and counted by LSR Fortessa flow cytometer. The analysed supernatants were supernatants of HNECs pre-treated with 10µg/L PolyI:C with or without Ze 339, a petasin isoform mixture, or isolated petasin, neopetasin and isopetasin (Figure 6).

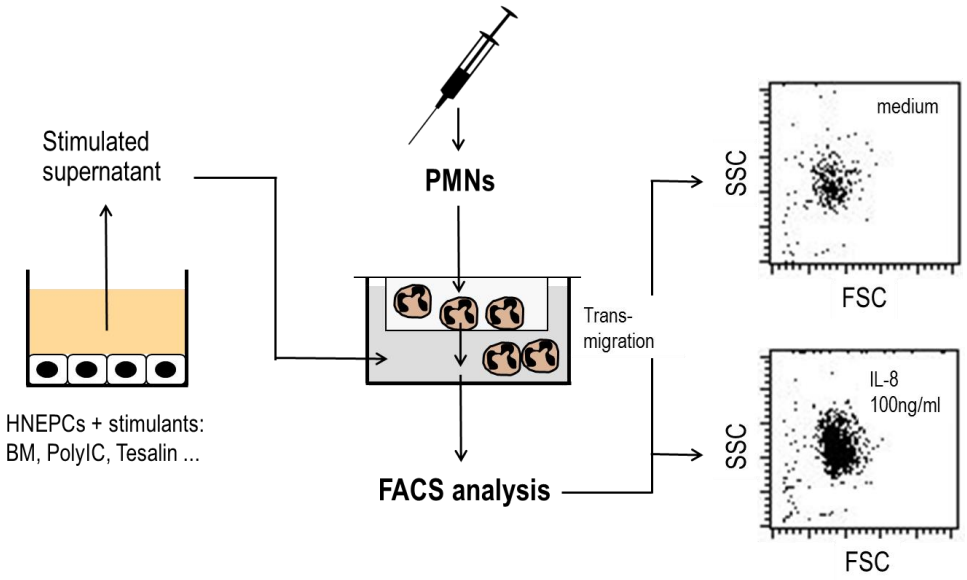


**Figure 6: Neutrophil migration assay**

## 3.12 Cell Fractionation

To analyse the single cell fraction of HNEC a cell fractionation kit was used. Cells were grown until 80% confluence before stimulation with stimuli for appropriate times. To generate the cytoplasmic, membrane and nuclear fraction of the whole cell lysates, the cell fractionation kit protocol was followed (Cell Signaling).

## 3.13 Measurement of cytokine and chemokine levels (Luminex)

Levels of CCL-3 (MIP-1α), CCL-4 (MIP-1β), MCP-1 (CCL-2), IP-10 (CXCL-10), IL-1α, IL-6, G-CSF, TNF-α were measured using a multiplex assay (MILLIPLEX MAP Human Cytokine/Chemokine Magnetic Bead Panel). The magnetic bead assay was run according to the manufacturers' instructions. Data were acquired on a validated and calibrated Bio-Plex 200 system (Bio-Rad) and analysed with Bio-Plex Manager 6.0 software (Bio-Rad).

## 3.14 Analysis of cytokine secretion by enzyme-linked immunosorbent assay (ELISA)

Cytokine concentration of IL-8 and CCL-5 in HNEC cell culture free supernatants was measured by ELISA. The assay was run according to the manufacturers' instructions using commercially available sandwich ELISA kits (BD and R&D Systems).

## 3.15 Western Blot

80% confluent HNECs were stimulated for 30 min (STAT1 and STAT3) and 60 min (STAT6) with medium, 800U/ml IL-6, 15 ng/ml IFN-γ, or 50 ng/ml IL-4 in the absence or presence of 3µM Ze 339 (Zeller). As PEG was used for solubilizing Ze 339, it was added to all wells at a dilution of 1:10,000. HNECs stimulated with PEG alone served as control. Total extracts and sub-cellular fractions were prepared from cells as described above. The protein samples, whole cell lysate and the cell fractionation lysates, were treated with 4x laemmli loading buffer before heating for 5 min at 95 °C. To separate proteins by size, samples were loaded to SDS-PAGE under reducing conditions before being transferred to a nitrocellulose membrane through an electric field by Western Blotting. The Wet-Blot-System consists of a methanol-activated PDVF-membrane, whatman paper, sponges and the gel runs for 1.5 h at 120 V before blotted PDVF-membrane is blocked with 1 % BSA, 0.1% Tween in 1x TBS buffer for 1 h. Afterwards specific primary antibodies were allowed to bind to the proteins of interest (concentrations listed below). A secondary peroxidase-linked antibody was used for detection of the bound primary antibody and chemiluminescence signals were quantitated with the ChemoStar$^{TM}$ software (Intas). To detect the phospho-STATs the membrane was cut into two pieces, the upper part was incubated with monoclonal anti-phospho-tyrosine-STAT3, polyclonal anti-phospho- tyrosine-STAT6 and monoclonal anti-phospho-tyrosine-STAT1, the low-

er part was incubated with anti-β-actin monoclonal antibody or polyclonal anti-Histone H3. The expression of the STATs was reported relative to the β-actin or histone signal.

| **Primary antibody** | **Dilution** |
|---|---|
| Polyclonal anti-phospho-tyrosine-STAT-1 mouse antibody | 1:1000 in 1% BSA, 0.1 % Tween in 1x TBS |
| Monoclonal anti-phospho-tyrosine-STAT-3 rabbit antibody | 1:1000 in 1% BSA, 0.1 % Tween in 1x TBS |
| Polyclonal anti-phospho-tyrosine-STAT-6 rabbit antibody | 1:1000 in 1% BSA, 0.1 % Tween in 1x TBS |
| Polyclonal anti-Histone H3 | 1:1000 in 1% BSA, 0.1 % Tween in 1x TBS |
| Monoclonal anti-β-actin antibody | 1:20000 in 1% BSA, 0.1 % Tween in 1x TBS |
| **Secondary antibody** | **Dilution** |
| Goat anti-rabbit IgG HRP | 1:5000 in 1% BSA, 0.1 % Tween in 1x TBS |
| Goat anti-mouse IgG HRP | 1:2000 in 1% BSA, 0.1 % Tween in 1x TBS |

## 3.16 Immunofluorescence

For immunohistochemistry experiments, HNECs were seeded onto chamber glass slides in complete medium and grown to 80% confluence. Then cells were stimulated with medium control, INF-γ, IL-6 or IL-4 +/- Ze 339 for 30 min (INF-γ, IL-6) or 60 min (IL-4). 4% formaldehyde in 1xPBS for 15 min at room temperature was used to fix the cells. For permeabilization, fixed cells on chamber slides were incubated with 100% ice-cold methanol for 20 min. Immunostaining of total and phospho-STATs was analysed using antibodies listed below and performed according to the manufacturers' instructions (Cell Signaling Technologie). Secondary fluorescent dye antibodies Alexa Fluor 488 goat anti-mouse IgG and Alexa Fluor 568 goat anti-rabbit IgG (Life Technology Inc.) were used. The cells were also stained with anti-tubulin antibody to visualise the shape of the cells and DAPI (1:1000) was used to visualize the nucleus of HNECs. The fluorescent images of STATs were captured by a Confocal Leica SP5 microscope.

| **Primary antibody** | **Dilution** |
|---|---|
| Polyclonal anti-phospho-tyrosine-STAT-1 mouse antibody | 1:100 in 1% BSA, 0.3 % Triton X-100 in 1x PBS |
| Monoclonal anti-phospho-tyrosine-STAT-3 rabbit antibody | 1:100 in 1% BSA, 0.3 % Triton X-100 in 1x PBS |
| Polyclonal anti-phospho-tyrosine-STAT-6 rabbit antibody | 1:100 in 1% BSA, 0.3 % Triton X-100 in 1x PBS 1:1000 in 1% BSA, 0.1 % Tween in 1x TBS |
| Anti-STAT-1 rabbit antibody | 1:100 in 1% BSA, 0.3 % Triton X-100 in 1x PBS |
| Anti-STAT-3 rabbit antibody | 1:100 in 1% BSA, 0.3 % Triton X-100 in 1x PBS |
| Anti-STAT-6 mouse antibody | 1:100 in 1% BSA, 0.3 % Triton X-100 in 1x PBS |
| α-Tubulin mouse antibody | 1:400 in 1% BSA, 0.3 % Triton X-100 in 1x PBS |
| **Secondary antibody** | **Dilution** |
| Alexa Fluor 488 goat anti-mouse IgG antibody | 1:600 in 1% BSA, 0.3 % Triton X-100 in 1x PBS |
| Alexa Fluor 568 goat anti-rabbit IgG antibody | 1:600 in 1% BSA, 0.3 % Triton X-100 in 1x PBS |

## 3.17 Real time (RT) - PCR

To quantify genes of interest on RNA level, a real time PCR was performed. For this a minimum of 10 ng RNA per sample was transcribed into cDNA using 10x RT buffer, 25x dNTPs, 10x RT random primer, MultiScribe Reverse Transkriptase and nuclear free water of the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Afterwards, a primer mastermix was set up with the primers listed below. GAPDH was used as the housekeeper gene.

Fast Start Universal SYBR Green Master was added to the prepared primer mastermix to create a PCR mastermix. The PCR mastermix was pipet into the PCR plate and centrifuged with quick run. Subsequently, cDNA samples were pipet into the wells to the PCR mastermix and centrifuged again before plate was sealed with a plastic foil.

| **PCR primer** | **Sequence** |
|---|---|
| IL2RG for | 5'TTG AAC CAC TGT TTG GAG CA3' |
| Il2RG rev | 5'AAA CGT GTA GCG TTT CTG CC3' |
| ADA rev | 5'ATC CCT CTC CTC CTG CCA TA3' |
| ADA for | 5'GCC TTC GAC AAG CCC AA3' |
| CXCR4 rev | 5'CTT GTC CGT CAT GCT TCT CA3' |
| CXCR4 for | 5'GAA CCC TGT TTC CGT GAA GA3' |
| IL 8 rev | 5'AAA TTT GGG GTG GAA AGG TT3' |
| IL 8 for | 5'TCC TGA TTT CTG CAG CTC TGT3' |
| CCL27 for | 5'ACT GTC ACC TCC AGG CTT TC3' |
| CCL27 rev | 5'TCC CAT GGA GCT TTC TCT CT3' |
| GAPDH for | 5'TGC ACC ACC AAC TGC TTA GC3' |
| GAPDH rev | 5'GGC ATG GAC TGT GGT CATGAG3' |
| IL 31 for | 5'GAT GAT GTA CAG AAA ATA GTC GAG GAA TT3' |
| IL 31 rev | 5'CTT CTC TTC CTC CAC ATC TTT CAAA3' |
| TSLP for | 5'GCT ATC TGG TGC CCA GGC TAT'3 |
| TSLP rev | 5'CGA CGC CAC AAT CCT TGT AAT'3 |

To analyse the target genes ViiA7 RvO Software was used.

## 3.18 Statistics

Data are presented as mean +/- SD (bar charts) or medians +/- 5-95 percentiles (box plots). To detect differences between treatment groups, samples were compared by Wilcoxon test. Differences between treatment groups were considered significant if $p<0.05$.

# 4 Results

## 4.1 Establishment of human primary nasal epithelial cells

As primary human nasal epithelial cells (HNECs) represent the first line of defence against microbes, they play a very important role in the immune system in the airways. The following cell culture experiments are therefore HNECs which were generated from ether biopsies of patients undergoing turbinoplastic surgery or from curettages (nasal scrapings). For the establishment of the culturing of primary HNECs, cells were treated and cultured under different conditions, thus different isolation models were tested. Therefore we isolated HNECs from biopsies as well as from curettages (Figure 7) to analyse the growth behaviours.

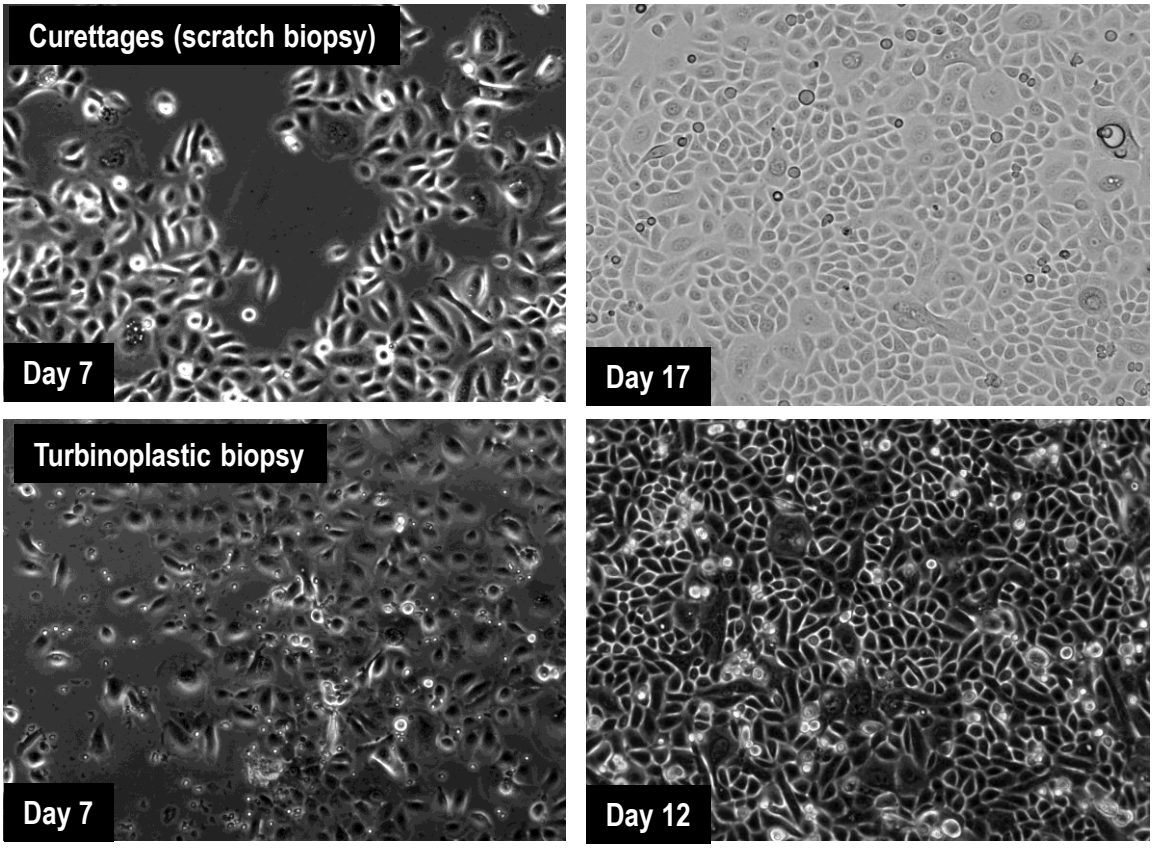


**Figure 7: The culturing of HNECs using different techniques.** Microscopial pictures of HNECs achieved through different generation processes including nasal scraping (curettages) and turbinoplastic biopsies.

Under cell culture conditions, HNECs from biopsies grew faster than HNECs from curettages. In some cases, it was even not possible to obtain viable HNECs from curettages, which could be explained by the low amount of cells received from nasal scrapings. Turbi-

noplastic biopsies were treated as described in 3.3 and HNECs reached confluence quicker than HNECs generated from curettages. Therefore we decided generate HNECs from biopsies for the upcoming experiments instead of curettages.

Additionally, different media were tested for the HNECs culturing. DMEM/Ham´s F12 compared to the Airway Epithelial Cell Growth Medium with supplements (3.1) was tested. The Airway Epithelial Cell Growth Medium showed better results regarding the growth of the cells (data not shown) and was used for the following experiments.

## 4.2 Characterisation of nasal epithelial cells via scanning electron micrograph

To characterise the HNECs, scanning electron micrograph images were recorded. HNECs were cultured as described in 3.3 and scanned with the JSM 6300 scanning microscope. With this method it is possible to demonstrate single HNECs and to show the exact composition of the surface of the cells such as microvilli on the surface or goblet cells (Figure 8).

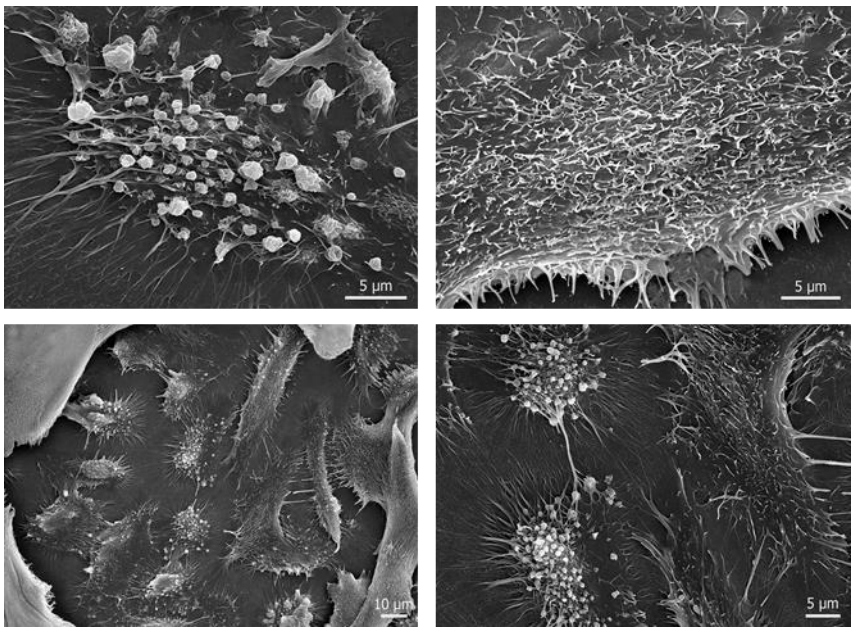


**Figure 8: Scanning electron micrograph** Phenotype of primary nasal epithelial cells Primary nasal epithelial cells were seeded onto glass slides and scanned with scanning electron microscope. The experiment is representative for one experiment.

## 4.3 Characterisation of nasal epithelial cells via fluorescence-activated cell sorting (FACS)

A FACS analysis was performed to stain two differentiation markers, involucrin and cytokeratin 14 (K14) on cultured HNECs. K14 is expressed on basal cells, whereas involucrin is present in highly differentiated cells. The cultured HNECs showed no staining for K14. However, the analysis shows the presence of involucrin on primary human nasal epithelial cells in the cell culture model (Figure 9). IgM and IgG are shown as second antibody control.

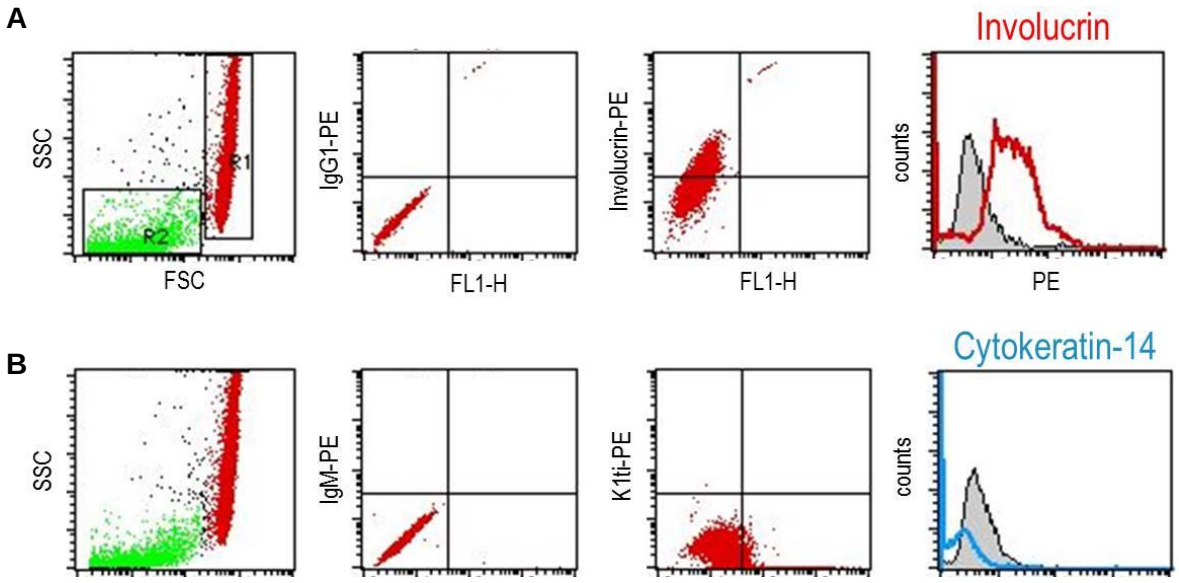


**Figure 9: Characterisation of HNECs via FACS analysis.** HNECs were cultured and stained with two differentiation markers, involucrin and cytokeratin 14 (K14). Distributions of analysed cells were illustrated in a side scatter and forward scatter. (A) Involucrin could be detected in cultured HNECs, whereas K14 (B) was not detectable. The experiment is representative for one experiment.

## 4.4 Stimulation of HNECs with Ze 339 or the single isoforms

The cultured HNECs were stimulated with Ze 339 (3µg/ml) or polyethylene glycol (PEG) only. PEG is the solvent of the extract Ze 339. IL-8 protein expression level was measured. In addition, the cytotoxicity level of HNECs pre-treated with Ze 339 or PEG only was analysed. Ze 339 is a complex of different components, 30% of this complex are petasins present in different isoforms, which are supposed to be the active elements. The cytotoxicity level of petasin isoform-treated HNECs and the IL-8 expression level were determined. The single isoforms, isopetasin, neopetasin and petasin, were used in the following concentrations: 0.01µg/ml, 0.03µg/ml, 0.1µg/ml, 0.3µg/ml or 1.0µg/ml.

There is a time dependent induction of IL-8 measured in PEG and Ze 339-treated HNECs (Figure 10). But, the overall levels of IL-8 are low compared to a viral-induced model, where the IL-8 response in airway epithelium reaches levels up to 50.000pg/ml.

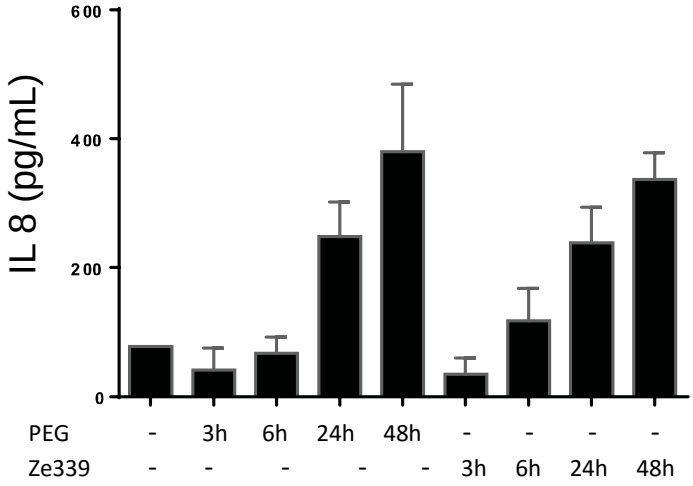


**Figure 10: IL-8 expression level of HNECs treated with Ze 339 (3µg/ml) or the solvent control PEG.** HNECs were cultured and treated with Ze 339 or PEG for 3h, 6h, 24h and 48h before collection of the supernatant. IL-8 expression level was measured with sandwich ELISA method. n=4.

The analysed IL-8 levels were also measured in the isoform-treated HNECs after 24h of stimulation. The results show a slight increase in IL-8 secretion with increasing isoform concentrations, but the overall levels of produced IL-8 are still low.

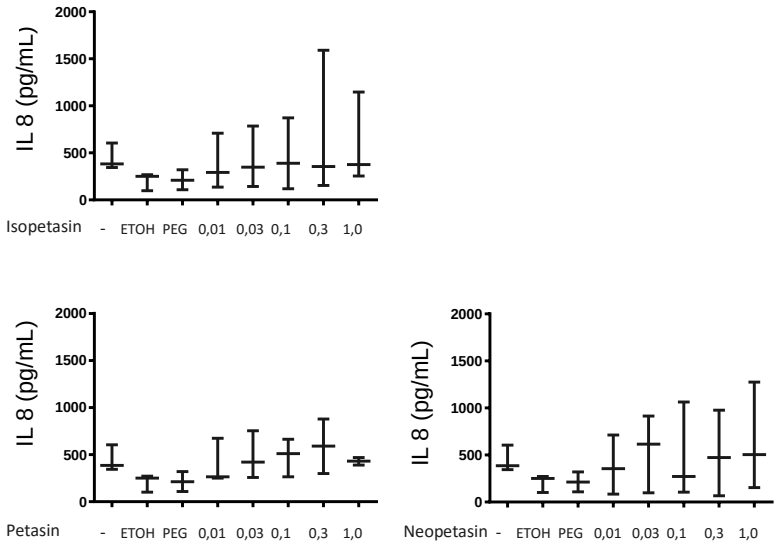


**Figure 11: IL-8 expression level of HNECs treated with different isoforms of petasin and the solvent controls.** HNECs were cultured and treated with the isoforms (0.01, 0.03, 0.1, 0.3, 1.0 µg/ml) isopetasin, neopetasin and petasin for 24h before collection of the supernatant. IL-8 expression level was measured with sandwich ELISA method. n=4.

To evaluate the viability of stimulated HNECs, the lactate dehydrogenase (LDH) activity in the supernatants of stimulated HNECs was quantified. Stimulation with Ze 339 or the petasin isoforms did not lead to a significant increased LDH production level (Figure 12).

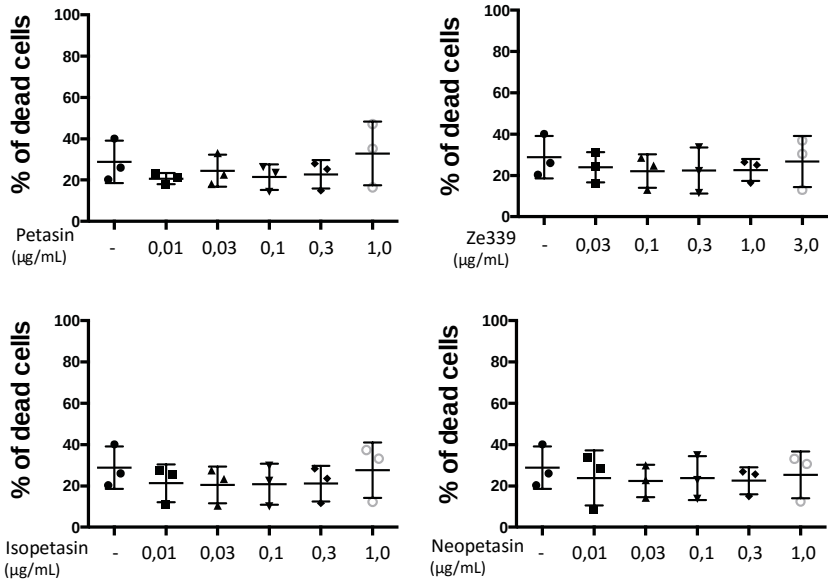


**Figure 12: Measurement of lactate dehydrogenase (LDH) activity in stimulated HNECs.** HNECs were cultured and stimulated with Ze 339, petasin, isopetasin and neopetasin in the known conditions for 24h. Then, supernatant was taken and LDH activity was measured. n=3.

## 4.5 Ze 339, but not the single isoforms, decreased the PolyIC-induced IL-8 expression in HNECs

A virus infection in epithelia cells results in the enhanced production of IL-8. To mimic a virus infection in airway epithelium, we stimulated HNECs with the dsRNA PolyIC (10µg/ml) for 24h. Additionally, HNECs were co-treated with Ze 339 (0.3µg/ml, 1µg/ml or 3µg/ml) or with the single isoforms (0.01µg/ml, 0.03µg/ml, 0.1µg/ml, 0.3µg/ml or 1.0µg/ml) or with a combined complex of all three isoforms together, the petasinmix (PM) (0.3µg/ml, 1µg/ml or 3µg/ml). Subsequently, the produced IL-8 levels were measured. The level of IL-8 is extremely enhanced, when HNECs are treated with PolyIC up to levels of more than 100ng/ml. There is a highly significant dose-dependent reduction of the PolyIC-induced secretion of IL-8 when HNECs are treated with Ze 339 for 24 h (Figure 13 a). Furthermore, when PolyIC-treated HNECs are co-treated with the petasinmix, there is also a significant, dose-dependent reduction of IL-8 secretion detectable (Figure 13 b).

HNECs were also stimulated with the single isoforms alone, in the presence of PolyIC. None of the single petasin isoforms mediated inhibition of PolyIC-induced IL-8 production in HNECs (Figure 13 c-e). Even with elevated concentration of the isoforms, there was no significant reduction of IL-8 expression detectable in HNECs.

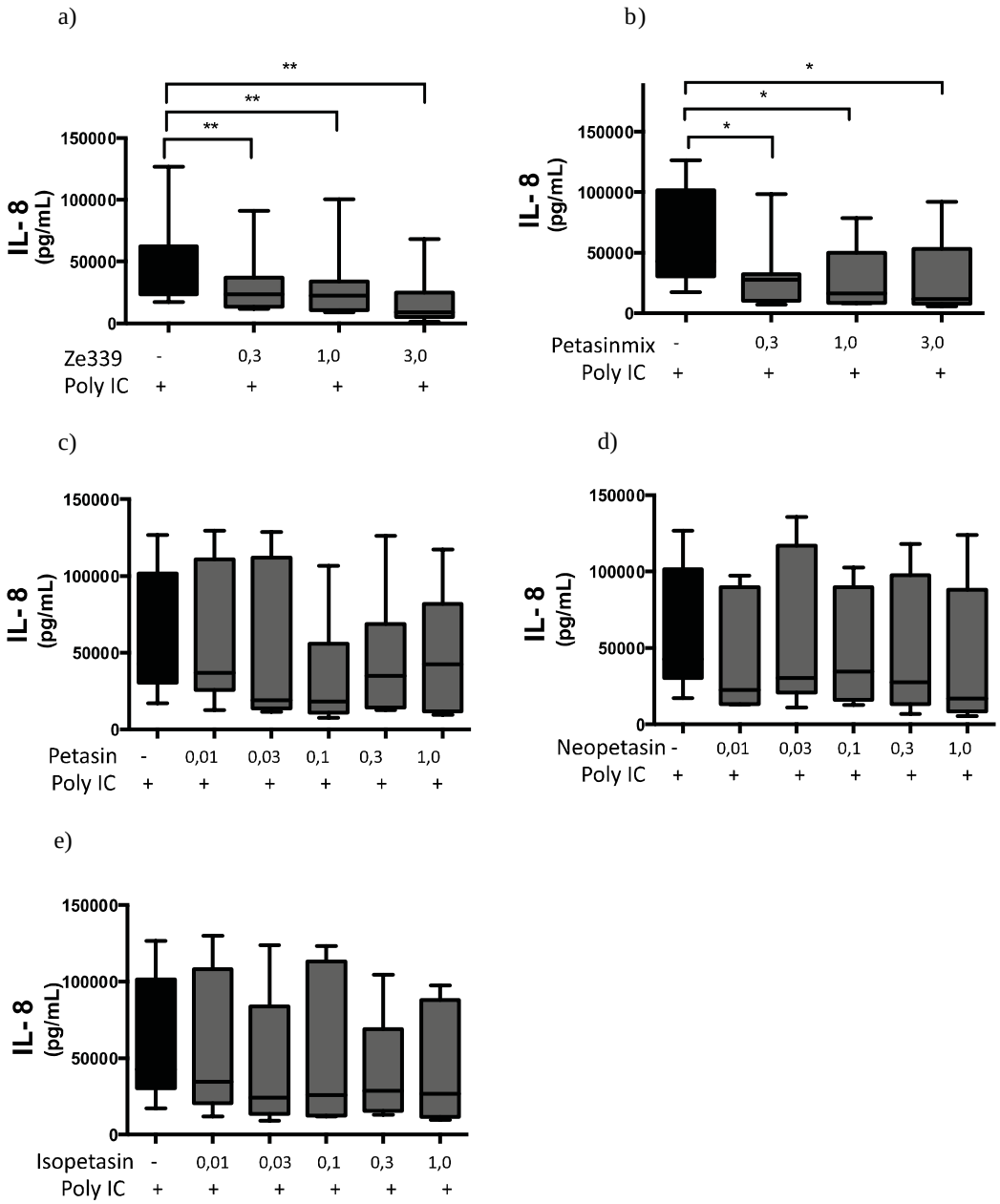


**Figure 13: IL-8 expression in PolyIC-treated HNECs in combination with Ze 339, petasinmix or the single isoforms.** There is a significant reduction of PolyIC-induced IL-8 production when co-treated with Ze 339 (0.3µg/ml, 1µg/ml, 3µg/ml). Additionally, the PolyIC-induced IL-8 is also significantly reduced when HNECs are co-stimulated with the petasinmix, a combined complex of all three isoforms together. Interestingly, the treatment with the single isoforms alone did not lead to a significant inhibition of PolyIC-induced IL-8 production. n=5, *: $p<0.05$; **: $p<0.01$, Wilcoxon test.

## 4.6 Ze 339, but not the single isoforms, reduced the PolyIC-induced neutrophil chemotaxis towards the supernatant

To address the question whether the reduction of the IL-8 response translates into reduced recruitment of inflammatory cells, neutrophil migration towards conditioned media from stimulated HNECs was assessed. There was enhanced neutrophil migration towards supernatants of PolyIC (10µg/ml) stimulated HNECs detectable as compared to supernatants of medium-treated HNECs. The supernatants of HNECs stimulated with a combination of PolyIC and Ze 339 were less potent in inducing neutrophil migration than supernatants of PolyIC-treated cells (Figure 14 a).

As shown in Figure 14 b the petasinmix was sufficient to inhibit the PolyIC-induced IL-8 response of HNECs. In line with this, HNEC supernatants stimulated with PolyIC and PM were less potent in mediating neutrophil chemotaxis than supernatants of cells stimulated with PolyIC only.

After 24h stimulation of HNECs with the single isoforms only in combination with PolyIC, supernatant was taken and neutrophil migration towards the supernatant was measured. The neutrophil migration towards the supernatant of the single isoforms stimulated cells did not reach a significant reduction compare to the PolyIC only stimulated cells (Figure 14 c-e). None of the supernatant of the single isoforms treated cells could show the effect of an enhanced neutrophil chemotaxis.

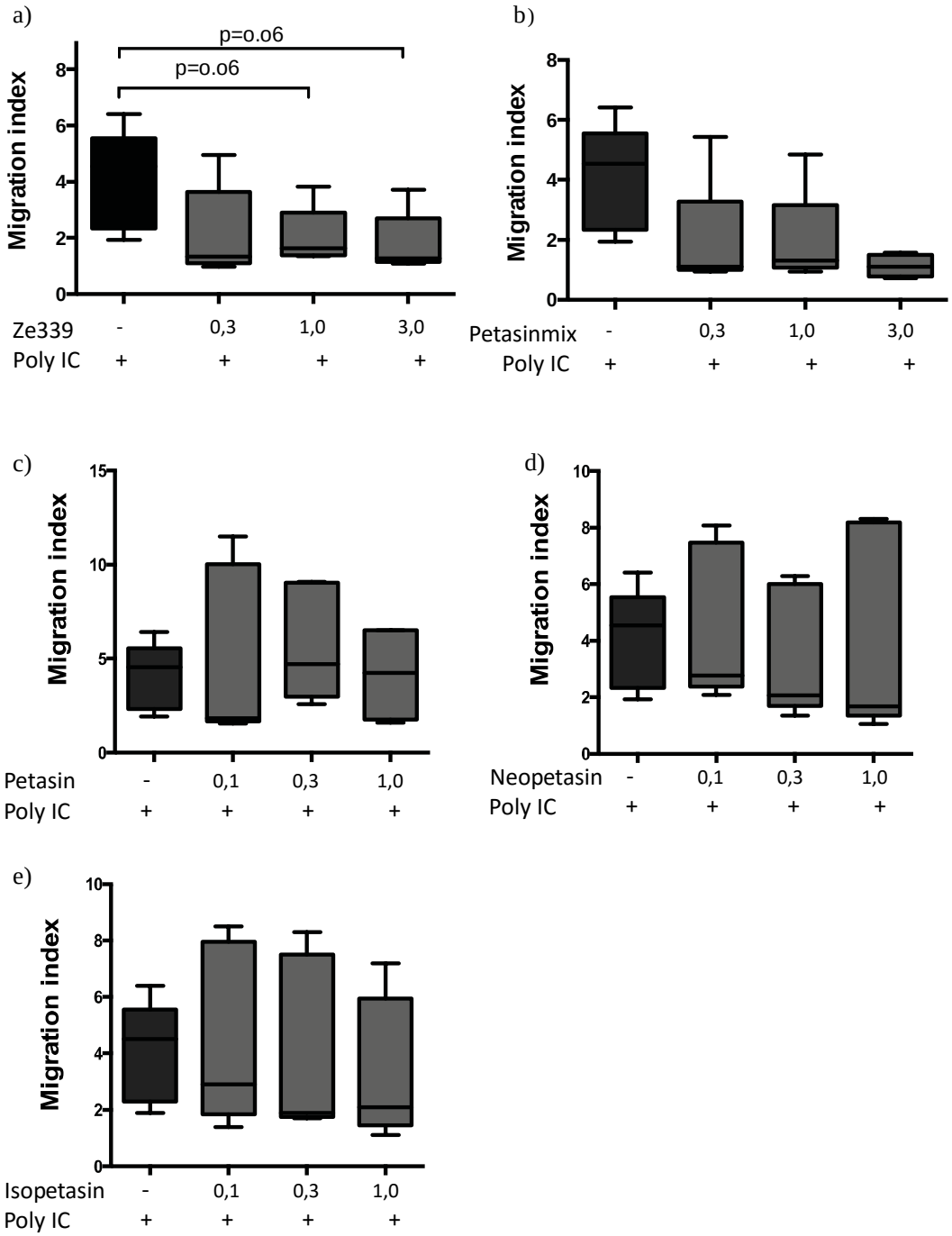


**Figure 14: Neutrophil migration** towards supernatants of Ze 339-incuded HNECs (0.3µg/ml, 1µg/ml, 3µg/ml), Petasinmix-induced HNECs (0.3µg/ml, 1µg/ml, 3µg/ml) or with single isoform-induced HNECs, isopetasin, neopetasin, petasin (0.3µg/ml, 1µg/ml, 3µg/ml) in a PolyIC stimulated model (n=5), compare to PolyIC stimulation alone. Ze 339 and petasin treatment reduced the neutrophil chemotaxis, whereas the single isoforms are not able to modulate the PolyIC induced neutrophil migration.

## 4.7 Anti-inflammatory potential of Ze 339 is not restricted to cells derived from atopic donors

The production of IL-8 and the neutrophil chemotaxis are hallmarks of allergic as well as non-allergic inflammation, and Ze 339 might act on the pro-inflammatory response of HNEC. To assess whether this beneficial effect of Ze 339 could be dependent on the atopic state of the patients, cytokine release by HNECs from both, atopic and non-atopic donors in response to Ze 339 plus PolyIC was measured. The atopy status of the HNEC donors was determined by Immuno-CAP assay, and results were calculated separately according to the atopy status. As shown in Figure 15, PolyIC stimulation of HNEC induced the secretion of IL-8 and other pro-inflammatory mediators (G-CSF, CCL-5, IL-6, and IL-1α), an effect that was most pronounced in cells derived from non-atopic donors. Ze 339 significantly reduced the PolyIC-induced cytokine and chemokine response in cells derived from both, non-atopic and atopic donors.

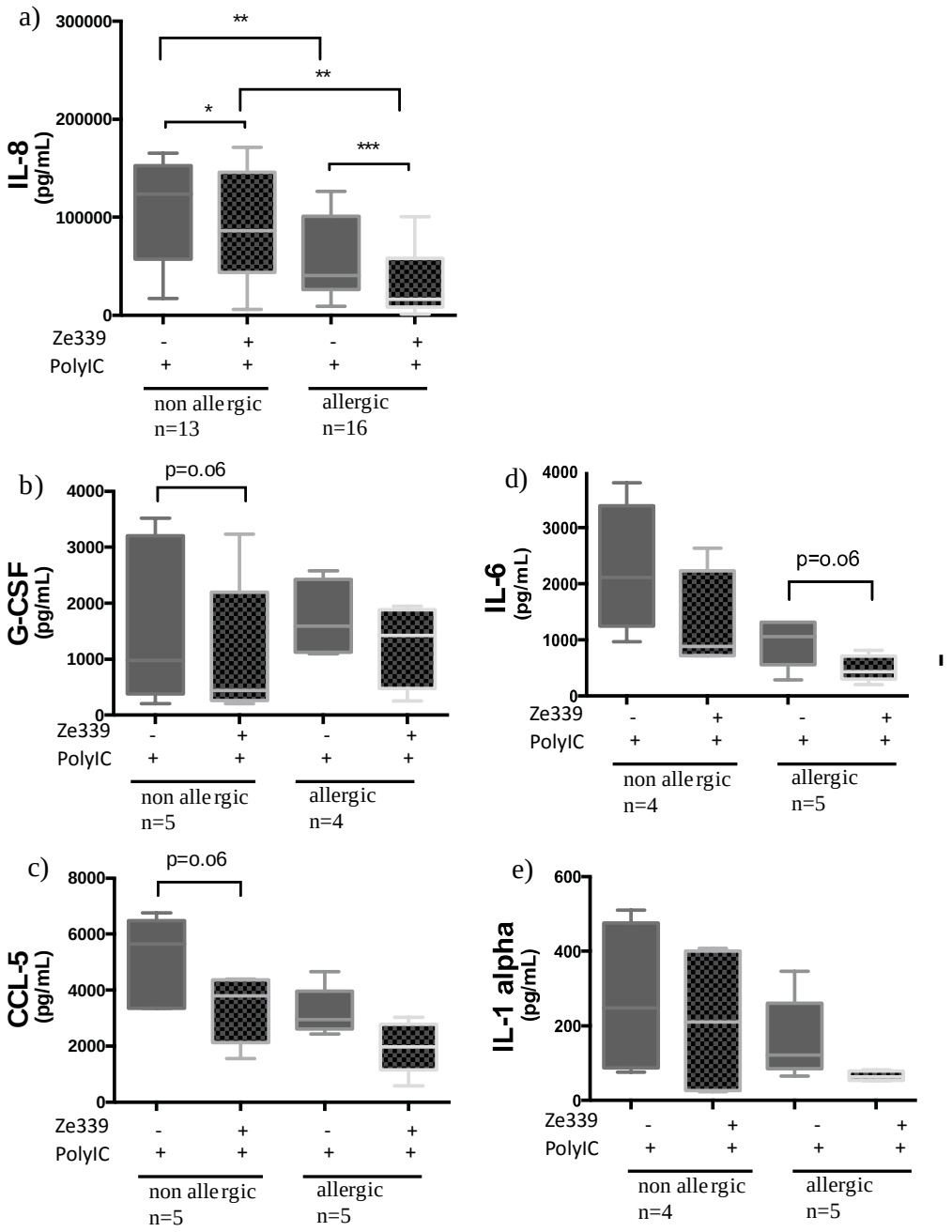


**Figure 15: Cytokine release of stimulated HNECs divided in atopic and non-atopic donors.**

HNEC of non-atopic (n=4) and atopic donors (n=5) were incubated for 24h medium, PolyIC (10µM) or PolyIC plus Ze 339 (3µM), and cell-culture supernatants were analysed for the pro-inflammatory mediators using Bioplex assay: IL-8 (a), G-CSF (b), CCL-5 (c), IL-6 (d), and IL-1α (e). *: $p<0.05$; **: $p<0.01$, Wilcoxon test.

## 4.8 Microarray analysis

The anti-inflammatory effect of Ze 339 on HNECs is based on its impact on the immune response such as modulation of the cytokine secretion. However, the mechanisms behind this regulation are unclear. To better understand the downstream mechanism of Ze 339 a microarray analyses was performed. As Montelukast is a leukotriene receptor antagonist and therefore used for the treatment of asthma or seasonal allergies, Ze 339 stimulation was compared to Montelukast stimulation in the microarray analysis. The solvent control of Montelukast is ethanol (EtOH), whereas PEG is the solvent control for Ze 339. HNECs were cultured and stimulated with Montelukast (1µg/ml), Ze 339 (3µg/ml), petasinmix (3µg/ml), EtOH or PEG plus the viral mimic PolyIC (10µg/ml) for 24h. Cells were collected after stimulation and RNA was processed for microarray analysis. Venn Diagrams on the entity list of the genespring analysis of the tested samples were created (Figure 16). In addition gene ontology analyses (GO terms) were performed to characterize the analysed genes in view of the immune response. GO terms included genes regulated in chemokine activity, cytokine activation, extracellular matrix proteins and cell-cell signalling. Using the specific GO terms reduces the amount of regulated genes and itemized the search for genes of interest.

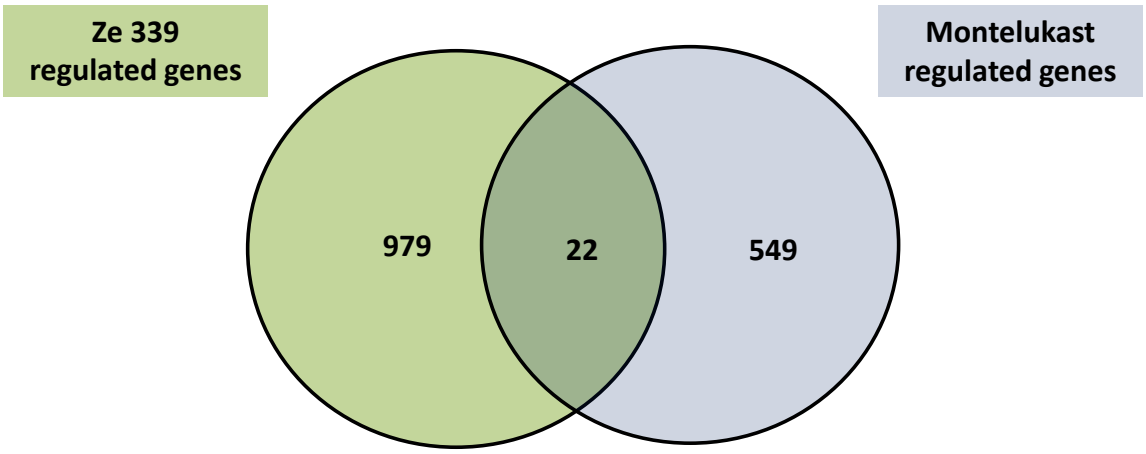


**Figure 16: Venn diagram of regulated genes: Ze 339/Montelukast;** Numbers of regulated analysed genes after Ze 339 and Montelukast stimulation in a PolyIC-stimulated model. 979 Ze 339-regulated genes could be detected, 22 genes are regulated of both, Ze 339 and Montelukast, whereas Montelukast alone regulates 549 genes based on microarray analysis. n=3

| Microarray analysis – Genes of interest | |
|---|---|
| **Ze 339 regulated genes** | **Function** |
| CXCR4 upregulation | Receptor for the C-X-C chemokine CXCL12, transducing a signal by increasing intracellular $Ca^{2+}$ and enhancing MAPK1/MAPK3 activation |
| IL-31 downregulation | Produced by activated TH2 T-cells; itch |
| TSLP downregulation | TH2 differentiating factor |
| CCL27 downregulation | Chemotactic for skin-associated memory T cells |
| IFNαR2 upregulation | Associates with IFNαR1 to form the type I IFN receptor |
| IL-2Rγ downregulation | Common gamma chain (ligands:IL-2, IL-4, IL-7, IL-9, IL-15, IL-21) |
| ADA upregulation | Positive regulator of T-cell co-activation |
| **Montelukast regulated genes** | **Function** |
| LIF upregulation | Pleiotropic cytokine; tolerance |
| IRF3 upregulation | Interferon response factor |
| CD74 upregulation | MHC II invariant chain |
| IL-17 RC downregulation | Binds both IL-17A and IL-17F with similar affinities in nonhematopoietic tissues |

As visible in the Venn diagram (Figure 16) there are 979 analysed genes, which are regulated by Ze 339 treatment in PolyIC-stimulated HNECs. 549 genes are regulated by the stimulation of Montelukast in PolyIC-stimulated HNECs. 22 genes are regulated by Ze 339 as well as by Montelukast. As mentioned before, we focused on genes involved in the regulation of the immune system and therefore selected typical GO terms. This targeted analysis of the genes regulated by either Ze 339 or Montelukast resulted in a list of genes, which are important for the immune function of the epithelial cells. Ze 339 upregulated the expression levels of CXCR4, Interferon-alpha receptor-1 (IFNAR-1) and adenosine deaminase (ADA). Furthermore, the epithelial expressed genes IL-31, TSLP and CCL-27 were downregulated in HNECs after treatment with Ze 339. The treatment of HNECs with Montelukast led to an upregulation of the Leukemia Inhibitory factor (LIF) gene, the IRF-3 gene as well as the CD74 gene. The IL-17 receptor C (IL17RC) gene was downregulated in Montelukast treated HNECs.

## 4.9 Real time PCR- Verification of microarray analysis results

The microarray analysis provided a large data set on gene regulation in the stimulated HNECs. The specific assessment of the array analysis identified immune modulating genes expressed and regulated in the epithelium. As described in 4.8, Ze 339 modulated amongst others the gene expression levels of IL-31, TSLP, CCL27, ADA and IL-2 receptor gamma (IL-2Rγ). To verify the analysed gene expression levels of the modulated cytokines, HNECs were stimulated for 24h before collection and preparation of RNA. The RNA samples were reverse transcribed and gene expression (IL-31, TSLP, CCL27, ADA and IL-2Rγ) was analysed (Figure 17).

As showed in 4.5, Ze 339 reduced the IL-8 protein production in PolyIC-stimulated HNECs. Therefore, the modulated expression profile of IL-8 on RNA level was analysed and quantified with RT-PCR as well.

The measured RNA expression levels of the cytokines were normalized to the respective medium control sample. The housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used for the RT-PCR.

Except for TSLP, PolyIC increased the expression level of all analysed cytokines compared to the medium control. In the case of IL-8 there was a strong induction on the RNA expression level, but in contrast to the protein data, levels of IL-8 are highly upregulated, when cells are treated with Ze 339 and PolyIC together. Levels of CCL27 were clearly downregulated when HNECs were stimulated with PolyIC in combination with Ze 339. Levels of TSLP could not be induced by PolyIC compared to the medium control. IL-31 is weakly induced by PolyIC, the treatment of the cells with PolyIC plus Ze 339 led to a slight reduction compare to the PolyIC alone stimulated cells. The expression level of ADA responding to PolyIC stimulation is relatively low, and the inhibitory effect of Ze 339 is moderate. In contrast, the expression of IL2Rγ was highly induced by PolyIC stimulation in HNECs. Treatment with PolyIC plus Ze 339 led to a strong inhibition of IL2Rγ expression on RNA level.

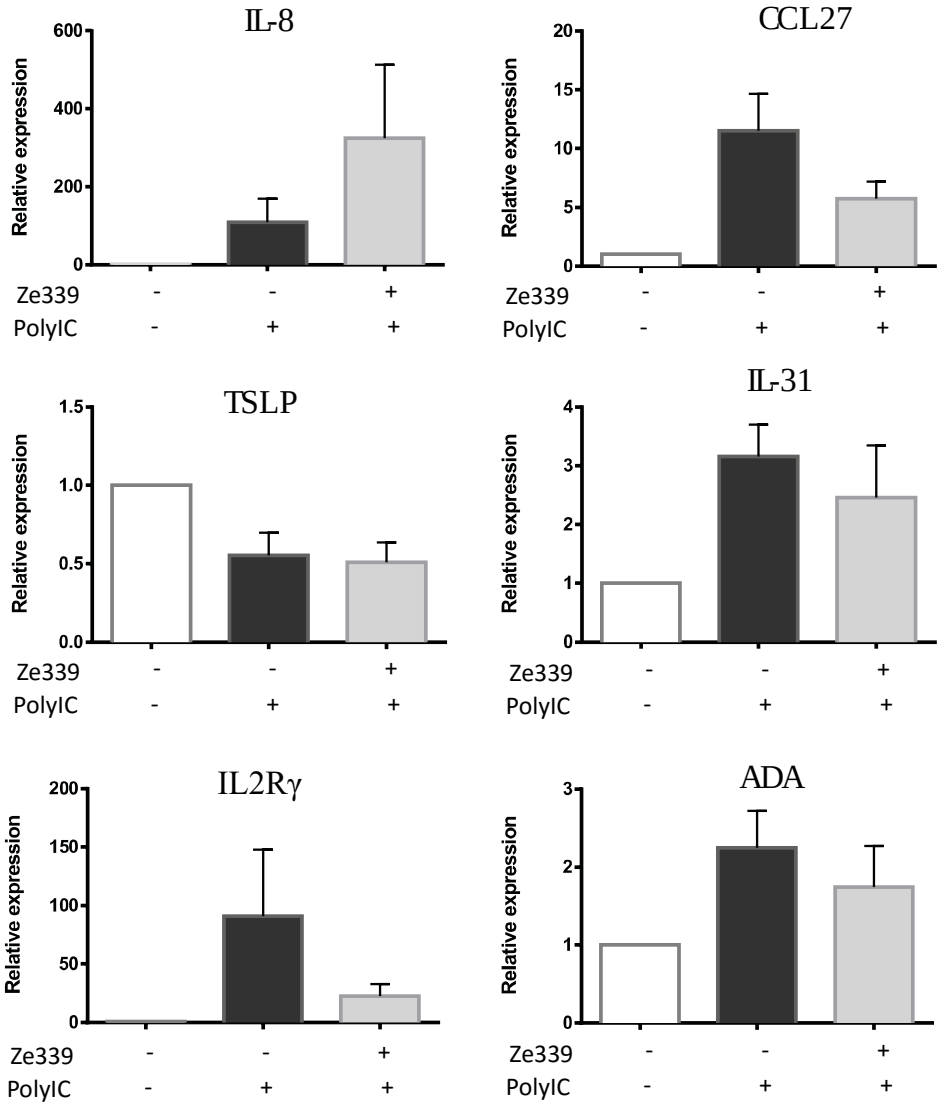


**Figure 17: Real time (RT)-PCR of selected cytokines in HNECs treated with PolyIC +/- Ze 339.** HNECs were cultured and stimulated with medium, PolyIC (10µg/ml) or a combination of PolyIC plus Ze 339 (3µg/ml) for 24h. After this, cells were collected and RNA was performed. A RT-PCR analysis measured cytokine expression level of IL-8, CCL27, TSLP, IL-31, ADA and IL2Rγ. GAPDH was used as the housekeeper gene. Expression level was normalized to the belonging medium control. IL-8, CCL27, TSLP, IL-31 n=5; ADA, IL2Rγ n=2.

## 4.10 Ze 339 inhibits viral-induced pro-inflammatory cytokine and chemokine response in HNECs

Based on the results that Ze 339 inhibits the IL-8 expression under PolyIC stimulation seen in 4.5, investigations were performed to find out whether this beneficial effect of Ze 339 is also present in other stimulation models. First, the effect of Ze 339 (3µg/ml) was studied in a viral mimic-induced model using PolyIC-LyoVec (4µg/ml), a RIG-I/MDA-5 ligand and imidaz-

oquinoline (R-848) (10µg/ml) a TLR7/8 ligand. For this purpose, HNECs were treated with the indicated viral stimuli in the absence or presence of Ze 339. After 24h, supernatants were collected and tested for a panel of pro-inflammatory cytokines and chemokines (IL-8, CCL2/MCP-1, CCL3/MIP-1α, CCL4/MIP-1β, CCL5/RANTES, CXCL10/IP-10, G-CSF, TNF-α, IL-1α, IL-6). Stimulation with PolyIC and PolyIC-LyoVec led to the induction of a pro-inflammatory response in HNECs, whereas the TLR7/8 ligand R-848 did not induce any measurable response (Figure 18). Ze 339 significantly reduced PolyIC-induced cytokine and chemokine response including G-CSF, CCL3, IL-8, CCL4, CCL2, TNF- α, CCL5, IL-6 and IL1- α. The expression level of IP-10 was not modulated by Ze 339 stimulation. The inhibitory effect of Ze 339 on the pro-inflammatory response to PolyIC-LyoVec was less pronounced than the effect on the response to PolyIC. Specifically, Ze 339 decreased only the PolyIC-LyoVec induced production of IL-8, CCL3, and IL-6, whereas the production of other cytokines and chemokines remained unaffected by Ze 339 (Figure 18).

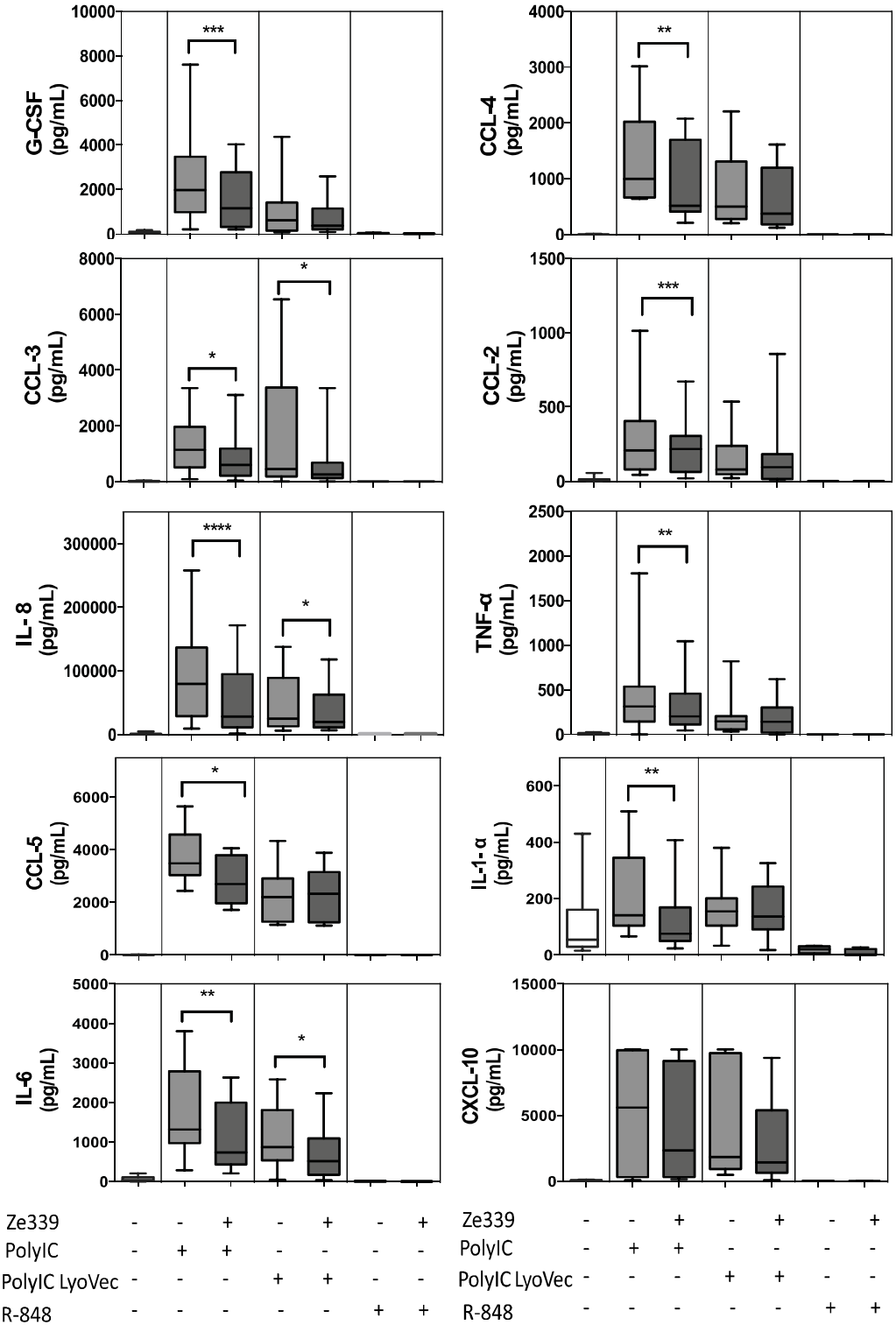


**Figure 18: Viral stimulation model in HNECs.** HNEC were stimulated for 24h with medium or the different viral mimics PolyIC (TLR-3; 10µg/ml), PolyIC-LyoVec (RIG-I/MDA-5; 4µg/ml), and R-848 (TLR-7/-8; 10µg/ml), in the absence or presence of Ze 339 (3µg/ml). After 24 hours, supernatants were collected and tested for the pro-inflammatory cytokines and chemokines by Bioplex analysis: IL-8, CCL-5, CCL-2, CCL-3, CCL-4, G-CSF, TNF-α, IL-6, IL-1α, CXCL-10: n=13; except for "medium" and "PolyIC" of IL-8: n=34. *: p<0.05; **: p<0.01; ***: p<0.001, Wilcoxon test.

## 4.11 Ze 339 does not inhibit bacterial induced pro-inflammatory cytokine and chemokine response in HNECs

To define whether Ze 339 exerts an anti-inflammatory effect on HNECs exposed to bacterial PAMPs, HNECs were incubated with CpG ODN 2006 (1µM) a TLR9 ligand, with a synthetic triacylated lipoprotein Pam3CSK4 (200ng/ml), a TLR2 ligand or flagellin (5µg/ml) from *Pseudomonas aeruginosa*, a TLR5 ligand with or without addition of Ze 339 (3µg/ml). HNECs were cultured and stimulated with the different ligands for 24h. Then, supernatants were collected for assessment of pro-inflammatory cytokines and chemokines expression profiles. Stimulation of HNECs with CpG, Pam3CSK4 and flagellin resulted in increased production of CCL2, TNF-α, IL-6, and IP-10 (Figure 19). G-CSF and IL-1α were not induced by the bacterial PAMPs tested. Flagellin and Pam3CSK4 increased the production of CCL2 and CXCL10 compare to the medium control, but Ze 339 did not modulate this expression. Ze 339 slightly inhibited the IL-6 production in Pam3CSK4-stimulated HNECs, but the overall induction of IL-6 expression compared to the unstimulated medium control was little.

Ze 339 did not mediate inhibition of pro-inflammatory mediator production in response to the bacterial stimuli. In contrast, Ze 339 significantly increased the CpG-induced production of G-CSF (Figure 19).

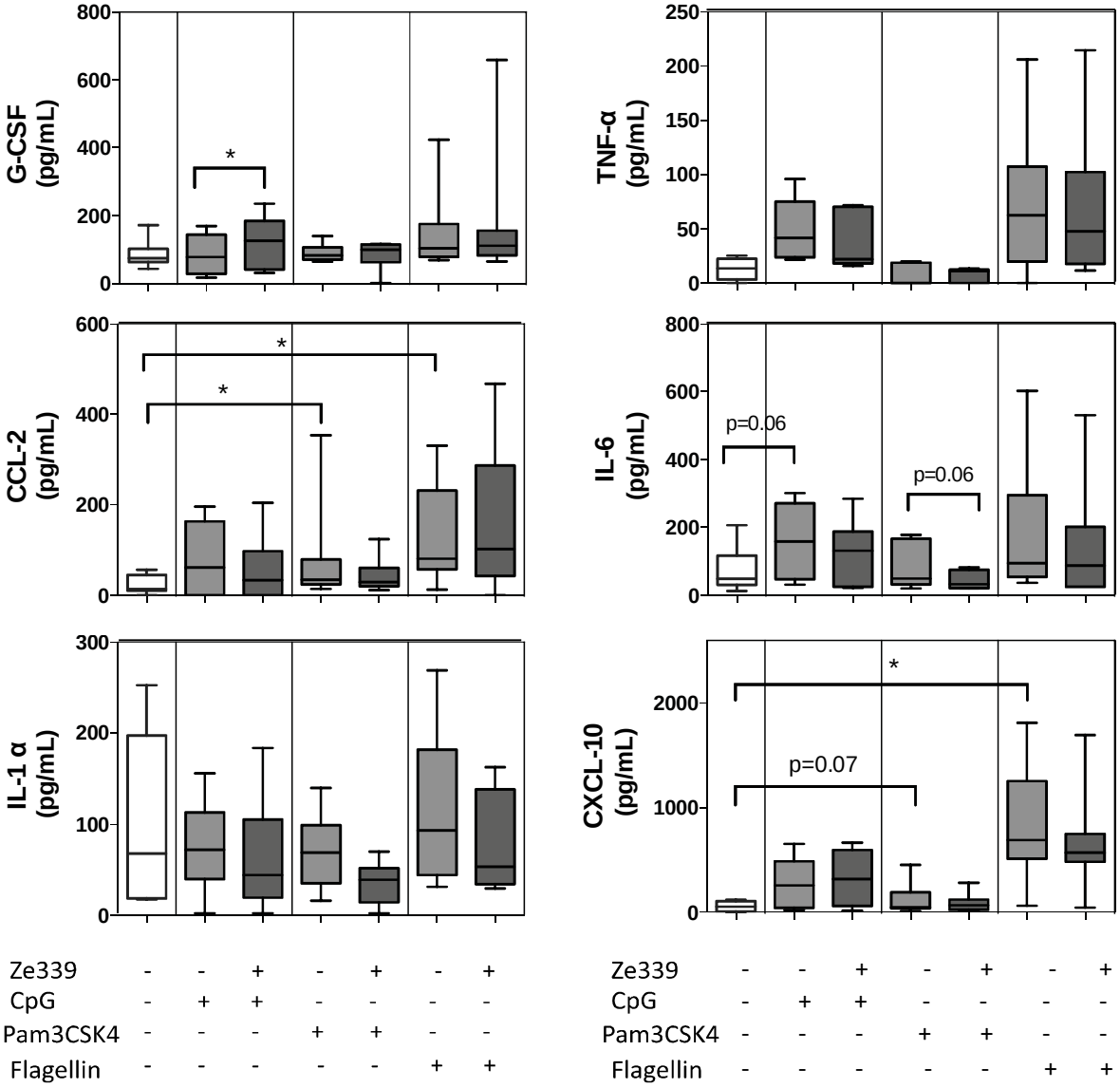


**Figure 19: Bacterial stimulation model in HNECs.** HNEC were stimulated for 24h with medium or the different bacterial mimics CpG-ODN 2006 (TLR-9; 5µg/ml), Pam3CSK4 (TLR-1/-2, TLR-2/-6; 200ng/ml), and Pseudomonas aeruginosa flagellin (TLR-5; 5µg/ml), in the absence or presence of Ze 339 (3µg/ml). After 24 hours, supernatants were collected and tested for the pro-inflammatory cytokines and chemokines G-CSF, CCL2, IL-1α, TNF-α, IL-6, CXCL-10. n=13; *: $p<0.05$, Wilcoxon test.

## 4.12 Ze 339 modulates the IL-4-, IL-6- and IFN-γ-induced pro-inflammatory cytokine and chemokine expression in HNECs

Ze 339 shows an anti-inflammatory effect in the viral-induced stimulation model, but not in the bacterial-induced stimulation model. In addition to the two tested models, the anti-inflammatory effect of Ze 339 in a model of HNECs under Th1-, Th2- or pro-inflammatory cytokine exposure was investigated. Therefore, we stimulated HNECs with IL-4 (50ng/ml) (Th2 condition), INF-γ (15ng/ml) (Th1 condition) or IL-6 (800U/ml) (pro-inflammatory condition) for 24h in the absence and presence of Ze 339 to assess the pro-inflammatory cytokine and chemokine response. Supernatant was taken to analyse the expressed cytokine levels in stimulated HNECs. IL-4 induced low-level secretion of CCL2 ($p<0.01$) and IL-6 ($p=0.1$), and Ze 339 significantly inhibited this response. IFN-γ stimulation, in contrast, led to significant induction of IP-10, which was decreased by Ze 339. Under IL-6 stimulation, Ze 339 decreased the expression levels of CCL2, IL-1α and IL-6; however, the effect did not reach statistical significance (Figure 20).

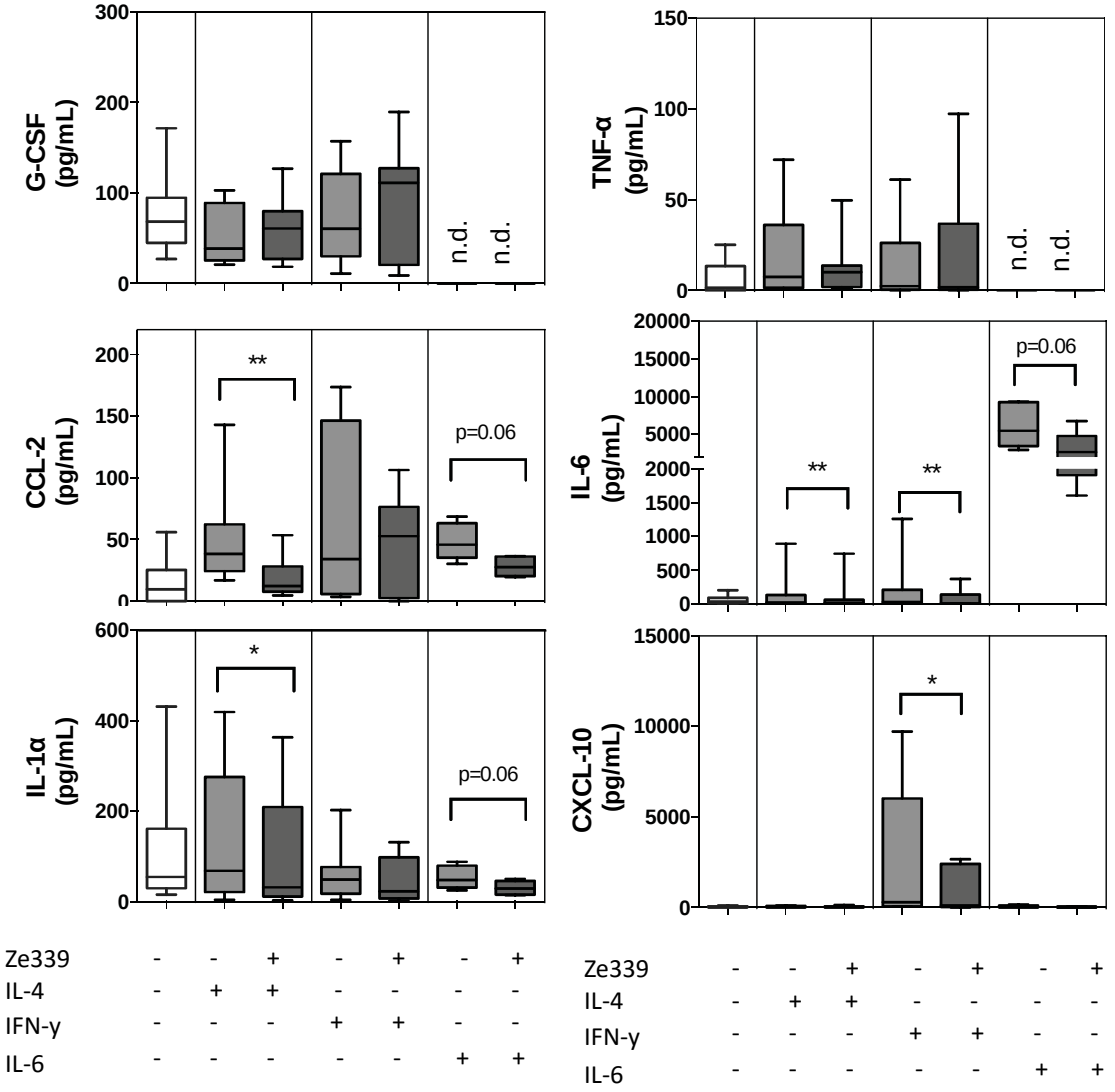


**Figure 20: Cytokine-induced stimulation model in HNECs.** HNECs were stimulated for 24h with medium or cytokines IL-4 (TH2 model; 50ng/ml), IFN-γ (TH1 model; 15ng/ml), and IL-6 (pro-inflammatory model; 800 U/ml), in the absence or presence of Ze 339 (3µg/ml). After 24 hours, supernatants were collected and tested for the pro-inflammatory cytokines and chemokines G-CSF, CCL2, IL-1a, TNF-a, IL-6 and CXCL-10. n=13; *: p<0.05; **: p<0.01, Wilcoxon test.

## 4.13 Ze 339 inhibits the cytokine-induced STAT signalling in HNECs

As shown in 4.12, Ze 339 modulates the cytokine-induced immune response reflected in an inhibition of the expression of various pro-inflammatory cytokines. Diverse cytokine pathways in the cell are dependent on STAT signalling. To assess whether the Ze 339 signalling pathway interferes with the STAT signalling pathway, the expression level of phospho-STAT1, phospho-STAT3 and phospho-STAT6 were analysed in HNECs. The cells were stimulated

with IFN-γ (15ng/ml) a STAT1 inducer, IL-6 (800U/ml) a STAT3 inducer and IL-4 (50ng/ml) a STAT6 inducer. HNECs were stimulated for 30 min with IL-6 and IFN-γ and for 60 min with IL-4 for the optimal STAT expression level. Western Blots were performed of whole cell lysates and subcellular fractions including membrane fraction, cytoplasmic fraction and nuclear fraction.

Both, in the medium-stimulated samples as well as in the Ze 339 only stimulated samples there is no phospho-STAT expression of all analysed phospho-STATs (p-STAT1, p-STAT3 and p-STAT6) detectable. In contrast, phospho-STAT expression was detectable when HNECs were stimulated with the respective cytokines; INF-γ stimulation induced phospho-STAT1 expression, IL-6 induced phospho-STAT3 expression and IL-4 induced phospho-STAT6 expression in all analysed fractions.

Ze 339 reduced the cytokine-induced phosphorylation of STATs. This is visible in the reduced expression level of p-STAT1 in the whole cell and slightly in the cytoplasmic fraction in IFN-γ induced HNECs. The expression level of p-STAT6 is also diminished by Ze 339, an effect which was most visible in the nuclear fraction, however less apparent in the whole cell lysate and the cytoplasmic fraction, in IL-6 induced HNECs. Ze 339 was also able to inhibit the expression of p-STAT6 in the membrane fraction, and little seen in the nuclear fraction, of IL-4 induced HNECs (Figure 21 a-c).

The reduced expression level of p-STAT3 in IL-6 induced HNECs by Ze 339 treatment reached statistical significance compared to the medium control (Figure 21 e). The induction of p-STAT3 expression compared to medium control after IL-6 stimulation is significant as well. Additionally, there is also a trend visible in the induction of p-STAT1 and p-STAT6 expression compare to the medium control after stimulation with IFN-γ or IL-6, respectively ($p=0.06$) (Figure 21 d and f).

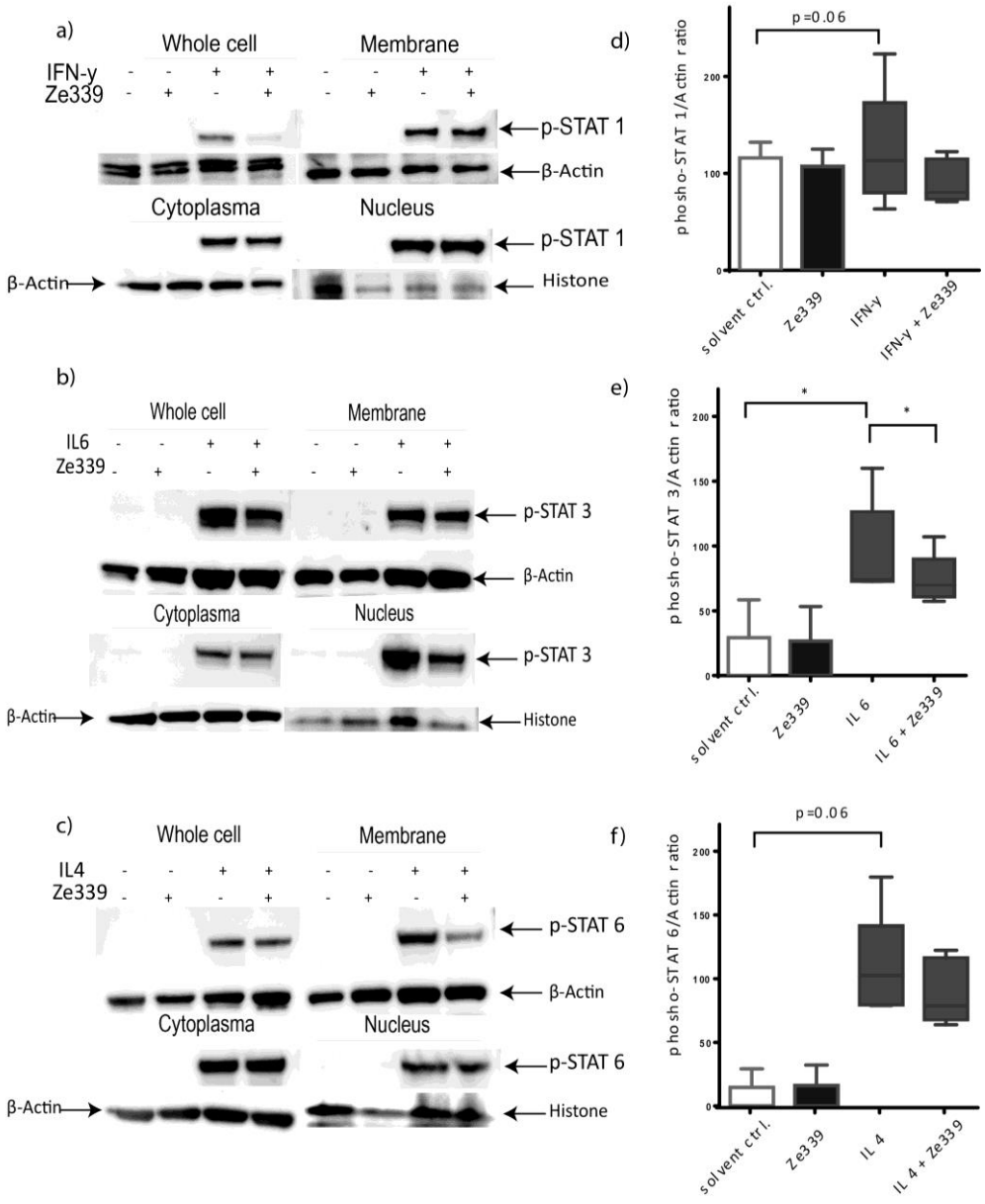


**Figure 21: Expression of cytokine-induced STAT1, STAT3 and STAT6 is reduced by Ze 339 in HNECs.** Cells were treated for 30 min with IFN-γ (a) and IL-6 (b) and for 60 min with IL-4 (c). Total cell lysates or subcellular fractions were subjected to reducing SDS-PAGE, and proteins were transferred on to a PVDF membrane by Western blotting. Total and tyrosine-phosphorylated forms of STATs were detected on the membrane using specific primary and HRP-conjugated secondary antibodies. Right panels: Relative expression levels of STATs in total cell lysates relative to β-Actin levels (n=5), statistics: Wilcoxon test.

## 4.14 Modulation of total STAT and cytokine-induced phospho-STAT expression and translocation in HNECs by Ze 339

As seen in 4.13 Ze 339 regulates the protein expression level of several STATs unto cytokine stimulation. To visualize the effect of Ze 339 on STAT signalling, HNECs were cultured and stimulated on chamber glass slides for fixation, staining and imaging by a confocal microscope.

First, HNECs were stimulated with Ze 339 (3µg/ml) for 1h to analyse the expression profiles of p-STAT1, p-STAT3, p-STAT6 and total STAT1, STAT3 and STAT6 under Ze 339 conditions only. As a control, tubulin was stained in HNECs to visualize the shape of the cells. As seen in Figure 22, treatment with Ze 339 without co-stimulation had nearly no effect on the expression level of p-STAT1, p-STAT3 and p-STAT6. But, the expression level of total STAT1, STAT3 and STAT6 is induced by the stimulation of Ze 339 alone in HNECs. Total STAT3 and total STAT1 were detectable in both, cytoplasmic and nuclear fractions, whereas total STAT6 seems to be only expressed in the cytoplasm of HNECs when stimulated with Ze 339 only.

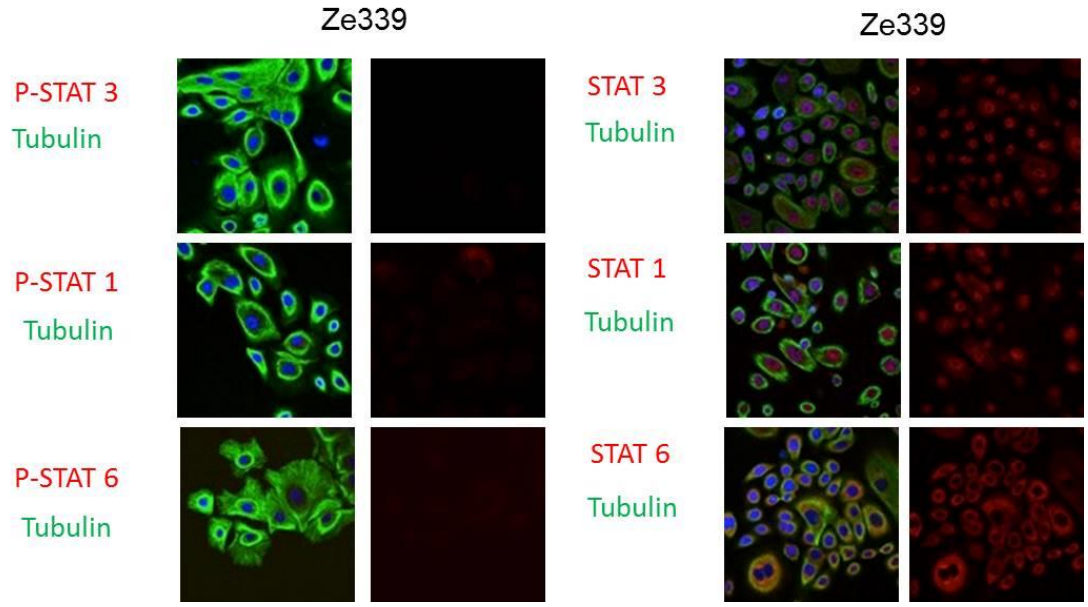


**Figure 22: Immunohistochemistry phospho-STATs/ total STATs in stimulated HNECs.** HNECs were cultured on glass chamber slides and stimulated with Ze 339 (3µg/ml) for 1h before immunostaining with STAT antibodies. Fluorescence images of p-STAT1, p-STAT3, p-STAT6 and total STAT1, STAT3 and STAT6 were captured by confocal microscope.

Additionally, HNECs were stimulated with the cytokines IL-4 (60 min), IFN-γ (30 min), and IL-6 (30 min). Stimulation of HNECs with cytokines led to the induction of total STATs and to nuclear translocation of phospho-STATs (Figure 23).

In IFN-γ-induced HNECs, p-STAT1 expression is weakly induced compared to solvent control and IFN-γ plus Ze 339 treated HNECs, in which no expression of p-STAT1 was detectable. Total STAT1 is expressed in every condition, but the nuclear expression is enhanced in the IFN-γ-treated cells. This nuclear expression is in turn also present, but reduced in IFN-γ plus Ze 339 stimulated cells.

IL-6 stimulated HNECs highly expressed p-STAT3 in the nucleus, whereas there was no expression in the solvent control. In IL-6 plus Ze 339 treated cells, there is a strongly reduced expression of p-STAT3 detectable. Total STAT3 is detected under all conditions. In the solvent control it is expressed in the cytoplasmic fraction of the cells, whereas in IL-6 induced HNECs it is highly upregulated in the nucleus. A combination of IL-6 with Ze 339 leads to a less present total STAT3 expression in the nucleus and nearly not expression of total STAT3 in the cytoplasmic fraction of HNECs.

Stimulation with IL-4 induced total and p-STAT6 expression in stimulated and control HNECs. P-STAT6 is expressed in the cytoplasmic and nuclear fraction of the cells when stimulated with IL-4 alone. In combination with Ze 339, p-STAT6 was less abundant, especially in the nucleus. Total STAT6 expression is induced in the solvent control sample, and was almost only detectable in the cytoplasmic fraction. The translocation into the nucleus of total STAT6 is increased in IL-4 stimulated HNECs. This translocation is less detectable when cells are stimulated with IL-4 together with Ze 339. Total STAT was more abundant in the cytoplasmic fraction of the stimulated HNECs.

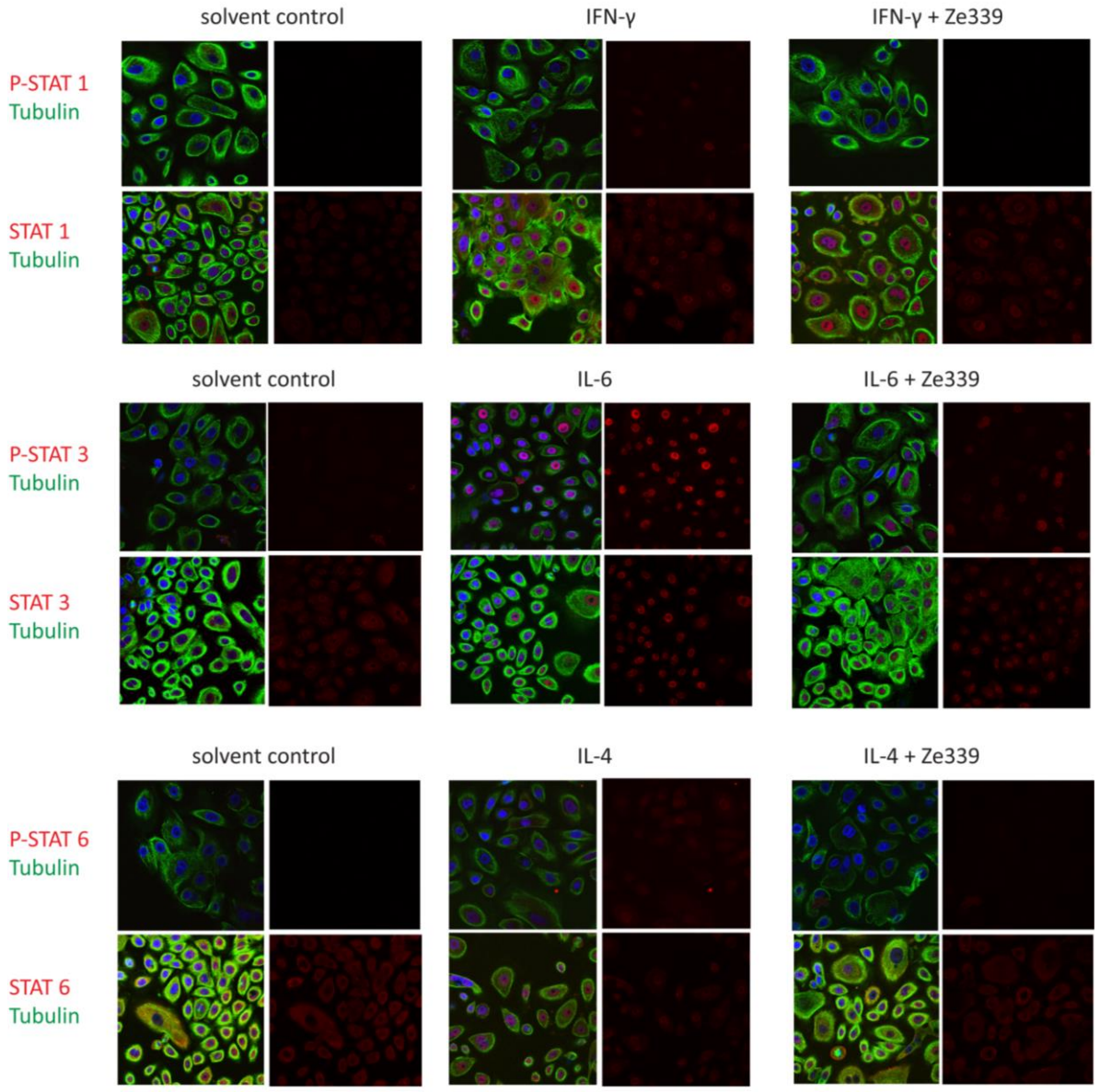


**Figure 23: Ze 339 inhibits cytokine-induced nuclear translocation of phospho-STATs.** HNECs were grown on glass chamber slides. At 80% confluence, cells were washed, stimulated and stained with PE-conjugated antibodies against total or tyrosine-phosphorylated STATs. FITC-conjugated anti-α-tubulin and DAPI blue were used as counter-stains for cytoskeleton and nucleus, respectively. As polyethylene glycol (PEG, 0.1 %0) served as solvent control for Ze 339, it was added to all wells. a) Cells incubated for 30 min with medium (unstim.), IFN-γ (15ng/ml) or IFN-γ (15 ng/ml) + Ze 339 (3µg/ml). b) Cells incubated for 30 min with medium (unstim.), IL-6 (800U/ml) or IL-6 plus Ze 339 (3µg/ml). c) Cells were stimulated for 60 min with medium (unstim.), IL-4 (50ng/ml) or IL-4 plus Ze 339 (3µM). Red: STATs/phospho-STATs, green: α-tubulin, blue: DAPI, yellow: overlay of STATs and α-tubulin. Representative images of n=3 experiments are shown.

# 5 Discussion

The aim of this thesis was to study the immune modulatory effects of Ze 339 in primary human nasal epithelial cells, to enlarge the knowledge and possibly extend the area of application of this phytodrug. The beneficial effects of the petasins, constituents of Ze 339, are known and were studied in clinical settings of airway inflammation and human myeloid cells such as eosinophils (Dumitru et al., 2011; Thomet et al., 2001). The exact mode of action on airway epithelial cells had not been studied before.

As IL-8 is expressed in airway epithelia cells in response to various stimuli such as respiratory viruses, bacteria, fungi, cytokines, allergens and mechanical strain (Cromwell et al., 1992; King et al., 1998; Shibata et al., 1996; Subauste et al., 1995; Tomee et al., 1997), HNECs are stimulated with dsRNA Poly IC as a viral mimic and to induce a immune response resulting in enhanced IL-8 expression. But to analyse whether Ze 339 itself induces an increased IL-8 secretion in HNECs, the IL-8 expression of Ze 339-only stimulated cells are analysed over a time period. PEG serves as the solvent control of Ze 339. There is a slightly induced IL-8 expression level visible, especially at the later time points. But compared to the IL-8 expression level responding to a viral stimulus, the expressed levels of IL-8 after Ze 339 treatment are insignificant. Thus, Ze 339 itself does not induce a considerable IL-8 expression level in cultured HNECs. The tested single isoforms all belong to the sesquiterpene ester family (Debrunner B, Meier B., 1998) that are known to modulate different intracellular signalling processes in human eosinophils (Thomet et al., 2001). To investigate whether the isoforms as a single stimulus play a role in the immune response in stimulated HNECs, cells are treated with the single isoforms only. First, IL-8 expression was tested. There is also no massive induced IL-8 expression level detectable when HNECs were treated with the petasin single isoforms alone.

As the treatment of HNECs with Ze 339 and the isoforms (separately and combined) could lead to an immune reaction of the cells also including the increased apoptosis of the cells due to the treatment, the cytotoxicity level of stimulated HNECs were analysed. Different concentrations were tested and the fact that the survival rate of treated HNECs did not significantly differ compare to the medium control, lead to the conclusion that the treatment with Ze 339 or the single isoforms has no effect on the survival rate of treated HNECs.

In a previous study, Dumitru et al. showed a significant reduction of IL-8 and $LTB_4$ in nasal fluids of allergic patients treated with Ze 339 in a human allergen challenge model (Dumitru et al., 2011). In cultured HNECs Ze 339 decrease the IL-8 release induced by PolyIC and this effect is also observed, when cells are treated with a mixture of the combined petasin

isoforms. Therefore, petasins, which make up 30% of Ze 339, are the active components in this readout. However, the treatment with the single petasin isoforms does not show the same effect. This discrepancy suggests that petasins can be recognized by several receptors or a hetero-multimeric receptor, but the combined action of three or maybe at least two petasin isoforms is needed for the inhibitory effect on the IL-8 expression level. Of note, several anti-inflammatory sesquiterpenes are ligands for the nuclear receptors peroxisome proliferator-activated receptor (PPAR)-γ (Lin, 2012), retinoid x receptor (RXR)-α (Zhang et al., 2011) or farnesoid X receptor (FXR) (Lin, 2015), classically acting as heterodimers in anti-inflammatory signalling. Thus, the heterodimerisation of two receptors could be essential for the modulation of the immune response regarding the IL-8 expression level.

Petasins were previously shown to act on human granulocytes, inhibiting cysteinyl-leukotriene and $LTB_4$ synthesis and intracellular calcium transient induction by PAF and C5a (Thomet et al., 2002). These effects could be related to the reduction of local $LTB_4$ release into nasal lining fluids of allergic patients under treatment with Ze 339 in a human randomized double placebo controlled clinical trial (Dumitru et al., 2011). This hypothesis is supported by the present study, which shows that Ze 339 has a clear effect not only on leukocytes but also on nasal epithelial cells under viral conditions. Therefore it could be possible that Ze 339 has a direct impact on infiltrating granulocytes and resident cells including epithelial cells, which can all release LTs in response to inflammatory stimuli (Jame et al., 2007). Leukotrienes play an important role in the pathogenesis of asthma, in allergic inflammation and in innate immunity (Hallstrand and Henderson, 2010). There are two receptors for $LTB_4$, the G-protein-coupled receptor leukotriene B4 receptor type 1 (BLT1) and type 2 (BLT2). BLT1 is mainly expressed in leukocytes (Tager and Luster, 2003), whereas BLT2 expression is present on the surface of epithelial cells (Ishii et al., 2015). LTs have the ability to bind on neutrophils, leading to a neutrophil recruitment (Kim et al., 2006). A potential “anti-neutrophil” effect of Ze 339 (due to its capacity to decrease $LTB_4$ synthesis) is supported by the finding that Ze 339 decreases IL-8 production by nasal epithelial cells. Thus, Ze 339 could have a beneficial effect in asthmatic patients through decreasing the level of $LTB_4$.

The finding that Ze 339 decreased neutrophil migration towards PolyIC-stimulated HNECs supernatants is most likely explained by the inhibitory effect of Ze 339 on IL-8 production, which is usually produced in higher amounts by epithelial cells as compared to the “leukocyte mediator” $LTB_4$. However, this would have to be tested by administration of an IL-8 neutralizing antibody to our chemotaxis assay. Again, the single isoforms are not able to inhibit the neutrophil migration towards the stimulated supernatant, but in combination, the petasinmix show reduced neutrophil migration in the tested concentrations. This finding encourages the

assumption that more than one petasin isoforms is needed to achieve immune modulation effects. Additionally, as mentioned before, BLT1 is present on neutrophils, resulting in neutrophil recruitment after the binding of $LTB_4$. The decreased production and release of $LTB_4$ due to Ze 339 could therefore reduce the recruitment of neutrophils, an effect, which could be addressed by performing chemotaxis assays in the presence of a BLT1 antagonist.

Notably, there is a very important role of neutrophils in multiple respiratory diseases including COPD, bronchiolitis, cystic fibrosis and asthma. Research data support the association between neutrophils and the severity and progression of inflammatory airway diseases such as COPD (Keatings et al., 1996). In asthma, neutrophils get recruited into the airways where they release several inflammatory mediators, including arachidonic acid metabolites such as $LTB_4$, which promote airway inflammation (Rabier et al., 1991; Radeau et al., 1990). The fact that Ze 339 decreases the neutrophil migration rate in the viral-induced PolyIC model could also identify Ze 339 as a possible tool to control the up-regulated neutrophil recruitment in inflammatory airway diseases such as asthma or COPD.

Montelukast is a leukotriene receptor antagonist, used as a treatment for chronic mild to moderate asthma and allergies (Knorr et al., 1998). Here, a microarray analysis of stimulated HNECs compared the treatments of Ze 339 with the treatments of Montelukast to identify differences in the gene expression profile. IL-8, TSLP, IL-31 and CCL27 are proteins secreted by the epithelium in response to a microbe or other pro-inflammatory stimuli (Cromwell et al., 1992; Neis et al., 2006; Sonkoly et al., 2006; Vestergaard et al., 2005; Ying et al., 2005). On the RNA level, Ze 339 reduces the gene expression of IL-31, TSLP and CCL27 and this result is corroborated by RT-PCR analysis. In atopic dermatitis (AD), levels of IL-31 are elevated and it leads i.e. to a reduced epidermal thickness and disturbed epidermal constitution (Kim et al., 2011). Thus a reduced IL-31 level by Ze 339 could be beneficial for AD patients. CCL27 is involved as a chemokine in the recruitment of T cells to the side of infection (Morales et al., 1999). Ze 339 reduces the expression of CCL27 and therefore might reduce the recruitment of T cells in a variety of diseases with a central role for T-cells. TSLP is a mediator in the development of allergic sensitization through the induction of DC-mediated Th2 differentiation (Omori and Ziegler, 2007; Rate et al., 2009), thus a reduction in the expression level of TSLP could prevent allergic sensitization.

The present study shows that Ze 339 reduces the expression of the common γ-chain in a viral-induced model on RNA level. The IL-2 receptor γ-chain, also known as common γ-chain composes a subunit of IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21 receptors (Asao et al., 2001). Hvid et al. showed a significant down-regulation of protease caspase-14 when keratinocytes were treated with IL-4. This leads to a modulation in the natural moisturizing process in the

skin, correlating with the induction of cornification (Denecker et al., 2008; Hoste et al., 2011; Hvid et al., 2011). Thus the reduced expression of the common γ-chain due to Ze 339 treatment may diminish the process of cornification by prohibiting the IL-4 pathway.

The beneficial effect of Ze 339 assessed in the human allergen challenge clinical trial (Dumitru et al., 2011) was only analysed in allergic patients. To find out, whether Ze 339 might also act in HNECs of healthy patients, PolyIC-stimulated HNECs form healthy as well as from allergic patients are analysed regarding their expression profile of selected chemokines and cytokines. IL-8 is significantly reduced in HNECs of allergic and non-allergic patients demonstrating that the effect of Ze 339 is not just present in HNECs of allergic patients but also in HNECS of non-allergic patients. G-CSF, IL-6, CCL5 and IL-1α showed a trend towards decreased expression of pro-inflammatory mediators in both allergic and non-allergic HNEC. Whereas the overall PolyIC-induced expression level of the measured cytokines and chemokines is much higher in the non-allergic HNECs. This could be due to the fact that HNECs from allergic patients have a higher level of pro-inflammatory mediators at baseline and therefore react less strongly to a viral mimic compared to HNECs from healthy donors.

Additionally, Ze 339 decreases the induced secretion of further pro-inflammatory chemokines, e.g. CCL2 and CCL3 in PolyIC-treated HNECs, all of which are implicated in neutrophil chemotaxis (Reichel et al., 2009). This broad capacity of inhibiting neutrophil chemotaxis suggests a potential of Ze 339 treatments of inflammatory states involving extensive recruitment of neutrophils such as neutrophilic asthma.

The treatment of HNECs with Ze 339 leads to a clear reduction in the pro-inflammatory cytokine and chemokine response to TLR3 stimulation, whereas the inhibitory effect on RIG-I and MDA-5 ligand-induced responses are present but less pronounced. This suggests that Ze 339 might act on the endosomal pathway or might be taken up by endosomes and therefore cannot bind to RIG-I and MDA-5. Being a CO2 extract of butterbur leafs, Ze 339 is mainly composed of hydrophobic compounds, among them terpenoids, lipids and glycolipids (Thomet et al., 2001). Therefore Ze 339 could also intercalate in cell membranes, which could decrease its bioavailability in the cytosol. Bound to the membrane it could interact or block receptors located in the membrane of epithelial cells and modulate the immune response.

In contrast to the inhibitory effect of Ze 339 in the viral models, comparable effects under stimulation with selected bacterial PAMPs are not observed. The used bacterial ligands are chosen on the basis of published data on TLR expression profiles of HNECs (Lin et al., 2007; van Tongeren et al., 2015). The TLR-4 ligand LPS does not induce any of the cytokines or

chemokines tested (data not shown). In contrast to a previous study that showed TLR-4 expression in HNECs on mRNA level (Lin et al., 2007), the present results argue against functionally active TLR-4 in HNECs. The finding that Ze 339 did not inhibit bacterial TLR ligand-induced responses therefore suggests that Ze 339 could be a promising tool for treatment of acute inflammatory symptoms during viral rather than bacterial infection.

Airway remodelling is present in airway diseases such as asthma. The according structural changes in the airways are characterized through modifications such as smooth muscle hyperplasia or the generation of myofibroblasts. The development of asthma and the comprised airway remodelling in early childhood can be promoted by a viral infection (Sigurs et al., 2000). The results of the present study suggest beneficial effects of Ze 339 in the prevention of virus-triggered asthma, due to its broad effects on pro-inflammatory mediator production. Ze 339 could therefore prevent a chronic inflammatory immune response due to viral infections, which may result in asthma later in life and therefore decrease the risk to develop airway remodelling (Crimi et al., 1998).

Under stimulation with the cytokines IL-4, IL-6 and IFN-γ, Ze 339 diminished the release of pro-inflammatory cytokines and chemokines from HNECs. The receptors for IL-4, IL-6 and IFN-γ act via signal transducers and activators of transcription (STATs), and the phosphorylation of STATs is important for nuclear translocation and activity. STAT signalling pathways are key regulators of various allergic and non-allergic inflammatory diseases (Darnell, 1997; Santos and Costa-Pereira, 2011) such as tumour defence. STAT 3 could demonstrate to be of benefit for the tumour development by inducing the production of VEGF, cyclin D1 or other chemokines in the tissue and therefore promote the growth of the tumour (Yue and Turkson, 2009). It could be demonstrated that Ze 339 significantly reduced the level of phospho-STAT3 in IL-6 stimulated HNECs. The STAT6 signalling pathway is activated by IL-4 binding, a cytokine which is overexpressed in patients suffering from asthma. The IL-4 induced STAT6 phosphorylation leads to the expression of various type 2 cytokines (Mathew et al., 2001). Ze 339 inhibited the expression level of phosphorylated STAT6 in IL-4 induced HNECs and could therefore represent an interesting therapeutic tool for patients suffering from type 2 cytokine driven nasal and potentially bronchial inflammation. The finding that Ze 339 modulated cytokine-induced expression and translocation of all analysed phospho-STATs suggests that the compound interferes with a signalling step common to all three cytokine receptors.

Cytokine receptor signalling via STATs involves the assembly of a multi-protein complex in lipid rafts or lipid microdomains, located in restricted membrane areas of the cell (Brown and London, 2000; Subramaniam and Johnson, 2002). Several glycosphingolipids, which are

important in the function as modulators of signal transduction pathways in the cell, such as cell proliferation, cell differentiation or cell survival, are located in the lipid microdomains (Kasahara and Sanai, 2000). The glycohydrolase β-hexosaminidase is involved in the degradation of glycosphingolipids. A recent *in vitro* study demonstrate that S-petasin inhibited the degranulation of the β-hexosaminidase in a rat basophilic cell line independently from intracellular calcium inhibition (Lee et al., 2015). Notably, β-hexosaminidase is localized in endosomal vesicles in epithelial cells and was found to be associated to lipid rafts in Jurkat T lymphocytes (Magini et al., 2012). Hydrophobic substances within Ze 339, including petasins, could be retained in the plasma membrane or become selectively enriched in lipid rafts where they might interfere with assembly of the JAK/STAT signalling complex. The natural oily monocyclic sesquiterpene α-bisabolol is a natural product, which can be found in different plants such as chamomile flowers. It is used in cosmetics for skin healing properties and it is reported to have anti-inflammatory and anti-microbial properties. Interestingly, the α-bisabolol lactone is incorporated in lipid rafts, where it induces apoptosis in tumour cells by interfering with the pro-apoptotic Bcl-2 family member Bid (Darra et al., 2008). Future studies need to show whether Ze 339 interferes with lipid raft signalling complexes.

The process of Th1 cell differentiation is amongst others dependent on the transcription factors STAT1, STAT4 and T-bet. The presence of IL-4 guides the differentiation process of T helper cells towards a Th2 differentiation (Kaplan et al., 1996; Thierfelder et al., 1996). In this process the transcription factors STAT6 and the trans-acting T cell-specific transcription factor GATA-3 (GATA-3) play a major role. The development of Th17 cells from naïve CD4+ is dependent on RAR-related orphan receptor gamma t (RORγt) but also on the transcription factor STAT3 and the presence of IL-6 (Bettelli et al., 2006). It could be shown that the level of IL-17 expression is increased in patients suffering from inflammatory bowel disease (Fujino et al., 2003). STAT3 is necessary for the Th17 differentiation process in response to stimulation with IL-6 together with IL-21. Chen et al. showed a direct binding of STAT3 to the IL-17A and IL-17F-promotor (Chen et al., 2006). The results of the present study show that Ze 339 exerted the most pronounced effects on the IL-6 induced nuclear translocation of phospho-STAT3 in HNECs. Therefore it can be speculated that Ze 339 could alter the priming of naïve T cells in the process of Th17 differentiation and Th17 mediated immune reactions. This mechanism could support a beneficial effect of Ze 339 via regulating phospho-STAT3. Ze 339 might therefore modulate disease-induced Th17 responses such as fatal intestinal inflammation or psoriasis (Chaudhry et al., 2009; Kikly et al., 2006). Additionally, STAT3 activity plays an important role in the IL-6 induced T cell migration (McLoughlin et al., 2005). Beside the question if Ze 339 has an impact on the priming of naïve T cells into Th17

cells, another interesting future research direction might be a possible modulation of the recruitment and migration of T cells by Ze 339.

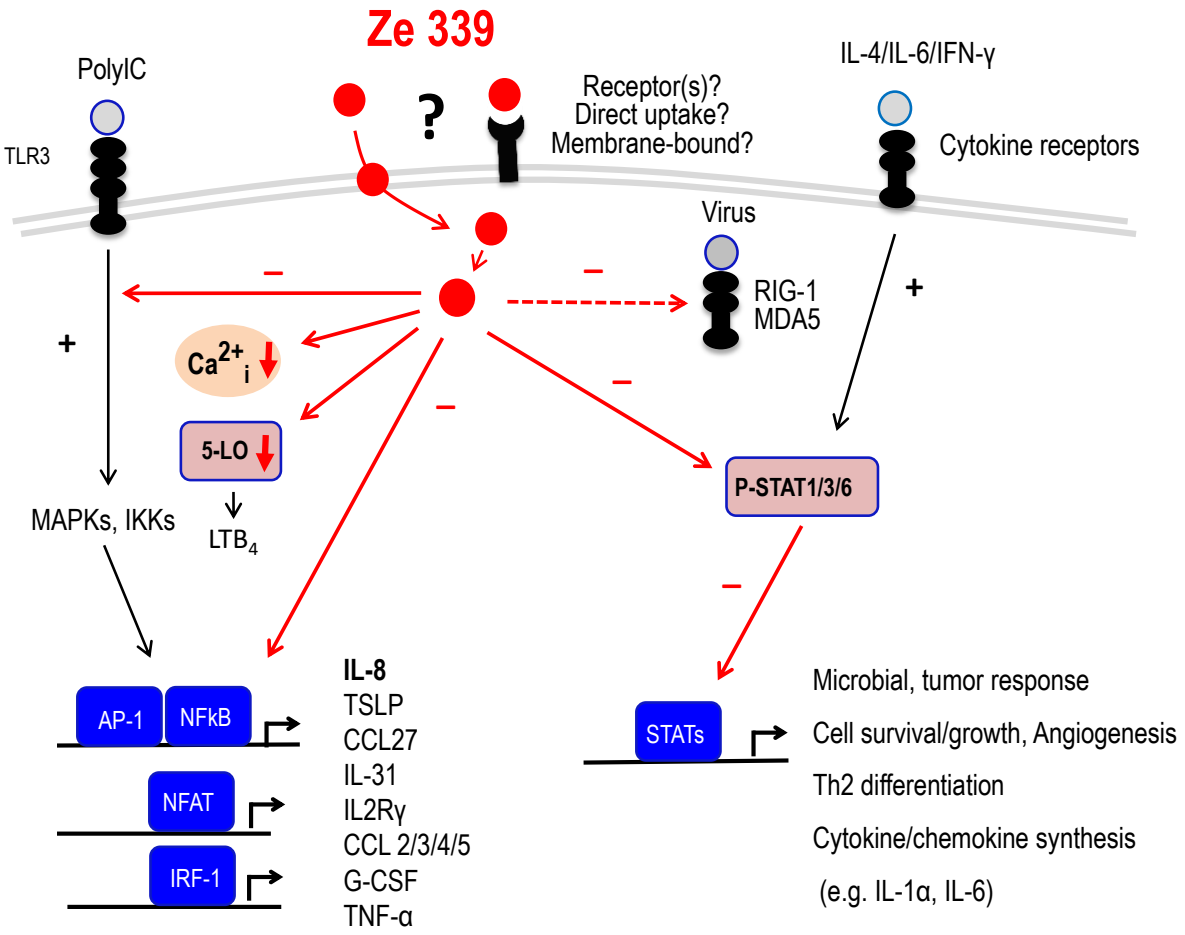


**Figure 24: Ze339 - Mode of action** The scheme illustrates the anti-inflammatory potential of Ze 339 on human nasal epithelial cells. Ze 339 modulates the expression level of several pro-inflammatory cytokines or chemokines such as IL-8, TSLP, CCL27, IL-31, CCL2/3/4/5, G-CSF, TNF-α, IL-1α and IL-6 in a viral induced model. Additionally, the translocation and expression of phosphorylated STAT1, STAT3 and STAT6 is modified by Ze 339 in cytokine stimulated HNECs.

The aim of the present study was to identify the mode of action of a carbon dioxide extract of the leaves of the butterbur plant, Ze 339, on human nasal epithelial cells under different treatment condition. The originated results do enlarge the knowledge about this phytodrug and extend the possible work field as a new treatment for various inflammatory diseases. The results of the study on human nasal epithelial cells imply that Ze 339, presently used as a treatment for allergic airway inflammation, might be applied for treatment of acute viral infections such as a common cold. In a viral-induced model Ze 339 showed a strong modifica-

tion in the expression level of important mediators such as IL-8, IL-6, IL-1α, CCL2, CCL3, CCL4, CCL5, TNF-α and G-CSF. Thus, Ze 339 could be of benefit in the treatment of immune deviations associated with continuous cytokine-induced inflammation. Additionally, the neutrophil migration rate towards conditioned medium from Ze 339-stimulated HNECs was strongly decreased. In addition to its established anti-inflammatory characteristics in the treatment of seasonal allergic rhinitis and the anti-chemotactic property in a viral stimulation model, Ze 339 appears to exert broad anti-cytokine effects by interfering with phospho-STAT nuclear translocation. The level of phospho-STAT3, a central transcription factor in the innate immune response, is significantly reduced in Ze 339-treated HNECs after IL-6 stimulation. STAT3 plays an important role in various diseases such as psoriasis, rheumatoid arthritis or other chronic inflammatory diseases via the Th17 pathway.

Taken together the results support the view that Ze 339 could be suitable for application in inflammatory diseases such as sinusitis. The mechanistic results provided in this thesis have to be translated in new clinical studies to confirm the potential of Ze 339 as a therapeutic agent exceeding allergic rhinitis.

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# Summary

Human nasal epithelial cells initiate the first line of defence against respiratory pathogens, leading to an inflammatory immune response. Chronic inflammatory airway diseases such as asthma or allergies like allergic rhinitis are nowadays a big public health issue worldwide. The use of phytodrugs beside common treatments is getting more and more popular. Ze 339, an herbal extract of the butterbur (*Petasites hybridus)* leafs has previously proven effective in the treatment of allergic rhinitis. This was shown in reduced local production of IL-8 and $LTB_4$ in nasal secretion of allergen-challenged patients, while the mechanisms underlying this successful treatment are still unknown. Aim of the present study was to assess the anti-inflammatory mechanism of Ze 339 on human primary nasal epithelium cells (HNEC) for potential expansion of the therapeutic spectrum.

As an experimental setup, HNEC were stimulated with viral mimics, bacterial TLR-ligands or cytokines, in absence or presence of Ze 339. Additionally, HNECs were also treated with a mixture of three different petasin isoforms, the potential active component of Ze 339. Pro-inflammatory mediators and neutrophil chemotaxis towards stimulated supernatants were quantified. A microarray analysis was performed to compare the gene expression profile of Ze 339 stimulated HNECs with Montelukast stimulated HNECs, a common leukotriene receptor antagonist. Furthermore, the impact of Ze 339 on total and phosphorylated STAT1, STAT3 and STAT6 in cytokine-stimulated HNECs was analysed.

Ze 339 reduced the PolyIC-induced production of pro-inflammatory mediator IL-8. The neutrophil chemotaxis towards supernatants of PolyIC/Ze 339-treated cells was decreased as well. The same result was visible when HNECs were treated with a combination of the three isoforms, but not when treated with just one single isoform alone. The analysed gene expression profile totally differs from Ze 339 treated cells compare to Montelukast treated cells, assuming a different activation profile of Ze 339 in the cell. Additionally, Ze 339 modulated HNEC responses to stimulation of RIG-1/MDA5, TLR9 and TLR3, but not to TLR1/2- and TLR5 stimulation, visible in the production of pro-inflammatory mediators such as IL-1α, TNF-α, IL-6, CCL2, CCL3 and CCL4. Likewise, Ze 339 reduced the nuclear translocation and expression levels of total and phospho-STATs. Especially the expression level of phospho-STAT3 was significantly reduced by Ze 339 in IL-6 stimulated HNECs.

The present study demonstrates a broad anti-inflammatory potential of the herbal drug and a potential to extend the therapeutic use of Ze 339. STAT signalling pathways are specifically targeted in nasal epithelial cells. Therefore, Ze 339 could be effective in the treatment of non-allergic rhinitis, sinusitis or other inflammatory diseases.

# Zusammenfassung

Humane nasale Epithelzellen stellen in der Abwehr gegen respiratorische Pathogene die erste immunologische Hürde dar. Das Pathogen trifft auf die Zellen der Atemwege wodurch eine entzündliche immunologische Antwort hervorgerufen wird. Chronische Atemwegserkrankungen, wie Asthma oder allergische Rhinitis, stellen heutzutage ein großes Problem in unserem Gesundheitssystem dar. Neben den bekannten Therapiemöglichkeiten erfreuen sich pflanzliche Arzneimittel, sogenannte Phytopharmaka, immer größerer Beliebtheit. Ze 339, ein pflanzliches Extrakt aus den Blättern der Pestwurz (*Petasites hybridus*), zeigt in einer humanen Allergenexpositionsstudie reduzierte IL-8 und $LTB_4$ Spiegel in nasalen Sekreten der Probanden. Ziel der gegenwärtigen Studie ist die Aufklärung der Mechanismen die dem anti-entzündlichen Potentials von Ze 339 in primären humanen nasalen Epithelzellen (HNEC) unterliegen. Dieses Wissen ist Voraussetzung für die Erweiterung des Applikationsfeldes.

Im vorliegenden Versuchsablauf werden HNEC sowohl mit verschiedenen viralen Stimulanzien, als auch mit verschiedenen bakteriellen TLR-Liganden sowie mit verschiedenen Entzündungs-relevanten Zytokinen, jeweils mit oder ohne Ze 339, stimuliert. Die vermutete aktive Komponente in Ze 339 sind die Petasine, die in drei verschiedenen Isoformen vorkommen und ebenfalls untersucht wurden. Gemessen wurden pro-entzündliche Mediatoren des Immunsystems und die Migrationsaktivität von Neutrophilen auf vorstimulierte Überstände. Um die Unterschiede im Genexpressionsprofil von Ze 339-stimuliverten Zellen im Vergleich mit einem Kontrollmedikament (Montelukast) festzustellen wurden genomweite Expressionsanalysen der unterschiedlich stimulierten Zellen durchgeführt. Zusätzlich wurde der Einfluss von Ze 339 auf das Expressionsmuster der Transkriptionsfaktoren STAT1, STAT3 und STAT6 in Zytokine-stimulierten Zellen untersucht.

Die Studie zeigt erstmals, dass Ze 339 die Poly-IC induzierte IL-8 Sekretion in HNEC verringert und die Migrationsaktivität von Neutrophilen hemmt. Dabei erwies sich die Kombination der drei Isoformen als ebenso effektiv wie der Gesamtextrakt, während die einzelne Isoformen keine signifikanten Effekte zeigten. Der Vergleich der durch Ze 339- und Montelukast regulierten Gene, zeigt ein grundlegend unterschiedliches Expressionsprofil. Somit scheinen die intrazellulären Wirkungen auf verschiedene Mechanismen zu beruhen. Zusätzlich kommt es zu einer Veränderung der Immunantwort in RIG-1/MDA5, TLR9 und TLR3 stimulierten HNECs in Kombination mit Ze 339. Dies kann in einem verringerten Sekretionsprofil der pro-entzündlichen Mediatoren IL-1α, TNF-α, IL-6, CCL2, CCL3 und CCL4 nachgewiesen werden. Eine Stimulation der TLR1/2 und TLR5 in Kombination mit Ze 339 ergibt keine signifi-

kante Veränderung der gemessenen Mediatoren. Ze 339 weißt ebenfalls einen Effekt auf die Translokation und Expression der Transkriptionsfaktoren STAT1, STAT3 und STAT6 auf. Dieses Resultat kann vor allem in dem Expressionslevel von Phospho-STAT3 in IL-6 stimulierten HNEC nachgewiesen werden.

Die erzielten Ergebnisse lassen darauf schließen, dass Ze 339, zusätzlich zum momentanen Anwendungsgebiet in der Therapie von anti-entzündlichen Erkrankungen, nicht-allergischer Rhinitis oder Sinusitis eine positive Auswirkung haben könnte. Ze 339 wirkt auf den STAT Signalweg in nasalen Epithelzellen und bestätigt zudem sein anti-entzündliches Potential, welches in den genomweiten Expressionsprofilen sichtbar wurde. Diese Fähigkeiten heben das vorhandene Potential von Ze 339 hervor und machen es somit zu einem vielversprechenden pflanzlichen Arzneimittel mit weitreichender therapeutischer Anwendung.

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